Chemical modification of p-hydroxybenzoate hydroxylase from
Pseudomonas fluorescens

Promotor: dr. F. Müller, hoogleraar in de Biochemie

Robert Adriaan Wijnands

Chemical modification of p-hydroxybenzoate hydroxylase from
Pseudomonas fluorescens

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op woensdag 28 november 1984
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

Voorwoord

Dit proefschrift had niet tot stand kunnen komen zonder hulp van een groot aantal mensen. Hoewel er vaak gezegd wordt dat deze mensen deze hulp wel moesten geven omdat dit nu eenmaal bij hun werk hoort wil ik ze toch allen bedanken voor de wijze waarop alles is gegaan.

Als eerste natuurlijk mijn promotor Franz Müller. Hij keek altijd een beetje bedenkelijk als ik op de vroege morgen weer eens met een hoop onzin op hem afkwam, maar hij heeft vast groen en geel van ergernis gezien als hij weer eens met oneindig veel rood door de door mij ingeleverde manuscripten ging. Het spijt me dat ook ik, net als mijn voorgangers, je weer van een rustige vakantie heb moeten afhouden.

Dan is daar natuurlijk de vaste ploeg van lab 6, Willem van Berkel en Willy van den Berg. Ook zij hebben het vast niet gemakkelijk gehad met mij. Steeds maar weer vroeg ik hoe het ook weer zat met die kolommen en waar die stofjes nou weer stonden. En dan spreek ik nog niet eens over al die keren dat ze mijn studenten moesten opvangen.

Ook de bijdrage van de andere mensen van de „groep Müller" moet niet onderschat worden. Chrit Moonen, Jacques Vervoort, Peter Bonants en Ben van Ommen,in de sfeer die mede door jullie gecreëerd werd voelde ik me altijd prima thuis. Chrit, jij wist mijn resultaten altijd zo mooi op te hemelen dat als ik jou moest geloven ik minstens de Nobel prijs verdien.

Johan van Leeuwen en Ton Visser wil ik bedanken voor hun bijdragen aan respectievelijk hoofdstuk 5 en 6 van dit proefschrift.

Bernard Klaassen, Jolanda van der Zee, Leon Frenken en op de valreep nog Bart Kleyn dank ik voor het onderzoek dat zij voor mij deden in het kader van hun studie.

Lyda Verstege en Jenny Toppenberg-Fang dank ik voor het typewerk. Steeds opnieuw moest zij de zaak afdraaien, wegens de eindeloze veranderingen in de manuscripten.

4

Stellingen

- 1 -

De methode voor het bepalen van de pK_a waarde van een tyrosine residue
in het actieve centrum van carboxypeptidase B, waarbij gebufferde op-
lossingen worden ingevroren m.b.v. vloeibare stikstof voor fosfores-
centie-metingen is onjuist.

Zisapel, N., Mallul, Y. and Sokolovsky, M. (1982) Int.J.Pept.
Protein.Res. *19*, 480-486.

- 2 -

Bij de chemische synthese van 2-amino-5-nitro-6-(1'-D-ribityl)amino-
pyrimidinon 5'-fosfaat waarbij gebruik wordt gemaakt van dimethyloxy-
tritylchloride voor de bescherming van de primaire alcohol groep en van
azijnzuuranhydride voor de bescherming van de secundaire alcohol groepen
kan het ontstaan van het 4'-fosfaat derivaat als bijprodukt ook worden
verklaard door acylmigratie na de detritylering.

Bacher, A. (1984) Z.Naturforsch. *39b*, 252-258.
Tsuda, Y. and Yoshimoto, K. (1981) Carbohydr.Res. *87*, C1-C4.

- 3 -

Het voorkomen van een 'charge transfer' band in het absorptiespectrum
van een flavoproteïne hoeft niet te betekenen dat er in de grondtoestand
sprake is van een charge transfer complex.

Williams, C.H. (1976) in The Enzymes (Boyer, ed.) 3rd ed., *13*,
pp. 89-172, New York, Academic Press.

- 4 -

De pK_a waarde toegeschreven aan een bij de NADPH binding betrokken amino-
zuur residue van p-hydroxybenzoaat hydroxylase uit *P.desmolytica* kan ook
worden toegeschreven aan de buffer waarin is gewerkt.

Shoun, H. Higashi, N., Beppu, T., Nakamura, S., Hiromi, K. and Arima,
K. (1979) J.Biol.Chem. *254*, 10944-10951.

- 5 -

Uit het feit dat bij chemische modificatie studies het aantal essentiële
residuen nog steeds vaak bepaald wordt met de zg. extrapolatie methode
blijkt dat veel biochemici bang zijn voor het gebruik van een paar ver-
gelijkingen.

Horiike, K., Tojo, H., Yamano, T. and Nozaki, M. (1983)
    J.Biochem. *95*, 605-609.

- 6 -

De reactiviteiten van de in membranen opgenomen fotoreactieve glycoli-
piden 2-(4-azido-2-nitrofenoxy)palmitoylglucosamine en 12-(4-azido-2-
nitrofenoxy)stearoylglucosamine met fosfatidylcholine transporterend
eiwit wijzen erop dat de onderscheiding in respectievelijk een grensvlak
en een diepte sonde hier niet terecht is.

    Berkhout, T.A. (1984) Proefschrift, Universiteit Utrecht.

- 7 -

Bij diefstal van een auto met een buitenlands kenteken kan de gedupeerde
zich een hoop ellende besparen door pas de politie in te lichten nadat
de dief ruim de gelegenheid heeft gehad de auto ergens rustig te
parkeren.

- 8 -

De manier waarop de meeste Nederlanders het woord 'hovercraft' uit-
spreken doet meer denken dat het gaat om een varende stofzuiger dan
om een luchtkussenvoertuig.

Robert Wijnands
Chemical modification of p-hydroxybenzoate hydroxylase from *Pseudomonas
fluorescens*.
Wageningen, 28 november 1984

Chapters 2 and 5 have been published separately.
Chapters 3 and 4 will be published separately.

Chapter 2 R.A. Wijnands and F. Müller (1982) Biochemistry 21,
           6639-6646.
Chapter 3 R.A. Wijnands and F. Müller, submitted to Eur.J.Biochem.
Chapter 4 R.A. Wijnands and F. Müller, submitted to Eur.J.Biochem.
Chapter 5 R.A. Wijnands, J. van der Zee, J.W. van Leeuwen, W.J.H.
           van Berkel and F. Müller (1984) Eur.J.Biochem. 139, 637-644.

6

Contents

Abbreviations

Mes	4-morpholineethanesulfonic acid
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hepps	4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
2',5'-ADP	adenosine 2',5'-bisphosphate
FAD	flavin adenine dinucleotide
NADH	nicotinamide-adenine dinucleotide (reduced)
NADP$^+$	nicotinamide-adenine dinucleotide 2'-phosphate (oxidized)
NADPH	nicotinamide-adenine dinucleotide 2'-phosphate (reduced)
t-NADPH	1,4,5,6-tetrahydronicotinamide-adenine dinucleotide 2'-phosphate
pFB	p-fluorobenzoate
pOHB	p-hydroxybenzoate
FPLC	fast protein liquid chromatography
HPLC	high performance liquid chromatography
NMR	nuclear magnetic resonance

Chapter 1   General introduction

p-HYDROXYBENZOATE HYDROXYLASE

p-Hydroxybenzoate hydroxylase is an external monooxygenase and has been obtained in crystalline form from four different species of *Pseudomonas*, *i.e. P.desmolytica* [1], *P.putida* A3.12 [2], *P.putida* M-6 [3] and *P.fluorescens* [4]. The enzymes from these sources show strikingly similar properties, but the hydroxylase from *P.fluorescens* is the most stable one. It can be kept for months as an ammonium sulphate precipitate, even in the absence of stabilizing agents, greatly facilitating experimental work.

Members of the genus *Pseudomonas* are known to metabolize aromatic compounds [5]. A large number of aromatic compounds is converted into either 3,4-dihydroxybenzoate (protocatechuate) or 1,2-dihydroxybenzene (catechol), which are degraded to succinate and acetyl-CoA *via* the β-ketoadipate pathway or to pyruvate and ethanol via the α-keto-acid pathway. In the β-ketoadipate pathway ring opening occurs between the two hydroxyl groups, whereas in the α-ketoacid pathway it occurs adjacent to the two hydroxyl groups.

p-Hydroxybenzoate hydroxylase catalyzes the hydroxylation of 4-hydroxybenzoate to 3,4-dihydrobenzoate using NADPH as an electron donor [2]. The electrons are first donated to FAD, which is tightly bound to the enzyme, the dissociation constant being 45 nM [6]. The rate of reduction of the enzyme-bound flavin is very much dependent on the presence of substrate. This will be discussed further on. In the presence of substrate, the electrons are passed from FAD to molecular oxygen of which one atom is incorporated into the substrate whereas the other is reduced to water. In the absence of substrate the product formed is H_2O_2.

p-Hydroxybenzoate hydroxylase from *P.fluorescens* has been shown to appear in several oligomeric forms [7], but it exists mainly as a

dimer. The dimeric enzyme has been studied in our laboratory using modern fluorescence techniques and it was shown that it is in equilibrium with the monomeric form with a dissociation contant of 1 μM [8]. The monomer has a molecular weight of approximately 44000 [7] and contains one molecule of FAD [4]. The FAD can easily be removed by binding the holoenzyme covalently to a Sepharose-5,5'-dithiobis(2-nitrobenzoate) column *via* a reactive sulfhydryl group and subsequent treatment with a high concentration of KBr and urea at neutral pH [6].

The crystal structure was also shown to contain dimers [9]. The enzyme was crystallized in a complex with its substrate and the structure was determined at a resolution of 0.25 nm. The amino acid sequence is also completely known [10] and the combination of primary, secondary and tertiary structures has given some very interesting results [11]. The parts of the polypeptide chain and the amino acids involved in the FAD and substrate binding sites have been identified, but the NADPH binding site is still unknown.

The substrate p-hydroxybenzoate is buried in the interior of the enzyme in an environment which is essentially hydrophobic, apart from some hydrophilic interactions at the carboxyl group and the hydroxyl group of the substrate. The carboxyl group is believed to form a salt bridge with Arg-214 and hydrogen bonds to the hydroxyl groups of Ser-212 and Tyr-222. The hydroxyl group of the substrate is believed to form a hydrophilic cluster with hydroxyl groups of Tyr-201 and Tyr-385. From kinetic and circular dichroism measurements it was found that the carboxyl moiety is necessary and sufficient for the enzyme-substrate binding, whereas the hydroxyl groups alone will not lead to binding [12]. The binding pocket is accessible *via* a deep cleft but it is shielded by Arg-214 when the substrate is bound. The three dimensional structure of the free enzyme is still not known, but a change of position of Arg-214 and a conformational change opening the cleft seem to be necessary for the substrate to bind and

the product to be released. A conformational change has indeed been observed by circular dichroism measurements in the UV region [3]. In the absence of substrate there is no countercharge for the positive charge of Arg-214. Moonen has proposed that this is the driving force for the conformational change (personal communication).

The FAD site has had special attention in an article where it was compared with the FAD and NADPH binding sites of glutathione reductase [13]. Although the amino acid sequences are rather different, a close resemblance between the three-dimensional structures of these three domains was found. The most striking correspondence was an α-helix pointing to the N(1)-O(2α) region of the isoalloxazine. The dipole field of this helix could influence the charge distribution in the ring or stabilize a negative charge at this position. ^{13}C-NMR studies in our laboratory have indeed shown that in the reduced state the enzyme-bound flavin is in the anionic form and that in the oxidized state substrate binding leads to an increased π electron density at C(2) [14]. This means that in the absence of substrate, this α-helix should have a different position in the oxidized state. Moonen has suggested that the conformation of the active site is the same in the oxidized ES complex, the free reduced enzyme and the reduced ES complex, but differs in conformation and mobility in free oxidized enzyme.

The reaction cycle of p-hydroxybenzoate hydroxylase can be divided into a reductive phase and an oxidative phase, which can be studied separately. Both phases have been studied extensively using rapid reaction techniques [15,16]. Before reduction of the enzyme-bound flavin takes place p-hydroxybenzoate and NADPH form a ternary complex with the enzyme. This can occur *via* the enzyme-substrate binary complex or *via* the enzyme-NADPH binary complex. Formation of these two binary complexes is accompanied by changes in absorbance, fluorescence and circular dichroism properties. For p-hydroxybenzoate from *P.des-molytica* it was shown that formation of both binary complexes consists

11

of two steps [17,18]: rapid bimolecular formation of a complex and its isomerization into a more tightly bound complex.

Anaerobic reduction of the free enzyme by NADPH was shown to be a slow monophasic process following first order kinetics [15,18]. No spectral intermediates with long wavelength absorbance were observed. In the presence of substrate however, the rate of reduction of the enzyme may be increased by a factor of 10^5 [19]. This increase, although less rigorous, is also found in the presence of other effectors which are not necessarily substrates [20]. The effector role of the substrate is an important property needed to avoid wasteful utilization of NADPH. Spectral intermediates absorbing in the 525 nm to 800 nm region indicated the existence of charge transfer complexes involving the oxidized FAD and the reduced pyridine nucleotide in one case and reduced FAD and oxidized pyridine nucleotide in another [4,18]. The last step of the reductive phase is the release of the oxidized pyridine nucleotide.

From kinetic studies on the oxidative phase using 2,4-dihydroxy-benzoate as a substrate it was concluded that at least three intermediates are involved. The first intermediate is believed to be a C(4a)-hydroperoxyflavin [21,22]. It is formed in a second order reaction with oxygen. The formation of the intermediate is independent of the subsequent hydroxylation reaction, since it is observed both in the presence and absence of a substrate or effector. It has been postulated that the reduced flavin transfers an electron to the oxygen to form a radical pair, which after spin inversion collapses to the hydroperoxyflavin [23].

In the absence of a substrate that can be hydroxylated, C(4a)-hydroperoxyflavin decays to oxidized flavin and H_2O_2, but in the presence of such a substrate a second intermediate is formed following the electrophilic attack of oxygen on the aromatic substrate. The structure of the second intermediate is still speculative. Experiments by Wessiak are in favour of an earlier proposed ring-opening between C(4a) and N(5) [24,25], but an analogue (model compound) with two

methyl groups at N(5) gave a completely different spectrum when compared with the second intermediate [26].

The third intermediate is thought to be a C(4a)-hydroxyflavin, because of the similarity of its spectrum with the first intermediate [21]. With the decay of this last intermediate the enzyme returns to its oxidized form, with the release of water and finally followed by the release of the product. It must be mentioned however, that one should be very cautious in ascribing structures to enzyme-bound intermediates solely on the basis of light absorbance data as has been done for these intermediates [26]. Other mechanisms involving a flavin-N(5)-nitroxyl radical [27] or C(4), C(4a)-dioxetane flavin derivative and an ester-like bond between C(4) and the substrate [28] have also been proposed.

The role of the protein surrounding of the flavin in the catalytic process is not yet clear. The effect of a dipole field of an α-helix pointing towards a certain region of the flavin has already been mentioned above. Modulation of the electronic structure of the flavin should play an important role in determining the type of reaction catalyzed by the flavin. Another important function of the protein is of course a role in catalysis itself. An ionizing residue in the vicinity of the N(5) of the flavin is required by the mechanism described above. This residue is probably a tyrosine and will be an important subject in chapter 4 of this thesis. Other amino residues may be involved in the binding of NADPH and the substrate. The main part of this thesis is dedicated to the amino acids involved in ligand binding. Chemical modification of these residues was performed to identify them. The roles of histidine, arginine and tyrosine residues are described in chapters 2, 3 and 4 respectively. Besides the separate amino acids, the protein as a whole must also be considered. This is done in chapter 5 where the dipole moment of the enzyme is calculated and used to explain NADPH and NADPH-analogue binding.

CHEMICAL MODIFICATION OF PROTEINS

Chemical modification is a widely used technique in structure-function relationship studies of enzymes [29-34]. It is used to identify the amino acid side chains involved in the activity of the enzyme. This can be achieved either by group specific or site specific modification. Most of the group specific modification reactions can be categorized into the following classes:
1. acylation
2. alkylation
3. ester- and amide-formation
4. reduction and oxidation
5. aromatic ring substitution.

Group specific reagents may be specific for one particular amino acid side chain [5,5'-dithiobis(2-nitrobenzoic acid) and p-chloromercuri-benzoic acid react specifically with cysteine residues], but in most cases side reactions may occur. No discussion of the results of chemical modification is therefore complete without attention to these possible side reactions. Detailed information on the applicability, specificity, reaction mechanism etc. of many reagents can be found in the literature cited above.

The conditions under which chemical modification is performed are very important. To prevent denaturation of the enzyme, much milder conditions are necessary than mostly used in general organic chemistry. The most important factor affecting reactions with amino acid residues in proteins is the pH as it controls the distribution of potentially reactive side chains between reactive and unreactive ionization states. A good example is discussed in chapter 4, where specific modification of some tyrosine residues is achieved by working at a low pH.

Initially, differences in the reactivities of amino acid side chains of the same type were interpreted in terms of the degree of

exposure of these groups. Later however, it became clear that other
features of the environment like polarity and charge of neighbouring
side chains are also of great importance for the reactivity. This
aspect too, will be discussed in chapter 4.

Site selective reagents can be catagorized into the following
classes:
1. substrate together with a chemical reagent to stabilize the labile
   and/or transistory bonds between the protein and its ligand
2. pseudosubstrate, giving a product that is a poor leaving group
3. classical affinity label
4. photoaffinity label
5. suicide label.
In classical affinity labelling the specificity of the modification
is determined by preferential binding of a reactive reagent to the
active site. The modification itself often involves an alkylation.
In the case of photoaffinity and suicide labelling, the reactive
groups are generated by exposure to light and by catalytic activity
of the enzyme itself, respectively. Photoaffinity labelling often in-
volves highly reactive nitrenes and carbenes, whereas suicide label-
ling often involves an allene. More information on site selective
modification can be found in [32,35,36].

A disadvantage of group specific modification as opposed to site
specific modification is the (average) number of residues that can be
modified. When inactivation indeed occurs upon chemical modification
of several or many amino acid residues of the same type the problem
encountered is the quantitive determination of the essential groups.
The most naive, but surprisingly also the most widely used method is
the so-called extrapolation method. In a plot of the remaining acti-
vity, a, against the number of residues modified, m, the linear part
of the plot is extrapolated to the m-axis and the intercept is then
referred to as the number of essential residues. It has been empha-
sized however that on the ground of the equations given by Tsou [37]

the extrapolation method is inapplicable in all but a few cases [38].

Another method has been introduced by Levy [39], but has been misinterpreted by many authors [40-44]. A logarithmic plot of the apparent first order rate constant of inactivation against the concentration of the reagent is used to determine the minimal order of this reaction from the slope. Although it has been noted that this method can only be applied to cases obeying simple n-order kinetics [44], it has been overlooked in all cases that the order of the reaction of inactivation cannot simply be correlated to the number of essential residues that are modified. If, for instance, modification of either one of two essential groups results in the complete loss of activity, inactivation will still obey first-order kinetics with respect to the concentration of reagent. On the other hand, a second-order reaction with respect to the concentration of reagent does not mean that both modified groups are essential.

Another kinetic method for the determination of the number of essential residues has been given by Ray and Koshland [45]. This method involves several theoretical models correlating pseudo-first order rate constants for the loss of enzyme activity and for modification of each reacting amino acid residue. These models can best be explained using a theoretical enzyme with two reactive dues. In the first model modification of either one of these two residues leads to inactivation. In the third model a second residue is susceptible to attack only after the first, unessential residue has been modified and in the fourth model modification of either one of the residues leads to partial inactivation. This last model has successfully been applied to the modification of histidines with diethylpyrocarbonate in chapter 2.

A graphical method for the determination of the number and type of essential groups has been described by Tsou [37]. It is a statistical method and does not explicitly contain a time factor like the method of Ray and Koshland. If the first-order rate constant for the

16

modification of p residues of which i are essential is k and for n-p non-essential residues is αk, then

$$\log\left(\frac{n-m}{a^{1/i}} - p\right) = \log (n-p) + \frac{\alpha-1}{i} \log a$$

where α is the residual activity, n the total number of reactive residues and m the number of residues modified. The best straight line in a suitable log plot will give the values for p, i and α. This has been done for the modification of tyrosines in chapter 4. Special cases where $\alpha \gg 1$, $\alpha = 1$ or $\alpha \ll 1$ have been considered [37,38]. One of these, in a slightly modified form has been applied to the modification of histidines in chapter 2.

Both methods given above have their advantages and their limitations [37,45,46]. The method of Ray and Koshland for instance, can only be applied in the case of strict pseudo-first order kinetics, where the kinetic data must be determined very accurately. When more than one essential group is involved, the total number of combinations compatible with a given set of experimental results can be very large. The same can be said for the number of combinations of p, i and α in the method of Tsou when n is large. Both methods require great accuracy for activity measurements. A small error in α will be greatly exaggerated when i>1. It is therefore advisable to apply both methods whenever possible.

A very useful method in cases where the essential groups are protected from the modifying reagent by ligands is the differential labelling technique. The enzyme is first modified in the presence of ligands and after removal of the ligands and excess reagent the enzyme is further modified with radioactive reagent. In favourable cases only the residues which were initially protected by ligands become isotopically labelled and can be determined. This method is especially useful when identification of the residue is wanted and too many other residues are also modified.

Besides group and site specific modification using chemical reagents there is another kind of modification which has become very popular, *i.e.* replacement modification. The best way to replace one amino acid by another nowadays, is not by chemical modification of the protein but by site directed mutagenesis at DNA level. This technique will become of great importance in structure-function relationship studies in the near future as absolute selectivity for one specific residue is possible. Chemical modification of amino acid residues however, will still be very useful in preliminary studies to determine which amino acids must be considered for replacement.

REFERENCES

[1] Yano, K., Higashi, N. & Arima, K. (1969) Biochem.Biophys.Res. Comm. *34*, 1-7.

[2] Hosokawa, K. & Stanier, R.Y. (1966) J.Biol.Chem. *241*, 2453-2460.

[3] Hesp, B., Calvin, M. & Hosokawa (1969) J.Biol.Chem. *244*, 5644-5655.

[4] Howell, L.G., Spector, T. & Massey, V. (1972) J.Biol.Chem. *247*, 4340-4350.

[5] Stanier, R.Y. & Ornston, L.N. (1973) in "Advances in Microbial Physiology" (Rose, A.H. and Tempes, D.W., eds.) pp. 89-151, Academic Press, London.

