Structure-function relationship of flavoproteins

With special reference to p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op dinsdag 9 mei 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

STELLINGEN

1. De enzym-nomenclatuur van FAD-bevattende monooxygenases dient nodig herzien en up to date gebracht te worden.

   - Enzyme Nomenclature (1984) IUB, Academic Press (USA).
   - Dit proefschrift.

2. Raju et al. gebruiken ten onrechte de resultaten, verkregen in hoofdstuk 5 van dit proefschrift, als argument om aan te geven dat cysteine residuen een essentiële rol spelen in de katalyse van 4-hydroxyphenylacetaat-3-hydroxylase uit Pseudomonas putida.

   - Raju, S.G., Kamath, A.V. and Vaidyanathan, C.S. (1988) Bioch. Biophys. Res. Commun. 154, 537-543.

3. Het is jammer te moeten constateren dat "de fabrikant van de beste biochemicaliën" anno 1989 de ratio lipoamide: diaphorase activiteit van lipoamide dehydrogenase slechts omschrijft als een maat voor de denaturatie van het enzym.

   - Massey, V. and Veeger, C. (1961) Biochim. Biophys. Acta 48, 33-47.
   - Sigma catalogus (1989).

4. Zolang een reactie-intermediair in de natuurlijke reactie gekatalyseerd door p-hydroxybenzoaat hydroxylase niet wordt gekarakteriseerd, laat staan waargenomen, blijft zijn voorkomen speculatief.

   - Anderson R.F., Patel, K.B. and Stratford, M.R.L. (1987) J. Biol. Chem. 262, 17475-17479.

5. Meer biochemische kennis omtrent de dehalogenering van halogeenhoudende aromatische verbindingen is dringend gewenst.

   - Castro, C.E., Yokoyama, W.H. and Belser, N.O. (1988) J. Agric. Food Chem. 36, 915-919.
   - Van den Tweel, W.J.J. (1988) Thesis, Wageningen.

6. Waterstofbruggen tussen enzym en substraat/produkt, zoals bepaald via de kristalstructuur van p-hydroxybenzoaat hydroxylase, moeten met een "korreltje" zout genomen worden.

- Schreuder, H.A. (1988) Thesis, University of Groningen.

7. Helaas werkt het "verkopen" van DNA-sequenties, coderend voor enzymen, ook het verkopen van onzin in de hand.

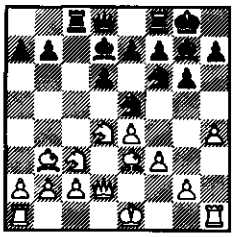
- Matsubara, Y., Kraus, J.P., Ozasa, H., Glassberg, R., Finocchiaro, G., Ikeda, Y., Mole, J., Rosenberg, L.E. and Tanaka, K. (1987) J. Biol. Chem. 262, 10104-10108.

8. Het in de voedingswereld al meer dan tien jaar consequent vermelden van de eenheid kilocalorie naast kiloJoule wekt de schijn dat de eerstgenoemde eenheid beter verteerbaar is.

- Duinker-Joustra, N. (1983) Kijk op joules en calorieën.

9. De ivoren toren der wetenschap begint aardig op een vuurtoren te lijken: de overheid steekt er de brand in !

10. Met deze stelling wordt het meest de draak gestoken:



Stellingen behorend bij het proefschrift van
Willem van Berkel, Wageningen, 9 mei 1989.

VOORWOORD

Dit proefschrift is het resultaat van een jarenlang leerproces temidden van vele collega's en studenten.

Iedereen die aan dit leerproces heeft bijgedragen wil ik bij deze hiervoor bedanken. Enkele personen wil ik met name noemen:

Mijn promotor, Franz Müller, die mij altijd de vrijheid heeft gegeven om naast de noodzakelijke ondersteunende werkzaamheden tijd te investeren in zelfstudie en eigen onderzoek.

Mijn meest directe collega's: Willy van den Berg, Martin Bouwmans, Berend Sachteleben, Jillert Santema, Yvonne Soekhram, Jenny Toppenberg, Cees Veeger, Lida Verstege, Jacques Vervoort, Ton Visser, Gerrit Voordouw, Adrie Westphal en Robert Wijnands. Jullie waren altijd bereid waren om praktische en theoretische problemen op te lossen.

Mijn wetenschappelijke vrienden uit Groningen: Jaap Beintema, Jan Drenth, Jan Hofsteenge, Wim Hol, Peter Jekel, Jan Metske van der Laan, Herman Schreuder, Johan Vereijken, Wicher Weijer en Rik Wierenga. Geweldig, om in zo'n sfeer te mogen samenwerken.

Tenslotte Anita, bedankt!

CONTENTS

ABBREVIATIONS

AcPyADP+	- oxidized 3-acetylpyridine adenine dinucleotide phosphate
ADP	- adenosine 5'-diphosphate
2',5' ADP	- adenosine 2',5'-bisphosphate
2' AMP	- adenosine 2'-monophosphate
cDNA	- complementary deoxyribonucleic acid
CoA(SH)	- coenzyme A
DTNB	- 5,5'-dithio-bis(2-nitrobenzoate)
EC	- enzyme code
EDTA	- ethylene diamine tetraacetate
ETF	- electron transferring flavoprotein
FAD(H2)	- flavin adenine dinucleotide (reduced)
FMN	- flavin mononucleotide
FPLC	- fast protein liquid chromatography
GSH	- glutathione reduced
GSSG	- glutathione oxidized
GuHCl	- guanidinium hydrochloride
HPLC	- high performance liquid chromatography
Kd	- dissociation constant
Km	- Michaelis Menten constant
kDa	- kilodalton
$LipS_2$	- lipoamide oxidized
Lip(SH)2	- lipoamide reduced
Mr	- relative molecular mass
$NAD(P)^+$	- nicotinamide dinucleotide (phosphate) oxidized
NAD(P)H	- nicotinamide dinucleotide (phosphate) reduced
nm	- nanometer
NMR	- nuclear magnetic resonance
PAGE	- polyacrylamide gelelectrophoresis
p-OHB	- para hydroxybenzoate
SDS	- sodiumdodecylsulfate
SE	- sulfoethyl
TCA	- trichloroacetic acid

Parts of chapters 1.3-1.8 will be published as reviews:

1a. Methods used to reversibly resolve flavoproteins into the constituents apoflavoprotein and prosthetic group.
Müller, F. and Van Berkel, W.J.H. (1989) in: Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed.) CRC Press, Boca Raton (Florida), in press.

1b. Flavin-dependent hydroxylases with special reference to p-hydroxybenzoate hydroxylase.
Van Berkel, W.J.H. and Müller, F. (1989) in: Chemistry and Biochemistry of Flavoenzymes (Müller, F., ed.) CRC Press, Boca Raton (Florida), in press.

Chapters 2-7 have been published separately:

2. Müller, F. and Van Berkel, W.J.H. (1982)
Eur.J.Biochem. 128, 21-27.
3. Van Berkel, W.J.H., Van den Berg, W.A.M. and Müller, F. (1988)
Eur.J.Biochem. 178, 197-207.
4. Van Berkel, W.J.H. and Müller, F. (1987)
Eur.J.Biochem. 167, 35-46.
5. Van Berkel, W.J.H., Müller, F., Weyer, W.J., Jekel, P.J. and Beintema, J.J. (1984)
Eur.J.Biochem. 145, 245-256.
6. Van Berkel, W.J.H., Müller, F., Jekel, P.J., Weyer, W.J., Schreuder H.A. and Wierenga, R.K. (1988)
Eur.J.Biochem. 176, 449-459.
7. Van Berkel, W.J.H. and Müller, F. (1989)
Eur.J.Biochem. 179, 307-314.

Chapter 1 General Introduction

1.1. Flavoproteins

Flavoproteins are proteins containing a tightly bound flavin (yellow) molecule usually associated in a non-covalent mode. Flavoproteins are involved in a wide variety of enzymatic reactions spread widely in nature.

The most occurring flavin prosthetic groups are flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) which both can be synthesized from riboflavin. Riboflavin (Vitamin B2) is the most abundant flavin compound found in nature being synthesized by all plants and many microorganisms. The structures of riboflavin, FMN and FAD are shown in Figure 1:

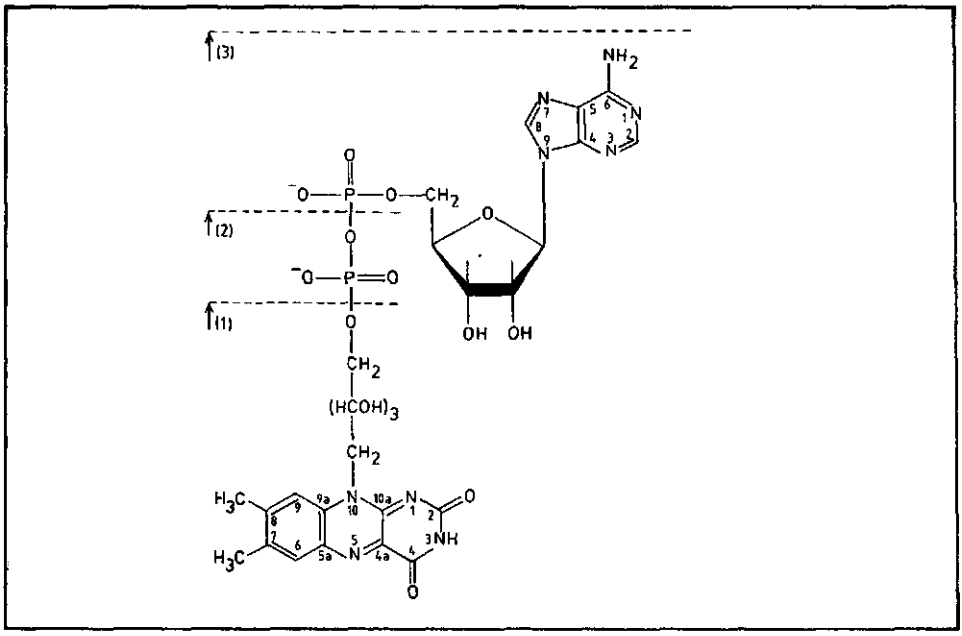


Figure 1. Structure of riboflavin (1), FMN (2) and FAD (3)

The isoalloxazine ring system which is the functional part of the flavin prosthetic group can undergo one- or two-electron transitions which are modulated by the surrounding protein structure. The biological important redox properties of the flavin molecule are outlined in Figure 2:

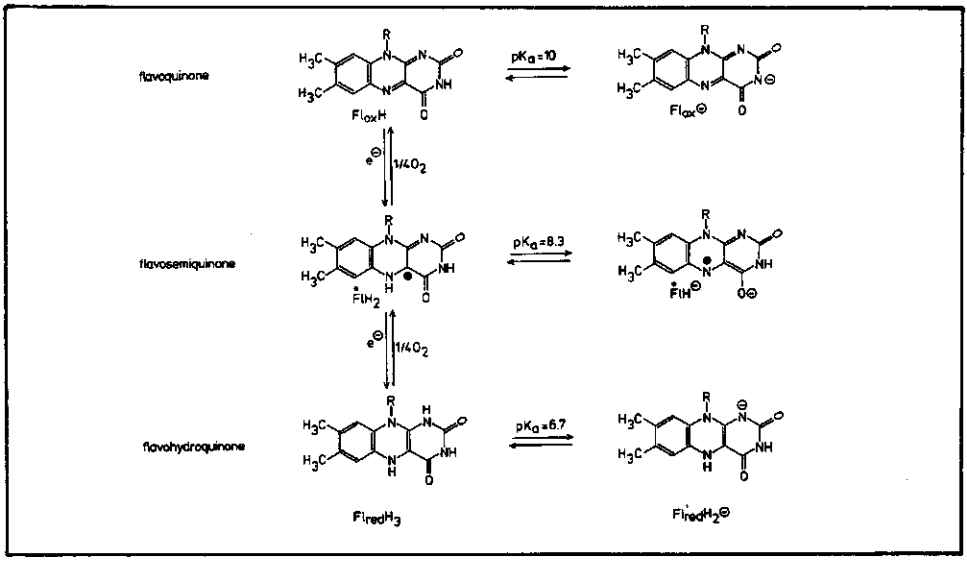


Figure 2. Redox states of flavin

One of the most fascinating aspects about flavins and flavoproteins is the visible spectral behaviour of the different redox states. As can be seen from Fig. 2 yellow oxidized flavin can be reduced by one electron either to the blue neutral or red anionic semiquinone or reduction can take place by a two-electron process yielding the pale yellow hydroquinone state. Moreover in flavoproteins all kind of typical coloured species can be observed due to the specific interaction of flavin and bound substrates or coenzymes.

Up to now more than 100 different flavoproteins, isolated from various organisms, have been characterized. From these about 25 different enzymes have been found to contain flavin covalently bound to a specific amino acid residue (histidine, tyrosine or cysteine), mostly through the 8α-methyl group of the flavin ring [1]. The functional significance of this covalent bond is as yet not clear [2]. For more details about action, mechanisms and classification of flavoproteins recent reviews [3-8] can be useful for reference.

Intensive research on the action and function of flavoproteins has started around 1960. At that time most information was obtained from kinetic data on both flavin enzymes and model flavin compounds [9,10]. After 1970 important progress was made with the elucidation of the primary and three-dimensional structures of some flavoproteins [11-14] and with the discovery and purification of more flavoproteins by using also newly developed techniques like for instance affinity chromatography [15]. The last decade the tremendous improvement of analytical techniques and instruments has opened new ways of studying flavoproteins in even more detail. At the moment most information about the structure-function relationship of flavoproteins is obtained by multidisciplinary research combining many different techniques. Some topics presented on the Ninth International Symposium on Flavins and Flavoproteins, Atlanta 1987 [16] are summarized in Table 1:

Table 1. Some Topics in Flavoprotein Research.

Technique	Information
HPLC, FPLC	Enzyme purification Amino acid sequence
Chemical modification	Role of specific amino acid residues
Cloning techniques	Amino acid sequence Production-clones
Site directed mutagenesis	Role of specific amino acid residues
Stopped flow techniques	Kinetic data Reaction intermediates
^{13}C, ^{15}N-NMR spectroscopy	Interactions between flavin and apoprotein π-electron density of flavin ring atoms
2D-NMR spectroscopy	Three-dimensional structure in solution
X-ray crystallography Computer graphics	Three-dimensional crystal structure

1.2. Aim of the Thesis

This thesis deals with different affinity studies on flavoproteins. The aim of the studies presented was two-fold:

1) within the scope to study different flavoproteins by ^{13}C and ^{15}N NMR technique it was desirable to explore the possible use of affinity chromatography methods in order to allow the large scale preparation and reconstitution of stable apo flavoproteins. For more information about the (enzymatic) synthesis of enriched flavins and ^{13}C or ^{15}N NMR studies on different flavoproteins the reader is referred to some recent papers [17-22].

2) within the scope of the Dutch multidisciplinary research project on p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens it was desirable to explore the possibilities of affinity labeling in order to get more insight in the involvement of different amino acid residues in the binding of the substrate (p-hydroxybenzoate) and coenzyme (NADPH). For more information about other aspects of this multidisciplinary approach the reader is referred to amino acid sequence [23,24], chemical modification [25] and X-ray crystallography [26-28] studies on this enzyme.

In the next part of the introduction, both subjects are reviewed.

1.3. Apo Flavoproteins

The reversible dissociation of flavoproteins into apoprotein and flavin prosthetic group has received much attention in the past [29,30]. In order to be able to study the properties of flavoproteins containing either artificial [6] or isotopically enriched [22] flavins it is important to remove the natural prosthetic group under conditions allowing a fully reversible association process between flavin and apoprotein yielding the same native structure again. Complete reversible removal of the non-covalently bound flavin prosthetic group is difficult to achieve because for most flavoproteins the dissociation constant of the equilibrium:

$$\text{E-flavin} \quad \rightleftarrows \quad \text{E} + \text{flavin}$$

is below 10^{-8} M under physiological conditions. Therefore conditions have to be found changing the equilibrium quite drastically without irreversibly altering the protein structure. For some flavoproteins this problem can easily be solved but in most cases very special treatments are required. Current methods to remove the flavin prosthetic group are classified into 3 categories:

- acid precipitation
- dialysis or gel filtration in the presence of high concentrations of monovalent anions
- dialysis or gel filtration in the presence of high concentrations of unfolding agents

Already in 1938 Christian and Warburg [31] reported on the removal of FAD from D-amino acid oxidase by acid treatment in the presence of high concentrations of ammonium sulfate. Although giving variable yields of reconstitutable apoprotein this method was adopted for a number of flavoproteins for a long period of time.

In 1961 Strittmatter [32] reported on an improvement of the acid ammonium sulfate precipitation procedure by extra adding high concentrations of KBr for a more efficient removal of the flavin prosthetic group by weakening electrostatic interactions between apoprotein and flavin prosthetic group. The concept of this method has been applied to a number of flavoproteins [33-35]. Only minor changes of the conditions of flavin resolution were introduced for maximal recovery of reconstitutable apoprotein. With some enzymes charcoal was added during apoprotein preparation in order to remove the prosthetic group more efficiently [36,37].

The main disadvantage of the different acid treatment procedures is the instability of most flavoproteins at pH values below 3. Flavodoxins which are a class of small electron carrying flavoproteins are a good exception of this rule. The prosthetic group FMN which is very tightly bound to the apoprotein [38] can easily be removed by precipitation in the presence of 5% TCA without damaging the protein structure [39]. After neutralization of the apoprotein solution the holoprotein can be reconstituted in a very fast association process [40].

A milder way of apoprotein preparation was first reported in 1966 by Massey and Curti [41] for D-amino acid oxidase. With this enzyme it is possible to remove FAD completely by simple dialysis against high concentrations of KBr at physiological pH. The same procedure can be applied to FMN-containing Old Yellow Enzyme at pH 5.3 [42] and to flavodoxins at pH 3.9 [43]. Unfortunately for most other flavoproteins this dialysis method is very time consuming leading to an irreversible denaturation of apoprotein. Though it was reported that this method can be applied to liver microsomal NADPH cytochrome P-450 reductase [44] and p-hydroxybenzoate hydroxylase from P.fluorescens [45] for these enzymes only small amounts of

apoprotein can be prepared in this way, still yielding considerable residual activity [46,47]. FMN could be more efficiently removed from NADPH-cytochrome P-450 reductase by an exchange procedure in the presence of apoflavodoxin, in combination with 2',5' ADP Sepharose affinity chromatography [46].

In principle the affinity of flavin and apoprotein can also be decreased by the addition of unfolding reagents like urea or guanidinium hydrochloride. Conditions have to be found allowing the prosthetic group to dissociate from the (partly) unfolded protein without losing the capability of the apoprotein to refold again, either in the absence or presence of flavin. The addition of GuHCl was first introduced in 1969 by Brady and Beychok [48] for the preparation of pig heart apo lipoamide dehydrogenase. With this enzyme the yield of reconstitutable protein is low [49]. With thioredoxine reductase from E. coli however, the low residual activity obtained has allowed stereochemical studies on 8-hydroxy-5-deaza-FAD reconstituted enzyme [51]. The extra addition of urea has proven to be useful in the preparation of apo p-hydroxybenzoate hydroxylase from P.fluorescens [45,47] and apo salicylate hydroxylase from P.cepacia [50].

It should be mentioned here that for all procedures described above optimal conditions can be found by changing the conditions systematically. For more experimental details and some specific procedures the reader is referred to the review of Husain and Massey [30]. An overview of general apoprotein preparation procedures is given in Table 2:

Table 2. Conventional Apo Flavoprotein Preparation Procedures.

Enzyme	Method[a]	Residual specific activity	Yield	Reconstitutable specific activity	Scale[b]	Ref.
		%	%	%		
glucose oxidase	1b	4	n.r.	75-90	M	35
from Aspergillus niger	1b	<0.1	40-50	100	L	36
[EC 1.1.3.4]						
acyl-CoA dehydrogenase	1b	0.7	70-80	90-100	M	37
from pig kidney						
[EC 1.3.99.3]						
D-amino acid oxidase	2a	<1	93-100	100	M	41
from pig kidney						
[EC 1.4.3.3]						
D-aspartate oxidase	1b	<1	24 ±3	79-99	S	59
from beef kidney						
[EC 1.4.3.1]						
microsomal	1b	1-2	75-90	100	L	32
cytochrome-b5						32a
reductase						
[EC 1.6.2.2]						
NADPH-cyt P-450 reductase	2b	10	70	85-95	L	44
from liver microsomes	2a	2	75	85-95	M	46
[EC 1.6.2.4]						
glutathione reductase	1b	8	n.r.	40	S	34
from human erythrocytes	1a	n.r.	n.r.	n.r.	n.r.	51
[EC 1.6.4.2]						
old yellow enzyme	2a	<5	90-100	90-100	L	42
from brewers yeast						55
[EC 1.6.99.1]						
lipoamide dehydrogenase	1b	4-8	90	80-90	M	33
from pig heart	3b	<1	30-40	90-100	n.r.	49
[EC 1.8.1.4]						
salicylate hydroxylase	1a	<1	n.r.	n.r.	S	52
from P.putida						
from P.cepacia	3a	n.r.	30-50	100	S	50
[EC 1.14.13.1]						

Table 2, continued

Enzyme	Method[a]	Residual specific activity	Yield	Reconstitutable specific activity	Scale[b]	Ref.
		%	%	%		
p-hydroxybenzoate hydroxylase from P. fluorescens [EC 1.14.13.2]	3a	1-3	60-70	60-70	S	45
	1b	<0.2	50-60	n.r.	S	54
melilotate hydroxylase from Pseudomonas sp. [EC 1.14.13.4]	2a	0.5	80-100	80-90	S	53
orcinol hydroxylase from P.putida [EC 1.14.13.6]	1b	15	n.r.	82	L	60
phenol hydroxylase from Trichosporon cutaneum [EC 1.14.13.7]	2a	0.3	50-100	n.r.	M	53
cyclohexanone monooxygenase from Acinetobacter sp. [EC 1.14.13.22]	1b	2	n.r.	71	S	58
flavodoxin from Megasphaera elsdenii	1a	<1	90-100	90-100	L	39
	2a			90-100	M	43
riboflavin-binding protein						
from egg white	1a	<1	80-90	95-100	L	56
from egg yolk	1a	<1	80-90	95-100	L	57

a) (1a) acid treatment; (1b) acid ammonium sulphate ± KBr (± charcoal); (2a) dialysis + KBr (± charcoal); (2b) ultrafiltration + KBr; (3a) dialysis + KBr+ urea (± charcoal); (3b) gel filtration + GuHCl.
b) (S) small scale, < 0.2 umol protein; (M) medium scale, 0.2 - 2 umol protein; (L) large scale, > 2 umol protein; n.r., not reported.

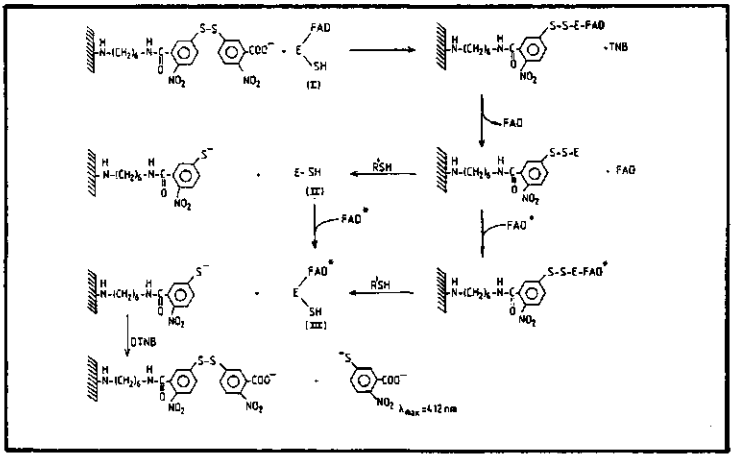
1.4. Large Scale Preparation of Apo Flavoproteins
(Affinity Chromatography)

Until about 1980 the aim of apoprotein preparation has mainly been to reconstitute the apoprotein with artificial flavins in order to study the chemistry of these modified flavins within the restricted region of a protein active site [6]. For these studies it is extremely important to have apoprotein preparations available showing low residual activity. However only relatively small amounts of apoprotein (200-400 nmol) are sufficient in order to allow mechanistic studies by stopped flow technique.

Around 1980 developments in NMR spectroscopy made it possible starting to study biological systems by ^{13}C and ^{15}N enrichment of ligands, nucleotides or cofactors. In the case of flavoproteins this technique allows the observation of π-electron density at atoms in the enriched flavin ring thereby yielding information about conformational structure, mobility, hydrogen bonding and charge of protein-bound flavin in different redox states either in the absence or presence of ligands [22].

Because for this type of studies large amounts (5-10 umol) of well defined preparations are required it was desirable to reconsider the current methods of apoprotein preparation. In **Chapters 2** and **3** of this thesis alternative ways of apoprotein preparation are presented based on affinity chromatography principles. The main advantages of affinity chromatography over conventional methods would be 1) production of large amounts of apoprotein by efficient elution of flavin 2) solvents may be changed very quickly under controlled conditions and 3) possible stabilization of the apoprotein structure by immobilization. Both covalent affinity chromatography and hydrophobic chromatography meet the requirements for resolution of flavin in the presence of high concentrations of salts. In the (exceptional) case of riboflavin-binding protein from both egg white [56] and egg yolk [57] it was possible to remove the neutral flavin prosthetic group by ion-exchange chromatography at pH 4 on SE-Sephadex A-50 at relatively low ionic strength. The stable apoprotein could be recovered by NaCl gradient elution.

The principle of the covalent affinity chromatography procedure is outlined in Scheme 1:



Scheme 1. Covalent affinity chromatography of (apo)flavoproteins.

The flavoprotein is bound to the activated thiolated matrix by thiol-disulfide exchange, with the formation of a mixed disulfide. After elution of flavin, the apoprotein is eluted by reduction of the disulfide bond. As an alternative the apoprotein may be reconstituted on the column with either artifical or isotopically enriched flavin. This kind of chromatography is limited to proteins posessing accessible sulfhydryl groups. Covalent chromatography has been successively applied to FAD-containing p-hydroxybenzoate hydroxylase from P.fluorescens (**Chapter 2**).

A more general application might be expected from hydrophobic interaction chromatography. In principle flavoproteins which are tightly bound on hydrophobic matrices at neutral pH can be tested for flavin dissociation behaviour by stepwise changing the composition of the elution buffer. This concept has been succesively applied to both FAD-containing disulfide oxidoreductases and FAD-containing fatty acid acyl-CoA dehydrogenases (**Chapter 3**).

Only for some apo flavoproteins the reconstitution process has been studied in detail [50]. For small monomeric stable apoproteins like flavodoxin [40] and riboflavin-binding protein [56] the association process of apoprotein and flavin prosthetic group can be described by unique well-defined second-order rate constants. For (multimeric) FAD-containing flavoproteins the reconstitution process may be quite complex. In the case of the monomeric apoproteins of glucose oxidase from A.niger [35], lipoamide dehydrogenase from pig heart [33,62] and D-amino acid oxidase from pig kidney [41,62-64] rapid reversible binding of flavin yielding (partially) inactive enzyme was followed by secondary slow conformational refinement with concomitant appearance of (full) catalytic activity and regaining of the dimeric structures. In the case of the dimeric apoprotein of salicylate hydroxylase from P.cepacia it was shown that rapid reappearance of catalytic activity was followed by slow conformational changes in the binding pocket of the adenosine part of FAD [50]. The question arises if these complex kinetics of holoprotein reconstitution are of physiological relevance or can be (partly) ascribed to the method of apoprotein preparation leading to different states of partially (ir)reversible unfolded protein structures.

With the exception of butyryl-CoA dehydrogenase from M.elsdenii the amino acid sequences of all apo flavoproteins prepared in **Chapters 2** and **3** are known. Moreover for p-hydroxybenzoate hydroxylase from P.fluorescens, glutathione reductase from human erythrocytes and lipoamide dehydrogenase from A.vinelandii crystal structures at high resolution are now available [65-67]. These studies nicely demonstrate that in all three enzyme structures FAD is bound in an extended conformation and held by many specific polar and hydrophobic interactions. The role of α-helixes in FAD binding and the presence of a $\beta\alpha\beta$ nucleotide binding fold in both p-hydroxybenzoate hydroxylase and glutathione reductase has been pointed out by Wierenga et. al [68,69]. In contrast to all other parts of FAD the adenine group in p-hydroxybenzoate hydroxylase is not involved in specific hydrogen bonding. but bound in a hydrophobic pocket [65]. In both p-hydroxybenzoate hydroxylase and glutathione reductase bound water molecules are involved in binding of the pyrophosphate moiety [65,66]. The flavin ring in glutathione reductase is in contact with the other subunit via hydrogen bonding of the N3 atom [66].

From these data it will be clear that apoproteins have to be prepared under well defined controlled conditions in order to achieve optimal catalysis of reconstituted enzymes. Therefore in **Chapters 2** and **3** the reconstitution process and biophysical properties of apo and reconstituted enzymes are described in some detail.

1.5. FAD-Containing Disulfide Oxidoreductases

The widespread pyridine nucleotide dependent FAD-containing disulfide oxidoreductases are one of the most extensively studied classes of flavoproteins [11].

All these enzymes are dimers built up by identical subunits with an M_r of about 50 kDa each containing both FAD and a redox-active disulfide bridge essential for catalysis.

In **Chapter 3** of this thesis the large scale preparation of the apoproteins of glutathione reductase from human erythrocytes, lipoamide dehydrogenase from Azotobacter vinelandii and mercuric reductase from Pseudomonas aeruginosa by hydrophobic interaction chromatography is described. Therefore a short outline of the properties of these enzymes will be given below.

Glutathione reductase [EC 1.6.2.4] catalyzes the NADPH-dependent reduction of glutathione disulfide [11]:

$$GSSG + NADPH + H^+ \rightleftarrows 2\ GSH + NADP^+$$

Glutathione is the most abundant low-molecular mass intracellular thiol compound found in aerobic biological species. Its reduced form is essential in keeping other thiol groups reduced [70].

(Dihydro)lipoamide dehydrogenase [EC 1.8.1.4] catalyzes the NAD^+ dependent oxidation of dihydrolipoyl groups which in vivo are covalently attached to the lipoate acyltransferase component of both the pyruvate and 2-oxoglutarate dehydrogenase multienzyme complexes [71]:

$$E\text{-}lip(SH)2 + NAD^+ \rightleftarrows E\text{-}lip(S)2 + NADH + H^+$$

In vitro free lipoamide or lipoic acid can act as substrates with different efficiency in catalytic turnover [11].

NADPH-dependent mercuric reductase [EC 1.16.1.1] plays an important role in the mainly plasmid encoded detoxification systems (mer genes) of mercury resistant bacteria by reducing highly toxic Hg^{2+} ions to metallic mercury which is volatile and can be excreted from the organism [72-74]:

$$Hg^{2+} + NADPH \rightleftarrows Hg^0 + NADP^+ + H^+$$

In vitro Hg^{2+} must be liganded by a thiol compound in order to yield efficient catalytic turnover [72].

The three enzymes as isolated from different organisms show strong homology in amino acid sequences around the redox-active disulfide bridge, the $NAD(P)^+$ binding site and near the end of the N-terminal region where the binding fold of the ADP part of FAD is situated [75-79]. In mercuric reductase an extra addition of about 85 residues at the N-terminal part can be removed by chymotrypsin treatment without loss of catalytic activity [79].

The genes encoding the bacterial enzymes have been cloned and their nucleotide sequences determined [80-86]. Recently also the amino acid sequences of human and yeast lipoamide dehydrogenase have been elucidated by cDNA methods [87-89]. These data now allow studies on mutant enzymes by protein engineering [90,91].

The high amino acid sequence homology between the enzymes suggest that the overall three-dimensional structures of glutathione reductase, lipoamide dehydrogenase and mercuric reductase have much features in common [92]. This has been confirmed by crystal structural analyses of glutathione reductase from human erythrocytes [66,93,94] and lipoamide dehydrogenase from both A.vinelandii [67] and yeast [95].

Until now most information comes from the crystal structure of glu-

tathione reductase. For the oxidized enzyme an 0.154 nm resolution map is available [66]. Each subunit can be divided into four structural domains: the FAD and NADPH binding sites, a central and an interface domain. The active site is constituted from both subunits and the substrate binds in a cleft between the two subunits. The two subunits are also held together by an intermolecular disulfide bridge, this in contrast to the non-covalently bound subunits of A.vinelandii lipoamide dehydrogenase [85]. In both enzymes FAD is rigidly bound in an extended conformation [66,67].

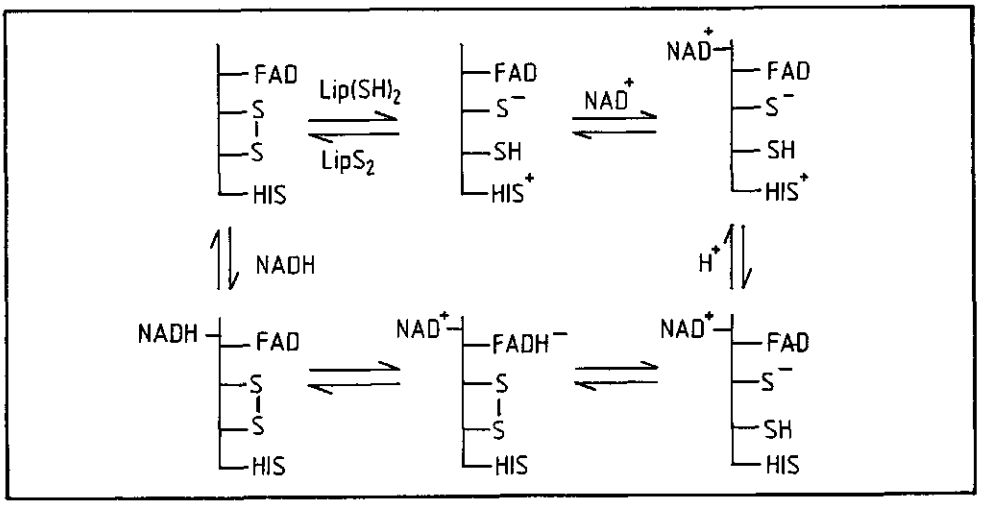
The reaction mechanisms of the different enzymes have been extensively studied by both conventional and (rapid scan) stopped flow absorption spectroscopy techniques [96-112]. During catalysis NAD(P)H binds to the enzyme thereby transferring electrons to FAD which in turn passes reducing equivalents onto the redox-active disulfide bridge, or the reaction can proceed in the other direction.

The spectral behaviour of protein-bound FAD upon reduction with either substrate, coenzyme or artificial reducing agents has been investigated in detail [11,105,111]. Anaerobic reduction of both lipoamide dehydrogenase and glutathione reductase with substrate yields red coloured two-electron disulfide reduced enzyme due to 'charge-transfer' between a protein-thiol anion as a donor and oxidized flavin as acceptor [11]. As contrasted with oxidized and fully reduced enzyme the visible absorption spectrum of two-electron reduced enzyme is strongly pH dependent and differs for the enzymes isolated from different sources [11,111]. Chemical modification studies strongly have contributed to the assignment of the different essential amino acid residues involved in "charge-transfer" complex formation [79,101,105]. It has been proposed that the pH-dependent stability of the 'charge-transfer' complex is influenced by a combination of different effects: (1) stabilization of the thiolate by a pKa shift of an active site histidine upon reduction, (2) stabilization of the thiolate by the positive dipole of an α-helix pointing towards both cysteine residues and (3) possible cooperative effects between the two subunits involved in substrate binding [111]. In this context it is important to note that in mercuric reductase the active site histidine is absent and replaced by a tyrosine residue [79]. An additional cysteine-pair at the C-terminal part of this enzyme [81] has been shown to be involved in Hg^{2+} ligandation in order to prevent too tight binding of the substrate initially and/or during catalytic reduction [90].

The pH-dependent visible absorption spectrum of NAD(P)H reduced enzymes is complex due to the different stability of possible 'charge transfer' intermediates between either thiolate and flavin or flavin and $NAD(P)^+$ [11,110-112]. A simplified reaction sequence for lipoamide dehydrogenase is depicted in Scheme 2.

Although the active site residues in lipoamide dehydrogenase from A.vinelandii are strongly conserved with respect to the enzymes from E.coli and pig heart the overall amino acid sequence homology is relatively low [85]. It has already been shown by Veeger et al. [98] that the visible absorption spectrum of NADH reduced enzyme from A.vinelandii is different as compared to related enzymes. The availability of large amounts of cloned lipoamide dehydrogenase from A.vinelandii [85] and the highly reproducible method of apoprotein preparation as described in **Chapter 3** should now allow characterization of the redox state of protein-bound FAD in the different enzyme NAD(H) complexes by ^{13}C and ^{15}N NMR technique [22].

It should be noted here that the apoprotein procedure described in **Chapter 3** can also be applied to lipoamide dehydrogenase from P.fluorescens (unpublished results). This enzyme, as recently purified from a production clone in E.coli, shows strong homology in amino acid sequence with the A.vinelandii enzyme [86].



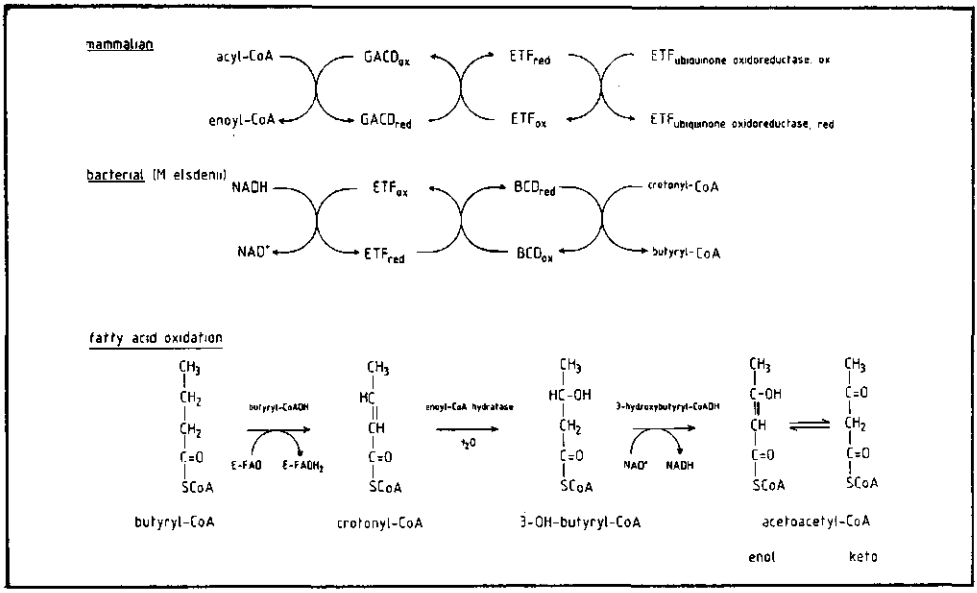
Scheme 2. Reaction cycle of lipoamide dehydrogenase

1.6. Butyryl-CoA Dehydrogenase

During β-oxidation in mammalian mitochondrial systems different soluble FAD-containing acyl-CoA dehydrogenases are involved in oxidizing saturated fatty acid acyl-CoA substrates of different chain-length to their corresponding trans-enoyl-CoA analogues [113]. The resulting 2,3-unsaturated products remain very tightly bound to the reduced enzymes preventing reoxidation by molecular oxygen [114]. The reduced enzyme-product complexes are specifically reoxidized in two one-electron steps by ETF (electron transferring flavoprotein) in an ordered mechanism in which enoyl-CoA product dissociates from the oxidized enzyme [115-117]. β-oxidation then proceeds by the successive action of enoyl-CoA hydratase, L-3-hydroxy-acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase to yield acetyl-CoA and a shortened acyl-CoA, ready for the next cycle. ETF in turn, transfers its electrons to the membrane-bound iron-sulfur flavoprotein, ETF ubiquinone oxidoreductase which then reduces Coenzyme Q, thus delivering electrons to the respiratory chain [114,118].

In the obligate anaerobic bacterium Megasphaera elsdenii butyryl-CoA dehydrogenase (EC 1.3.99.2) is the only occurring acyl-CoA dehydrogenase catalyzing the reverse reaction in vivo [119-121]. Here the enzyme functions in funneling excess of reducing equivalents into the production of saturated short-chain fatty acids which are excreted from the organism. Again the enzyme is specific for ETF which in this system acts as electron donor receiving reducing equivalents from NADH [114,122] (Scheme 3):

All acyl-CoA dehydrogenases purified to homogeneity [61,113,121,123-130c] are tetrameric enzymes built up by identical subunits of about 40 kDa, each containing one equivalent of FAD. Although the mammalian short-, medium- and long-chain enzymes have remarkably similar isoelectric points and amino acid compositions, they are immunologically distinct [130c].



Scheme 3. Function of FAD-containing acyl-CoA dehydrogenase.

Recently the gene sequences of both the human and rat liver medium-chain acyl-CoA dehydrogenases have been reported [130d, 130e]. Comparison of the amino acid sequence predicted from the human medium-chain enzyme cDNA with the partial amino acid sequence, as derived from tryptic peptide characterization of the porcine liver medium-chain enzyme, revealed 88% of interspecies sequence identity [130d]. From a comparable procedure for the rat liver medium-chain enzyme it was revealed that the obtained cDNA clone encodes a precursor protein of 421 amino acids including a 25-amino acid leader peptide [130e]. The human and rat liver mature proteins show 86% amino acid sequence homology. Comparison of the medium-chain acyl-CoA dehydrogenase sequence to other flavoproteins and enzymes which act on coenzyme A ester substrates did not lead to unambiguous identification of a possible FAD-binding site nor a coenzyme A binding domain [130e].

The reaction mechanisms of both mammalian and bacterial enzymes have been studied in considerable detail by using substrate analogues [124,130-134], chemical modification reagents [130f, 135-139] as well as by isotopic and kinetic methods [117,130g, 140-143]. The importance of tightly bound substrates/products in the regulation of the reduction potential of the different enzymes has been shown by electrochemical studies [129,144,145].

Both medium-chain acyl-CoA dehydrogenase from pig kidney [128] and butyryl-CoA dehydrogenase from M.elsdenii [146] show intrinsic crotonyl-CoA hydratase activity. For the mammalian enzyme FAD was found to be essential for the hydratation reaction which probably takes place at the substrate dehydrogenation site [128]. The very slow hydratation turnover however suggests that the reaction is of no physiological significance.

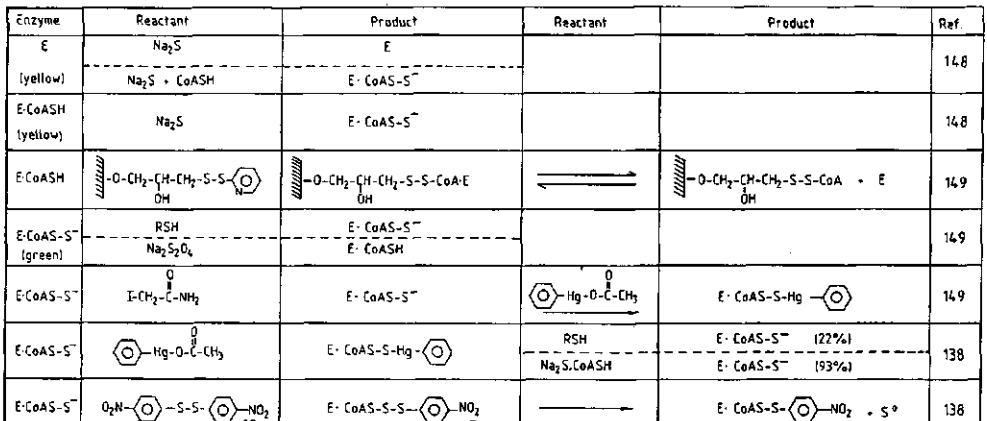
In contrast to most mammalian enzymes butyryl-CoA dehydrogenase from M.elsdenii shows considerable oxidase activity [121]. It has been argued that the different oxygen reactivity might reflect a genetic adaptation of the mammalian enzymes to prevent acces of oxygen to the flavin prosthetic group [142].

The isolation of an active-site peptide from pig kidney medium-chain acyl-CoA dehydrogenase labeled with 2-octynoyl-CoA has been described very recently [139a]. As already suggested for the inactivation of butyryl-CoA dehydrogenase from M.elsdenii by 3-pentynoyl-pantetheine [136] a glutamate residue was found to be the target amino acid residue. The modified Glu-401 may be the base involved in abstraction of the pro-R α-proton during the dehydrogenation of normal substrates [139a].

The recent advances in protein purification- and (DNA) sequencing techniques should now give an impuls to study the structural features of these metabolic important enzymes. In this context it is interesting to note that a preliminary account on the crystal structure of general acyl-CoA dehydrogenase at 0.55 nm resolution was given recently [147].

The most striking property of short-chain acyl-CoA dehydrogenases purified from various sources is their green colour related to an extra absorption band around 700 nm [119-121,127]. It has been shown by Williamson et al. [148] that the green colour of butyryl-CoA dehydrogenase from M.elsdenii is due to tight binding of a CoA persulfide ligand at the substrate binding site with the persulfide moiety situated in the vicinity of the flavin nucleus. It has been speculated by the same authors that the tightly bound CoA persulfide in vivo could serve as a regulatory ligand blocking the dehydrogenase activity unless a sufficient amount of substrate is available.

Upon anaerobic reduction by dithionite the absorption band around 700 nm dissappears yielding yellow liganded enzyme after reoxidation [120,121]. At physiological pH values the reduced CoA ligand can only be removed by covalent affinity chromatography using Thiopropyl-Sepharose 6B [129,149]. This now allows reliable binding studies with different substrate analogues. For instance regreening of yellow unliganded enzyme could be achieved by incubation with a mixture of CoASH and Na_2S [148]. The preparation of the different un(liganded) forms of butyryl-CoA dehydrogenase from M.elsdenii is summarized in Scheme 4:



Scheme 4. Identification of different (un)liganded forms of butyryl-CoA dehydrogenase from M.elsdenii.

As noted above in **Chapter** 1.2 one of the goals of this thesis was to explore the possibilities of preparing large amounts of stable apoproteins in order to perform NMR studies on, with isotopically enriched flavin, reconstituted enzymes. As stable highly pure butyryl-CoA dehydrogenase from M.elsdenii can be obtained in very good yield [61], among the acyl-CoA dehydrogenases this enzyme meets the requirements for this technique the best. However, until now for this enzyme no apoprotein preparation has been reported yet. Unfortunately, the acid ammonium sulfate procedure as described for general acyl-CoA dehydrogenase from pig kidney [37] (Table 2) irreversibly damages the protein-structure of the bacterial enzyme. This is in line with results obtained for the different purified acyl-CoA dehydrogenases from rat liver. With these enzymes FAD is very tightly bound by the short-chain acyl-CoA dehydrogenase but relatively weakly associated with the medium- and long-chain acyl-CoA enzymes [130, 130c, 130f]. As described in **Chapter 3** we have tried to prepare the apoprotein of butyryl-CoA dehydrogenase from M.elsdenii by performing hydrophobic interaction chromatography. Preliminary NMR results on both yellow unliganded and green liganded 2,4^{A}-^{13}C FAD-enriched enzyme have been reported elsewhere [22].

1.7. External Flavoprotein Monooxygenases

The external flavoprotein monooxygenases are members of a class of inducable enzymes catalyzing the insertion of one atom of molecular oxygen into the substrate, using pyridine nucleotide as external electron donor [150]. The overall reaction is shown below:

$$RH + NAD(P)H + H^+ + O_2 \rightleftharpoons ROH + NAD(P)^+ + H_2O$$

Until now about 25 external flavin-dependent monooxygenases have been isolated and (partially) characterized (Table 3). The enzymes listed in Table 3 show considerable differences in both molecular mass and subunit composition. Except for the $\alpha 2\beta 2$ tetrameric 2,4-dichlorophenol hydroxylase from Alcaligenes eutrophus [175] all multimeric external flavin-dependent monooxygenases are built up by identical subunits, each containing non-covalently bound FAD as a prosthetic group. With some enzymes FAD is partly lost during purification. Only in the case of cyclohexanone 1,2-monooxygenase from a cyclohexane degrading Xanthobacter species [201] it was reported that protein-bound FMN replaces FAD as electron acceptor.

Most FAD-dependent monooxygenases have been isolated from aerobic prokaryotes able to degrade aromatic or cyclic ketone compounds [206-209]. These bacteria (especially soil pseudomonads) can grow on the different substrates as sole carbon source thereby inducing the corresponding flavoprotein monooxygenases. Through the initial action of these enzymes the resulting products are readily subject to further catabolism allowing microbes to grow. In this way breakdown products of lignin as well as many pollutants and toxicants (e.g. herbicides) can be metabolized. It should be noted here that non-activated aromatic compounds like for instance benzene or benzoate cannot be hydroxylated by the FAD-dependent monooxygenases. Metabolism of this type of compounds requires the initial action of both flavin and non-heme iron containing enzyme systems showing a more potent oxygenating capacity [209].

As can be seen from Table 3 most FAD-dependent monooxygenases prefer either NADH or NADPH as reductant, though some enzymes can utilize both pyridine nucleotides with different efficiency in catalytic turnover. All FAD-dependent monooxygenases studied sofar show preference for abstraction of the pro-R hydrogen of the C-4 prochiral center of the dihydronicotinamide ring [210,211].

Two other (no iron containing) flavin-dependent monooxygenases have been reported receiving their reducing equivalents in a different way. Firstly, luciferase from bioluminiscent marine bacteria binds external reduced FMN, most probably generated by a FMN-reductase [212,213]. Luciferase is a heterodimer with a single active center that resides primarily on the α-subunit [214]. The enzyme catalyzes the oxygenation of long-chain aldehydes to the corresponding acids and yields a photon of blue-green light as one of the products. Secondly, in the degradation of camphor by Pseudomonas putida three distinct monooxygenase systems are present. Besides Cytochrome P-450 dependent camphor hydroxylase and the recently purified FAD-dependent 2-Oxo-Δ^5-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase (no. 26 in Table 3), another monooxygenase system is present catalyzing the lactonization of 2,5-diketocamphane.
This monooxygenase has recently reported to be a homodimer of 78 kDa, binding one FMN molecule per dimer, and receiving reducing equivalents from a (weakly) associated 36 kDa monomeric NADH oxidase [215].

Table 3. Origin, Electron Donor Selectivity and Molecular Mass of FAD-dependent External Monooxygenases.

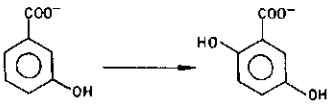
Origin	Reaction Catalyzed: Electron Donor	Enzyme Code: Molecular Mass	Refs.
(1) 4-Hydroxybenzoate-3-hydroxylase [EC 1.14.13.2]			
Pseudomonas putida	NADPH>>NADH	88 kDa (dimer)	151,152
Pseudomonas desmolytica	NADPH>>NADH	88 kDa (dimer)	153,154
Pseudomonas fluorescens	NADPH>>NADH	88 kDa (dimer)	155-158
Corynebacterium cyclohexanicum	NADH, NADPH	47 kDa (monomer)	159
(2) 3-Hydroxybenzoate-4-hydroxylase [EC 1.14.13.23]			
Aspergillus niger	NADPH>>NADH	145 kDa (?)	160
Pseudomonas testosteroni	NADPH>>NADH	145 kDa (?)	161
(3) 3-Hydroxybenzoate-6-hydroxylase [EC 1.14.13.24]			
Pseudomonas aeruginosa	NADH>NADPH	65 kDa (?)	162
Pseudomonas cepacia	NADH>NADPH	45 kDa (monomer)	163,164
(4) 4-Hydroxyphenylacetate-3-hydroxylase [EC 1.14.13.4]			
Pseudomonas ovalis	NADH	260 kDa (tetramer)	165
Pseudomonas putida	NADH		166

Table 3 (continued)

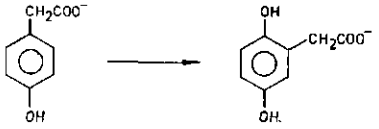
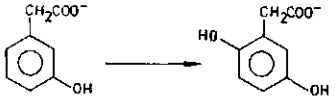
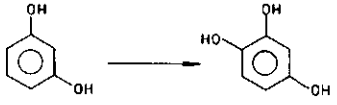
Origin	Reaction Catalyzed / Electron Donor	Enzyme Code / Molecular Mass	Refs.
(5) 4-Hydroxyphenylacetate-1-hydroxylase		[EC 1.14.13.18]	
			
Pseudomonas acidovorans	NADH>NADPH	?	167
(6) 3-Hydroxyphenylacetate-6-hydroxylase		[EC 1.14.13.x][a]	
			
Flavobacterium sp.	NADPH, NADH	250 kDa (tetramer)	168,169
(7) Phenol hydroxylase		[EC 1.14.13.7]	
			
Trichosporon cutaneum	NADPH	152 kDa (dimer)	170
Candida tropicalis	NADPH	?	171
(8) Orcinol hydroxylase		[EC 1.14.13.6]	
			
Pseudomonas putida	NADH>NADPH	68 kDa (monomer)	60,172
(9) Resorcinol hydroxylase		[EC 1.14.13.x]	
			
Pseudomonas putida	NADH	68 kDa (monomer)	172

Table 3 (continued)

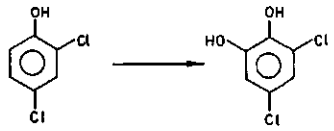
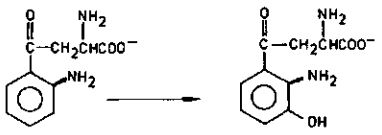
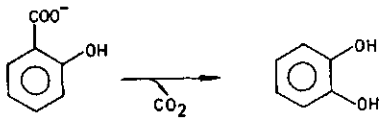
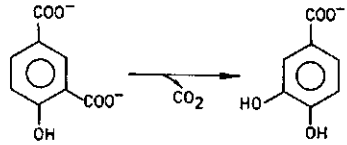
Origin	Reaction Catalyzed: Electron Donor	Enzyme Code: Molecular Mass	Refs.
(10) 2,4-Dichlorophenol-6-hydroxylase		[EC 1.14.13.20]	
			
Acinetobacter sp.	NADPH>NADH	250 kDa (tetramer)	173,174
Alcaligenes eutrophus	NADPH>NADH	45 kDa, 67 kDa (tetramer)	175
(11) Melilotate hydroxylase		[EC 1.14.13.4]	
			
Athrobacter sp.	NADH>NADPH	?	176
Pseudomonas sp.	NADH	260 kDa (tetramer)	177,178
(12) l-Kynurenine-3-hydroxylase		[EC 1.14.13.9]	
			
liver mitochondria	NADPH, NADH	150 kDa (monomer)	179,180
(13) Salicylate hydroxylase (decarboxylating)		[EC 1.14.13.1]	
			
Pseudomonas putida	NADH>>NADPH	57 kDa (monomer)	181,182
Pseudomonas sp.	NADH>NADPH	90 kDa (dimer)	183,184
Pseudomonas cepacia	NADH>NADPH	90 kDa (dimer)	185

Table 3 (continued)

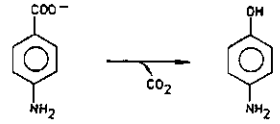
Reaction Catalyzed		Enzyme Code		
Origin	Electron Donor	Molecular Mass	Refs.