[6] Müller, F. & Van Berkel, W.J.H. (1982) Eur.J.Biochem. *128*, 21-27.

[7] Müller, F., Voordouw, G., Van Berkel, W.J.H., Steenis, P.J., Visser, S. & Van Rooijen, P.J. (1979) Eur.J.Biochem. *101*, 235-244.

[8] Visser, A.J.W.G., Penners, G., Van Berkel, W.J.H. & Müller, F. (1984) Eur.J.Biochem., in press.

[9] Wierenga, R.K., De Jong, R.J., Kalk, K.H., Hol, W.G.J. & Drenth, J. (1979) J.Mol.Biol. *131*, 55-73.

[10] Hofsteenge, J., Weijer, W.J., Jekel, P.A. & Beintema, J.J. (1983) Eur.J.Biochem. *133*, 91-108.

[11] Weijer, W.J., Hofsteenge, J., Beintema, J.J., Wierenga, R.K. & Drenth, J. (1983) Eur.J.Biochem. *133*, 109-118.

[12] Teng, N., Kotowycz, G., Calvin, M. & Hosokawa, K. (1971) J.Biol. Chem. *246*, 5448-5453.

[13] Wierenga, R.K., Drenth, J. & Schulz, G.E. (1983) J.Mol.Biol. *167*, 725-739.

[14] Moonen, C.T.W. (1983) Thesis, Agricultural University, Wageningen.

[15] Husain, M. & Massey, V. (1979) J.Biol.Chem. *254*, 6657-6666.

[16] Hiromi, K. (1979) Kinetics of Fast Enzyme Reactions Theory and Practice pp. 287-320, Halsted Press Book.

[17] Higashi, N., Shoun, H., Yano, K., Arima, K. & Hiromi, K. (1972) Z.Naturforsch. *27b*. 1172-1175.

[18] Shoun, H., Higashi, N., Beppu, T., Nakamura, S., Hiromi, K. & Arima, K. (1979) J.Biol.Chem. *254*, 10944-10951.

[19] Nakamura, S., Ogura, Y., Yano, K. Higashi, N. & Arima, K. (1970) Biochemistry *9*,3235-3242.

[20] Spector, T. & Massey, V. (1972) J.Biol.Chem. *247*, 4679-4687.

[21] Entsch, B., Ballou, D.P. & Massey, V. (1976) J.Biol.Chem. *251*, 2550-2563.

[22] Ghisla, S., Entsch, B., Massey, V. & Husain, M. (1977) Eur.J. Biochem. *76*, 139-148.

[23] Kemal, C., Chan, T.W. & Bruice, T.C. (1977) J.Am.Chem.Soc. *99*, 7272-7286.

[24] Wessiak, A., Schopfer, L.M. & Massey, V. (1984) in "Flavins and Flavoproteins" (Bray, R.C., Engel, P.C. & Mayhew, S.G., eds.) Walter de Gruyter & Co., Berlin.

[25] Hamilton, G.A. (1971) in "Progress in Bioorganic Chemistry" (Kaiser, E.T. and Kezdy, F.J., eds.) Vol. 1, p. 83, Wiley, New York.

[26] Wessiak, A. & Bruice, T.C. (1981) J.Am.Chem.Soc. *103*, 6996-6998.

[27] Frost, J.W. & Rastetter, W.H. (1981) J.Am.Chem.Soc. *103*, 5242-5245.

[28] Visser, C.M. (1983) Eur.J.Biochem. *135*, 543-548.

[29] Means, G.E. & Feeney, R.E. (1971) "Chemical Modification of Proteins", Holden-Day, Inc., San Francisco.

19

[30] Hirs, C.H.W. & Timasheff, S.N., eds. (1972) Methods Enzymol. *25*.

[31] Hirs, C.H.W. & Timasheff, S.N., eds. (1977) Methods Enzymol. *47*.

[32] Glazer, A.N., DeLange, R.J. & Sigman, D.S. (1975) "Chemical Modification of Proteins; Selected Methods and Analytical Procedures" Elsevier/North-Holland, Amsterdam.

[33] Freedman, R.B. (1978) "Determination of Essential Residues in Enzymes by Chemical Modification" Elsevier/North-Holland, Amsterdam.

[34] Feeney, R.E., Yamasaki, R.B. & Geoghegan, K.F. (1982) Adv.Chem. Ser. *198*, no. Modification of Proteins, 3-55.

[35] Benisek, W.F., Ogez, J.R. & Smith, S.B. (1982) Adv.Chem.Ser. *198*, no. Modification of Proteins, 267-323.

[36] Jakoby, W.B. & Wilchek, M., eds. (1977) Methods Enzymol. *46*.

[37] Tsou, C.L. (1962) Sci.Sin. (Engl. Ed.) *11*, 1535-1558.

[38] Horiike, K. & McCormick, D.B. (1979) J.Theor.Biol. *79*, 403-414.

[39] Levy, H.M., Leber, P.D. & Ryan, E.M. (1963) J.Biol.Chem. *238*, 3654-3659.

[40] Takuya, N., Toraya, T. & Fukui, S. (1981) Eur.J.Biochem. *119*, 273-277.

[41] Wu, L.N.Y. & Fisher, R.R. (1982) Biochim.Biophys. Acta *681*, 388-396.

[42] Kuno, S., Toraya, T. & Fukui, S. (1981) Arch.Biochem.Biophys. *211*, 722-730.

[43] Narayanan, C.S. & Krakow, J.S. (1982) Biochemistry *21*, 6103-6111.

[44] Jabalquinto, A.M., Eyzaguirre, J. & Cardemil, E. (1983) Arch. Biochem.Biophys. *225*, 338-343.

[45] Ray, W.J. & Koshland, D.E. (1961) J.Biol.Chem. *236*, 1973-1979.

[46] Rakitzis, E.T. (1984) Biochem.J. *217*, 341-351.

20

Chapter 2   Chemical modification of histidine residues

Robert A. Wijnands and Franz Müller*

ABSTRACT: The flavoprotein *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is inactivated by diethyl pyrocarbonate. Below pH 7, diethyl pyrocarbonate reacts specifically with histidine residues. The inactivation reaction is biphasic and follows pseudo-first-order kinetics. Four of the nine histidine residues of the enzyme are modified. During the first phase of the reaction, one histidine residue is modified and leads to a loss of about 30% of the activity. Modification of the additional three histidine residues during the second phase leads to complete loss of activity. Two of the latter histidine residues are essential for activity and are involved in the binding of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The activity can be restored almost quantitatively upon treatment of modified enzyme with hydroxylamine. The modified enzyme is still capable of binding NADPH. The dissociation constant of the enzyme–NADPH complex is larger by a factor of 10 for the modified enzyme as compared to that for the native enzyme. The modification does not affect the affinity of the enzyme for the substrate, although effectors protect two histidine residues from chemical modification by diethyl pyrocarbonate. The rate of inactivation of the enzyme is pH dependent and increases with increasing pH values. From the pH dependence of the rate constant, it is calculated that two cooperative histidine residues participate in the reaction with diethyl pyrocarbonate. Both histidine residues possess a pK_a' value of 6.2. At pH >7, other reactions take place which are completely abolished in the presence of an effector (substrate) of the enzyme.

$\mathbf{T}$he inducible enzyme *p*-hydroxybenzoate hydroxylase (EC 1.14.13.2) belongs to the class of external flavoprotein monooxygenases. It can be obtained from four different species of *Pseudomonas*, but the enzyme present in *Pseudomonas fluorescens* is the most stable one. It catalyzes the following reaction:
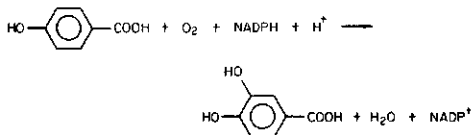
Significant progress has been made in recent years concerning the structure and catalytic mechanism of *p*-hydroxybenzoate hydroxylase (Shoun et al., 1979a; Müller et al., 1979; Husain & Massey, 1979). We have devoted ourselves to the study of the chemical modification of amino acid residues important for the catalytic activity of the enzyme. This is now a promising approach since the entire sequence of the enzyme is known (Hofsteenge et al., 1980; Vereijken et al., 1980; Weijer et al., 1982). In addition, the existing three-dimensional model of the enzyme–substrate complex at a resolution of 0.25 nm (Wierenga et al., 1979) allows a more detailed interpre-

tation of the data of chemical modification. Furthermore, modification studies will yield results important for understanding the catalytic mechanism of the enzyme.

The work that has been done in the field of chemical modification so far includes arginine and histidine modification of the enzyme from *Pseudomonas desmolytica* (Shoun et al., 1979b, 1980) and cysteine modification of the enzyme from *P. fluorescens* (F. Müller and W. J. H. Van Berkel, unpublished experiments). Histidine modification by Shoun and his colleagues (Shoun et al., 1979b) was carried out by photooxidation at pH 6, and their conclusion was that a histidine residue with a pK_a' of 7.0 is involved in substrate binding by forming a hydrogen bridge with the phenolic OH of *p*-hydroxybenzoate. However, from the low-resolution three-dimensional model of the enzyme, it can be concluded that histidine is not involved directly in the binding of the substrate, at least not as far as the enzyme from *P. fluorescens* is concerned. This apparent discrepancy could be related to the fact that photooxidation reactions are not very specific, as tyrosine, methionine, and tryptophan can also be modified by photooxidation (Westhead, 1972).

The aim of this paper is to elucidate the role of histidine residues in *p*-hydroxybenzoate hydroxylase from *P. fluorescens*. Our results clearly indicate that histidine residues are not involved in the binding of the substrate *p*-hydroxybenzoate, but rather in the binding of the pyridine nucleotide.

Materials and Methods

Diethyl pyrocarbonate was from Fluka, L-histidine monochloride was from the British Drug Houses Ltd., reduced nicotinamide adenine dinucleotide phosphate (NADPH)[1] was

21

from Boehringer, Mes, Hepes, and Tris were from Sigma, and all other chemicals were from Merck.

Spectrophotometric measurements were done on a Zeiss PMQII, a Cary 16, or an Aminco DW2A spectrophotometer, and fluorometric measurements were performed on an Aminco SPF-500 spectrofluorometer.

p-Hydroxybenzoate hydroxylase was purified from *P. fluorescens* as described previously (Müller et al., 1979). The purified enzyme was kept as an ammonium sulfate precipitate (70%) at 4 °C, pH 6–7. Prior to use, a desired amount of the precipitate was dialyzed against 20 mM Mes buffer, pH 6, ionic strength 20 mM, unless stated otherwise. The enzyme concentration was determined spectrophotometrically on the basis of the FAD content by assuming a molar absorption coefficient of 11 300 M^{-1} cm^{-1} at 450 nm (Müller et al., 1979).

The enzyme activity was assayed by adding 4 μL of a 20 μM enzyme solution to 1 mL of 0.1 M Tris–H_2SO_4, pH 8.0. The buffer solution contained 0.15 mM *p*-hydroxybenzoate and 0.15 mM NADPH. The activity was determined by recording the decrease of absorbance of NADPH at 340 nm vs. time. The temperature was 25 °C.

Ethoxyformylation of the enzyme was achieved by treating a solution of 20 μM *p*-hydroxybenzoate hydroxylase with 0.2–0.8 mM diethyl pyrocarbonate at 4 or 25 °C. The diethyl pyrocarbonate was added as an ethanol solution (15–25 mM). This stock solution was kept at −20 °C and before use checked for its diethyl pyrocarbonate content by adding 10 μL to 1 mL of a 10 mM histidine solution, pH 6.0. A molar difference absorption coefficient of 3200 M^{-1} cm^{-1} at 240 nm for ethoxyformylhistidine was used to calculate the concentration of diethyl pyrocarbonate (Ovádi et al., 1967). The reaction of diethyl pyrocarbonate with the enzyme was stopped at desired times either by addition of a solution of 10 mM imidazole to the reaction mixture or by dilution (100–500-fold) of an aliquot of the reaction mixture into the assay mixture.

The pH dependence studies of inactivation were conducted similarly with the pH values adjusted from 5.5 to 8.5 and at a constant ionic strength of 20 mM. The pH did not change during the course of the experiment, and the enzyme lost negligible activity on standing in the absence of inactivator under the conditions used. In the pH range from 5.5 to 7.0, Mes was used as a buffer and Hepes in the range from pH 7.0 to 8.5.

De-ethoxyformylation (Melchior & Farney, 1970) of modified enzyme was achieved by adding an equal volume of 200 mM hydroxylamine in 20 mM Mes or Hepes to a solution of inactivated enzyme at a given pH and following the return of activity with time in an assay mixture at pH 8, to which an aliquot of the reaction mixture had been added. A similar procedure was carried out with an unmodified enzyme solution as a control.

The extent of ethoxyformylation of histidine residues of the enzyme was determined by plotting difference absorption spectra of the enzyme at different times after the addition of diethyl pyrocarbonate to one cuvette and the same volume of ethanol to the other one. Peak values at 244 nm ($\Delta\epsilon = 3600$ M^{-1} cm^{-1}) were used for kinetic calculations. At the same time intervals, 4-μL samples were withdrawn from the reaction

mixture for the determination of the residual activity in order to relate the chemical modification reaction to that of the inactivation of the enzyme. The $\Delta\epsilon$ of 3600 M^{-1} cm^{-1}, at 244 nm, was determined at pH 6 by measuring the absorbance difference produced by solutions containing 20 μM histidine and multiples thereof in the presence of 0.8 mM diethyl pyrocarbonate. This $\Delta\epsilon$ value is larger than that usually used (Ovádi et al., 1967) owing to the fact that, as pointed out by Roosemont (1978), the $\Delta\epsilon$ value of ethoxyformylated histidine depends on whether an excess of diethyl pyrocarbonate or an excess of histidine is used in the determination. Therefore, in the modification reaction of the enzyme, an excess of diethyl pyrocarbonate was always used. When inactivation had to be related to chemical modification of the enzyme–*p*-fluorobenzoate complex, this could only be done indirectly. Diethyl pyrocarbonate (0.91 mM) was added to a solution of 33.9 μM *p*-hydroxybenzoate hydroxylase in the presence of 15.4 mM *p*-fluorobenzoate at a given pH and temperature. At given time intervals, 1-mL samples were withdrawn, added to 10 μL of 1 M imidazole, and purified by gel chromatography. After determination of the enzyme concentration and activity, the residual amount of histidines that could still be carbethoxylated in each sample was determined as described above.

Dissociation constants of different enzyme complexes were determined fluorometrically or spectrophotometrically by titration experiments. Quenching of the protein-bound FAD fluorescence emission at 525 nm (excitation at 450 nm) was monitored as a function of the concentration of NADPH or *p*-hydroxybenzoate added. For the enzyme–*p*-fluorobenzoate complex, absorption differences at 500 or 384 nm were monitored. From Benesi–Hildebrand plots (Benesi & Hildebrand, 1949), the dissociation constants of the complexes of native and modified enzyme were calculated.

The determination of the concentration of diethyl pyrocarbonate in reactions with free NADPH was carried out by the method described by Berger (1975); i.e., aliquots (50 μL) of the incubation mixture were added to 350-μL solutions of thionitrobenzoate, and the change of absorbance at 412 nm was recorded. The decrease of absorbance at 412 nm is proportional to the diethyl pyrocarbonate consumed in the reaction mixture.

The reaction of diethyl pyrocarbonate with *p*-hydroxybenzoate at a given pH value was followed by measuring the decrease of absorbance of *p*-hydroxybenzoate at 245 nm. Gel chromatography was performed by using Bio-Gel P-6DG (Bio-Rad) packed in a column (1 × 9 cm).

Results and Discussion

When ethoxyformylation of free *p*-hydroxybenzoate hydroxylase is carried out at pH 5.8 and 4 °C, the enzyme rapidly becomes inactivated (Figure 1). The inactivation reaction follows pseudo-first-order kinetics when an excess of diethyl pyrocarbonate is used, but clearly two or more reactions are involved. Extrapolation of the curves of the slower reaction to zero time yields an intercept at about 0.7 on the ordinate (Figure 1). From a secondary plot of the pseudo-first-order rate constants against the diethyl pyrocarbonate concentration, second-order rate constants of 500 and 245 M^{-1} min^{-1}, respectively, are obtained at pH 5.8 and 4 °C (Figure 1, insert).

The two-phase reaction was not observed at 20 °C. At this temperature, only the second reaction could be observed because the first reaction was too fast to be followed by conventional methods. At 20 °C, the slow reaction exhibits a second-order rate constant of 1000 M^{-1} min^{-1} at pH 6.1.

Both the fast and the slow reactions are slowed down when NADPH or 2′,5′-ADP is included in the reaction mixture
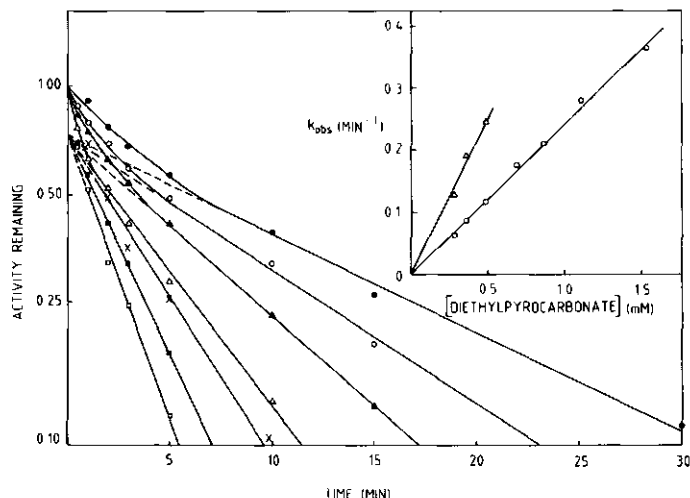

22

FIGURE 1: Semilogarithmic plot of the time-dependent inactivation of p-hydroxybenzoate hydroxylase as a function of the diethyl pyrocarbonate concentration. Each incubation mixture contained 20 μM p-hydroxybenzoate hydroxylase in 20 mM Mes, pH 5.8, and various concentrations of diethyl pyrocarbonate: 0.42 mM ($\bullet$); 0.50 mM (O); 0.63 mM ($\blacktriangle$); 0.83 mM ($\triangle$); 1.00 mM ($\times$); 1.25 mM ($\blacksquare$); 1.67 mM ($\square$). The reactions were conducted at 4 °C. Aliquots were withdrawn 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 insert shows the linear relationship between the apparent pseudo-first-order rate constants (k_{obsd}) of the two inactivation reactions and the diethyl pyrocarbonate concentration.
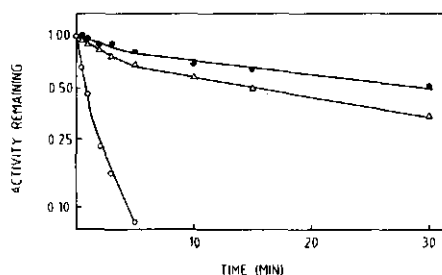

FIGURE 2: Effect of NADPH and 2',5'-ADP on the rate of inactivation of p-hydroxybenzoate hydroxylase. A solution of 20 μM p-hydroxybenzoate hydroxylase in 20 mM Mes and at 4 °C was treated with 0.8 mM diethyl pyrocarbonate in the presence of 25 mM NADPH ($\triangle$) (pH 6.2) or in the presence of 25 mM 2',5'-ADP ($\bullet$) (pH 5.9). The inactivation of the free enzyme under identical conditions is also shown (O). The pH of the solution of the free enzyme was 6.0. The data are presented as a semilogarithmic plot (cf. Figure 1).

(Figure 2). The latter compound inhibits the enzyme competitively with respect to NADPH (Santema et al., 1976). The substrate p-hydroxybenzoate did not influence the rates of inactivation (data not shown). Neither NADPH nor p-hydroxybenzoate reacts with diethyl pyrocarbonate under these conditions (about pH 6) as judged by control experiments in the absence of the enzyme. The reaction of diethyl pyrocarbonate with p-hydroxybenzoate actually occurs with the phenoxy form. The pK_a' value for the hydroxyl group of the substrate is 9.3 (Dawson et al., 1969), so little or no reaction is expected below pH 7, as found. Reaction between diethyl pyrocarbonate and NADPH or 2',5'-ADP, which could be expected because diethyl pyrocarbonate reacts with adenosine (Leonard et al., 1971), was found, however, to occur at a negligible rate under the conditions used in the protection

experiments. Our results therefore strongly indicate that histidine residues are involved in the binding of NADPH but not in the binding of the substrate, as claimed by Shoun et al. (1979b). The possibility of modification of other amino acid residues with unusually reactive groups, e.g., lysine or arginine, can be excluded because it has been shown (Little, 1977) that the imidazole ring of histidine is by far the preferred target for diethyl pyrocarbonate at pH 6 or lower pH values.

The experiments of Figure 2 further show that the interaction between histidine residues and NADPH most probably occurs with the 2',5'-ADP moiety of NADPH because 2',5'-ADP itself exhibits about the same protective effect as NADPH. It should be noted that the apparent difference in the protective effect between NADPH and 2',5'-ADP (Figure 2) is due to a difference in the pH of the solutions.

The reaction was followed with time simultaneously both by spectrophotometric (at 244 nm) and by activity measurements (Figure 3) to see if the two different rates of inactivation could be correlated to the chemical modification reaction of histidine residues. So that the reactions could be followed as accurately as possible, the experiment was done at pH 5.6 and 4 °C, where the rate of inactivation is decreased as compared to that at higher pH values (cf. below). Both monitoring systems show two reaction rates with the point of inflection at about 3.5 min (Figure 3). According to Ray & Koshland (1961), the pseudo-first-order rate constants for the loss of activity can be calculated from the slopes of Figure 3:

$$k_1 = \frac{\text{initial slope} - k_2}{1 - F} \tag{1}$$

and

$$k_2 = \text{final slope} \tag{2}$$

where k_1 is the pseudo-first-order rate constant of the reaction of the faster modified group(s), k_2 is the pseudo-first-order rate constant of the reaction of the slower modified group(s),
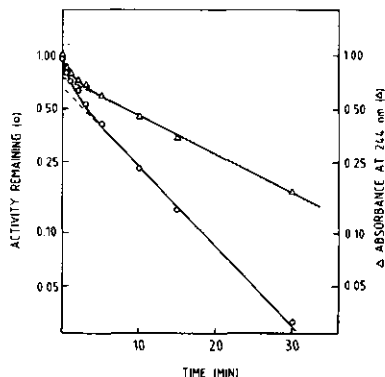
23

FIGURE 3: Kinetics of inactivation and chemical modification of p-hydroxybenzoate hydroxylase by diethyl pyrocarbonate. 20 μM enzyme in 20 mM Mes, pH 5.6, was treated with 0.81 mM diethyl pyrocarbonate at 4 °C. Aliquots of the solution were assayed at time intervals for residual activity (O) and for the increase of absorbance at 244 nm (Δ). The increase of absorbance was normalized by giving the difference of absorbance at zero time a value of 1.00 and at t = 60 min a value of 0.

FIGURE 4: Ultraviolet difference spectrum for the inactivation of p-hydroxybenzoate hydroxylase by diethyl pyrocarbonate. Both cuvettes contained 0.6 mL of a 20 μM enzyme solution in 20 mM Mes, pH 5.6. Before inactivation, a base line (A) was recorded. Then 27 μL of a 18.8 mM diethyl pyrocarbonate solution in ethanol was added to the sample cuvette. An equivalent volume of ethanol was added to the reference cuvette. After 60 min at 4 °C, the difference spectrum (B) was recorded, and the enzyme solution in the sample cuvette exhibited less than 3% of the activity of the enzyme solution in the reference cuvette. The difference in absorbance corresponds to four histidine residues modified per mole of FAD bound. A similar experiment was performed in 20 mM Hepes, at pH 8 and at 20 °C. The spectrum shown (C) was recorded after 5-min incubation of the solution with diethyl pyrocarbonate. The activity of the modified enzyme solution was 0% of that in the reference cuvette.

and F is the fractional residual activity of the enzyme belonging to the two reactions. Since the fractional activity after the slow reacting groups are modified is very small, F represents the fractional residual activity of the enzyme after only the fast reacting groups have been modified. F is determined from the extrapolation of the lines of the slow reaction (cf. Figures 1 and 3). The values calculated from Figure 3 for inactivation with 0.81 mM diethyl pyrocarbonate at pH 5.6 and 4 °C are k_1 = 0.80 min^{-1}, k_2 = 0.10 min^{-1}, and F = 0.71. The values for the chemical modification, calculated in the same way from Figure 3, are k_1 = 0.80 min^{-1}, k_2 = 0.05 min^{-1}, and F = 0.75.

In the case of the chemical modification reaction, F represents the fraction of the slower modified histidine residues. According to the theory of Ray & Koshland (1961), from these kinetic results the following conclusions can be drawn. First, F indicates that there are at least two sets of histidine residues differing in reactivity toward diethyl pyrocarbonate. The ratio of the two sets of histidine residues is 1:3. The histidine residue(s) of the fast reacting set is (are) responsible for the loss of about 30% of the total activity. The equivalence of the rate constant k_1 for the inactivation and the chemical modification reaction suggests that modification of one histidine residue is responsible for the rapid loss of some activity. From this, it follows that the set of the less reactive histidine residues is composed of three residues. Of these three residues, only two are apparently essential for activity, since the rate of loss of activity is equal to the sum of the rate of the chemical modification reaction (Ray & Koshland, 1961), i.e., 0.10 min^{-1} = 2 × 0.05 min^{-1}.

The difference spectrum produced when all accessible histidine residues of the enzyme have reacted with diethyl pyrocarbonate is shown in Figure 4. Using a molar extinction coefficient of 3600 M^{-1} cm^{-1} for N-ethoxyformylated histidine residues (cf. Materials and Methods), it is calculated that four histidine residues of the enzyme are modified. The difference spectrum also clearly indicates that under the experimental conditions used, no amino acid residues other than histidines are modified (cf. also below). From the spectral data, the number of equivalents of histidine residues modified as a function of time can also be determined. When the rate of
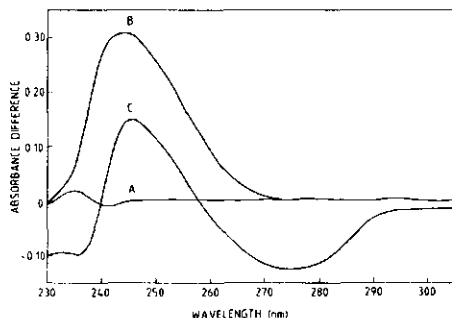
loss of activity of the enzyme is measured under conditions identical with those for the rate of the chemical modification of histidine residues, as has been done in Figure 3, the loss of activity can be correlated to the number of equivalents of histidine residues reacted. The experimental data of Figure 3 were used to obtain such a correlation (Figure 5A). Extrapolation of the line to zero activity suggests that three histidine residues per enzyme monomer (expressed as moles of His per mole of FAD) are apparently responsible for the loss of activity and that a further residue is responsible for the loss of some activity.

An alternative and more reliable approach for correlating the specific modification with the loss of activity is the statistical method developed by Tsou (1962). This method was later improved by Horiike & McCormick (1979). The relation is shown by eq 3 and 4:

$$a^{1/i} = \frac{p + s - m}{p} \qquad m > s \qquad (3)$$

$$a^{1/i} = 1 \qquad m < s \qquad (4)$$

where s is the number of rapidly reacting residues causing little or no loss of activity of the enzyme, p is the number of residues that react slower, but of which i residues are essential for activity, and m is the number of groups modified, which is correlated with the residual activity a. A plot of $a^{1/i}$ against m will give a straight line with a slope of $-1/p$. The value of i can be determined by plotting a, $a^{1/2}$, $a^{1/3}$, etc. against m until the best straight line fit is obtained. The value obtained in this way indicates the number of modified residues that are essential for catalysis. In our case, there is one rapidly reacting residue that is probably not essential, but which does cause about 30% inactivation. This prevented the use of the more sophisticated approach (Horiike & McCormick, 1979) which was developed for cases only where one of the kinetic constants is not involved in the inactivation of an enzyme. Therefore, the value of 70% activity was set to 100%, and the residue
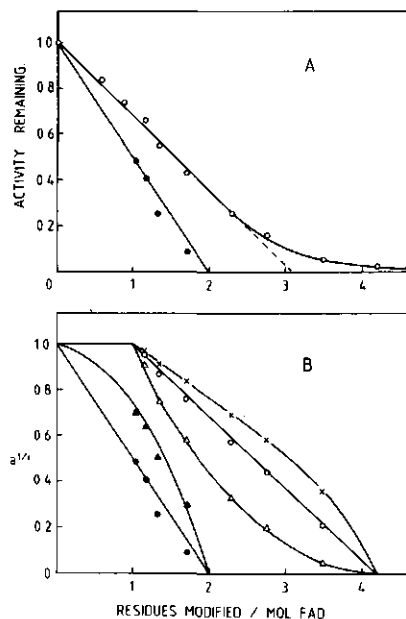
24

FIGURE 5: Correlation between the number of histidine residues of *p*-hydroxybenzoate hydroxylase modified by diethyl pyrocarbonate and the fractional activity remaining. (A) The open symbols represent the same data as in Figure 3 except that the change in absorbance is converted into the amount of histidines modified. The closed symbols represent the data for the enzyme–*p*-fluorobenzoate complex, obtained by the differential labeling technique (cf. Materials and Methods), in 20 mM Mes, pH 5.7 at 4 °C. The samples were withdrawn at 0, 1, 3, 10, and 30 min after the addition of diethyl pyrocarbonate. (B) The data of Figure 5A are presented in the form of a Tsou plot (see text) for $i = 1$ ($\triangle$), $i = 2$ (O), and $i = 3$ (X) (free enzyme) and for $i = 1$ (●) and $i = 2$ (▲) (*p*-FB-complexed enzyme).

Table I: Comparison of the Affinity of Native and Modified Enzyme for NADPH and *p*-Hydroxybenzoate[a]

	dissociation constant (mM)	
enzyme preparation	native enzyme	modified enzyme
enzyme–NADPH complex	0.49	5.0
enzyme–*p*-hydroxybenzoate complex	0.06	0.07

[a] The titration experiments were conducted fluorometrically at pH 6 and 20 °C. For the modification of *p*-hydroxybenzoate hydroxylase, a 20 μM solution of the enzyme was allowed to react with 0.8 mM diethyl pyrocarbonate for 45 min at 4 °C, pH 6.0. Before use in titration studies, the sample was purified by gel chromatography.

Table II: Average Number of Histidine Residues Protected in a 30 μM Solution of *p*-Hydroxybenzoate Hydroxylase in 20 mM Mes, pH 5.7[a]

	conditions of differential labeling		no. of accessible residues remaining after preincubation (mol of His/ mol of FAD)[b]
addition	preincubation time (min)	activity remaining (%)	
none	15	12	~1.4
none	30	0	0
10 mM NADPH	15	67	~3.0
10 mM NADPH	30	51	~2.0
15 mM *p*-FB	15	14	~1.9
15 mM *p*-FB	30	3.5	~1.7

[a] The solution was preincubated in the presence of NADPH or *p*-fluorobenzoate with 0.88 mM diethyl pyrocarbonate, for a certain period of time at 4 °C. After the preincubation, an excess of a solution of imidazole was added to the reaction mixture and the preparation purified by gel chromatography. An aliquot of the purified solution, pH 5.7, was then treated again with 0.88 mM diethyl pyrocarbonate and the reaction followed at 244 nm at 13 °C. From the increase in absorbance, the number of histidine residues protected by the differential labeling technique was then determined. [b] The value given is an average of three independent measurements.

responsible for the fast loss of activity was added to *s*. The result is shown in Figure 5B. The best straight line is obtained for $i = 2$, indicating that only two of the four histidine residues modified are essential for catalysis. This conclusion would be in agreement with the results presented above if it is assumed that modification of the more reactive histidine residue exerts an indirect effect on the catalytic activity of the enzyme. That this is most likely can be seen from the following experiments.

The affinity of the modified enzyme for the substrate *p*-hydroxybenzoate and NADPH was studied with free enzyme preparations modified at pH 6 and purified by gel chromatography. The dissociation constants were determined by titration experiments monitoring the quenching of the fluorescence emission of enzyme-bound FAD. It must be noted that the dissociation constants for the enzyme–NADPH complex determined in this way are less accurate than those determined kinetically (Entsch et al., 1976). The values presented are, however, quite satisfactory for the purpose of comparison (Table I). The results clearly indicate that the affinity of the modified enzyme for NADPH is decreased, whereas the affinity for the substrate remains unaltered. Moreover, an enzyme preparation exposed to diethyl pyrocarbonate for a short period of time, resulting in the loss of about 30% of the original activity, exhibited the same affinity for NADPH as native enzyme. In addition, the light absorption spectrum in the visible region and the circular di-

chroism spectrum in the ultraviolet region of native and modified enzyme are identical, indicating that the conformation of the enzyme is little or not at all affected upon chemical modification of histidine residues.

Modification studies in the absence or presence of these compounds were carried out to obtain more insight into the possible involvement and interaction of the four histidine residues with the substrate *p*-hydroxybenzoate or NADPH. The enzyme was modified in the presence or absence of *p*-fluorobenzoate (see below) or NADPH for a certain period of time, the treated enzyme solution was purified, and the number of the protected histidine residues was determined by reaction with diethyl pyrocarbonate from the increase of absorbance at 244 nm. The results of the differential labeling technique are presented in Table II. The results strongly indicate that two histidine residues are essential for activity, as already concluded above from the Tsou plot (Figure 5B), and that the substrate analogue protects one to two histidine residues which are not essential for catalytic activity. Since it follows from Table I that enzyme preparations in which the four accessible histidine residues were modified retained about the same affinity for the substrate as native enzyme, it must be concluded that the histidine residues protected by *p*-fluorobenzoate are involved neither in the binding of substrate nor in the binding of NADPH. Obviously, occupation of the substrate binding site diminishes the accessibility of two his-

25

Table III: Time Course of the Reactivation Reaction of Modified Enzyme by Hydroxylamine[a]

time (h)	% activity
0	~2
0.5	9
1	14
1.5	75
2	86
2.5	92

[a] A 20 μM solution of enzyme was inactivated in the presence of 0.8 mM diethyl pyrocarbonate at pH 6 for 30 min at 4 °C. The solution was then purified by gel chromatography. A 10 μM solution of this preparation was incubated with hydroxylamine (116 mM) at pH 7 and the reactivation of the enzyme followed with time.   An identical sample of native enzyme was also incubated with hydroxylamine and served as a reference sample.

tidine residues for diethyl pyrocarbonate. It is possible that the conformational change induced by the binding of the substrate to the enzyme (Teng et al., 1971) is responsible for the protective effect.

It is surprising that the capability of the modified enzyme to interact with NADPH is not completely abolished; however, histidine residues are obviously not solely responsible for the binding of NADPH, and other amino acid residues are also involved.

The inactivated enzyme can be reactivated by hydroxylamine. Also, in the absence of hydroxylamine, a very slow reactivation of the enzyme can be observed; allowing a completely inactivated enzyme solution to stand for 12 h at 4 °C restores about 30% of the original activity. However, hydroxylamine shows a clear catalytic effect on the reactivation reaction. As shown in Table III, the reactivation reaction is rather slow, but more than 90% of the activity can be reconstituted as compared with a control. In accordance with these observations, the affinity of the reactivated enzyme for NADPH is the same as that of the native enzyme. These data also indicate that no irreversible conformational change occurs on modification of essential histidine residues of the enzyme. This conclusion is in agreement with the spectral data (cf. above).

For determination of the pK_a' values of the essential histidine residues, the pseudo-first-order rate constant of inactivation by diethyl pyrocarbonate was measured at different pH values (Figure 6). When the free enzyme is inactivated, the pH-dependent rate constant seems to approach a maximum value at about pH 7, but it increases again at pH >7. This second increase is not observed when instead of the free enzyme the complex with p-fluorobenzoate is ethoxyformylated (Figure 6). It should be noted that the apparent pseudo-first-order rate constant for the inactivation of the enzyme is not affected by the presence of p-fluorobenzoate (Figure 6). p-Fluorobenzoate instead of p-hydroxybenzoate was used as a protecting agent because of the possible reaction of the hydroxyl group of the substrate with diethyl pyrocarbonate at pH >7. p-Fluorobenzoate induces the same changes in the CD spectrum of p-hydroxybenzoate hydroxylase upon binding as the natural substrate and also functions as an effector (Teng et al., 1971). This molecule is therefore a good substitute for the natural substrate while in addition it cannot react with the modification reagent. The observed increase in rate at higher pH values suggests that diethyl pyrocarbonate reacts with amino acid residues other than histidine in the free enzyme and that the substrate analogue protects the enzyme from this modification. Preliminary experiments at pH 8 suggest that this reaction is due to the modification of a tyrosine residue in the substrate binding site (Wijnands & Müller, 1982). In
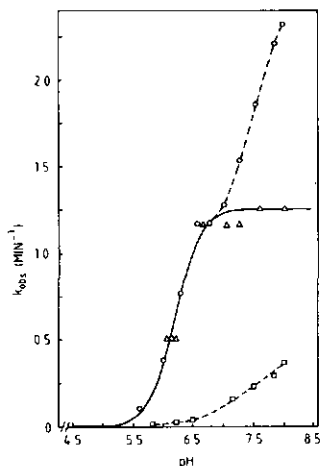

FIGURE 6: pH dependence of the rate of inactivation of p-hydroxybenzoate hydroxylase by diethyl pyrocarbonate in the absence or presence of NADPH and/or p-fluorobenzoate. The pH-dependent pseudo-first-order rate constant k_{obsd} for the inactivation of the enzyme due to the slow reacting histidine residues was calculated from the slope of curves as shown in Figures 1 and 2. The temperature was 4 °C. (O) 20 μM free enzyme; ($\triangle$) 20 μM enzyme + 25 mM p-fluorobenzoate; ($\square$) 20 μM enzyme + 25 mM NADPH. In all cases, 0.8 mM diethyl pyrocarbonate was added at $t = 0$. The solid line is a theoretical curve fitted to the experimental data by using eq 8 (see text). The theoretical line was calculated by using $k_{max} = 1.25$ min^{-1} and $pK_1 = 6.2$.

fact, free enzyme modified at pH 8 had lost the capability to bind p-hydroxybenzoate. In addition, the difference absorption spectrum of enzyme modified at pH 8 (Figure 4) shows the typical decrease of absorption at 278 nm caused by tyrosine modification (Mühlrad et al., 1967). The inactivation of free enzyme at high pH values is currently under active investigation.

Attempts failed to fit the data of the pH-dependent inactivation rate constants of the free enzyme at pH <7 and of the enzyme–p-fluorobenzoate complex by assuming a single, ionizing group, indicating once more that we are indeed dealing with more than one essential histidine residue. Scheme I shows the equilibrium and ethoxyformylation rate constants involved when dealing with two ionizing groups. All these microscopic values cannot be distinguished from one another because ethoxyformylation of either histidine leads to inactivation. Scheme II, on the other hand, shows the macroscopic or molecular ionization constants that are involved. According to these schemes, K_3' and the second ethoxyformylation reaction ($^1/_2 k_{max}$, Scheme II) are not important because they have no influence on the inactivation; therefore, the following relationships can be deduced:

$$K_1' = K_{1x} + K_{1y} \qquad \frac{1}{K_2'} = \frac{1}{K_{2x}} + \frac{1}{K_{2y}}$$
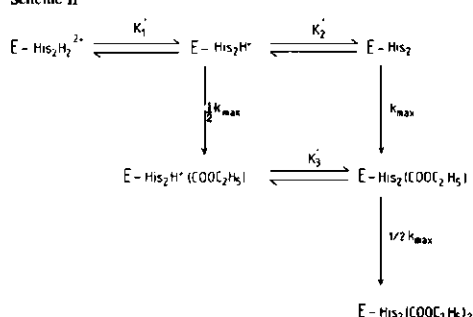
$$k_{max} = k_{x_{max}} + k_{y_{max}}$$

As $k_x = k_y$ (Figure 3), it can easily be shown that $K_{1x} = K_{1y}$ ($=K_1$), which means that $K_{2x} = K_{2y}$ ($=K_2$). The relations between the macroscopic and microscopic ionization constants can therefore be simplified as follows:

$$K_1' = 2K_1 \qquad K_2' = \frac{1}{2}K_2$$

26
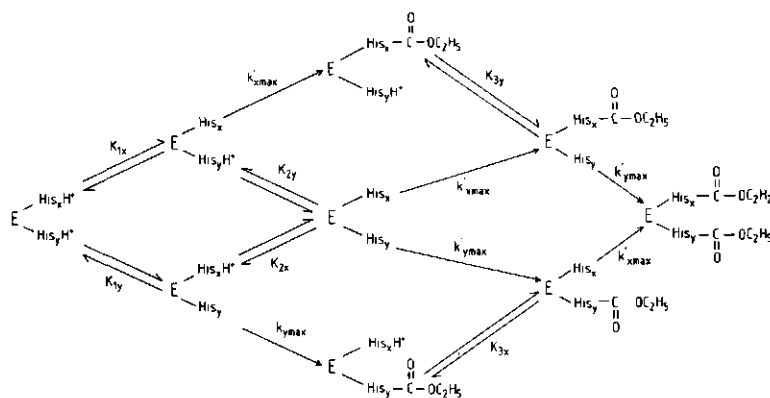
Scheme I

Scheme II

The relation between the observed inactivation rate constant, k_{obsd}, and the pH then becomes

$$k_{obsd} = k_{max}\left[\frac{1}{2 + [H^+]/K_1 + K_2/[H^+]} + \frac{1}{1 + 2[H^+]/K_2 + [H^+]^2/(K_1K_2)}\right] \quad (5)$$

Let us now consider three extreme situations. (1) Protonation of one histidine does not influence protonation of the other, or $K_1 = K_2 = K$. Equation 5 simplifies to $k_{obsd} =$

$$k_{max}\left[\frac{1}{2 + [H^+]/K + K/[H^+]} + \frac{1}{(1 + [H^+]/K)^2}\right] \quad (6)$$

(2) Protonation of one histidine prevents protonation of the other, or $K_2 \ll K_1$. Equation 5 now becomes

$$k_{obsd} = \frac{k_{max}}{2 + [H^+]/K_1} \quad (7)$$

(3) Protonation of one histidine forces protonation of the other, or $K_2 \gg K_1$. This situation can be described by Hill's model for cooperativity (Hill, 1910) with two binding sites:

$$k_{obsd} = \frac{k_{max}}{1 + ([H^+]/K_1)^2} \quad (8)$$

Equation 8 implies that either both histidine residues are protonated or both are deprotonated. Our data can best be

fitted by eq 8, with $k_{max} = 1.25$ min^{-1} and p$K_1 = 6.2$, as shown in Figure 6. This result is in agreement with the experimental data given above which show that p-fluorobenzoate indeed protects two histidine residues from modification but that these residues are not essential for activity. From Figure 6, it also follows that NADPH protects the essential histidine residues. These residues react in a cooperative manner. From this result, it is not clear whether both residues, which react in a cooperative manner, are indeed essential or only one is essential. For further clarification of this point, the differential labeling technique in the presence of p-fluorobenzoate was employed, and the chemical modification and the inactivation reactions were followed with time. The results are presented in Figure 5A and show the expected relation, i.e., two histidine residues responsible for the loss of activity. Application of the method of Tsou (1962) to these data, however, reveals that only one histidine residue is apparently essential for the activity of the enzyme (Figure 5B). This seems in contradiction with the results presented in Figures 5 and 6 for the free enzyme. It should be noted, however, that in cases of cooperativity the conclusions derived from Tsou plots concerning the number of essential residues may be quite erroneous (Rakitzis, 1978; Horiike & McCormick, 1979).

With the results presented at hand, one would like to indicate which histidine residues in the sequence are the essential ones. This is not yet possible because diffraction data on the enzyme–substrate complex only are available. Nevertheless, p-hydroxybenzoate hydroxylase from *P. fluorescens* contains nine histidine residues at positions 22, 72, 130, 162, 197, 204, 278, 289, and 351 in the sequence of the enzyme (Weijer et al., 1982). The three-dimensional model shows that not a single histidine residue is present in the substrate binding site. This seems in contradiction with our results showing that p-fluorobenzoate protects two histidine residues from chemical modification (Figure 5, Table II), but the model also shows that two pairs of histidine residues are located in the neighborhood of the substrate binding site. Therefore, the protection of two histidine residues from chemical modification is most probably related to the conformational change induced by the substrate (effector). The protection could also be caused by the substrate itself preventing access to the histidine residues in question. It is believed that only His-162 is involved in the binding of NADPH (J. Drenth and R. K. Wierenga, unpublished experiments). This proposal would explain our results of Figure 5 where it was concluded that only one of the two

27

histidine residues modified in the presence of *p*-fluorobenzoate is essential for activity. But this proposal does not explain the fact that the two histidine residues react in a cooperative manner (Figure 6). In the three-dimensional model, the closest neighboring histidine residue of His-162 is His-289. The C_α–C_α distance between His-162 and His-289 is 1.2 nm (J. Drenth and R. K. Wierenga, unpublished experiments). It might be that the reactivity of His-289 is influenced in a cooperative manner upon chemical modification of His-162. This proposal would explain our results, but a correlation between the results of chemical modification of histidine residues and their position in the sequence and the three-dimensional model cannot be offered yet. Modification of the histidine residues by chemical groups not susceptible to easy hydrolysis should make it possible to identify the essential histidine residues in the sequence of the enzyme and to elucidate their role in the catalytic mechanism. Such attempts have failed so far, but this line of research is currently further followed up.