(14) 4-Hydroxyisophtalate hydroxylase (decarboxylating) [EC 1.14.13.x]



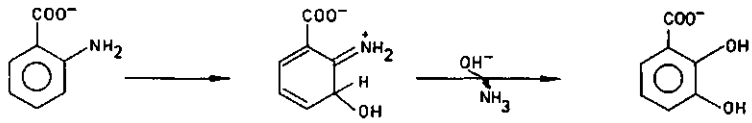
Pseudomonas putida	NADPH, NADH	115 kDa (dimer)	186

(15) 4-Aminobenzoate hydroxylase (decarboxylating) [EC 1.14.13.x]



Agaricus bisporus	NADH>NADPH	49 kDa (monomer)	187

(16) Anthranilate hydroxylase (deaminating) [EC 1.14.12.2]

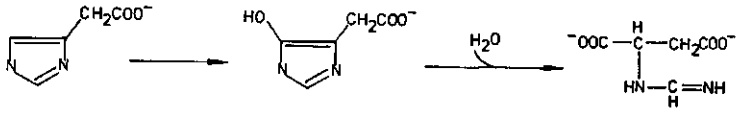


Trichosporon cutaneum	NADPH	100 kDa (dimer)	188

(17) 2,6-Dihydroxypyridine-3-hydroxylase [EC 1.14.13.10]

Athrobacter oxidans	NADH>NADPH	89 kDa (?)	189

(18) Imidazolacetate hydroxylase [EC 1.14.13.5]



Pseudomonas sp.	NADH>>NADPH	90 kDa (?)	190

Table 3 (continued)

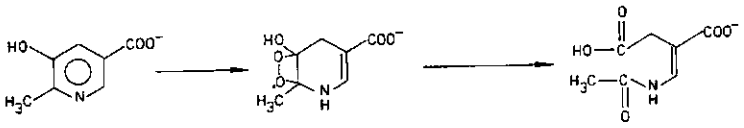
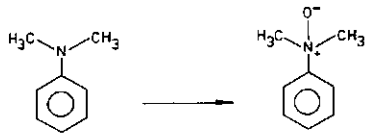
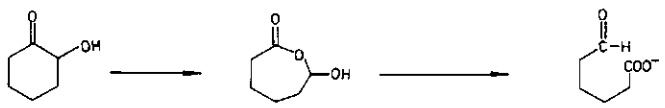
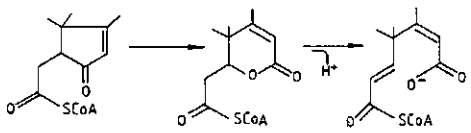
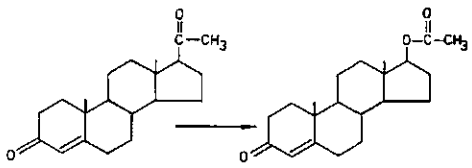
Origin	Reaction Catalyzed: Electron Donor	Enzyme Code: Molecular Mass	Refs.
(19) 2-Methyl-3-hydroxypyridine-5-carboxylic acid oxygenase		[EC 1.14.12.4]	
			
Pseudomonas sp.	NADH	168 kDa (tetramer)	191,192
(20) Microsomal flavin containing monooxygenase		[EC 1.14.13.8]	
			
liver, lung and kidney microsomes from mouse, rat, rabbit and pig	NADPH	56-59 kDa (monomer)	193-197
Trypanosomi cruzi		52 kDa	195
(21) 2-Hydroxycyclohexanone-monooxygenase		[EC 1.14.12.6]	
			
Acinetobacter sp.	NADPH	57 kDa (monomer)	198
(22) Cyclohexanone monooxygenase (1,2-lactonizing)		[EC 1.14.13.22]	
			
Acinetobacter sp.	NADPH	59 kDa (monomer)	58,199
Nocardia globerula	NADPH	53 kDa (monomer)	199,200
Xanthobacter sp. (FMN)	NADPH	50 kDa (monomer)	201

Table 3 (continued)

Origin	Reaction Catalyzed: Electron Donor	Enzyme Code: Molecular Mass	Refs.
(23) Cyclopentanone monooxygenase		[EC 1.14.13.x]	
			
Pseudomonas sp.	NADPH	200 kDa (tetramer)	202
(24) 2-Tridecanone monooxygenase		[EC 1.14.13.x]	
			
Pseudomonas cepacia	NADPH	110 kDa (dimer)	203
(25) 2-Oxo-Δ^3-4,5,5-trimethylcyclopentenyl-acetyl-CoA monooxygenase		[EC 1.14.13.x]	
			
Pseudomonas putida	NADPH	112 kDa (dimer)	204
(26) Progesterone monooxygenase		[EC 1.14.99.4]	
			
Cylindrocarpon radicicola	NADPH	112 kDa (dimer)	205

a) X indicates that this enzyme has not yet been numbered according to the International Enzyme Nomenclature.

The enzymatic monooxygenation reaction of an organic compound under physiological conditions requires activation of oxygen. For the external FAD-dependent monooxygenases it has now generally been accepted that this activation is achieved by production of a reduced flavin-(C4A) hydroperoxide intermediate, capable to attack the various substrates [178,194,216,217]. Depending on the type of substrate oxygenated the enzymes listed in Table 3 can be divided into two subclasses catalyzing the following reactions:

1) Hydroxylations of substituted aromatic compounds (no. 1-19)

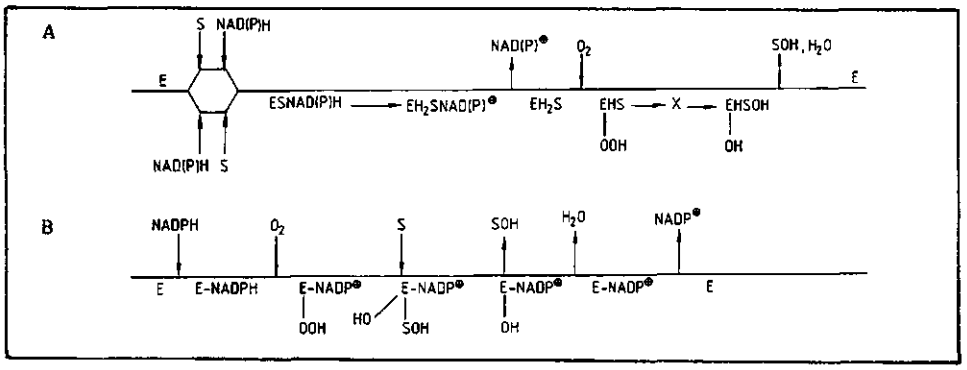
2) Peroxide oxygenations of either nucleophilic or electrophilic substrates (no. 20-26)

In 1) substituted aromatic substrates are hydroxylated at positions ortho or para to the already present hydroxyl or amino group. The only exception to this rule is p-hydroxyphenylacetate-1-hydroxylase from Pseudomonas acidovorans [167] where the oxygen atom is inserted at C-1 of the substrate with simultaneous displacement of the side chain to C-2. Moreover this is the only FAD-containing aromatic hydroxylase reported requiring Mg^{2+} ions to get maximal turnover. The eukaryotic phenol hydroxylase is unusual among this group of enzymes by showing a quite broad substrate specificity [170].

In 2) the microsomal enzymes show a very broad substrate specificity in catalyzing the oxidation of aromatic as well as aliphatic nucleophilic sulfur and nitrogen-containing compounds [195,197]. All cyclo-alkanone monooxygenases also show a broad substrate specificity yielding either esters or lactones in so-called biological Bayer-Villiger reactions [199]. Besides the natural substrates cyclohexanone monooxygenase from Acinetobacter NCIB 9871 has been shown to catalyze the oxidation of hetero-atom substrates like for instance thia-cyclohexane as well as the conversion of boronic acid derivatives to the corresponding alcohols. These reactions demonstrate the ambivalent character of the flavinhydroperoxide capable of catalyzing the conversion of both electronrich and electrondeficient substrates [199].

Although 2-Methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (no.19 in Table 3) belongs formally to the class of dioxygenating enzymes, the mechanism of oxygenation seems to be comparable to the external FAD-dependent monooxygenases [192,218].

The two subclasses mentioned above show remarkable differences in their reaction sequences (Scheme 5):



Scheme 5. Reaction sequences of external flavoprotein monooxygenases.

With the phenolic hydroxylases (Scheme 6A) reduction of protein-bound FAD by the pyridine nucleotide is strongly stimulated by bound substrate. Except for melilotate hydroxylase [219] the enzymes studied sofar [157,220,221] show random order mechanisms in their reductive half-reactions. The binding of molecular oxygen is preceded by the release of $NAD(P)^+$. In the absence of substrate molecular oxygen is only slowly reduced by the NAD(P)H oxidase activity of the enzyme, yielding H_2O_2. After $NAD(P)^+$ release reduced flavin reacts with molecular oxygen forming the labile flavin-(C 4A) hydroperoxide intermediate. Then attack of the substrate takes place in a complicated process, finally yielding product and H_2O (see **Chapter 1.8**).

For the aromatic hydroxylases the lifetime of the flavinhydroperoxide can be increased in the presence of effectors and monovalent anions [150]. However, until now, the instability of this intermediate has not allowed a detailed structural analysis. In the related bacterial luciferase the structure of the stabilized flavin-(C 4A) hydroperoxide has been confirmed by 13C-NMR experiments [222].

With the microsomal FAD-containing monooxygenase [194,223] and cyclohexanone monooxygenase [217] the flavin-(C4A)hydroperoxide intermediate is more stable when generated in the absence of the substrate (Scheme 5B). Moreover with these enzymes $NADP^+$ remains bound during the oxygenation reaction. In the microsomal pig liver enzyme the stability of the flavin-hydroperoxide could be considerably increased by the addition of phosphatidylserine [224]. In contrast to the aromatic hydroxylases the reactivity of these enzymes, though in a less efficient way, can be mimicked by a variety of peroxides including 4a-hydroperoxide-N(5)-ethyl-3-methyl-lumiflavin as a model flavin compound [225,226].

The lability of the flavinhydroperoxide in the aromatic hydroxylases as compared to the enzymes of the other subclass might be indicative for the fact that in the aromatic hydroxylases the flavinhydroperoxide is not the actual oxygenating species. For p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens an additional intermediate has been detected by stopped flow technique [216] which will be discussed in **Chapter 1.8**.

It is now generally accepted that with all FAD-dependent monooxygenases studied product formation results in the appearance of a flavin-(C4A)-hydroxide intermediate. This flavin-adduct finally is dehydrated yielding oxidized enzyme and H_2O (Scheme 5).

Until now little is known about the amino acid sequence homology between the different FAD-dependent monooxygenases. For the FAD-dependent aromatic hydroxylases only the amino acid sequence of p-hydroxybenzoate hydroxylase from P.fluorescens has been elucidated by chemical methods [12,228]. Moreover this enzyme is the only FAD-dependent monooxygenase for which a high resolution three-dimensional model is available [14,28]. More recently the gene encoding for cyclohexanone monooxygenase from Acinetobacter NCIB 9871 has been cloned and its sequence determined [229]. From the derived amino acid sequence no strong homology between these two enzymes can be observed. It is obvious that at least more amino acid sequences are needed in order to relate general secondary structural features to properties like binding of FAD, substrates, effectors and coenzymes. In this context it would be also interesting to know more about aspects like gene regulation and molecular mechanisms of gene repression allowing the differential induction of FAD-dependent monooxygenases by one micro-organism species [163].

1.8 p-Hydroxybenzoate Hydroxylase

1.8.1 Introduction

p-Hydroxybenzoate hydroxylase from Pseudomonas fluorescens (no. 1 in Table 3, Chapter 1.7) is one of the most extensively studied flavoproteins. The reaction mechanism of the enzyme has been studied in considerable detail by Massey and coworkers (Ann Arbor, USA), using rapid kinetics techniques. Dutch multidisciplinar research has revealed the complete amino acid sequence, using chemical methods (group Beintema, Groningen), a three-dimensional model at high resolution, using X-ray diffraction techniques (group Drenth/Hol, Groningen) and much information about the dynamic structure of both the enzyme and its prosthetic group by different biochemical and biophysical techniques (group Müller, Wageningen).

1.8.2 Enzyme purification

In 1966 p-hydroxybenzoate hydroxylase was first isolated and crystallized from Pseudomonas putida [151]. In 1969 the crystallization of the enzyme from Pseudomonas desmolytica was reported [153]. The enzyme from Pseudomonas fluorescens was first isolated in 1972 by conventional chromatographic techniques [155]. The enzymes from P.putida, P.desmolytica and P.fluorescens are almost strictly dependent on NADPH as external electron donor.
More recently it was reported that the purified enzyme from Corynebacterium cyclohexanicum can both use NADH and NADPH in a relative efficient way [159]. Although the enzymes from the various pseudomonas species show similar characteristics, the enzyme from P.fluorescens has been reported to be the most stable one [155]. Therefore this enzyme was chosen to study in more detail by a Dutch multidisciplinar approach [230].

Upon purification the yield of enzyme was considerably improved by introducing Cibacronblue-Sephadex G-100 affinity chromatography [156]. From SDS-PAGE experiments a mininum molecular mass of 44 kDa was estimated [156]. The enzyme mainly exists as a dimer in solution, each subunit containing one equivalent of FAD [156]. Under certain conditions the enzyme tends to aggregate, yielding different higher-order quaternary structures [155,156]. In the crystalline state the enzyme is also present as a dimer [14].

From preperative iso-electric focusing experiments the iso-electric point of the main fraction (about 85%) of enzyme molecules was found to be 5.8 [156]. The minor fraction of enzyme molecules (about 15%) eluted at lower pH values (i.e. 5.7 and 5.4). Performing DTNB-Sepharose covalent affinity chromatography (Chapter 2) the microheterogeneity in charge could be related to the rather easily air-oxidation of the only accessible sulfhydryl group [47]. The reduced state of this sulfhydryl group is essential in order to obtain large amounts of apoenzyme showing negligible residual activity [47]. Even better resolution of different states of oxidized enzyme molecules could be achieved by performing anion-exchange chromatography either in the absence [231] or presence of dithiothreitol [232,233].

The addition of reducing agents was also found to prevent the formation of higher order quaternary structures [231-234]. The presence of these higher order quaternary structures inhibits enzyme crystallization [27] and limits the enzyme solubility [235].

In Chapter 4 of this thesis the observed microheterogeneity of the enzyme has been studied in detail using various (bio)chemical techniques. A kinetic method has been developed to study the stability of dimeric enzyme

molecules by FPLC analysis. Also some recommendations are given in order to obtain large amounts of well-defined enzyme preparations suitable for both NMR [235] and crystallization experiments [27,28].

1.8.3 Substrate specificity

All bacterial FAD-dependent aromatic hydroxylases studied sofar show a relatively narrow substrate specificity. However many substrate analogs can act as non-hydroxylatable effectors, highly stimulating the rate of reduction of protein-bound FAD. As a consequence of the uncoupling of the hydroxylating reaction stoichiometric amounts of H_2O_2 are produced after oxygen attack [183]. In the case of p-hydroxybenzoate hydroxylase from P.fluorescens only a few analogs can serve as substrates (Table 4). Except for p-mercaptobenzoate all substrates are hydroxylated at positions ortho to the hydroxyl function. p-Mercaptobenzoate undergoes oxygen attack at the thiol substituent resulting in 4,4'-dithiobisbenzoate as an unusual product [236]. Besides p-hydroxybenzoate the monofluorinated derivatives of p-hydroxybenzoate can be hydroxylated quite efficiently [237]. In the case of the di- and tetrafluoro derivatives two equivalents of NADPH are consumed, most probably due to the non-enzymatic reduction of a quinoid intermediate species [237]. For more details about the dissociation constants of the different substrates/effectors and the rates of NADPH oxidation the references in Table 4 can be helpful.

Table 4. Substrate and effector specificity of p-hydroxybenzoate hydroxylase

Compound	Product	Ref.
4-hydroxybenzoate	3,4-dihydroxybezoate	238
2,4-dihydroxybenzoate	2,3,4-trihydroxybenzoate	238
3,4-dihydroxybenzoate	H_2O_2	238
benzoate	H_2O_2	238
4-aminobenzoate	3-hydroxy-4-aminobenzoate	216
4-fluorobenzoate	H_2O_2	238
4-mercaptobenzoate	4,4'-dithiobisbenzoate	236
2-fluoro-4-hydroxybenzoate	2-fluoro-3,4-dihydroxy-benzoate	237
3-fluoro-4-hydroxybenzoate	5-fluoro-3,4-dihydroxy-benzoate	237
3,5-difluoro-4-hydroxy-benzoate	5-fluoro-3,4-dihydroxy-benzoate	237
2,3,5,6-tetrafluoro-4-hydroxybenzoate	2,5,6-trifluoro-3,4-dihydroxybenzoate	237
6-hydroxynicotinate	H_2O_2	216
5-hydroxypicolinate	H_2O_2	216

1.8.4 Electron Donor Specificity

Protein-bound FAD can be reduced in a two-electron process by NADPH, dithionite or EDTA in the presence of light [155]. Stabilization of the neutral blue flavin semiquinonic state has only been observed in the presence of tetrafluoro-p-hydroxybenzoate [237]. It has been proposed by Müller [7] that in this case the exceptional stability of the neutral semiquinone might be due to hydrogen bond formation between the N(5)H group of flavin and either one of the fluoro atoms at position 2 or 6 of the ligand.

The slow reduction of free enzyme by NADPH is enormously stimulated in the presence of p-hydroxybenzoate, acting both as a substrate and effector [155]. At pH 8, 25 °C a 40.000 fold stimulation in rate of reduction was observed (k_{red} = 1.5 x 10^4 min^{-1}) [155]. At pH 6.6, 4 °C the stimulation is even much higher (150.000 fold) [157]. As the affinity of NADPH for the oxidized enzyme is only slightly affected in the presence of effectors [241,242] these effector molecules must induce substantial conformational changes in the enzyme allowing an rapid positioning of the nicotinamide moiety of NADPH for reduction of the flavin.

Upon addition of effectors, pertubations in absorption, fluorescence and circular dichroism spectra of protein-bound FAD are indicative for these differences in conformation [152,155,157,243].

Upon anaerobic reduction in the presence of p-hydroxybenzoate a transient long-wavelength species has been observed, most probably reflecting a 'charge-transfer' complex between reduced enzyme and bound $NADP^+$ [157]. In the presence of p-hydroxybenzoate the enzyme can also be reduced by NADH, though in a much less efficient way [234].

The binding of NADPH to oxidized enzyme is strongly dependent on both pH and ionic strength [244]. Optimal binding is observed at pH 6.4 and low ionic strength (K_d = 200 µM, I = 45 mM). At pH 8, the optimum pH for catalytical turnover, the binding of NADPH is relatively weak. Comparable results have been obtained for the binding of NADH [244] and for the binding of both reduced pyridine nucleotides to the apoenzyme (**Chapter 2**) [47]. Therefore, especially when performing studies on chemically modified enzyme preparations (**Chapter 6** and **7**), well-defined conditions are required in order to obtain reliable dissociation constants for NAD(P)H binding. The very weak binding of NADPH at high ionic strenght might explain the difficulties encountered in crystallization of enzyme-NAD(P)H complexes [27].

1.8.5 Enzyme inhibition

The enzyme shows mixed type inhibition in the presence of high concentrations of p-hydroxybenzoate or substrate analogs [157,216,238]. Competitive inhibition with respect to p-hydroxybenzoate is observed with all compounds shown in Table 3 and to a lesser degree with some other structurally related compounds [157,216,236-238].

The enzyme is inhibited competitively with respect to NADPH by a range of negatively charged compounds. The binding of most of these compounds is strongly stimulated in the presence of p-hydroxybenzoate or substrate analogs.

The strongest competitive inhibition with respect to NADPH is observed with $Au(CN)_2^-$ or $Ag(CN)_2^-$ [245,246]. These compounds bind in the vicinity of the flavin nucleus as deduced from both crystallization [14,28] and fluorescence quenching studies [245]. The affinity of both free and p-hydroxybenzoate complexed enzyme for the metal-cyanide complexes is constant in the pH range 6-7 and decreases at higher pH values (unpublished results).

Halogen ions (Cl^-,I^-,F^-) are also competitive inhibitors with respect

to NADPH, binding in the vicinity of the flavin nucleus as well [245,247]. The binding of monovalent anions might explain the very weak binding of $NADP^+$ to both oxidized and reduced enzyme. Repulsive forces in the binding pocket of the nicotinamide moiety of NADP+ probably enhance catalytical turnover. In this respect the role of helix H10, pointing with the positive end of its dipole moment towards the active site [28] should also be mentioned here.

Among the pyridine nucleotide analogs tested 2',5'-ADP and 2'-AMP show the strongest inhibition, indicating that the 2' phosphate group of the ribose moiety plays an important role in binding of NADPH [244-246].

1.8.6 The overall reaction sequence

The overall reaction mechanism of the enzyme has been studied in detail at pH 6.6, 4 °C by both steady state kinetics and stopped flow absorption spectrophotometry [157]. As can be seen from Scheme 5A (**Chapter 1.7**) p-hydroxybenzoate and NADPH can bind at random to the free oxidized enzyme yielding the first ternary complex (E-FAD-p-OHB-NADPH). After release of $NADP^+$ the reduced enzyme-p-hydroxybenzoate complex reacts with oxygen yielding the second ternary complex ($E\text{-}FADH_2\text{-}p\text{-}OHB\text{-}O_2$). The intermediates involved in the subsequent oxygen reaction will be discussed below. After product formation the resulting flavin-(C4A)hydroxide finally is dehydrated yielding oxidized enzyme and H_2O. According to the nomenclature of Cleland [248] the enzyme follows a Bi uni uni uni ping pong mechanism [157]. The overall reacton cycle is given below:

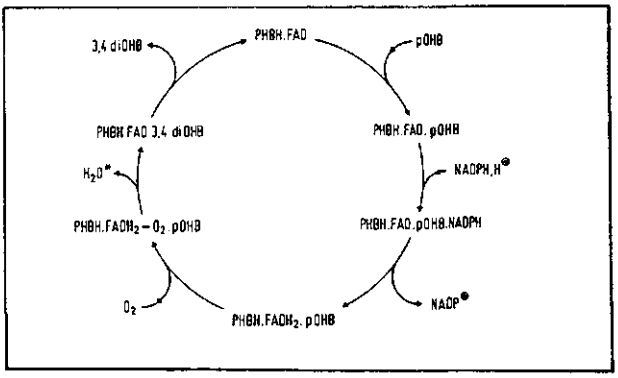


Figure 3. Overall reaction cycle of p-hydroxybenzoate hydroxylase

1.8.7 The oxidative half-reaction

The reaction of reduced flavin and molecular oxygen proceeds via fast one-electron transfer from reduced flavin to oxygen [7]. After radical recombination, formation of the reduced flavin-(C4A)hydroperoxide can then occur as shown with both glucose oxidase and model flavin compounds [249].

The reaction of reduced flavin with oxygen is extremely enhanced in the FAD-dependent monooxygenases yielding stoichiometric amounts of the reduced flavin-(C4A)hydroperoxide intermediate (**Chapter 1.7**). For instance, at pH 6.6, 4° C the second-order rate constant for the formation of this intermediate in p-hydroxybenzoate hydroxylase is about $5x10^5$ $M^{-1}s^{-1}$ [216]. This rate constant is about 4 orders of magnitude higher as compared to the

less specific reaction of free reduced flavin with oxygen [250] and relatively independent of the nature of substrate/effector present [216].

With 2,4-dihydroxybenzoate as a substrate three spectral distinct intermediates can be observed during the oxygen reaction [216]. Intermediate I (E_{max} = 380 nm, ϵ = 8.5 $mM^{-1}cm^{-1}$) and intermediate III (E_{max} = 385 nm, ϵ = 9 $mM^{-1}cm^{-1}$) represent respectively the flavin-(C4A)-hydroperoxide and flavin-(C4A)hydroxide already mentioned above. The structure of the strongly absorbing intermediate II (E_{max} = 400 nm, ϵ = 13 $mM^{-1}cm^{-1}$) is as yet unclear (see below). With both p-hydroxybenzoate and 2,4-dihydroxybenzoate as a substrate the decay of intermediate III is rate limiting in overall catalysis. With p-hydroxybenzoate as a substrate, most probably for kinetic reasons, only intermediates I and III are detectable. In the presence of p-aminobenzoate again three intermediates are observed [216].

The decay of intermediate I is dependent on the reactivity of the different substrates. With tetrafluoro-p-hydroxybenzoate as a substrate the decay of this intermediate is about 100 times slower as compared to the reaction in the presence of p-hydroxybenzoate and becomes rate limiting in overall catalysis [237]. However with p-mercaptobenzoate oxygen transfer is strongly stimulated as a consequence of the highly reactive thiol substituent [236].

The reactivity of the reduced flavinhydroperoxide has also been probed using N5-alkyl-4A-hydroperoxide as a model compound [225,226]. In N- or S-oxidations (cf. liver microsomal monooxygenase, **Chapter 1.7**) the reaction rate of this model compound could be correlated with the pKa value of the corresponding reduced (C4A)-hydroxyflavin. Though the reactivity of the different FAD-dependent monooxygenases (**Chapter 1.7**) cannot simply be explained by these observations this model study shows the importance of the electronic distribution in the flavin ring (see also below).

The pH-dependence of the oxidative half reaction has been studied in detail using 2,4-dihydroxybenzoate as a substrate [251]. For a better separation of the individual reaction steps the experiments were performed at 4 °C in the presence of 100 mM NaN_3. Both the decay of intermediate I and III were found to be base catalyzed whereas the decay of intermediate II was found to be acid catalyzed. Upon raising the pH the spectrum of intermediate II shifts to longer wavelengths. Except for the decay of intermediate III a pKa of about 7.8 could be calculated for the pH-dependent transitions. This pKa was tentatively assigned either to the product or to a tyrosine residue present in the active site [251]. Chemical modification studies of the oxidized free enzyme have indicated the presence of an active site tyrosine residue showing a drastically lowered pKa value [242,252].

Since hydroxylation of the substrate most probably occurs after formation of intermediate II [216] the elucidation of the structural features of this intermediate has received much attention [251]. Until now however no direct experimental evidence has been obtained for the various proposed structures [226,251].

The substrate specificity of both p-hydroxybenzoate hydroxylase [216] and phenol hydroxylase [253] are consistent with an electrophilic aromatic substitution reaction of phenolic compounds. Therefore it seems plausible that the distal peroxide oxygen leaves intermediate I as an oxene species [5,216] yielding a positively charged oxygenated substrate intermediate. From modelling studies using the refined crystal structure at 0.19 nm resolution [28] it was argued that such an intermediate might be stabilized by the carbonyl oxygen atoms of both Pro-293 and Thr-294.

Recently a spectrum closely resembling intermediate II was constructed by combining the spectrum of intermediate I (or III) with the spectrum of the •OH radical adduct of the substrate as generated by pulse radiolysis

[254]. It was suggested that polarization of the peroxide bond in combination with deprotonation of the 4-hydroxylgroup of p-hydroxybenzoate or 2,4-dihydroxybenzoate might favour the formation of both a C(4a)-alkoxyl radical and a substituted hydroxycyclohexadienyl radical (after attack of the substrate by the leaving •OH radical). However, until now it has not been possible to detect any radical formation with these phenolic hydroxylases. The possible ionization of the 4-hydroxyl group of the substrate will be discussed below.

The above kinetic data clearly show the complexity of the hydroxylation reaction. The complex reaction sequence is modulated by a close cooperation of flavin, bound substrate and (active site) amino acid residues. The electronic structure of the flavin in the different redox states and the role of different amino acid residues in catalysis is described below.

1.8.8 Amino acid sequence

The amino acid sequence of the enzyme from Pseudomonas fluorescens has been determined by Beintema and co-workers using chemical methods [12,228,255-257]. The availability of the amino acid sequence has been of utmost importance in both the refinement of the three-dimensional structure [28,258] and the assignment of chemically modified amino acid residues [246,252,257]. For reference the complete amino acid sequence is given below (Scheme 6):

```
  1               5                  10                  15                  20
met-lys-thr-gln-val-ala-ile-ile-gly-ala-gly-pro-ser-gly-leu-leu-leu-gly-gln-leu-
 21              25                  30                  35                  40
leu-his-lys-ala-gly-ile-asp-asn-val-ile-leu-glu-arg-gln-thr-pro-asp-tyr-val-leu-
 41              45                  50                  55                  60
gly-arg-ile-arg-ala-gly-val-leu-glu-gln-gly-met-val-asp-leu-leu-arg-glu-ala-gly-
 61              65                  70                  75                  80
val-asp-arg-arg-met-ala-arg-asp-gly-leu-val-his-glu-gly-val-glu-ile-ala-phe-ala-
 81              85                  90                  95                 100
gly-gln-arg-arg-arg-ile-asp-leu-lys-arg-leu-ser-gly-gly-lys-thr-val-thr-val-tyr-
101             105                 110                 115                 120
gly-gln-thr-glu-val-thr-arg-asp-leu-met-glu-ala-arg-glu-ala-cys-gly-ala-thr-thr-
121             125                 130                 135                 140
val-tyr-gln-ala-ala-glu-val-arg-leu-his-asp-leu-gln-gly-glu-arg-pro-tyr-val-thr-
141             145                 150                 155                 160
phe-glu-arg-asp-gly-glu-arg-leu-arg-leu-asp-cys-asp-tyr-ile-ala-gly-cys-asp-gly-
161             165                 170                 175                 180
phe-his-gly-ile-ser-arg-gln-ser-ile-pro-ala-glu-arg-leu-lys-val-phe-glu-arg-val-
181             185                 190                 195                 200
tyr-pro-phe-gly-trp-leu-gly-leu-leu-ala-asp-thr-pro-pro-val-ser-his-glu-leu-ile-
201             205                 210                 215                 220
tyr-ala-asn-his-pro-arg-gly-phe-ala-leu-cys-ser-gln-arg-ser-ala-thr-arg-ser-arg-
221             225                 230                 235                 240
tyr-tyr-val-gln-val-pro-leu-thr-glu-lys-val-glu-asp-trp-ser-asp-glu-arg-phe-trp-
241             245                 250                 255                 260
thr-glu-leu-lys-ala-arg-leu-pro-ala-glu-val-ala-gly-lys-leu-val-thr-gly-pro-ser-
261             265                 270                 275                 280
leu-glu-lys-ser-ile-ala-pro-leu-arg-ser-phe-val-val-glu-pro-met-gln-his-gly-arg-
281             285                 290                 295                 300
leu-phe-leu-ala-gly-asp-ala-ala-his-ile-val-pro-pro-thr-gly-ala-lys-gly-leu-asn-
301             305                 310                 315                 320
leu-ala-ala-ser-asp-val-ser-thr-leu-tyr-arg-leu-leu-leu-lys-ala-tyr-arg-glu-gly-
321             325                 330                 335                 340
arg-gly-glu-leu-leu-glu-arg-tyr-ser-ala-ile-cys-leu-arg-arg-ile-trp-lys-ala-glu-
341             345                 350                 355                 360
arg-phe-ser-trp-trp-met-thr-ser-val-leu-his-arg-phe-pro-asp-thr-asp-ala-phe-ser-
361             365                 370                 375                 380
gln-arg-ile-gln-gln-thr-glu-leu-glu-tyr-tyr-leu-gly-ser-glu-ala-gly-leu-ala-thr-
381             385                 390             394
ile-ala-glu-asn-tyr-val-gly-leu-phe-tyr-glu-glu-ile-glu
```

Scheme 6. Amino acid sequence of p-hydroxybenzoate hydroxylase.

1.8.9 Three-dimensional structure

The three-dimensional crystal structure of the enzyme-p-hydroxybenzoate complex was first elucidated at 0.25 nm resolution by Wierenga et al. using heavy atom derivatives [14]. This structure has recently been improved to 0.19 nm resolution using synchroton radiation for data collection and computer graphics methods for model refinement [28]. The crystallization of free enzyme (0.29 nm resolution) and enzyme complexed with NAD(P)H (analogs) is much more difficult to achieve as compared to the crystallization of the p-hydroxybenzoate complexed enzyme [27]. A model of the enzyme-3,4-dihydroxybenzoate complex at 0.23 nm resolution has been obtained by allowing the catalytical cycle to proceed in crystals of the enzyme-p-hydroxybenzoate complex [259]. In the same way anaerobic reduction of crystals of the enzyme-p-hydroxybenzoate complex has revealed a model of the reduced enzyme-p-hydroxybenzoate complex at 0.23 nm resolution [28]. This structure, lacking the presence of $NADP^+$ or NADPH, is very similar to the structure of the oxidized enzyme-p-hydroxybenzoate complex [28].

A schematic drawing, made by Jane Richardson, of the overall folding of the enzyme-p-hydroxybenzoate complex at 0.25 nm resolution is given below:

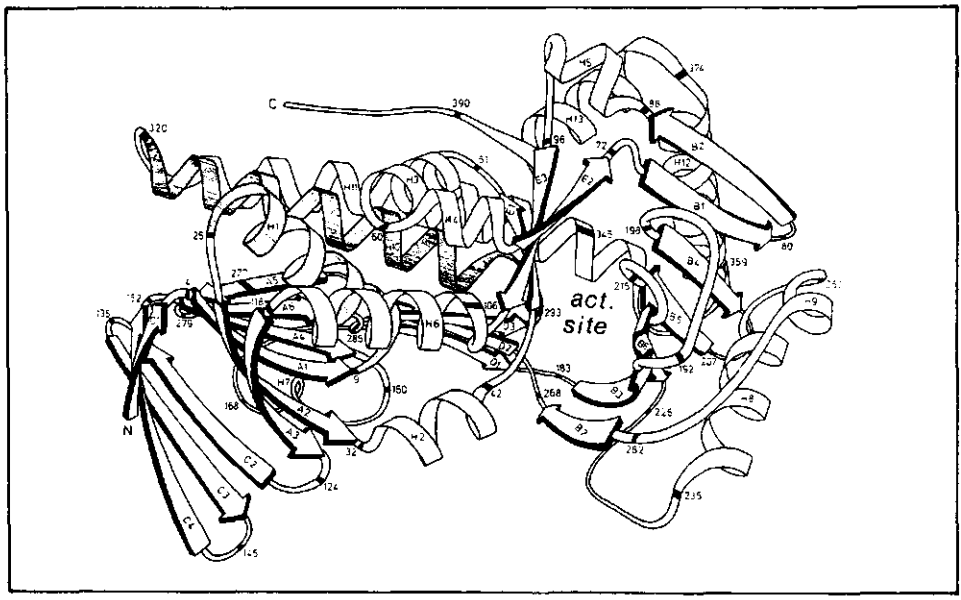


Figure 4. Overall folding of p-hydroxybenzoate hydroxylase.

The enzyme structure can be divided into three domains [27]: 1) the FAD binding domain (residues 1-175), 2) the p-hydroxybenzoate binding domain (residues 176-290) and 3) the interface domain (residues 291-394). The active site is built up by amino acid residues from all three domains [14,28].

The position of p-hydroxybenzoate and FAD, both deeply buried in the interior of the protein, are depicted in **Chapter 6** of this thesis. For more details about different aspects of overall FAD binding the reader is referred to **Chapter 2** and X-ray diffraction studies [28,68,258-260].

The refined crystal structure has revealed many interesting details about the binding pocket of p-hydroxybenzoate [28,260]. The substrate is

bound tightly via its carboxylic moiety by formation of a salt bridge with the guanidinium group of Arg-214 [14,258]. The carboxyl group is also in (hydrogen bonding) contact with Tyr-222 and Ser-212. The side chains of Tyr-201, Pro-293 and Thr-294 are within hydrogen bonding distance of the hydroxyl moiety of the substrate. Tyr-385 points its side chain towards the hydroxyl group of Tyr-201. After product formation the side chain of Tyr-201 still makes an excellent hydrogen bond with the 4'-hydroxyl group of the product whereas the carbonyl moiety of Pro-293 now is in hydrogen bonding distance of the inserted 3'-hydroxyl group [259]. The 3'-hydroxyl moiety of the product is located quite close to the C4A and N5 position of the flavin ring supporting the role of this part of the flavin in the proposed reaction sequence described above.

A difference of about one order of magnitude has been reported for the binding constants of substrate and product respectively [238]. The crystal stucture of the enzyme-3,4-dihydroxybenzoate complex at 0.23 nm resolution still needs further refinement in order to give a satisfactory explanation for the different binding constants observed.

1.8.10 The isoalloxazine ring

Besides the kinetic studies discussed above the role of the isoalloxazine ring of FAD in catalysis has been probed by using the following techniques:

1) X-ray diffraction studies (computer graphics modelling).
2) ^{13}C and ^{15}N NMR studies on apoprotein reconstituted with FAD, isotopically enriched in the isoalloxazine ring.
3) Kinetic studies on apoprotein reconstituted with FAD, chemically modified in the isoalloxazine ring.

1) X-ray diffraction

The refined crystal structure at 0.19 nm resolution has yielded detailed information about the static conformation of the isoalloxazine ring, the possible interactions of the flavin-moiety with surrounding amino acid residues and the position of fixed solvent molecules in the active site of the oxidized enzyme-p-hydroxybenzoate complex [28].

Restrained refinement shows a small twisting of the nearly planar flavin ring, consistent with the planarity found from NMR results [235]. The nitrogens of the active site loop from residue 295-300 (comprising the nitrogens of Gly-298 and Leu-299 at the beginning of helix H-10) are within hydrogen bonding distance of the flavin N1 atom. The N3-O4 region of the flavin ring is in close contact with the peptide groups of Gly-46 and Val-47. No side chains of amino acid residues are involved in flavin binding. Two fixed solvent molecules (Sol-579 and Sol-717) are supposed to bind in the entrance of the proposed nicotinamide binding pocket (just above Pro-293) at the reface of the flavin ring.

In the reduced state of the enzyme-p-hydroxybenzoate complex part of the xylene ring (C6-C8) is solvent accessible [28]. From modelling studies using 4a,5-epoxyethano-3-methyl-4a,5-dihydrolumiflavin as a model compound it has been argued that both the stability and reactivity of the reduced flavin-(C4A)hydroperoxide intermediate might be enhanced by an increased strength of the hydrogen bonds between flavin O4 and the nitrogen groups of Gly-46 and Val-47 [260].

In a more recently study the most likely position of the distal oxygen of the reduced flavin C4A-hydroperoxide intermediate was obtained by com-

puter rotation of the distal peroxide oxygen around the C4A-proximal oxygen bond [28]. The peroxide anion fits best in a position above the reface of the flavin ring, close to Pro-293. It should be remembered here that the nicotinamide moiety of NADPH is also believed to bind in this region near the flavin ring.

In order to arrive at the hydroxylation site it is postulated that the distal peroxide oxygen takes up a proton from a nearby situated solvent molecule and moves towards the hydroxylation site by a rotation around the C4A-proximal oxygen bond [28]. In contrast to the proposal mentioned above [260], polarization of the oxygen-oxygen bond is now believed to be stimulated by the partially negatively charged carbonyl oxygens of Pro-293 and Thr-294.

2) ^{13}C and ^{15}N NMR studies

The reactivity of flavin coenzymes within the restricted region of a protein active site is strongly dependent on the electronic distribution of the isoalloxazine ring during catalysis. To date, ^{13}C NMR is the most powerful technique to study this electronic distribution in detail [22]. Moreover ^{15}N NMR studies yield information about hydrogen bonding interactions. It should be noted here that only flavin intermediates can be studied which are stable during the time scale of the NMR experiments.

With p-hydroxybenzoate hydroxylase from P.fluorescens the apoprotein has been reconstituted with both C2,4A, C4,10A and N1,3,5,10 isotopically enriched FAD. NMR studies have been performed on both oxidized and reduced enzyme either in the absence or presence of p-hydroxybenzoate [235]. The results obtained have been interpreted by comparison the chemical shifts observed with results obtained for FMN in water, tetraacetylriboflavin in chloroform and results obtained with a set of other flavoproteins [19,20,261,262].

From the resonances observed for free oxidized enzyme it can be concluded that in this state the isoalloxazine ring is fairly solvent accessible. Upon binding of p-hydroxybenzoate the chemical shift observed indicates that the N(5) atom becomes shielded from solvent. The upfield shifts observed for all other enriched atoms are consistent with the conformational change observed on p-hydroxybenzoate binding [238] and are probably related to a repositioning of helix H-10 pointing with its positive end towards the active site [258].

In the reduced state of free enzyme the N(1) atom becomes ionized. This has also been observed with most other flavoproteins studied sofar [19,20,262]. Reduced flavin is bound in a more planar configuration as compared to free flavin in solution. Upon binding of p-hydroxybenzoate the resonances of N(1), C(10A) and N(10) shift sharply upfield, most probably as a consequence of a conformationial change comparable to oxidized enzyme.

From the NMR results it is concluded that in the reduced state the N(5) atom is in a planar and the N(10) atom in a slightly bended configuration. Comparable results have been obtained with the related bacterial luciferase [19]. In contrast, both 6-hydroxy-L-nicotine oxidase [262] and glucose oxidase [263] show the appearance of a bended N(5) and a sp2 hybridized N(10) atom.

Delocalization of electron density from N(10) can be accomodated by both carbonyl groups and by C(4A), C(5A), C(7) and C(9) respectively. On the other hand, delocalization of electron density from N(5) can be accomodated by C(6), C(8), C(9A) and C(10A) [261]. It is believed that the opposite hybridization states of both the N(5) and N(10) atom in hydroxylases and oxidases respectively are an important factor in regulating the reactivity of the isoalloxazine ring during catalysis [235].

The C(4A)hydroperoxide intermediate in bacterial luciferase, as studied by ^{13}C NMR, shows electronegative character as a consequence of its almost planar structure [222]. The C(4A) atom can easily attain sp2 character due to electron delocalization via the planar N(5) atom (see also above).

The chemical reactivity of reduced flavin C(4A)hydroperoxide has also been probed using model flavin compounds [226]. These studies indicate that oxygen transfer from flavinhydroperoxides is dependent on the possible stabilization of the anionic C(4A)hydroxyflavin. Moreover, the high reactivity of reduced flavin C(4A)hydroperoxide is a consequence of the electronegative character of the C(4A) atom [226].

In oxidases attack of substrate reduced flavin by oxygen is believed to occur also via formation of a reduced flavin C(4A)hydroperoxide intermediate [249]. The decay of the intermediate to form oxidized enzyme and H_2O_2 however is much faster as compared to the monooxygenases. The instability of the reduced flavin C(4A)hydroperoxide might be related to the less electronegative character of C(4A) as a consequence of the bended configuration of N(5) and increased electron density at C(4) due to electron delocalization from N(10).

In contrast to the oxidases in both p-hydroxybenzoate hydroxylase and luciferase the N(3)H-C(4)=O part of the flavin nucleus seems not to be involved in electron delocalization from C(4A). This part of the flavin is believed only to be responsible for binding to the apoprotein moiety.

From the above-mentioned results it is concluded that in p-hydroxybenzoate hydroxylase polarization of the flavin C(4A)hydroperoxide by charge delocalization from C(4) towards O(4a) [260] seems very unlikely. Polarization of the reduced flavin C(4A) hydroperoxide by the nearby situated partially negatively charged carbonyl oxygens from Pro-293 and Thr-294 [28] might be a more attractive alternative and is also in accordance with an oxene mechanism.

3) Replacement by artificial FAD

The active site of p-hydroxybenzoate hydroxylase has also been probed with a variety of chemically modified FAD analogues. The most important analogues as summarized in Table 5 can be divided in either chemically reactive or mechanistic probes [6].

Chemically reactive probes have been used to study the solvent accessibility of different parts of the flavin nucleus in either the free enzyme or the enzyme-p-hydroxybenzoate complex. Using 8-chloro- and 8-mercapto-FAD as reactive probes (Table 5) it has been shown that this part of the xylene moiety of the flavin ring is in close contact with bulk solvent in both the free enzyme and the enzyme-p-hydroxybenzoate complex [269]. These results are in good agreement with the X-ray data obtained from the corresponding crystal structures [27,28]. Accessibility of the 8-position of FAD to solvent has also been observed in both free and substrate-complexed phenol hydroxylase from T.cutaneum [271].

In free enzyme reconstituted with 2-thio-FAD the 2-thiol anion reacts readily with methyl methanethiosulfonate to yield the corresponding flavin disulfide. However the reaction with methyl methanethiosulfonate is considerably inhibited in the presence of p-hydroxybenzoate [264]. These results indicate that the 2-position of the pyrimidine subnucleus of the modified flavin ring is solvent accessible in the free enzyme but relatively inaccessible in the enzyme-p-hydroxybenzoate complex [264]. The difference in reactivity is most probably related to the repositioning of helix H-10 upon substrate binding. In contrast with these results 2-thio-FAD reconstituted phenol hydroxylase hardly reacts with methyl methane thiosulfonate both in the absence or presence of phenol [271].

Table 5. *Chemically modified FAD analogues used to probe the active site of p-hydroxybenzoate hydroxylase.*

Compound	Chemical agent	Mechanism	Ref.
1-deaza-FAD	no	oxidase	54
2-thio-FAD	Methylmethane-thiosulfonate H_2O_2	oxidase	264 265
4-thio-FAD	Sulfite	oxidase	266
6-hydroxy-FAD	no	hydroxylase oxidase	267
7-bromo-FAD	Photoreduction	hydroxylase	268
8-chloro-FAD	Photoreduction Na_2S Thiophenol	hydroxylase	268
8-mercapto-FAD	Iodoacetamide Iodoacetic acid	n.d.	269
8-sulfonyl-FAD	no	hydroxylase	270
8-hydroxy-5-deaza-FAD (reduced)	no	n.d.	51

The formation of N(5)sulfite adducts is one of the classical parameters used to probe the reactivity of the flavin prosthetic group within different flavoproteins [272]. With native p-hydroxybenzoate hydroxylase no N(5)sulfite adduct is formed [272]. 4-Thio-FAD-reconstituted enzyme slowly forms 4-hydroxy-4-sulfonyl-FAD with sulfite like free 4-thioriboflavin under aerobic conditions [266]. In contrast 4-thio-FAD D-amino acid oxidase, like native enzyme, readily forms the N(5)sulfite adduct [266]. However, from these results no conclusions can be drawn for the accessibility of this part of the flavin moiety.

Both 7-bromo- and 8-chloro-FAD reconstituted enzymes can be dehalogenated upon photoreduction yielding the corresponding 7- and 8-nor-FAD enzymes [268]. All four enzymic forms show catalyical activity comparable to native enzyme with p-hydroxybenzoate acting also as an effector. Upon photoreduction of the 7- or 8-halogen-substituted enzymes in the absence of p-hydroxybenzoate, in contrast to native enzyme, the red anionic flavin semiquinone is stabilized kinetically. Although this observation might be solely a result of the intrinsic properties of the chemically modified FAD analogues, it could also reflect different kinetic effects on stabilization of the radical as compared to native free enzyme [268].

Recently 8-demethyl-8-hydroxy-5-deaza-5-carba analogues of FMN and FAD have been used to probe the stereochemical reduction of the prosthetic group within different flavoproteins [51,274]. Stereochemically reduced flavin can be obtained by reduction of 8-hydroxy-5-deaza FAD reconstituted

general acyl-CoA dehydrogenase with tritium labeled sodium borohydride [51]. After binding to apo p-hydroxybenzoate hydroxylase the stereochemical reduced flavin analogue could be reoxidized by $AcPyADP^+$ in the presence of p-hydroxybenzoate. Analytical gel filtration of the reaction mixture revealed that most of the radioactivity co-eluted with $AcPyADP^+$ [51]. Comparable results were obtained with glutathione reductase from humann erythrocytes [51].

In glutathione reductase [94] it is known that $NADP^+$ binds at the re face of the flavin ring. Therefore it is concluded that in p-hydroxybenzoate hydroxylase NADPH also binds at the re face of the flavin ring [51]. As already discussed above these results are in good agreement with the crystal structure data of the enzyme-p-hydroxybenzoate complex at 0.19 nm resolution [28].

It should be noted here that all FAD-dependent disulfide oxidoreductases (**Chapter 1.5**), FAD-dependent fatty acid acyl-CoA dehydrogenases (**Chapter 1.6**) and FAD-dependent monooxygenases (**Chapter 1.7**) tested sofar show re side stereospecificity [51]. Until now si side stereospecificity has only been observed with L-lactate oxidase from Mycobacterium smegmatis and D-lactate dehydrogenase from M.elsdenii [274].

Different chemically modified FAD analogues have been used to probe the reaction mechanism of p-hydroxybenzoate hydroxylase from P.fluorescens. Despite an overwhelming amount of kinetic data these studies have not allowed a definitive assignment of the different intermediates found in the reaction sequence of native enzyme [267].

*With 1-deaza-FAD reconstituted enzyme the affinity of NADPH and p-*hydroxybenzoate for oxidized enzyme is comparable with native enzyme [54]. The rate of NADPH reduction, in the presence of p-hydroxybenzoate, is about 11% as compared to native enzyme.

In the oxidative half reaction the reduced flavin 1-deaza-C(4A)hydroperoxide can be produced but the ability to hydroxylate p-hydroxybenzoate and other substrates is lost [54]. The inability to hydroxylate the substrate might be a consequence of either (or both) a slightly misorientation or a difference in the electronic distribution of the oxygenating species. Similar results have been obtained with other FAD-dependent aromatic hydroxylases [6]. In contrast both 1-deaza-FMN reconstituted bacterial luciferase [275] and 1-deaza-FAD reconstituted cyclohexanone monooxygenase [217] are fully competent in oxygen transfer.

2-Thio-FAD reconstituted enzyme has also lost the ability to hydroxylate p-hydroxybenzoate [265]. However, the rate of reduction of the modified enzyme, in the presence of p-hydroxybenzoate, is increased by a factor 7 as compared with native enzyme. The very slow reaction of reduced enzyme with oxygen can be explained by the very high Km (O_2) value found from steady state kinetics. Again, as with 1-deaza-FAD modified enzyme, most probably the reduced C(4A) peroxide intermediate can be formed but readily decomposes to yield H_2O_2 and oxidized flavin [265].

With 4-thio-FAD reconstituted enzyme, most probably reduced flavin 4-thio-C(4A)hydroperoxide is produced [273]. In the presence of the effector 6-hydroxynicotinate the intermediate undergoes slowly desulfurization upon single turnover yielding native oxidized enzyme again. However, in the presence of 2,4-dihydroxybenzoate as the substrate, the intermediate breaks down to yield H_2O_2 and 4-thio-FAD oxidized enzyme [273]. Again, with 4-thio-FAD reconstituted enzyme, no hydroxylation is observed.

The reaction mechanism of 6-hydroxy-FAD reconstituted p-hydroxybenzoate hydroxylase has been studied in considerable detail [267]. This flavin occurs naturally in glycollate oxidase and electron transferring flavoprotein [276] but its biological function is still unknown [277]. In solution the transition of the yellow neutral to the greenish anionic form of 6-hydroxy-FAD shows a pKa value of 7.1 [276]. Upon binding

to apo p-hydroxybenzoate hydroxylase the pKa value shifts to 6.2 in the free enzyme and to 6.8 in the enzyme-p-hydroxybenzoate complex respectively [267].

The binding of NADPH to oxidized modified enzyme, the reduction pathway of modified enzyme in the presence of substrate and the oxidative half reaction of modified enzyme in the absence of substrate are all comparable to native enzyme. However, the affinity of p-hydroxybenzoate for oxidized modified enzyme (pH 7.0) is decreased by a factor of about ten [267].

With p-hydroxybenzoate, p-aminobenzoate and p-mercaptobenzoate as a substrate the rate of catalytical turnover is about 15% as compared to native enzyme (pH 6.6, 4 °C). With these substrates an increased rate of nonproductive decomposition of the reduced flavin 6-hydroxy-C(4A)hydroperoxide intermediate, yielding different ratio of both the normal products and H_2O_2, was observed. Moreover with p-hydroxybenzoate as a substrate product release becomes rate limiting in catalytical turnover. No new spectral evidence could be obtained for the process of oxygen transfer [267].

In the presence of 2,4-dihydroxybenzoate as a substrate, the entire reaction pathway is different from native enzyme. Although the affinity of oxidized modified enzyme for 2,4-dihydroxybenzoate is comparable to native enzyme, upon NADPH reduction, the effector role of 2,4-dihydroxybenzoate is lost. These results indicate that the introduction of an additional hydroxyl group at specific positions in both flavin and substrate must prevent the effector induced correct orientation between flavin and NADPH [267].

Almost no hydroxylation occurs in the oxidative half-reaction of reduced modified enzyme in the presence of a high concentration of 2,4-dihydroxybenzoate. This is most probably a consequence of the very weak interaction between 2,4-dihydroxybenzoate and reduced modified enzyme [267]. From these results it has been argued that the conformation of the active site in the substrate-complexed reduced enzyme must differ from the substrate-complexed oxidized enzyme [267]. From NMR and X-ray data on both native enzyme complexes, however no indications for such differences are found [28,235]. Steric interference of the 6-hydroxyl group of the reduced modified flavin with the 2'-hydroxyl moiety of 2,4-dihydroxybenzoate however cannot be excluded [267].

Finally the reaction mechanism of p-hydroxybenzoate hydroxylase has also been probed using 8-sulfonyl-FAD reconstituted enzyme [270]. In the presence of 2,4-dihydroxybenzoate the pH dependence and reaction rates of the different steps in oxygen transfer are comparable to native enzyme. The absorption spectrum of intermediate II in 8-sulfonyl-FAD reconstituted enzyme [270] is blue shifted as compared to the corresponding spectrum of native enzyme [216]. Upon raising the pH the spectrum becomes red shifted, showing a pKa value of 7.8. The spectrum has been discussed with respect to the ring open structure as originally suggested by Entsch [216]. From the absorption spectrum alone however no conclusions can be drawn about the electron distribution of the modified flavin ring in the different transition states [22].

1.8.11 Chemical modification studies

In addition to the various techniques described above chemical modification studies can yield valuable information about:

1) the amino acid residues involved in binding of both p-hydroxybenzoate and NADPH
2) differences in the dynamic structure of free enzyme and enzyme-ligand complexes.

In general chemical modification studies can be performed by using either group-specific or site-selective reagents. For more details about the general applicability of the various reagents the reader is referred to the thesis of Wijnands [25] and references therein.

The chemical modification of p-hydroxybenzoate hydroxylase has been studied by applying both group-specific and affinity labeling reactions:

1) Group-specific modification

Group-specific modification studies have both been performed with the enzymes from P.fluorescens and P.desmolytica. The different reagents used are summarized in Table 6.

A short review of the results obtained with both enzymes is given below:

Enzyme activity of the enzyme from P.fluorescens is not affected by sulfhydryl reagents like N-ethylmaleimide and DTNB [156]. In the native enzyme only one cysteine residue is accessible for these reagents [156]. Unfolding in the presence of GuHCl yields five DTNB-titrable cysteine residues [156] which is in accordance with the amino acid sequence [12]. The assignment in the sequence of the only accessible cysteine residue is described in Chapter 5.

Enzyme activity of the enzyme from P.desmolytica can be blocked by covalent modification using phenylglyoxal [278]. It was concluded that phenylglyoxal reacts with an active site arginine residue interacting with the carboxylic moiety of p-hydroxybenzoate. This is in agreement with the three-dimensional structural data of the enzyme from P.fluorescens [14,258].

Arginine modification of the enzyme from P.fluorescens is quite complex [279]. Modification by phenylglyoxal leads to loss of the enzyme capacity to bind both p-hydroxybenzoate and NADPH. From quantitation and protection experiments it was concluded that two arginine residues are essential for catalysis. One essential arginine residue is protected from modification in the presence of p-hydroxybenzoate whereas the other essential arginine residue is protected from modification in the presence of NADPH.