References

Benesi, H. A., & Hildebrand, J. H. (1949) *J. Am. Chem. Soc. 71*, 2703–2707.
Berger, S. L. (1975) *Anal. Biochem. 67*, 428–437.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1979) *Data for Biochemical Research*, pp 82–83, Oxford University Press, Oxford.
Entsch, B., Ballou, D. P., Husain, M., & Massey, V. (1976) *J. Biol. Chem. 251*, 7367–7379.
Hill, A. V. (1910) *J. Physiol. (London) 40*, 4–8.
Hofsteenge, J., Vereijken, J. M., Weijer, W. J., Beintema, J. J., Wieringa, R. K., & Drenth, J. (1980) *Eur. J. Biochem. 113*, 141–150.

Horiike, K., & McCormick, D. B. (1979) *J. Theor. Biol. 79*, 403–414.
Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254*, 6657–6666.
Leonard, N. J., McDonald, J. J., Henderson, R. E., & Reichmann, M. E. (1971) *Biochemistry 10*, 3335–3342.
Little, C. (1977) *Biochem. J. 167*, 399–404.
Melchior, W. B., & Farney, D. (1970) *Biochemistry 9*, 251–258.
Mühlrád, A., Hegyi, G., & Tóth, G. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung. 2*, 19–29.
Müller, F., Voordouw, G., Van Berkel, W. J. H., Steennis, P. J., Visser, S., & Van Rooijen, P. J. (1979) *Eur. J. Biochem. 101*, 235–244.
Ovádi, J., Libor, S., & Elödi, P. (1967) *Acta Biochim. Biophys. Acad. Sci. Hung. 2*, 455–458.
Rakitzis, E. T. (1978) *J. Theor. Biol. 70*, 461–465.
Ray, W. J., & Koshland, D. E. (1961) *J. Biol. Chem. 236*, 1973–1979.
Roosemont, J. L. (1978) *Anal. Biochem. 88*, 314–320.
Santema, J. S., Müller, F., Steennis, P. J., Jarbandhan, T., Drenth, J., & Wierenga, R. K. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 155–160, Elsevier Scientific Publishing Co., Amsterdam.
Shoun, H., Higashi, N., Beppu, T., Nakamura, S., Hiromi, K., & Arima, K. (1979a) *J. Biol. Chem. 254*, 10944–10951.
Shoun, H., Beppu, T., & Arima, K. (1979b) *J. Biol. Chem. 254*, 899–904.
Shoun, H., Beppu, T., & Arima, K. (1980) *J. Biol. Chem. 255*, 9319–9324.
Teng, N., Kotowycz, G., Calvin, M., & Hosokawa, K. (1971) *J. Biol. Chem. 246*, 5448–5453.
Tsou, C.-L. (1962) *Sci. Sin. (Engl. Ed.) 11*, 1535–1558.
Vereijken, J. M., Hofsteenge, J., Bak, H. J., & Beintema, J. J. (1980) *Eur. J. Biochem. 113*, 151–157.
Weijer, W. J., Hofsteenge, J., Vereijken, J. M., Jekel, P. A., & Beintema, J. J. (1982) *Biochim. Biophys. Acta 704*, 385–388.
Westhead, E. W. (1972) *Methods Enzymol. 25*, 401–409.
Wieringa, R. K., de Jong, R. J., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1979) *J. Mol. Biol. 131*, 55–73.
Wijnands, R. A., & Müller, F. (1982) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Jr., Eds.) pp 318–322, Elsevier/North-Holland, New York.

Chapter 3   Chemical modification of arginine residues

SUMMARY

The flavoprotein p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* as inactivated by several arginine specific reagents. Modification by 2,3-butanedione had little or no influence on the binding of NADPH and p-hydroxybenzoate, although in contrast with the binding of the latter to native enzyme, the binding increased the fluorescence of the FAD moiety. Modification by 4-hydroxy-3-nitrophenylglyoxal reduced the capacity to bind substrate and NADPH by a factor 2-3. The ligands offered no protection against inactivation and the correlation between residual activity and the number of arginines modified was not influenced either. It is concluded that one arginine residue is essential in a further stage of catalysis. Modification by phenylglyoxal, however, led to almost complete loss of the capacity to bind substrate and NADPH. Quantitation and protection experiments revealed that two arginines are essential and a model is given to explain the results.

Sequential modification studies confirmed the findings that the reagents react with different sets of arginines. This may be explained by differences in charge and hydrophobicity.

INTRODUCTION

p-Hydroxybenzoate hydroxylase is an external flavoprotein monooxygenase that catalyzes the hydroxylation of p-hydroxybenzoate in the presence of NADPH and molecular oxygen. It has been obtained from four different species of *Pseudomonas*, but the enzymes isolated from *Pseudomonas desmolytica* and from *Pseudomonas fluorescens* have been studied most extensively. The role of the flavin in the catalytic mechanism, as revealed mainly by kinetic and spectroscopic analyses, has had much attention [1,2]. As far as the role of amino acid residues in the reaction mechanism is concerned studies on the pH dependence of binding of NADPH

to the protein [3] and chemical modification [4-9] have given interesting results, especially when related to the known primary and tertiairy structure [10,12].

The amino acid residues that have been modified so far include cysteine [9], histidine [5,7,8] and arginine [6]. The modification of these residues leads to loss of activity in all three cases.

Shoun and his colleagues [6] have used phenylglyoxal to modify arginine residues in the enzyme from *P.desmolytica*. Their conclusion was that phenylglyoxal was incorporated into the substrate binding site and reacted with one arginine residue that has interaction with the carboxylate group of the substrate in the enzyme-substrate complex. This agrees well with results obtained from the three-dimensional structure of p-hydroxybenzoate hydroxylase from *P.fluorescens*. The electron density map of the enzyme-substrate complex suggests that a salt bridge exists between the carboxylate group of the substrate and the guanidinium group of Arg-214 [12].

The modification of histidine residues by diethyl pyrocarbonate indicated that one or two histidine residues are involved in NADPH binding [8]. As ethoxyformylated histidine residues are not stabile towards even mild hydrolysis we were unable to identify the histidine residues in the NADPH binding site. This binding site is still not known, but a site containing two arginine residues (Arg-166 and Arg-269) and one histidine residue (His-162) has been suggested [13]. These amino acid residues could form salt bridges with the pyrophosphate moiety of NADPH.

The aim of this paper is to elucidate the possible role of arginine residues in the binding of NADPH to p-hydroxybenzoate hydroxylase from *P.fluorescens*. Identification of the arginine residues would be helpful in the elucidation of the actual NADPH binding site.

MATERIALS AND METHODS

Phenylglyoxal was from Aldrich, 2,3-butanedione and 1,2-cyclo-hexanedione were from Fluka, [7-^{14}C]phenylglyoxal (3,69 MBq/mg) was from

Amersham, 4-hydroxy-3-nitrophenylglyoxal was synthesized according to Borders [14], L-arginine monohydrochloride was from the British Drug Houses Ltd., Cibacron blue was a gift from Ciba-Geigy (Arnhem, The Netherlands), $KAu(CN)_2$ was from Drijfhout & Zoon's Edelmetaalbedrijven, B.V. (Amsterdam, The Netherlands), NADPH and NADH were from Boehringer, 2',5'-ADP, 2'-AMP, 5'-AMP, Mes, Hepes, Hepps and Tris were from Sigma and all other chemicals were from Merck.

Spectrophotometric measurements were done on a Zeiss PMQ II, a Varian Cary 16 or an Aminco DW2A spectrophotometer. Fluorometric measurements were performed on an Aminco SPF-500 spectrofluorometer. Circular dichroism spectra were recorded on a Roussel-Jouan Dichrograph II.

p-Hydroxybenzoate hydroxylase was purified from *P.fluorescens* as described previously [15]. The purified enzyme was kept as an ammonium sulphate precipitate (70%) at 4^OC, pH 6-7. Fresh solutions of the enzyme in the appropriate buffer were prepared daily by gel filtration over Biogel P-6DG. The buffers were brought to a constant ionic strength of 25 mM with Na_2SO_4 whenever NADPH was present in the incubation mixtures [3]. The enzyme concentration was determined spectrophotometrically on the basis of the FAD content by the use of a molar absorption coefficient of 11.3 $mM^{-1}cm^{-1}$ at 450 nm [15].

The enzyme activity was assayed by adding 8 µl of a 2-3 µM enzyme solution to 0.8 ml of 0.1 M Tris-H_2SO_4, pH 8.0. The buffer solution contained 0.15 mM p-hydroxybenzoate and 0.15 mM NADPH. The activity was determined by recording the decrease of absorbance of NADPH at 340 nm *vs.* time at 25^OC.

The modification of arginine residues by 2,3-butanedione and 1,2-cyclohexanedione was performed in 50 mM borate buffer, pH 8.0, at 25^OC, unless stated otherwise. A 22.8 mM solution of 2,3-butanedione in borate buffer was prepared each day by diluting it 500 times into 50 mM borate buffer, pH 8.0. The pH decrease (to ∿7.5) occurring upon mixing was compensated by adding small volumes of a 5 M NaOH solution in order to obtain a solution of the desired pH value of 8.0. Equal volumes of the 2,3-butanedione solution, or of a solution further diluted

if desired, and of a 4-10 μM enzyme solution in the same buffer were mixed. In some cases the reaction was started by addition of a smaller volume of a more concentrated enzyme solution in a different buffer (Mes, Hepes or Hepps). The reaction was stopped either by diluting an aliquot of the reaction mixture into the assay mixture for the determination of the residual activity or by passing the incubation mixture over a Biogel P-6DG column for the spectrophotometric analysis of the product, binding or reactivation studies. Reactivation of modified enzyme in the borate buffer was accomplished by incubation with 100 mM hydroxylamine. In the absence of borate the reactivation of the enzyme was spontaneous if 2,3-butanedione was removed by dialysis or gel chromatography.

Modification by phenylglyoxal was carried out in 40 mM Hepps, pH 8.0, 25°C, unless stated otherwise. A fresh solution of 0.5 M phenylglyoxal in methanol was prepared each day. The phenylglyoxal concentration in the reaction mixture was 10 mM, except when the inactivation reaction was studied in dependence on the phenylglyoxal concentration. The enzyme concentration varied from 2 - 90 μM depending on the kind of experiment. The reaction was stopped as described for the butanedione modification experiments. After modification, the product was kept in 20 mM Mes, pH 6.5, to avoid reactivation. [7-^{14}C]Phenylglyoxal was used for quantitation experiments. 7.06 mg Of cold phenylglyoxal was added to 0.50 mg of 3.69 MBq/mg [7-^{14}C]phenylglyoxal and dissolved in 150 ml of methanol resulting in a 0.33 M solution of 37 MBq/mmole. The modification was carried out as described above using a 90 μM enzyme solution. Aliquots were analysed for radioactivity and residual activity at time intervals. A 2:1 stoichiometry for the arginine modification was assumed [16,6]. The radioactivity measurements were performed with Scintillator 299^{tm} from United Technologies Packard using a Packard Tri-carb model 2450 liquid scintillation spectrometer.

Identification of the modified arginines was attempted twice. In the first experiment the enzyme was modified by radioactive phenylglyoxal in the absence and presence of p-fluorobenzoate and NADPH and in the

second experiment the free enzyme was modified by radioactive phenyl-
glyoxal after premodification by cold phenylglyoxal in the absence
and presence of p-hydroxybenzoate and/or Cibacron blue. After denatura-
tion by urea (pH 4) the modified enzyme was cleaved by trypsine and
analysed by HPLC as has been described elsewhere [9].

Modification by 4-hydroxy-3-nitrophenylglyoxal was carried out in
40 mM Hepps, pH 8.5, 25°C, unless stated otherwise. A 100 mM solution
of the reagent in the same buffer was prepared each day. The pH was re-
adjusted to 8.5 using 5 M and 0.1 M NaOH solutions. Further procedures
were as described for the phenylglyoxal reaction. A molar difference
absorption coefficient of 21.8 $mM^{-1}cm^{-1}$ at 316 nm was used for the quan-
titation of arginine modification with 4-hydroxy-3-nitrophenylglyoxal,
assuming a 2:1 stoichiometry [14].

Sequential labelling experiments were performed under the same
conditions as described above. After each step the reaction was stopped
by passing it over a Biogel P-6DG column equilibrated in the desired buf-
fer for the next modification step. When Cibacron blue was present during
the first step of modification, it was necessary to pass the mixture
over Biogel P-6DG equilibrated with 40 mM Mes, 0.5 M KCl, pH 6.5 first
in order to remove the Cibacron blue.

Dissociation constants of different enzyme complexes were determined
fluorometrically by titration experiments or spectrophotometrically by
inactivation experiments in the presence of various concentrations of
ligand. For fluorometric titration the quenching of the protein-bound
FAD fluorescence at 525 nm (excitation at 450 nm) was monitored as a
function of the ligand concentration. From Benesi-Hildebrand plots [17]
the dissociation constants of the complexes were calculated. In those
cases where a ligand offered protection against inactivation by chemical
modification, the dissociation constants were also derived from plots
of the reciprocal difference between pseudo-first-order rate constants of
inactivation in the absence and the presence of the ligand against the
reciprocal ligand concentration [18].

The rate constant of modification of the enzyme-substrate complex

by phenylglyoxal was also determined by measuring the catalytic activity in the presence of the modifier. p-Hydroxybenzoate hydroxylase (5 nM) was added to a mixture of 100 nM FAD, 3 mM p-hydroxybenzoate, 10 mM phenylglyoxal and a varying amount of NADPH in 20 mM Hepps, pH 8.0., 30°C. The reaction was monitored at 360 nm using the mixture without the enzyme as a reference. The enzyme concentration was chosen such that the change in the NADPH concentration was small during the whole reaction course, not significantly changing the degree of saturation of the enzyme by the substrate. In practice the change in the steady-state rate, had there been no phenylglyoxal present, was less than 10%. It has been shown for a unireactant system [19,20] that the following equation can be applied:

$$\ln(P_{\infty} - P) = \ln P_{\infty} - AYt \tag{1}$$

where P is the product concentration at time t, P_{∞} the product concentration a time approaching infinity, Y the concentration of the irreversible inhibitor and A the apparent modification rate constant in the presence of the substrate.

A is given by:

$$A = \frac{kK_m + k'S}{K_m + S} \tag{2}$$

where K_m is the Michaelis-Menten constant of the catalytic reaction, k the second-order rate constant of the modification reaction of the free enzyme, k' the second-order rate constant of the modification reaction of the enzyme-substrate complex and S the concentration of the substrate. When the modification reaction is classified as competitive (k' = 0), noncompetitive (k = k') or uncompetitive (k = 0), in analogy to reversible inhibition, equation (2) can be simplified to the following expressions:

competitive
$$A = \frac{k \, K_m}{K_m + S} \tag{3}$$

noncompetitive $\quad A = k \tag{4}$

uncompetitive
$$A = \frac{k' \, S}{K_m + S} \tag{5}$$

34

It is easily seen that the different types of inhibition can be distinguished by suitable A and S plots. A plot of $\frac{1}{A}$ $vs.$ S or $\frac{1}{S}$ gives straight lines for competitive and uncompetitive inhibition, respectively, while for noncompetitive inhibition A is independent of S. The above equations were derived by Tsou and originally published in Chinese [19]. Later the salient points of these papers appeared in English [20].

RESULTS AND DISCUSSION

Modification by 2,3-butanedione

When p-hydroxybenzoate hydroxylase is added to 2,3-butanedione in 50 mM borate buffer (pH 8.0, 25 $^{\circ}$C) the enzyme is inactivated at a rate dependent on the butanedione concentration. The decrease of activity to 85% of the initial activity within the first minute is caused by borate, as judged by the omittance of 2,3-butanedione. The straight lines obtained in a plot of the natural logarithm of the residual activity against time indicate that the inactivation reaction follows pseudo-first-order kinetics (Fig. 1). From the slope of a plot of the pseudo-first-order rate constants as a function of the 2,3-butanedione concentration a second-order rate constant of 11.6 $M^{-1}min^{-1}$ is calculated (Fig. 1, inset). This value decreases when butanedione solutions are used which have been allowed to stand for more than one day at room temperature. It has been reported [21] that butanedione reacts with itself in mildly alkaline aqueous solution (pH 8.2) to form a bicyclic trimer, which can still react with arginine residues. From the fact that butanedione solution older than one day showed a decreased rate of modification it is concluded that the monomer is more reactive with p-hydroxybenzoate hydroxylase than the trimer, as was also found for carboxypeptidase A [22]. From a similar treatment with 1,1 cyclohexanedione a second-order rate constant of 1.6 $M^{-1}min^{-1}$ was found (data not shown).

Butanedione reacts quite specifically with arginine residues [22],
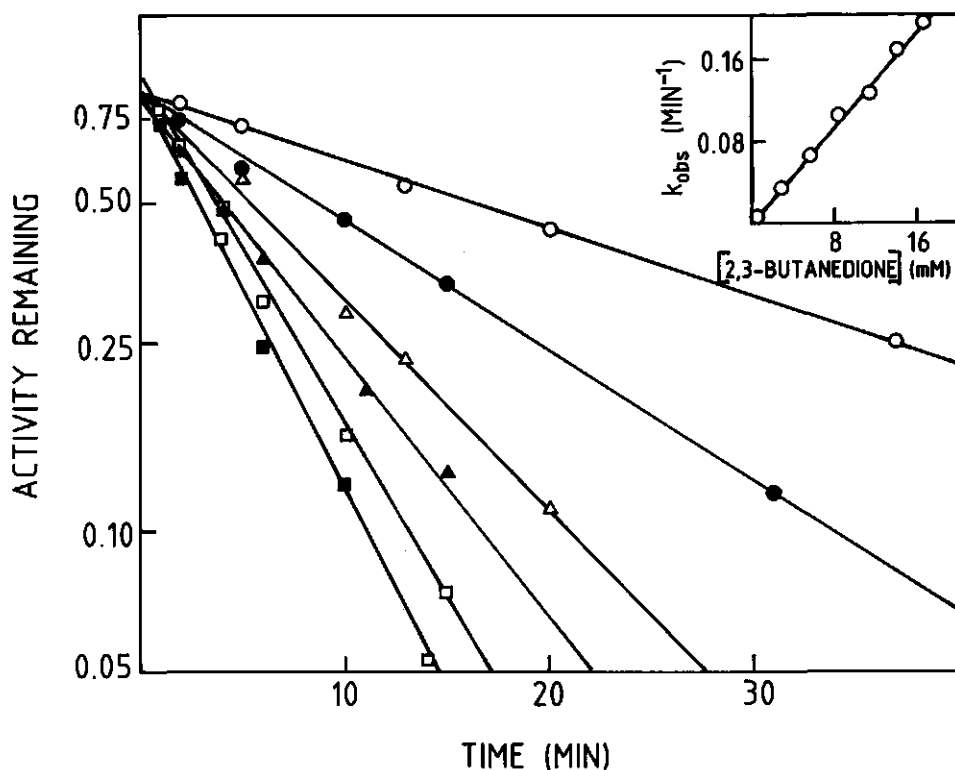
Figure 1. *Inactivation of p-hydroxybenzoate hydroxylase by 2,3-butanedione.* The enzyme concentration was 2 μM in 50 mM borate buffer pH 8.0 and the 2,3-butanedione concentrations were 2.85 mM (o), 5.70 mM (●), 8.55 mM (Δ), 11.40 mM (▲), 14.25 mM (□) and 17.10 mM (■). In the inset the pseudo-first-order rate constants determined from the slopes are plotted *vs.* the concentration of butanedione.

but other residues can react with butanedione as well. It has been shown that under the same conditions used in this paper modification of lysine residues was neglegible in carboxypeptidase A [22]. Therefore we assume that little or no lysine residues are modified in our enzyme, although this has not been proven directly. Inactivation due to photosensitized modification of tryptophan or tyrosine residues [23] can be eliminated since the rate constants of inactivation in the presence and absence of light were identical. Finally, it was checked if the only essential

cysteine residue [9] was stil accessible for p-chloromercurinitrophenol after inactivation by 2,3-butanedione, which was the case. These results indicate that the loss of enzymic activity is most probably due to modification of arginine residues.

Inactivation was also achieved in the absence of borate. When the reaction was performed in 40 mM Hepps, pH 8.0, the $t_{\frac{1}{2}}$ value for the reaction was identical with that found in 50 mM borate, pH 8.0, but the reaction did not obey first-order kinetics (data not shown). This is not surprising since the reaction product is not stable in the absence of borate [22]. The reversal of the reaction was demonstrated either by adding 100 mM hydroxylamine to trap the dicarbonyl compound or by passing the reaction mixture over a Biogel P-6DG column equilibrated with 20 mM Mes, pH 6.5. In both cases reactivation up to 80% of the original activity was found.

The loss of activity by butanedione modification in 50 mM borate is accompanied by a twofold increase of the original fluorescence of protein-bound FAD. The pseudo-first-order rate constant of this process is the same as that found for the loss of activity. In the absence of borate, an increase of only 30% of the fluorescence of FAD was found but the rate constant was roughly six times higher than that in the presence of borate. The residual activity of the modified enzyme was about 30% when the fluorescence had reached its maximum. Addition of borate to such a reaction mixture causes a slow further increase in the fluorescence. As the complex formation of borate with 4,5-dimethyl-4,5-dihydroxy-2-imidazoline is reported to be rapid [24,25], we conclude that the observed difference between the fluorescence in the presence and absence of borate is not merely due to complexation of the modified arginine residues with borate. The results therefore strongly suggest that more arginines are modified in the end in the presence of borate than in its absence. And because the additional 70% increase in fluorescence still occurs after activity is lost the arginines responsible for the twofold increase of the FAD fluorescence when reacting with butanedione only in the presence of borate are not the same arginines that are essential

for activity and also react with butanedione in the absence of borate.

The circular dichroism spectrum in the region of 250-320 nm of enzyme modified in borate buffer was identical with that of native enzyme in the same buffer. This suggests that modification does not cause any conformational changes that could account for the loss of activity. In addition the dissociation constants of the complexes between native enzyme and NADPH, and butanedione-modified enzyme and NADPH are very similar. The dissociation constant of the modified enzyme-NADPH complex is 165 μM at pH 6.5 and I = 25 mM (20°C), while the dissociation constant of the native enzyme-NADPH complex is 130 μM under the same conditions. On the other hand the corresponding dissociation constants of the p-hydroxybenzoate-enzyme complexes are 150 μM and 35 μM, respectively. More striking however, is the fact that binding of the substrate to the modified enzyme leads to 20% enhancement of the original FAD fluorescence while substrate binding to native enzyme causes 70% quenching of the FAD fluorescence. Enhancement of the fluorescence caused by ligand binding to p-hydroxybenzoate hydroxylase has been reported by others [26]. A 1.8 fold enhancement was found in the case of 6-hydroxynicotinate, which is also an effector. Two other spectroscopic methods used to monitor substrate binding to the enzyme, were also employed i.e. circular dichroism and light absorption in the visible region. Changes found in the circular dichroism spectrum between 250 and 320 nm upon the binding of p-hydroxybenzoate to the native enzyme [27] were also found in the case of substrate binding to the modified enzyme, indicating that the conformational change is still induced by substrate binding. The absorption spectrum of modified enzyme-bound FAD in the region 320 to 600 nm however, did not change at all upon substrate binding.

In Table 1 relative pseudo-first-order rate constants of inactivation by 2,3-butanedione in the presence of NADPH, p-hydroxybenzoate and the effector p-fluorobenzoate are shown. It is clear that all three ligands offer protection against inactivation by butanedione. The protection by NADPH however, is not a true protection because NADPH merely reduces the effective butanedione concentration in the reaction mixture. Butane-

38

Table 1. *Relative pseudo-first-order rate constants of inactivation of p-hydroxybenzoate hydroxylase by 11.4 mM 2,3-butanedione as observed in the presence of several ligands.* The enzyme concentration was 3 µM in 50 mM borate buffer, pH 8.0 and 24°C.

Ligand(s)	Concentration (mM)	k'/k_o' [a]
p-OHB	3.1	0.40
p-OHB	25.0	0.45
NADPH	25.0	0.65
NADPH + p-FB	25.8 each	0.31

[a] The relative pseudo-first-order rate constant k'/k_o' is defined by the ratio of the pseudo-first-order rate constants in the presence and absence of the ligand.

dione is probably reduced by NADPH since the absorbance of an NADPH solution in borate buffer slowly decreased at 340 nm when butanedione was added (data not shown).

When the results of the protection experiments are combined with those of the binding experiments we must conclude that butanedione does not react with any arginines that are involved in either NADPH or substrate binding directly. There seems to be some influence of the arginine modification by butanedione on the binding of the substrate. The binding is reduced by a factor of 5 and it does not seem to have the same influence on the environment of the modified enzyme-bound flavin as on the environment of the native enzyme-bound flavin. Because the modification itself also causes a change in the environment of the enzyme-bound flavin, as judged by the increase of the fluorescence of 30% without borate and 100% with borate, the arginines that are modified probably are close to the flavin, especially the ones that react when borate is present.

The accessibility of the arginine residues that only react in the presence of borate and cause the twofold increase of the enzyme-bound FAD fluorescence is also changed in the presence of the substrate. The

pseudo-first-order rate constant as monitored by the fluorescence increase is reduced by 75%. As the protection against inactivation is demonstrated by only a 60% decrease of the pseudo-first-order rate constant (Table 1), the substrate appears to offer better protection against modification by butanedione for the arginines close to the flavin than for those more essential for the activity .

The above clearly demonstrates that protection by a ligand on its own is not enough to prove the direct involvement of a residue in ligand binding.

Modification by phenylglyoxal

When phenylglyoxal is added to p-hydroxybenzoate hydroxylase in 40 mM Hepps, pH 8.0, 25°C, the enzyme is also inactivated at a rate dependent on the phenylglyoxal concentration. As with 2,3-butanedione and 1,2 cyclohexanedione the inactivation reaction obeys pseudo-first-order kinetics, but it is biphasic. in contrast with chemical modification of p-hydroxybenzoate hydroxylase from *P.desmolytica* by phenylglyoxal [6]. The pseudo-first-order rate constants of the two processes can be calculated from the slopes of Fig. 2 by the following equations [28]:

$$k_1' = \frac{\text{initial slope} - k_2'}{1 - F}$$

and

$$k_2' = \text{final slope}$$

where k_1' and k_2' are the pseudo-first-order rate constants of the fast and slow process respectively, and F is the fractional residual activity after only the fast process has taken place. F is determined by extrapolation of the lines of the slow reaction to t=0. From Fig. 2 a value of 0.75 for F is calculated. The second-order rate constants can be determined from Fig. 2 (inset) as described for arginine residues modification by 2,3-butanedione. Values of 91 $M^{-1}min^{-1}$ and 40 $M^{-1}min^{-1}$ are thus calculated for the fast and slow process, respectively. A double reciprocal plot of the same data (not shown) demonstrates that the enzyme and

40
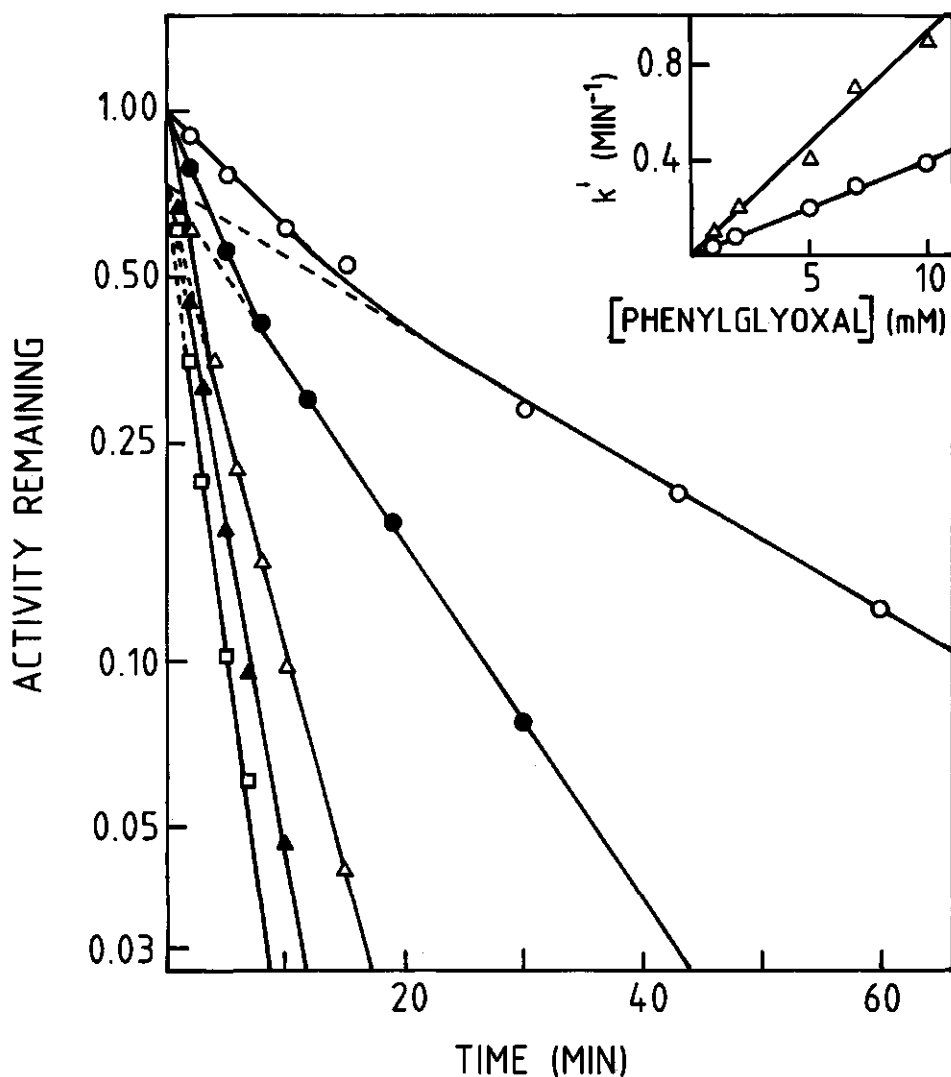
Figure 2. *Inactivation of p-hydroxybenzoate hydroxylase by phenylglyoxal.*
The enzyme concentration was 2 µM in 40 mM Hepps, pH 8.0, 26°C and the
phenylglyoxal concentrations were 1 mM (o), 2 mM (•), 5 mM (Δ), 7 mM (▲)
and 10 mM (□). In the inset the pseudo-first-order constants determined
from the initial (Δ) and final (o) slopes are plotted *vs.* the concen-
tration of phenylglyoxal.

Table 2. *The influence of several ligands on the relative pseudo-first-order rate constant for the modification of p-hydroxybenzoate hydroxylase by phenylglyoxal.* The enzyme concentration was 2 µM. Other conditions are given under Materials and Methods.

Ligand(s)	Concentration (mM)	k'/k_oa)
none	–	1
p-OHB	1	0.3
NADPH	20	0.4
NADH[b]	20	0.7 (0.4)[b]
2',5'-ADP	20	0.4
2'-AMP[b]	21	0.7 (0.4)[b]
5'-AMP[b]	21	0.7 (0.4)[b]
Cibacron blue	0.1	0.2
Cl^-	250	0.4
SO_2^{2-}	500	1.3
phosphate[b]	330	0.8
$Au(CN)_2^-$	5	0.3
p-OHB + 2',5'-ADP	1 and 20	0.09
p-OHB + Cibacron blue	1 and 0.1	0.06
p-OHB + Cl^-	1 and 250	0.17

a) The relative pseudo-first-order rate constant is defined in Table 1.

b) Complex formation is not maximal, the constants shown in parentheses have been calculated for maximal protection assuming that under the given conditions about 50% of the enzyme is complexed [3].

phenylglyoxal do not form a complex prior to either the fast or the slow inactivation reaction, as no saturation kinetics are observed.

Like in the case of modification by 2,3-butanedione, the inactivation reaction is accompanied by a strong increase in the fluorescence of FAD. With phenylglyoxal however, the reaction rate of the increase of the fluorescence (179%) is four to five fold slower than the rate of the slow

inactivation process.

Table 2 demonstrates the protection of several ligands against inactivation. It is clear that the substrate and NADPH give partial protection against inactivation. Practically the same protection is observed for all competitive inhibitors [29,30] of enzyme-NADPH complex formation. The protection is strongly increased when ternary complexes are subjected to modification. The results therefore suggest that different arginines are protected from modification by the substrate on one side, and NADPH and its analogues or inhibitors on the other. As ligand binding in the NADPH binding site has been shown to be dependent on the ionic strength [3], sulphate, which is not a competitive inhibitor with respect to NADPH was included to demonstrate that the protection by e.g. chloride is not merely caused by an ionic strength effect. In fact it shows that an increase of the ionic strength disfavours protection and increases the inactivation rate.

No reaction of phenylglyoxal with NADPH could be detected as was the case with butanedione. $Au(CN)_2^-$ (a competitive inhibitor of the enzyme with respect to NADPH [30]) however, appeared to form a complex with phenylglyoxal as shown by a yellow colour when mixing the two substances at pH 8, which dissappeared when the pH was lowered to 6.5.

Dissociation constants of complexes of the enzyme with p-hydroxybenzoate and chloride were determined by modification with phenylglyoxal in the presence of different concentrations of these ligands as described under Materials and Methods. Values of 52 μM for the dissociation constant of the enzyme-p-hydroxybenzoate complex and 50 mM for the dissociation constant of the enzyme-chloride complex, both at pH 8 and 25°C, were calculated. These values agree reasonably well with data determined by fluorescence titration methods [30,31].

Protection by binary complex formation is also observed in the increase of the fluorescence of the enzyme-bound FAD upon modification with phenylglyoxal, as can be seen in Table 3. As was clearly shown for modification with butanedione, this does not necessarily mean that the residues involved in this reaction are the same residues that are involved

Table 3. *Pseudo-first-order rate constants of inactivation and of the increase of the fluorescence at 525 nm for the modification of p-hydroxybenzoate hydroxylase with phenylglyoxal.* The conditions were as mentioned under Materials and Methods except for the temperature, which was 20°C. The ligand concentrations were as given in Table 2.

Sample	Pseudo-first-order rate constant (min^{-1})	
	activity	fluorescence
free enzyme	0.183	0.037
enzyme + p-OHB	0.048	0.013
enzyme + 2',5'-ADP	0.067	0.013

Table 4. *The affinity of p-hydroxybenzoate hydroxylase for several ligands after modification with phenylglyoxal in the absence and presence of saturating amounts of these ligands.* Modification was performed in 40 mM Hepps pH 8.0 until the activity was less than 1%. The binding studies were performed in 20 mM Mes pH 6.5 at 20°C.

Ligand present during modification	Ligand binding after modification			
	NADPH	Cl^-	Au(CN)_2^-	p-OHB
none	−	−	−	−
2.5 mM p-OHB	±	+	+	+
250 mM Cl^-	±	+	+	+
5 mM Au(CN)_2^-	±	+	+	−
.15 mM Cibacron blue	±	nd	nd	−
.15 mM Cibacron blue + 3 mM p-OHB	+	nd	nd	+

+ = normal binding; ± = reduced binding   − = no binding   nd = not determined.

in ligand binding or that are essential for the activity. Additional information can be obtained from binding studies. Table 4 shows the results of binding studies of p-hydroxybenzoate, NADPH and some competitive inhibitors of the enzyme with respect to the latter. The enzyme was modified in the absence and presence of saturating amounts of these ligands. After modification of the free enzyme by phenylglyoxal none of the ligands examined were able to form a complex with it. While NADPH and the competitive inhibitors offer protection against the loss of the ability to form a complex between the enzyme and NADPH, they do not prevent the loss of the ability to bind the substrate. The substrate however, offers protection against the loss of the ability to form a complex between p-hydroxybenzoate hydroxylase and all the ligands investigated. The latter observation means that the protection is at least partly caused by the conformational change induced by substrate binding. Although the arginine(s) involved in substrate binding may be directly protected, at least one of the arginine(s) involved in NADPH binding must have been protected by a change in conformation.

A third way of characterizing the modification reaction is by measuring the catalytic reaction in the presence of the modifier. Equations 2-5 have been derived for inhibition with respect to unireactant systems only [19,20]. From the above it is clear that in our case inhibition occurs with respect to both p-hydroxybenzoate and NADPH. If it is assumed for example that both inhibition reactions are competitive it can be shown that

$$A = \frac{(K_d^n + N) \ K_m^S \ k_1 + (K_d^S + S) \ K_m^n \ k_2}{K_d^S \ K_m^n + K_m^S N + K_m^n S + NS} \tag{6}$$

where K_d^n and K_d^S are the dissociation constants of the binary complexes of the enzyme with NADPH and the substrate, respectively, K_m^n and K_m^S the Michaelis-Menten constants for NADPH when the substrate is saturating and for the substrate when NADPH is saturating, respectively, k_1 and k_2 are the second-order rate constants of modification at the NADPH and

substrate binding sites, respectively, N the concentration of NADPH
and S the concentration of p-hydroxybenzoate. Equation (6) can be
simplified when the concentration of S or N is large compared to the
other constants:

$$A = \frac{k_2 K_m^n}{K_m^n + N} \qquad (7)$$

$$A = \frac{k_1 K_m^S}{K_m^S + S} \qquad (8)$$

Equations (7) and (8) are the same as equation (3), which means that in
the case of competitive inhibition with respect to two substrates each
inhibition site can be studied separately by saturating the other site
with the appropriate substrate. In a similar way the following expres-
sions for the apparent modification rate constant in the case of non-
competitive inhibition (equation (9)) and in the case of uncompetitive
inhibition (equation (10)) can be derived:

$$A = k_1 + k_2 \qquad (9)$$

$$A = k_1 + k_2 - \frac{k_1 K_m^n}{K_m^n + N} \qquad (10)$$

Equation (10) is valid when the substrate concentration is large. It is
clear that also in the case of inhibition with respect to two substrates,
the different types of inhibition can easily be distinguished.

In Fig. 3 the course of NADPH oxidation by p-hydroxybenzoate hydroxy-
lase in the presence of phenylglyoxal and a saturating amount of sub-
strate is plotted according to equation (1) for several concentrations
of NADPH. Like the inactivation of the free enzyme shown in Fig. 2 the
reaction is biphasic, so two rate constants can be calculated in each
case. In the inset of Fig. 3 the reciprocal values of these rate constants
are plotted against the NADPH concentration. This plot shows that the
inhibition by phenylglyoxal is noncompetitive with respect to NADPH where
the fast modification reaction is concerned and competitive with respect
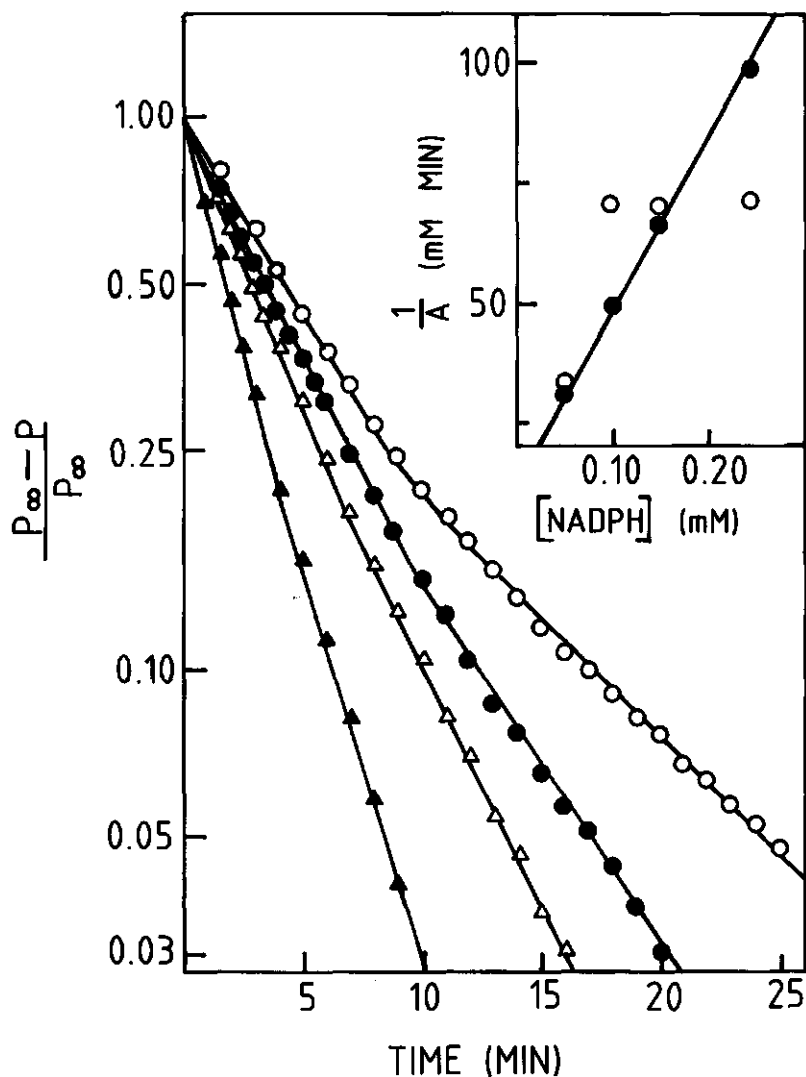
Figure 3. *NADPH oxidation by p-hydroxybenzoate hydroxylase in the presence of phenylglyoxal and a saturating amount of p-hydroxybenzoate.* The NADPH concentrations were 0.05 mM (o), 0.10 mM (•), 0.15 mM (Δ) and 0.25 mM (▲). Other conditions are given under Materials and Methods. In the inset the reciprocal values of the rate constants determined from the initial (o) and final (•) slopes are plotted *vs.* the concentration of NADPH.

to NADPH where the slow modification reaction is concerned. Using the reciprocal form of equation (7) values of 74 $M^{-1}min^{-1}$ for k_2 and 39 μM for K_m^n were calculated ($30^\circ C$, pH 8.0, 3 mM p-hydroxybenzoate). K_m^n was also determined by the conventional method under the same conditions. NADPH was varied from 20 to 200 μM and a value of 37 μM for K_m^n was thus found, which agrees very well with the value given above. k_2 Of the fast phase cannot be calculated from eq. (10) without k_1.

Characterization of the modification reaction with saturating a- mounts of NADPH was not possible as the degree of saturation with NADPH at pH 8.0 cannot exceed 86%. Addition of NADPH increases the ionic strength which in its turn increases the dissociation constant of the complex [3]. An equation to calculate the dissociation constant at any ionic strength has been published [3]. Using this equation and the data from the same paper a $\frac{N}{K_d}$ vs N plot was made (not shown) which had a maximal value of 6.23 for $\frac{N}{K_d}$ at 12.8 mM NADPH. The data used were for $20^\circ C$ and as NADPH binding at higher temperature is decreased [3], the maximal degree of saturation at $30^\circ C$ will be even less than 86%.

Phenylglyoxal is known to also react with lysine and cysteine resi- dues [16]. Lysine has already been excluded as a possible point of attack [6]. Although strictly speaking cysteine is not eliminated as a site for modification by phenylglyoxal the only essential cysteine in p-hydroxy- benzoate hydroxylase was shown to be still susceptible to attack by 2- chloromercuri-4-nitrophenol.