Unfortunately the labile modified amino acid residues could not be identified by sequencing of radioactive labeled peptides.

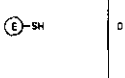
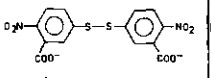
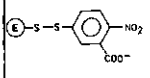
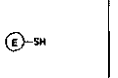
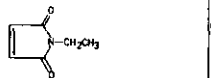
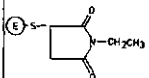
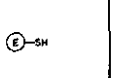
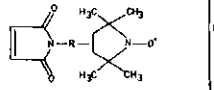
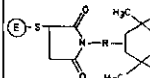
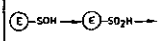
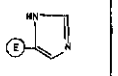
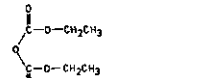
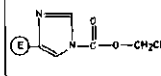
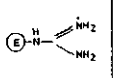
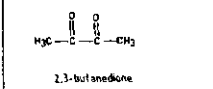
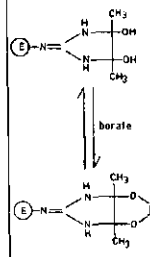
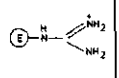
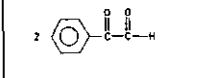
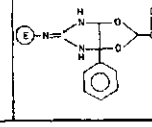
Arginine modification of the enzyme from P.fluorescens by 2,3-butanedione shows pseudo-first-order inactivation but does not influence the binding of both p-hydroxybenzoate and NADPH [279]. Sequential modification studies confirmed the findings that phenylglyoxal and 2,3-butanedione react with different sets of arginine residues [279].

From photo-oxidation of the enzyme from P.desmolytica it was concluded by Shoun et al. [281] that a histidine residue with a pKa value of 7.0 is involved in substrate binding by hydrogen bonding of the phenolic moiety of p-hydroxybenzoate. This however is not in agreement with the three-dimensional data of the enzyme from P.fluorescens [28]. Results obtained from modification studies with the enzyme from P.fluorescens [242,252,257] indicate that the reported loss of activity most probably is related to modification of a tyrosine residue present in the p-hydroxybenzoate binding site.

With both the enzymes from P.desmolytica [280] and P.fluorescens [242] inactivation by diethyl pyrocarbonate at pH values below 7.0 can be related to the modification of histidine residues.

In the case of the enzyme from P.desmolytica the inactivation was correlated with the modification of a single histidine residue showing a pKa value of about 6.6 [280]. Enzyme activity could be restored by treat-

Table 6. Group-specific modification of p-hydroxybenzoate hydroxylase.

Target enzyme amino acid residue	Modification reagent	Enzyme product	Ref.
	 5,5' -dithiobis (2-nitrobenzoate)		47,156 233
	 N-ethylmaleimide		156,233 252
	 3-(maleimido-R)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl		233
	 hydrogen peroxide		233
	 diethyl pyrocarbonate		242,280
	 2,3-butanedione	 borate	279
	 phenylglyoxal		278,279

ment of modified enzyme with hydroxylamine. The inactivation reaction is inhibited in the presence of NADPH. The modified enzyme, as compared to native enzyme, showed the same affinity for p-hydroxybenzoate but a drastically decreased affinity for NADPH.

With the free enzyme from P.fluorescens four out of nine histidine residues can be modified in a biphasic process [242]. During the first phase of the inactivation, one histidine is modified yielding about 70 % residual activity. During the second phase of the inactivation, the additional three histidine residues are modified with complete loss of activity. Two of the latter histidine residues, both showing a pKa value of

6.2, were found to be essential for activity and are modified in a cooperative process. Inactivation was completely reversible on addition of hydroxylamine. Modified enzyme showed strongly decreased affinity for NADPH. Though effectors protect two histidine residues from modification, the affinity of p-hydroxybenzoate for modified enzyme is comparable to native enzyme.

The instability of ethoxyformylated histidine residues has not allowed their assignment in the amino acid sequence. From the three-dimensional model of the enzyme-p-hydroxybenzoate complex at 0.25 nm resolution [14] His-162 and His-289 were tentatively assigned to be involved in the cooperative inactivation process [242]. His-162 is believed to be the only histidine residue directly involved in binding of the 2',5'-ADP moiety of NADPH [27].

With both the free enzymes from P.desmolytica [280] and P.fluorescens [242] it has been reported that inactivation by diethyl pyrocarbonate is strongly enhanced at pH values above 7.0. From protection experiments it was concluded for the enzyme from P.desmolytica that either arginine, tyrosine or histidine residues, situated in the binding site of p-hydroxybenzoate, were modified [280].

Diethyl pyrocarbonate inactivation of the enzyme from P.fluorescens at pH values above 7.0 is inhibited by p-fluorobenzoate [242]. From UV difference spectra the inactivation reaction was related to tyrosine modification at the p-hydroxybenzoate binding site. This interpretation has been confirmed by performing sequential modification studies [257].

2) Affinity labeling

The application of affinity labeling for studying the structure-function relation of enzymes has received much attention. A review about the strategy of active site directed reagent 'design' and directives for the interpretation of obtained results is given by Plapp [282].

Affinity labels used for the chemical modification of p-hydroxybenzoate hydroxylase from P.fluorescens are summarized in Table 7.

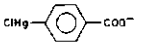
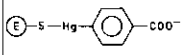
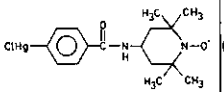
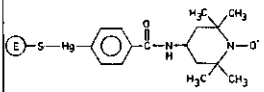
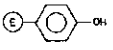
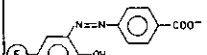
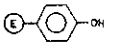
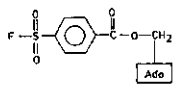
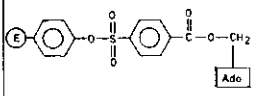
It is well-known that protein sulfhydryl groups are very sensitive towards mercurial reagents [283]. The inactivation by p-chloromercuribenzoate is a general applied method to indicate the presence of cysteine residues in an enzyme active site [284]. In the case of p-hydroxybenzoate hydroxylase this reagent is especially interesting as its structure closely resembles the structure of p-hydroxybenzoate.

The enzyme from P.fluorescens is almost completely inactivated by a small excess of p-chloromercuribenzoate [156]. Details about the chemical modification of sulfhydryl groups by mercurial reagents, the assignment in the sequence and the implications for enzyme activity are described in **Chapter 5**.

Chemical modification of the enzyme from P.fluorescens by both diethyl pyrocarbonate [242] and p-chloromercuribenzoate (**Chapter 5**) have indicated the presence of catalytically important tyrosine residues in the p-hydroxybenzoate binding site. These results are in agreement with data obtained from the X-ray diffraction pattern of the enzyme-p-hydroxybenzoate complex [14]. Fitting of the amino acid sequence to the electron-density map at 0.25 nm resolution has revealed that both Tyr-201, Tyr-222 and Tyr-385 are pointing their side chain towards bound substrate [258].

Interestingly, monitoring p-chloromercuribenzoate modification of free enzyme by UV difference spectroscopy (**Chapter 5**) indicates that probably one of these tyrosine residues shows a pKa value of about 7.6.

Table 7. Affinity labeling of p-hydroxybenzoate hydroxylase.

Target enzyme amino acid residue	Affinity label	Enzyme product	Ref.
	 p-chloromercuribenzoate		252
	 4-(4-chloromercuribenzamido)-2,2,6,6,-tetramethylpiperidine-1-oxyl		252
	 p-diazoniumbenzoate		257
	 5-(p-fluorosulfonylbenzoyl)-adenosine		246

The reactivity of the different tyrosine residues has been probed by using the affinity label p-diazoniumbenzoate [257]. Modification by p-diazoniumbenzoate abolishes the affinity of the enzyme for p-hydroxybenzoate. The inactivation reaction is inhibited by the effector p-fluorobenzoate. From sequencing radioactive labeled peptides it was concluded that loss of enzymic activity is caused most probably by modification of Tyr-222.

Chemical modification by diethyl pyrocarbonate at pH values above 7.0 is also inhibited by p-fluorobenzoate [242] and prevents the binding of p-hydroxybenzoate [257]. Prior reaction with diethyl pyrocarbonate at pH 8.0 doesnot prevent the enzyme from azo coupling by p-diazoniumbenzoate. Moreover sequential labeled enzyme is not reactivated by hydroxylamine indicating that diethyl pyrocarbonate reacts most probably with either Tyr-201 or Tyr-385.

Both these tyrosine residues are involved in the binding of the hydroxyl moiety of the substrate [258]. From p-chloromercuribenzoate labeling it has been proposed that in the free enzyme one of the active site tyrosine residues is easily ionized (**Chapter 5**). Diethyl pyrocarbonate modification at pH 6.0 and diethyl pyrocarbonate modification at pH 8.0 in the presence of p-fluorobenzoate do not prevent the production of the UV difference spectrum generated by p-chloromercuribenzoate labeling [257]. The UV difference spectrum however, is not produced anymore by prelabeling with diethyl pyrocarbonate at pH 8.0. These results strongly indicate that either Tyr-201 or Tyr-385 posesses the observed pKa value of about 7.6. It should be noted here that in the refined three-dimensional models of both the enzyme-substrate and enzyme-product complex [28] Tyr-201 is in hydrogen bonding distance of the p-hydroxyl moiety of both ligands.

From kinetic studies on the pH dependence of the overall reaction it has been proposed for both the enzymes from P.desmolytica [281] and

P.fluorescens [157] that the hydroxyl group of p-hydroxybenzoate might ionize upon enzyme-substrate complex formation. It should be remembered here that pH 8.0 is the optimum pH for overall catalysis. From a chemical point of view ionization of the substrate could facilitate the hydroxylation reaction by favouring electrophilic attack at the 3'-position of the substrate [216].

As noted above (**Chapter 1.8.7**) the different steps of the oxidative half reaction in the presence of 2,4-dihydroxybenzoate are strongly pH dependent [251]. All pH-dependent transitions could be fitted by pKa values between 7.5 and 7.9 and were attributed to either the product or a tyrosine residue in the active site [251].

Ionization of the 4-hydroxyl group of the substrate is not expected to be very favourable in case of ionization of Tyr-201.
Therefore, in order to possibly discriminate between the different proposals mentioned above, in **Chapter 7** the pH-dependent properties of both free enzyme and enzyme-substrate(analogs) have been studied in more detail by the use of different spectroscopic techniques.

Unfortunately, until now both group-specific modification studies [242,279] and crystallization experiments [27] have not allowed a detailed description of the NADPH binding site. The possible involvement of His-162 and different arginine residues in NADPH binding have been discussed by van der Laan [27].

In many nucleotide-dependent enzymes either NAD or FAD are bound in a characteristic $\beta\alpha\beta$ (Rossmann) fold [68,69,287,288]. For instance, in glutathione reductase both FAD and NADPH are associated with such a fold [66,94,289]. In p-hydroxybenzoate hydroxylase however, only a single Rossmann fold, involved in FAD binding, is present [28,68].

As pointed out by van der Laan [27], the quite different topology of NADP-dependent enzymes with known X-ray structure considerabbly hampers the prediction of the NADPH binding site in p-hydroxybenzoate hydroxylase.

(Photo)affinity labeling using chemically reactive pyridine nucleotide analogs [290] can yield information about amino acid residues possibly involved in NADPH binding. In **Chapter 6** the covalent modification of the enzyme by 5'-(p-fluorosulfonylbenzoyl)adenosine has been studied in more detail.

From the above-mentioned results it will be clear that, for even better understanding the complete catalytical event, conditions have to be found, considerably decreasing the rates of the different reaction steps, without altering the active site geometry too dramatically. In native enzyme, in the presence of p-hydroxybenzoate (pH 6.6, 4°C), the speculative intermediate II is kinetically invisible. Under the same conditions in the presence of 2,4- dihydroxybenzoate or p-aminobenzoate the lifetime of intermediate II is in the order of seconds [216]. Assignment of intermediate II by ^{13}C NMR by stabilization at subzero temperatures therefore will be difficult.

It will be a tedious but challenging task to see if stabilizing conditions can be created by preparing mutant enzymes [291]. As the use of binding energy is the important factor in catalysis, nondisruptive mutations might give more information about hydrogen-bonding site chains in the active site important for binding and catalysis. In this respect it should be remembered here that both X-ray and chemical modification experiments show that all three domains are involved to achieve optimal active site geometry.

The preparation of mutant enzymes might also give more information about other structural features (i.e. the 'unusual' mode of NADPH binding) as well.

Finally, better characterization of other FAD-dependent aromatic hydroxylases is required as well, in order to get more insight in the fine tuning of the active sites of the different enzymes.

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Chapter 2

A Study on *p*-Hydroxybenzoate Hydroxylase from *Pseudomonas fluorescens*

A Convenient Method of Preparation and Some Properties of the Apoenzyme

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Conventional methods to prepare the apoenzyme of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* yield an inhomogeneous apoenzyme with respect to the reconstitutable activity. To overcome this problem the holoenzyme was bound covalently to a Sepharose–5,5′-dithio-bis(2-nitrobenzoate) column via the reactive sulfhydryl group of the enzyme. The prosthetic group FAD was removed at neutral pH by addition of a high concentration of KBr and urea to the elution buffer. The holoenzyme can be reconstituted while the apoenzyme is still bound to the column or the apoenzyme can be isolated in the free state. The yield of apoenzyme was between 90% and 95% of the starting enzyme. The degree of reconstitution of holoenzyme is better than 95% of the original activity. The apoenzyme is stable for a long period of time as an ammonium sulfate precipitate.

The relative molecular mass of the apoenzyme is 43000 per polypeptide chain. The apoenzyme exists mainly as a dimer in solution.

During the preparation of the apoenzyme the substrate *p*-hydroxybenzoate protects the apoenzyme from inactivation, indicating complex formation. The dissociation constant for the apoenzyme-substrate complex was determined to be about 30 μM.

The apoenzyme also forms a complex with NADPH. The dissociation constant (≈ 12 μM) of this complex is about a factor of ten smaller than that of the holoenzyme (≈ 120 μM). The dissociation constant of the apoenzyme-NADPH complex is strongly dependent on pH and ionic strength of the solution.

The dissociation constant of the holoenzyme into its constituents is 45 nM. The association rate constant calculated from kinetic experiments is $4.1 \times 10^4\ M^{-1}\ s^{-1}$. The calculated dissociation rate constant is $1.85 \times 10^{-3}\ s^{-1}$, indicating that the holoenzyme possesses a high stability which is governed by the low dissociation rate constant.

The method of preparation of apoenzyme can also be used to separate enzyme molecules where the reactive SH group has been oxidized by air from enzyme molecules still containing the original SH group. This separation technique is in particular useful when quantitative labelling of the SH group is required.

The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is an external monooxygenase catalyzing the conversion of *p*-hydroxybenzoate into 3,4-dihydroxybenzoate. The enzyme is strictly dependent on NADPH. The reaction mechanism of the enzyme has been studied in detail [1].

The enzyme contains FAD as a prosthetic group which is non-covalently but tightly bound to the apoenzyme. Several methods have been used to prepare the apoenzyme. Acid ammonium sulfate precipitation resulted in a 50–70% recovery of the holoenzyme [2]. Ghisla et al. [3] used a dialyzing technique at high pH values in solutions containing 2 M KBr and 1 M urea. This method takes, dependent on the concentration of the enzyme used, from several days up to more than one week to remove FAD completely from the enzyme. It is not surprising that this lengthy method yields an apoenzyme preparation of variable reconstitutable activity [3]. In studies where the FAD of the enzyme is replaced by a chemically modified analogue to investigate the mechanism and the active center of the enzyme in more detail [2, 3], it is of the utmost importance to have a reconstituted enzyme of high quality available, allowing a thorough physical characterization of such preparations. This approach of studying the enzyme containing modified prosthetic groups is now very promising since the sequence of the enzyme is known [4, 5] and also a low-resolution three-dimensional model of the enzyme exists [6]. In this context we wish to investigate the specific interactions between the apoenzyme and the prosthetic group by nuclear magnetic resonance using ^{13}C-labeled FAD as previously described for flavodoxins [7]. For such a study a large amount of highly reconstitutable enzyme is needed.

In a previous paper [8] we described the complex hydrodynamic and some other physical and chemical properties of the enzyme. In the present paper we wish to report on a simple and fast method of preparation of the apoenzyme of *p*-hydroxybenzoate hydroxylase. The method is highly reproducible and the apoenzyme is almost quantitatively reconstitutable. The enzyme contains one sulfhydryl group accessible

Abbreviations. Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Epps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Nbs_2, 5,5′-dithio-bis(2-nitrobenzoic acid).

Enzymes. *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2); alcohol dehydrogenase (EC 1.1.1.1); *Naja naja* snake venom phosphodiesterase (EC 3.1.4.1).

to *N*-ethylmaleimide [8]. We made use of this sulfhydryl group to bind the enzyme covalently to Sepharose chemically substituted with 5,5'-dithio-bis(2-nitrobenzoate). This method allows us to prepare a large amount of apoenzyme (100 – 150 mg) in high yields in a very short period of time. Some physical properties of the apoenzyme are also described.

MATERIALS AND METHODS

General

NADPH, NADH, FAD, ovalbumin, α-chymotrypsinogen, bovine serum albumin, alcohol dehydrogenase and myoglobin were products of Boehringer A.G. (Mannheim, FRG). AH-Sepharose was a product of Pharmacia (Uppsala, Sweden) and Bio-Gels (P-2 and P-6-DG) were from Bio-Rad (California, USA). *Naja naja atra* (snake venom) was purchased from Sigma. All other chemicals were products of Merck A.G. (Darmstadt, FRG) and were the purest grade available.

Large-scale production of *P. fluorescens* was performed by Diosynth B. V. (Oss, The Netherlands). The isolation and purification procedure of the enzyme has been described earlier [8]. The assay conditions have been reported previously [9]. The apoenzyme was assayed under standard conditions [8,9] in the presence of 10 μM FAD. In order to obtain reliable reference activities the assay mixture for the holoenzyme also contained 10 μM FAD.

Light absorption spectra were recorded with a Varian Cary model 16 spectrophotometer. Circular dichroic spectra were obtained with a Jouan Dichrograph model DC 185. Fluorescence emission spectra were recorded on an Aminco SPF-500 fluorimeter connected to a Hewlett Packard calculator interface accessory. The degree of polarization of the fluorescence was determined as described previously [10]. All spectrometers were equipped with thermostated cell holders. All spectra were obtained at 20 °C.

The relative molecular mass of the apoenzyme was determined by sedimentation analysis as previously reported [8]. A MSE analytical ultracentrifuge was used. The temperature was 20 °C. Samples of apoenzyme (0.1 – 1.0 mg/ml) were dialyzed against a solution of 0.1 M K_2HPO_4, pH 7.5, containing 0.3 mM EDTA and 0.1 mM *p*-hydroxybenzoate. Sodium dodecyl sulfate gel electrophoresis was performed using the phosphate buffer system as described by Weber et al. [11]. Polyacrylamide gels of 7.5% were used.

Sulfhydryl groups were determined according to the method of Ellman [12] in 0.1 M K_2HPO_4, pH 8.0.

The protein concentration was determined by a modification of the biuret method as described by Brumby and Massey [13].

Preparation of Sepharose Substituted with Nbs_2

AH-Sepharose (CNBr-activated Sepharose coupled with 1,6-diaminohexane) was coupled with Nbs_2 according to the procedure described by Lin and Foster [14]. This procedure yielded a degree of coupling of 3.8 μmol Nbs_2/ml gel. To improve the degree of coupling the procedure was repeated twice yielding a preparation with 5.8 μmol Nbs_2/ml gel. The degree of coupling was determined by reacting 1 ml of settled column material with 5 ml of a 5 mM solution of dithioerythritol in 0.1 M phosphate buffer, pH 8.0, and washing with the same buffer until the effluent was colorless, yielding Sepharose – 2-nitro-5-thiobenzoate (orange-colored) material. This material was treated with 5 ml of a 5 mM solution of Nbs_2 in 0.1 M K_2HPO_4, pH 8.0, for 10 min and then washed with the same buffer. The eluate was collected and the concentration of 2-nitro-5-thiobenzoate determined from the absorbance at 412 nm ($\varepsilon = 13600\ M^{-1}\ cm^{-1}$ [12]). The color of the regenerated column material is faint yellow.

Preparation of the Apoenzyme of p-Hydroxybenzoate Hydroxylase

A suspension of the enzyme in 70% ammonium sulfate was centrifuged and the precipitate dissolved in a minimum volume of a mixture consisting of 0.1 M K_2HPO_4, 0.5 M KBr, 1 mM *p*-hydroxybenzoate and 0.3 mM EDTA, pH 7.5 (in the following called the coupling buffer). About 15 mg of enzyme can be covalently bound per ml column material. The enzyme solution was brought onto the column pre-equilibrated with the coupling buffer at about 20 °C. Then the column was washed with two volumes of coupling buffer. During this washing some activity is eluted from the column. This eluate contains enzyme molecules in which the reactive sulfhydryl group has been oxidized (cf. Table I). The prosthetic group was removed from the column-bound enzyme with a mixture consisting of 0.1 M K_2HPO_4, 2 M KBr, 2 M urea, 1 mM *p*-hydroxybenzoate and 0.3 mM EDTA, pH 7.5. The release of FAD can be followed by fluorescence measurements. After removal of FAD from the enzyme the column was washed with 10 volumes of the coupling buffer to remove urea and excess KBr from the column. The apoenzyme was then eluted from the column with 10 volumes of coupling buffer containing 5 mM dithioerythritol. The elution of the apoenzyme was followed by activity measurements in an assay mixture containing 10 μM FAD. The eluted apoenzyme was dialyzed at 4 °C against a large volume of a solution containing 0.1 M K_2HPO_4, 1 mM *p*-hydroxybenzoate, 0.3 mM EDTA, pH 7.0. During dialysis a small amount (about 2% of the total protein) of an orange-brown colored precipitate was formed which was removed by centrifugation. For storage the apoenzyme was precipitated with ammonium sulfate (70% saturation). As an ammonium sulfate suspension the apoenzyme can be kept at 4 °C for several months without loss of reconstitutable activity.

Regeneration of the Column Material

After use the column was washed with several bed volumes of 0.1 M K_2HPO_4 buffer, pH 8.0. Then the column was washed with the same buffer containing 5 mM Nbs_2 followed by washing with the phosphate buffer alone. The regenerated column material exhibits a yellowish color and was stored at 4 °C in H_2O containing 0.02% sodium azide.

Determination of Kinetic and Thermodynamic Properties of the Apoenzyme-FAD Complex

In all experiments commercial FAD purified on a Bio-Gel P-2 column was used. A solution of 50 mM potassium acetate, pH 4.9, was used as elution buffer. The purification was performed at 4 °C and in the dark. The collected fractions were checked for purity by measuring the increase of fluorescence emission upon treatment of aliquots with snake venom, as described by Wassink and Mayhew [15]. Only samples yielding the expected increase of fluorescence emission were used in this study. These samples are considered, according to published analytical standards, to be of the highest purity achievable.

The kinetic K_m value for the interaction of the apoenzyme with FAD was estimated by addition of various concentrations of FAD to a solution of 0.1 M Tris/SO_4, pH 8.0, containing 7.5 nM or 15 nM apoenzyme, 0.2 mM *p*-hydroxybenzoate and 0.2 mM NADPH. The activity of the reconstituted enzyme was determined for each concentration of added FAD and the K_m value for FAD estimated graphically by a double-reciprocal plot.

The association rate constant for the interaction between FAD and apoenzyme was determined by addition of an excess of FAD over the apoenzyme in the above assay mixture. The activity was then followed with time until the maximal velocity was reached. From the kinetic curve the pseudo-first-order rate constant was obtained by plotting the natural logarithm of the activity versus time. The second-order rate constant was estimated from a secondary plot of the pseudo-first-order rate constants versus the concentration of FAD.

Estimation of the Molar Absorption Coefficient of the Apoenzyme

The absorbance at 280 nm was determined for several samples containing about 30 μM of the apoenzyme in 0.1 M K_2HPO_4, pH 7.0. The samples were then titrated with FAD until an excess of FAD was present. 1.0 ml of the reconstituted enzyme samples was brought on a Bio-Gel P-6-DG column (Bio-Rad economy column, $V_0 = 2.0$ ml, $V_t = 7.0$ ml), preequilibrated with the phosphate buffer. The enzyme was then eluted with 3.0 ml phosphate buffer. The eluate (3.0 ml) contained all of the enzyme and no free FAD. The absorbance at 450 nm ($\varepsilon_{450} = 11\,300\ M^{-1}\,cm^{-1}$ [8]) of the enzyme solution was then determined and the molar absorption coefficient for the apoprotein, after appropriate corrections for dilutions, estimated. The value of ε_{280} given for the apoprotein is an average of several determinations.

RESULTS AND DISCUSSION

Preparation of the Apoenzyme

The enzyme possesses five sulfhydryl groups of which one is accessible to *N*-ethylmaleimide [8]. We made use of this property of the enzyme to couple it covalently to the Sepharose-Nbs_2 column. Although rather drastic conditions are used to remove the prosthetic group (2 M urea, 2 M KBr) the structure of the enzyme is apparently not affected by this procedure (cf. also below), owing to the protection by the gel and probably also the substrate. The substrate was present in all solutions at all times because the free apoenzyme in solution is less stable than the complexed one during the preparation. As described in Materials and Methods, the preparation of the apoenzyme of *p*-hydroxybenzoate hydroxylase is very simple and gives almost quantitative yields. The preparation can be done at room temperature since no difference was noticed in activity of reconstituted apoenzyme prepared at 4 or 20 °C. Dependent on the amount of enzyme used the procedure takes only about 1–2 h.

The binding capacity of the column material depends on the degree of coupling of the Sepharose gel. We have taken care to obtain a degree of coupling as high as possible. With our best preparation it was possible to bind about 150 mg of enzyme/10 ml of column material. Besides the large-scale production of apoenzyme, there is another reason to achieve a high degree of coupling. The commercial AH-Sepharose is produced by CNBr activation followed by substitution with 1,6-diaminohexane. Depending on the degree of coupling, the column material possesses some ion-exchange properties due to the terminal free ammonium group of diaminohexane. To suppress the residual ion-exchange property of the column material, 0.5 M KBr was added to the coupling buffer.

Table 1. *Percentage activity and sulfhydryl content of various fractions collected during the preparation of the apoenzyme*
80 mg of enzyme (= 100% activity) in 0.1 M K_2HPO_4, pH 7.5, was loaded onto a Sepharose-Nbs_2 column (8 ml) and then washed with several bed volumes of the given solutions. n.d. = not determined

Solution	Volume	Activity of eluate		Sulfhydryl groups
		− FAD	+ FAD	
	ml	% original		mol/mol FAD
Phosphate buffer	50	0	0	–
Phosphate buffer + 0.5 M KBr	50	5–10	5–10	< 0.05
Phosphate buffer + 2 M KBr + 2 M urea	200	0– 5	0– 5	n.d.
Phosphate buffer + 0.5 M KBr + 5 mM dithioerythritol	100	< 2	85–98[a]	0.95–1.0

[a] The yield of apoenzyme depends on the amount of enzyme molecules containing the oxidized sulfhydryl group in a particular preparation of native enzyme, i.e. the actual yield of apoenzyme is 95–98%.

The accessible sulfhydryl group of *p*-hydroxybenzoate hydroxylase is rather easily oxidized by air at pH > 7, but this reaction does not affect the activity of the enzyme [8]. The oxidation of the reactive sulfhydryl group by air can be prevented by storage of the enzyme as an ammonium sulfate suspension at pH < 7 and 4 °C. The molecules possessing an oxidized sulfhydryl group do not bind to the column via covalent linkage but may be bound via electrostatic interactions. To prevent this we decided to remove these molecules from the column during the coupling of the enzyme to the column. This was achieved by the addition of 0.5 M KBr to the coupling buffer. The properties of a typical preparation of apoenzyme including activity and SH content of different fractions are summarized in Table 1. The results show that the molecules containing an oxidized SH group are bound to the column but can be easily separated from the unmodified molecules. This technique is therefore also valuable for labeling experiments of the SH group where quantification is desired or necessary. Table 1 also shows that the recovery of the enzyme is high and that the original activity is almost quantitatively restored. The original activity returns immediately after addition of FAD to the apoenzyme indicating easy reconstitution of the holoenzyme. It should be noted that the yield of apoenzyme (Table 1) is given with respect to the total amount of native enzyme used in a particular preparation. Correcting the yield for the percentage of enzyme molecules possessing the oxidized SH group, the actual yield is 95–100%, depending on the residual activity of the preparation and the small loss of apoenzyme. Furthermore an almost quantitative removal of FAD (≪ 1% of residual activity) does not influence the reconstitutability of the apoenzyme.

This method of apoenzyme preparation could also be applied to other flavoproteins containing accessible sulfhydryl groups. The method is a valuable tool especially in cases where a large amount of apoenzyme is required.

Table 2. *The stability of 1 μM apoenzyme in 0.1 M K_2HPO_4 buffer (pH 7.0) containing 0.3 mM EDTA in the presence and absence of* p-*hydroxybenzoate at 4 °C*

The activity is given as a percentage of an identical solution of native enzyme in the absence of substrate; 10 μM FAD was added to all assay mixtures

Time	Remaining activity of		
	native enzyme	apoenzyme in the presence of	
		none	substrate
days	%		
0	100	100	100
5	97	52	73
10	95	35	62
15	92	23	54

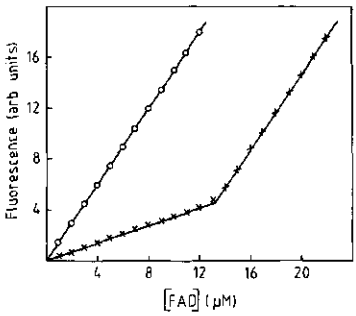

Fig. 1. *Fluorescence titration of apoenzyme of* p-*hydroxybenzoate hydroxylase at pH 7.0 and 20 °C.* The fluorescence was measured in arbitrary units upon the addition of 5–50 μl of 172 μM FAD to a cuvette containing 13.0 μM apoenzyme (×) and to a cuvette containing no apoenzyme (○). The buffer solution consisted in both cases of 100 mM K_2HPO_4, 0.3 mM EDTA and 1.0 mM *p*-hydroxybenzoate. Fluorescence emission was observed at 525 nm upon excitation at 450 nm

Small amounts of apoenzyme can also be prepared from dilute solutions of holoenzyme in the presence of phosphodiesterase (*Naja naja atra*, snake venom). The reaction is slow but goes to completion as judged from measurements of activity with time. The reaction can conveniently be followed by fluorimetry. The fluorescence increase parallels the activity decrease during the entire reaction.

The hydrolysis of FAD in *p*-hydroxybenzoate hydrolase was followed with time. The following times for the loss of 50% activity were found for 1 μM, 5 μM and 25 μM enzyme solutions in 0.1 M Hepes buffer, pH 7.0, in the presence of 1 mM *p*-hydroxybenzoate and 0.1 mg/ml *Naja naja* snake venom: 21 min, 33 min and 60 min, respectively. The results indicate that the reaction is complex with respect to the dependence on the concentration. The complexity of the reaction is probably related to the complex hydrodynamic properties of the enzyme [8]. The reconstitutability of the apoenzyme obtained in this way is the same as that described above for the large-scale production. However, to purify the apoenzyme the Nbs_2 column should be used to remove the phosphodiesterase quantitatively.

Stability of the Apoenzyme

The apoenzyme can be stored as an ammonium sulfate suspension, pH 6, at 4 °C for several months without appreciable loss of activity. In solution, however, the apoenzyme loses activity with time as shown in Table 2. The loss of activity with time depends very much on the composition of the solution. From Table 2 it follows that the substrate *p*-hydroxybenzoate stabilizes the apoenzyme considerably, whereas the presence or absence of dithioerythritol has no influence on the stability. The stability of the apoenzyme decreases with increasing pH and decreasing apoenzyme concentrations.

Subunit Composition of the Apoenzyme

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yielded a single band of relative molecular mass 43000, as found previously for the holoprotein [8]. Sedimentation equilibrium experiments and gel filtration on Sephadex G-150, as described previously for the native enzyme [8], gave the following results. At room temperature the apoenzyme is present almost quantitatively as a dimer (M_r = 79000–80000). Under identical conditions the holoenzyme is also present largely as a dimer (M_r = 83000–90000) but tetramers and hexamers are also formed, in contrast to the behaviour of the apoenzyme. Similarly to the native enzyme, the dimer of the apoenzyme is on the average more dissociated at 4 °C than at room temperature (M_r = 65000–67000); however, the higher-order quaternary structures are not present [8]. The apoenzyme elutes from the Sephadex G-150 column as a single, almost symmetrical peak, in contrast to the elution profile of the native enzyme (cf. Fig. 2 and 4B in [8]).

Other Physical Properties

The molar absorption coefficient of the apoenzyme at 280 nm is $7.4 \pm 0.3 \times 10^4$ M^{-1} cm^{-1}. This is an average value of three determinations.

The circular dichroic spectrum of the apoenzyme is, as far as the far-ultraviolet region is concerned, very similar, if not identical, to that of the holoenzyme indicating that no gross conformational change occurs upon removal of FAD from the holoenzyme.

Binding of FAD to the Apoprotein

Different fluorescence methods were used to determine the dissociation constant for the apoenzyme-FAD complex. The holoenzyme of *p*-hydroxybenzoate hydroxylase possesses a fluorescence yield of 65% of that of free FAD [16]. In the presence of substrate the residual fluorescence yield of the enzyme-substrate complex is 13% of that of free FAD [16]. Titration of a fixed concentration of FAD by the apoenzyme in the presence of substrate showed a linear decrease of the fluorescence of FAD, but the final fluorescence yield was larger than expected, i.e. about 20% instead of 13%. This indicates that commercial FAD, even after purification over a Bio-Gel P-2 column, still contains impurities other than riboflavin and FMN, which have been removed by column chromatography. Our results indicate that FAD purified on Bio-Gel P-2 still contains an FAD analogue (see below) not capable of binding to the apoenzyme. Therefore the relative fluorescence yield of various concentrations of FAD in the presence and absence of apoenzyme was determined [17]. The results show (Fig. 1) that FAD is rather strongly bound to the apoenzyme and that one mol of FAD is bound per mol

monomer apoenzyme, as expected. These data do not however allow us to calculate a dissociation constant. In principle the dissociation constant of the complex could also be determined by a fluorescence titration experiment which followed the degree of polarization (p) [10]. The native enzyme-substrate complex possesses a degree of polarization of 0.42 – 0.43 [9]. Titration of apoenzyme by FAD, or vice versa, always yielded a value of p which was 20% smaller than expected. This value could not be improved by addition of *Megasphaera elsdenii* apoflavodoxin or apoprotein from riboflavin-binding protein from eggs. This strongly indicates that the flavin impurity causing the depolarization is neither FMN nor riboflavin. That these fluorescence effects are not due to a possible slow conformational change of the apoprotein to acquire a conformation needed to bind FAD, as observed with apoglucose oxidase for example [18], is deduced from the following. The fluorescence yield and the degree of polarization of titrated samples remained constant over a very long period of time (1 day). The expected values were, however, observed after dialysis or gel chromatographic purification of the samples.

Since fluorescence techniques are often used in flavin and flavoprotein research we paid some additional attention to the nature of the impurity in purified FAD. A fixed concentration of FAD was incubated at 20 °C with a large excess of apoenzyme for several hours. The mixture was then chromatographed on a Bio-Gel P-6-DG column and the fractions of enzyme and free flavin collected. The free flavin obtained in this way showed no activity when added to apoenzyme, but it must possess an FAD-like structure since the fluorescence yield increases by a factor of two upon treatment with phosphodiesterase (*Naja naja* venom). Analysis of our fluorescence results according to the method described by Wassink and Mayhew [15] showed that FAD purified on Bio-Gel still contains an impurity, which amounts to 2% of the purified FAD. The fact that the fluorescence yield of modified FAD increases by a factor of only two on treatment with phosphodiesterase instead of ten, as observed for FAD [15], indicates that the quantum yield of the modified FAD is larger than that of 'natural' FAD. The structure of this impurity has not been elucidated. Nevertheless from these results it is clear that one has to be very cautious in using purified, commercially available FAD for analytical purpose employing fluorescence techniques.

To estimate the dissociation constant of the apoenzyme-FAD complex various samples of a fixed concentration of apoenzyme were incubated in the presence of different concentrations of FAD and the activity measured. The results are shown in Fig. 2. The calculated kinetic K_m value for FAD is 45 ± 5 nM. This value should not be considered as an absolute number since the dissociation constant is determined indirectly. The value of about 45 nM is in the range of dissociation constants of many other flavoproteins.

The association rate constant for formation of the complex was studied by using in various experiments a fixed concentration of apoenzyme in an assay mixture without FAD. The reaction was initiated by the addition of an excess of FAD (pseudo-first-order conditions) and the activity followed with time as a decrease of absorbance at 340 nm. p-Hydroxybenzoate and NADPH were present in concentrations of 0.2 mM to ensure maximal velocity conditions during the full time of recombination. At 10-s intervals the slope of the kinetic curve was drawn and the pseudo-first-order rate constant calculated from a plot of ln activity versus time. The second-order rate constant was obtained from a plot of the

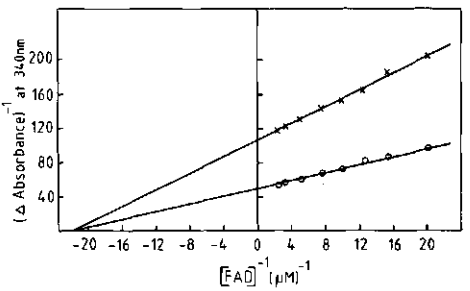

Fig. 2. *Relation of activity of apo-p-hydroxybenzoate hydroxylase to FAD concentration.* Assays were performed in 0.1 M K_2HPO_4 buffer, pH 8.0, as described in Materials and Methods. NADPH and p-hydroxybenzoate were both 0.2 mM; apoprotein concentrations were 7.5 (×) and 15 (○) nM, respectively. Velocity is expressed as NADPH oxidized min^{-1} ml^{-1} and plotted as the change in absorbance at 340 nm

pseudo-first-order rate constant against the concentration of FAD. From this slope the second-order rate constant was calculated to be $4.1 \pm 0.4 \times 10^4$ M^{-1} s^{-1}. This rate constant seems small for a second-order rate constant but lies in the range of values determined for other flavoproteins, e.g. flavodoxin from *M. elsdenii* [19].

From the dissociation constant for the apoenzyme-FAD complex and the association rate constant the dissociation rate constant was calculated to be 1.85×10^{-3} s^{-1}. This value indicates that the complex has a long lifetime and that the stability of the complex is determined by the dissociation rate constant, under the conditions of the experiments (pH, ionic strength).

Binding of NADPH and NADH to the Apoenzyme

The fact that the structure of the holoenzyme seemed to be preserved in the apoenzyme, as deduced from circular dichroic spectra, raised the question whether the affinity of the apoenzyme for NADPH was also preserved. Titration of a fixed concentration of NADPH by a solution of apoenzyme revealed that the fluorescence of NADPH was augmented by the apoenzyme, indicating complex formation between the two species. The effect of the apoenzyme on the fluorescence emission of NADPH is illustrated in Fig. 3A. The spectra have been corrected for small but significant fluorescence contributions of the buffer and the apoenzyme, and for dilution. It can be seen that the fluorescence emission increases by more than a factor of two upon complex formation. In addition the fluorescence emission maximum is shifted approximately 10 nm to shorter wavelengths as compared to that of free NADPH (461 nm). This behavior of NADPH is similar to that observed for, for example, malate dehydrogenase [17] and muscle lactate dehydrogenase [20]. The average values of several titrations are presented in a double-reciprocal plot in Fig. 3A. The dissociation constant calculated from this plot is 12.5 ± 3 μM at pH 6.5, an ionic strength of 0.025 M and 20 °C. Similar results were obtained in titration experiments where NADPH was added to a fixed concentration of apoenzyme. At infinite concentration of apoenzyme the fluorescence yield of NADPH increases by a factor of 3.2 at 445 nm and a factor of 3.8 at 430 nm as compared to those of free NADPH at the corresponding wavelengths. The dissociation constant determined under the same conditions of pH and ionic strength for the holoenzyme-

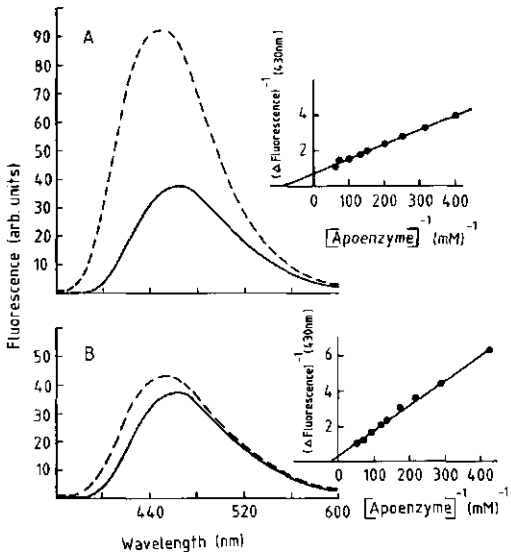

Fig. 3. *Fluorescence emission spectra of NADPH in the absence (* —— *) and the presence (----) of apo-*p*-hydroxybenzoate hydroxylase at pH 6.5 and 20 °C.* In all cases the concentration of NADPH was 5.0 μM and that of the apoenzyme was 8.22 μM. The solutions were activated with light at 350 nm and contained Mes buffer. (A) In 20 mM Mes buffer, $I = 0.025$ M; (B) in 80 mM Mes buffer, $I = 0.1$ M. The insets show the results of titration experiments in a double-reciprocal plot (fluorescence difference versus apoenzyme concentration) from which the dissociation constant of the NADPH-apoenzyme complex was calculated. All spectra, and data used in the insets were corrected for dilution, and for buffer and apoenzyme fluorescence

NADPH complex is 115 μM. This value is larger by a factor of then than that determined for the apoenzyme. This observation suggests that removal of FAD from the holoenzyme influences somewhat the structure of the NADPH binding site. The circular dichroism spectra suggests, however, that the structural change must be small (cf. below).

In principal the tryptophan fluorescence emission of the apoenzyme (excitation at 295 nm, emission maximum at 335 nm) could also be monitored to follow the formation of the complex with NADPH. However, it was found that this method gave less reliable results due to energy transfer to the pyridine nucleotide.

The interaction between the apoenzyme and NADPH is strongly dependent on the pH of the solution. The affinity of the apoenzyme for NADPH decreases very rapidly below pH 6.3 and above pH 6.7. Therefore the pH dependence of the dissociation constant was not determined over a larger pH range, but the affinity of the apoenzyme for NADPH is optimal at pH 6.5. The pH-dependent interaction of NADPH with the holoenzyme of *p*-hydroxybenzoate hydroxylase from *Pseudomonas putida* has been investigated in detail [21]. The dissociation constant was determined to be 71 μM at pH 6.0 and 670 μM at pH 8.0 [21]. The apoenzyme studied in this paper thus exhibits properties similar to those of the holoenzyme from *P. putida* with respect to the interaction with NADPH. In addition it is interesting to note that the apoenzyme of the flavoprotein salicylate hydroxylase also shows a strong interaction with the reduced form of pyridine nucleotide [22]. These observations suggest that the structure of the holoenzymes of flavoprotein hydroxylases is better maintained in their apoenzyme compared to other flavoproteins, e.g. glucose oxidase [18].

We observed another interesting effect. The affinity of the apoenzyme for NADPH is strongly dependent on the ionic strength of the solution. As demonstrated in Fig. 3 B where the same experimental conditions were used as in Fig. 3 A, except for the ionic strength of the solution, the fluorescence increase of NADPH upon binding to the apoenzyme is very small and the dissociation constant is calculated to be 43 ± 5 μM. The published dissociation constants of the NADPH-holoenzyme complexes from *P. fluorescens* [1] and *P. putida* [21] were determined at different ionic strengths. Preliminary experiments with the holoenzyme from *P. fluorescens* indicate that the interaction with NADPH is also strongly dependent on the ionic strength (unpublished results). Therefore the published values cited above for the holoenzyme-NADPH complexes cannot be compared directly with the dissociation constants of the apoenzyme.

With the holoenzyme it has been observed that the kinetic K_m value for NADPH at pH 6.6 increases in the presence of the substrate and decrease with increasing pH [1, 21]. It was not possible to study the effect of *p*-hydroxybenzoate on the dissociation constant of the apoenzyme-NADPH complex because of the small residual catalytic activity of the particular apoenzyme preparation used in this study (cf. Table 1). It should be noted that the residual NADPH oxidase activity of the apoenzyme, which is a very slow reaction in the absence of the substrate [1], did not prevent an accurate determination of the dissociation constant of the NADPH-apoenzyme complex, as also judged by a control experiment.

In analogy to the holoenzyme from *P. putida* [21], the apoenzyme from *P. fluorescens* is also capable of binding NADH. The dissociation constant for the apoenzyme-NADH complex, determined in the same way as that for the NADPH complex, was calculated to be 23 ± 3 μM at pH 6.5 and $I = 0.025$ M. At infinite concentration of apoenzyme the fluorescence yield of bound NADH increased by a factor of 3.3 at 450 nm with respect to that of free NADH. Neither the dissociation constant nor the fluorescence emission maximum of the apoenzyme-NADH complex were affected by the presence of substrate.

Binding of the Substrate to the Apoenzyme

The preservation of the NADPH binding site in the apoenzyme suggested that possibly the binding site for the substrate is also unaltered in the apoprotein. That this is the case could be best demonstrated with circular dichroism. The apoenzyme shows a positive Cotton band at 290 nm (Fig. 4). In fact the pattern of the circular dichroic spectrum of the holoenzyme is very similar to that shown for the apoenzyme. Addition of substrate to the apoenzyme induces a reduction of the intensity of the band at 290 nm in the spectrum and a new band is formed with a peak at about 303 nm. The curves produced in the presence of various concentrations of the substrate form an isosbestic point at 281 nm (Fig. 4). From a double-reciprocal plot of titration data (Fig. 4, inset) the dissociation constant for the apoenzyme-substrate complex was calculated to be 30 ± 3 μM. This value compares favourably with that for the holoenzyme-substrate complex (25 μM) [1]. The same value was found by monitoring the decrease of the fluorescence emission of the apoenzyme upon addition of substrate. The fluorescence yield of the apoenzyme-substrate

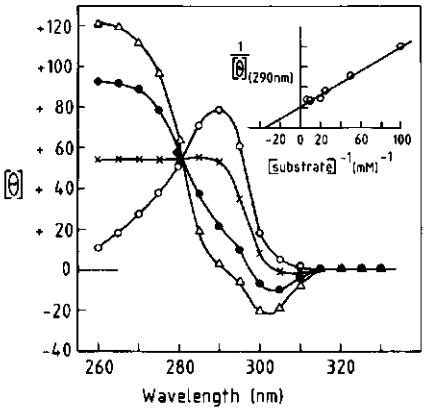

Fig. 4. *Circular dichroism spectra of apo*-p-*hydroxybenzoate hydroxylase in the absence and presence of the substrate* p-*hydroxybenzoate.* The mean residue (394 amino acid residues [5]) ellipticity [θ] in degrees/cm^2 decimol is plotted against the wavelength (nm). The concentration of the apoenzyme was 13.0 μM in 80 mM Epps buffer, pH 8.05, $I = 0.1$ M. The curves represent free enzyme (○) and in the presence of 10 μM (×), 40 μM (○) and 200 μM (△) substrate. All curves were corrected for the base line and for dilution. The inset shows the data of titration experiments in a double-reciprocal plot

complex is decreased by 10% as compared to that of free apoenzyme.

The results presented in this paper strongly indicate that the structure of the apoenzyme is very similar, if not identical, to that of the holoenzyme with respect to the binding sites for the substrate, NAD(P)H and FAD. This explains the easy reconstitutability of the activity of the holoenzyme. The fact that the hydrodynamic properties of the holoenzyme differ from those of the apoenzyme, in that the holoenzyme forms higher-order quaternary structures, suggests that it is mainly the conformation of amino acid residues on the surface of the protein that is influenced upon removal of the prosthetic group.

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Chapter 3

Large-scale preparation and reconstitution of apo-flavoproteins with special reference to butyryl-CoA dehydrogenase from *Megasphaera elsdenii*

Hydrophobic-interaction chromatography

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A new method is described for the large-scale reversible dissociation of flavoproteins into apoprotein and prosthetic group using hydrophobic-interaction chromatography.

Lipoamide dehydrogenase from *Azotobacter vinelandii* and butyryl-CoA dehydrogenase from *Megasphaera elsdenii* are selected to demonstrate the usefulness of the method. In contrast to conventional methods, homogeneous preparations of apoproteins in high yields are obtained. The apoproteins show high reconstitutability.

The holoenzymes are bound to phenyl-Sepharose CL-4B at neutral pH in the presence of ammonium sulfate. FAD is subsequently removed at pH 3.5–4.0 by addition of high concentrations of KBr. Large amounts of apoenzymes (200–500 mg), showing negligible residual activity, are eluted at neutral pH in the presence of 50% ethylene glycol.

The holoenzyme of lipoamide dehydrogenase can be reconstituted while the apoprotein is still bound to the column or the apoenzyme can be isolated in the free state. In both cases the yield and degree of reconstitution of holoenzyme is more than 90% of starting material. Apo-lipoamide-dehydrogenase exists mainly as a monomer in solution and reassociates to the native dimeric structure in the presence of FAD. The apoenzyme is stable for a long period of time when kept in 50% ethylene glycol at – 18°C.

Steady-state fluorescence-polarization measurements of protein-bound FAD indicate that reconstituted lipoamide dehydrogenase possesses a high stability which is governed by the low dissociation rate constant of the apoenzyme-FAD complex.

The holoenzyme of butyryl-CoA dehydrogenase cannot be reconstituted when the apoenzyme is bound to the column. However, stable apoprotein can be isolated in the free state yielding 50–80% of starting material, depending on the immobilization conditions. The coenzyme A ligand present in native holoenzyme is removed during apoprotein preparation. The apoenzyme is relatively stable when kept in 50% ethylene glycol at – 18°C.

From kinetic and gel filtration experiments it is concluded that the reconstitution reaction of butyryl-CoA dehydrogenase is governed by both the pH-dependent hydrodynamic properties of apoenzyme and the pH-dependent stability of reconstituted enzyme. At pH 7, the apoenzyme is in equilibrium between dimeric and tetrameric forms and reassociates to a native-like tetrameric structure in the presence of FAD. The stability of reconstituted enzyme is strongly influenced by the presence of CoA ligands as shown by fluorescence-polarization measurements.

The degree of reconstitution of butyryl-CoA dehydrogenase is more than 80% of the original specific activity under certain conditions. Unliganded reconstituted enzyme is easily regreened in the presence of CoASH and Na_2S.

The large-scale preparation and reconstitution of the apoproteins of glutathione reductase from human erythrocytes and mercuric reductase from *Pseudomonas aeruginosa* is also described.

Lipoamide dehydrogenase and butyryl-CoA dehydrogenase are both representative members of different important classes of flavoproteins [1, 2]. Lipoamide dehydrogenase is a member of the flavin-containing disulfide oxido-reductases catalyzing the re-oxidation of dihydrolipoamide by NAD^+. *In vivo*, the enzyme functions in the oxidative decarboxylation of both 2-oxoglutarate and pyruvate as part of multi-enzyme complexes [1].

Butyryl-CoA dehydrogenase is a member of the fatty-acid-acyl-CoA dehydrogenases catalyzing the oxidation of saturated acyl-CoA thioesters to give the corresponding *trans*-enoyl-CoA analogue. *In vivo*, the bacterial enzyme from *Megasphaera elsdenii* acts in the reverse direction producing the reduced butyryl-CoA [2].

Both enzymes contain FAD as a prosthetic group which is non-covalently, but tightly, bound to the apoenzymes. Though lipoamide dehydrogenase from *Azotobacter vinelandii* and butyryl-CoA dehydrogenase from *M. elsdenii* can be obtained in very high yield [3, 4], no methods have been

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Enzymes. Lipoamide dehydrogenase, NADH:lipoamide oxidoreductase (EC 1.8.1.4); glutathione reductase, NADPH:glutathione oxido-reductase (EC 1.6.4.2); mercuric reductase, NADPH:mercuric ion oxidoreductase (EC 1.16.1.1); butyryl-CoA dehydrogenase, butyryl-CoA:(acceptor) oxidoreductase (EC 1.3.99.2).

Abbreviation. Cl_2Ind, 2,6-dichloroindophenol.

reported for the preparation of stable apoproteins in large amounts to allow mechanistic studies on reconstituted enzymes.

Several methods have been described for the preparation of reconstitutable apo-flavoproteins [5]. Reconstitution depends very much on the protein used and most methods are only suitable for small-scale preparations.

When performing biophysical studies on reconstituted apo-flavoproteins where the non-covalently bound prosthetic group is replaced by artificial flavins [6], it is very important to use preparations showing negligible residual activity. Moreover, in NMR studies where the prosthetic group is replaced by ^{13}C- or ^{15}N-enriched flavins [7] only large amounts of well-defined reconstituted enzymes will allow a thorough physical characterization.

We have already shown that affinity chromatography can be a potent alternative for apoprotein preparation [8]. In the particular case of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* both low residual activity and high yield of fully reconstitutable apoenzyme could be achieved by using Sepharose-5,5'-dithiobis(2-nitrobenzoate) covalent affinity chromatography [8]. Because the use of this kind of chromatography is limited to flavoproteins showing the presence of well-characterized, accessible sulfhydryl groups [9] we looked for a more generally applicable method, taking advantage of the immobilization procedure.

In this paper we report on the large-scale reconstitution of several apo-flavoproteins by using hydrophobic-interaction chromatography. It is shown that immobilization of the different enzymes used allows resolution of apoprotein and flavin under relatively mild pH conditions, in contrast to conventional methods. Some physical properties of the apo and reconstituted enzymes, with special attention to butyryl-CoA dehydrogenase, are also described.

MATERIALS AND METHODS

General

FAD (F-6625), FMN (F-6750), lipoamide (oxidized), glutathione (oxidized), butyryl-CoA, acetoacetyl-CoA, Bis-tris, Mes, Hepes, Hepps, phenazine ethosulfate and *Naja naja atra* snake venom were from Sigma. Dithiothreitol, Tris, NAD^+, NADH, NADPH, ovalbumin, bovine serum albumin, alcohol dehydrogenase, α-chymotrypsinogen and myoglobin were products of Boehringer. 2,6-Dichloroindophenol was purchased from BDH.

DEAE-Sepharose CL-6B, phenyl-Sepharose CL-4B, thiopropyl-Sepharose 6B, Sepharose 6B, Sephacryl-S200, Superose 12 preparative grade, prepacked Mono Q HR 5/5 and prepacked Superose 12 HR 10/30 were all products of Pharmacia. Bio-Gel P-6DG was obtained from Bio-Rad. All other chemicals were from Merck and the purest grade available.

Transformed *Escherichia coli* TG-2 cells containing the lipoamide-dehydrogenase gene from *Azotobacter vinelandii* [3] were kindly provided by Mr A. H. Westphal. Cells of *Megasphaera elsdenii*, strain LC 1 (ATCC 25940), were maintained and grown in iron-poor media as described by Mayhew and Massey [10]. Lipoamide dehydrogenase from transformed *E. coli* cells was purified as described by Westphal and De Kok [3]. Butyryl-CoA dehydrogenase was purified from *M. elsdenii* essentially according to the method described by Engel [4], as modified by Fink et al. [11]. The DEAE-cellulose column was replaced by a DEAE-Sepharose CL-6B column of the same size. Mercuric reductase from *Pseudomonas aeruginosa* PAO 9501 (pVS1) was a gift of Dr S. Lindskog. Glutathione reductase from human erythrocytes was a gift of Dr B. Mannervik.