Inactivation experiments with [7-^{14}C]phenylglyoxal showed that at least eight arginines were modified, but that the residual activity was zero after five arginines had been modified. A plot of the residual ac- tivity vs. the amount of arginines modified is given in Fig. 4. Analysis of the data according to Tsou [32] gave no decisive results as to the number of essential arginines. The data could be fitted by assuming either one, two or three essential arginine residues. The reason for this is the large number of non-essential, slow-reacting residues. Inactivation experiments with [7-^{14}C]phenylglyoxal were therefore also performed in the presence of p-hydroxybenzoate, 2',5'-ADP or both. The results are
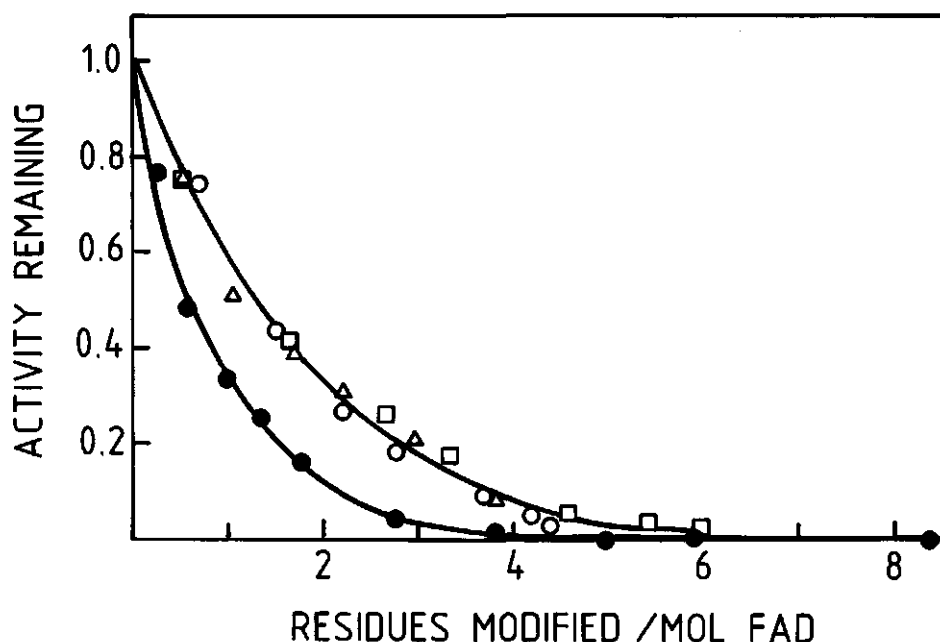
48

Figure 4. *Correlation between the number of arginine residues in p-hydroxybenzoate hydroxylase modified by phenylglyoxal and 4-hydroxy-3-nitrophenylglyoxal and the fractional activity remaining.* Samples were withdrawn at 0.05, 2, 7, 10, 20, 30, 45, 60 and 120 min. after the addition of [7-^{14}C] phenylglyoxal at 20°C (●) and at 0, 2, 10, 20, 30, 60, 90, and 120 min after the addition of 4-hydroxy-3-nitrophenylglyoxal at 22°C (o). The latter reaction was also done in the presence of 3.8 mM p-hydroxybenzoate (Δ) or 30 mM 2',5'-ADP (□). 0.1 ml Aliquots (90-100 μM p-hydroxybenzoate hydroxylase) were withdrawn from the incubation mixture and passed over a Biogel P-6DG column equilibrated with 20 mM Mes pH 6.5, I= 0.025 mM. All other conditions are given under Materials and Methods.

Table 5. *The inactivation of p-hydroxybenzoate hydroxylase by phenyl-glyoxal in the absence and presence of p-fluorobenzoate and/or NADPH, and the number of arginine residues modified.* The samples have been analyzed at various time intervals. Reaction conditions are given under Materials and Methods. The concentrations of p-fluorobenzoate and NADPH were both 25 mM.

Reaction time	Presence or absence of		Relative residual activity	Arginine residues modified/ mol protein-bound FAD
	p-FB	NADPH		
7	−	−	0.27	1.4
	+	−	0.58	1.0
	−	+	0.65	0.9
	+	+	0.89	0.7
20	−	−	0.043	2.8
	+	−	0.40	2.3
	−	+	0.57	2.0
	+	+	0.7	1.7
30	−	−	0.016	3.8
	+	−	0.28	3.1
	−	+	0.42	2.9
	+	+	0.73	2.2
45	−	−	0	5.0
	+	−	0.19	4.1
	−	+	0.33	3.8
	+	+	0.47	3.0

given in Table 5. It can be seen that the substrate and 2',5'-ADP each protect one arginine residue. As protection of either residue results in a decrease of the rate of inactivation it can be concluded that both residues are essential. This is confirmed by the quantitation experiment using the ternary complex which shows that two residues are protected. The correlation between the residual activity and the number of modified arginine residues of the free enzyme is given in Fig. 4.

The results of the experiments with phenylglyoxal can be explained by the following model. Arginine 1 and arginine 2 must both be free for 100% activity and normal NADPH and substrate binding. When either one of these two residues is modified by phenylglyoxal, p-hydroxybenzoate hydroxylase exhibits a reduced binding capacity towards NADPH and when both are modified, NADPH does not bind at all. When arginine 2 is modified there is no binding of p-hydroxybenzoate. When the NADPH binding site of the enzyme is occupied, arginine 1 is shielded and when p-hydroxybenzoate is complexed to the enzyme, arginine 2 is shielded from phenylglyoxal modification. As the pseudo-first-order rate constants of inactivation in the presence of either p-hydroxybenzoate or an NADPH analogue do not add up to give the rate constant observed for the free enzyme (see Table 3), either partial protection against phenylglyoxal modification occurs by p-OHB and NADPH with respect to arginine 1 and 2 respectively, or other arginines are also in some way protected. The latter idea is confirmed by the protection against the increase of the fluorescence upon modification by phenylglyoxal by both p-hydroxybenzoate and 2',5'-ADP.

One essential arginine at the substrate binding site has been reported for p-hydroxybenzoate from *P.desmolytica* [6]. This is in agreement with our results. In the model presented above this arginine would be arginine 2.

As the primary and tertiary structure of p-hydroxybenzoate hydroxylase is known [10-12] it would be interesting to identify arginines 1 and 2. Phenylglyoxal has been shown to be a suitable reagent for identification experiments [16]. In our case, however, radioactivity was both quantitatively and qualitatively spread identically over the HPLC chro-

matograms of the tryptic digests of each sample, whether the substrate(s) were present or not (See Materials and Methods, results not shown). Because a lot of radioactivity (30%) was lost during denaturation of the enzyme in urea, the obtained patterns cannot represent the situation as it was just after completion of the modification. Lability of modified active centre arginyl residues has been found before [33] and it was pointed out that unless conditions are particularly favourable, labeled proteins will not survive fragmentation and isolation.

Sequential modification by 2,3-butanedione and phenylglyoxal

The results of modification by 2,3-butanedione and phenylglyoxal indicate that different arginines are involved in each case. Binding experiments using p-hydroxybenzoate hydroxylase first modified by 2,3-butanedione in the presence of borate, followed by modification by phenylglyoxal in the absence of borate and subsequent removal of 2,3-butanedione were carried out to confirm this finding. No binding of either NADPH or p-hydroxybenzoate was found, while normal binding was found when the phenylglyoxal modification step was omitted. The difference in selectivity may be explained by the aromatic ring of phenylglyoxal.

Modification by 4-hydroxy-3-nitrophenylglyoxal

4-Hydroxy-3-nitrophenylglyoxal is a chromophoric reporter group found to react analogously to phenylglyoxal [14]. When inactivation studies were undertaken with concentrations of the reagent in the range from 10 to 50 mM at different pH values no pseudo-first-order kinetics were observed. This can be explained by Borders observation that 4-hydroxy-3-nitrophenylglyoxal undergoes a spontaneous acid-releasing process when maintained at pH 8 or above [14]. The pH of a 50 mM reagent solution in 20 mM Hepps pH 8.5 was indeed found to decrease to 7 within 5 hours. Kinetic constants can therefore not be given. Under the conditions given under Materials and Methods, enzymatic activity decreased to 8 and 3% after 1 and 2 hours, respectively. The presence of ligands, however, was of very little influence on the half time of the reaction,

Table 6. *Half times ($t_{\frac{1}{2}}$) of inactivation of p-hydroxybenzoate hydroxylase by 4-hydroxy-3-nitrophenylglyoxal in the presence of several ligands.* The concentrations of the enzyme and the reagent were 2 μM and 10 mM, respectively. Other conditions are given under Materials and Methods.

Ligand	Concentrations (mM)	$t_{\frac{1}{2}}$ (min)
none	--	10
p-OHB	3	15
p-FB	25	15
NADPH	20	15
Cibacron blue	0.1	10
p-FB and NADPH	25 and 20	35
p-OHB and Cibacron blue	3 and 0.1	40

as can be seen in Table 6. It can also be seen that better protection is found when both the substrate and NADPH binding site are occupied.

Determination of the amount of modified arginines was performed in the absence and presence of p-hydroxybenzoate or 2',5'-ADP. Residual activity as a function of the number of arginine residues modified is shown in Fig. 4. It is clear that the presence of either p-hydroxybenzoate or 2',5'-ADP has no influence, in contrast with analogous experiments using phenylglyoxal. When the same samples were used to check p-hydroxybenzoate and NADPH binding it was found that binding of both compounds was reduced by a factor of 2 to 3, again independent of whether p-hydroxybenzoate or 2',5'-ADP were present during modification. This means that inactivation by 4-hydroxy-3-nitrophenylglyoxal does not involve the same arginines as are involved in inactivation by phenylglyoxal. This was confirmed by a differential sequential labelling experiment. In the first step p-hydroxybenzoate hydroxylase was modified by phenylglyoxal in the absence or presence of both p-hydroxybenzoate and Cibacron blue. After

$2\frac{1}{2}$ minutes the reagent and the ligands were removed leaving the partly inactivated, free enzyme. The activities of the enzyme samples modified in the absence or presence of the ligands were 10 and 75% respectively. The number of arginines that could still be modified by 4-hydroxy-3-nitrophenylglyoxal however, was 3.4 in both cases.

The difference in selectivity and reactivity between phenylglyoxal and 4-hydroxy-3-nitrophenolglyoxal may be explained by the fact that the latter has a negative charge due to the deprotonation of the phenolic hydroxyl group (pK_a = 4.8 at $25^{O}C$ [14]).

From analysis of the data in Fig. 4 according to Tsou [32] it was found that one of the six arginines that were modified is essential and that the other five are three times less reactive. As this essential arginine is not involved in either p-hydroxybenzoate and NADPH binding it must be of some importance during catalysis itself. Here again it may well be the negative charge of 4-hydroxy-3-nitrophenylglyoxal that causes the enzyme to become inactive in one of its reactions of catalysis.

ACKNOWLEDGEMENTS

54

REFERENCES

[1] Husain, M., Entsch, B., Ballou, D.P., Massey, V. and Chapman, P.J. (1980) J.Biol.Chem. *255*, 4189-4197 (and references therein).

[2] Nakamura, S., Ogura, Y., Yano, K., Higashi, N. and Arima, K. (1970) Biochemistry *9*, 3235-3242.

[3] Wijnands, R.A., Van der Zee, J., Van Leeuwen, J.W., Van Berkel, W.J.H. and Müller, F. (1984) Eur.J.Biochem. *139*, 637-644.

[4] Shoun, H., Higashi, N., Beppu, T., Nakamura, S., Hiromi, K. and Arima, K. (1979) J.Biol.Chem. *254*, 10944-10951.

[5] Shoun, H., Beppu, T. and Arima, K. (1979) J.Biol.Chem. *254*, 899-904.

[6] Shoun, H., Beppu, T. and Arima, K. (1980) J.Biol.Chem. *255*, 9319-9324.

[7] Shoun, H. and Beppu, T. (1982) J.Biol.Chem. *257*, 3422-3428.

[8] Wijnands, R.A. and Müller, F. (1982) Biochemistry *21*, 6639-6646.

[9] Van Berkel, W.J.H., Müller, F., Weijer, W.J., Jekel, P.A. and Beintema, J.J. (1984) Eur.J.Biochem., in press.

[10] Wierenga, R.K., De Jong, R.J., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1979) J.Mol.Biol. *131*, 55-73.

[11] Weijer, W.J., Hofsteenge, J., Vereijken, F.M., Jekel, P.A. and Beintema, J.J. (1982) Biochim.Biophys.Acta *704*, 385-388.

[12] Weijer, W.J., Hofsteenge, J., Beintema, J.J., Wierenga, R.K. and Drenth, J. (1983) Eur.J.Biochem. *133*, 109-118.

[13] Wierenga, R.K., Kalk, K.H., Van der Laan, J.M., Drenth, J., Hofsteenge, J., Weijer, W.J., Jekel, P.A. Beintema, J.J., Müller, F. and Van Berkel, W.J.H. (1982) in Flavins and Flavoproteins (Massey, V. & Williams, C.H., eds) pp. 11-18, Elsevier/North Holland, New York.

[14] Borders, C.L., Pearson, L.J., McLaughlin, A.E., Gustafson, M.E., Vasiloff, J., An, F.Y. and Morgan, D.J. (1979) Biochim.Biophys. Acta *568*, 491-495.

[15] Müller, F., Voordouw, G., Van Berkel, W.J.H., Steennis, P.J. Visser, S. and Van Rooijen, P.J. (1979) Eur.J.Biochem. *101*, 235-244.

[16] Takahashi, K. (1968) J.Biol.Chem. *243*, 6171-6179.

[17] Benesi, H.A. and Hildebrand, J.H. (1949) J.Am.Chem.Soc. *71*, 2703-2707.

[18] Ehrlich, R.S. and Colman, R.F. (1978) Eur.J.Biochem. *89*, 575-587.

[19] Tsou, C.L. (1965) Sheng Wu Hua Hsueh Yu Sheng Wu Wu Li Hsueh Pao *5*, 398-408, 409-417.

[20] Tian, W.X. and Tsou, C.L. (1982) Biochemistry *21*, 1028-1032.

[21] Yankeelov, J.A.Jr. Kochert, M., Page, J. and Westphal, A. (1966) Fed.Proc. *25*, 590.

[22] Riordan, J.F. (1973) Biochemistry *12*, 3915-3923.

[23] Gripon, J.C. and Hofmann, T. (1981) Biochem.J. *193*, 55-65.

[24] Roy, G.L., Laferriere, A.L. and Edwards, J.O. (1957) J.Inorg. Nucl. Chem. *4*, 106-110.

[25] Riordan, J.F. (1979) Mol.Cel.Biochem. *26*, 71-92.

[26] Howell, L.G. and Massey, V. (1970) Biochem.Biophys.Res.Commun. *40*, 887-893.

[27] Hesp, B., Calvin, M. and Hosokawa, K. (1969) J.Biol.Chem. *20*, 5644-5655.

[28] Ray, W.J. and Koshland, D.E. (1961) J.Biol.Chem. *236*, 1973-1979.

[29] Steennis, P.J., Cordes, M.M., Hilkens, J.G.H. and Müller, F. (1973) FEBS Lett. *36*, 177-180.

[30] Müller, F., Van Berkel, W.J.H. and Steennis, P.J. (1983) Biochem. Int. *8*, 115-122.

[31] Howell, L.G., Spector, T. and Massey, V. (1972) J.Biol.Chem. *247*, 4340-4350

[32] Tsou, C.L. (1962) Sci.Sin. (Engl.Ed.) *11*, 1535-1558.

[33] Jörnvall, H., Lange, L.G.Riordan, J.F. and Vallee, B.L. (1977) Biochem.Biophys.Res.Commun. *77* 73-78.

56

Chapter 4   Chemical modification of tyrosine residues

SUMMARY

Tyrosine residues were modified by diethylpyrocarbonate and by p-diazobenzoate. Both modifications led to loss of enzymic activity due to reactions with tyrosine residues in the substrate binding site, but different tyrosine residues were involved in each case. Tyr-201 is most probably involved in inactivation by p-diazobenzoate as shown by sequence studies on the modified enzyme. Judging from X-ray data of the enzyme-substrate complex, a reaction with this particular tyrosine residue is possible if p-diazobenzoate is bound exactly like p-hydroxybenzoate. Tyr-222, which is modified both in the absence and presence of the effector p-fluorobenzoate, was shown to be non-essential and cannot therefore be involved in acid-base catalysis.

INTRODUCTION

p-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is a flavoprotein belonging to the class of monooxygenases. It catalyzes the hydroxylation of p-hydroxybenzoate in the presence of molecular oxygen using NADPH as an electron donor. The reaction cycle can be divided into a reductive phase and an oxidative phase. Both phases have been studied using rapid reaction techiques [1,2]. In the reductive phase NADPH and substrate bind randomly to the enzyme, the flavin is reduced and $NADP^+$ dissociates. In the oxidative phase molecular oxygen binds, the substrate is hydroxylated and then the product dissociates. From isotopic effects it was concluded that general acid-base catalysis is involved in the oxidative phase [3].

From the correlation between the rate of reduction of the enzyme-bound flavin and the pK_a of anion formation in the para position of the substrate it was concluded that the reduction of the flavin of p-hydroxy-

benzoate hydroxylase is channeled through a ternary complex containing the dianion of the substrate [4]. This interpretation is in contradiction with pH dependence studies on the enzyme from *Pseudomonas desmolytica* showing that the maximum rate of reduction of the oxidized enzyme-substrate complex with NADPH is almost constant in the pH range from 5.8 to 9.1 [5]. Moreover the binding of the substrate to the oxidized enzyme in this pH region was shown to be influenced by two ionizations: ionization of the phenolic group of the bound substrate with a pK_a value of 7.1 and ionization of a basic residue with a pK_a value of 8.2 [6]. As the constants of the free substrate and the basic residue in the free enzyme are 9.3 [7] and 7.03 [6] respectively, the binding of substrate would lead to large pK_a shifts. The two ionization equilibria were believed to reflect on the pH dependence of the K_m for NADPH in the reduction of the oxidized enzyme-substrate complex and not on the reduction rate itself like mentioned above.

In this paper our main interest is the identification of the basic residue that seems to be involved in substrate binding and/or the catalytic reaction. Histidine has been suggested, based on the pK_a value and photooxidation experiments [6]. Chemical modification studies in our laboratory, however, indicate that tyrosine is a more probable candidate [8,9]. The three dimensional structure of the enzyme-substrate complex as determined by X-ray experiments indeed shows three tyrosine and no histidine residues in the active site [10-12]. Preliminary experiments also strongly indicated that at least one of these tyrosine residues plays a role in the catalyis by the enzyme. Therefore chemical modification of tyrosine residues by diethylpyrocarbonate and p-diazobenzoate was performed. It is shown that different tyrosine residues are modified by these two reagents but that both modifications lead to loss of enzymic activity. The tyrosine residue modified by p-diazobenzoate is tentatively assigned in the sequence.

MATERIALS AND METHODS

p-Amino[^{14}C-carboxyl] benzoic acid (58.5 mCi/mmole) was from the
Commissariat a l'énergie atomique (Saclay, France), diethylpyrocarbonate
was from Fluka, 2-chloromercuri-4-nitrophenol was from Eastman, NADPH
was from Boehringer, Mes, Hepes, Tris and p-chloromercuribenzoic acid
were from Sigma and all other chemicals were from Merck.

p-Diazobenzoate was prepared freshly before each experiment as was
described by Riordan and Vallee [13], giving a final concentration of
either 20 or 40 mM diazotized p-aminobenzoate. To obtain a 20 mM solu-
tion of p-diazobenzoate, 1 ml of a 0.72 M solution of sodium nitrite was
slowly added to 20 ml of a stirred 25 mM solution of p-aminobenzoate
in 0.15 M HCl at 0-4°C. After 15 min the pH of the solution was careful-
ly brought to a value between 5 and 6 with 5 M NaOH. After the addition
of 1 ml of a 0.72 M solution of urea the final volume was brought to 25
ml with water. When a 40 mM solution of p-diazobenzoate was needed, the
same additions were done to 10 ml of a 50 mM solution of p-aminobenzoate
in 0.3 M HCl, with a final volume of 12.5 ml. For the preparation of a
40 mM p-diazo[^{14}C-carboxyl]benzoate solution the above was scaled down
by a factor of 20. The specific activity of the p-amino[^{14}C-carboxyl]
benzoate was decreased to 1 mCi/mmole by adding cold p-aminobenzoate.
The stability of p-diazobenzoate was measured by taking 20 µl time sam-
ples of 0.2 or 2 mM solutions in the appropriate buffer and adding them
to 1 ml of a 4 mM p-hydroxybenzoate solution in 0.1 M Tris/HCl pH 8.8.
The absorbance at 440 nm after 5 minutes was used directly as a measure
of the concentration of p-diazobenzoate.

Spectrophotometric measurements were done on a Zeiss PMQII, a Cary
16 or an Aminco DW2A spectrophotometer and fluorometric measurements on
an Aminco SPF-500 spectrofluorometer. Radioactivity measurements were
carried out using Pico-fluor^{TM} 30 from United Technologies Packard and
a Packard Tri-carb 2450 liquid scintillation spectrometer.

p-Hydroxybenzoate hydroxylase was purified from *Pseudomonas fluores-*
cens as described previously [14]. The purified enzyme was kept as an

ammonium sulphate precipitate (70%) at 4°C pH 6-7. Fresh solutions of the enzyme in the appropriate buffer were prepared each day by gel filtration over Biogel P-6DG. The enzyme concentration was determined spectrophotometrically on the basis of the FAD content by assuming a molar absorption coefficient of 11300 $M^{-1}cm^{-1}$ at 450 nm [14]. The enzyme activity was assayed by adding 8 µl of a 2-3 µM enzyme solution to 0.8 ml of 0.1 M Tris-H_2SO_4, pH 8.0 containing 0.15 mM p-hydroxybenzoate and 0.15 mM NADPH. The activity was determined from the decrease of absorbance at 340 nm at 25°C.

Dissociation constants of the binary complexes between the enzyme and the NADPH or the substrate were determined fluorometrically or spectrophotometrically as published before [9,15].

Azo coupling was performed in 40 mM Hepes pH 7.0 at 0-4 °C, unless stated otherwise. At low enzyme concentrations (< 20µM) 0.2 mM p-diazobenzoate was added from a freshly prepared stock solution of 20 mM and at higher enzyme concentration (preparative experiments) four portions of 1.25 mM p-diazobenzoate were added at 15 minutes time intervals. Time samples were taken to check activity. The reaction was stopped by passing the mixture over Biogel P-6DG equilibrated with the appropriate buffer. For quantitation experiments the latter was also done with time samples.

The number of residues that were modified by p-diazobenzoate was determined spectrophotometrically and also by incorporation of p-diazo-[^{14}C-carboxyl]benzoate. An ε value of 20,000 at 325 nm (pH < 7) was used for monoazotyrosine [16]. Identification of the modified tyrosines was performed by sequencing the radioactive peptides of the tryptic digest as has been described previously [17,18].

Modification of p-hydroxybenzoate hydroxylase by N-ethylmaleimide, p-chloromercuribenzoate and diethylpyrocarbonate was performed as has been described earlier [18,9] and modification by 2-chloromercuri-4-nitrophenol was performed by adding 50 µM of this reagent to a 10 µM solution of azoenzyme in 40 mM Hepes pH 7.0. The reaction of the 2-chloromercuri-4-nitrophenol sensitive cysteine was followed fluorometrically at the emission wavelength of the flavin, which is 525 nm (excitation

at 450 nm).