The enzyme activities of lipoamide dehydrogenase, mercuric reductase and glutathione reductase were measured using standard conditions [3, 12, 13]. Butyryl-CoA dehydrogenase activity was assayed as described by Williamson and Engel [14] except that, for better buffering capacity, the phosphate buffer was replaced by 100 mM Tris/sulfate, pH 8.0, giving 90% activity of that in the phosphate buffer system. When dithiothreitol was present the enzyme samples were first gel-filtered over Bio-Gel P-6DG in 100 mM K_2HPO_4, pH 7.0, containing 0.5 mM EDTA.

Enzyme concentrations were determined by measuring the absorbance of protein-bound FAD using the following molar absorption coefficients: lipoamide dehydrogenase, $\varepsilon_{458} = 11.3\ mM^{-1}\ cm^{-1}$ [3]; mercuric reductase, $\varepsilon_{455} = 11.3\ mM^{-1}\ cm^{-1}$ [12]; glutathione reductase, $\varepsilon_{462} = 11.3\ mM^{-1}\ cm^{-1}$ [13]; green butyryl-CoA dehydrogenase, $\varepsilon_{430} = 10.4\ mM^{-1}\ cm^{-1}$ [14]; yellow liganded butyryl-CoA dehydrogenase, $\varepsilon_{450} = 14.2\ mM^{-1}\ cm^{-1}$ [14] and yellow unliganded butyryl-CoA dehydrogenase, $\varepsilon_{450} = 14.4\ mM^{-1}\ cm^{-1}$ [14].

Protein concentrations were determined by the method of Lowry [15] using bovine serum albumin as an standard.

Molar absorption coefficients of apoenzymes were determined at 280 nm by comparison of the absorbance of apo and reconstituted enzyme using the gel-filtration procedure described earlier [8].

Enzyme activity measurements were performed at 25°C using a Zeiss PMQ II spectrophotometer. Absorption spectra were recorded on an Aminco DW-2A spectrophotometer at 25°C and fluorescence measurements on an Aminco SPF-500 fluorimeter at 20°C. Both instruments were equipped with thermostatted cell holders.

Enzyme purity was checked by both sodium dodecyl sulfate gel electrophoresis using 15% slab gels [16] and Mono Q analytical anion-exchange chromatography using an FPLC system [9].

Phenyl-Sepharose CL-4B was regenerated by successively washing the column with 2 vol H_2O, ethanol, butanol, ethanol and H_2O. For longer periods the column was stored in 25% ethanol (by vol.) at 4°C.

Analytical methods

Apoprotein samples were stored at a concentration of about 10 mg/ml at −18°C in 100 mM K_2HPO_4, pH 7.0, containing 0.5 mM EDTA and 50% ethylene glycol. Before storage the samples were concentrated at 4°C with aid of an Amicon ultrafiltration apparatus using YM-30 filters.

Sedimentation velocity experiments were performed at 20°C in 50 mM K_2HPO_4, pH 7.0, containing 0.5 mM EDTA as described earlier [17], using an MSE Centriscan 75 analytical ultracentrifuge.

Analytical gel filtration was performed, by injection of 100-μl samples, on a Superose 12 HR 10/30 column in 50 mM K_2HPO_4, pH 7.0, containing 150 mM KCl, 0.5 mM EDTA. The flow rate was 0.5 ml/min. The absorbance was monitored at 280 nm. Gel-filtration experiments to monitor the reconstitution of apo-butyryl-CoA dehydrogenase were performed either in 100 mM Mes, pH 6.0, 100 mM Hepes, pH 7.0, 100 mM K_2HPO_4, pH 7.0 or 100 mM Hepps, pH 8.0. For these particular experiments, the apoenzyme used was first gel-filtered over Bio-Gel P-6DG in the appropriate buffer to remove ethylene glycol.

Steady-state fluorescence polarization of native and reconstituted enzymes was determined at 20°C, as described previously [18]. The excitation wavelength was 450 nm while KV 515 filters were used to cut off the emission light. A spectral band width of 3 nm was used in all experiments. High concentrations of native or reconstituted enzymes (approximately 50 μM) were freshly gel-filtered over Bio-Gel P-6DG in the appropriate buffer and then diluted stepwise. A solution of FMN (0.5 μM), freshly prepared each day, was used as a standard to estimate the relative fluorescence quantum yield of the different enzyme samples. FAD used for polarization experiments was purified by gel filtration on Bio-Gel P-2, as reported earlier [8].

Large-scale reconstitution of apo-butyryl-CoA dehydrogenase was perfomed by incubation of 100 μM apoprotein with a fivefold molar excess of FAD overnight at 4°C. Excess FAD was removed by gel filtration over Bio-Gel P-6DG in 100 mM K_2HPO_4, pH 7.0, containing 0.5 mM EDTA, unless stated otherwise.

Reconstitution of apo-butyryl-CoA dehydrogenase on an analytical scale was done by treatment of 13.5 μM apoprotein with various concentrations of FAD (100–300 μM), either in the absence or presence of 1 mM CoASH or 0.5 mM acetoacetyl-CoA. The time-dependent increase of enzyme activity was followed by the standard assay procedure. In the presence of acetoacetyl-CoA the time-dependent increase of absorbance was followed at 580 nm, using a molar absorption coefficient of 4.5 mM^{-1} cm^{-1} [11] or by recording the visible absorption spectrum. These parameters were also used to quantify the reaction. Samples were also withdrawn from the incubation mixtures in order to check the increase in enzyme activity with time.

Regreening of reconstituted butyryl-CoA dehydrogenase was quantified by time-dependent recording of the absorption spectrum, using a molar absorption coefficient at 710 nm of 5.6 mM^{-1} cm^{-1} [19].

RESULTS

Preparation of apo-flavoproteins

Lipoamide dehydrogenase (10 μmol, 500 mg in 70 ml) was loaded on a phenyl-sepharose column (2.5 × 10 cm) at 20°C in 50 mM K_2HPO_4, containing 1.7 M ammonium sulfate, 1 M KBr and 0.5 mM EDTA (final pH 7.0) at a flow rate of 1 ml/min. Under these conditions the capacity of phenyl-Sepharose (Table 1) can easily be checked by eye, owing to the bright yellow band of protein-bound FAD. All further steps were performed at a constant flow rate of 3 ml/min. The column was then washed with 2 vols starting buffer. No protein or FAD was eluted under these conditions. FAD was eluted with 180 ml of the same buffer adjusted to pH 4 with phosphoric acid. The release of FAD was followed by monitoring the fluorescence emission of the eluate at 525 nm. After FAD elution the column was immediately washed with 2 vols starting buffer, omitting 1 M KBr, and 2 vols 0.5 mM EDTA in 100 mM K_2HPO_4, pH 7.0. The apoprotein was eluted by the final buffer containing 50% ethylene glycol and stored at −18°C, if storage over a long period of time was needed. Under these conditions the apoprotein can be kept for months without loss of reconstitutable activity.

The apoproteins of butyryl-CoA dehydrogenase, mercuric reductase and glutathione reductase were prepared essentially by the same procedure as described above for lipoamide dehydrogenase. In order to obtain both low residual activity and high yield of apoprotein the composition of the FAD eluting buffer was varied slightly for each individual enzyme used (Table 1). The apoproteins of mercuric reductase and glutathione reductase were not isolated in the free state. Large-scale production of reconstituted enzymes was performed on the column as described below.

Table 1. *Preparation of apo flavoproteins using hydrophobic-interaction chromatography*

100 mg enzyme in 100 mM K_2HPO_4, 1.7 M ammonium sulfate, pH 7.0, was loaded onto a phenyl Sepharose CL-4B column (10 ml) and treated as described under Results. All experiments were performed at 20°C. n.d. = not determined

Enzyme	Column capacity (bed vol.)	FAD elution buffer	Yield apoprotein
	mg/ml	pH	%
Lipoamide dehydrogenase	25 ± 5	4.0	95 ± 5
Butyryl-CoA dehydrogenase	12 ± 4	3.7	50 ± 5
			80 ± 5[a]
Glutathione reductase	25 ± 5	3.5	n.d.
Mercuric reductase	25 ± 5	3.5	n.d.

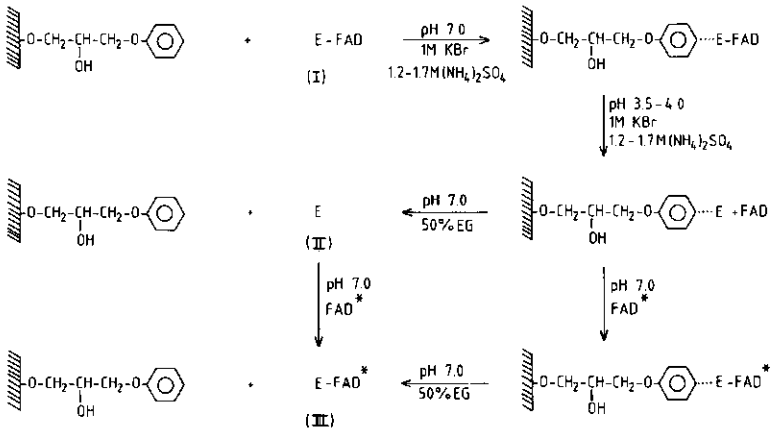
[a] Apoprotein was prepared using 1.2 M ammonium sulfate.

Butyryl-CoA dehydrogenase is usually isolated in a green form [4]. This green colour is due to 'charge-transfer' interaction between a CoA persulfide ligand and protein-bound FAD [19]. Reduction of green liganded enzyme by dithionite yields yellow liganded enzyme after reoxidation [19]. The reduced CoA ligand can be removed by thiopropyl-Sepharose, yielding yellow unliganded enzyme [11, 20].

Both green and yellow liganded enzymes can be used for apoprotein preparation. The capacity of phenyl-Sepharose for these enzymes is lower than that for lipoamide dehydrogenase (Table 1). For large-scale production of apo-butyryl-CoA dehydrogenase 6 μmol (240 mg in 40 ml) enzyme were loaded onto the column. Apo-butyryl-CoA dehydrogenase is relatively stable when kept at −18°C in 50% ethylene glycol. After six months, 80% of the original activity could be restored on addition of excess FAD.

The residual activity of both apo-lipoamide dehydrogenase and apo-butyryl-CoA dehydrogenase was less than 1% as compared to the activities of the corresponding native holoenzymes. The yield of apo-lipoamide dehydrogenase was more than 90% of starting material (Table 1). Using the standard procedure of apoprotein preparation, as described above, the yield of apo-butyryl-CoA dehydrogenase was only 50% of starting material (Table 1). Upon elution of FAD at pH 3.7, about 50% of apoprotein became too tightly bound to the hydrophobic matrix, preventing elution at pH 7.0, even in the presence of high concentrations of ethylene glycol. The strongly immobilized apoprotein could only be eluted under denaturing conditions using high concentrations of ethanol as confirmed by both protein determination and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. In contrast, native holoenzyme could be eluted with full recovery from the column with 50 mM K_2HPO_4 containing 0.5 mM EDTA, pH 7.0.

In order to increase the yield of apo-butyryl-CoA dehydrogenase, the conditions of apoprotein preparation were studied in more detail. Optimal recovery (≈ 80%) of apoprotein was obtained using 1.2 M ammonium sulfate in all steps where ammonium sulfate was included in the buffer (Table 2). Under



Scheme 1. *Schematic representation of the preparation of apoflavoproteins by immobilization on phenyl Sepharose CL-4B.* FAD* denotes the free flavin used in the reconstitution reaction

these conditions the capacity of phenyl-Sepharose for butyryl-CoA dehydrogenase was about 10 mg/ml column material. Similar results were obtained at 4 °C.

Reconstitution of matrix-bound apoenzymes

Lipoamide dehydrogenase could easily be reconstituted while the apoprotein was still bound to the column. Passing a small molar excess of FAD in 100 mM K_2HPO_4, 0.5 mM EDTA, pH 7.0, over the coumn spontaneously yielded a very fluorescent band, characteristic for the relatively high fluorescence quantum yield of this enzyme [18]. After washing the column with 100 mM K_2HPO_4, 0.5 mM EDTA, pH 7.0 to remove excess of free flavin, the holoenzyme was eluted in a sharp band using the same buffer containing 50% ethylene glycol (Table 2).

Mercuric reductase and glutathione reductase could be reconstituted essentially by the same procedure as described above for lipoamide dehydrogenase. However, for these enzymes the yield of reconstitutable enzyme was lower, as can be seen from Table 2. The absorption spectra and specific activities of the reconstituted disulfide oxidoreductases were identical with those of the starting holoenzymes.

Butyryl-CoA dehydrogenase could not be reconstituted while the apoprotein was bound to the column. Phenyl-Sepharose showed a very high affinity for the apoprotein under the conditions used preventing reconstitution. The principle of apoprotein preparation on phenyl-Sepharose CL-4B and reconstitution with FAD either on the matrix or in solution is summarized in Scheme 1.

Molar absorption coefficients of apoenzymes

The molar absorption coefficient of highly pure apolipoamide-dehydrogenase subunit at 280 nm is 27 ± 1 mM^{-1} cm^{-1}. This value is much lower than reported earlier [21] but is in good agreement with the low content of aromatic amino acid residues, as found by sequence analysis [3].

The molar absorption coefficient of highly pure apobutyryl-CoA dehydrogenase subunit at 280 nm is 49 ± 1 mM^{-1} cm^{-1}. From the ultraviolet absorption spectrum (Fig. 1 A) it can be concluded that the coenzyme-A ligand is removed during apoprotein preparation (see also below).

Table 2. *Reconstitution of apo flavoproteins prepared by hydrophobic-interaction chromatography*
Apoprotein, bound to phenyl Sepharose CL-4B in 100 mM K_2HPO_4, pH 7.0, was reconstituted by recycling a fivefold molar excess of FAD over the column. Apoprotein, eluted from the column in the presence of 50% ethylene glycol, was reconstituted by the addition of a fivefold molar excess of FAD and checked for time-dependent activity increase by the standard assay procedures. All experiments were performed at 20 C. n.d. = not determined

Enzyme	Reconstitution on matrix		Reconstitution in solution
	yield	specific activity	specific activity
	%		
Lipoamide dehydrogenase	95 ± 5	100	100
Butyryl-CoA dehydrogenase	[a]	–	80 ± 5
			85 ± 5 [b]
Glutathione reductase	60 ± 5	90 ± 5	n.d.
Mercuric reductase	75 ± 5	90 ± 5	n.d.
	85 ± 5	90 ± 5 [b]	n.d.

[a] Not reconstitutable on the column.
[b] Apoprotein was prepared using 1.2 M ammonium sulfate.

Subunit composition of apo and reconstituted enzymes

The relative molecular mass of holo, apo and reconstituted enzymes was determined at 20 C by gel filtration through Superose-12, sedimentation analysis in an analytical ultracentrifuge and sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. The results summarized in Table 3 show that the apoprotein of lipoamide dehydrogenase exists mainly as a monomer in solution which was also reported for the wild-type *A. vinelandii* enzyme [21]. The reconstituted enzyme showed the same dimeric structure as native enzyme.

Table 3. *Relative molecular mass of apo and reconstituted enzymes*
The relative molecular mass of lipoamide dehydrogenase and butyryl-CoA dehydrogenase was determined by FPLC on a Superose-12 column (M_r) sedimentation velocity ($s_{20,w}$) and SDS/PAGE (M_r) analysis. For details see Materials and Methods. For gel filtration and ultracentrifugation the enzyme concentration ranged over 1–5 mg/ml

Enzyme	$10^{-3} \times M_r$ gel filtration	$s_{20,w}$ by ultracentrifugation	$10^{-3} \times M_r$ by SDS/PAGE
		S	
Lipoamide dehydrogenase			
Holo	112	5.9	–
Apo	59 (85%) 112 (15%)	3.7	58
Reconstituted	112 (91%)	5.9	–
Butyryl-CoA dehydrogenase			
Holo (green)	160	8.1	–
Apo	160 (30%) 75 (70%)	5.6	40
Reconstituted (yellow)	160 (90%) 75 (10%)	8.0	–
Reconstituted (green)	160	8.0	–

The apoprotein of butyryl-CoA dehydrogenase exists mainly as a dimer in solution (Table 3). Upon addition of excess FAD, the enzyme reassociates to the native tetrameric structure yielding the yellow unliganded form (Table 3). The subunit composition of apo-butyryl-CoA dehydrogenase is strongly dependent on both pH and enzyme concentration. For instance, in 100 mM K_2HPO_4, pH 7.0, the apoenzyme is in equilibrium between the dimeric and tetrameric forms showing a higher dissociation at lower enzyme concentrations (Table 4). From the data of Table 4, an apparent dissociation constant of about 25 μM is estimated for the dimer/tetramer equilibrium of apoenzyme molecules at pH 7.0.

The presence of KCl in the buffer favours, to some degree, association to the tetrameric form, suggesting hydrophobic interaction between dimeric molecules (Table 4). Apoprotein kept for one month at −18°C in 50% ethylene glycol at pH 7.0 showed almost complete formation of dimers, as judged by chromatography on Superose-12.

At pH 6.0, 100 mM Mes, the subunit composition of apo-butyryl-CoA dehydrogenase is much more complex showing the presence of, at least, monomeric, dimeric, tetrameric and polymeric forms (Table 4). Increasing the concentration of apoenzyme strongly favours the formation of polymers eluting in the void volume of the Superose-12 column.

Some properties of reconstituted lipoamide dehydrogenase

In contrast to observations made when the apoprotein was prepared at pH 1.5 [21] the FAD absorption spectrum of reconstituted lipoamide dehydrogenase, prepared either on the matrix or in solution (Table 2), was identical with that of native holoenzyme, with maxima in the visible region at 360 nm and 458 nm and characteristic shoulders at 430 nm and 485 nm.

The kinetics of the reconstitution reaction have not been studied in detail. However, upon incubation of the apoprotein with excess FAD either at 0°C or 25°C, under conditions

Table 4. *Analytical gel chromatography of apo butyryl-CoA dehydrogenase on Superose 12*
Freshly prepared apoprotein was first gel-filtered over Bio-Gel P-6DG in the appropriate buffer. Aliquots of 100 μl were injected onto the column. The flow rate was 0.5 ml/min. All experiments were done at 20°C

Buffer	Species at apoprotein concentration of			
	15 μM		67 μM	
		%		%
50 mM K_2HPO_4 150 mM KCl, pH 7.0	tetramer dimer	30 70	tetramer dimer	60 40
100 mM K_2HPO_4, pH 7.0	tetramer dimer	30 70	tetramer dimer	50 50
100 mM Mes, pH 6.0	polymer tetramer dimer monomer	25 25 40 10	polymer tetramer dimer monomer	50 20 20 10

described elsewhere [21], the lipoamide activity is fully regained within 5 min at both incubation temperatures. Therefore, our results indicate that the apoprotein prepared on the hydrophobic matrix at pH 4.0 has a more native structure than the apoprotein prepared by the classical acid/ammonium sulfate procedure. For such preparations, a relatively slow biphasic process of reconstitution was reported at 0°C [21].

The properties of reconstituted lipoamide dehydrogenase have also been studied by steady-state flavin-fluorescence-polarization experiments. At pH 7.0, 100 mM K_2HPO_4, freshly gel-filtered reconstituted enzyme posesses a degree of polarization of 0.43–0.44. These values agree with those found for native holoenzyme and are in accordance with results obtained for wild-type *A. vinelandii* enzyme [22]. Upon dilution of reconstituted enzyme to catalytic concentrations, no change was observed in the degree of polarization of protein-bound FAD, indicating very tight binding of the prosthetic group. The quantum yield of freshly gel-filtered reconstituted enzyme was the same as that found for native enzyme [22] and comparable to that of free FMN.

Light absorption spectra of reconstituted butyryl-CoA dehydrogenase

The ultraviolet and visible absorption spectra of reconstituted butyryl-CoA dehydrogenase (Fig. 1 B and C) compare favorably with those of native unliganded enzyme [11]. The suggestion that the coenzyme-A ligand [19] is removed during apoprotein preparation is confirmed by regreening of reconstituted enzyme. In the presence of a preincubated mixture of CoASH and Na_2S, reconstituted enzyme can be 80% regreened within 30 min (Fig. 1 D), while in the presence of Na_2S alone no regreening occurs. Regreening of the enzyme leads to a shift of the maximum at 450 nm to 430 nm and an isosbestic point at 513 nm is observed. From the inset of Fig. 1 E a pseudo-first-order rate constant, $k' = 0.46\ min^{-1}$, is obtained which compares well with the regreening rate of native enzyme [19].

Kinetics of reconstitution of apo-butyryl-CoA dehydrogenase

Reconstitution of apo-butyryl-CoA dehydrogenase by FAD, as monitored by activity measurements, is a relatively

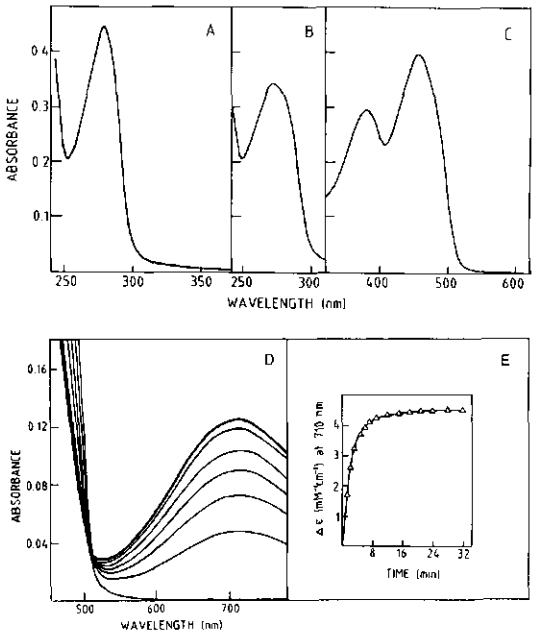


Fig. 1. *Light absorption spectra of apo and reconstituted butyryl-CoA dehydrogenase.* All spectra were recorded in 100 mM K_2HPO_4, pH 7.0, 0.5 mM EDTA. The temperature was 25°C. Cells of 10 mm path length were used. All other details are given under Materials and Methods. (A) Ultraviolet absorption spectrum of 9 μM apo-butyryl-CoA dehydrogenase. (B) Ultraviolet absorption spectrum of 4.9 μM unliganded, reconstituted butyryl-CoA dehydrogenase. (C) Visible absorption spectrum of 27.8 μM unliganded, reconstituted butyryl-CoA dehydrogenase. (D) Light absorption spectra observed during regreening of 27.8 μM unliganded, reconstituted butyryl-CoA dehydrogenase. The reaction was initiated by 20-fold dilution of a mixture of 4 mM CoASH and 20 mM Na_2S (preincubated at pH 7 for 15 min) into the enzyme solution. The absorbance changes were followed by scanning the spectra respectively at 60-s intervals until the reaction was completed. For clarity, only a few spectra are shown, recorded at t = 0, 1, 2, 3, 4.5, 9 and 32 min after mixing. (E) The inset shows the increase of absorbance at 710 nm with time

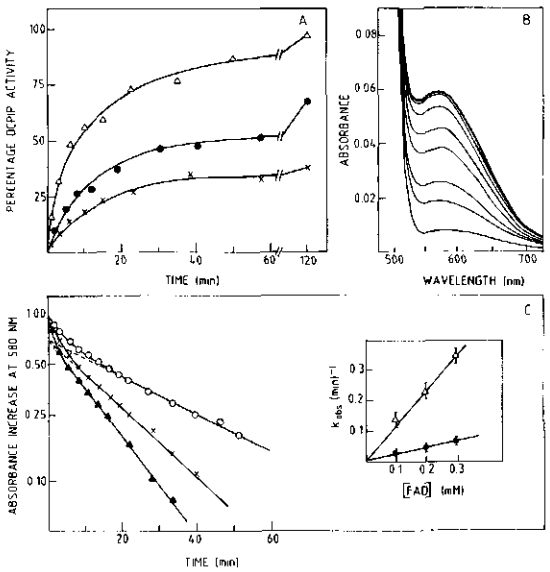
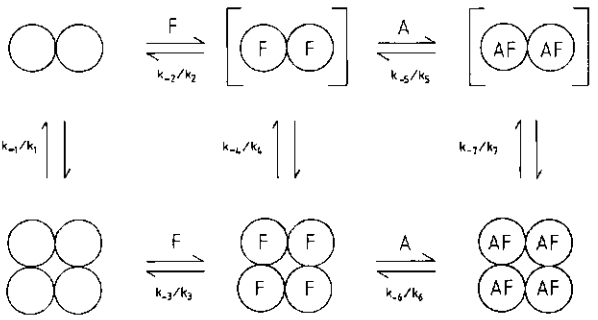


Fig. 2. *Reconstitution of apo-butyryl-CoA dehydrogenase by FAD in the absence and presence of CoA derivatives.* All experiments were performed in 100 mM K_2HPO_4, 0.5 mM EDTA, pH 7.0 at 25°C. (A) The time-dependent reactivation of 13.5 μM apo-butyryl-CoA dehydrogenase in the presence of 200 μM FAD: in the absence (●——●) and presence of 1 mM CoASH (Δ——Δ), or the presence of 0.5 mM acetoacetyl-CoA (×——×). Aliquots were withdrawn from the incubation mixtures at intervals and assayed for Cl_2Ind activity after dilution. The relative rate was determined by correction for the blank rate in the absence of phenazine ethosulfate. The plotted values are expressed relative to the Cl_2Ind activity of native liganded enzyme (50% green). The lower curve has not been corrected for the competitive inhibition effect of acetoacetyl-CoA (see text). DCPIP = Cl_2Ind. (B) Light absorption spectra observed during the reconstitution of 13.5 μM apo-butyryl-CoA dehydrogenase in the presence of 200 μM FAD and of 0.5 mM acetoacetyl-CoA. Before initiating the reaction, a base line was recorded in the absence of FAD. The absorbance changes were followed by repetitive scanning until the reaction was completed. For clarity, only a few spectra are shown, recorded at t = 1, 3.5, 6, 14, 22, 33.5, 52 and 65 min (at 580 nm) after mixing. (C) Kinetics of the reconstitution of 13.5 μM apo-butyryl-CoA dehydrogenase in the presence of 0.5 mM acetoacetyl-CoA, as a function of the concentration of FAD: 100 μM (○——○), 200 μM (×——×) and 300 μM (▲---▲) FAD. The time-dependent increase of absorbance at 580 nm was normalized by giving the difference of absorbance at zero time a value of 1.00 and at t = 90 min a value of 0. The inset shows the relationship between the apparent pseudo-first-order rate constants (k_{obs}) of the reconstitution reactions and the FAD concentrations

slow process taking about 2 h for completition at pH 7.0 and 25°C. As can be seen from Fig. 2A reactivation is stimulated in the presence of CoASH. The apparently slow reactivation in the presence of acetoacetyl-CoA (lower curve in Fig. 2A) can be explained by the following observations. In control experiments, using native liganded enzyme (50% green) as a reference, the addition of 1 mM CoASH to the incubation mixture did not influence the activity measurements (after correction for the blank reaction of CoASH with Cl_2Ind). However the addition of 0.5 mM acetoacetyl-CoA to the incubation mixture caused 60% inhibition, in agreement with the reported K_i value of 0.12 μM [2]. Therefore, when the lower curve in Fig. 2A is corrected for this competitive inhibition effect, acetoacetyl-CoA mimics the stimulating effect observed for CoASH.

In order to study the binding of FAD in more detail, the kinetics of the reconstitution reaction were followed in the presence of acetoacetyl-CoA by monitoring 'charge-transfer complex' formation between protein-bound FAD and protein-bound acetoacetyl-CoA (Fig. 2B). At pH 7.0, the kinetics at 580 nm are biphasic (Fig. 2C) and parallel those of the reactivation reaction as monitored by activity measurements (cf. Fig. 2A).

'Charge-transfer complex' formation follows pseudo-first-order kinetics when using different amounts of excess FAD (Fig. 2C). Extrapolation of the curves of the slower reaction to zero time yields an intercept at about 0.7 on the ordinate, indicating that the fast step of 'charge-transfer complex' formation is related to a fixed amount of enzyme. Moreover, monitoring 'charge-transfer complex' formation as described above, using 27 μM enzyme yields an intercept at about 0.5 on the ordinate (results not shown). The pseudo-first-order



Scheme 2. *Possible mechanisms of reconstitution of apo-butyryl-CoA dehydrogenase by FAD (F) and in the presence of coenzyme A derivatives (A).* No experimental evidence is available for the species between brackets

rate constants for 'charge-transfer complex' formation can be calculated from the slopes of Fig. 2C according to [23]:

$$k_1 = \frac{\text{initial slope} - k_2}{1 - F} \qquad (1)$$

where k_1 is the pseudo-first-order rate constant of the reaction of the more rapidly reactivated fraction of apoprotein, k_2 is the pseudo-first-order rate constant of the reaction of the more slowly reactivated fraction of apoprotein and F represents the fraction of the more slowly reactivated apoprotein. From a secondary plot of the pseudo-first-order rate constants against the FAD concentration, second-order rate constants of 19.2 $M^{-1}\,s^{-1}$ and 3.7 $M^{-1}\,s^{-1}$, respectively, are obtained at pH 7.0 and 25°C (inset Fig. 2C).

As already shown above (Table 4), apo-butyryl-CoA dehydrogenase is present as a mixture of dimers and tetramers at pH 7.0 (100 mM K_2HPO_4). Using 15 μM apoprotein, about 70% of the apoenzyme molecules are in the dimeric state (Fig. 3A). These results suggest that the dimeric molecules are responsible for the slower reconstitution reaction, as observed in Fig. 2. Therefore, the properties of apo and reconstituted enzyme were studied in more detail by gel filtration experiments.

Apoprotein reconstituted in the presence of acetoacetyl-CoA at pH 7.0 is almost quantitatively present as a tetramer (Fig. 3B). The small amount of dimer present did not show any activity nor the presence of bound FAD. The same effect was seen when apoprotein, incubated for 20 min in the presence of 200 μM FAD and 1 mM CoASH (cs. Fig. 2A), was subjected to gel filtration (Fig. 3C). In this case, all activity and protein-bound FAD could be related to the amount of tetramer formed. The peaks eluting at 15 ml and 16 ml correspond to acetoacetyl-CoA (Fig. 3B) and CoASH (Fig. 3C), respectively, as checked by the use of authentic samples alone. The peaks eluting at 18.2 ml and 21 ml (Fig. 3B and C) correspond to FAD and its impurity, riboflavin, respectively. The possibility that CoASH influences the aggregation state of apoprotein was tested by running an apoenzyme sample preincubated in the presence of 1 mM CoASH. These chromatograms were identical with those of untreated apoenzyme. This result also indicates that no or very weak binding of CoASH to the apoprotein occurs.

From the above results the following scheme of reconstitution is proposed where F and A denote FAD and CoA derivatives, respectively (Scheme 2).

The rate-limiting step of the fast process in 'charge-transfer complex' formation (cf. Fig. 2C) is represented by k_3 while the rate-limiting step of the slow process is represented by either k_1 or k_2. Since no yellow dimer was observed on gel filtration of partly reconstituted enzyme (cf. Fig. 3C), it can be concluded that either k_4 and k_6 or k_5 and k_7, or both, must be fast if these reactions occur at all.

Fig. 3D shows the gel filtration elution pattern of apoenzyme incubated for 66 min with FAD in the absence of CoA derivatives. From this elution pattern, it is clear that less tetramer has been formed, though it should be kept in mind that bound CoA contributes to the absorbance of the tetrameric peaks observed in Fig. 3B and C.

The peak eluting at 16.4 ml in Fig. 3D corresponds to FMN, another impurity in FAD. This peak could not be observed in Fig. 3B and C due to the severe overlap of excess CoA. The inset of Fig. 3D shows the time-dependent increase of the tetrameric form, due to formation of reconstituted enzyme. The results of Fig. 3B–D indicate that FAD binding is inducing the binding of CoA derivatives, thereby stabilizing the tetrameric holoprotein structure. Moreover, when the apoenzyme was preincubated for 30 min with FAD and then monitored for 'charge-transfer complex' formation by adding acetoacetyl-CoA, the absorbance at 580 nm produced instantaneously was only 50% that of the results shown in Fig. 2B, but was consistent with the results shown in Fig. 2A. From the data presented, it can be stated that the slow and fast step in both holoenzyme reconstitution and 'charge-transfer complex' formation are rate-limited by k_1 and/or k_2 and k_3, respectively.

Finally it should be mentioned that reconstitution of apobutyryl-CoA dehydrogenase is dependent on aging. As already mentioned above, apoprotein kept for six months at −18°C in 50% ethylene glycol could be 80% reactivated on addition of excess FAD. However, the rate of reconstitution decreases with increasing storage time. After storage for one month at −18 C, the rate of the slow process in 'charge-transfer complex' formation (cf. Fig. 2C) was decreased by about a factor of two.

Stability of reconstituted butyryl-CoA dehydrogenase

It has already been observed by others [11] that unliganded holoenzyme is most stable at pH 6.0. The observed decreasing stability with time, as monitored by Cl_2Ind activity, could be related to increased dissociation of FAD with increasing pH. These results are in full agreement with our observation that at pH 6.0 the overall reconstitution rate of apoenzyme is about twice that at pH 7.0 (results not shown). However, the kinetics

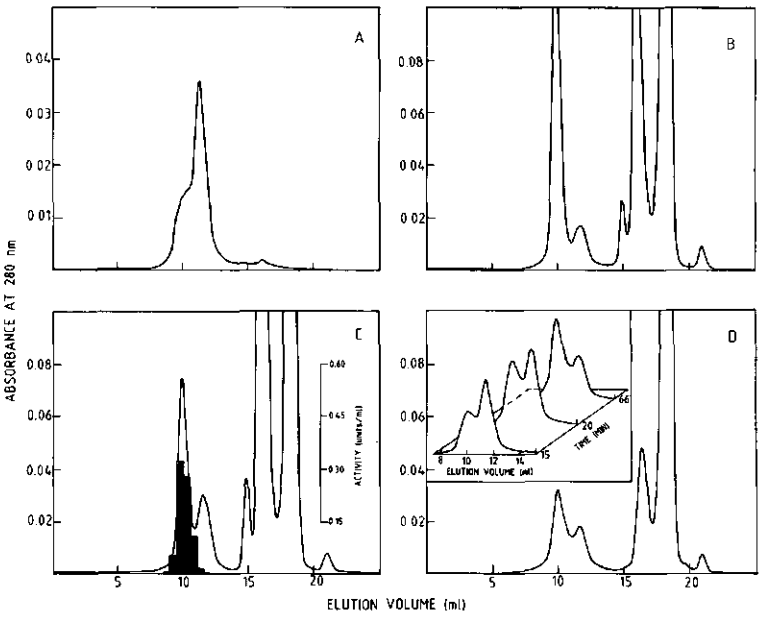


Fig. 3. *Reconstitution of apo-butyryl-CoA dehydrogenase by FAD at pH 7.0 as followed by gel filtration on Superose-12.* 100-µl samples were loaded onto the column. Elution was performed in 100 mM K_2HPO_4, pH 7.0. The flow rate was 0.5 ml/min. For other details see Materials and Methods. (A) 13.4 µM apo-butyryl-CoA dehydrogenase. (B) 15 µM apo-butyryl-CoA dehydrogenase incubated for 90 min in the presence of 200 µM FAD and 200 µM acetoacetyl-CoA. (C) 14.2 µM apo-butyryl-CoA dehydrogenase incubated for 20 min in the presence of 200 µM FAD and 1 mM CoASH. 0.5-ml fractions were collected and checked for Cl_2Ind activity by adding a 100-µl aliquot to the standard assay mixture (vertical bars). The fractions were also tested for their fluorescence polarization behaviour (see text). (D) 13.4 µM apo-butyryl-CoA dehydrogenase incubated for 66 min in the presence of 200 µM FAD. The inset shows the protein-elution profiles of comparable mixtures chromatographed after 5, 20 and 66 min incubation

of the reconstitution reaction are complicated by the fact that at pH 6.0 the apoenzyme shows complex quaternary structure behaviour (Fig. 4A, Table 4). Nevertheless, gel filtration of an apoenzyme sample at pH 6.0, reconstituted at pH 6.0 in the presence of 100 µM acetoacetyl-CoA, yielded a very homogeneous tetrameric form (Fig. 4B). Moreover, the relatively high intensity of the protein peak indicates strong binding of acetoacetyl-CoA. The fact that the elution patterns of Fig. 4A and B are shifted to a lower elution volume compared to the elution patterns of Fig. 3A – D can be ascribed to the different swelling properties of Superose-12 in Mes, pH 6.0, and K_2HPO_4, pH 7.0, respectively. This was checked by control experiments using standard proteins and free coenzymes.

The question arose as to whether the pH-dependent dissociation of FAD could be related to the dissociation of tetramers into dimeric molecules as suggested by Scheme 2. Therefore, native green and unliganded enzyme were subjected to gel filtration at pH 8.0 (100 mM Hepps) at different time intervals. It should be noted here that native green liganded enzyme showed complete tetrameric structure at pH 7.0 (cf. Table 3). As can be seen from Fig. 4C, green liganded enzyme is relatively stable at pH 8.0 but the unliganded enzyme (Fig. 4D) is readily dissociated into dimeric and also monomeric forms, due to subsequent release of FAD (Fig. 4D). At longer time intervals, the apoprotein formed tends to aggregate to polymers eluting in the void volume of the Superose column. The results obtained at pH 8.0 could explain the relatively low activity observed for reconstituted unliganded enzyme (Fig. 2A).

Fluorescence properties of native and reconstituted butyryl-CoA dehydrogenase

In order to obtain a more quantitative insight into the FAD-binding properties of different (un)liganded enzymic forms, the apparent equilibrium constants of FAD dissociation were determined as a function of the pH by means of steady-state-fluorescence-polarization measurements.

The relative fluorescence excitation and emission spectra of green liganded, yellow liganded and free enzyme are all comparable to those of free FAD (results not shown), allowing reliable determination of the relative quantum yields of the enzyme solutions by excitation at 450 nm.

Assuming that the rotational relaxation times of both dimer and tetramer are much longer than the fluorescence lifetime of FAD bound to the protein [24] and that FAD is strongly bound to the apoprotein, the fraction of free FAD can be calculated according to Chien and Weber [25]:

$$x = 1 - \frac{I}{I_f}\left(\frac{P_b - P_{obs}}{P_b - P_f}\right) \qquad (2)$$

where x represents the fraction of free FAD, I/I_f the relative intensity of FAD fluorescence of the enzyme solution (fluorescence of FAD bound to the enzyme plus fluorescence of free FAD) to that of free FAD, P_b, P_f and P_{obs} the degree of polarization of FAD bound to the enzyme, free FAD and the experimental enzyme solution, respectively.

From Eqn (2) and assuming a single binding site/subunit, the dissociation constant of the equilibrium

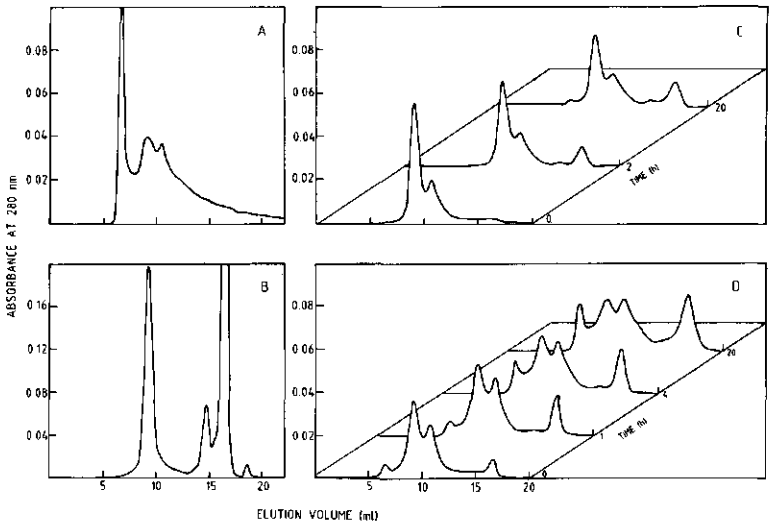


Fig. 4. *Time-dependent gel filtration of different forms of butyryl-CoA dehydrogenase.* 100-µl samples were loaded onto Superose 12 equilibrated in 100 mM Mes, pH 6.0 or 100 mM Hepps, pH 8.0. The flow rate was 0.5 ml/min. Before use, the samples were freshly gel-filtered over Bio-Gel P-6DG in the elution buffer. All experiments were performed at 20 °C. (A) 67 µM apo-butyryl-CoA dehydrogenase at pH 6.0. (B) 15 µM apo-butyryl-CoA dehydrogenase incubated at pH 6.0 for 90 min in the presence of 200 µM FAD and 100 µM acetoacetyl-CoA. (C) 15 µM native green liganded butyryl-CoA dehydrogenase at pH 8.0 and recorded at 0, 2 and 20 h after gel filtration over Bio-Gel P-6DG. (D) 15 µM native unliganded butyryl-CoA dehydrogenase at pH 8.0, recorded at 0, 1, 4 and 20 h after gel filtration over Bio-Gel P-6DG

Table 5. *Relative fluorescence quantum yield and dissociation constant of native and reconstituted butyryl-CoA dehydrogenase*
The relative quantum yield (I/I_f) and dissociation constant (K_d) were determined at 20 °C according to Eqns (2) and (4) (see text)

Enzyme	pH	Concn	I/I_f	K_d	Concn	I/I_f	K_d
		µM		nM	µM		nM
Native 50% green	6.0	10	0.136	< 0.1	2	0.155	< 0.1
		5	0.136	< 0.1	1	0.160	0.4
	7.0	10	0.152	< 0.1	2	0.179	0.2
		5	0.160	0.3	1	0.186	1.0
	8.0	10	0.197	7.8	2	0.249	5.4
		5	0.242	8.3	1	0.288	13.0
Native unliganded	6.0	10	0.263	0.7	2	0.319	0.4
		5	0.421	1.3	1	0.386	2.2
	7.0	10	0.314	5.9	2	0.600	29
		5	0.421	5.6	1	0.719	44
	8.0	10	0.299	100	2	0.483	130
		5	0.509	190	1	0.536	160
Reconstituted 80% green	6.0	10	0.20	16	2.3	0.31	10
		6	0.25	16	1.1	0.34	7.8
	7.0	10	0.30	75	2	0.43	46
		6	0.34	72	1	0.53	37
Reconstituted unliganded	6.0	20	0.29	13	2	0.37	12
		5	0.34	11	1	0.39	10
	7.0	22.5	0.43	500	2.2	0.62	250
		7.5	0.52	280	0.6	0.77	670

$$E + FAD \leftrightarrows E - FAD \tag{3}$$

can be inferred from [26]:

$$K_d = E_o\left[\frac{(1-x)^2}{x}\right] \tag{4}$$

where E_o represents the total concentration of free and bound FAD.

At pH 6.0 and 20 µM enzyme concentration, both native green and yellow liganded holoenzyme possess a very low fluorescence yield of about 12% that of free FAD, which in

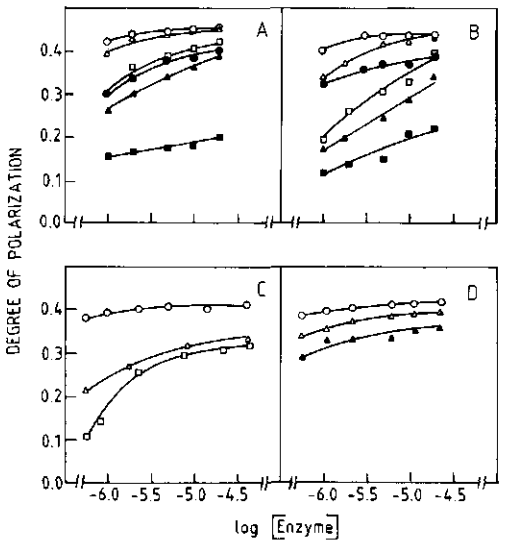


Fig. 5. *The dependence of the steady state fluorescence polarization of native and reconstituted butyryl-CoA dehydrogenase on pH and enzyme concentration.* Enzyme solutions were freshly gel-filtered over Bio-Gel P-6DG in the desired buffer (100 mM) and diluted stepwise. All experiments were performed at 20°C. For other details see Materials and Methods. (A) Native green liganded (50% green) butyryl-CoA dehydrogenase in Mes, pH 6.0 (○——○), Hepes, pH 7.0 (△——△) and Hepps, pH 8.0 (□——□). The filled symbols represent dilutions of the different concentrated enzyme solutions after 100-min incubation at room temperature. (B) Native unliganded butyryl-CoA dehydrogenase in Mes, pH 6.0 (○· · ○), Hepes, pH 7.0 (△——△) and Hepps, pH 8.0 (□——□). The filled symbols represent dilutions of the different concentrated enzyme solutions after 100-min incubation at room temperature. (C) Reconstituted unliganded butyryl-CoA dehydrogenase in Mes, pH 6.0 (○——○), Hepes, pH 6.8 (△——△) and K_2HPO_4, pH 7.0 (□——□). (D) Reconstituted green liganded (80% green) butyryl-CoA dehydrogenase in Mes, pH 6.0 (○——○) and K_2HPO_4, pH 7.0 (△——△). The filled symbols represent dilutions of the concentrated enzyme solution in K_2HPO_4, pH 7.0 after standing for one night at 4°C

turn is about ten times less fluorescent than free FMN [8]. Native unliganded holoenzyme prepared by thiopropyl-Sepharose [11, 20] is somewhat more fluorescent (16% as compared to free FAD) but bound FAD is still strongly quenched by the apoprotein.

Native liganded enzyme (50% green) shows a high degree of polarization of protein-bound FAD indicating strong immobilization of flavin. As can be seen from Fig. 5A, the degree of polarization at pH 6–7 remains almost constant upon dilution, indicating tight binding of FAD. At pH 8.0 depolarization at lower enzyme concentrations is accompanied by an increase of the relative fluorescence quantum yield indicating dissociation of protein-bound FAD (Table 5). Depolarization becomes more pronounced after incubation of the concentrated enzyme samples for 100 min at room temperature (Fig. 5A) and is in accordance with the gel filration patterns of Fig. 4C and D.

The apparent dissociation constant for FAD dissociation from native liganded enzyme was calculated according to Eqns (2) and (3), using a value of 0.45 for P_b, obtained after extrapolation to infinite enzyme concentration and a value of 0.04 for free FAD [27].

From the data of Fig. 5A and Table 5, an apparent dissociation constant of about 10 nM, at pH 8.0, can be calculated for native liganded enzyme. It can be concluded that, although depolarization is observed at low enzyme concentrations, even at pH 8.0 more than 90% of FAD is bound to native liganded enzyme. The relative low polarization values observed are a consequence of the very low fluorescence quantum yield of the enzyme and illustrate the high sensitivity of the technique used to monitor FAD dissociation. At pH 6.0, the apparent dissociation constant for FAD dissociation from reconstituted enzyme (about 10 nM) is at least a factor of 100 higher than that of native liganded enzyme which shows very tight binding of FAD ($K_d < 0.1$ nM). Comparable differences between native and reconstituted enzyme are observed at pH 7.0.

DISCUSSION

Until now two procedures have been described for the preparation of the apo form of pig-heart lipoamide dehydrogenase [28, 29]. Both procedures have their own limitations, as already mentioned by several authors [30–32]. In the case of mercuric reductase and glutathione reductase, only qualitative information was given recently [33] about a modified procedure of the original method described by Staal et al. [13].

For butyryl-CoA dehydrogenase from *M. elsdenii* no procedure for apoprotein preparation has been reported previously. On an analytical scale, related pig-kidney general-acyl-CoA dehydrogenase apoprotein could be prepared by a modified acid/ammonium sulfate procedure [34].

The use of hydrophobic-interaction chromatography made it possible to remove FAD quantitatively from all enzymes tested, at relatively mild pH values compared to known procedures. This and the immobilization of the enzymes are the main advantages of this technique, strongly stabilizing the protein structures and allowing large-scale production of apo flavoproteins.

The main disadvantage of the technique used is obvious in the case of butyryl-CoA dehydrogenase and to a lesser degree with glutathione reductase. For full recovery of apoprotein, conditions have to be found which prevent irreversible interaction of enzymes with the column material during apoprotein preparation. In principle these conditions can be found by varying the following parameters: (a) type of support ligand; (b) type of salts; (c) eluent pH values; (d) temperature and (e) addition of organic solvents or chaotropic agents.

A. vinelandii apo-lipoamide dehydrogenase prepared by acid/ammonium sulfate treatment is more stable as compared to the pig-heart enzyme [21]. However, from the absorption spectrum of reconstituted enzyme, prepared by the classical method, it can be concluded that small changes in the enzyme structure had been generated upon apoprotein preparation. The use of hydrophobic-interaction chromatography overcomes this problem. All properties of the reconstituted enzyme tested are identical to those of native enzyme. The fact that the tertiary structure of the enzyme was kept intact could be demonstrated by the very fast reactivation process, even at low temperature. Reactivation was accompanied by dimerization of enzyme and tight binding of FAD.

In the case of *M. elsdenii* butyryl-CoA dehydrogenase, the situation is much more complicated. From the results obtained, it can be concluded that apoprotein preparation

strongly depends on the conditions used, i.e. the apoprotein can become too tightly bound to the hydrophobic matrix preventing reconstitution or elution. Using less hydrophobic conditions, the yield of apoprotein could be increased considerably, although it was still impossible to reconstitute the apoenzyme on the matrix. Moreover, when testing the properties of apo and reconstituted butyryl-CoA dehydrogenase it became clear that more specific conditions are required for the preparation of apo and reconstituted butyryl-CoA dehydrogenase than for lipoamide dehydrogenase from *A. vinelandii*. Nevertheless, under suitable conditions it is now possible to prepare large amounts of reconstituted enzyme showing nearly the same properties as native enzyme. These preparations are suitable for biophysical studies using either isotopically enriched or artificial flavins.

Reconstituted butyryl-CoA dehydrogenase is somewhat less stable than native enzyme. By performing pH-dependent gel filtration and fluorescence-polarization studies, it could be demonstrated that the reconstituted enzyme is most stable at pH 6.0. At higher pH values FAD dissociates more easily than from the native enzyme, indicating that even under relatively mild conditions some small structural changes occur during apoprotein preparation. Moreover, as already noticed by others [19], this paper again shows the important role of CoA derivatives in stabilizing the protein structure of fatty acid acyl-CoA dehydrogenases.

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Erratum

1.7 M and 1.2 M ammonium sulfate should be read as 1.2 M and 0.8 M ammonium sulfate respectively (Results, Table 1 and 2, Scheme 1).

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Chapter 4

The elucidation of the microheterogeneity of highly purified *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* by various biochemical techniques

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Highly purified *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* can be separated into at least five fractions by anion-exchange chromatography. All fractions exhibit the same specific activity and the enzyme exists mainly in the dimeric form in solution. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of a mixture of the different fractions reveals two apparent forms of enzyme molecules, while isoelectric focusing experiments, on the other hand, reveal six apparently different forms of enzyme molecules. It is shown that the different forms of enzyme molecules are due to the (partial) oxidation of Cys-116 in the sequence of the enzyme. This interpretation of the data is supported by kinetic measurements of the formation of hybrid dimeric molecules monitored by fast protein liquid chromatography, using purified enzyme containing Cys-116 either in the native and or the fully oxidized (sulfonic acid) state. By chemical modification studies using maleimide derivatives, 5,5′-dithiobis(2-nitrobenzoate) and H_2O_2, it is shown that sulfenic, sulfinic and sulfonic acid derivatives of Cys-116 are products of oxidation. The results are briefly discussed with respect to the possibility that this isolation artifact might also be partially responsible for the appearance of multiple forms of enzyme molecules in other biochemical preparations.

The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* belongs to the class of external monooxygenases and catalyzes the conversion of *p*-hydroxybenzoate into 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil bacteria. The enzyme contains FAD as a prosthetic group and is predominantly dependent on NADPH as an external electron donor.

The reaction mechanism of the enzyme has been studied in detail [1]. A model (0.25-nm resolution) of the three-dimensional structure of the enzyme is available [2]. In addition the complete amino acid sequence of the protein is known [3, 4]. In order to understand the reaction mechanism in more detail it is necessary to know the amino acid residues involved in the catalysis. Recently we have initiated a program to identify amino acid residues involved in the binding of the substrates by chemical modification studies [5, 6]. In such studies it is of utmost importance to use a well-defined enzyme preparation in order to arrive at a safe interpretation of the often complex chemical kinetics. In our studies we always used one particular fraction of the final purification step. This fraction consists of dimeric enzyme molecules, as has previously been shown [7]. However, we found that all other fractions of the final purification step consisted of protein showing the same specific enzymatic activity but showed partially different properties when analyzed further. Since it is rather disturbing to observe this unexplained behaviour of the physically and kinetically well-characterized flavoprotein, we decided to investigate this apparent basic property of the enzyme in more detail. In this paper we describe the isolation of various fractions of highly purified, dimeric enzyme molecules and the characterization of the various fractions using various biochemical techniques. It is shown that the highly purified enzyme preparation consists of enzyme molecules differing in sodium dodecyl sulfate gel electrophoresis and isoelectric focusing behaviour. The results are interpreted in terms of a (partial) oxidation of a particular cysteine residue in the enzyme. Preliminary results have been published elsewhere [8]. The present study is probably also of general relevance for the purification of other proteins.

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Abbreviations. Bistrispropane, *N,N*-bis[2-(hydroxymethyl)-2-propyl-1,3-diol]-1,3-diaminopropane; Me_2SO, dimethylsulfoxide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); Nbs, 5-thio-2-nitrobenzoate; E-Nbs, 5-thio-2-nitrobenzoate-labeled enzyme; MalNEt, *N*-ethylmaleimide; E-MalNEt, *N*-ethylmaleimide-labeled enzyme.

Enzymes. *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2); catalase, hydrogenperoxide oxidoreductase (EC 1.11.1.6).

MATERIALS AND METHODS

General

NADPH and catalase were from Boehringer (Mannheim, FRG), sodium dodecyl sulfate from BDH; dithiothreitol, Tris, Bistris, Bistrispropane, Hepes and agarose were from

Sigma. Acrylamide, bisacrylamide and Coomassie brilliant blue R250 were from Serva (Heidelberg, FRG). The maleimide spin-label derivatives were products of Syva (Palo Alto, CA, USA). Bio-Gel P-6DG and economy columns were from Bio-Rad (California, USA). DEAE-Sepharose 6B, Pharmalyte ampholines, Sephadex G-150 as well as the prepacked columns Mono Q HR 5/5 and Superose 12 were from Pharmacia (Uppsala, Sweden). All other chemicals were products of Merck (Darmstadt, FRG) and were the purest grade available.

Large-scale production of *Pseudomonas fluorescens* was performed by Diosynth BV (Oss, The Netherlands). The purification procedure of the enzyme as described earlier [9] was followed up to the Sephadex-Cibacron blue affinity chromatography with one slight modification. Cell-free extract was brought to 42% ammonium sulfate with 0.72 vol. saturated ammonium sulfate, centrifuged for 2 h at $20000 \times g$ (4 °C) and the supernatant then brought to 47% ammonium sulfate by slow addition of solid ammonium sulfate. Enzyme activity was determined as described previously [9]. The enzyme concentration was determined spectrophotometrically on the basis of FAD content by assuming a molar absorption coefficient of 11.3 $mM^{-1}\,cm^{-1}$ at 450 nm [9].

Sedimentation velocity and equilibrium experiments were done with an MSE Centriscan 75 analytical ultracentrifuge and runs were analyzed with a home-made minicomputer program using an HP85 (Hewlett Packard). Calculations for the molecular mass were done as described previously [9]. The enzyme concentration was varied over 0.1 – 10 mg/ml.

Polyacrylamide discontinuous sodium dodecyl sulfate gel electrophoresis was carried out mainly according to Laemmli [10] using 12.5% or 15% slab gels (13 × 13 cm, thickness 1 or 2 mm).

Flatbed isoelectric focusing was performed using the LKB 2117 Multiphorsystem (LKB, Stockholm, Sweden). Temperature was kept at 10 °C according to conditions described by Laas et al. [11]. Gels were 1 mm thick. pH gradients were measured after equilibration using small pieces of gel in a solution of 0.15 M KCl. Ultrathin (200 µm) isoelectric focusing gels were coated on silanized polyester sheets. These gels were cast by using the 'flap' technique according to Radola [12]. After prefocusing samples (0.5 – 5 µg protein in 1 – 10 µl of the appropriate buffer, I = 25 mM) were loaded at about pH 7.0 Two-dimensional gel electrophoresis was done using a system virtually according to Görg et al. [13].

Analytical gel filtration at 4 °C was performed as described earlier [9]. Sepharose – 5,5′-dithio-bis(2-nitrobenzoate) covalent chromatography was performed in the same way as described for the preparation of the apo-protein [14]. 10 – 20 mg holoenzyme were eluted in a 4-ml bed-volume column. Enzyme which binds to the column is called E-SH, enzyme which does not bind to the column is called E-S-ox.

The number of sulfhydryl groups in the enzyme were estimated as described earlier [9].

Desalting or buffer exchange of small enzyme solutions (0.5 – 10 ml) was performed on Bio-Gel P-6DG economy columns of different sizes (2 – 40 ml) depending on the sample volume.

Enzyme labeling studies

Labeling of Cys-116 with *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoate) or maleimide spin-label derivatives was done as described earlier [6, 9]. Excess of reagent was removed by Bio-Gel P-6 filtration in Hepes buffer, pH 7.0 (I = 0.1 M), unless stated otherwise. SDS-PAGE of Nbs_2-modified enzyme was performed in the absence of 2-mercaptoethanol. Before denaturation of the samples 10 mM MalNEt was added in order to block the remaining sulfhydryl groups [6] accessible on denaturation. pH-dependent modification studies with H_2O_2 were performed at constant ionic strength (I = 25 mM) in buffer systems described earlier [6] at 21 °C.

10 µM enzyme (10 nmol) was incubated with 20 mM H_2O_2 in the presence of 0.5 mM EDTA and the reaction was stopped at different time intervals by mixing a 200-µl aliquot with a catalytic amount (1 µg) of catalase. After 10 min, samples were incubated either in the absence or presence of dithiothreitol; 10 min thereafter the samples were frozen in liquid N_2 until used for monitoring either the FPLC (100 µl), SDS-PAGE or IEF pattern.

Anion-exchange chromatography

Preparative anion-exchange chromatography of the enzyme purified up to the Sephadex-Cibacron blue affinity column was performed with a $K_{25/100}$ column (Pharmacia, 2.6 × 85 cm, 450 ml bed volume) packed with DEAE-Sepharose 6B. The column was preequilibrated at 4 °C with 10 mM KH_2PO_4, pH 7.0, containing 0.3 mM EDTA and 0.3 mM dithiothreitol. Then a sample of *p*-hydroxybenzoate hydroxylase in the same buffer was loaded on top of the column and the column was washed with 2 vol. starting buffer. The enzyme was eluted from the column with a 0 – 0.3 M KCl linear gradient in 2 l of the same buffer. A constant flow of buffer was maintained with the aid of a LKB-12000 Varioperpex peristaltic pump (40 ml/h). The elution profile was monitored with the aid of an Isco US-5 monitor. After eluting of about 1200 ml of the gradient, 9.0-ml fractions were collected with an LKB 2070 Ultrorac II fraction collector. In this way 650 mg flavoprotein (out of 2 kg bacteria) could be run at one time. The column was regenerated by washing with the same buffer, containing 1 M KCl to elute some impurities strongly bound to the exchanger. Different peaks of the preparative DEAE-Sepharose 6B column were kept separately as 70% ammonium sulfate precipitates in 50 mM phosphate buffer, pH 7.0, as indicated in Fig. 1.

Conductivity of the fractions was measured with a CDM 80 conductivity meter (Radiometer Copenhagen) equipped with a CDC 114 flow cell electrode.

Fast protein liquid chromatography

To analyze small samples of the enzyme the fast protein liquid chromatography (FPLC) system of Pharmacia was used, consisting of two P500 dual-piston pumps, a GP 250 gradient programmer, a UV-1 monitor with HR flow cell, a V-7 injection valve and a REC-482 recorder. Absorbance was monitored either at 280 nm or 405 nm and peak widths and retention volumes were stored and recovered from the memory of a Pharmacia FRAC-100 fraction collector. 100-µl samples of 0.1 – 3 mg/ml protein were injected on a Mono Q 5/5 column, equilibrated in 20 mM Bistris, pH 6.4, containing 0.3 mM EDTA and 0.3 mM dithiothreitol (start buffer). Dithiothreitol was omitted from the start buffer when analyzing enzyme samples modified with MalNEt, Nbs_2 or H_2O_2, unless otherwise stated. Elution gradients were made with either 0.5 M Na_2SO_4 or 1 M KCl in start buffer (= elution buffer). Although Cl^- is a competitive inhibitor of the enzyme with respect to NADPH [15], we did not see any

significant difference in elution behaviour with respect to the 0.5 M Na_2SO_4 elution buffer, suggesting that the NADPH site does not influence the affinity of the enzyme for the anion exchanger. The flow rate varied over 1.0 – 2.0 ml/min. After each run the column was washed with 2 ml elution buffer and reequilibrated with 5 vol. start buffer. Total run time for one experiment varied over 20 – 40 min. All buffers were filtered before use through 0.22-μm Millipore filters and degassed under vacuum.

Analytical gel filtration at 20°C was performed on a Superose 12, HR 10/30 column in 50 mM K_2HPO_4, pH 7.0, containing 150 mM KCl. The flow rate was 0.2 ml/min. The enzyme concentration was varied over 0.1 – 1.0 mg/ml (100-μl samples). Calibration proteins used were catalase (240 kDa), alcohol dehydrogenase (141 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), myoglobin (17.6 kDa) and cytochrome *c* (12.4 kDa).

The electron spin resonance spectra were recorded on a Bruker ER 200 spectrometer. The instrument was connected to a Data General NOVA 3 computer for storage and handling of the experimental data. The spectra were obtained at 20°C using quartz capillary tubes with an inside diameter of 1 mm. The concentration of the spin-labeled enzyme was about 100 μM.

RESULTS AND DISCUSSION

Two purification procedures have been published for *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* [9, 16]. The procedure of Howell et al. [16] gave a yield of 16%, the purified enzyme possessed an apparent molecular mass of 65 kDa which was at that time considered to represent the minimal molecular mass of the protein. This purification procedure was improved by us [9] using a Cibacron blue affinity column giving a final yield of 45%. Furthermore, it was found that the enzyme exists mainly as a dimer in solution having a molecular mass of about 88 kDa. 10 – 20% of the enzyme was found to be in the form of higher-order quaternary structures at room temperature and polymerization tends to increase with decreasing temperature [9]. Crystallographic studies [17] have revealed that the enzyme crystallizes as a dimer and sequence studies [18] have confirmed that the minimum molecular mass of the enzyme is about 44 kDa.

In the past few years we have observed repeatedly that purified enzyme showed heterogeneity on anion-exchange chromatography, polyacrylamide gel electrophoresis and isoelectric focusing, although the enzyme could be considered as very pure as judged by its specific activity. We have already previously observed and reported [14] that at least two forms of enzyme molecules can be distinguished by the preparation of apo-*p*-hydroxybenzoate hydroxylase using a 5,5'-dithiobis(2-nitrobenzoate) affinity column. These results were explained in terms of (partial) oxidation of Cys-116 in the sequence of the enzyme [3, 4]. This amino acid residue is known to be very reactive towards dioxygen and sulfhydryl group reagents [6]. A more serious problem was posed by the more recent observation that enzyme purified according to our purification scheme [9] no longer crystallized or the crystals obtained were of poor quality not suitable for X-ray studies [19]. These facts necessitate a more detailed investigation to characterize the enzyme. In addition, since *p*-hydroxybenzoate hydroxylase is one of the better characterized flavoproteins, both with respect to the biochemical and biophysical properties, the elucidation of the cause of the heterogeneity observed in highly purified enzyme is desirable.

Modified purification procedure

As described under Materials and Methods, the purification of the enzyme was followed up to the Cibacron blue affinity column as described previously [9]. Two batches of 1 kg (wet weight) bacteria were processed and combined after the affinity column. The total combined volumes of 2 l contained 1.1 g total protein and 35000 units of total activity giving a specific activity of 31.8 units/mg and a recovery of 58%. The recovery is considerably higher than that previously published (45%), while the specific activity at this step was about the same as reported. The improved recovery of the enzyme is due to the modified ammonium sulfate fractionation step mentioned under Materials and Methods and also a fact of experience. In contrast to the previously used procedure, the enzyme was loaded on a DEAE-Sepharose 6B column in the presence of dithiothreitol. The elution profile of this purification step is shown in Fig. 1. About 50% of the total enzyme, based on the absorbance at 450 nm, is eluted in one almost symmetrical peak (F_1 in Fig. 1), while the residual enzyme is distributed over four or probably five peaks. The total volume of the eluates is about 600 ml containing 650 mg protein and a total activity of 32500 units. The final recovery is 54% with respect to the starting material. All fractions shown in Fig. 1 exhibit a specific activity of 50 units/mg (based on flavin content), indicating that the enzyme is highly pure.

Since the enzyme isolated by the previously published procedure [9] showed complex hydrodynamic properties, fraction F_1, F_4 and F_6 of the DEAE-Sepharose column were investigated by the ultracentrifugation method at 20°C. Both sedimentation velocity ($s_{20,w} = 5.3 \pm 0.25$ S) and sedimentation equilibrium ($M_r = 79 \pm 9$ kDa) experiments indicate that the enzyme exists in the dimeric form. Only fraction F_4 shows the presence of a small amount ($\leq 16\%$) of higher-order quaternary structures. An identical set of experiments was done at 4°C yielding essentially the same results. Performing analytical gel filtration at 4°C either in the absence or presence of dithiothreitol the fractions F_1 and F_6 elute as a symmetrical peak ($R_s = 3.7$ nm) while again fraction F_4 shows the presence of about 15% of polymeric forms. The fact that dithiothreitol prevents the formation of the higher-order quaternary structures suggests that intermolecular disulfide bridge formation is involved in the formation of higher-order aggregates of the enzyme. This is most probably not the case since sodium dodecyl sulfate/polyacrylamide gel electrophoresis in the absence of 2-mercaptoethanol of samples obtained from the Cibacron blue affinity column did not reveal the presence of any polymeric forms of the enzyme samples tested. This is in accordance with previous gel filtration and ultracentrifugation studies [9] and suggests that dithiothreitol prevents the formation of polymeric forms, probably by rather specific polar interactions with some part(s) of the enzyme. An identical observation has been made recently with xanthine oxidase isolated from cow milk omitting the proteolytic treatment of the crude extract (W. A. M. van den Berg and F. Müller, unpublished observations). Nevertheless all enzyme fractions isolated by the modified procedure could be crystallized [19], indicating that the presence of higher-order quaternary structures in the previously isolated enzyme [9] inhibits the crystallization of the enzyme. Therefore dimeric native enzyme molecules have to be used for crystallization purposes. Consequently the modified puri-

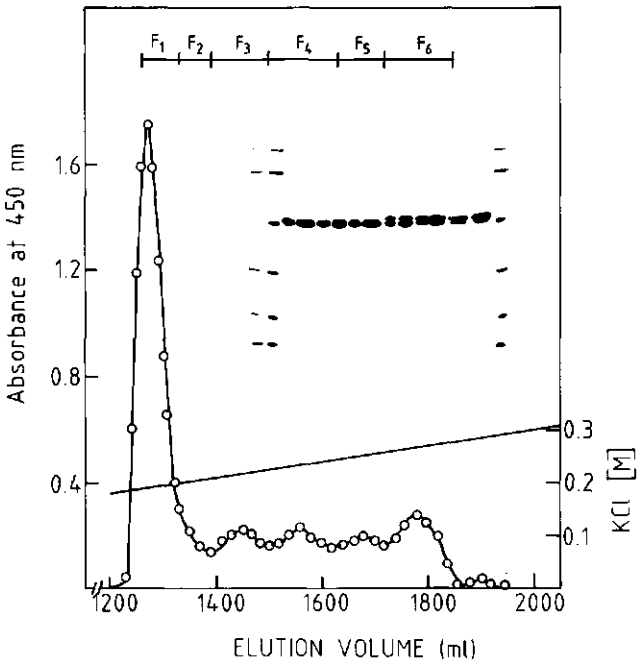

Fig. 1. *Preparative anion-exchange chromatography of* p-*hydroxybenzoate hydroxylase on DEAE-Sepharose 6B.* 650 mg of partially purified enzyme, obtained from 2 kg of wet weight bacteria and processed as described under Materials and Methods, was dialyzed against a solution of 10 mM KH_2PO_4, pH 7.0, containing 0.3 mM EDTA and 0.3 mM dithiothreitol and loaded on the column. After washing the column with 2 vol. buffer solution, the enzyme was eluted using a linear gradient of 0 – 0.3 M KCl in the same buffer. The flow rate was 40 ml/h. Fractions F_1 to F_6 were pooled as indicated. *Inset:* sodium dodecyl sulfate/polyacrylamide gel electrophoresis of fractions of *p*-hydroxybenzoate hydroxylase obtained from anion-exchange column chromatography. Lanes 1, 2 and 15, calibration protein (from top to bottom: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa and α-lactalbumin, 14.4 kDa). Lanes 3 and 4, fraction F_1; lanes 5 and 6, fraction F_2; lanes 7 and 8, fraction F_3; lanes 9 and 10, fraction F_4; lanes 11 and 12, fraction F_5; lanes 13 and 14, fraction F_6

fication procedure described above should be used in the isolation of the enzyme. In addition we found that the enzyme can be stored as an ammonium sulfate precipitate at pH 6.5 in the presence of dithiothreitol over long periods of time without oxidation of Cys-116.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis

All fractions obtained from the DEAE-Sepharose 6B column were subjected to gel electrophoresis under denaturing conditions (Fig. 1). At least two forms of enzyme molecules are present in the highly purified enzyme. One form of the enzyme is apparently exclusively present in the first three fractions eluting from the DEAE-Sepharose column. Fraction 4 contains both enzyme molecules in about the same amount, whereas fractions 5 and 6 exhibit increasingly the other form of the enzyme. Enzyme molecules apparently more negatively charged (tighter binding to the DEAE-Sepharose column) are probably binding less sodium dodecyl sulfate and therefore move more slowly in the polyacrylamide gel. A comparable effect was observed for the various phosphorylated forms of human and monkey phenylalanine hydroxylase [20]. Similarly Tung and Knight [21] described the effects of introducing additional charges into proteins on the electrophoretic behaviour of such proteins. These data strongly support our interpretation of the gel electrophoresis results. That the difference in electrophoretic mobility is not due to a difference in molecular mass will be shown below.

Analysis of sulfhydryl groups

The enzyme contains five sulfhydryl groups [9], but only Cys-116 is accessible to 5,5′-dithiobis(2-nitrobenzoate) in the native enzyme [6]. We suspected that the apparent two forms of the enzyme could be related to the fact that Cys-116 is (partially) oxidized in some enzyme molecules. This oxidation has little or no effect on the specific activity of the enzyme [9]. To check if the fractions shown in Fig. 1 contain molecules with a modified Cys-116, the fractions were analyzed for the SH content of the enzyme in the native and denatured state. The results are summarized in Table 1. It is seen that the SH content of the enzyme decreases with increase in fraction number. It is also obvious that the enzyme in F_6 shows the loss of one SH group. These results are in perfect agreement with the gel electrophoresis pattern of the same fractions (Fig. 1).

Similarly labeling of the fractions F_1, F_4 and F_6 by 3-(maleimido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (for

Table 1. *The sulfhydryl content of native and denatured enzyme of the different fractions obtained by anion-exchange chromatography (cf. Fig. 1)*
The values for SH content are from three independent determinations. n.d. = not determined

Fraction	Affinity chromatography		SH content	
	binding	not binding	native	denatured
	% activity		mol/mol FAD	
F_1	96.1	1.1	≈1	5.0
F_2	n.d.	n.d.	0.90	4.87
F_3	n.d.	n.d.	0.85	4.83
F_4	55.7	36.9	0.61	4.55
F_5	n.d.	n.d.	0.41	4.40
F_6	4.6	97.6	0.20	4.15

structure see Fig. 6A) revealed the incorporation of 1 mol spin label/mol FAD into F_1, whereas F_4 contained about 0.5 mol spin label/mol FAD and F_6 less than 0.1 mol spin label/mol FAD.

Moreover when the fractions were put on the covalent affinity column of Sepharose–5,5′-dithiobis(2-nitrobenzoate) [14], about 96% of the enzyme in F_1 was retained on the column whereas only about 5% of the enzyme in F_6 was bound (Table 1).

The above results strongly suggest that the (partial) oxidation of Cys-116 is responsible for the gel electrophoresis behaviour of the enzyme. This is in agreement with the observations of Smith et al. [20] and Tung and Knight [21] who showed that the gel electrophoretic mobility of proteins of the same molecular mass is influenced by the relative charges of these proteins.

Since the purified enzyme exists mainly as a dimer in solution the sodium dodecyl sulfate gel electrophophoresis pattern (Fig. 1) indicates that the monomeric enzyme molecules are better defined with respect to the oxidation state of Cys-116 than the dimeric molecules. Obviously the dimeric enzyme molecules consist of pure $(E\text{-}SH)_2$ (both Cys-116 unmodified) and $(E\text{-}S_{ox})_2$ [both Cys-116 (partially) oxidized], and of a mixture of both. This interpretation could explain the results obtained from DEAE-Sepharose column chromatography and sodium dodecyl sulfate gel electrophoresis and is in accord with the sulfhydryl content of the enzyme in the different fractions (Table 1).

The fact that the elution pattern shown in Fig. 1 varies from preparation to preparation somewhat with respect to the concentration of the various fractions suggests that there must exist an equilibrium among the different dimeric enzyme molecules depending on the degree of oxidation. The simplest model to explain the results is the following:

$$(E\text{-}SH)_2 + (E\text{-}S_{ox})_2 \rightleftarrows 2\,(E\text{-}SH \ldots E\text{-}S_{ox}). \qquad (1)$$

Fast protein liquid chromatography

In order to analyze the various fractions in more detail they were investigated by high-resolution anionic chromatography on a Mono Q column using the FPLC system. In Fig. 2 the FPLC behaviour of fraction 1 (Fig. 2A), fraction 4 (Fig. 2B) and fraction 6 (Fig. 2C) from the DEAE-Sepharose column (see Fig. 1) is shown. The results are in good agreement with those of the preparative column and confirm the gel electrophoresis pattern of the corresponding fraction (see Fig. 1). However, Fig. 2 demonstrates that none of the fractions contains only one particular type of dimeric enzyme molecules. The FPLC chromatogram indicates that at least six different types of enzyme molecules can be distinguished. Therefore the peak with the shortest (Fig. 2A) and that with the longest (Fig. 2C) retention time were purified to homogeneity by repeated FPLC chromatography. The FPLC chromatograms of the two fractions are shown in Fig. 3A and B. As also judged by gel electrophoresis the two fractions are apparently homogeneous and correspond to lane 3 and lane 13 in Fig. 1, i.e. $(E\text{-}SH)_2$ and $(S\text{-}S_{ox})_2$. These two purified fractions have been used to check the validity of the proposed equilibrium of Eqn (1) and to study the kinetics of the formation of the mixed dimer $E\text{-}SH \ldots E\text{-}S_{ox}$.

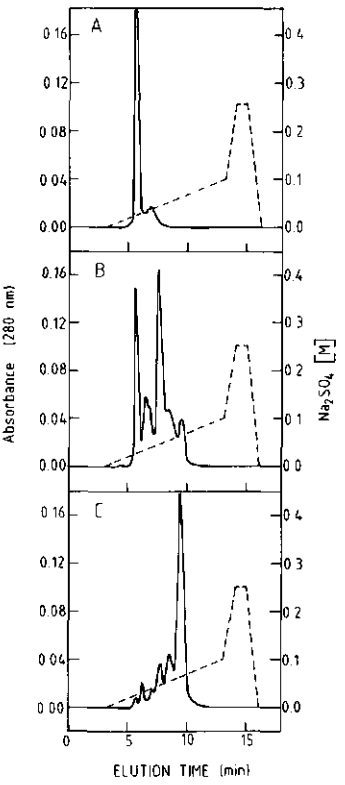

Fig. 2. *Fast protein liquid chromatography of purified* p-*hydroxybenzoate hydroxylase.* (A) Fraction F_1 in Fig. 1. (B) Fraction F_4 in Fig. 1. (C) Fraction F_6 in Fig. 1. A 200-µl aliquot of each fraction containing about 400 µg protein was analyzed using a Mono Q column. Gradient composition: buffer A, 20 mM Bistris pH 6.4; buffer B, buffer A containing 0.5 M Na_2SO_4. Flow rate 2 ml/min. The gradient was made as indicated by the dashed line

Thus on mixing the two preparations a new peak is formed with time in the elution diagram (Fig. 3C–H). Sodium dodecyl sulfate gel electrophoresis showed that the new peak consists of the two forms of the enzyme in about equal concentrations. This result is in agreement with Eqn (1). The kinetics of the reaction was investigated at different temperatures by determining the concentration of the three enzyme forms by integration of the three peaks in Fig. 3C–H. Using an equal concentration of the starting materials yields a ratio of 1:2:1

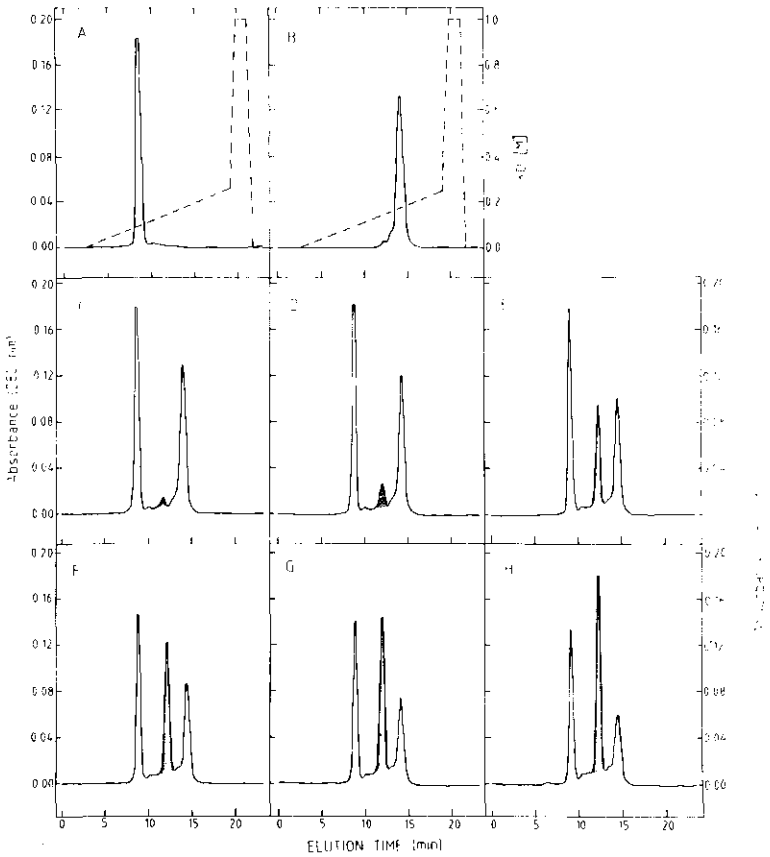

Fig. 3. *The formation of (E-SH ... E-S_{ox}) from pure (E-SH)$_2$ and pure (E-S_{ox})$_2$.* The reaction was followed in time after mixing the starting materials using an FPLC apparatus (Mono Q column). (A) Chromatogram of pure (E-SH)$_2$, 20 μM. (B) Chromatogram of pure (E-S_{ox})$_2$, 20 μM. A 100-μl aliquot of the mixture was analyzed in the following time intervals after mixing: 1.5 h (C), 4.5 h (D), 22.5 h (E), 47 h (F), 73 h (G) and 143 h (H). The reaction was carried out at 0°C in 10 mM K_2HPO_4, pH 7, containing 0.3 mM EDTA and 0.3 mM dithiothreitol. The analysis was done at 20°C. Gradient composition: buffer A, 20 mM Bistris, pH 6.4, containing 0.3 mM EDTA, 0.3 mM dithiothreitol; buffer B, buffer A containing 1 M KCl. Flow rate 1.5 ml/min. The gradient was made as indicated by the dashed line

for (E-SH)$_2$, the hybrid molecule and (E-S_{ox})$_2$ at the equilibrium position. These ratios are maintained when one of the starting materials is used in excess over the other. However such experiments are limited by the fact that the determination of the concentration of the hybrid dimeric form becomes less accurate with increasing concentration of one of the starting materials due to severe overlap with the central band. From the results shown in Fig. 3 an apparent dissociation constant of 0.25 μM is calculated for the hybrid dimeric enzyme. This dissociation constant is little dependent on the temperature in the range of 0 – 20°C.

The rate of the formation of the hybrid dimeric molecule from its constituents is very slow and it takes several days to reach the equilibrium position at pH 7 and 0°C. The rate of the reaction is only dependent on the concentration of the starting material when one of the components is used in excess over the other. This has been studied in the range of 1 – 8 μM. The rate of the reaction is however strongly dependent on the temperature (Fig. 4). From several similar experiments as shown in Fig. 3 a second-order rate constant for the formation of the hybrid dimeric enzyme is calculated and found to be 89.4 $M^{-1}\,s^{-1}$ at 20°C and 31.2 $M^{-1}\,s^{-1}$ at 0°C. The activation energy of the reaction is 34.9 kJ mol^{-1}. The rather slow reaction suggests that the formation of the hybrid molecule is governed by the dissociation of the starting materials into the corresponding monomeric molecules, as shown in Eqn (2):

$$(\text{E-SH})_2 + (\text{E-S}_{ox})_2 \rightleftarrows 2\,\text{E-SH} + 2\,\text{E-S}_{ox} \rightleftarrows 2\,(\text{E-SH} \ldots \text{E-S}_{ox}). \quad (2)$$

On the other hand the dissociation rate constant for the hybrid molecule is calculated to be 7.8×10^{-6} s^{-1} at 0°C. This indicates that the stability of the hybrid molecules is governed by the dissociation rate constant.

The dissociation constant for (E-SH)$_2$ has previously been estimated to be about 1 μM [7]. The dissociation constant for (E-S_{ox})$_2$ is unknown but assumed to be of the same order of magnitude. We have previously shown [7] that (E-SH)$_2$ dissociates to a higher degree in an aqueous solution contain-

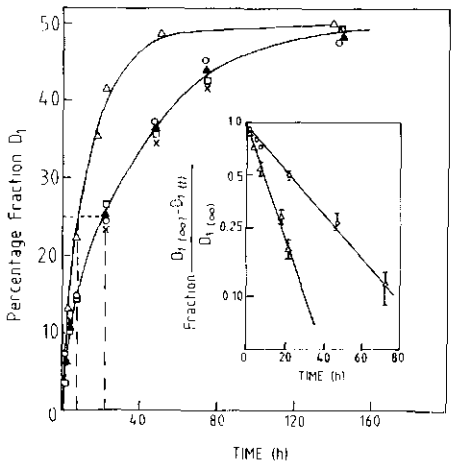

Fig. 4. *Kinetics of the formation of (E-SH ... E-S_{ox}) from (E-SH)$_2$ and (E-SO_{ox})$_2$.* The results are plotted as percentage of (E-SH ... E-S_{ox}) formed in dependence on time. The starting components were mixed in various concentrations at 0°C (1 μM, □; 4 μM, ○; 20 μM, ▲; 50 μM, ×) and at 20°C (1 μM, △) *in 10 mM* KH_2PO_4, pH 7.0, containing 0.3 mM EDTA and 0.3 mM dithiothreitol. The formation of the hybrid molecule (D_1) was followed by FPLC analysis of the mixture with time using a Mono Q column. The inset shows a semilogarithmic plot of the same data

ing Me_2SO. When the reaction of Eqn (2) was done in 20% Me_2SO, the observed rate for the formation of the hybrid dimer was more than ten-times increased as compared to that in aqueous solution, supporting the validity of Eqn (2). In addition the reaction is sowewhat dependent on the ionic strength of the solution. This observation has however not been studied in further detail.

Chemical modification by maleimide derivatives and 5,5'-dithiobis(2-nitrobenzoate)

The remarkable effects observed on both SDS-PAGE and anion-exchange chromatography by introducing only one additional negative charge to the overall protein charge have prompted us to study this effect in more detail by chemical modification studies. Cys-116 can be specifically blocked by either MalNEt or Nbs_2 [6, 9]. Treatment of (E-SH)$_2$ (Fig. 5A) with MalNEt does not alter the retention on either FPLC (Fig. 5C) or SDS-PAGE (Fig. 5F) with respect to that of the native protein. However when (E-SH)$_2$ is treated with Nbs_2 the FPLC pattern is shifted to a longer retention time (Fig. 5B) resembling the position of (E-S_{ox})$_2$ (Fig. 2C), while the SDS-PAGE pattern of (E-Nbs)$_2$ is shifted to a shorter retention (Fig. 5F). It should be mentioned that in this particular SDS-PAGE experiment (see Materials and Methods) 2-mercaptoethanol in the denaturation buffer was replaced by MalNEt in order to avoid regeneration of native Cys-116 by 2-mercaptoethanol or the remaining sulfhydryl groups in the protein [6]. Moreover when (E-MalNEt)$_2$ was treated with Nbs_2 no significant changes were observed in either the FPLC

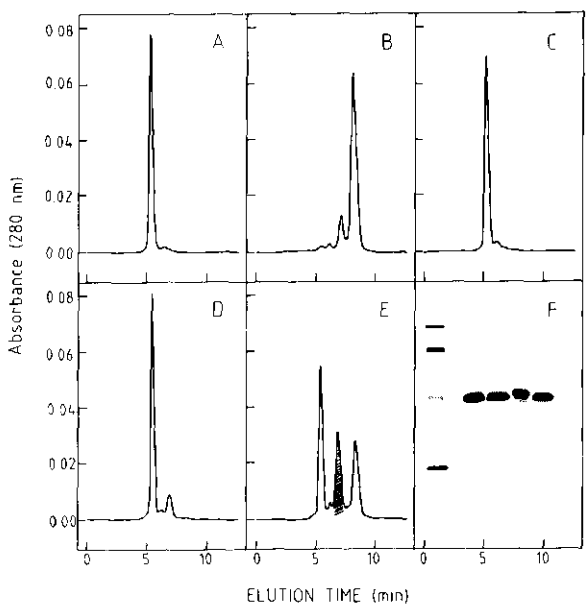

Fig. 5. *Chemical modification of (E-SH)$_2$ by 5,5'-dithiobis(2-nitrobenzoate) and N-ethylmaleimide followed by FPLC and SDS-PAGE analysis.* The modified samples were prepared as described under Materials and Methods. (A) Purified (E-SH)$_2$. (B) (E-SH)$_2$ modified by Nbs_2. (C) (E-SH)$_2$ modified by MalNEt. (D) E-MalNEt modified by Nbs_2. (E) Equimolar amounts of E-MalNEt and E-Nbs after reaction for 72 h at 0°C. (F) SDS-PAGE (15% gel): pattern (from left to right): lane 1, standard proteins (see Fig. 1); lane 2, (E-SH)$_2$; lane 3, E-MalNEt; lane 4, E-Nbs; lane 5, E-MalNEt modified with Nbs_2. SDS-PAGE was performed in the absence of 2-mercaptoethanol as described under Materials and Methods. For FPLC conditions see the legend of Fig. 2

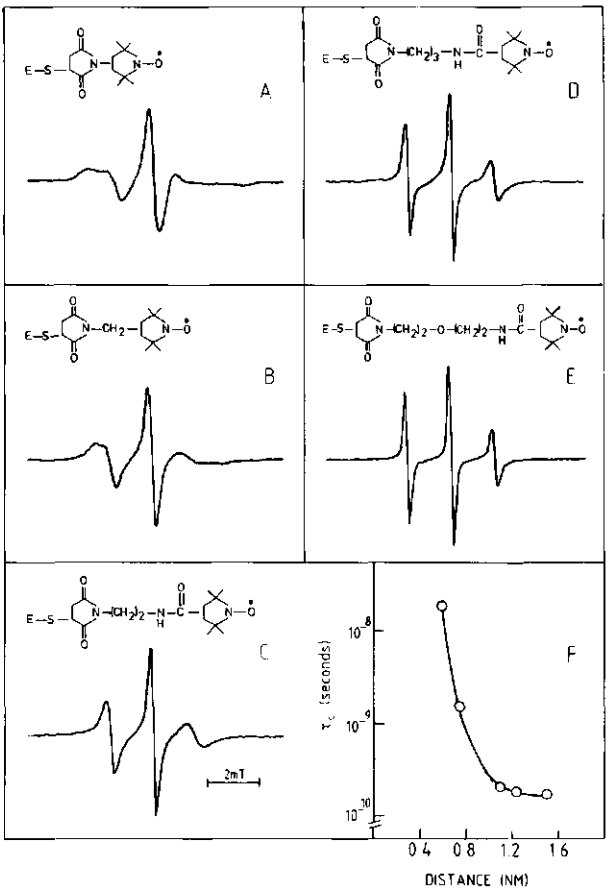

Fig. 6. *ESR spectra of* p-*hydroxybenzoate hydroxylase labeled with various maleimide spin-label derivatives as indicated.* Samples of 100 μM spin-labeled enzyme in 80 mM Hepes, pH 7.0, (I = 0.1 M) were used. The measurements were done at 20°C. The correlation between the calculated rotational correlation time (τ_c) and the chain length of the spin label is shown in F

(Fig. 5D) or SDS-PAGE (Fig. 5F) pattern as expected from earlier studies [6]. When $(E\text{-MalNEt})_2$ (Fig. 5C) and $(E\text{-Nbs})_2$ (Fig. 5B) were mixed in about equal concentration again a hybrid molecule is formed with time (Fig. 5E) according to Eqn (3):

$$(E\text{-MalNEt})_2 + (E\text{-Nbs})_2 \rightleftarrows 2\,(E\text{-MalNEt} \ldots E\text{-Nbs}). \quad (3)$$

Again the hybridization rate is about ten-times faster in the presence of 20% Me_2SO (v/v) than in its absence. Though we have not performed the kinetics of this reaction in detail, an apparent dissociation constant of 1 μM is calculated for this hybrid molecule (Fig. 5E) indicating that the stability of this particular molecule is somewhat less than that of E-SH ... $E\text{-}S_{ox}$ (i.e. K_d = 0.25 μM). The lower stability of the hybrid molecule in Eqn (3) is probably a consequence of the introduction of the bulky Nbs group into the enzyme.

The above results clearly demonstrate that the introduction of a negatively charged group into the enzyme yields the same FPLC and SDS-PAGE pattern as observed with $(E\text{-}S_{ox})_2$, strongly suggesting that oxidation of Cys-116 in native enzyme leads to the observed FPLC and SDS-PAGE pattern.

In this context it is of interest to obtain some information about the topography of Cys-116 in the enzyme because Cys-116 is not accessible for modification in the crystal state [4, 17, 19]. In addition X-ray diffraction studies [19] show that Cys-116 is localized on the opposite site of the monomer-monomer interface and could easily form a disulfide bridge with Cys-116 in another dimeric molecule yielding higher-order quaternary structures.

In order to estimate the distance between the SH group of Cys-116 and the surface of the protein, we used a series of maleimide spin labels and the ESR technique. The results are shown in Fig. 6. The ESR spectrum of the enzyme labeled with-3-(maleimido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (Fig. 6A) indicates that the label possesses a low mobility. The mobility of the spin label increases gradually from Fig. 6B to Fig. 6E. A measure for the mobility of the spin label is the rotational correlation time (τ_c), which was determined from the spectra by the method of Freed [22]. The results are

Table 2. *The distance between the SH group of Cys-116 and the N-O group of the covalently bound spin label, and the corresponding calculated rotational correlation time (τ_c) of the spin labels in p-hydroxybenzoate hydroxylase*

Compound in Fig. 6	Distance between the SH and the N-O group	τ_c
	nm	ns
A	0.6	30 3
B	0.75	8 2
C	1.1	4
D	1.25	3
E	1.5	2

summarized in Table 2. It should also be noted that the low-field resonance lines of the spin labels in Figs 6A and B show two components, i.e. the mobility of these spin labels is heterogeneous. The reason for this is not yet clear but it is possible that the conformation of the enzyme in the vicinity of Cys-116 exists in different states. Nevertheless the rotational correlation time of native dimeric enzyme is about 50 ns [23] and that of the low-mobility form of the spin label in Fig. 6A is about 30 ns. This indicates that the spin label is localized in a cavity of the enzyme and possesses some freedom of mobility. From Fig. 6F it is seen that the mobility of the spin label gradually increases with the chain length. From these results we estimate that the distance between the SH group of Cys-116 and the surface of the protein is roughly 0.6 nm. Considering this fact, it is not very likely that disulfide bridges are formed in solution between dimeric enzyme molecules, in agreement with our observations.

Chemical modification by hydrogen peroxide

Although the results presented above are in excellent agreement with Eqn (2), the additional bands observed on both the preparative (Fig. 1) and analytical (Fig. 2) anion exchangers cannot be fully explained by this equation. It has been shown by model studies [24] that sulfenic acid, sulfinic acid and sulfonic acid are products of oxidation of thiols and also occur in proteins [25, 26]. They can be generated by oxidizing agents [26]. Only sulfenic acid oxidation products are reducible to the original thiol group [25]. Therefore it is not unlikely that the following monomeric species are constituents of the isolated dimeric molecules of *p*-hydroxybenzoate hydroxylase: E-SH, E-SOH, E-SO_2^- and E-SO_3^-.

In order to check this hypothesis the oxidation of Cys-116 was investigated by chemical modification with oxidizing agents. Because the enzyme activity is not influenced by relatively high concentrations of hydrogen peroxide [27] this reagent was chosen. When $(E\text{-}SH)_2$ is incubated with hydrogen peroxide at least two new species are formed as observed by FPLC analysis (Fig. 7A). The reaction shows pseudo-first-order kinetics and is strongly pH-dependent (Fig. 7B). To check whether the reaction indeed involves Cys-116 a similar experiment was done using $(E\text{-}MalNEt)_2$. Since this treatment did not alter much the FPLC elution pattern of $(E\text{-}MalNEt)_2$ (results not shown), the result strongly suggests that the new peaks observed in Fig. 6A are related to the oxidation of

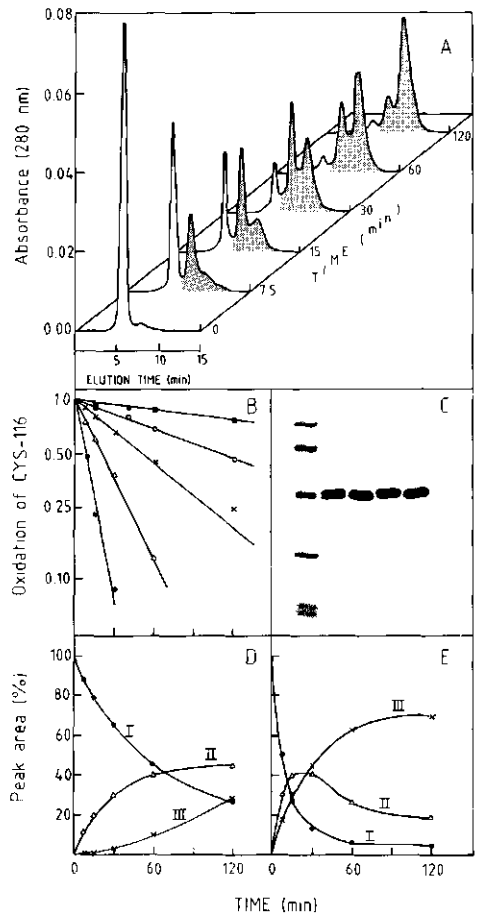

Fig. 7. *Chemical modification of $(E\text{-}SH)_2$ by hydrogren peroxide as monitored by FPLC and SDS-PAGE analysis.* The conditions of modification are described under Materials and Methods. (A) The time-dependent reaction of $(E\text{-}SH)_2$ with H_2O_2 at pH 8.5 as analyzed by FPLC (100-µl samples). (B) Kinetics of oxidation of Cys-116 by H_2O_2: (■) E-MalNEt, pH 8.5; ○——○ $(E\text{-}SH)_2$, pH 6.8; ×——× $(E\text{-}SH)_2$, pH 7.5; △——△ $(E\text{-}SH)_2$, pH 8.0; ●——● $(E\text{-}SH)_2$, pH 8.5. (C) SDS-PAGE (15% gel) pattern of H_2O_2 modification products of $(E\text{-}SH)_2$ and $(E\text{-}MalNEt)_2$ after 120-min reaction time: lane 1, standard proteins (see Fig. 1); lane 2, $(E\text{-}SH)_2$ at pH 7.5; lane 3, $(E\text{-}MalNEt)_2$ at pH 7.5; lane 4, $(E\text{-}SH)_2$ at pH 8.5; lane 5, $(E\text{-}MalNEt)_2$ at pH 8.5. (D) Time-dependent formation of FPLC peaks at pH 7.5. (E) Time-dependent formation of FPLC peaks at pH 8.5. For FPLC conditions see legend of Fig. 2

Cys-116. This statement is further supported by the fact that treatment of $(E\text{-}SH)_2$ and $(E\text{-}MalNEt)_2$ at pH 7.5 and pH 8.5 by H_2O_2 for 120 min and subsequent SDS-PAGE analysis of the samples revealed that the $(E\text{-}MalNEt)_2$ samples remained unaffected while the $(E\text{-}SH)_2$ samples showed partial (pH 7.5) and complete (pH 8.5) conversion (Fig. 7C). The two new peaks generated by H_2O_2 treatment of $(E\text{-}SH)_2$ show the same SDS-PAGE pattern as fractions F_4 and F_6 in Fig. 1, i.e. the first product observed in Fig. 7A consists of the hybrid molecule and the second product represents oxidized enzyme.

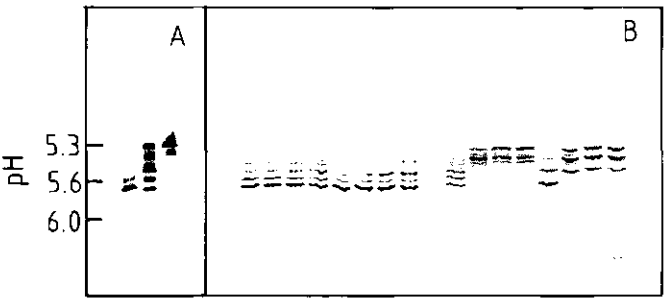

Fig. 8. *Isoelectric focusing of purified p-hydroxybenzoate hydroxylase.* (A) Isoelectric focusing of (from left to right) fractions F_1, F_4 and F_6 (Fig. 1) in a 1-mm gel. Before use samples were dialyzed overnight at 4 °C in 10 mM KH_2PO_4, pH 7.0, containing 0.3 mM EDTA. The pH gradient was measured on both sides of the gel. (B) Ultrathin (200 μm) isoelectric focusing of fraction F_1 (Fig. 1) modified by H_2O_2 for 0, 10, 30 and 90 min, respectively (see Materials and Methods). Lanes 1–4, pH 6.6; lanes 5–8, pH 6.6 treated with dithiothreitol; lanes 9–12, pH 9.3; lanes 13–16, pH 9.3 treated with dithiothreitol

Moreover treatment of the oxidized sample with dithiothreitol did not change the FPLC pattern, indicating that no detectable amount of intermolecular disulfide bridge formation has occurred during the oxidation reaction.

The time-dependent formation of the new peaks at pH 7.5 and pH 8.5 is plotted in Fig. 7D and E, respectively. These plots suggest that the formation of III is dependent on the intermediate formation of II. This suggests that we are dealing with a consecutive reaction: $A \xrightarrow{k_1} B \xrightarrow{k_2} C$. The value for k_1 (pH 8.5) is known from the plot of Fig. 7B. The value for k_2 was estimated from the time-dependent formation of III in Fig. 7E and found to be 0.03 min^{-1}. With these rate constants the curves in Fig. 7E can be reasonably simulated, although it should be mentioned that curves II and III are much less accurately calculated than curve I. This is due to the fact that the time-dependent concentrations of II and III are difficult to estimate accurately because of severe overlap of the bands and the formation of a small amount of additional products. Nevertheless the time-dependent concentration of II can be calculated using the equation:

$$[II] = \frac{k_1[A_0]}{k_1 - k_2} \cdot e^{-k_2 t}(1 - e^{-(k_1 - k_2)t}) \qquad (4)$$

where $[A_0]$ is the initial concentration of native enzyme. The concentration of III at any time is obtained by the material balance:

$$[III] = [A_0] - [I] - [II]. \qquad (5)$$

Further support for the consecutive reaction is obtained by the rather good prediction of the time needed for II to reach the maximal concentration:

$$t_{max} = \frac{\ln(k_2/k_1)}{k_2 - k_1} \approx 20 \text{ min}. \qquad (6)$$

These results suggest that the two Cys-116 in a dimeric molecule are oxidized one by one. This interpretation is in accord with the fact that the formation of the hybrid molecule from its constituents is a very slow process (see above).

The pseudo-first-order rate constant belonging to Fig. 7B (using 10 μM enzyme, 20 mM H_2O_2 and 20 mM buffer at 20 °C) were calculated to be 0.006 min^{-1} (pH 6.8), 0.014 min^{-1} (pH 7.5), 0.034 min^{-1} (pH 8.0) and 0.086 min^{-1} (pH 8.5). Although the rate constant increases with increasing pH value, a pK_a value for a group participating in the reaction could not be deduced from these results.

It should be mentioned that the kinetics of the oxidation of Cys-116 by hydrogen peroxide are not affected by the presence of the substrate *p*-hydroxybenzoate (results not shown). Furthermore identical FPLC patterns are also observed when the $(E\text{-}SH)_2$-substrate complex is treated with *o*-iodosobenzoate (50–200 μM) leading to complete oxidation of Cys-116. The modification does not affect the enzyme activity if the substrate is present. The kinetics of this reaction was not studied in more detail however.

Isoelectric focusing

As already mentioned above, it can be expected that the oxidation reaction proceeds via the stepwise formation of E-SOH, $E\text{-}SO_2^-$ and $E\text{-}SO_3^-$. The sulfenic acid intermediate in the H_2O_2-oxidation reaction could not be identified by FPLC, because either it has a short lifetime or its properties are too similar to those of the native enzyme. This is in contrast to observations made with glyceraldehyde-3-phosphate dehydrogenase [25] and papain [26]. On the other hand, FPLC of fraction F_4 (Fig. 2) suggests the presence of several species. Therefore an attempt was undertaken to obtain a deeper insight into the composition of the various bands by isoelectric focusing.

Fig. 8A shows the IEF gels of the fractions of F_1, F_4 and F_6 of Fig. 1. The relatively pure fractions F_1 and F_6 exhibit two and three bands, respectively, whereas F_4 shows at least five bands. These results are in good agreement with the FPLC data of the same samples (cf. Fig. 2). The main band of F_1 has an isoelectric point of 5.7. This value is in good agreement with an earlier determination using native enzyme [9] and is assigned to $(E\text{-}SH)_2$. The main band of F_6 shows an isoelectric point of 5.3 and is assigned to $(E\text{-}SO_3^-)_2$ and/or $(E\text{-}SO_2^-)_2$. In this context it is reasonable to propose that the hybrid molecule E-SH ... $E\text{-}SO_3^-$ possesses an isoelectric point of 5.5. Such a band is present in the IEF gel of F_4, which contains a considerable amount of the hybrid molecule (cf. Figs 1 and 2). It should also be noted that F_6 also contains a weak band representing the hybrid molecule in accord with the presence of a small amount of E-SH in SDS-PAGE (Fig. 1). The above results were also confirmed by two-dimensional gel electrophoresis.

The time-dependent oxidation of Cys-116 in the enzyme by H_2O_2 was investigated at pH 6.6 and pH 9.3 at 20°C. Both samples were incubated with H_2O_2, aliquots were withdrawn after 10, 30 and 90 min of incubation and treated with catalase to remove excess H_2O_2. The samples were divided into two equal volumes, to one of which dithiothreitol was added (see Materials and Methods). This approach should yield some information on the formation of species which are reducable by dithiothreitol. The results are shown in Fig. 8B where the first eight lanes refer to the reaction mixture at pH 6.6. The first four lanes represent samples at 0, 10, 30 and 90 min of incubation with H_2O_2. The second four lanes show the samples treated with dithiothreitol. Both samples of the starting material already show some oxidation products due to storage over a long period of time. The treatment with dithiothreitol decreases mainly the intensity of the second band from the bottom and diminishes some bands of weak intensity showing a low isoelectric point. With time the intensity of the second band and that due to the starting material decrease slowly and the intensity of the other bands increases. These results are in good agreement with the results shown in Fig. 7D where it is seen that the species I and II are present in higher concentrations after a 90-min reaction time than species III. At pH 9.3 the starting material already contains a considerable amount of oxidation products due to the higher reactivity of Cys-116 towards O_2. In contrast to the results at pH 6.6, the addition of dithiothreitol diminishes the intensity of the second band completely owing to the stronger reducing power of dithiothreitol at higher pH values. At the same time the intensities of the first and third band are increased. Adding H_2O_2 to the sample converts the first three bands in a very fast reaction into at least three new bands. Treatment of these samples with dithiothreitol leads to the formation of an intense band with an isoelectric point of 5.5. Simultaneously the band with a higher isoelectric point is decreased.

From these results we propose the following assignments for the observed IEF bands. The first band (isoelectric point of 5.7) is assigned with certainty to the native enzyme molecule containing an unmodified Cys-116. The second band represents most probably the sulfenic acid derivative of Cys-116 (isoelectric point of $\approx$ 5.6). This assignment is based on the fact that the corresponding enzyme molecules are easily converted to native enzyme molecules by dithiothreitol or further oxidized by H_2O_2. The third band (isoelectric point of 5.5) is assigned to the hybrid molecule E-SH ... E-S_{ox} (E-S_{ox} = E-SO_2^- or E-SO_3^-) because purified samples of this species show two bands of equal intensity on SDS-PAGE. The fourth band (isoelectric point of $\approx$ 5.4) yields on treatment with dithiothreitol the hybrid molecule, suggesting that it contains a reductable group. Therefore it is suggested that it represents either E-SOH ... E-SO_2^- or E-SOH ... E-SO_3^-. The fifth and the sixth bands (isoelectric point 5.3) exhibit only the faster-moving band on SDS-PAGE, i.e. native enzyme is absent. Therefore the fifth band is assigned to E-SO_2^- and the sixth band to E-SO_3^-. Although these assignments cannot be verified it is not unlikely that the different oxidation states of the sulfur atom in the two species could lead to a different isoelectric focusing behaviour. This idea is in fact supported by the observation that the sulfenic acid derivative of Cys-116 also shows a distinct isoelectric focusing band.

If we now accept that E-SOH, E-SO_2 and E-SO_3 are oxidation products of E-SH and considering the fact that the enzyme exists in a dimer-monomer equilibrium, then ten different enzyme dimer molecules can be expected as shown in Fig. 9. Only six of the ten species can be identified by IEF,

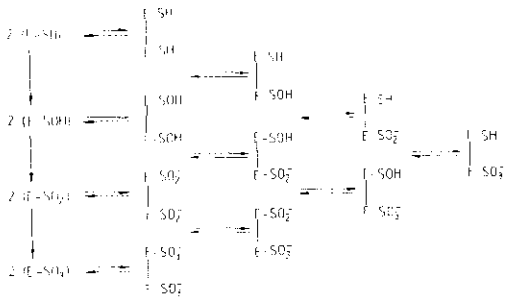

Fig. 9. *Hypothetical scheme of dimeric forms of* p-*hydroxybenzoate hydroxylase*. The forms containing E-SOH could not be identified by the techniques used or are partially converted to the SH group by dithiothreitol

i.e. (E-SH)$_2$, E-SH ... E-SOH and/or (E-SOH)$_2$, (E-SO_2^-)$_2$, (E-SO_3^-)$_2$, E-SH ... E-SO_2^- and/or E-SH ... E-SO_3^-, and E-SOH ... E-S_{ox}. The residual four species could not be observed, probably because their isoelectric focusing properties are similar with those of the corresponding mono- and divalently charged dimeric molecules.

CONCLUSIONS

Although the scheme shown in Fig. 9 seems to explain the experimental observations rather well, it should be kept in mind that other modifications than oxidation of Cys-116 alone in the sequence of the enzyme could also contribute to the various observed species of the enzyme. Since however the modification of the enzyme occurs rather easily and can in part be reversed by the addition of sulfhydryl-containing organic molecules to enzyme solutions, we believe that the various forms of the enzyme are mainly generated by (partial) oxidation of Cys-116. Using pure (E-SH)$_2$ it could in fact be shown that several species observed in IEF gels could be generated by incubation of the enzyme with H_2O_2 and that a few of these bands disappeared upon incubation of these samples with dithiothreitol.

In conclusion, it can be stated that the availability of high-resolution biochemical techniques will probably reveal similar effects with other enzymes. The enzyme *p*-hydroxybenzoate hydroxylase can be considered as a relatively simple dimeric protein. This fact, and the fact that oxidation of Cys-116 does not affect the catalytic properties of the enzyme [6, 9], made it possible to unravel the cause of the heterogeneity of the enzyme rather easily. In other enzymes the situation may become more complex due to the involvement of cysteine or other amino acid residues in the catalysis, which may become altered during the isolation of a particular enzyme. In such cases the catalytic properties of an enzyme may either become completely abolished or the enzyme may become partially inactivated or even show an altered substrate specificity. To distinguish in such cases whether the altered activities are due to apparent isozymes or to isolation artifacts will be a very tedious task and will require a broad combination of techniques to establish the real properties of a particular enzyme. The degree of complexity will increase with the complexity of a particular system where nature has already provided a large number of isozymes as for instance in cytochrome P-450.

Our results unambiguously show that the microheterogeneity in *p*-hydroxybenzoate hydroxylase is due to isolation

artifacts. This statement is further supported by the fact that Western blotting of a cell-free extract followed by incubation with antiserum against purified *p*-hydroxybenzoate hydroxylase and autoradiography using ^{125}I-protein A showed only the presence of $(E\text{-}SH)_2$. This observation also strongly supports our interpretation that microheterogeneity in *p*-hydroxybenzoate hydroxylase is caused by a time- dependent (partial) oxidation of Cys-116.