FPLC analysis was performed with Pharmacia Fine Chemicals (Uppsala, Sweden) equipment using Mono Q as an anion exchange bed.

RESULTS

Chemical modification of tyrosines can be achieved either by a nucleofilic attack on the phenolate group or an electrofilic attack on the aromatic ring, ortho to the hydroxyl function. The most widely used nucleofilic reagent for tyrosine is N-acetylimidazol and the most widely used electrofilic reagents are iodine and tetranitromethane [19]. In two preceding papers we have shown that tyrosine residues in p-hydroxybenzoate hydroxylase are modified by diethylpyrocarbonate at pH 8, which is accompanied by an increase of the fluorescence of the protein-bound flavin [8,9]. We have reinvestigated the tyrosine modification at pH 7.5 and it was found that the increase of the fluorescence was biphasic. The two pseudo-first order rate constants using 0.9 mM diethylpyrocarbonate at $4^{o}C$ were 0.40 min^{-1} and 0.36 min^{-1}. In the presence of an effector however, only the slow reaction was observed. Whereas the substrate binding capacity of the enzyme was lost after modification of the free enzyme at pH 8, this was not the case when the enzyme was modified in the presence of an effector i.e., p-fluorobenzoate. It is therefore concluded that the fast phase of the increase of the fluorescence is directly related to the modification of a residue involved in substrate binding.

Reactivation by 0.1 M hydroxylamine at pH 6 or 8 was possible, but much slower than reactivation of the enzyme carbethoxylated at pH 6 reported previously [9]. Whereas reactivation of the enzyme carbethoxylated at pH 6 was 92% after $2\frac{1}{2}$ h [9], reactivation of the enzyme carbethoxylated at pH 8 was 80% after 18 h. The only other amino acid that can be decarbethoxylated besides histidine is tyrosine. O-Carbethoxy-N-acetyltyrosineethylester was reported to be four times less reactive with hydroxylamine than N-carbethoxyimidazole [20]. As spectral changes after modification by diethylpyrocarbonate indicated that tyrosines were

indeed modified [9] it can also be concluded that the residue involved in substrate binding mentioned above is a tyrosine residue.

As carbethoxylated tyrosine is not stabile under the conditions needed for protein hydrolysis another approach was needed to identify this tyrosine residue. Considering the fact that the aim of this study is associated with the phenolic groups of essential tyrosines, N-acetylimidazole would be the most obvious reagent to use. However, there is one reagent, which, although it is electrophilic, is far more suitable. The reagents mentioned above could react with any tyrosine that is accessible. As monomeric p-hydroxybenzoate hydroxylase contains 17 tyrosines [21] this will not favour clarity. p-Diazobenzoate, however, is a substrate analogue and although it is not very specific for tyrosines in general, this is a big advantage. It is derived from p-aminobenzoate, which acts as an effector of the enzyme so it is very likely that it is bound to the enzyme in a similar way as the substrate. If complex formation precedes the electrophilic substitution, the reaction conditions need not be optimal and if reaction conditions are not optimal reactions at other sites than the active site are less probable. Optimal conditions for azo coupling are a compromise between favoured acidic conditions for the existence of the diazotized species that is able to couple and favoured alkaline conditions for the existence of the ionized phenolic group of tyrosine. In practice this means a pH value of 9. By working at pH 7, tyrosines outside the active centre probably will not react while tyrosines inside the active center of which at least one is believed to have a relatively low pK_a value [8,9] should.

When azo coupling of p-diazobenzoate to p-hydroxybenzoate hydroxylase is carried out at pH 7 the enzyme is slowly inactivated. The rate of inactivation is dependent on the p-diazobenzoate concentration and the pH as can be seen in Fig. 1A. The reaction does not obey first order kinetics, which can be explained by the instability of the reagent, which is demonstrated in Figs. 1B and 1C. p-Diazobenzoate is hydrolysed to p-hydroxybenzoate under neutral and alkaline conditions, which in turn can react with the remaining p-diazobenzoate forming 3-benzoazo-4-hydroxy-
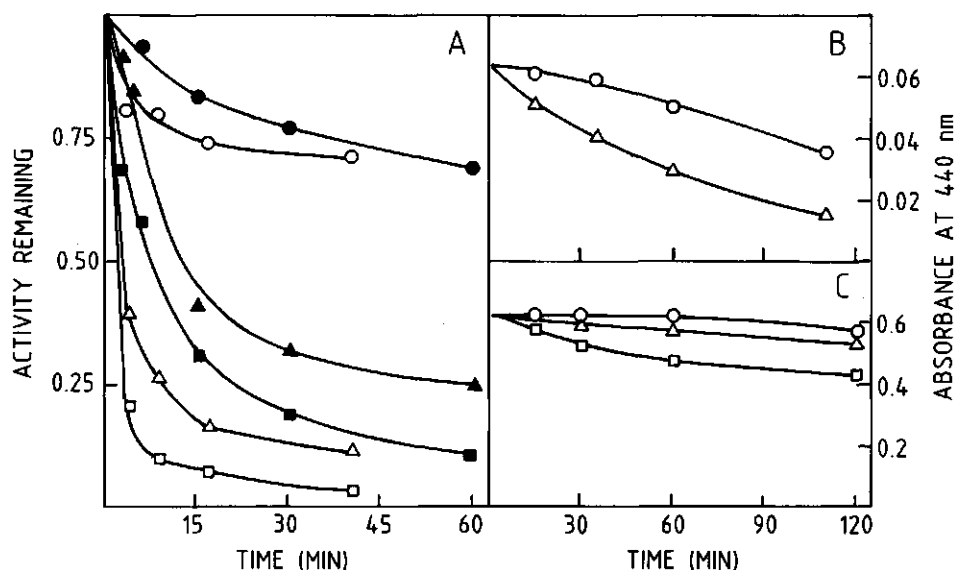
62

Fig. 1. *Inactivation of p-hydroxybenzoate hydroxylase by and stability of p-diazobenzoate*. (A) Azocoupling took place at pH 7 (filled symbols) and at pH 8.7 (open symbols) with 0.018 (o), 0.091 (▲) and 0.18 (■) mM p-diazobenboate. The enzyme concentration was 2 µM. The stability of 0.2 mM (B) and 2 mM (C) diazobenzoate solutions was determined at pH 7 (o), pH 8 (Δ) and pH 8.5 (□). The temperature was 0-4°C and all other conditions are given under Materials and Methods.

benzoate [19]. Time-dependent changes in the absorption spectrum of the enzyme upon azo coupling at pH 7 are shown in Fig.2. These difference spectra correspond well with the spectrum of the monoazochloroacetylty-rosine derivative of p-diazobenzoate [16]. Azocoupling to histidine does not seem to occur under these conditions. Not even a shoulder is found at 370 nm, the maximum wavelength of absorbance of the monoazohistidine derivatives with an ε value of 21100 [16]. Carbethoxylation of the enzyme with diethylpyrocarbonate at pH 6 [9] did indeed not reveal any protection against inactivation by p-diazobenzoate (data not shown). The same was found for the p-chloromercuribenzoate modified enzyme [18]. Moreover, the only essential cysteine was still accessible for 2-chloromercuri-4-nitrophenol as could be seen by the decrease of the fluorescence of the enzyme-bound flavin (data not shown). FPLC analysis (anion exchange
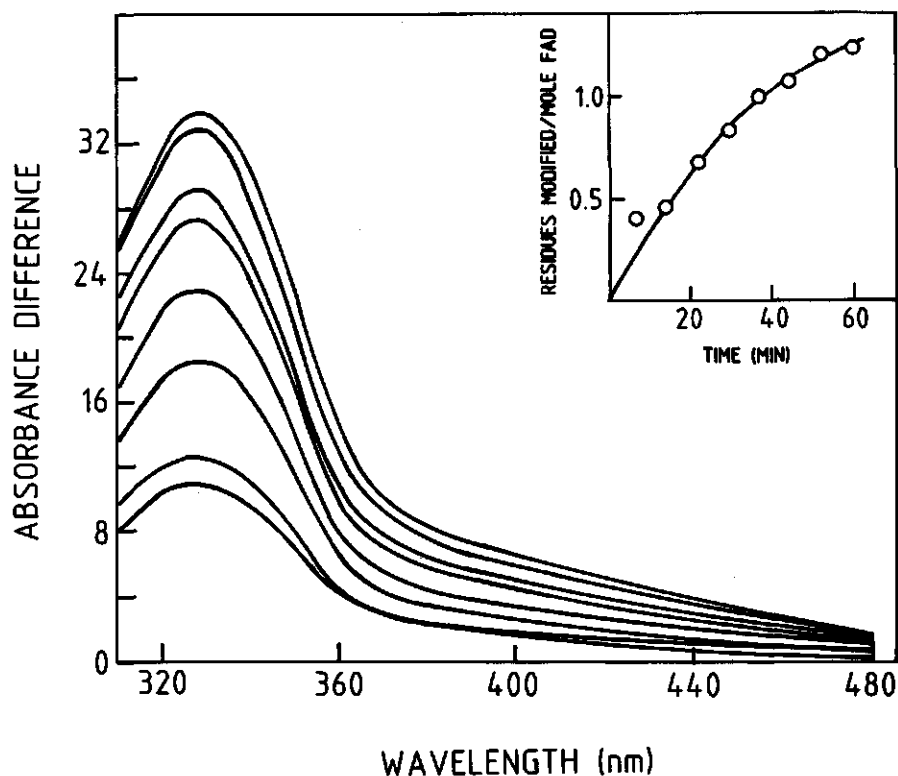
63

Fig. 2. *Time dependent difference spectra of azocoupled p-hydroxybenzoate hydroxylase and the native enzyme.* The modification was performed as described under Materials and Methods with a 0.2 mM enzyme solution. Time samples were taken at 7.5 min intervals between 7.5 and 60 min after the first addition of p-diazobenzoate. The enzyme concentration in the cuvet was 12.8 μM in each case.

column) of the azo coupled enzyme revealed two new peaks. Enzymic activity and absorbance at 320 nm of these two fractions were the same as those of the fraction with a peak coinciding with the peak of the starting material (unmodified enzyme). When N-ethylmaleimide modified enzyme was used for the azo coupling reaction, no new peaks were found. As N-ethylmaleimide reacts specifically with Cys-116 [17,18] it was concluded that p-diazobenzoate also reacts with Cys-116 at pH 7. This cysteine residue was shown to be of no importance for enzymic activity [14] but for quantitation experiments using p-diazo[14C-carboxyl] benzoate it was decided to prelabel the enzyme with N-ethylmaleimide. A fourth amino

acid that is able to react with diazonium salts in general is lysine
[22-24]. Like the azo coupling of sulfhydryls, the azo coupling of free
amines does not interfere with spectral analysis due to the low molar
absorbance of the product [24]. As the degree of tyrosine modification
determined spectrophotometrically coincides with the degree of modifica-
tion of N-ethylmaleimide modified p-hydroxybenzoate hydroxylase with
p-diazo[^{14}C-carboxyl]benzoate (see below) it is concluded that no lysines
react under the conditions given. From the above it is clear that when
Cys-116 is protected by N-ethylmaleimide, only tyrosines in the enzyme
react with p-diazoniumbenzoate at pH 7. Although it is possible that bis-
azotyrosines are formed it has been reported that this reaction does not
occur easily in proteins due to steric constraints [24]. This was con-
firmed for the case of p-hydroxybenzoate hydroxylase by the absorbance
spectrum of the modified enzyme at pH 12, exhibiting an absorption band
only at 490 nm. Since no absorbance at 550 nm, was observed it can safely
be concluded that no bisazoderivative is formed in the enzyme [16].

The benzoazotyrosines in the enzyme could not be reduced to 3-amino-
tyrosines with dithionite or borohydride as no loss of absorbance at 325
nm was found upon the addition of either of these reagents.

Carbethoxylation of the enzyme with diethylpyrocarbonate at pH 8
[8,9] did not reveal any protection against inactivation by p-diazobenzo-
ate. Decarbethoxylation of the carbethoxylated enzyme with 0.1 M hydroxyl-
amine gave 80% reactivation within eighteen hours, whereas decarbethoxy-
lation of the enzyme which had been azo-coupled after modification by di-
ethylpyrocarbonate was not accompanied by reactivation at all. It is
therefore concluded that different tyrosine residues are involved in
these modification reactions.

When p-hydroxybenzoate hydroxylase was inactivated by p-diazobenzoate,
the dissociation constant of the azoenzyme-NADPH complex was 0.14 mM at
pH 7 and I= 20 mM. As the K_d value of the native enzyme-NADPH complex is
also 0.14 mM [15], it is concluded that the inactivation due to modifi-
cation of tyrosines is not caused by a loss of the ability of the enzyme
to bind NADPH. p-Hydroxybenzoate, however, did not bind to its primary

binding site in the azoenzyme as only binding to the secondary binding site [1] could be detected after inactivation. Benzoate and p-fluorobenzoate were not able to bind either. Protection experiments in the pre-
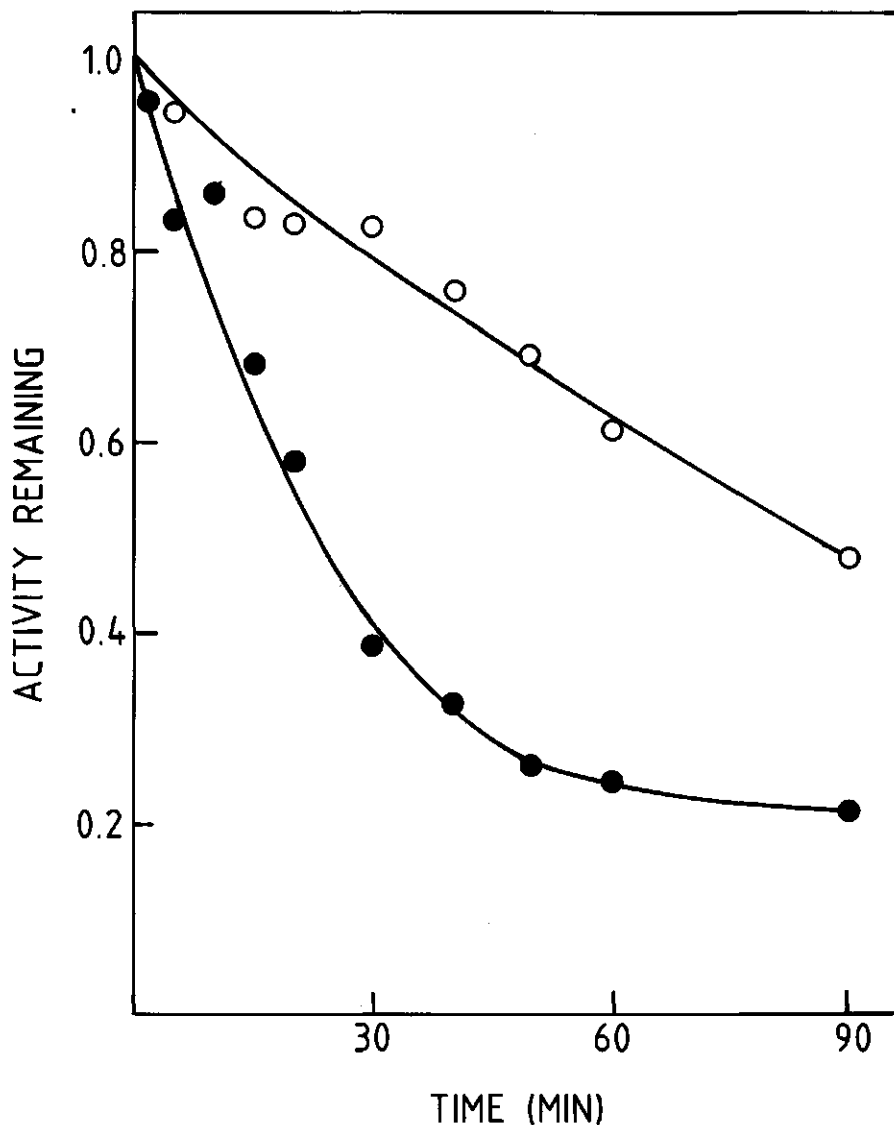

Fig. 3. *Protection by p-fluorobenzoate against inactivation of p-hydroxybenzoate hydroxylase by p-diazobenzoate.* The modification was performed with 0.2 mM p-diazobenzoate in the presence (o) and absence (●) of 20 mM p-fluorobenzoate at pH 7 (0-4°C). The enzyme concentration was 2 μM.

sence of p-hydroxybenzoate are impossible due to its reactivity with p-diazobenzoate. We found it to be $2\frac{1}{2}$ times as reactive as N-acetyltyrosine-ethylester at pH 8.8 (25 $^{\circ}$C). This is of course due to the lower pK_a value of the phenolic group of p-hydroxybenzoate (9.3) compared to that of tyrosine (10.1) [7]. Instead, therefore p-fluorobenzoate was added to the reaction mixture during inactivation by p-diazobenzoate. From Fig.3 it can be seen that indeed some protection against inactivation occurs.

To determine the number of essential tyrosines that are modified by p-diazobenzoate time samples were taken during inactivation and monitored for residual activity and the extent of chemical modification. In one experiment the latter was determined spectrophotometrically (Fig. 2) and in another with the aid of p-diazo[^{14}C-carboxyl]benzoate. As has already been explained above, N-ethylmaleimide labeled p-hydroxybenzoate hydroxy-
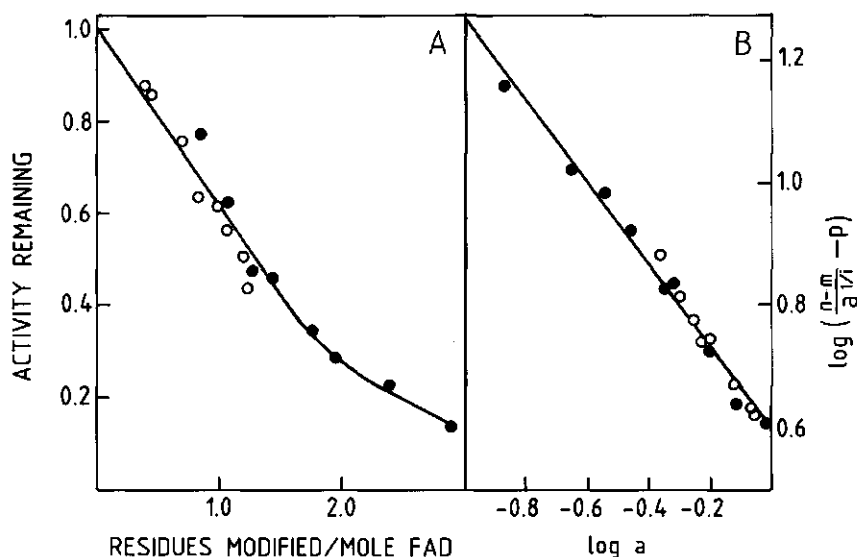

Fig. 4. *Correlation between the residual activity and the amount of residues modified by p-diazobenzoate.* The results of two separate experiments are shown. In the first experiment (o) m was determined spectrophotometrically (see Fig. 2) and in the second (•) it was determined by the incorporation of ^{14}C. Conditions of the first experiment are given under Fig. 2. The enzyme concentration during the second modification experiment was 0.1 mM. 4.9 nmoles of p-hydroxybenzoate hydroxylase were used for counting. All other data are given under Materials and Methods. The results are plotted direcly (A) and according to the equation given in the text (B). The meaning of the symbols is also given in the text.

lase was used in the case of radioactive chemical modification. The correlation between the residual activity (a) and the number of residues (m) that are modified is shown in Fig. 4A. It can be seen that the number of tyrosine residues modified coincides with the amount of ^{14}C incorporated. It has been shown by Tsou [25] that 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 equation is valid:

$$\log \frac{n-m}{a^{1/i}} - p = \log (n-p) + \frac{\alpha-1}{i} \log a$$

where n is the total number of residues that is modified. A straight line is obtained when $\log (n-m)/a^{1/i} - p$ is plotted against log a. In Fig. 4B the data of Fig. 4A are plotted in this manner with n=5 and p=i=1, which was the best fit possible to a straight line. An α value of 0.32 was determined from the slope.

The samples that were analysed spectrophotometrically were also used to determine the maximal amount of quenching of the fluorescence of the enzyme-bound FAD that could be induced by p-hydroxybenzoate. It can be seen in Fig. 5 that the decrease in maximal quenching is correlated directly to inactivation. When a quantitation experiment, comparing inactivation and ^{14}C incorporation in the absence and presence of p-fluorobenzoate, was performed a difference of 0.8 tyrosine residues per mol FAD was found (Table 1).

It is therefore concluded that inactivation is caused by the inability of the enzyme to bind its substrate, which in turn is caused by azo coupling of p-diazobenzoate to one essential tyrosine residue. To identify this tyrosine residue the in the absence and presence of p-fluorobenzoate azo coupled enzyme samples were denatured in 6 M urea and subjected to trypsine digestion. Quite surprisingly, however, most of the radioactive label (80%) was lost during denaturation. An identical observation was made in an earlier attempt to identify the tyrosine residue using the absorption maximum of the azo coupled product as a monitor. When the labeled peptides were isolated by reversed-phase HPLC, most of the maining label in the case of the azo coupled free enzyme was found
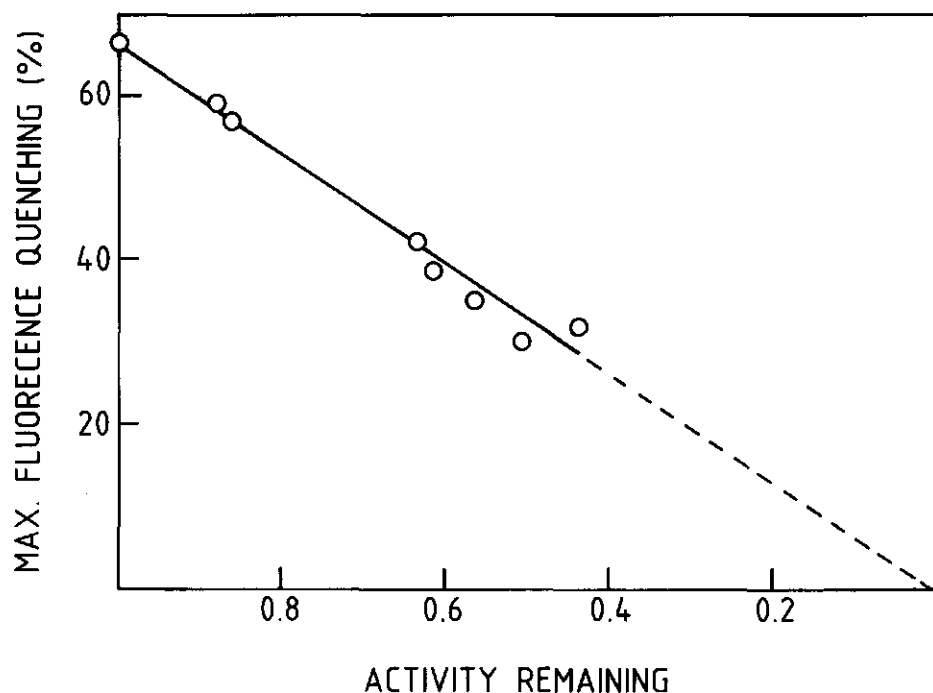
68

Fig. 5. *Correlation between the maximal quenching of the fluorescence of enzyme-bound FAD by p-hydroxybenzoate and the residual activity after azo-coupling.* All conditions are given under Fig. 2 and under Materials and Methods.