Finally it should be mentioned that scaling-up of enzyme purifications with classical purification methods can possibly lead to this kind of isolation artifacts. When the enzyme is purified under semi-anaerobic conditions (by flushing all solutions with argon) in the presence of 5 mM 2-mercaptoethanol the oxidation of Cys-116 can be suppressed, but not completely abolished.

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Chapter 5

Chemical modification of sulfhydryl groups in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

Involvement in catalysis and assignment in the sequence

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The cysteine residues in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* were modified with several cysteine reagents. One of the five sulfhydryl groups reacts rapidly and specifically with *N*-ethylmaleimide without inactivation of the enzyme. Cysteine-116 was found to be the reactive cysteine by isolation of a labeled tryptic peptide.

The enzyme is easily inactivated by mercurial compounds. The original activity can be fully restored by treatment of the modified enzyme with sulfhydryl-containing compounds. The rate of incorporation of mercurial compounds is pH-independent and is pseudo-first-order up to 90–95% loss of activity. The reaction shows saturation kinetics. The substrate *p*-hydroxybenzoate protects the enzyme from fast inactivation. The mercurial compounds themselves inhibit the inactivation reaction at concentrations higher than 80 μM. A spin-labeled derivative of *p*-chloromercuribenzoate reacts fairly specifically with only Cys-152 on use of enzyme prelabeled with *N*-ethylmaleimide, in contrast to *p*-chloromercuribenzoate which reacts with additional cysteine residues, i.e. Cys-211 and Cys-158. From these results it is concluded that modification of Cys-152 decreases drastically the affinity of the enzyme for the substrate. The results strongly indicate that the substrate binding site and Cys-152 are interdependent. This observation is not obvious when the three-dimensional data only are considered. The modified enzyme exhibits a somewhat higher affinity for NADPH than the native enzyme.

Modification of *N*-ethylmaleimide-prelabeled enzyme by *p*-chloromercuribenzoate leads to absorbance difference spectra showing maxima at 250 nm, 290 nm and 360 nm. The intensities of the absorbance difference maxima at 290 nm and 360 nm are strongly dependent on the pH value of the solution. The intensities are very low at low pH values and increase with increasing pH values, reaching a maximum at about pH = 9. The ionizing group shows a p*K* value of about 7.6. The maximal molar difference absorption coefficient at 290 nm is 3200 $M^{-1}cm^{-1}$ at pH 9, suggesting that tyrosine residues ionize under the conditions of modification of the enzyme.

The results are discussed in the light of the known three-dimensional structure.

The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is an external mono-oxygenase that catalyzes the conversion of *p*-hydroxybenzoate into 3,4-dihydroxybenzoate. NADPH serves as an electron donor. The reaction mechanisms of the enzymes from *P. fluorescens* [1] and *Pseudomonas desmolytica* [2] have been studied in detail.

A low-resolution (0.25-nm) three-dimensional structure of the enzyme from *P. fluorescens* is available [3]. In addition, the complete sequence of the same enzyme has been elucidated very recently [4, 5]. These results form a good basis for an investigation to elucidate the essential amino acid residues of the enzyme involved in the catalytic reaction. Studies on the active-site region by chemical modification so far include arginine [6] and histidine [7] residues in the enzyme from *P. desmolytica* and histidine residues in the enzyme from *P. fluorescens* [8].

It has been mentioned in the literature for the enzyme from *P. desmolytica* that the enzyme reacts with *p*-chloromercuribenzoate leading to inactivation and that NADPH protects this enzyme from inactivation by mercurial reagents [7, 9]. Unfortunately no data have been published yet on this subject.

p-Hydroxybenzoate hydroxylase from *P. fluorescens* contains five cysteine residues [10]. The enzyme contains no cystine residues [10]. It has already been shown [10] that one of the five cysteine residues reacts specifically with *N*-ethylmaleimide. This modification does not affect the catalytic activity of the enzyme. This observation led us to study the possible effects of mercurial reagents on the activity of the enzyme.

In this paper the inactivation of the enzyme by mercurial reagents is described. It is shown that these reagents easily inactivate the enzyme and that modification of one particular cysteine residue is responsible for the loss of activity. This cysteine residue and that specifically reacting with *N*-ethylmaleimide have been tentatively identified in the sequence of the enzyme. Some preliminary results have been reported elsewhere [11].

Abbreviations. Bistrispropane, *N*,*N*-bis[2-(hydroxymethyl)-2-propyl-1,3-diol]-1,3-diaminopropane; ClHgBzOH, *p*-chloromercuribenzoate; ClHgBzsl, spin-labeled *p*-chloromercuribenzoate; MalNEt, *N*-ethylmaleimide; E-MalNEt, *N*-ethylmaleimide-labeled enzyme; ESR, electron spin resonance; HPLC, high-performance liquid chromatography.

Enzyme. *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2).

MATERIALS AND METHODS

Reagents

Trifluoroacetic acid (spectroscopic grade), *p*-hydroxybenzoate and EDTA were from Merck (Darmstadt, FRG),

acetonitrile (HPLC grade) from J. T. Baker Chemicals (Deventer, The Netherlands), NADPH from Boehringer (Mannheim, FRG), *p*-chloromercuri[*carboxy*-^{14}C]benzoate and *N*-[^{14}C]ethylmaleimide from ICN, California (USA) and all other chemicals were from Sigma. Spin-labeled *p*-chloromercuribenzoate was synthesized by condensation of *p*-chloromercuribenzoylchloride with 4-amino-2,2,6,6-tetramethylpiperidinooxy (Aldrich) in freshly distilled pyridine as described by Zantema et al. [12].

Analytical methods

Difference light absorption spectra were recorded on an Aminco DW-2A spectrophotometer. Fluorescence emission spectra were obtained on an Aminco SPF-500 fluorometer. Activity measurements were done on a Zeiss PMQII spectrophotometer. All instruments were equipped with thermostated cell holders.

Dissociation constants of complexes between enzyme and substrate, and enzyme and NADPH were determined fluorometrically as described previously [13].

Radioactivity determinations were carried out either in hydroluma or in emulsifier scintillator 299 scintillation fluid using a Packard model 2450 or model 3375 Tri-carb liquid scintillation spectrometer.

Acrylamide gels for fluorography were prepared as described elsewhere [14].

Succinylcysteine, formed upon acid hydrolysis of cysteine alkylated with *N*-ethylmaleimide [15], was identified and quantitatively determined as described by Gehring and Christen [16]. Succinylcysteine preceded carboxymethylcysteine on the Kontron Liquimat III amino acid analyser. The amounts of protein were determined from amino acid analysis, unless otherwise mentioned.

Enzyme labeling for kinetic studies

The enzyme used was isolated and purified as described previously [10]. The assay mixture contained 0.15 mM *p*-hydroxybenzoate, 0.15 mM NADPH in 0.1 M Tris/H_2SO_4, pH 8.0 (25°C). Prior to use the enzyme was poured over a Bio-Gel P-6DG (Bio-Rad, California, USA) column (1 × 8 cm) equilibrated with 80 mM Hepes buffer, pH 7.0 (I = 0.1 M), unless otherwise stated. The pH-dependent modification studies were performed at constant ionic strength (I = 0.1) in 80 mM Mes (pH 6 – 7), 80 mM Hepes (pH 7 – 8), 80 mM Hepps (pH 8 – 8.7) and 50 mM Bistrispropane (pH 8.8 – 9.3). Buffers were brought to constant ionic strength with 0.5 M Na_2SO_4 as described earlier [17]. The concentration of the enzyme was determined using a molar absorption coefficient of 11.3 $mM^{-1}cm^{-1}$ at 450 nm [1, 10].

Labeling of the reactive sulfhydryl group of the enzyme was done by addition of a tenfold excess of *N*-ethylmaleimide to a 100 μM enzyme solution, pH 7.0. The reaction was allowed to proceed for 10 min at 25°C, then the reaction was stopped by addition of a solution of dithiothreitol to a final concentration of 5 mM. The enzyme was poured over a Bio-Gel column to remove excess reagent. The resulting fully active MalNEt-enzyme was usually used in this study for labeling experiments with mercurial-containing compounds. To identify the cysteine residue in the sequence of the enzyme, a similar sample was prepared using radioactive *N*-ethylmaleimide.

Mercuration of the MalNEt-enzyme was done by treatment of an enzyme solution with various concentrations of the reagent (5 – 100 μM) at 22°C. Incubation mixtures contained about 2 μM enzyme when the time-dependent loss of activity was followed by the standard assay procedure. The reaction mixture contained about 10 μM enzyme when the time-dependent incorporation of mercurial compounds into the enzyme was followed by spectrophotometric methods.

The concentration of free *p*-chloromercuribenzoate was determined by measuring the absorbance at 232 nm of a diluted sample in 0.1 M KOH, using a molar absorption coefficient of 16.9 $mM^{-1}cm^{-1}$ [18]. The incorporation of *p*-chloromercuribenzoate into the enzyme was followed spectrophotometrically at 250 nm, using a molar difference absorption coefficient of 7.6 $mM^{-1}cm^{-1}$ [18] to quantify the reaction. The length of the two-compartment cell used was 0.875 cm.

The spectrophotometric method could not be used when the enzyme was modified in the presence of the substrate or NADPH. In these cases the enzyme was incubated with various concentrations of radioactive *p*-chloromercuribenzoate in the presence of a high excess of substrate or NADPH for 4 h at 22°C. The samples (1.0 ml) were then purified by gel filtration over a Bio-Rad economy column (V_o = 2.0 ml, V_t = 7.0 ml). The activity and radioactivity of the eluate (3.0 ml) were determined thereafter. 1.0 ml of the sample was diluted to 10 ml with scintillation fluid for radioactivity measurements.

Radioactive *p*-chloromercuribenzoate was kept as a 1 mM solution (6.3 nCi/ml) in a 25 mM KOH solution at 4°C. Before use the radioactive solution was diluted with a conventional 1 mM *p*-chloromercuribenzoate solution to yield a 1 mM solution with a specific activity of 1.89 nCi/ml.

Reactivation of the modified enzyme was achieved by addition of a 1 mM solution of dithiothreitol to a 1.1 μM solution of the modified enzyme. After a 15-min incubation the activity of the enzyme was fully restored.

Spin-labeled *p*-chloromercuribenzoate was dissolved in ethanol (≈ 1 mM). The concentration of the spin-labeled compound was determined spectrophotometrically using a molar absorption coefficient of 20.7 $mM^{-1}cm^{-1}$ at 237 nm [12]. A molar difference absorption coefficient of 6.1 $mM^{-1}cm^{-1}$ at 257 nm was published [12] for the quantification of the reaction between glutamate dehydrogenase and the spin-labeled mercurial compound. However, $\Delta\varepsilon$ for *p*-hydroxybenzoate hydroxylase was found to be 7.6 $mM^{-1}cm^{-1}$. This value was determined by combined spectrophotometric and ESR measurements.

All electron spin resonance spectra were recorded on a Bruker ER 200D spectrometer at 22°C. The instrument was connected to a Data General NOVA 3 computer for storage and handling of the experimental spectra. The amount of incorporated spin-label in the enzyme was determined by running the spectrum in a quartz capillary with an inside diameter of about 1 mm. After recording the spectra, an excess of dithioerythritol was added to the enzyme solution and the spectrum of the free spin-label was recorded. This spectrum was compared with that of a standard solution of the free spin-label under identical conditions. The areas of both spectra were determined by double integration and the amount of spin-label incorporated was calculated by comparison with the spectrum of the free-label standard.

Enzyme labeling for sequence studies

Materials and methods, except those described below, were the same as used previously [4]. Approximately 100 nmol

p-hydroxybenzoate hydroxylase was modified with *N*-[^{14}C]-ethylmaleimide (specific radioactivity 10 Ci/mol). After removal of the reagent, a product was obtained which had incorporated about 1 mol reagent/mol of subunit (protein amount determined from the flavin content of the enzyme). The protein so obtained was supplemented next with protein that had been modified with unlabeled *N*-ethylmaleimide. Approximately 2000 nmol of protein with a specific activity of 1100 dpm/nmol was used for preparative experiments.

Modification of *p*-hydroxybenzoate hydroxylase with the mercurial compounds was done with protein that had been pretreated with unlabeled *N*-ethylmaleimide. 47 μM E-MalNEt (188 nmol) was incubated with 340 μM ClHgBzOH for 2 h at 21 °C in 80 mM Hepes buffer, pH 7.0 (I = 0.1 M). The residual activity was 10%. An identical enzyme solution was also incubated with 175 μM ClHgBzsl under the same conditions. The residual activity was 6%. The samples were then poured over a Bio-Gel P-6 column (1.5 × 10 cm) and the eluates lyophilized. Next the protein samples were denatured in 7.2 M urea in 0.1 M potassium phosphate, pH 7.2, in the presence of 10 mM unlabeled *N*-ethylmaleimide in order to block the remaining sulfhydryl groups. This treatment prevented a rapid release of mercurials from the cysteine residues which was observed when the enzyme was denatured in the absence of *N*-ethylmaleimide, probably caused by the other cysteine residues of the protein. The reaction mixtures were allowed to stand for 17.5 h at 21 °C. Then the samples were dialyzed for 6 h against a solution consisting of 8 M urea and 1 mM MalNEt in 0.1 M potassium phosphate, pH 7, and for 16 h against the same solution, but omitting MalNEt. Modification of cysteine residues with the mercurials was subsequently reversed by incubation of the samples for 4 h in the phosphate buffer containing 8 M urea and 20 mM dithiothreitol. After removal of mercurials and dithiothreitol by column chromatography (Bio-Gel P-6, 1 × 10 cm), the regenerated sulfhydryl groups were modified with 400 μM MalNEt containing 100 μM *N*-[^{14}C]ethylmalaimide (specific radioactivity 2.5 Ci/mol) under denaturing conditions. Excess of reagent was removed by exhaustive dialysis against 8 M urea followed by dialysis against the buffer (0.1 M ammonium bicarbonate, pH 8.0) that was used in the subsequent digestion with trypsin. The protein samples (about 100 nmol each) that were originally treated with ClHgBzsl and ClHgBzOH had specific activities of 3400 dpm/nmol (0.62 mol label/mol subunit) and 4200 dpm/nmol (0.78 mol label/mol subunit), respectively. A separate sample (100 nmol) which underwent the same procedure without mercurial treatment had a specific activity of 1200 dpm/nmol (0.22 mol label/mol subunit). Any label in such a sample (in the following referred to as control sample) would show the effectiveness by which cysteine residues were blocked by unlabelled *N*-ethylmaleimide and any increase of the specific activity of a particular cysteine residue in the mercurial-treated samples as compared with the control sample implies that it had originally reacted with mercurial in the native enzyme. All four samples were prepared and analyzed in parallel under exactly the same conditions.

Numbering of amino acid residues and nomenclature of peptides

Amino acids are numbered according to the numbering used for the complete primary structure of *p*-hydroxybenzoate hydroxylase [4, 5]. Except for peptides T6C1, T6C2, T6C3 and T6C4, the nomenclature of all other peptides is the same as originally used in studies on the primary structure of *p*-hydroxybenzoate hydroxylase [4, 5]. All tryptic and chymotryptic peptides mentioned in this paper are indicated in Fig. 2 by their amino acid sequences.

Enzymic digestions

Tryptic digests of modified *p*-hydroxybenzoate hydroxylase were made by incubation at 37 °C in 0.1 M ammonium bicarbonate, pH 8.0, at a trypsin/substrate ratio of 1:100 (w/w) for 1 h; the incubation was continued for another 3 h after addition of the same amount of trypsin. Because of the low solubility of denatured *p*-hydroxybenzoate hydroxylase in 0.1 M ammonium bicarbonate, pH 8.0, a finely divided suspension of the substrate was made by sonification prior to digestion. After digestion, remaining insoluble material (containing about 8% of the total radioactivity) was removed by centrifugation. The insoluble material consisted for the greater part, if not exclusively, of large partial tryptic cleavage products as was shown by analysis by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (major peptide bands near 10000 Da).

Digestion of peptide T6 with chymotrypsin was performed at 37 °C in 0.1 M ammonium bicarbonate, pH 8.0, at an enzyme/substrate ratio of 1:20 (w/w) during 4 h.

RESULTS AND DISCUSSION

Labeling with N*-ethylmaleimide and assignment of the cysteine residue in the sequence*

p-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* possesses five sulfhydryl groups of which only one is reactive towards *N*-ethylmaleimide [10]. This modification affects the activity of the enzyme only slightly. In order to identify this sulfhydryl group in the sequence of the enzyme the protein was labeled using *N*-[^{14}C]ethylmaleimide. This yields a preparation which has incorporated about 1 mol label/mol subunit, confirming previous results [10]. The reaction is rather fast, not allowing characterization of the kinetics of the reaction.

The choice of the peptide isolation procedure was largely dictated by the experience acquired earlier from the determination of the complete amino acid sequence of the enzyme by CNBr cleavage of the protein [19]. This procedure produces five fragments, viz. CB1 – CB5, accounting for the entire polypeptide chain [5]. CNBr fragment CB1 contains four cysteine residues, whereas CB2 contains a single cysteine residue. After CNBr cleavage of the protein modified with *N*-[^{14}C]-ethylmaleimide, analysis by sodium dodecyl sulphate/polyacrylamide gel electrophoresis and fluorography indicated exclusive location of the label on CB1 (data not shown). In order to determine the modified cysteine residue in peptide CB1, the following procedure was followed: (a) purification of labeled CB1 from a CNBr digest of *p*-hydroxybenzoate hydroxylase of which the remaining sulfhydryl groups had been carboxymethylated under denaturing conditions; (b) tryptic cleavage at arginine residues of citraconylated CB1 and (c) subsequent purification of the smaller labeled fragments.

In order to obtain a suitable amount of protein (approximately 2000 nmol) for the preparative isolation of peptides, the amount of protein was increased 20-fold by addition of *p*-hydroxybenzoate hydroxylase that had been treated with unlabeled reagent. The CNBr peptides generated from this material were gel-filtered by Sephadex G-100 chromatography [20]. On the basis of sodium dodecyl

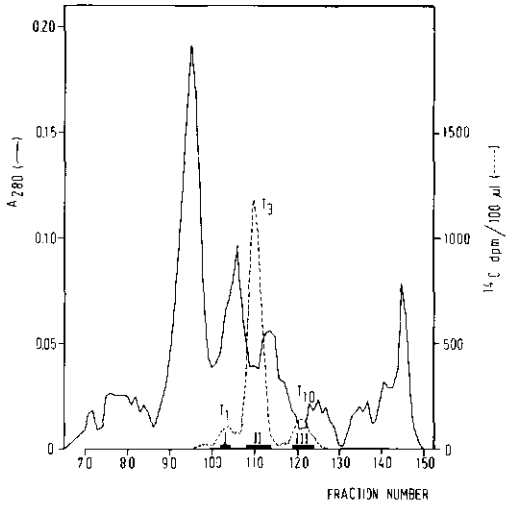

Fig. 1. *Fractionation of a tryptic digest of the pool of CB1 obtained by Sephadex G-100 chromatography.* About 250 nmol of citraconylated CB1 was digested with trypsin, applied to two columns (2.0 × 200 cm) of Sephadex G-50F in series and eluted in 2.0-ml fractions with 30% acetic acid at a flow rate of 4.7 ml/h. Peptides were detected by their absorbance at 280 nm and by the radioactivity in 0.1-ml aliquots of the eluted fractions. The fractions were pooled as indicated by the horizontal bars and only radioactive peptides of interest were subsequently isolated from these pools by thin-layer chromatography (T1 and T3) and high-voltage paper electrophoresis (pH 3.5) (T10)

sulphate/poyacrylamide gel patterns, CB1-containing fractions were pooled. Results of amino acid analysis and eight cycles of Edman degradation on the CB1 Sephadex pool were compatible with the known amino acid composition and the N-terminal sequence Glu-Ala-Arg-Glu-Ala-Cys-Gly-Ala- (residues 111 – 118) of peptide CB1 from the protein [4]. Liquid scintillation counting of the phenylthiohydantoin derivatives of the amino acids released during automatic Edman degradation on the unfractionated CNBr digest, as well as on peptide CB1, showed a clear peak of radioactivity at cycle 6 only, indicating that Cys-116 had been modified with N-ethylmaleimide.

To see whether other cysteine residues in peptide CB1 also had been modified by *N*-ethylmaleimide, this peptide was further digested with trypsin. The resulting tryptic peptides were fractionated on a Sephadex G-50F column as shown in Fig. 1. The radioactivity pattern of the eluted fractions revealed one major peak flanked by two minor peaks. The peptide almost solely responsible for the major radioactive peak in Fig. 1 was obtained after an additional purification step by thin-layer chromatography of pool II. Sequence analysis and amino acid analysis of this peptide (yield: 63 nmol) indicated a pure peptide which was identified as the tryptic peptide T3 in the primary structure of CNBr fragment CB1 [4] (see Fig. 2 for the sequence of the various fragments). Five cycles of dansyl-Edman degradation and measurement of the radioactivity of the derivatives cleaved off in each degradation cycle, revealed a pronounced peak of radioactivity for Cys-116, which definitely established that this residue had been modified with *N*-ethylmaleimide.

We did not obtain conclusive evidence for the identity of all the peptides which contained relatively small amounts of radioactivity in the two pools I and III in Fig. 1. Nevertheless a small amount of peptide T1 was suspected to be present in pool I (yield: 2 nmol after the final purification step). This peptide, which results from incomplete cleavage at Arg-113, also contains Cys-116 alkylated with *N*-ethylmaleimide. The cysteine-containing peptide T10 was isolated from the third peak with radioactivity (pool III) with about the same yield as peptide T3, i.e. 55 nmol. This peptide, together with a mixture of undefined peptides, was responsible for the radioactivity present in pool III. Therefore Cys-211 seems to be the most likely candidate for a slight modification by *N*-ethylmaleimide, although this has not been proven directly. A peptide which encompasses both Cys-152 and Cys-158 does not seem to be notably labeled by *N*-ethylmaleimide since it was not recovered from any of the pools in Fig. 1 by screening for radioactive peptides.

The above-mentioned results clearly establish that Cys-116 is the preferentially labeled residue in the modification by *N*-[^{14}C]ethylmaleimide. From the specific activities of peptides T3 and T1, as well as that of the phenylthiohydantoin derivative of Cys-116 (as estimated from the recoveries of the derivatives of the neighbouring alanine residues released during automatic Edman degradation on peptide CB1) we find values representing 0.6 – 0.7 mol fraction of the original sulfhydryl group. In the amino acid analysis of peptide T3 74% of Cys-116 is accounted for by succinylcysteine. This also indicates that Cys-116 has largely, although not completely, been modified by *N*-ethylmaleimide. About 5% of the originally incorporated radiolabeled reagent has been recovered in pool III containing peptide T10 with Cys-211. No appreciable substitution by *N*-ethylmaleimide could be demonstrated for Cys-152, Cys-158 and Cys-332.

In the three-dimensional structure of *p*-hydroxybenzoate hydroxylase from *P. fluorescens* [3], Cys-116 is positioned at the C-terminus of helix H3 [5] and is far away (about 2.8 nm) from the active site. The side chain points towards the solvent. These results are compatible with the chemical reactivity of Cys-116 towards *N*-ethylmaleimide, 5,5′-dithio-bis(2-nitrobenzoate) [10] and molecular oxygen at pH > 7 [13]. Cys-116 is modified easily by *p*-chloromercuribenzoate as expected, but mercurial compounds also react with other cysteine residues of the enzyme (cf. below).

In this context it is interesting to note that the preparation of heavy atom derivatives of the enzyme for crystallographic studies [3] using mercurial compounds, did not lead to the modification of Cys-116 but mainly of Cys-332 [5]. This strongly indicates that Cys-116 had been oxidized by air in the single crystals used. This is in line with the observed chemical reactivity of Cys-116 towards molecular oxygen [13].

Labeling with mercurial compounds

Protein preparations in which Cys-116 is alkylated with *N*-ethylmaleimide, designated E-MalNEt, are usually used to study the possible chemical modification of the other sulfhydryl groups of the enzyme.

We have observed that the complexity of the kinetics of the inactivation reaction depends on the mercurial compound. In the following we therefore first present the results obtained with a spin-label derivative of *p*-chloromercuribenzoate (ClHgBzsl), yielding the simplest kinetics.

Treatment of the enzyme (E-MalNEt) with ClHgBzsl leads to inactivation of the enzyme. The reaction is dependent on

Peptide	Sequence
T1	111 116 128 Glu-Ala-Arg-Glu-Ala-CYS-Gly-Ala-Thr-Thr-Val-Tyr-Gln-Ala-Ala-Glu-Val-Arg
T3	114 116 128 Glu-Ala-CYS-Gly-Ala-Thr-Thr-Val-Tyr-Gln-Ala-Ala-Glu-Val-Arg
T6	148 152 158 166 Leu-Arg-Leu-Asp-CYS-Asp-Tyr-Ile-Ala-Gly-CYS-Asp-Gly-Phe-His-Gly-Ile-Ser-Arg
T6C1	148 152 158 161 Leu-Arg-Leu-Asp-CYS-Asp-Tyr-Ile-Ala-Gly-CYS-Asp-Gly-Phe
T6C2	162 166 His-Gly-Ile-Ser-Arg
T6C3	148 152 154 Leu-Arg-Leu-Asp-CYS-Asp-Tyr
T6C4	155 158 161 Ile-Ala-Gly-CYS-Asp-Gly-Phe
T10	207 211 214 Gly-Phe-Ala-Leu-CYS-Ser-Gln-Arg
CB2aT12	328 332 334 Tyr-Ser-Ala-Ile-CYS-Leu-Arg
CB2bT2	353 362 Phe-Pro-Asp-Thr-Asp-Ala-Phe-Ser-Gln-Arg

Fig. 2. *Amino acid sequences of tryptic and chymotryptic peptides mentioned in this paper.* Cysteine residues are enclosed in boxes

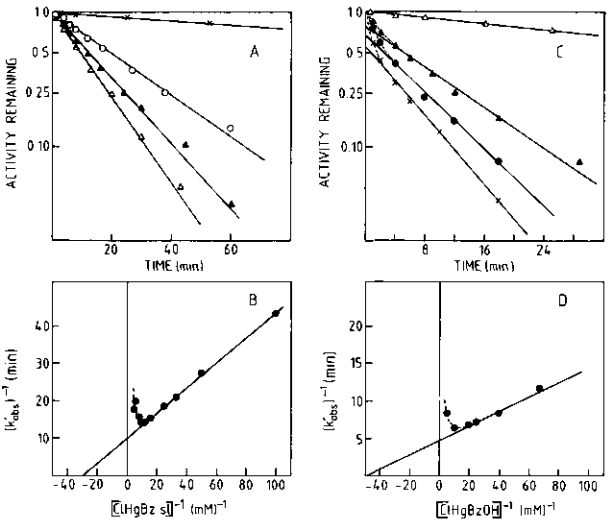

Fig. 3. *The time-dependent inactivation of p-hydroxybenzoate hydroxylase as a function of the concentration of ClHgBzsl and ClHgBzOH.* Aliquots were withdrawn from the incubation mixtures at intervals and assayed after dilution. The relative rate was determined by comparison with an identical enzyme sample in the absence of the inactivator. The temperature was 21 °C. (A) 1.6 μM E-MalNEt in 80 mM Hepes buffer, pH 7.0, $I = 0.1$ M, was incubated with the following concentrations of ClHgBzsl: 20 μM (○); 40 μM (▲); 80 μM (△); 40 μM in the presence of 800 μM *p*-hydroxybenzoate (×). (B) Plot of the reciprocal slopes of A against the reciprocal inhibitor concentration. (C) 2.4 μM E-MalNEt in 80 mM Hepes buffer, pH 7.5, $I = 0.1$ M, was incubated with the following concentrations of ClHgBzOH: 15 μM (▲); 25 μM (●); 50 μM (×); 40 μM in the presence of 1 mM *p*-hydroxybenzoate (△). (D) Plot of the reciprocal slopes of C against the reciprocal inhibitor concentration

the concentration of the modifying reagent. The reaction is a pseudo-first-order process up to about 93–95% loss of activity. The calculated kinetic constants, as derived from semilogarithmic plots (Fig. 3A), have been corrected for the residual activity. Further incubation (up to 4–6 h) leads to little increase in modification of the enzyme. The residual activity is most probably due to native enzyme (see below). Efforts to separate modified from native molecules possibly

present in the reaction mixture by ion-exchange chromatography failed.

The inactivation of the enzyme by all mercurial compounds tested is irreversible. Dialysis or gel chromatography of modified enzyme does not lead to recovery of activity. However, the enzymatic activity of all modified enzyme preparations reported in this paper can be quantitatively restored by treatment with sulfhydryl-containing compounds (results not shown).

It is apparent from Fig. 3A that the rate of inactivation does not linearly increase with the concentration of ClHgBzsl used. This suggests that the enzyme-inhibitor complex (E . . . I) is an intermediate in the reaction leading to an irreversible loss of activity giving an inactive enzyme (E-I) where the inhibitor is covalently bound to the enzyme, according to the scheme [21]:

$$E + I \overset{K_i}{\rightleftarrows} E \ldots I \overset{k_2}{\rightarrow} E\text{-}I$$

and

$$\frac{1}{k_{app}} = \frac{K_i}{k_2[I]} + \frac{1}{k_2}. \qquad (1)$$

The concentration dependence of the inactivation reaction is shown in Fig. 3B. Linearity and intercepts are compatible with saturation kinetics, supporting the above-mentioned proposal. An apparent dissociation constant, K_i = 33 μM, and a first-order rate constant at saturation, k_2 = 0.1 min^{-1} at pH 7.0 and I = 0.1 M, are calculated from the intercepts. The K_i value is almost identical with the dissociation constant of the enzyme-substrate complex (see below). From these data the second-order rate constant of 50 $M^{-1}s^{-1}$ is calculated. The rate of inactivation is almost independent of the pH value of the solution (pH 6.1 – 8.9).

Fig. 3B also shows another remarkable effect, i.e. the inactivation reaction is inhibited by the mercurial compound at concentrations larger than about ~ 80 μM at the enzyme concentration used. The plot of Fig. 3B resembles very much that obtained in Lineweaver-Burk plots when the enzyme is assayed at high substrate concentrations, i.e. inhibition by substrate [1]. The observed effect is not due to substrate inhibition in the assay mixture. This has been tested by using an identical assay mixture but varying the concentration of native enzyme, approaching that present in inactivation mixtures containing low enzymatic activities. A plot of activity versus the enzyme concentration was linear. Therefore it is suggested that the mercurial compound inhibits the inactivation reaction by binding to the substrate binding site at higher concentrations. This idea is substantiated by the fact that the substrate exerts a strong protective effect on the enzyme against inactivation by ClHgBzsl, as shown in Fig. 3A. Similar effects are observed using 2,4-dihydroxybenzoate, 3,4-dihydroxybenzoate or benzoate. Enzyme preparations that had been almost fully inactivated showed no affinity for the substrate, as judged by fluorometric titration experiments (Table 1).

The inactivation reaction of the enzyme by ClHgBzOH and the properties of the modified enzyme show similar features as observed with ClHgBzsl, except that the inactivation is faster and biphasic (Fig. 3C). The reason for the biphasic reaction is not clear but it has also been observed in the modification of the enzyme by diethylpyrocarbonate [8]. As will be shown below, ClHgBzOH is a less specific reagent for the modification of the enzyme than ClHgBzsl, i.e. several SH groups are modified. This fact could be responsible for the observation of the biphasic kinetic. Extrapolation of the slow reaction to t = 0 shows that the initial loss of activity depends on the concentration of ClHgBzOH used. The initial loss of activity varies over 20 – 45%, as seen in Fig. 3C. An apparent dissociation constant for ClHgBzOH, K_i = 22 μM, and a first-order rate constant at saturation, k_2 = 0.204 min^{-1} at pH 7.5 and I = 0.1 M, are calculated from the intercepts from Fig. 3D. The second-order rate constant calculated from these data is found to be 163 $M^{-1}s^{-1}$.

The incorporation of ClHgBzsl has been followed by difference spectroscopy at 257 nm (Fig. 4). It was found that there is a linear relationship between the loss of activity and the development of the difference absorbance at 257 nm (see inset Fig. 4). A bimolecular rate constant of 43 $M^{-1}s^{-1}$ is

Table 1. *Dissociation constants of complexes between various enzyme preparations and* p-*hydroxybenzoate, NADPH or* $KAu(CN)_2$
The dissociation constants were determined fluorimetrically at 22 °C, I = 0.1 M, pH 6.5

Sample	Dissociation constant of the complex		
	NADPH	*p*-hydroxybenzoate	$KAu(CN)_2$
	μM		
Native enzyme	280	38	95
E-MalNEt	300	45	94 (18)[a]
E-MalNEt-ClHgBzsl	105	> 2000	62
E-MalNEt-ClHgBzOH	110	> 2000	63

[a] Dissociation constant between the enzyme-substrate complex and $KAu(CN)_2$.

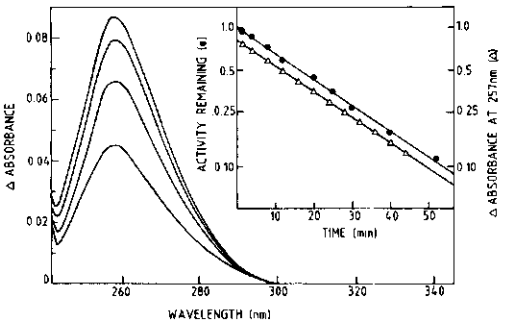

Fig. 4. *The time-dependent incorporation of ClHgBzsl into* p-*hydroxybenzoate hydroxylase prelabeled with* N-*ethylmaleimide (E-MalNEt).* The reaction was followed by recording absorbance difference spectra or following the development of the absorbance at 257 nm. The two (two-compartment cells (total path length of each cell = 0.875 cm) contained 20 μM E-MalNEt in 80 mM Hepes buffer, pH 7.0, I = 0.1 M, in one compartment and 80 μM ClHgBzsl in the same buffer in the other compartment. A baseline was recorded from these solutions and the reaction was then initiated by mixing the content of the sample cell. The spectra shown were recorded at 9 min, 20 min, 36 min and 50 min after initiation of the reaction. The results are also presented as a semilogarithmic plot of the time-dependent absorbance changes at 257 nm (△, inset). Aliquots of the same reaction mixture were assayed for activity at intervals. These results are also shown in the inset (●) as a semilogarithmic plot. The temperature was 21 °C

Table 2. *Quantification of the incorporation of ClHgBzsl into* N-*ethylmaleimide-prelabeled enzyme (E-MalNEt)*
The two reaction mixtures each consisted of 40 μM E-MalNEt in 0.5 ml 80 mM Hepes pH 7.0, $I = 0.1$ M, and 120 μM ClHgBzsl. One sample contained in addition 1.5 mM *p*-hydroxybenzoate. The reaction mixture was incubated for 1 h at 21 °C. The mixtures were then poured over a Bio-Gel P-6DG column and the residual activity of the eluate determined (corrected for enzyme dilution by measuring the flavin absorption of the enzyme at 450 nm). The ESR spectra of the eluates were then recorded, an excess dithiothreitol added to the solutions and after full reactivation of the enzyme the spectrum again recorded. These spectra were doubly integrated and compared with that of a known solution of the free spin label (see Materials and Methods)

Sample	Residual activity	Spin label incorporated
	%	mol/mol FAD
E-MalNEt + 5 mM dithiothreitol	100	0
E-MalNEt + ClHgBzsl	≈8	0.95
E-MalNEt + substrate + ClHgBzsl	66	0.38

calculated for both the time-dependent loss of activity and the development of the difference absorbance at 257 nm. This value is in good agreement with that calculated from Fig. 3B, strongly suggesting that the chemical modification reaction and the reaction leading to loss of activity occur at the same site. From the molar difference absorption coefficient ($7.6\ mM^{-1}cm^{-1}$) it is calculated that 1 mol ClHgBzsl reacts/mol protein-bound FAD. This result is fully confirmed by ESR measurements (Table 2).

The incorporation of ClHgBzOH into the enzyme was quantified by combined spectrophotometric and activity measurements. The results are presented in Fig. 5. Native enzyme was used for these experiments to demonstrate that Cys-116 is labeled very fast and reacts quantitatively when using an equimolar amount of reagent per protein-bound FAD, not affecting the activity of the enzyme. It can be concluded from Fig. 5 that a total of three cysteine residues can be modified in native enzyme by ClHgBzOH, whereas only two residues are modified by ClHgBzsl, including Cys-116 (data not shown). Furthermore only one of the labeled residues is responsible for the loss of activity. This conclusion is drawn from the fact that the lines in Fig. 5 are parallel for the three concentrations used and show a slope of one [22]. The results are fully confirmed using radioactive labeled ClHgBzOH and E-MalNEt (Table 3).

Other sulfhydryl-modifying compounds such as iodosobenzoate, mercurichloride and iodoacetamide also inactivate the enzyme. However, we have not done any detailed kinetic studies on these compounds. Furthermore using a glycine buffer, pH 8.5, instead of the other buffers used in this study, it was found that ClHgBzOH reacts rather slowly with the enzyme, leading to the labeling of all five cysteine residues. The slow reaction of ClHgBzOH with the enzyme is probably due to the fact that the mercurial compound forms a complex with glycine, as shown by Boyer [18].

The protection of the enzyme from inactivation by the substrate and derivatives thereof is surprising considering the published low-resolution X-ray data on the enzyme [3]. According to these data and the known sequence of the

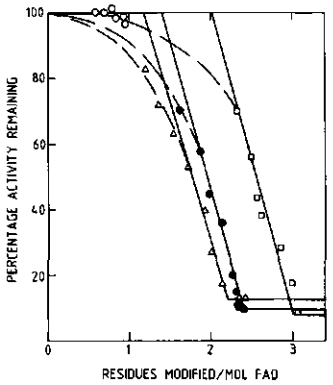

Fig. 5. *Remaining activity as a function of ClHgBzOH incorporation into p-hydroxybenzoate hydroxylase.* Reaction of the enzyme with one equivalent ClHgBzOH leads to the almost quantitative labeling of Cys-116, with little loss of activity. The time-dependent incorporation of the mercurial compound was followed at 250 nm using $\Delta\varepsilon = 7600\ M^{-1}cm^{-1}$ for the formation of the mercaptide bond and compared with the time-dependent inactivation of the enzyme. 10 μM native enzyme in 80 mM Hepes buffer pH 7.0, $I = 0.1$ M, was incubated with the following concentrations of ClHgBzOH: 10 μM (○); 30 μM (△); 50 μM (●); 100 μM (□). For the latter three concentrations of ClHgBzOH a slope of 1 is obtained, indicating that one cysteine residue is responsible for the loss of activity

Table 3. *Quantification of the incorporation of radioactive ClHgBzOH into p-hydroxybenzoate hydroxylase prelabeled with* N-*ethylmaleimide*
A solution of 10 μM E-MalNEt in 80 mM Hepes buffer, pH 7.0, $I = 0.1$ M, 22 °C, was incubated for 240 min with various concentrations of the reagent. 1.0 ml of the enzyme mixture was then poured over a Bio-Gel column as described under Materials and Methods. Where appropriate the concentration of NADPH and the substrate was 6 mM and 0.6 mM, respectively. An aliquot of the incubation mixture was assayed for activity

ClHgBzOH incubated	ClHgBzOH incorporated (residual activity) in the presence of		
	nil	substrate	NADPH
	mol/mol FAD (%)		
0	0 (100)	0 (100)	0 (100)
0.33	0.26 (85)	–	–
0.50	–	0.32 (93)	0.42 (82)
0.67	0.54 (73)	–	–
1.0	0.80 (43)	0.58 (77)	0.87 (48)
1.33	1.03 (27)	–	–
1.50	–	0.75 (54)	1.16 (22)
1.67	1.23 (17)	–	–
2.0	1.26 (13)	0.99 (46)	1.23 (18)
3.0	1.48 (10)	1.21 (33)	1.25 (17)
5.0	1.50 (9)	1.44 (22)	1.25 (15)
10.0	1.69 (8)	1.67 (14)	1.51 (15)

enzyme [4, 5] no cysteine residue is located in the active center. Cys-211 is located closest to the active center. Its Cα atom is at a distance of about 0.75 nm from the substrate. It should however be noted that the three-dimensional data were obtained on the enzyme-substrate complex, whereas our results had to be obtained on the free enzyme. It is known that

binding of the substrate to the enzyme induces a conformational change [23], facilitating reduction of enzyme-bound FAD by NADPH, i.e. the substrate functions also as an effector. The observation that the two sites mutually influence each other is interesting and tempting to speculate upon. It is reasonable to suggest that the conformational change induced by the substrate also affects the conformation of the cysteine residue in question. This cysteine residue becomes probably more buried by the conformational change and becomes almost inaccessible for the mercurial compound. On the other hand, alkylation of the cysteine residue by the mercurial compounds prevents the substrate from inducing the conformational change needed for its optimal binding and for generating optimal activity. It is not yet clear if these facts are related to a possible regulation mechanism of the activity of the enzyme in the cell. In addition the fact that the mercurial compounds themselves can inhibit the alkylation of the cysteine residue (Fig. 3) strongly suggests that these compounds also bind to the substrate binding site. This is not surprising considering the close structural relationship between the substrate and in particular ClHgBzOH. However, it is also possible that the non-covalent interaction between the enzyme and the mercurial compounds induces a less active conformation. This could explain the observation that assays of inactivation mixtures showed a lag period when the concentration of the mercurial compounds exceeded 80 μM. The lag period is dependent on the concentration of the mercurial compound.

NADPH and its competitive inhibitors adenosine 2′,5′-bisphosphate, 2′AMP and $KAu(CN)_2$ [24] do not protect the enzyme from inhibition by ClHgBzsl. The affinity of the inactivated enzyme for NADPH is even somewhat increased as compared to that of the free enzyme (Table 1). This is also true for $KAu(CN)_2$. Previously [24] we have shown that the enzyme-substrate complex shows a much higher affinity for $KAu(CN)_2$ than the free enzyme. The results given in Table 1 suggest therefore that labeling by the mercurial compounds exerts a similar effect on the $KAu(CN)_2$ binding site as the substrate, though to a much lesser degree. Since the circular dichroic spectra of the free and the mercurial-labeled enzyme are very similar, the structural perturbation by the labeling must be small.

Assignment in the sequence

Although the mercurials react specifically with cysteine residues in proteins, the modification may readily be reversed under the relatively harsh conditions of sequence analysis, which poses difficulties on the assignment of the site of attack of mercurials in proteins [15, 25]. Therefore we have developed a scheme by which these residues could be labeled and identified as described in the Materials and Methods section.

Fig. 6 shows the reversed-phase HPLC peptide mapping analyses of tryptic digests of approximately the same amounts of the ClHgBzsl treated enzyme (A) and that of the control sample (B), together with the radioactivity content determined for each pool. The overall patterns of the two fractionation profiles are very similar; minor differences are probably due to small changes in the column characteristics between each run. The radioactivity patterns reveal the presence of three major labeled peaks at corresponding elution volumes (29, 32 and 37 ml), in addition to several small peaks common to both digests. As it could be demonstrated that the radioactivity in a separate peak eluting just before the 37-ml peak originated from the same peptide that causes the radioactivity of this major labeled peak, this will therefore be left out of consideration in the following. Among the three major labeled peaks in the digest of the ClHgBzsl-treated enzyme, the 32-ml and the 37-ml peaks show a significant increase in their radioactivity content as compared with the control sample, the increase for the 37-ml peak being the most notable.

Since each of the three major radiolabeled peaks from both digests in Fig. 6A and Fig. 6B contained a mixture of peptides, they were rechromatographed on the same C_{18} reversed-phase column using a different eluent system (see legend to Fig. 6). Each of the major radiolabeled peaks contained a single major radiolabeled peptide as indicated in Fig. 6. Amino acid analysis and determination of the N-terminal amino acid by dansylation allowed their identification as known cysteine-containing peptides in the primary structure of CNBr fragment CB1 [4]: peptide T3 was isolated from the 29-ml peaks from both digests (still contaminated with a non-cysteine-containing peptide known as CB2bT2 in the primary structure of CNBr fragment CB2 [20]), peptide T10 from the 32-ml peaks and peptide T6 from the 37-ml peaks.

As already apparent from the results presented in Fig. 6, the specific activity of peptide T3 isolated from the ClHgBzsl-treated enzyme had almost the same value (450 dpm/nmol) as that calculated for peptide T3 isolated from the control sample (400 dpm/nmol). Peptide T3 contains Cys-116 and pretreatment of the enzyme with unlabeled *N*-ethylmaleimide prior to modification with the mercurial compound should have largely blocked this residue for further modification. An incorporation of about 0.1 mol *N*-[^{14}C]ethylmaleimide/mol peptide could be calculated from the specific activity of the labeled reagent used in these studies and that of peptide T3. This indicates that about 90% of Cys-116 had been blocked by the pretreatment with *N*-ethylmaleimide which agrees very well with the results obtained in the previous section. Peptides T10 and T6 isolated from the ClHgBzsl-treated protein showed a 3.7-fold and a 13-fold increase in their specific activities, respectively, as compared with the specific activities of these peptides (160 dpm/nmol and 180 dpm/nmol, respectively) isolated from the control sample. The incorporation values determined for the various characterized peptides are summarized in Table 4.

The results demonstrate that the cysteine residues contained within peptides T10 and T6 had originally reacted with ClHgBzsl and that peptide T6 is the major radiolabeled peptide. However, peptide T6 contains two cysteine residues (Cys-152 and Cys-158). An effort was undertaken to determine whether one of them preferentially reacts with ClHgBzsl. Fig. 7 shows a reversed-phase HPLC chromatogram of a chymotryptic digest of peptide T6 from the ClHgBzsl-treated enzyme together with the radioactivity pattern. Unfortunately, cleavage at Tyr-154 had proceeded very incompletely and peptides T6C3 and T6C4, each containing a single cysteine residue, were isolated with very low recoveries (0.8 nmol and 1.3 nmol, respectively). Because of the low yield and corresponding radioactivity content for peptides T6C3 and T6C4, the experimentally determined values for their specific activities of 1400 dpm/nmol and 600 dpm/nmol, respectively, remain somewhat uncertain. Nevertheless these figures suggest that Cys-152 was preferentially modified by ClHgBzsl (see Table 4).

The cysteine-containing peptide CB2aT12 did not cause a major radiolabeled peak in Fig. 6. This peptide was normally isolated in good yield by reversed-phase HPLC chromatography from tryptic digests of carboxymethylated *p*-hydroxybenzoate hydroxylase (at an elution volume of about 28 ml).

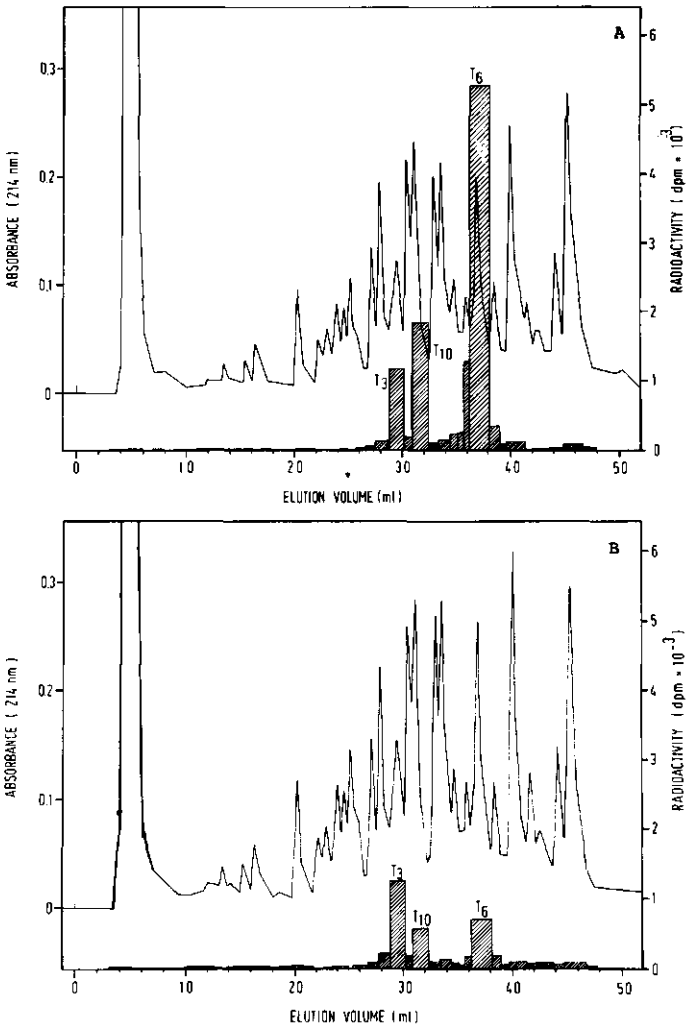

Fig. 6. *HPLC chromatograms of tryptic digest of* N-[^{14}C]*ethylmaleimide containing* p*-hydroxybenzoate hydroxylase that had originally been modified with ClHgBzsl in the native state (A) or that had not been subjected to this modification (B).* The protein samples were prepared as described in Materials and Methods. Approximately 5 nmol of the tryptic digest of each sample was chromatographed on a Nucleosil $10C_{18}$ column (300 × 4.6 mm) with a 60-min linear gradient from 0% to 67% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Peptide elution was monitored by the absorbance at 214 nm. Vertical bars represent the total radioactivity content associated with pools of eluent collected for each peptide peak. Preparative isolation of the major radio-labeled peaks at elution volumes of 29 ml, 32 ml and 37 ml was done by three separate runs of approximately 30 nmol of the tryptic digest of each sample. Rechromatography of each of the major radiolabeled peaks was done on the same column with a 36-min linear gradient from 0% to 40% acetonitrile in 0.1% ammonium acetate at a flow rate of 1.0 ml/min. Only the radioactive peptides subsequently isolated by these rechromatographies are indicated

In the present work we have isolated only relatively small amounts of this peptide in the rechromatography of the 32-ml peaks in Fig. 6. Its low recovery and radioactivity content and its contamination with several other peptides (most probably including radioactive ones) allowed neither reliable calculations nor further purification.

Cys-332 contained within peptide CB2aT12 could not have been heavily labeled in the ClHgBzsl-treated protein anyhow since the specific activities found for peptides T3, T6 and T10 could almost completely account for the specific activity of the starting material (≈ 3400 dpm/nmol) (Table 4).

Samples treated with ClHgBzOH were analyzed in exactly the same way as described above for the ClHgBzsl-treated protein. The results are also included in Table 4. As can be seen from Table 4, the material treated with ClHgBzOH shows incorporation values significantly higher for Cys-211, slightly higher for Cys-158 and lower for Cys-152, indicating no preference for any of these cysteine residues.

Effects of modification

The ESR spectrum of ClHgBzsl-labeled E-MalNEt is shown in Fig. 8. The spectrum indicates that the protein-bound spin label possesses a fairly high mobility suggesting that the label is located in a cleft or close to the surface of the protein. On the other hand, the spin label must be more than 1 nm distanced from the prosthetic group. This conclusion is drawn from the fact that the quantum yields of the fluorescence emission of both native and ClHgBzsl-labeled enzyme are almost identical.

These interpretations are in agreement with the three-dimensional data [3, 5], showing that Cys-152 is situated at a far distance from the prosthetic group (see below). Even so the protein-bound spin label is reduced in a slow reaction in the presence of NADPH. This reduction is caused by the residual activity of the enzyme because the reduction is stimulated by the addition of benzoate and inhibited by $KAu(CN)_2$,

Table 4. *Incorporation of* N-[^{14}C]*ethylmaleimide in native, ClHgBzsl-treated and ClHgBzOH-treated* p-*hydroxybenzoate hydroxylase and their cysteine-containing peptides*
For details see Materials and Methods, and text. n.d., not determined

Protein or peptide		Incorporation		
		control	ClHgBzsl-treated	ClHgBzOH-treated
		dpm/nmol		
Holoenzyme		1200	3400	4200
T3	(Cys-116)	400	450	550
T6	T6C3 (Cys-152)	180 (40)	2300 (1400)	2000 (960)
	T6C4 (Cys-158)	(130)	(600)	(810)
T10	(Cys-211)	160	590	970
Sum of T3, T6 and T10		740	3340	3520
CB2aT12	(Cys-332)	n.d.	n.d.	n.d.

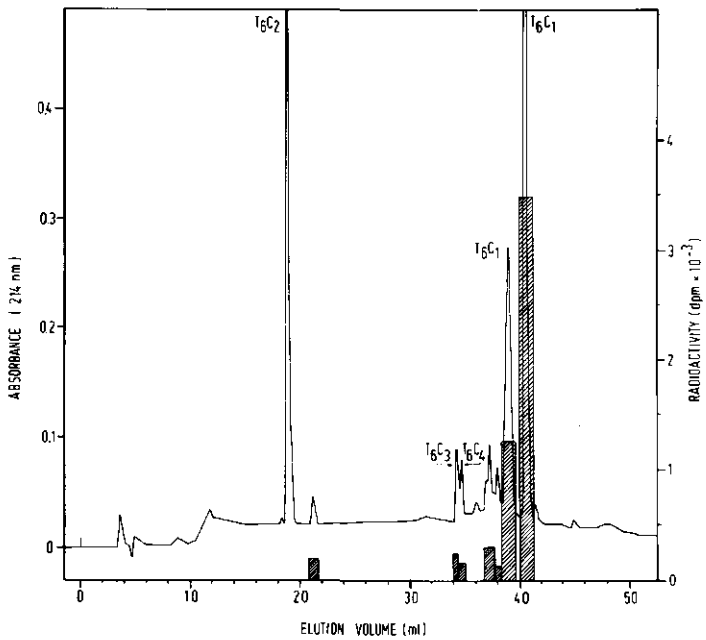

Fig. 7. *Reversed-phase HPLC chromatogram of a chymotryptic digest of about 20 nmol of peptide T6.* Chromatography was done on a Nucleosil $10C_{18}$ column (300 × 4.6 mm) with a 54-min linear gradient from 0% to 60% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min. Peptides were detected by their absorbance at 214 nm. Vertical bars represent the radioactivity content determined for 20% of the total amount of peptide material isolated from each peak. Only peptides which could be correlated with known sequences in the primary structure of peptide CB1 [4] from the results of amino acid analysis and determination of the N-terminal amino acid by dansylation have been indicated

a very potent competitive inhibitor with respect to NADPH [24]. The reduction is much slower under aerobic than anaerobic conditions suggesting that the reduced prosthetic group, and not reduction products of molecular oxygen, is responsible for the reduction of the spin label.

The time-dependent development of the difference spectrum observed during the chemical modification of the enzyme by ClHgBzOH is remarkable because the expected absorbance changes at 250 nm are also accompanied by an absorbance change at 290 nm and in the flavin-absorbing region (Fig. 9). Isosbestic points are observed at 386nm, 320 nm and 278 nm. The kinetics at 250 nm are biphasic and parallel those of the inactivation reaction (cf. Fig. 3C), whereas the absorbance changes at 290 nm and 360 nm correspond to the slow part of the inactivation and the modification reaction (Fig. 9, inset), monitored at 250 nm. From the fact

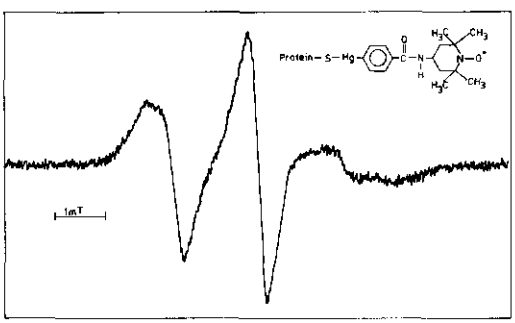

Fig. 8. *The electron spin resonance spectrum of* p-*hydroxybenzoate hydroxylase (E-MalNEt) labeled with ClHgBzsl.* 50 μM enzyme in 80 mM Hepes, pH 7.0, I = 0.1 M, was incubated with 140 μM ClHgBzsl for a period of 1 h. The incubation mixture was then poured over a Bio-Gel P-6 column prior to the measurement. Final enzyme concentration was about 30 μM. The chemical structure of ClHgBzsl is also shown

that selective modification of Cys-116 (cf. Fig. 5) does not yield difference absorbances at 290 nm and 360 nm it is concluded that these difference absorbances are caused by the modification of cysteine residues other than Cys-116. Although the rate of inactivation of the enzyme by ClHgBzOH is, as monitored at 250 nm, almost independent of the pH value of the solution, the intensity of the maxima at 360 nm and 290 nm in the difference spectrum are strongly dependent on the pH value of the solution. The inset in Fig. 9 shows that the absorbance difference at 290 nm (the same holds for the band at 360 nm) increases with increasing pH values, reaching an optimum at about 9. The first spectra recorded after mixing consistently showed a small wavelength-dependent and rapid increase in the difference absorbance.

Since this absorbance change was not related to the rate of the chemical reaction it is ascribed to the formation of a small amount of denatured enzyme. Consequently the pH-dependent absorbance changes shown in the inset of Fig. 9 have been corrected for the initial absorbance changes. The molar difference absorption coefficient at pH 9 is 3200 $M^{-1}cm^{-1}$ at 290 nm and about 1600 $M^{-1}cm^{-1}$ at 360 nm. A pK value of about 7.6 is calculated from the pH dependence of the difference absorbance at 290 nm (inset of Fig. 9). Since the pH-dependent absorbance changes are not related to a pH-dependent rate of the chemical modification of the enzyme, the observed spectral changes are most probably caused by an indirect effect. In addition, the ionizing group leading to the pH-dependent spectral changes at 290 nm must be located in the vicinity of the prosthetic group affecting also the absorption properties of bound FAD. It is known from three-dimensional data [3, 5] that three tyrosine residues (Tyr-201, Tyr-222 and Tyr-385) are located in the vicinity of the prosthetic group. It is therefore tentatively suggested that the ionizing group is due to at least one of the three tyrosine residues.

The different shape observed for the difference spectra of ClHgBzsl- and ClHgBzOH-labeled enzyme needs some further comment. First, the different spectrum of the ClHgBzsl-labeled enzyme (Fig. 4) is much broader in the

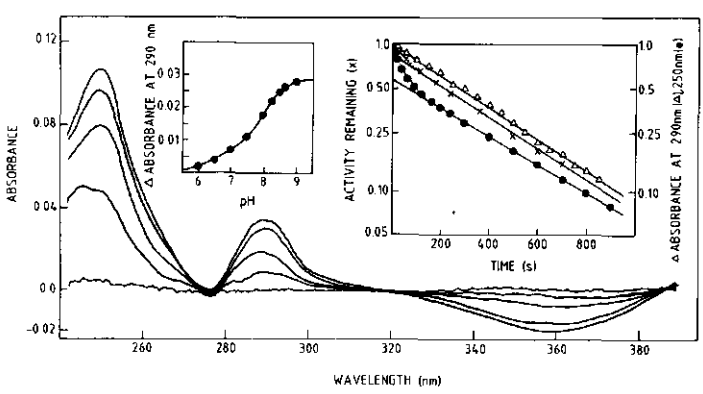

Fig. 9. *Ultraviolet difference spectra observed during the inactivation of* p-*hydroxybenzoate hydroxylase by ClHgBzOH.* Both cuvettes contained 1.0 ml of 20 μM enzyme (E-MalNEt) in 80 mM Hepps buffer, pH 8.5, I = 0.1 M, in one compartment and 1.0 ml of 80 μM ClHgBzOH in the same buffer in the other compartment. Before initiating the reaction, a base line was recorded. The solutions in the two compartments of the sample cell were then mixed. The absorbance changes were followed by automatically scanning a spectrum at 30-s intervals until the reaction was completed. For clarity only a few spectra are shown, recorded 15 s, 130 s, 520 s and 1010 s after mixing. The intensities of the absorbance changes at 290 nm and 360 nm are pH-dependent. In the inset the pH-dependent absorbance difference is shown for the peak at 290 nm. The time-dependent chemical modification reaction, monitored at 250 nm and 290 nm, is compared with the time-dependent inactivation reaction in a semilogarithmic plot in the other inset. The path length of the cells was 0.875 cm and the temperature 21°C

ultraviolet region than that of the ClHgBzOH-labeled enzyme. The spectrum of the ClHgBzsl-labeled enzyme is also dependent on the pH value of the solution, but the pH dependence is not as pronounced as that in ClHgBzOH-labeled enzyme. At low pH values the intensity in the 280–300-nm region decreases and a clear shoulder is formed around 275 nm. No attempts were therefore undertaken to unravel the complex difference spectrum.

In the electron density map of the oxidized enzyme-substrate complex [3, 5], Cys-152 is situated at the loop between β strands C3 and A4 at a distance of about 3.5 nm from the active site. Its Cα atom is at the molecular surface, whereas its side chain is more or less buried within the enzyme structure forming part of the hydrophobic cluster A between β sheets A and C. Loss of enzymatic activity by modification with mercurial compounds can therefore only be explained by some conformational change which affects the substrate-binding site through long-distance effects. On the other hand, the conformational change induced by the substrate leads to a lesser accessibility of Cys-152 in particular and probably of others as well. A similar effect was also observed for another flavin-dependent monooxygenase. It has been reported for phenol hydroxylase that the sulfhydryl groups become less accessible in the enzyme-substrate complex [26, 27].

In fact all of the five cysteine residues present in *p*-hydroxybenzoate hydroxylase seem to be in a position not expected to be crucial for the enzymatic activity. Although the Cα atom of cysteine-211 (at β strand B3) is at 0.75 nm distance from the substrate, its side chain points away from the active site and is part of the hydrophobic cluster D between sheet B and helix H4. All other cysteine residues (Cys-116, Cys-152, Cys-158 and Cys-332) are situated at a distance of 2.2 nm or more away from the active site. All cysteine residues, except for Cys-116, have their side chains more or less buried within the protein.

Crystals of the enzyme-substrate complex however bind ClHgBzOH at positions which presumably are near Cys-332 and Cys-211 [3]. The cysteine residues that are reactive towards ClHgBzOH in the substrate-free enzyme are therefore different from those of the enzyme-substrate complex as derived from the interpretation of the crystal structure. This indicates that the substrate *p*-hydroxybenzoate affects the reactivity of the sulfhydryl groups of the enzyme, as shown by the present modification studies. In this context it would be interesting to know the three-dimensional structure of the free enzyme.

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Chapter 6

Chemical modification of tyrosine-38 in *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* by 5′-*p*-fluorosulfonylbenzoyladenosine: A probe for the elucidation of the NADPH binding site?

Involvement in catalysis, assignment in sequence and fitting to the tertiary structure

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p-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* was covalently modified by the nucleotide analog 5′-(*p*-fluorosulfonylbenzoyl)-adenosine in the presence of 20% dimethylsulfoxide. The inactivation reaction is pH-dependent and does not obey pseudo-first-order kinetics, due to spontaneous hydrolysis of the reagent. The kinetic data further indicate that a weak, reversible enzyme-inhibitor complex is an intermediate in the inactivation reaction and that only one amino acid residue is responsible for the loss of activity.

The inactivation is strongly inhibited by NADPH and 2′,5′ADP. Steady-state kinetics and 2′,5′ADP bioaffinity chromatography of the modified enzyme suggest that the essential residue is not directly involved in NADPH binding.

Sequence studies show that Tyr-38 is the main residue protected from modification in the presence of NADPH. From crystallographic studies it is known that the hydroxyl group of Tyr-38 is 1.84 nm away from the active site. Model-building studies using computer graphics show that this distance can be accommodated when FSO_2BzAdo binds in an extended conformation with the sulfonylbenzoyl portion in an orientation different from the nicotinamide ring of NADPH.

The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is an external monooxygenase catalyzing the conversion of *p*-hydroxybenzoate into 3,4-dihydroxybenzoate, an intermediate step in the degradation of aromatic compounds in soil bacteria. The reaction mechanism of the enzyme from *P. fluorescens* has been studied in detail by stopped-flow technique [1, 2]. The enzymes from *P. fluorescens* and *P. desmolytica* prefer NADPH over NADH as electron donor [3, 4], while the enzyme from *Corynebacterium cyclohexanicum* [5] can use both NADPH and NADH efficiently.

X-ray diffraction studies [6–8] have revealed the three-dimensional model of the enzyme–*p*-hydroxybenzoate complex from *P. fluorescens* at a resolution of 0.25 nm. More recently this structure has been refined to 0.19 nm (Schreuder et al., unpublished results). The complete amino acid sequence of the enzyme from *P. fluorescens* is also known [9–11].

Chemical modification of cysteine [12] and tyrosine residues [11] has provided information about differences in the dynamic structures of free and *p*-hydroxybenzoate-complexed enzyme.

These data are the basis for the study of the amino acid residues involved in the binding of NADPH.

Chemical modification by diethyl pyrocarbonate at pH values < 7 have indicated that one or two histidine residues are involved in NADPH binding [13, 14]. Because of the instability of ethoxy-formylated-histidine residues, even towards mild hydrolysis, it was not possible to assign the modified histidine residues in the sequence.

The possible role of arginine residues in the binding of NADPH to the enzyme from *P. fluorescens* has been studied using several arginine reagents [15]. From these studies it was concluded that the reagents react with different arginine residues. Unfortunately it was not possible to assign the modified residues in the sequence of the enzyme because the incorporated radioactivity was spread equally over the HPLC peaks of the peptides.

The inhibition of various adenine nucleotide analogs, competitive with respect to NADPH [16], has prompted us to investigate the covalent modification of the enzyme with the adenine nucleotide analog 5′-(*p*-fluorosulfonylbenzoyl)-adenosine. The properties of FSO_2BzAdo are well documented, as reviewed by Colman [17].

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Abbreviations. FSO_2BzAdo, 5′-(*p*-fluorosulfonylbenzoyl)-adenosine; SO_3BzAdo^-, 5′-(*p*-sulfonatobenzoyl)-adenosine; 2′,3′AMP, adenosine 2′ and 3′-monophosphate (mixed isomers); Me_2SO, dimethylsulfoxide; MalNEt, *N*-ethylmaleimide; E-MalNEt, *N*-ethylmaleimide labeled enzyme; DABITC, 4-(*N*,*N*-dimethylamino)azobenzene-4′-isothiocyanate; DABTH, 4-(*N*,*N*-dimethylamino)azobenzene-4′-thiohydantoin; 2′,5′ADP-Sepharose, N^6-(6-aminohexyl)adenosine 2′,5′-bisphosphate coupled to Sepharose 4B; Bistrispropane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

Enzyme. *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2).

It is shown that the inactivation of the enzyme can be explained by the modification of one amino acid residue and this reaction is protected by NADPH and 2′,5′ADP but not by *p*-hydroxybenzoate. This residue has been identified as Tyr-38 by sequencing tryptic peptides of modified enzyme. Model building of the modified enzyme using computer graphics suggests a possible mode of binding for NADPH which is in agreement with the observed modification of Tyr-38 by FSO_2BzAdo.

MATERIALS AND METHODS

General

5′-(*p*-Fluorosulfonylbenzoyl)-[*adenine*-8-^{14}C]adenosine (FSO_2Bz[^{14}C]Ado; 43.2 Ci/mol) was from New England Nuclear (Boston, USA). NADPH, $NADP^+$ and dithiothreitol were from Boehringer. *N*-Ethylmaleimide, FSO_2BzAdo, 2′,5′ADP, 2′,3′AMP, FAD, Hepes, Hepps, Mes, Tris, Bistris and Bistrispropane were from Sigma. 2′,5′ADP-Sepharose was from Pharmacia and BioGel P-6 DG was obtained from Bio-Rad. $KAu(CN)_2$ was from Drijfhout & Zoon's Edelmetaalbedrijven B.V. (Amsterdam, The Netherlands). All other chemicals were from Merck and of the best grade available.

Analytical methods

Analytical methods, including measurement of absorbance, fluorescence and (radio)activity, have been described elsewhere [11, 12].

Dissociation constants of the binary complexes between enzyme and substrate (analogs) or NADPH (analogs) were determined fluorimetrically as described previously [4].

Rate constants for the hydrolysis of FSO_2BzAdo were determined essentially according to Pettigrew [18] using an F 1052 F fluoride-specific electrode in combination with an Ag/AgCl reference electrode (Radiometer/Copenhagen). The hydrolysis of 1 mM FSO_2BzAdo was monitored under exactly the same conditions as those used for enzyme modification.

Bioaffinity chromatography analyses were by FPLC (Pharmacia Biotechnology Products) [19] using a 2′,5′ADP-Sepharose (1 × 6 cm) column. Enzyme samples were loaded onto the column, equilibrated in 50 mM Mes pH 6.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol (start buffer). Elution gradients were made as indicated in Results and Discussion. Absorbance was monitored at 280 nm or, when performing nucleotide gradient elution, at 405 nm. The flow rate varied over 0.5 – 1.0 ml/min. After each run the column was washed with 2 vol. 1 M NaCl and reequilibrated with 5 vol. start buffer.

Kinetic data of the inactivation of enzyme by FSO_2BzAdo were transferred to and evaluated on a Micro VAX II computer.

Modification studies

Large scale production of *Pseudomonas fluorescens* was performed by Diosynth BV (Oss, The Netherlands).

p-Hydroxybenzoate hydroxylase was isolated and purified as described previously [19]. Prelabeling of the reactive sulfhydryl group of the enzyme (Cys-116) with *N*-ethylmaleimide was performed as reported earlier [12]. Fresh solutions of approximately 100 μM *N*-ethylmaleimide-labeled enzyme (E-MalNEt) in the appropriate buffer were prepared each day by gel filtration over BioGel P-6 DG. The concentration of E-MalNEt was determined using a molar absorption coefficient of 11.3 mM^{-1} cm^{-1} at 450 nm [20]. The enzyme activity was assayed spectrophotometrically at 340 nm by measuring the oxidation of NADPH in 0.1 M Tris/H_2SO_4, pH 8.0, at 25°C, as previously reported [20].

Steady-state kinetics of NADPH oxidation were performed at 340 nm in either 50 mM Mes, pH 6.5, or 50 mM Hepes, pH 7.5, at 25°C, in the presence of 100 nM FAD and 0.5 mM EDTA [16]. All determinations were performed in triplicate. At high NADPH concentrations the enzyme activity was monitored at 375 nm using a molar absorption coefficient of 1.85 mM^{-1} cm^{-1}. The NADPH-dependent anaerobic reduction of enzyme-bound FAD in either native or modified E-MalNEt – *p*-hydroxybenzoate complex was performed in 100 mM Hepes, pH 7.5, at 20 °C, essentially according to Husain and Massey [1].

Stock solutions of 10 – 20 mM FSO_2BzAdo were prepared in dimethylsulfoxide and stored at −18 °C under silica gel. The concentration of FSO_2BzAdo was determined by measuring the absorbance at 259 nm of a diluted sample, using a molar absorption coefficient of 13.5 mM^{-1} cm^{-1} [21]. For radioactivity experiments the specific activity of FSO_2Bz[^{14}C]Ado was diluted to 1.47 Ci/mol by adding unlabeled FSO_2BzAdo in the following way: the concentrated radioactive solution (43.2 Ci/mol) in 2.5 ml 95% ethanol was dried on ice by flushing with a gentle stream of nitrogen gas for 1 h. Then, 20 mg unlabeled FSO_2BzAdo was added in 2.5 ml Me_2SO, yielding a 13.1 mM stock solution. Experimentally, a specific activity of 2.26 cpm/pmol was calculated from a standard curve using 0 – 65 nmol FSO_2BzAdo.

Covalent modification of E-MalNEt by FSO_2BzAdo was performed in closed glass bottles at 30 °C in 40 mM Hepes, at pH 7.0, 7.5 and 8.1 (I = 50 mM), containing 20% (by vol.) Me_2SO. Buffers were brought to constant ionic strength with 0.5 M Na_2SO_4 as described earlier [4]. On an analytical scale ($\leq$ 10 μM enzyme) FSO_2BzAdo was added from a freshly prepared stock solution of 12.5 mM. For preparative experiments (pH 7.5, 10 – 200 μM enzyme) two portions of 1.5 mM FSO_2BzAdo were added at 130 min intervals from a 13.5 mM stock solution. The reaction of FSO_2BzAdo with E-MalNEt was stopped at the desired times either by passing the mixture, or an aliquot, over BioGel P-6 DG or by dilution (100 – 1 000 fold) of an aliquot of the reaction mixture into the assay mixture. Before gel filtration the samples were centrifuged to remove precipitated reagent. The recovery of enzyme was more than 95% as checked by the flavin absorption spectrum. The residual activities of modified enzyme are reported, relative to that of native enzyme.

The number of residues which were modified by FSO_2BzAdo was determined by incorporation of FSO_2Bz[^{14}C]Ado using the preparative procedure described above. Measurement of covalent attachment of FSO_2Bz[^{14}C]Ado to E-MalNEt was performed essentially according to the chromatographic assay of Penefsky [22]. Under the conditions used, no loss of label due to spontaneous hydrolysis of the ester linkage between the adenosine and benzoyl moieties of FSO_2BzAdo occurs [23].

Materials and methods for the identification of chemically modified residues in tryptic peptides of *p*-hydroxybenzoate hydroxylase have been described elsewhere [11].

Model building

Model building was on an Evans & Sutherland PS2 computer graphics system using GUIDE software [24] and on an

Evans & Sutherland PS390 computer graphics system using FRODO software [25].

RESULTS AND DISCUSSION

Kinetic studies

On an analytical scale, optimal conditions for inactivation were found in the pH range 7–8 in 20% (by vol.) Me_2SO at 30°C (Fig. 1A). It should be noted that, under the conditions used, native enzyme is in the stable monomeric form [19, 28], showing no loss of activity for at least 8 h.

The inactivation of E-MalNEt by FSO_2BzAdo does not obey pseudo-first-order kinetics during its whole course (Fig. 1B), mainly owing to the instability of the reagent. The hydrolysis of the fluorosulfonyl moiety of the reagent [26] is accompanied by an increase in turbidity of the incubated samples with increasing time and pH.

The inactivation of E-MalNEt by FSO_2BzAdo could not be reversed by the addition of excess dithiothreitol indicating that thiosulfonated derivatives of cysteine, which are eventually formed [27], are not responsible for enzyme inactivation.

The activity of E-MalNEt reaches a plateau value after an incubation of about 400 min. Further treatment of freshly gel-filtered, modified E-MalNEt with FSO_2BzAdo again leads to the same inactivation rate as observed initially, indicating that the residual activity observed is due to native E-MalNEt.

The most simple scheme to describe the inactivation kinetics is:

$$\begin{array}{l} E + FSO_2BzAdo \xrightarrow{k_i} E\text{-}SO_2BzAdo \\ \qquad\qquad\quad \downarrow k_h \\ \qquad\qquad\quad SO_3BzAdo^- \end{array}$$

where k_i is the second-order rate constant for inactivation and k_h represents the rate constant due to hydrolysis of the reagent.

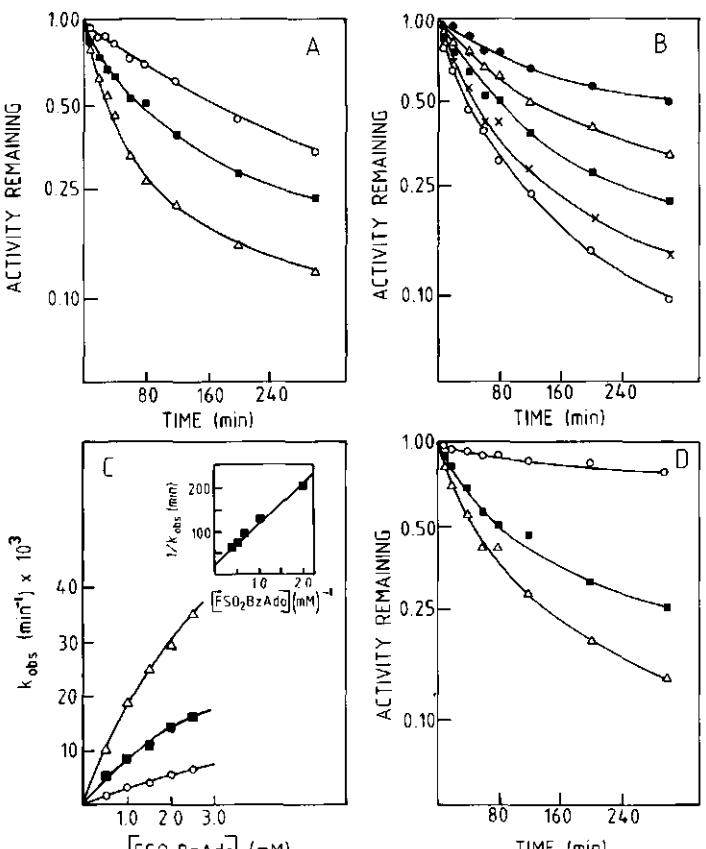

Fig. 1. *Inactivation of* N-*ethylmaleimide prelabeled* p-*hydroxybenzoate hydroxylase by FSO_2BzAdo under various conditions.* (A) 2 μM E-MalNEt (2 nmol) was incubated with 1.5 mM FSO_2BzAdo in 40 mM Hepes buffer (I = 50 mM) containing 20% Me_2SO. pH 7.0 (○——○); pH 7.5 (■——■); pH 8.1 (△——△). (B) 2 μM E-MalNEt (2 nmol) was incubated in 40 mM Hepes buffer pH 7.5 (I = 50 mM) containing 20% Me_2SO with different concentrations FSO_2BzAdo: 0.5 mM (●——●); 1.0 mM (△——△); 1.5 mM (■——■); 2.0 mM (×——×); 2.5 mM (○——○). (C) Plot of fitted initial rate constants (see text) against the inactivator concentration. pH 7.0 (○——○); pH 7.5 (■——■); pH 8.0 (△——△). Inset, plot of the reciprocal initial rate constants at pH 7.5 against the reciprocal inactivator concentration. (D) 2 μM E-MalNEt (2 nmol) was incubated with 2.0 mM FSO_2BzAdo in 40 mM Hepes pH 7.5 (I = 50 mM) containing 20% Me_2SO. Free enzyme (△——△); in the presence of 1 mM *p*-hydroxybenzoate (■——■); in the presence of 10 mM NADPH (○——○)

This scheme can be expressed by the following equations [26]:

$$\ln\frac{E_t}{E_0} = I_0 \cdot \frac{k_i}{k_h}(e^{-k_h \cdot t} - 1). \quad (1)$$

At

$$t \rightarrow \infty : \ln\frac{E_\infty}{E_0} = -I_0 \cdot \frac{k_i}{k_h} \quad (2)$$

where (E_t/E_0) respresents the residual activity at time t and I_0 is the concentration of FSO_2BzAdo used. The second-order rate constants for inactivation (k_i) were obtained from Eqn (2) using k_h values determined independently from fluoride release experiments (Table 1).

The fitted initial-rate constants for inactivations (k_{obs}, Fig. 1C) suggest that a weak non-covalent enzyme-inhibitor complex between E-MalNEt and FSO_2BzAdo is an intermediate in the inactivation reaction according to:

$$E + FSO_2BzAdo \overset{K_d}{\rightleftarrows} E...FSO_2BzAdo \xrightarrow{k_2} E\text{-}SO_2BzAdo$$

and

$$k_{obs} = \frac{k_2}{1 + \left(\frac{K_d}{I_0}\right)} \quad (3)$$

where K_d is the dissociation constant of the enzyme-reagent complex and k_2 is the first-order rate constant at saturation.

Under this situation Eqn (2) becomes [29]:

$$\ln\frac{E_0}{E_\infty} = \frac{k_2}{k_h}\left[\ln\left(\frac{K_d}{I_0} + 1\right) - \ln\frac{K_d}{I_0}\right]. \quad (4)$$

From the intercept in the inset of Fig. 1C an apparent dissociation constant, $K_d = 4$ mM, and a first-order rate constant at saturation, $k_2 = 0.04\ min^{-1}$ at pH 7.5 are calculated. It should be noted that no protection by the hydrolysis product SO_3BzAdo^- [18] was observed, probably because this product precipitates during the inactivation reaction.

The inactivation reaction is only slightly influenced by the presence of the substrate *p*-hydroxybenzoate or its competitive inhibitor *p*-fluorobenzoate (Table 1, Fig. 1D).

When the inactivation of E-MalNEt by FSO_2BzAdo was performed in the presence of NADPH a strong protection against inactivation was observed (Fig. 1D, Table 2), indicating that the reaction of FSO_2BzAdo takes place at the NADPH binding site.

The inactivation reaction of the E-MalNEt–*p*-hydroxybenzoate complex was further studied at pH 7.5, either in the presence of 2′,5′ADP or $KAu(CN)_2$. As already reported earlier [16], both 2′,5′ADP and $KAu(CN)_2$ are competitive inhibitors of the enzyme with respect to NADPH, but interact with the enzyme at different sites of the NADPH-binding pocket. In the presence of $KAu(CN)_2$ no protection against inactivation was observed, while in the presence of 2′,5′ADP protection occurred depending on the amount of enzyme-ligand complex present in the incubation mixtures (Table 2). In the presence of a competing ligand the observed inactivation rate constant can be expressed as [29]:

$$k_{obs} = \frac{k_2}{1 + \frac{K_d}{I_0}\left(1 + \frac{L}{K_l}\right)} \quad (5)$$

Table 1. *Inactivation of E-MalNEt by FSO_2BzAdo in the absence and presence of* p*-hydroxybenzoate or* p*-fluorobenzoate*
2 μM (2 nmol) of E-MalNEt was incubated with various concentrations of FSO_2BzAdo (cf. Fig. 1B) at 30°C in 20% Me_2SO in the absence of both substrates or the presence of 1 mM *p*-hydroxybenzoate or 20 mM *p*-fluorobenzoate alone. All other details are given in Materials and Methods. k_h was determined from fluoride release experiments. The rate constants for inactivation (k_i) were fitted using Eqn (2) (see text)

Sample	pH	$10^3 \times$ Rate constants	
		k_h	k_i
		min^{-1}	$mM^{-1}\ min^{-1}$
E-MalNEt	7.0	4.0	3.2
	7.5	6.0	8.0
	8.0	11.0	21.0
E-MalNEt– *p*-hydroxybenzoate	7.0	4.0	1.4
	7.5	6.0	5.4
	8.0	11.0	9.2
E-MalNEt–*p*-fluorobenzoate	7.5	6.0	4.0

Table 2. *Inactivation of E-MalNEt by FSO_2BzAdo in the presence of NADPH, 2′,5′ADP or $KAu(CN)_2$*
2 μM (2 nmol) of E-MalNEt was incubated with 2 mM FSO_2BzAdo at 30°C in 40 mM Hepes pH 7.5 ($I = 50$ mM) containing 20% Me_2SO. The concentrations of the protecting ligands were 10 mM NADPH, 0.24 mM, 2.4 mM and 12 mM 2′,5′ADP and 1 mM $KAu(CN)_2$ respectively. All other details are given in Materials and Methods. k_{obs} was estimated from the initial slopes; E_∞/E_0 is the apparent residual activity as a fraction of the original activity

Sample	$10^3 \times k_{obs}$	E_∞/E_0
	min^{-1}	
E-MalNEt	16.6	0.10
E-MalNEt-NADPH	2.4	0.60
E-MalNEt-2′,5′ADP (0.24 mM)[a]	10.2	0.20
E-MalNEt-2′,5′ADP (2.4 mM)[a]	4.4	0.54
E-MalNEt-2′,5′ADP (12 mM)[a]	1.7	0.67
E-MalNEt-$KAu(CN)_2$	14.0	0.09

[a] In the presence of 1 mM *p*-hydroxybenzoate.

Table 3. *Quantification of the incorporation of radioactive FSO_2BzAdo into E-MalNEt in the absence of both substrates or presence of* p*-hydroxybenzoate or NADPH alone*
200 μM (250 nmol) of E-MalNEt in 40 mM Hepes buffer pH 7.5, $I = 50$ mM, was incubated with the reagent at 30°C in the presence of 1 mM *p*-hydroxybenzoate or 10 mM NADPH alone, or the absence of both. Two portions of 1.5 mM FSO_2BzAdo were added at 125-min intervals. All reactions were stopped after 250 min by gel filtration of the incubation mixtures over BioGel P-6 DG. All other details are given in Materials and Methods

Sample	Residual activity	FSO_2BzAdo incorporated
	%	mol/mol FAD
E-MalNEt	10	2.7
E-MalNEt–*p*-hydroxybenzoate	28	2.3
E-MalNEt–NADPH	85	0.6

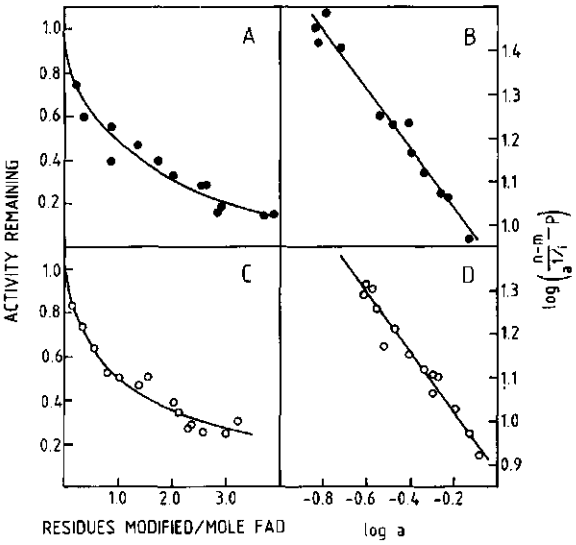

Fig. 2. *Correlation between the residual activity of* N-*ethylmaleimide-prelabeled* p-*hydroxybenzoate hydroxylase and the number of residues modified by* FSO_2BzAdo. 20 μM (40 nmol) of E-MalNEt was modified by $FSO_2Bz[^{14}C]Ado$. Two portions of 1.5 mM FSO_2BzAdo were added, at 150 min intervals, from a 13.5 mM stock solution in Me_2SO. 100-μl aliquots were withdrawn from the reaction mixture at time intervals comparable with those in Fig. 1A and gel-filtered on BioGel P-6 DG. Aliquots of 1.6 nmol enzyme were used for scintillation counting. All other data are given in Materials and Methods. The results were plotted directly (A, C) and according to Eqn (7) given in the text (B, D). (A, B) Free E-MalNEt; (C, D), E-MalNEt–*p*-hydroxybenzoate complex

where L is the concentration of 2′,5′ADP and K_i is the dissociation constant for the enzyme-substrate-2′,5′ADP complex.

When the data of Table 2 are used for the calculation of K_i using Eqn (5), values of 500–850 μM are obtained. As the value obtained from fluorescence quenching studies, K_i = 300 μM [4], is lower by about a factor of 2 it seems that the affinity of the native enzyme-substrate complex for 2′,5′ADP is slightly decreased in the presence of 20% Me_2SO.

From the results of inactivation in the presence of ligands, it can be concluded that chemical modification of E-MalNEt by FSO_2BzAdo most probably takes place at the binding site of the adenosine part of the NADPH molecule.

Incorporation studies

In order to determine the number of amino acid residues which are modified by FSO_2BzAdo, E-MalNEt was incubated for 250 min with radioactive FSO_2BzAdo either in the absence of both substrates or in the presence of NADPH or *p*-hydroxybenzoate alone. Table 3 demonstrates that NADPH strongly protects the enzyme from inactivation (and incorporation of the radioactive label), while *p*-hydroxybenzoate has only a slight effect on modification as compared to native E-MalNEt.

To check if cysteinyl residues are involved in covalent binding of FSO_2BzAdo an aliquot of the gel-filtered sample was treated with excess dithiothreitol. After a second gel filtration step, no reactivation was found and only a very small amount of label was lost, suggesting that cysteine residues are not involved in the modification reaction.

To determine the number of essential amino acid residues which are modified by FSO_2BzAdo, samples were withdrawn from an independent incubation mixture at time intervals during inactivation and assayed for residual activity and extent of incorporation of label into the enzyme. Because, in the presence of NADPH (Table 3), only a small modification occurs with almost no loss in activity, these experiments were done with E-MalNEt (Fig. 2A) and E-MalNEt–*p*-hydroxybenzoate complex (Fig. 2C). The correlation between the residual activity (a) and the number of residues that are modified (m) was obtained by use of the statistical method developed by Tsou [30]. When p residues of which i are essential react with a rate constant k and $n-p$ residues react with a rate constant αk, the following expression is valid:

$$\log [(n-m)/a^{1/i}]-p = \log (n-p) + [(\alpha-1)/i]\log a \quad (6)$$

where n is the total number of residues that are modified. In fact this approximation has also been used to estimate the number of essential residues of this enzyme modified by *p*-diazobenzoate [11]. When $\log [(n-m)/a^{1/i}]-p$ was plotted against $\log a$, using the data of Fig. 2A and Fig. 2C, the best fit to a straight line was obtained (Fig. 2B and 2D) with $n = 8$ and $p = i = 1$. For $n = 8$, and α value of 0.32 was determined from the slope. The fits were unacceptable for $p = i = 2$ indicating that the loss of activity is related to the modification of only one residue. The strong protection against inactivation and incorporation of label by NADPH suggests that this residue must be located at the NADPH-binding site.

Properties of modified E-MalNEt

The visible absorption spectrum of flavin of modified E-MalNEt is identically to that of native E-MalNEt. The quantum yield of fluorescence of modified E-MalNEt is 125 ± 5%, compared to that of native E-MalNEt. As can be

Table 4. *Dissociation constants of complexes between native or modified E-MalNEt and p-hydroxybenzoate, NADPH, 2′,5′ADP or KAu(CN)₂*
The dissociation constants were determined fluorimetrically at 20 °C, I = 50 mM, pH 6.4. Data for native E-MalNEt and native E-MalNEt-*p*-hydroxybenzoate complex are from elsewhere [4, 12]. For experiments with modified E-MalNEt, 100 μM (200 nmol) E-MalNEt was inactivated by adding two portions of 1.8 mM FSO_2BzAdo from a 20 mM stock solution at 270-min intervals (see Materials and Methods). The residual activity was 5%

Sample	Dissociation constant (maximal quenching) of the complex			
	p-hydroxybenzoate	NADPH	2′,5′ADP	$KAu(CN)_2$
	μM (%)			
Native E-MalNEt	45 (80)	130 (50)		94 (87)
Native E-MalNEt-*p*-hydroxybenzoate			200 (30)	18 (87)
Modified E-MalNEt	50[a] (51)	400[a] (15)		100 (50)
Modified E-MalNEt – *p*-hydroxybenzoate			200[a] (5)	22 (50)

[a] The same values were obtained when E-MalNEt was modified by FSO_2BzAdo in the presence of 1 mM *p*-hydroxybenzoate (residual activity = 6%).

seen from Table 4, the dissociation constant of the modified enzyme – *p*-hydroxybenzoate complex is 50 μM at pH 7 (I = 50 mM), as found for native E-MalNEt – *p*-hydroxybenzoate complex [12]. These results strongly support our conclusion that FSO_2BzAdo modification of the protein does not involve the substrate-binding site.

Using the fluorescence-quenching technique it was difficult to estimate the dissociation constants of the modified E-MalNEt-NADPH and E-MalNEt-*p*-hydroxybenzoate – 2′,5′-ADP complexes. As can be seen from Table 4, the percentages of maximal quenching of the modified E-MalNEt complexes were much lower compared to those of native E-MalNEt. Because of this and ionic strength limitations [4], the calculated dissociation constants must be considered as approximations.

The affinity of the modified enzyme for $KAu(CN)_2$ remains unaffected (Table 4). Both in free and *p*-hydroxybenzoate-complexed E-MalNEt the very strong quenching of protein-bound FAD by $KAu(CN)_2$ is less pronounced in the modified enzyme. The same effect is observed with *p*-hydroxybenzoate quenching (Table 4) indicating that modification by FSO_2BzAdo leads to a small change in the microenvironment of protein-bound FAD.

At low NADPH concentrations the residual activity of modified E-MalNEt can be attributed to native enzyme (Fig. 3) as can be concluded from both the K_m value (30 μM) and k_{cat} values (2000 min^{-1} at pH 6.5; 3140 min^{-1} at pH 7.5) after correction for residual activity (5.3%). However, when the NADPH concentration is raised to 1 mM a clear increase of activity is observed indicating that NADPH competes with covalently bound FSO_2BzAdo for the adenosine-binding pocket.

The same effect is also seen when the modified E-MalNEt – *p*-hydroxybenzoate complex is reduced anaerobically with NADPH. Native *p*-hydroxybenzoate-complexed enzyme is rapidly reduced by only a small excess of NADPH [1]. Using the modified E-MalNEt – *p*-hydroxybenzoate complex (residual activity = 6%) the amount of NADPH needed to reach complete reduction of enzyme-bound FAD was about ten times more than that required for native E-MalNEt – *p*-hydroxybenzoate complex (i.e. 19 and 1.9 mol NADPH/mol enzyme, respectively). Although it is not possible to calculate the dissociation constant for NADPH from these results it is clear that, although the affinity for NADPH is decreased, NADPH still interacts with the modified enzyme.

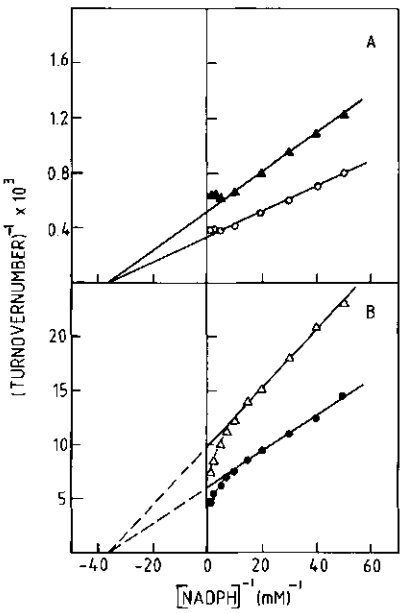

Fig. 3. *NADPH-dependent steady-state kinetics of* N-*ethylmaleimide-prelabeled* p-*hydroxybenzoate hydroxylase modified by* FSO_2BzAdo. The incubation temperature was 25 °C. (A) 16 nM native E-MalNEt, pH 6.5 (▲——▲) and pH 7.5 (○——○); (B) 180 nM modified E-MalNEt, pH 6.5 (△——△) and pH 7.5 (●——●). Enzyme samples were freshly gel-filtered over BioGel P-6 DG. All other details are given in Materials and Methods

Because the inactivation of E-MalNEt by FSO_2BzAdo is strongly inhibited by NADPH and 2′,5′ADP (Table 2), it was worth investigating the elution behaviour of both native and modified E-MalNEt on 2′,5′ADP-Sepharose. As a first approach, the chromatographic behaviour of E-MalNEt, was tested using different elution conditions [31]. Native E-MalNEt binds to 2′,5′ADP Sepharose at pH 6.5 and I = 50 mM. The enzyme could be eluted from the column in one symmetrical peak with either a salt, pH or nucleotide

gradient (Table 5), which is in accordance with results obtained from binding studies [4].

FSO_2BzAdo-modified E-MalNEt also showed affinity for 2',5'ADP-Sepharose. Fig. 4 shows the elution pattern of E-MalNEt, modified by radioactive FSO_2BzAdo in the presence of *p*-hydroxybenzoate. In order to minimize the amount of non-specific labeling (cf. Fig. 2) only 1.02 mol label (residual activity = 43%) was incorporated into this particular sample. About 10% of the protein, containing 15% of the total radioactivity, was eluted in the breakthrough volume of the column. Most of the protein (about 90%) and the radioactivity (85%) was bound to the column and recovered by 2',3'AMP gradient elution. Further analyses of the fractions showed that the radioactivity pattern was shifted to a lower elution volume, compared to the enzyme activity pattern (Fig. 4).

These results indicate that under the conditions used for this specific experiment about 10% of the label is bound to essential amino acid residue(s) directly involved in nucleotide binding, about 50% of the label is bound to amino acid residues not directly involved in nucleotide binding but inhibiting binding due to their bulkiness and about 40% of the label is bound to non-essential amino acid residues. These values could not be predicted directly, either from kinetic or incorporation studies (see above), because of experimental limitations.

Assignment of the modified amino acid residues

Radioactive samples, prepared as indicated in Table 3, where used for the assignment of the amino acid residues modified by FSO_2BzAdo. Denaturation and subsequent dialysis of gel-filtered, labeled E-MalNEt (approximately 225 nmol) prior to tryptic digestion [11] led to the loss of about 50% of the radioactive label, probably due to the spontaneous hydrolysis of the ester linkage between adenosine and *p*-fluorosulfonylbenzoyl moieties of FSO_2BzAdo [23]. Despite this we have assumed that the distribution of the remaining label reflects the sites in the native enzyme, modified by FSO_2BzAdo.

Fig. 5 shows the reversed-phase HPLC peptide mapping analysis of tryptic digests of the enzyme, modified in the absence and the presence of NADPH, together with their radioactivity profiles. The distribution of radioactive peaks of the tryptic digest of E-MalNEt, modified in the presence of *p*-hydroxybenzoate, was quite similar to that found for free E-MalNEt. Six significant peaks were observed in the unprotected sample (at 14 ml, 19.5 ml, 25 ml, 33.5 ml, 34.5 ml and 35 ml), which disappeared when NADPH was included in the labeling mixture. These peaks were further purified by HPLC (see legend to Fig. 5) yielding one major labeled peak in each case (data not shown). In addition, several radioactive peaks, adjacent to the three major labeled peaks eluting after 30 ml in Fig. 5, were rechromatographed. It was found that several peptides were resolved, none of which appeared to have an appreciable amount of radioactivity. Amino acid analyses of the purified radioactive material from the major labeled peaks eluting before 30 ml in Fig. 5 showed that no peptides were associated with radioactivity. These peaks, therefore, probably represent decomposition products of the labeling compound arising during trypsin treatment of the modified protein. Decomposition products (adenosine and the sulfonic and sulfonamide derivatives of 5'-benzoyladenosine) from FSO_2BzAdo-modified peptides have been described by others [32–35]. The first radioactive peak in Fig. 5 is most probably free [^{14}C]adenosine, since free adenosine co-chromatographed with this radioactive peak.

Table 5. *Elution behaviour of N-ethylmaleimide prelabeled p-hydroxybenzoate hydroxylase on 2',5'ADP-Sepharose*
50 µM E-MalNEt (50 nmol) in 50 mM Mes, pH 6.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol was adsorbed onto the matrix. After washing with one bed volume of the buffer the enzyme was eluted with a linear gradient over 40 min at a flow rate of 1 ml/min. All other details are given in Materials and Methods

Sample	Eluent	Elution volume
		ml
E-MalNEt	1 M Mes, pH 6.5	22[a]
	50 mM Hepps, pH 8.5	25[b]
	5 mM $NADP^+$	17
	10 mM 2',3'AMP	20
E-MalNEt – *p*-hydroxybenzoate	10 mM 2',3'AMP[c]	27

[a] At 0.42 M.
[b] At pH 7.8.
[c] In the presence of 1 mM *p*-hydroxybenzoate.

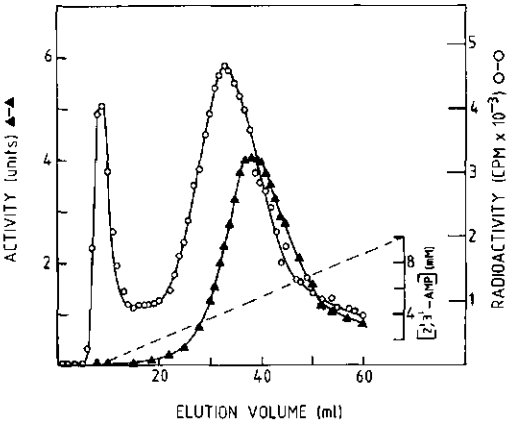

Fig. 4. *2',5'ADP-Sepharose chromatogram of N-ethylmaleimide-prelabeled p-hydroxybenzoate hydroxylase, modified by radioactive FSO_2BzAdo in the presence of p-hydroxybenzoate.* A solution of 77 µM E-MalNEt (116 nmol) in 40 mM Hepes pH 7.5, I = 50 mM, was incubated for 45 min at 30°C with 1.57 mM radioactive FSO_2BzAdo in the presence of 1 mM *p*-hydroxybenzoate (12% Me_2SO, by vol.). The enzyme was then poured over BioGel P-6 DG equilibrated with 50 mM Mes, pH 6.5, containing 0.5 mM EDTA and 0.5 mM dithiothreitol. 55.7 µM (100 nmol) of modified E-MalNEt (residual activity = 43%) was then chromatographed on 2',5'ADP-Sepharose with a linear gradient of 0–10 mM 2',3'AMP over 120 min at a flow rate of 0.5 ml/min. 1.0-ml fractions were collected and tested for activity (▲——▲) and radioactivity (O——O). The gradient is indicated by the dashed line. For all other details see Materials and Methods

Determination of the N-terminal amino acid by dansylation [36] and amino acid analysis of the purified peptides from the three major labeled peaks eluting after 30 ml in Fig. 5 were all compatible with the known amino acid composition of peptide CB3T4 in *p*-hydroxybenzoate hydroxylase spanning residues 34–42 [37]. The sequence of this peptide is Gln-Thr-Pro-Asp-Tyr-Val-Leu-Gly-Arg [37]. The only difference

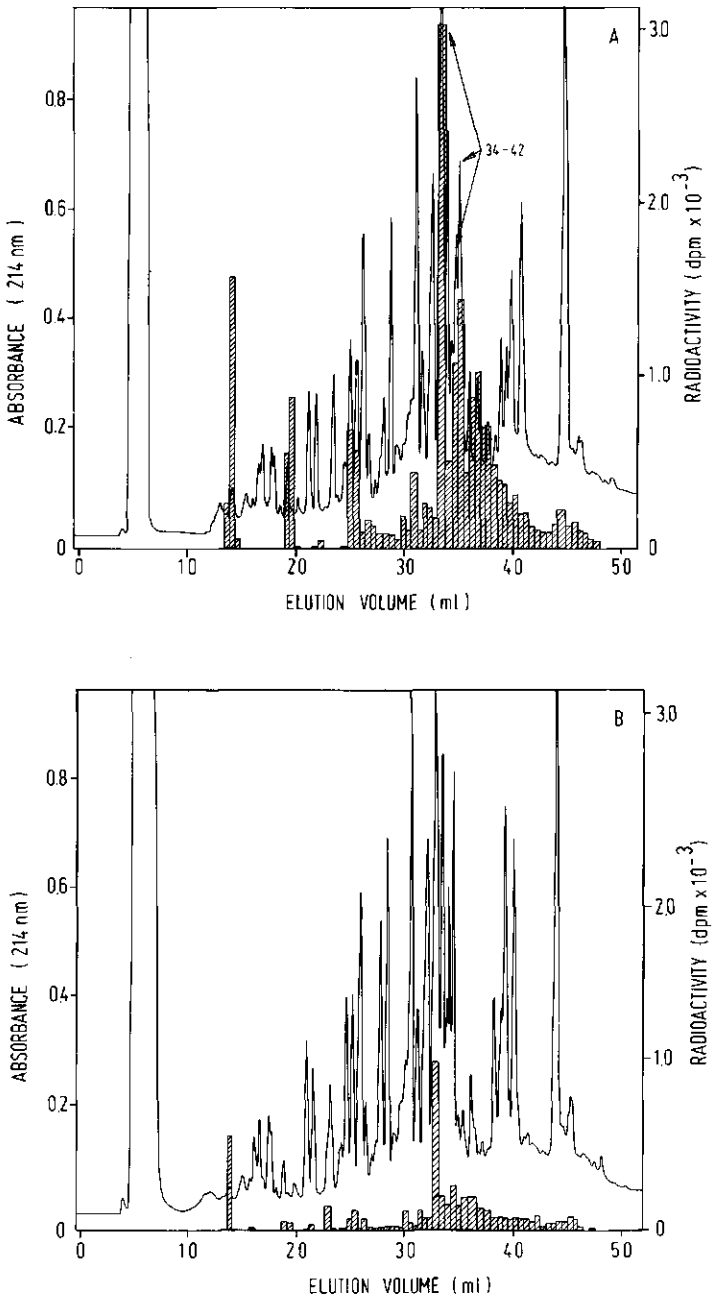

Fig. 5. *HPLC chromatogram of tryptic digests of* N-*ethylmaleimide-prelabeled* p-*hydroxybenzoate hydroxylase, modified by* FSO_2BzAdo *in the absence (A) and presence (B) of NADPH.* 5% of the tryptic digest of each sample was chromatographed with a linear gradient of 0 – 67% acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min. Peptide elution was monitored by the absorbance at 214 nm. Vertical bars represent the total radioactivity content associated with pools of eluent collected at 0.5-ml intervals. The radioactive peaks at 14 ml, 19.5 ml, 25 ml, 33.5 ml, 34.5 ml and 35 ml were preparatively isolated by a few separate runs of the tryptic digest of each sample. Each of the pools collected for the unprotected sample was further purified by HPLC with a linear gradient of 0 – 67% acetonitrile in 0.1% ammonium acetate, pH 6.0. The peptide which could subsequently be correlated with a known sequence in the primary structure of *p*-hydroxybenzoate hydroxylase is indicated by its N- and C-terminal residue number

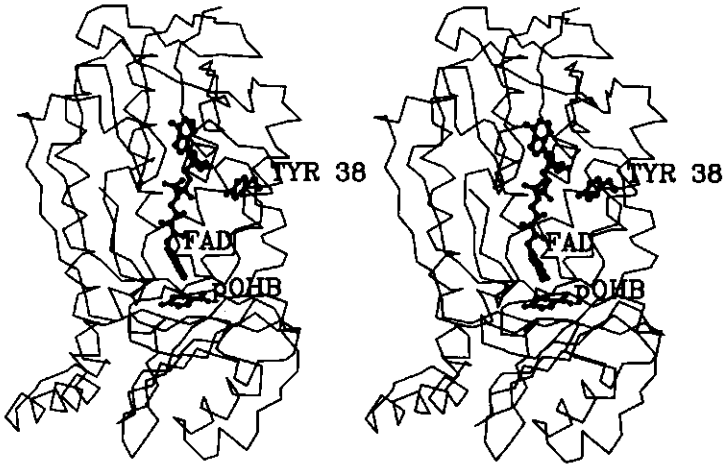

Fig. 6. *Stereo drawing of the C_α-skeleton of* p-*hydroxybenzoate hydroxylase based on a refined 0.19-nm structure.* The positions of FAD, *p*-hydroxybenzoate (pOHB) and Tyr-38 are shown

between the composition of the FSO_2BzAdo-modified peptides and that of CB3T4 is that no tyrosine was recovered in the modified peptides suggesting that Tyr-38 was the site of covalent attachment of FSO_2BzAdo. There were, however, no unknown peaks detected on the chart of the amino acid analysis which could correspond with acid-stable *O*-(4-carboxybenzenesulfonyl)-tyrosine which is formed upon hydrolysis of the ester linkage between the benzoyl and adenosine moiety of FSO_2BzAdo-modified tyrosine [23]. No N-terminal residue could be detected in the modified peptides, eluting at 34.5 ml and 35 ml in Fig. 5, by dansylation. These peptides were probably blocked by cyclization of the glutaminyl residue forming pyroglutamic acid. The reason for the different elution times of this blocked peptide in HPLC analysis is not clear at present. The non-blocked form of the modified peptide, eluting at 33.5 ml (Fig. 5), was sequenced up to the last residue by the DABITC method [38, 39]. Since the ester bond joining [^{14}C]adenosine to the benzoyl moiety in the SO_2Bz[^{14}C]Ado derivative of peptide 34 – 42 does not survive the relatively harsh conditions of sequence analysis [35], the labeled residue could not be determined by monitoring the release of radioactivity, during Edman degradation with DABITC. Results of sequence analysis were in full agreement with the known sequence of residues 34 – 42 of *p*-hydroxybenzoate hydroxylase [37], except at position 38 where DABTH-Tyr was detected on polyamide sheets to a minor extent, together with a major product running close to the position of DABTH-Tyr [38] and most probably corresponding to the DABTH derivative of *O*-(4-carboxybenzenesulfonyl)-tyrosine.

Based on the recovery of peptides and radioactivity, a value of about 0.8 mol FSO_2BzAdo/mol peptide was calculated indicating that no other residues in peptide 34 – 42 were covalently modified. Moreover, peptide 34 – 42 does not contain other amino acids which have been shown to react with the electrophilic reagent FSO_2BzAdo, and Tyr-38 is the sole residue in this peptide which can form a stable derivative with this reagent. Therefore it is concluded that Tyr-38 is the main residue protected by NADPH during modification of E-MalNEt by FSO_2BzAdo, although the modified residue was not directly identified in peptide 34 – 42.

Structural studies

The modification of Tyr-38 was analyzed using the 0.19-nm-crystal structure of the enzyme-*p*-hydroxybenzoate complex. This structure has been refined to a crystallographic *R*-factor of 15.6% (Schreuder et al., unpublished results).

Fig. 6 shows that Tyr-38 is on the same side of the enzyme as the active-site region, indicated by the positions of FAD and *p*-hydroxybenzoate. The distance between the hydroxyl group of Tyr-38 and the N5 of the flavin ring is 1.84 nm.

To date, crystallographic binding studies of NADPH with crystals of *p*-hydroxybenzoate hydroxylase have not been successful. Experiments to soak NADPH into crystals of the enzyme – *p*-hydroxybenzoate complex were not successful, probably due to ionic strength limitations [4]. Soaking in 200 mM NADPH yielded crystals of reduced enzyme but no NADPH binding was detected [40]. Therefore, a model building study was undertaken in an attempt to correlate the results from the present study with structural features of the structure of the enzyme-*p*-hydroxybenzoate complex.

The model building was initiated by placing the nicotinamide portion of the NADPH molecule in a cavity parallel to the flavin ring. This is supported by several lines of evidence: (a) binding of $Au(CN)_2^-$, a very strong competitive inhibitor of NADPH binding [16], near this position close to the flavin ring [6]; (b) analogy with the flavoprotein, glutathione reductase, where the crystal structure of the complex with NADPH is known [41]; (c) using a tritium-labeled flavin analog Manstein et al. showed that the nicotinamide ring is bound at the *re* side of the flavin ring [42]; (d) during the reduction process an intermediate with long-wavelength absorbance was detected indicating the existence of a charge-transfer complex between reduced flavin and stacked $NADP^+$ [1].

The proposed nicotinamide-binding pocket in the crystal structure of the enzyme – *p*-hydroxybenzoate complex is too

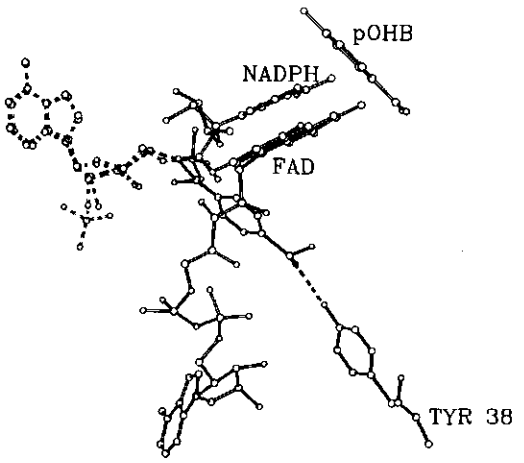

Fig. 7. *Schematic drawing of a potential mode of binding of NADPH and FSO_2BzAdo with respect to FAD, p-hydroxybenzoate and Tyr-38.* The adenosine moiety of both NADPH and FSO_2BzAdo is drawn with dotted lines to indicate uncertainties of the proposed binding site. The sulfur of the reactive group of FSO_2BzAdo (solid lines) comes close to Tyr-38 as is indicated with a dotted line. pOHB, *p*-hydroxybenzoate

narrow for a good fit of the nicotinamide ring. However, the evidence mentioned above, and also the fact that small rearrangements of residues can take place during enzyme reduction, suggests strongly that the nicotinamide ring will bind in the above-mentioned cavity on the *re* side of the flavin ring.

The remaining part of the NADPH molecule was fitted to the structure. Some side chains were rotated to provide enough space, but a few short contacts still existed. Since no experimental data concerning the position of the adenosine moiety are available yet, we are not certain if the position of the adenosine moiety is correct. A model of FSO_2BzAdo was fitted by superimposing the adenosine moiety of FSO_2BzAdo onto the adenosine part of the NADPH model and rotating the sulfonylbenzoyl moiety towards Tyr-38. After rotation of the side chain of Tyr-38 the distance between the hydroxyl moiety and the sulfur of FSO_2BzAdo is approximately 0.3 nm (Fig. 7). This short distance is in complete agreement with the observed covalent modification of Tyr-38.

Conclusions

The reaction of *p*-hydroxybenzoate hydroxylase with FSO_2BzAdo results in modification of Tyr-38. Protection from labeling by NADPH and by 2′,5′ADP and formation of a weak, reversible enzyme-inhibitor complex suggest that the adenosine moiety of FSO_2BzAdo mimicks the binding of the adenosine moiety of NADPH. Although we cannot exclude the possibility that FSO_2BzAdo binds in another orientation, the binding studies and bioaffinity chromatography indicate that covalently bound label is pushed aside by NADPH. This is in agreement with the model, where Tyr-38 is situated outside the NADPH-binding pocket itself.

Examination of the crystal structure of the enzyme–*p*-hydroxybenzoate complex reveals that Tyr-38 is far away from the active site; the distance between the hydroxyl moiety of Tyr-38 and the N5 atom of FAD is 1.84 nm. Nevertheless, Tyr-38 is positioned on the same side of the protein molecule as the active site. Moreover model-building studies indicate that a mode of binding can be proposed which is consistent with the experimental data.

In conclusion it can be stated that FSO_2BzAdo is a probe more useful for design of the enzyme NAD(P)H-binding pocket than for an identification of an amino acid residue directly involved in coenzyme binding. As only a very weak enzyme-inhibitor complex is found in the modification reaction, the use of other site-specific probes such as 2′,5′ADP analogs [43, 44] might be useful to provide further evidence for the proposed NADPH-binding site. We, however, intend to tackle this problem by site-directed mutagenesis in combination with X-ray crystallography studies.

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Chapter 7

The temperature and pH dependence of some properties of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*

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The free and complexed flavoprotein, *p*-hydroxybenzoate hydroxylase, was studied by light-absorption, circular-dichroism and fluorescence techniques as a function of the pH. The following compounds served as ligands for the enzyme: *p*-hydroxybenzoate, *p*-fluorobenzoate, benzoate, *p*-aminobenzoate and tetrafluoro-*p*-hydroxybenzoate. Depending on the technique used, the various ligands exhibit pH-dependent physical properties and dissociation constants. The data can be fitted with pK_a values in the range 7.7–7.9. It is suggested that this pK_a value belongs to a tyrosine residue in the active center of the enzyme. This assignment is supported by published data and additional experiments.

The flavoprotein *p*-hydroxybenzoate hydroxylase is classified among the external monooxygenases and catalyzes the conversion of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate. The substrate acts also as an effector, facilitating the reduction of protein-bound FAD by NADPH and, to a lesser degree, also by NADH.

p-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is one of the best studied flavoproteins. The catalytic mechanism of the enzyme has been investigated in detail (e.g. [1]). Many compounds are known to inhibit the enzyme competitively with respect to the substrate or NADPH [2–4]. The amino acid sequence has been elucidated [5, 6] and the three-dimensional model of the enzyme–*p*-hydroxybenzoate complex at 0.19 nm resolution is available [7, 8]. The possible involvement of amino acid residues in the binding of the substrate and ligands and in the enzymic mechanism has also been studied. Chemical modification of the following amino acid residues was shown to influence the catalysis of the enzyme: histidine [9], tyrosine [10, 11], arginine [12] and cysteine [13].

Only a few pH- and temperature-dependent studies on the enzyme have been performed. The pH-dependent interaction between the enzyme and NADPH was investigated in detail [14]. Chemical modification of Cys-152 [13] of the enzyme yields pH-dependent difference spectra indicating the presence of an ionizing group with a pK_a of 7.6 in the enzyme [13]. This pK_a value was tentatively assigned to a tyrosine residue [13]. An ionizing group with a similar pK_a value was also observed by a kinetic study on the oxidative half reaction of the enzyme [15].

In one single case it has also been observed [1] that the shape of the light-absorption difference spectra of enzyme-substrate/effector complexes differ with pH. It was suggested that the difference may be due to perturbation of the pK_a of the hydroxyl group of *p*-hydroxybenzoate to a lower value on binding to the enzyme [1]. The possibility that the pH-dependent shape of the difference spectra might be an intrinsic property of the enzyme was not considered. If this were the case, the observed difference would have to be re-interpreted. Consequently, a comparison of such spectra recorded at different pH values in different laboratories would be difficult, if not impossible. This prompted us to study the biophysical properties of the free and substrate/effector-complexed enzyme as a function of pH. In this context it was also desirable to study the dependence of the stability of the enzyme on pH and temperature.

The results show that the thermostability optimum of the enzyme is in the pH range 5.5–6.5, this in contrast to the optimum for the enzymic activity which is found in the range pH 7.5–8.5. The pH-dependent light-absorption difference, fluorescence emission and circular-dichroism spectra all indicate the presence of an ionizing group in the enzyme exhibiting a pK_a value of about 7.7. The results are interpreted in terms of the participation of the ionizing group in the binding of the substrate.

MATERIALS AND METHODS

NADPH, FAD, *p*-fluorobenzoate and pentafluorobenzoate were products of Sigma, Chemical Co. *p*-Hydroxytetrafluorobenzoate was prepared from pentafluorobenzoate by a published method [16].

The isolation and purification procedure for the enzyme was described previously [17]. The assay conditions were reported in an earlier paper [3].

Dissociation constants were determined from light-absorption difference, fluorescence emission and circular-dichroism spectra by titration of a known concentration of enzyme with the desired compound and were graphically evaluated by the method of Benesi and Hildebrand [18].

Light-absorption spectra were obtained on an Aminco DW 2 A spectrophotometer. The difference spectra were obtained by placing an equal volume of the same concentration of the enzyme into both the reference and sample cell. After temperature equilibration of the samples a baseline was recorded and, if necessary, adjusted to zero absorption with

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Enzyme. *p*-Hydroxybenzoate hydroxylase (EC 1.14.13.2).

the multipot system. A known concentration of the reagent was added to the sample cell; to the other cell an equal volume of buffer solution was added. After every addition a difference spectrum was recorded. This procedure was repeated until no further changes in absorbance occurred.

Fluorescence spectra were obtained on an Aminco SPF-500 recording fluorimeter. All instruments were equipped with thermostated cell holders. The experiments were performed at 22 – 23 °C.

pH-dependent studies were performed using the following buffers: 80 mM Mes (pH 5 – 7), 80 mM Hepes (pH 7 – 8), 80 mM Hepps (pH 8 – 8.7) and 50 mM Bistrispropane (pH 8.8 – 9.5). Buffers were brought to constant ionic strength (I = 0.1 M) with 0.5 M Na_2SO_4 as described earlier [14]. The pH of the solutions was checked before and after the measurements. The deviation of the pH values did not exceed ± 0.05 pH units.

The pH-dependent initial-velocity measurements of enzyme activity were performed in air-saturated buffers (I = 25 mM) with varying concentrations of either NADPH or *p*-hydroxybenzoate. V and K_m values were obtained by plotting the data according to Lineweaver-Burk. The temperature was 25 °C.

The temperature-dependent studies on the stability of the enzyme as a function of pH were performed by incubating the enzyme at the desired temperature. At time intervals, aliquots were withdrawn from the incubation mixture and assayed at pH 8.0 and 25 °C.

The pH-dependent light-absorption difference spectra were obtained using an enzyme solution at pH 5.6 as a reference sample. The other cell contained an enzyme solution at the same concentration at different pH values. In both cases, an equal aliquot of an enzyme stock solution was diluted into the appropriate buffer. In the case of enzyme-ligand complexes, the same concentration of ligand was added to both cells.

Circular-dichroic spectra were measured with a Jobin Yvon mark V auto-dichrograph, equipped with Silex microcomputer for data aquisition. The measurements are expressed as molar ellipticity [θ], in deg · cm^2 · dmol^{-1}. Spectra were recorded at 22 °C in 80 mM Mes pH 6.5 (I = 0.1 M) or as otherwise indicated. The enzyme concentrations were about 10 µM in the range 250 – 320 nm and 50 µM in the range 320 – 550 nm.

RESULTS AND DISCUSSION

Thermal stability and pH dependence of the enzyme activity

The kinetics of inactivation of the enzyme was investigated as a function of the temperature and pH of the solutions. The enzyme is inactivated in a first-order reaction. As can be seen from Fig. 1, the enzyme is rather stable at 40 °C in the pH range 5.5 – 7.5. At higher and lower pH values, the rate of inactivation increases rapidly with increasing temperature and the pH range, within which the stability of the enzyme remains little affected, becomes narrower. At 60 °C, the enzyme is still rather stable in the pH range 5.5 – 6.5. When the enzyme-substrate complex was subjected to the same treatment, the enzyme was somewhat more stable against thermal inactivation (Fig. 1). From these results it can be concluded that pH 6.0 represents the optimal condition under which the enzyme should be stored over a long period of time. It is noteworthy that the related enzyme from *P. desmolytica* shows a lower thermal stability [19] than that from *P. fluorescens*.

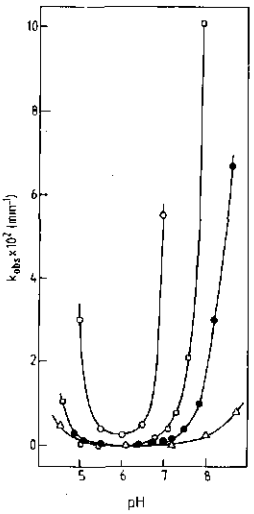


Fig. 1. *The pH dependence of the temperature stability of* p-*hydroxybenzoate hydroxylase in the presence and absence of* p-*hydroxybenzoate.* The apparent first-order rate constant for enzyme inactivation is plotted against pH. The rate constants obtained by the usual graphical methods were determined by incubation of 2.2 µM enzyme at different pH values (for buffers see Materials and Methods). Aliquots were taken from the incubation mixture at time intervals (t = 0, 2.5, 5, 10, 20, 40, 80 and 160 min) and assayed at pH 8.0 and 25 °C. The incubations were performed at 40 °C (△), 50 °C in the presence (●) and absence (□) of 1 mM *p*-hydroxybenzoate and 60 °C (○)

It is interesting to compare the temperature stability data with the pH dependence of the activity of the enzyme. The pH optimum for enzyme activity (Fig. 2A) is about 8, whereas the stability curve exhibits an optimum at about pH 6 (Fig. 1). The observed pH optimum of the activity is in agreement with work published by others [1]. Replotting the results obtained at 25 °C in the form of log activity against pH (Fig. 2B) or log stability against pH (results not shown), two apparent pK_a values are observed for each plot exhibiting slopes of 1 and − 1. The apparent pK_a values are about 5 and 8 for the stability curve and about 6.5 and 9 for the activity curve. The destabilization of the enzyme at low pH values can be attributed to protonation of amino acid residues, leading to denaturation (conformational changes) of the protein. This interpretation is in agreement with the pI value of 5.7 of the protein [17]. The pK_a value of 8 could be due to a particular amino acid residue. This point will be discussed below in the context of other pH-dependence studies.

The apparent pK_a value of 9 in the pH-dependent activity curve (Fig. 2A) is most probably due to the ionization of the substrate, which has a pK_a value of 9.3 [20]. This interpretation is supported by both the pH-dependent measurements of V and K_m as a function of the substrate concentration (Fig. 2A). The K_m value for the substrate is relatively independent of pH in the range 5.8 – 7 but increases at higher pH values, yielding an apparent pK_a of about 8.8 (Fig. 2B). At infinite substrate concentration, V is only slightly influenced at high pH values (Fig. 2A). For the enzyme from *P. desmolytica*, Shoun et al. [19] assigned the dissociating group on the basic side of the overall activity curve to either a lysine or cysteine residue.

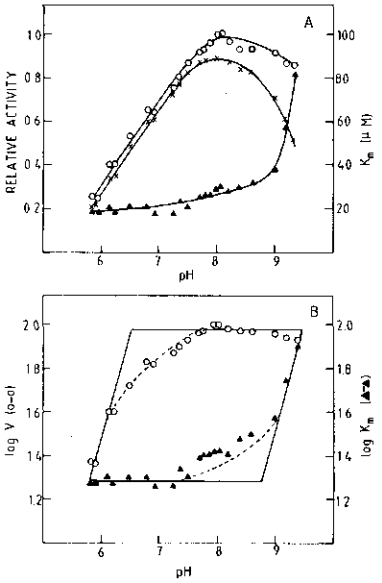


Fig. 2. *The pH dependence of the activity of* p-*hydroxybenzoate hydroxylase*. The pH-dependent activity was determined at constant ionic strength, I = 25 mM (for buffers see Materials and Methods) at 25°C and varying concentrations of *p*-hydroxybenzoate (20–200 μM). (A) Plots of the relative activity at 40 μM *p*-hydroxybenzoate (×), extrapolated to infinite *p*-hydroxybenzoate concentration (○), and of K_m for the substrate (▲) against pH. (B) Logarithmic plot of V (○) and K_m (▲) against pH

Since these amino acid residues are not involved in the binding of the substrate [8] and since V is only slightly influenced at high pH values (Fig. 2A), the observed pK_a value must be assigned to the substrate and also implies that ionized substrate does not bind to the enzyme.

The pK_a value of about 6.5 is difficult to assign. It could reflect the ionization of His-162, probably involved in the binding of NADPH [9, 14, 21].

The activation energy of the overall catalytic reaction has been determined, using the standard assay procedure, at pH 8.0. A plot of ln K versus $1/t$ in the range 4–25°C yielded a straigth line, from which an activation energy of 49 kJ/mol was determined (results not shown). The temperature coefficient, Q_{10}, was found to be 2.0. At temperatures above 30°C enzyme activity decreased significantly, due to denaturation of the enzyme during catalysis.

pH-Dependent light-absorption difference spectra

Fig. 3A shows the pH-dependent changes of difference spectra of the free enzyme, with enzyme solution at pH 5.6 as reference sample. The difference in absorbance increases with increasing pH value. A relatively intense and two less intense positive peaks are observed at 495 nm, 463 nm and 350 nm, respectively. An intense negative peak is formed at 388 nm. Isobestic points are observed at 520 nm, 453 nm and 361 nm. When the difference in absorbance is plotted against pH, an apparent pK_a value of 7.5 is calculated (Fig. 3B). The curve in Fig. 3B was obtained theoretically, using a pK_a value of 7.5 and a maximal absorbance difference ($\Delta\varepsilon_{max}$ = 1.5 mM^{-1} cm^{-1}) of 0.039 at 388 nm. The calculated curve in Fig. 3B represents the expression

$$\Delta\varepsilon_{obs} = \frac{\Delta\varepsilon_{max}}{\frac{[H]}{K} + 1} \qquad (1)$$

suggesting that ionization of a group in the enzyme causes the flavin spectrum to change with pH. Similar experiments were performed with the enzyme-substrate complex (Fig. 3C). The difference spectra exhibit a different pattern to those of the free enzyme. A pK_a value of 7.67 was calculated from the experimental data ($\Delta\varepsilon_{max}$ = 1.89 mM^{-1} cm^{-1} at 440 nm), as shown by the fitted curve in Fig. 3D.

It is tempting to speculate about the nature of the ionizing group in the enzyme. Previously, we reported on an ionizing group in Cys-152–*p*-chloromercuribenzoate-modified enzyme exhibiting a pK_a value of 7.6 [13]. It was suggested that a tyrosine residue in the active center of the enzyme possesses such a pK_a value. For the time being, the apparent pK_a value is assigned to one of the three tyrosine residues (Tyr-201, Tyr-222 and Tyr-385) [6] in the active site of the enzyme (see also below). The above interpretation seems to be supported by the observation that ethoxycarboxylated enzyme, leading to the alkylation of a tyrosine residue in the active site [9], no longer showed the pH-dependent difference spectrum at high pH values [10].

The difference spectra shown in Fig. 3A and C are remarkably different from those published [1, 22, 23]. The pH-dependent difference spectra of both the free and the substrate-complexed enzyme almost maintain their shape over the whole pH range tested, except that the intensity increases with increasing pH. This is not the case when the free enzyme is used as a reference against the enzyme-substrate complex. This is demonstrated in Fig. 3E and F where difference spectra between free and substrate-complexed enzyme at pH 6.1 and pH 8.7, respectively, are shown. These spectra differ from those published and recorded at 4°C at pH 6.6 (Fig. 6 in [1]), pH 7.6 (Fig. 1 in [22]), and at 20°C at pH 8 and 5.7 (Figs 1 and 4 in [23]). Although our spectra are not identical with the published difference spectra, the two sets of spectra are similar with respect to shape at a particular pH range. Therefore, the differences reflect the pH-dependence of the spectra, i.e. are composed of pH-dependent contributions of both solutions. It is, therefore, not justified to conclude from such spectra alone, obtained at two pH values, that the changes indicate ionization of the enzyme-bound substrate, as was done in [1, 23].

Circular-dichroism spectra

The CD spectra of *p*-hydroxybenzoate hydroxylase in the free and substrate-complexed state are shown in Fig. 4. These spectra are identical with those published by Spector and Massey [2] as far as the spectral region 320–550 nm is concerned. Until now only the CD spectra of the apoprotein of this enzyme in the ultraviolet region have been reported [24]. The spectrum of the free holo enzyme exhibits a positive Cotton effect at about 290 nm and this band is of moderate intensity. Addition of substrate to the enzyme has a drastic effect on the CD spectrum in the region 250–300 nm. The positive band at 290 nm in the free enzyme disappears completely from the spectrum and a strong negative band with a minimum at about 280 nm is formed. This band is further intensified by the addition of Cl^-, which inhibits enzyme activity competitively with respect to NADPH [3]. CD spectra

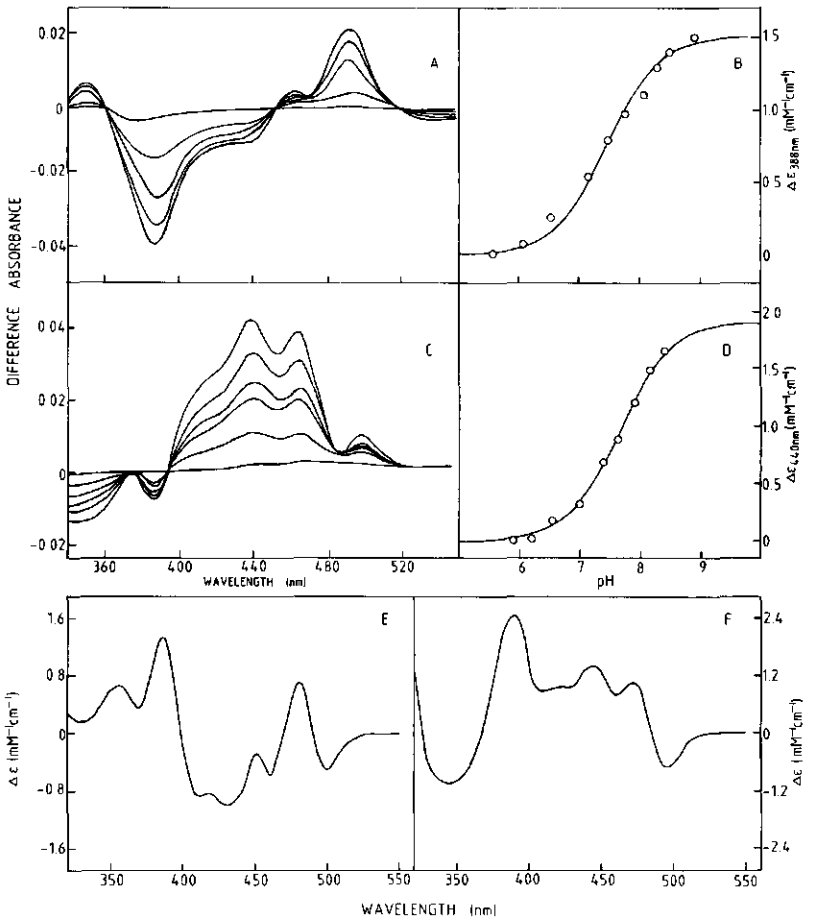


Fig. 3. *The pH dependence of the light-absorption difference spectra of* p-*hydroxybenzoate hydroxylase in the presence and absence of* p-*hydroxybenzoate.* (A) Difference spectra between a reference solution of 25.6 μM free enzyme, pH 5.6, and solutions of 25.6 μM free enzyme at pH 7.2, pH 7.8, pH 8.3 and pH 8.95, respectively. The temperature was 20°C. For buffers used see Materials and Methods. (B) The absorbance differences of A are plotted against pH. (C) Difference spectra between a reference solution of 26.5 μM enzyme in the presence of 0.5 mM *p*-hydroxybenzoate, pH 6.1, and solutions of 26.5 μM enzyme in the presence of 0.5 mM *p*-hydroxybenzoate at pH 7.0, pH 7.4, pH 7.6, pH 7.9 and pH 8.45 respectively. The temperature was 22°C. For buffers used see Materials and Methods. (D) The absorbance differences of C were extrapolated to infinite *p*-hydroxybenzoate concentration (cf. Fig. 5D) and plotted against pH. (E) Difference spectrum between a solution of 40 μM free enzyme, pH 6.1, and the same solution containing 0.5 mM *p*-hydroxybenzoate as a reference. The temperature was 20°C. (F) Difference spectrum between a solution of 40 μM free enzyme, pH 8.7, and the same solution containing 0.5 mM *p*-hydroxybenzoate as a reference. The temperature was 20°C

of the enzyme from *P. putida* in the region 250 – 300 nm have been published by Hesp et al. [25]. The two sets of data are in fair agreement with each other. The strong negative Cotton effect seen in Fig. 4 is probably due to protein-bound FAD, which is strongly perturbed in the presence of the substrate. It is known that the substrate induces a conformational change in the enzyme [1, 2, 22]. This effect is most clearly shown in the 250 – 300-nm region of the CD spectrum. Therefore, the strong negative band in this region is probably caused by opposite contributions of protein-bound FAD and aromatic amino acid residues and, possibly, of bound *p*-hydroxybenzoate. That the observed band is mainly due to perturbation of the flavin is supported by the fact that the CD spectra of the free and substrate-complexed apoprotein do not show this band, although the CD spectrum of the free apoprotein is affected in the presence of the substrate [24].

The CD spectrum of the free enzyme is relatively independent of the pH value of the solution (results not shown), in contrast to that of the substrate-complexed enzyme (Fig. 5A). The CD spectra of the enzyme at pH 6.0, as a function of the substrate concentration, are shown in Fig. 5B. The dissociation constant determined from these data is in good agreement with those obtained by other methods (see below). The pH-dependence spectra (Fig. 5A) show that the intensity of the negative band at 275 nm decreases with increasing pH, reaching a low intensity at pH 8.4 and even becomes zero at

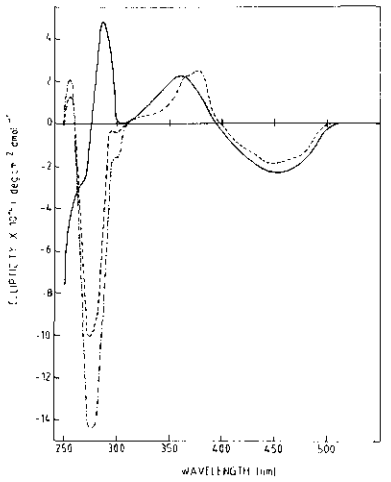


Fig. 4. *Circular-dichroic spectra of* p-*hydroxybenzoate hydroxylase in the absence and presence of* p-*hydroxybenzoate.* The molar ellipticity, $[\theta]$ in deg · cm^2 · dmol^{-1} is plotted against the wavelength. The concentration of the enzyme was 10 μM over the range 260–320 nm and 50 μM over the range 300–550 nm in 80 mM Mes buffer, pH 6.5, I = 0.1 M. The curves represent free enzyme (——), in the presence of 200 μM *p*-hydroxybenzoate (– – – –) and in the presence of 200 μM *p*-hydroxybenzoate and 50 mM KCl (·—·—·). All curves were corrected for the base line, recorded in the absence of enzyme

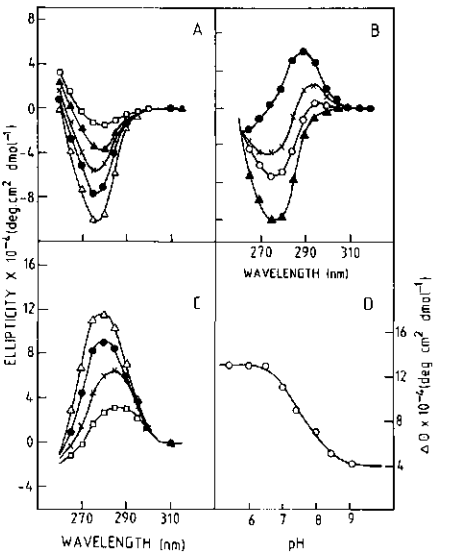


Fig. 5. *The pH dependence of the circular-dichroic spectra of* p-*hydroxybenzoate hydroxylase in the absence and presence of* p-*hydroxybenzoate.* The molar ellipticity, $[\theta]$ in deg · cm^2 · dmol^{-1} is plotted against the wavelength. The concentration of the enzyme was 10 μM in different buffers, I = 0.1 M, as indicated under Materials and Methods. All curves were corrected for the base line recorded in the absence of enzyme and for dilution. (A) The curves represent enzyme in the presence of 200 μM *p*-hydroxybenzoate at pH 6.0 (Δ——Δ), pH 7.0 (●——●), pH 7.5 (×——×), pH 8.0 (▲——▲) and pH 8.5 (□——□). (B) The curves represent free enzyme (●——●) at pH 6.0 and in the presence of 20 μM (×——×), 60 μM (○——○) and 120 μM (Δ——Δ) *p*-hydroxybenzoate. (C) Difference spectra between free enzyme and enzyme in the presence of 200 μM *p*-hydroxybenzoate at pH 6.0 (Δ——Δ), pH 7.0 (●——●), pH 8.0 (×——×) and pH 9.1 (□——□). (D) The absorbance differences obtained from Fig. 3C were extrapolated to infinite *p*-hydroxybenzoate concentration and plotted against pH

higher pH values. Concomitantly, the minimum at 275 nm moves to longer wavelengths. In order to evaluate the pH-dependent intensity of the CD spectra, difference spectra were generated by substraction of the CD spectra of the complexed enzyme from those of the free enzyme. Such difference spectra in the pH range 5.4–9.1 are shown in Fig. 5C. When the data of Fig. 5C are plotted against the pH value according to the expression:

$$\Delta\varepsilon_{obs} = \frac{\Delta\varepsilon_{max}}{\frac{K}{[H]} + 1} + \frac{\Delta\varepsilon_{min}}{\frac{[H]}{K} + 1} \qquad (2)$$

an apparent pK_a value of 7.6 is obtained (Fig. 5D). This value is in good agreement with those obtained from light-absorption difference spectra. Similar data were obtained using other ligands for the enzyme (Table 1).

Fluorescence studies

The quantum yield of protein-bound FAD is about 60% that of free FAD. The pH-dependent fluorescence of free enzyme is shown in Fig. 6A. The fluorescence yield remains constant over the pH range 6.0–8.0. At higher pH values the fluorescence yield decreases, most probably due to ionization of the N(3)H group of protein-bound FAD. The calculated apparent pK_a value is 9.35.

On the other hand, the quantum yield of the substrate-complexed enzyme is pH-independent, if extrapolated to infinite substrate concentration (Fig. 6A). These results indicate that binding of the substrate prevents the flavin becoming ionized. In free FAD the N(3)H group ionizes with a pK_a = 10.4 (Fig. 6A); this value is in excellent agreement with that determined by Massey and Ganther [26]. The observed shift

Table 1. *The pH-dependent behaviour of some complexes between* p-*hydroxybenzoate hydroxylase and ligands, as determined by circular-dichroic spectra*

Ligand	pH	K_d	pK_a	λ_{max}	$\Delta[\theta]_{max} \times 10^{-4}$
		μM		nm	deg · cm^2 · dmol^{-1}
Benzoate	6.5	90	–	275	8.3
	7.8	130			
p-Aminobenzoate	6.0	20	–	287	12.5
	8.0	38			
p-Fluorobenzoate	6.2	99	7.9	275	9.9
	8.2	265			
p-Hydroxy-tetra-fluorobenzoate	6.0	140	7.6	285	11.6
	8.0	600			

of the pK_a by one pH unit in the free enzyme is not unusual for a protein-bound flavin and has also been observed in glycolate oxidase for example [27]. The ionized protein-bound FAD in *p*-hydroxybenzoate hydroxylase is non- or only

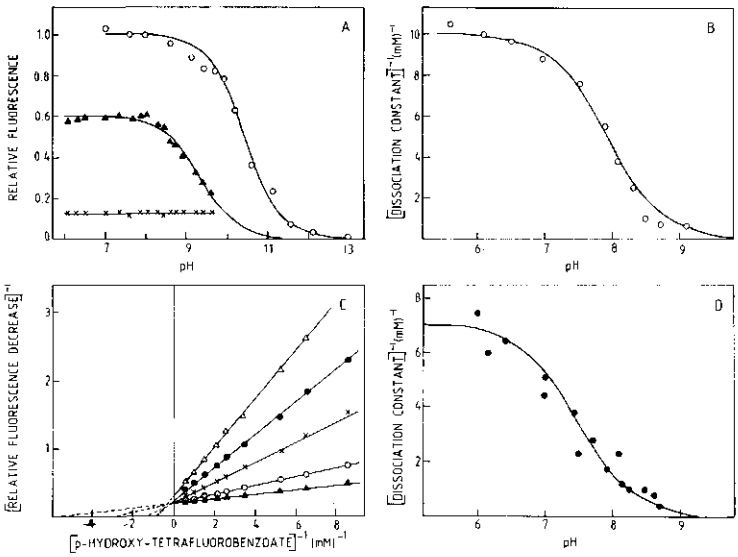


Fig. 6. *The pH dependence of the fluorescence properties of* p-*hydroxybenzoate hydroxylase in the absence and presence of* p-*hydroxybenzoate and analogs.* (A) pH Dependence of the relative fluorescence quantum yield of 10 μM FAD (○——○), 10 μM free enzyme (▲——▲) and 10 μM enzyme—*p*-hydroxybenzoate complex (×——×). The relative fluorescence quantum yield of the enzyme—*p*-hydroxybenzoate complex was obtained from the Y-axis intercept of Benesi-Hildebrand plots (cf. Fig. 6C and Table 2). (B) pH Dependence of the dissociation constant of the enzyme—*p*-fluorobenzoate complex. (C) Benesi-Hildebrand plot of the titration of enzyme with *p*-hydroxy-tetrafluorobenzoate at pH 6.15 (▲), pH 7.7 (○), pH 8.15 (×), pH 8.45 (●) and pH 8.62 (∧). (D) pH Dependence of the dissociation constant of enzyme—*p*-hydroxy-tetrafluorobenzoate complex

weakly fluorescent as ionized free FAD. Ionization of N(3)H of protein-bound FAD in *p*-hydroxybenzoate hydroxylase does not lead to dissociation of the prosthetic group from the protein, since the degree of fluorescence polarization (0.42) [24] remained unaffected at pH 9.5 and 20°C. If FAD were released one would expect a decrease in the degree of polarization value, because some of the free FAD would not ionize at pH 9.5 and thereby affect the degree of polarization.

Ionization of the N(3)H group of FAD in the free enzyme requires solvent accessibility of the functional group. Although the pyrimidine subnucleus of FAD seems to be buried in the protein, as indicated by three-dimensional data [7, 8], it has been shown that the 2 position of the 2-thio-FAD—*p*-hydroxybenzoate hydroxylase complex can be chemically modified [28, 29]. Similar results were obtained with the analogous complex of riboflavin-binding protein, where it is known that the pyrimidine subnucleus is accessible to bulk water. In the presence of the substrate *p*-hydroxybenzoate, the prosthetic group in the above mentioned complex of *p*-hydroxybenzoate hydroxylase is not easily modified anymore. These results correspond exactly with our fluorescence data. These observations can be explained by a substrate-induced conformational change.

In contrast to the fluorescence properties of the enzyme—*p*-hydroxybenzoate complex, the dissociation constant of the complex is pH dependent. As can be seen from Table 2, *p*-hydroxybenzoate-binding is favoured at low pH. At pH values 8—9, binding is somewhat decreased and at pH values above 9 binding decreases dramatically. These effects resemble those observed for the pH-dependent K_m value for substrate (Fig. 2). The same effects are observed with 6-hydroxynicotinate, benzoate and *p*-aminobenzoate (Table 2). It is suggested that the decreased affinity of the enzyme for the ligands at pH 8, as compared to pH 6, reflects the ionization of one of the tyrosine residues in the active site, whereas at pH values above 9 affinity decreases dramatically due to different effects, i.e. ionization of hydroxylated ligands and/or ionization of the free enzyme. In contrast to NADPH-binding [14], the binding of *p*-hydroxybenzoate is only slightly dependent on the ionic strength of the solution (Table 2).

In order to explore the possibility of arriving at a safer assignment of the pK_a values, we also investigated complexes of *p*-fluorobenzoate and *p*-hydroxy-tetrafluorobenzoate with the enzyme [2, 16]. The former compound possesses only the carboxyl group as an ionizing moiety, whereas in the latter the hydroxyl group shows a pK_a of 5.3 [16]. The pH-dependent K_d values of the enzyme—*p*-fluorobenzoate complex are shown in Fig. 6B. An apparent pK_a value of 7.9 is found. Titration of the enzyme with *p*-fluorobenzoate influences the quantum yield of the enzyme to a much lesser extent than titration with the substrate *p*-hydroxybenzoate. At pH values below 7.5 the quantum yield of *p*-fluorobenzoate-complexed enzyme is about 55%, compared to free enzyme (Table 3). At pH values around 8, the quantum yield is even less affected than at lower pH values. This has also been observed by Spector and Massey [2]. The observed effect at pH 8 can be mainly attributed to the pH-dependent increase in the quantum yield of the enzyme—*p*-fluorobenzoate complex. At pH 8.4, this effect is responsible for the unique situation that no change in fluorescence is observed at all, although *p*-fluorobenzoate binds to the enzyme. Moreover, at pH values above 8 the binding studies are complicated by another effect, i.e. at high concentrations *p*-fluorobenzoate binds also at a different site, yielding an increase in fluorescence. This devi-

Table 2. *The pH-dependent dissociation constants of some complexes between* p-*hydroxybenzoate hydroxylase and ligands, as determined by fluorimetric titration experiments*
nd, not determined; –, no or very weak binding; ?, experimental limitations (see text)

Ligand	*I*	Dissociation constants of the complexes at pH									
		5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.3	9.5
	mM	μM									
p-Hydroxybenzoate	25	22	20	25	29	37	40	40	70	nd	107
	100	29	35	38	40	45	50	50	nd	nd	nd
6-Hydroxynicotinate	100	150	146	155	172	273	324	350	>1000	–	–
Benzoate	100	83	95	86	148	135	279	?	?	?	?
p-Aminobenzoate	100	19	20	21	25	27	38	50	75	97	nd

Table 3. *The pH-dependent quantum yield of some complexes between* p-*hydroxybenzoate hydroxylase and ligands, as determined by fluorimetric titration experiments*

Ligand	Fluorescence quantum yield relative to free enzyme at pH				
	6.0	7.0	8.0	8.5	9.0
Benzoate	0.44	0.44	0.71	1.00	1.32
p-Aminobenzoate	0.11	0.11	0.11	0.12	0.14
p-Fluorobenzoate	0.56	0.53	0.70	1.00	2.11
6-Hydroxynicotinate	1.48	1.38	1.51	1.58	2.10

ation from 1:1 binding has also been observed by others for *p*-hydroxybenzoate and 6-hydroxynicotinate [30]. Because, at pH values above 8.5, the quantum yield of free enzyme is lowered (cf. Fig. 6A) binding of *p*-fluorobenzoate at a different site leads to an apparent strong increase in fluorescence. The dissociation constant of the second binding site, which is pH independent in the range 6–9, is about 2.3 mM. Similar effects are observed with benzoate (Table 3).

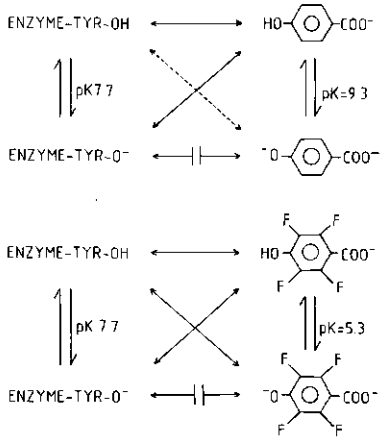
Fig. 6C shows the fluorimetric titration of the enzyme with *p*-hydroxy-tetrafluorobenzoate as a function of pH. The quantum yield of the enzyme–*p*-hydroxy-tetrafluorobenzoate complex is pH independent and about 45% that of free enzyme. The pH dependence of the dissociation constant is shown in Fig. 6D, yielding a pK_a value of about 7.6. The dissociation constants of several enzyme complexes as a function of pH are summarized in Table 2. The results are in good agreement with the results obtained from circular-dichroic spectra (cf. Table 1).

Conclusions

The data presented in this paper clearly show that, in the oxidized enzyme, the pK_a values of 7.5–7.9 are not due to ionization of the ligands bound to the enzyme and must therefore be assigned to an amino acid residue, probably located in the active site.

Chemical modification studies have already indicated that, in the oxidized enzyme, either Tyr-201 or Tyr-385 possesses a pK_a value of 7.6 [10]. The present study now supports this earlier tentative assignment of the pK_a value.

The presently existing three-dimensional model of the enzyme–*p*-hydroxybenzoate complex, refined to 0.19-nm resolution [31], and the three-dimensional model of the enzyme–3,4-dihydroxybenzoate complex [8] show that the 4-hydroxyl



Scheme 1. *Schematic representation of the interaction of* p-*hydroxybenzoate hydroxylase with* p-*hydroxybenzoate or* p-*hydroxytetrafluorobenzoate*

group of the substrate (or product) is located close to the hydroxyl group of Tyr-201. Therefore, Tyr-201 is a likely candidate showing this low pK_a value.

pH-Dependent kinetic studies of the reoxidation reaction of the enzyme–2,4-dihydroxybenzoate complex have also revealed pK_a values in the range 7.7–7.9 [15]. It was suggested that the pK_a is due either to a tyrosine residue in the active center or to the substrate or product.

The results presented in this paper can be explained by Scheme 1. As long as Tyr-201 is not ionized, all compounds tested can interact strongly with the enzyme, even *p*-hydroxytetrafluorobenzoate which possesses a relatively low pK_a value. At higher pH values, where the ionization of Tyr-201 and of the ligands come into play, the affinity of the ionized enzyme for an ionized ligand is decreased, due to repulsion.

The hydroxylation of the substrate by the enzyme occurs probably by the addition of an oxenium ion [32]. Ionization of the hydroxyl group of the substrate would therefore facilitate the monooxygenation reaction [33, 34]. In Scheme 1 the hydroxyl group of the substrate does not becomes ionized, but the hydrogen-bond formation between the ionized Tyr-201 and the hydroxyl group of the substrate will also facilitate the catalysis by weakening the O-H bond of the substrate.

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SUMMARY

In this thesis different studies probing the structure-function relationship of some flavoproteins are dealt with. The attention has been focused on two central themes:

The first part of the thesis deals with studies concerning the application of affinity chromatography in order to allow the large scale preparation of apo flavoproteins.

In the second part of the thesis, different studies are presented concerning the biophysical properties of p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens.

Conventional methods for the preparation of apo flavoproteins and general properties of FAD-dependent external monooxygenases are reviewed in **Chapter 1**. Special attention has been paid to the different studies performed with p-hydroxybenzoate hydroxylase from P.fluorescens.

Conventional methods to prepare the apoenzyme of p-hydroxybenzoate hydroxylase from P.fluorescens yield relatively low amounts of apoenzyme showing both variable residual and reconstitutable activity. In **Chapter 2** a new method is described to overcome this problem.

Large amounts of stable apoprotein, showing almost no residual activity, have been obtained by use of DTNB-Sepharose covalent affinity chromatography.

The enzyme can be reconstituted on the column or the dimeric apoprotein can be isolated in the free state. The degree of reconstitution of almost completely recovered enzyme is better than 95% of the original activity.

The affinity of p-hydroxybenzoate for the apoprotein is comparable to native holoenzyme. The substrate protects the apoprotein from inactivation.

The apoenzyme also forms a complex with NADPH. The dissociation constant of this complex is even lower than that of the holoenzyme and is strongly dependent on pH and ionic strength of the solution.

Kinetic experiments show that the enzyme is reconstituted in a fast process, FAD being tightly bound by the apoprotein.

In **Chapter 3** a new and more general applicable method for the large scale preparation of apo flavoproteins is described. Two classes of flavoproteins have been selected to demonstrate the usefulness of the applied hydrophobic interaction chromatography method. In contrast to conventional methods, homogeneous preparations of apoproteins in high yields are obtained.

The holoenzyme of lipoamide dehydrogenase from Azotobacter vinelandii can be reconstituted while the apoprotein is still bound to the column or the apoenzyme can be isolated in the free state. The biophysical properties of completely recovered reconstituted lipoamide dehydrogenase compare favorable with the properties of native holoenzyme.

The holoenzyme of butyryl-CoA dehydrogenase from Megasphaera elsdenii cannot be reconstituted when the apoenzyme is bound to the column. However, this is the first report where stable apoprotein can be isolated in the free state. The yield of apoprotein is more than 50% of starting material. The coenzyme A ligand present in native holoenzyme is removed during apoprotein preparation.

At pH 7.0 apo butyryl-CoA dehydrogenase is in equilibrium between dimeric and tetrameric forms and reassociates to a native-like tetrameric structure in the presence of FAD.

Fluorescence-polarization experiments show that the pH-dependent stability of reconstituted enzyme is strongly influenced by the presence of CoA ligands. Unliganded reconstituted enzyme is easily regreened in the presence of a mixture of coenzyme A and sodium sulfide.

In **Chapter 4** the large scale purification of p-hydroxybenzoate hydroxylase from P.fluorescens is described. The highly purified enzyme can be separated into at least five fractions by anion-exchange chromatography. All enzyme molecules exhibit the same specific activity and exist mainly in the dimeric form in solution. The observed microheterogeneity of the enzyme can be explained by the (partial) oxidation of Cys-116 in the sequence of the enzyme.

The separation of the different enzymic forms has allowed the development of a kinetic FPLC method to describe the dissociation behaviour of the dimeric enzyme.

By chemical modification studies using maleimide derivatives, DTNB and H_2O_2, it is shown that sulfenic, sulfinic and sulfonic acid derivatives of Cys-116 are the main products of oxidation.

In **Chapter 5** the chemical modification of cysteine residues in p-hydroxybenzoate hydroxylase from P.fluorescens by several reagents is described. Differential labeling and sequencing radioactive labeled tryptic peptides have allowed the assignment of different cysteine residues involved in enzyme modification.

Cys-116 is found to react rapidly and specifically with N-ethylmaleimide without inactivation of the enzyme.

The enzyme is easily inactivated by mercurial reagents. Enzyme activity can be fully restored upon addition of dithiothreitol. p-Hydroxybenzoate and also the mercurial compounds themselves inhibit the inactivation reaction.

A spinlabeled derivative of p-chloromercuribenzoate reacts fairly specifically with Cys-152 in N-ethylmaleimide prelabeled enzyme. Modification of Cys-152 decreases drastically the affinity of the enzyme for the substrate p-hydroxybenzoate. The modified enzyme exhibits a somewhat higher affinity for NADPH than the native enzyme.

Modification by p-chloromercuribenzoate leads to absorption difference spectra showing pH-dependent maxima at 290 and 360 nm. The observed pKa value of about 7.6 is tentatively ascribed to at least one of the three tyrosine residues located in the substrate binding site.

From the three-dimensional structure of the enzyme-p-hydroxybenzoate complex it can be deduced that Cys-152 is far away from the active site. The modification results strongly indicate that the substrate binding site and Cys-152 are interdependent.

Both group-specific chemical modification studies and crystallization experiments have not (yet) led to the elucidation of the NADPH binding site of p-hydroxybenzoate hydroxylase from P.fluorescens. In **Chapter 6** the NADPH binding site has been probed using the affinity label p-(fluorosulfonylbenzoyl) adenosine.

The enzyme is slowly inactivated by the reagent in the presence of 20% dimethylsulfoxide. The inactivation, strongly inhibited by NADPH and 2',5' ADP, can be related to the modification of one amino acid residue.

Steady state kinetics and 2',5' ADP Sepharose affinity chromatography of modified enzyme suggest that the essential residue is not directly involved in NADPH binding.

From sequencing radioactive labeled peptides it is shown that Tyr-38 is the main residue protected from modification in the presence of NADPH.

The refined crystal structure of the enzyme-p-hydroxybenzoate complex at 0.19 nm resolution shows that Tyr-38 is far away from the active site. From model-building studies using computer graphics a potential mode of binding of both NADPH and 5'-(p-sulfonylbenzoyl)adenosine is presented.

Chemical modification studies (**Chapter 1.8** and **5**) have indicated the presence of an ionized tyrosine residue in the vicinity of the flavin prosthetic group of p-hydroxybenzoate hydroxylase from P.fluorescens.

In **Chapter 7** therefore, the pH-dependent spectral properties of free oxidized enzyme in the absence or presence of substrate (analogues) have been studied by various spectroscopic techniques.

The observed pH-dependent transitions are explained by (de)protonation of a tyrosine residue involved in binding of the hydroxyl group of the substrate. From the crystal structure it is deduced that Tyr-201 is the most likely candidate showing this low pKa value.

The exact mechanism of the FAD-dependent aromatic hydroxylation reaction is still unclear. The possible role of ionization of Tyr-201 during catalysis is discussed.

SAMENVATTING

Dit proefschrift beschrijft verschillende studies ter verkrijging van meer inzicht in de structuur-functie relatie van flavoproteïnen.

Het eerste deel van het proefschrift behandelt de toepassing van verschillende soorten affiniteitschromatografie voor het in handen krijgen van grote hoeveelheden aan apo flavoproteïnen.

In het tweede deel van het proefschrift wordt in verschillende studies de aandacht gevestigd op de biofysische eigenschappen van p-hydroxybenzoaat hydroxylase uit Pseudomonas fluorescens.

Een overzicht van de klassieke methoden voor de bereiding van apo flavoproteinen en een overzicht van de algemene eigenschappen van FAD-afhankelijke externe mono-oxygenasen wordt gegeven in **hoofdstuk 1**. Speciale aandacht wordt besteed aan de verschillende studies verricht aan p-hydroxybenzoaat hydroxylase uit P.fluorescens.

Klassieke methoden om het apo-eiwit te bereiden van p-hydroxybenzoaat hydroxylase uit P.fluorescens leveren relatief lage hoeveelheden eiwit op. Bovendien vertoont het relatief labiele apo-eiwit variaties in restactiviteit. In **hoofdstuk 2** wordt een nieuwe methode beschreven om dit probleem te verhelpen.

Grote hoeveelheden stabiel apo-eiwit, met een zeer lage restactiviteit, kunnen verkregen worden door gebruik te maken van DTNB-Sepharose covalente chromatografie.

Het apo-eiwit is een dimeer en bindt het substraat p-hydroxybenzoaat net zo goed als het holo-enzym. Het substraat heeft een beschermende invloed op de structuur van het apo-eiwit.

Het apo-eiwit vormt ook een complex met NADPH. De binding van het coenzym is sterk afhankelijk van de pH en ionsterkte.

Het is mogelijk om het holo-enzym zowel op de kolom als vrij in oplossing te reconstitueren. De opbrengst en specifieke activiteit van het gereconstitueerde enzym zijn bijzonder hoog. Kinetische experimenten geven aan dat het reconstitutie-proces zeer snel verloopt, waarbij FAD zeer stevig gebonden wordt.

In **hoofdstuk 3** wordt een meer algemeen toepasbare methode beschreven voor de bereiding van grote hoeveelheden stabiel apo-eiwit. De methode, waarbij gebruik wordt gemaakt van hydrofobe interactie chromatografie, is toegepast op twee verschillende klassen van flavoproteïnen.

Het holo-enzym van lipoamide dehydrogenase uit Azotobacter vinelandii kan zowel op de kolom als in oplossing gereconstitueerd worden. Het gereconstitueerde enzym is van zeer goede kwaliteit.

Het holo-enzym van butyryl-CoA dehydrogenase kan niet op de kolom gereconstitueerd worden. Het is echter voor de eerste keer mogelijk relatief stabiel apo-eiwit in handen te krijgen. De opbrengst aan apo butyryl-CoA dehydrogenase is meer dan 50%. De stevig gebonden coenzym A ligand wordt tijdens de apo-bereiding verwijderd.

Bij pH 7.0 is het apo-eiwit in evenwicht tussen dimere en tetramere vormen. Tijdens de reconstitutie reassocieert het eiwit tot een met holoenzym vergelijkbare tetramere structuur met goede specifieke activiteit.

Fluorescentie-polarisatie experimenten geven aan dat de pH-afhankelijke stabiliteit van het gereconstitueerde enzym sterk wordt beïnvloed door de aanwezigheid van coenzym A liganden. Niet geligandeerd gereconstitueerd enzym wordt gemakkelijk "groen" in aanwezigheid van een mengsel van coenzym A en natriumsulfide.

In **hoofdstuk 4** wordt de zuivering op grote schaal van p-hydroxybenzoaat hydroxylase uit P.fluorescens beschreven. Het zeer zuivere enzym kan met behulp van anionenwisselingschromatografie gescheiden worden in tenminste vijf fracties. Alle enzymvormen vertonen dezelfde specifieke activiteit en komen vnl. als dimeer voor in oplossing. De waargenomen microheterogeniteit van het enzym kan verklaard worden door de (gedeeltelijke) oxidatie van Cys-116.

De scheiding van de verschillende enzym-vormen heeft geleid tot de ontwikkeling van een kinetische chromatografische methode (FPLC) om het dissociatiegedrag van het enzym te beschrijven.

D.m.v. chemische modificatie studies zijn verschillende oxidatie-toestanden van Cys-116 aangetoond.

In **hoofdstuk 5** wordt de chemische modificatie beschreven van cysteïne residuen in p-hydroxybenzoaat hydroxylase uit P.fluorescens m.b.v. verschillende reagentia. D.m.v. differentiële labeling en aminozuurvolgorde bepaling van radio-actief gelabelde proteolytische eiwitfragmenten zijn de vershillende gemodificeerde cysteine residuen aangetoond.

Cys-116 reageert zeer snel en specifiek met N-ethylmaleimide zonder de enzymactiviteit te beïnvloeden.

Het enzym wordt gemakkelijk geïnactiveerd door kwikverbindingen. Met dithiothreitol is het mogelijk de enzymactiviteit snel te herstellen. p-Hydroxybenzoaat maar ook de kwikverbindingen zelf beschermen de inactiveringsreactie.

Een spinlabel-derivaat van p-chloromercuribenzoaat reageert tamelijk specifiek met Cys-152 als het enzym voorgelabeld is met N-ethylmaleimide. Onder dezelfde condities reageert p-chloromercuribenzoaat naast Cys-152 ook gedeeltelijk met Cys-158 en Cys-211. Modificatie van Cys-152 leidt tot een drastische verlaging van de affiniteit van het enzym voor p-hydroxybenzoaat. NADPH echter bindt goed aan het gemodificeerde enzym.
Modificatie met p-chloromercuribenzoaat leidt tot absorptie-verschilspectra met pH-afhankelijke maxima bij 290 en 360 nm. De waargenomen pKa waarde van 7.6 wordt voorlopig toegekend aan een van de tyrosine residuen in het actieve centrum.

Hoewel uit de kristalstructuur van het enzym-substraat complex blijkt dat Cys-152 ver verwijderd zit van het actieve centrum lijkt er toch een onderlinge afhankelijkheid te bestaan tussen de substraat bindingsplaats en Cys-152.

Groep-specifieke modificatie studies en kristallisatie-experimenten hebben (nog) niet geleid tot de opheldering van de NADPH bindingsplaats van p-hydroxybenzoaaat hydroxylase uit P.fluorescens. In **hoofdstuk 6** is gepoogd meer inzicht te verkrijgen in de NADPH binding d.m.v. affiniteitslabeling van het enzym met p-(fluorosulfonylbenzoyl)adenosine.

Het enzym wordt langzaam geinactiveerd door het reagens in aanwezigheid van 20% dimethylsulfoxide. De inactivering, sterk geremd door NADPH en 2',5' ADP, kan toegekend worden aan de modificatie van een enkel aminozuurresidu.

Enzymkinetiek en 2',5' ADP Sepharose affiniteitschromatografie van het gemodificeerde enzym geven aan dat de modificatie vnl. plaatsvindt buiten de NADPH-bindingsplaats.

Uit de aminozuurvolgorde bepaling van radio-actief gelabelde proteolytische eiwitfragmenten blijkt dat in aanwezigheid van NADPH vnl. de modificatie van Tyr-38 wordt geremd.

Uit de kristalstructuur gegevens blijkt dat Tyr-38 ver weg zit van het actieve centrum. M.b.v. modelbouw op een grafisch computersysteem wordt een mogelijke manier van binding van zowel NADPH als 5'-(p-sulfonylbenzoyl)adenosine voorgesteld.

Chemische modificatie studies (**hoofdstuk** 1.8 en 5) wijzen op de aanwezigheid van een negatief geladen tyrosine residu in het actieve centrum van p-hydroxybenzoaat hydroxylase uit P.fluorescens. In **hoofdstuk** 7 zijn daarom de pH-afhankelijke spectrale eigenschappen van het geoxideerde enzym in aan- en afwezigheid van substraat(analogen) bestudeerd m.b.v. verschillende spectroscopische technieken.

De pH-afhankelijke overgangen worden toegeschreven aan de (de)protonering van een tyrosine residu betrokken bij de binding van de hydroxyl groep van het substraat. Op basis van de positie in de kristalstructuur wordt afgeleid dat het hier waarschijnlijk om Tyr-201 gaat.

Het precieze mechanisme van de FAD-afhankelijke aromatische hydroxyleringsreactie is nog steeds onduidelijk. De mogelijke rol van de ionisatie van Tyr-201 tijdens de katalyse wordt besproken.

CURRICULUM VITAE

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