Table 1. *Inactivation of p-hydroxybenzoate hydroxylase by diazo[^{14}C-carboxyl]benzoate and incorporation of ^{14}C in the absence and presence of p-fluorobenzoate.* 175 Nmoles of enzyme in the absence and presence of p-fluorobenzoate were first labelled with N-ethylmaleimide and then modified by diazo[^{14}C-carboxyl]benzoate for identification experiments (see text). During the coupling the concentrations of enzyme and p-fluorobenzoate were 90 μM and 20 mM respectively. Both reactions were stopped after 2 hours. All other details are given under Materials and Methods. Only 4.6 nmoles of each sample were used for quantitation and activity measurements.

p-fluorobenzoate	relative enzymic activity	Tyr residues modified per mole FAD
–	0.25	2.0
+	0.60	1.2

69

in a peptide containing Tyr-222. Little radioactivity was found in a peptide containing Tyr-201. In the case of the enzyme azo coupled in the presence of p-fluorobenzoate, the only difference found was 50% less radioactive label in the peptide containing Tyr-201. It is therefore concluded that the loss of enzymic activity upon azo coupling of p-diazobenzoate to p-hydroxybenzoate hydroxylase is caused by modification of Tyr-201.

DISCUSSION

Although diethylpyrocarbonate is a widely used reagent for the modification of histidine residues [26], it is also known to react with tyrosine residues [27]. The two reactions can be discriminated by the different wavelengths at which the change of absorbance occurs upon carbethoxylation [27]. When histidine residues are carbethoxylated, there is an increase of absorbance at 240 nm ($\varepsilon = 3200 \; cm^{-1}M^{-1}$) [28] and when tyrosine residues are modified, there is a decrease at 278 nm ($\varepsilon = 1310 \; cm^{-1}M^{-1}$) [30]. Although sulfhydryl, arginyl, α-amino and ε-amino groups are also reactive [27], only the reactions with tyrosine and histidine are known to be reversible [20].

Diazonium salts have been shown to be useful reagents in structure-function relationship studies [30,31]. Although diazonium compounds were shown to couple to $N(\alpha)$-acetylarginine, -proline and -tryptophan as well [22,23], a more recent study revealed that protein reactivity to azo coupling can be attributed to tyrosine, histidine, cysteine and lysine residues [24]. In the case of p-hydroxybenzoate hydroxylase we have shown above that at pH 7 coupling only occurs to Cys-116 and several tyrosine residues including Tyr-201 and -222 in the active site. Coupling to either histidine or lysine residues could be excluded. Attempts to restore activity by adding either sodium dithionite or sodium borohydryde to reduce the benzoazotyrosines to 3-aminotyrosines were unsuccessful as no reduction took place. Although reduction is expected to take place its failure has been reported as well [30].

The finding that azocoupling to Cys-116 gave rise to two new peaks when passed over an anion exchange column can be explained by our earlier finding that p-hydroxybenzoate hydroxylase exists mainly as a dimer [14]. The first peak would then account for a dimer with one of its cysteines modified and the second peak for a dimer with both subunits modified at Cys-116. Apparently the change of charge that occurs when the tyrosines are modified is not sufficient to separate the modified enzyme from the native enzyme by anion exchange chromatography. Isoelectric focussing experiments gave the same results (data not shown).

The graphical method for the determination of the number of essential residues modified introduced by Tsou [25] has again proved to be very useful. The true number of residues that actually reacts is not known exactly, because there may be many residues that react very slowly and are thus not seen. Although nearly all activity was lost after 2-3 tyrosine residues had been modified (Fig. 4A) a minimum amount of five was able to explain our data. Higher values for n merely result in lower values for α, as α can also be regarded as an average value of α for each reacting residue. Extension of the basic model with only two types of residues reacting with a rate constant k or αk to a more general case where modifiable residues can be divided into more than two types on the basis of their reactivities has been published [32].

The result of differential labeling in the presence and absence of p-fluorobenzoate is in good agreement with the results of the graphical method. Only one of the modified tyrosine residues is essential for enzymic activity and can be protected from modification by a ligand in the substrate binding site. Based on the three dimensional structure of the substrate binding site it can be seen that the para position of the substrate is close to Tyr-201 and Tyr-385 [12], so it may be expected that one of these two residues is the modified essential residue. Although the results of the identification experiments may be disputable because of the large amount of label lost during denaturation, Tyr-201, was identified as the residue in question.

It has been concluded above that different essential active site tyrosine residues are involved in modification by p-diazobenzoate and diethylpyrocarbonate. As inactivation by p-diazobenzoate was ascribed to the modification of Tyr-201, inactivation by diethylpyrocarbonate under alkaline conditions must involve the modification of Tyr-385. Due to the lability of O-carbethoxytyrosine this cannot be proved unambiguously.

As Tyr-222 was modified equally well by p-diazobenzoate in the presence and absence of the effector p-fluorobenzoate it cannot be involved in substrate binding and because only one of the modified tyrosines was shown to be essential it cannot be involved in any other step of the enzymic reaction either. Any role in the acid-base mechanism proposed by Entsch *et al.*[3] can therefore be excluded. The only candidates left for the postulated residue are now Tyr-201 and Tyr-385.

Tyr-222 is situated near the carboxyl binding site of p-hydroxybenzoate hydroxylase [12]. Modification of this residue must therefore take place at a stage where p-diazobenzoate is not properly complexed to the substrate binding site. This is in agreement with the finding that p-fluorobenzoate does not protect against the azo coupling to this residue.

ACKNOWLEDGEMENTS

REFERENCES

[1] Husain, M. & Massey, V. (1979) J.Biol.Chem. *254*, 6657-6666.
[2] Hiromi, K. (1979) Kinetics of Fast Enzyme Reactions. Theory and Practice pp. 287-320, Halsted Press Book.

[3] Entsch, B., Ballou, D.P. and Massey, V. (1976) J.Biol.Chem. *251*, 2550-2563.

[4] Entsch, B., Ballou, D.P., Husain, M. & Massey, V. (1976) J.Biol. Chem. *251*, 7367-7379.

[5] Shoun, H., Higashi, N., Beppu, T., Arima, K. and Nakamura, S. (1974) FEBS Lett. *49*, 13-17.

[6] Shoun, H. Beppu, T. and Arima, K. (1979) J.Biol.Chem. *254*, 899-904.

[7] Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969) Data for Biochemical Research, pp. 82-83, Oxford University Press, Oxford.

[8] Wijnands, R.A. and Müller, F. (1982) in Flavins and Flavoproteins (Massey, V. and Williams, C.H. jr. eds.) pp. 318-322, Elsevier/ North Holland, New York.

[9] Wijnands, R.A. and Müller, F. (1982) Biochemistry *21*, 6639-6646.

[10] Wierenga, R.K., De Jong, R.J., Kalk, K.H., Hol, W.G.J. and Drenth, J. (1979) J.Mol.Biol. *131*, 55-73.

[11] Wierenga, R.K., Kalk, K.H., Van der Laan, J.M., Drenth, J., Hofsteenge, J., Weijer, W.J., Jekel, P.A., Beintema, J.J., Müller, F. and Van Berkel, W.J.H. (1982) in Flavins and Flavoproteins (Massey, V. and Williams, C.H. eds) pp. 11-18, Elsevier/North Holland, New York.

[12] Weijer, W.J., Hofsteenge, J., Beintema, J.J., Wierenga, R.K. and Drenth, J. (1983) Eur.J.bioch. *133*, 109-118.

[13] Riordan, F.J. and Vallee, B.L. (1972) Methods Enzymol. *25*, 521-531.

[14] Müller, F., Voordouw, G., Van Berkel, W.J.H., Steennis, P.J., Visser, S. and Van Rooijen, P.J. (1979) Eur.J.Biochem. *101*, 235-244.

[15] Wijnands, R.A., Van der Zee, J., Van Leeuwen, J.W., Van Berkel, W.J.H. and Müller, F. (1984) Eur.J.Biochem. *139*, 637-644.

[16] Tabachnick, M. and Sobotka, H. (1959) J.Biol.Chem. *234*, 1726-1730.

[17] Weijer, W.J. (1983) Thesis, Univ. Groningen.

[18] Van Berkel, W.J.H., Müller, F., Weijer, W.J., Jekel, P.A. and Beintema, J.J. (1984) Eur.J.Biochem. in press.

[19] Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day, Inc., San Francisco.

[20] Melchior, W.B. and Farney, D. (1970) Biochemistry *9*, 251-258.

[21] Weijer, W.J., Hofsteenge, J., Vereijken, J.M. Jekel, P.A. and Beintema, J.J. (1982) Biochim.Biophys.Acta *704* 385-388.

[22] Howard, A.N. and Wild, F. (1957) Biochem.J. *65*, 651-659.

[23] Higgins, H.G. and Harrington, K.J. (1959) Arch.Biochem.Biophys. *85*, 409-425.

[24] Pielak, G.J., Urdea, M.S. and Legg, J.I. (1984) Biochemistry *23*, 596-603.

[25] Tsou, C.L. (1962) Sci.Sin. (Engl.Ed.) *11*, 1535-1558.

[26] Miles, E.W. (1977) Methods Enzymol. *47*, 431-442.

[27] Mühlrad, A., Hegyi, G. and Tóth, G. (1967) Acta Biochim.Biophys. Acad.Sci.Hung. *2*, 19-29.

[28] Ovádi, J., Libor, S. and Elödi, P. (1967) Acta Biochim.Biophys. Acad.Sci. Hung. *2*, 455-458.

[29] Burnstein, Y., Walsh, K.A. and Neurath, H. (1974) Biochemistry, *13*, 205-210.

[30] Lemke, H.D., Bergmeyer, J., Straub, J. and Oesterhelt, D. (1982) J.Biol.Chem. *257*, 9384-9388.

[31] Mäkinen, K.K., Mäkinen, P.L., Wilkes, S.H., Bayliss, M.E. and Prescott, J.M. (1982) Eur.J.Biochem. *128*, 257-265.

[32] Horiike,K., Tojo, H., Yamano, T. and Nozaki, M. (1984) J.Biochem. *95*, 605-609.

Chapter 5 The importance of monopole-monopole and monopole-dipole interactions on the binding of NADPH to the enzyme

Robert A. WIJNANDS, Jolanda VAN DER ZEE, Johan W. VAN LEEUWEN, Willem J. H. VAN BERKEL, and Franz MÜLLER

Department of Biochemistry, Agricultural University, Wageningen

NADPH binding to *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is found to be strongly dependent on pH and ionic strength. In the ionic strength range of 0.02 – 0.15 M, optimal NADPH binding is observed at a pH value of 6.4. Extrapolation of the dissociation constants to infinite ionic strength shows that under these conditions optimal binding occurs at pH values > 8. Similar results were obtained for complexes between the enzyme and two NADPH analogues in the presence or absence of the substrate.

The experimental data can be explained by a theoretical model in which monopole-monopole or monopole-dipole interactions between the enzyme and the ligand are dominant. Changes in the former interaction prevail at low ionic strength and low pH values while the changes in the latter prevail at high ionic strength and high pH values. The dipole moment of the enzyme in the direction of the NADPH binding site was calculated from the ionic strength and pH dependence of the complex formation. The calculated dipole moment of the enzyme is about 2000 Debye at pH 6 and decreases to about 1100 Debye at pH 8.5. The results are discussed with respect to published results, including data obtained from the enzyme from a different source.

p-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* is an external flavoprotein monooxygenase catalyzing the hydroxylation of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate. The reaction requires NADPH as an electron donor [1]. It has been observed that the reduction of the protein-bound FAD by NADPH is accelerated enormously upon substrate binding [2]. This 'effector' role was shown to be a property of several substrate analogues, which are not necessarily transformed to a product [3]. The enhancement of the rate of reduction of FAD cannot be explained by an increase in NADPH binding since the dissociation constant of the enzyme-NADPH complex decreases only slightly at pH 6.6 and about 10-fold at pH 7.5 in the presence of the substrate or effector [1]. It has been suggested, for the enzyme from *Pseudomonas desmolytica*, that the substrate *p*-hydroxybenzoate not only stimulates the rate of reduction of FAD by NADPH but also shifts the pH optimum of the binding of NADPH to more alkaline pH values [4]. This proposal is related to the fact that optimum binding of NADPH or NADH to the free enzyme occurs at about pH 6 whereas the enzyme possesses its optimal activity at a pH value of about 8. The data of the apparent pH-dependent dissociation constants for the complex between enzyme and NADPH or NADH

Abbreviations. 1-NADPH, 1,4,5,6-tetrahydronicotinamide adenine dinucleotide 2'-phosphate; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; 2',5'-ADP, adenosine 2',5'-bisphosphate.

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

Definition. Debye, unit of dipole moment = 3.34×10^{-30} ml.

served as further support for this proposal. They parallel those of the apparent pH-dependent rate of reduction of the enzyme by NADPH and NADH in the presence of the effector benzoate [4]. From these results one could conclude that a particular amino acid residue of the enzyme plays an important role in the binding of the substrate and the catalytic mechanism of the enzyme. Shoun et al. [5] reported that this particular amino acid residue is a histidine in the enzyme from *P. desmolytica*, which would be in agreement with the postulate. However, more recently Shoun and Beppu [6] have shown that there is no histidine residue involved in the binding of the substrate. The latter finding is in agreement with a similar study on the role of histidines of the enzymes from *P. fluorescens* [7]. As more data on the enzyme from the two different bacterial sources become available it becomes more and more evident that the two enzymes are very similar with respect to both the physical and biochemical properties. This is also supported by our recent finding (unpublished results) that NADH can also serve as an electron donor, as was also shown by Shoun et al. [8] for the enzyme from *P. desmolytica*.

In our opinion, therefore, the hypothesis of Shoun et al. [4] can no longer serve as an explanation for the observed effects. In a previous study [9] on the apoenzyme from *P. fluorescens* we observed that the interaction between the apoenzyme and the pyridine nucleotide depends critically on the ionic strength and the pH value of the solution. This and the fact that the published results of Shoun et al. [4] were obtained under varying ionic strengths in dependence of the pH values prompted us to investigate the interaction between the enzyme and the pyridine nucleotide in dependence of the pH and the

638

ionic strength, under well defined conditions. It is shown that the experimental data can theoretically be fully explained by the consideration of monopole-monopole and dipole-monopole interactions.

MATERIALS AND METHODS

NADPH and NADH were from Boehringer, 2',5'-ADP, Tris, Hepes, Mes and Hepps were from Sigma, and all other chemicals from Merck.

Spectrophotometric measurements were performed on a Zeiss PMQ II or a Varian Cary model 16 spectrophotometer and fluorometric measurements on an Aminco SPF-500 spectrofluorometer. All spectrometers were equipped with thermostated cell-holders.

p-Hydroxybenzoate hydroxylase was isolated from *Pseudomonas fluorescens* and purified as described previously [10]. The purified enzyme was kept as an ammonium sulphate precipitate (70 %) at 4 °C, pH 6 − 7. Stock solutions of the enzyme were prepared in the appropriate buffers by gel filtration over Bio-Gel P-6DG. Enzyme solutions of different pH values were obtained by dilution of an aliquot of a stock solution into Mes (pH < 7), Hepes (7 < pH < 8) or Hepps (pH > 8) buffer with the desired ionic strength. The buffers were brought to the desired pH values and ionic strengths with solutions of NaOH and Na_2SO_4, respectively, taking into consideration the association constant of $10^{0.72}$ for the formation of $NaSO_4^-$ [11]. NaCl cannot be used to adjust the ionic strength of the solution because chloride ions inhibit the catalytic reaction competitively with respect to NADPH [12]. The pH values were measured before and after each experiment.

t-NADPH was synthesized as described by Bielmann and Jung [13], except that the DEAE-cellulose column (4 × 10 cm) was equilibrated with a 4 mM solution of NH_4HCO_3 at 4 °C under nitrogen gas. The column was first washed with 150 ml of a 4 mM NH_4HCO_3 solution after which t-NADPH was eluted with 300 ml of a 4 − 500 mM NH_4HCO_3 gradient. The purity of t-NADPH was checked by both ultraviolet absorption and NMR spectroscopy [13]. No by-products were detected.

The dissociation constants were determined fluorometrically by titration experiments at 20 °C unless stated otherwise. Aliquots (8 µl) of 2 mM, 5 mM, 20 mM and 50 mM solutions of NADPH or NADPH analogue in water were added to 800 µl of 5 µM enzyme solutions in the appropriate buffer. Care was taken to affect the ionic strength of the solutions as little as possible during the titration experiments. Quenching of the protein-bound FAD fluorescence emission at 525 nm (excitation at 450 nm) was monitored as a function of the NADPH or NADPH-analogue concentration. The dissociation constants of the complexes were calculated from Benesi-Hildebrand plots [14].

The net charge of p-hydroxybenzoate hydroxylase was calculated from pH-dependent titration experiments (20 °C). The ammonium sulphate precipitate of the enzyme was taken up in 10 mM potassium phosphate buffer (pH 7) and dialyzed against deionized water. The water was boiled before use to remove dissolved CO_2 and was then kept under nitrogen gas. After dialysis, the enzyme solution was passed over a Bio-Rad AG 501-X8 mixed bed column, the conentration determined (50 − 150 µM) and 4.5 ml of the solution brought in a water-jacketed titration vessel. At this stage the pH of the enzyme

equals the isoionic point pI of the enzyme (pI = 6.0). The pH value of the solution was measured with a glass electrode connected to a Metrohm 632 pH meter. 0.5 ml of a 336 mM Na_2SO_4 solution was then added to the enzyme solution to acquire an ionic strength of 84 mM. The pH-dependent titration was done by adding 10-µl portions of either a 0.125 M or a 0.25 M sodium hydroxide solution containing 33.6 mM Na_2SO_4. At pH 9 the solution was titrated back to pH 5.8 with one equivalent hydrochloric acid solution. Thereafter a similar titration was performed again with the same enzyme solution. During all operations the surface of the enzyme solution was flushed with a stream of N_2 to prevent dissolution of CO_2. Prior to use N_2 was passed through an NaOH solution.

RESULTS

The influence of ionic strength and pH on NADPH binding

The pH and ionic strength dependence of the dissociation constant of the p-hydroxybenzoate-hydroxylase − NADPH complex is shown in Fig. 1A. For the three different ionic strengths used, an optimal binding for NADPH is observed at a pH value of about 6.4 and the K_d values increase with increasing ionic strength. Considering the fact that the isoelectric point of the enzyme is 5.8 [10], so that the enzyme is negatively charged at pH values > 5.8, and considering that the net charge of NADPH varies from −3 at pH 4 to −4 at pH 8, one would expect a decrease in the K_d values by increasing the ionic strength. This is clearly not the case. Therefore the electrostatic interaction between the net charges (monopole-monopole interaction) cannot account for the observed ionic strength dependence, i.e. increasing the ionic strength should decrease the repulsion forces. It can be accounted for if it is assumed that one side of the enzyme molecule contains more positively charged groups than the other side and that NADPH interacts with the positively charged side of the enzyme. This asymmetry in charge distribution on the surface of the enzyme can be expressed in terms of a dipole moment. The ionic strength dependence of the monopole-monopole and monopole-dipole interaction energy has been derived elsewhere [15,16]. The dissociation constant and its ionic strength dependence are given by:

$$K_{d(I)} = K_{d(\infty)} \cdot \exp\left[V(R)/kT\right] \qquad (1)$$

with

$$\frac{V(R)}{kT} = \frac{q^2}{4\pi\varepsilon\varepsilon_0 kTR} \left\{ \frac{Z_1 Z_2}{(1+\kappa R)} + \frac{Z_2 P_1 \cos\theta_1}{qR} \right\} f(\kappa) \qquad (1a)$$

and

$$f(\kappa) = \frac{(1+\kappa R)\left[1 - \exp\left(-2\kappa R_2\right)\right]}{(1+\kappa R_1)\, 2\kappa R_2} \qquad (1b)$$

where $V(R)/kT$ is the electrostatic interaction energy at the encounter radius R, normalized to kT, $K_{d(I)}$ the dissociation constant at ionic strength I, $K_{d(\infty)}$ the dissociation constant at infinite ionic strength, Z_i and R_i the charge and radius of the enzyme ($i = 1$) and NADPH ($i = 2$), respectively, R the sum of the radii, $\kappa = 3.3 \sqrt{I} \cdot nm^{-1}$ (at 294 K), $P_1 \cos\theta_1$ the component of the dipole moment of the enzyme in the direction of the binding site, ε the dielectric permittivity and q the elementary charge. In Eqn (1a) the energy of interaction between the monopole term of the enzyme and the dipole moment of NADPH and the dipole-dipole interaction energy
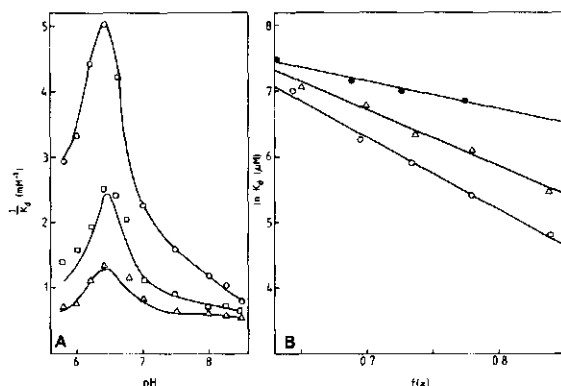
76

Fig. 1. *Dissociation constant of the p-hydroxybenzoate-hydroxylase – NADPH complex as a function of pH and ionic strength.* (A) The experimentally determined values of $1/K_d$ are plotted against pH at three different ionic strengths: $I = 45$ mM (O), 56 mM (□) and 115 mM (△). The curves represent theoretical data obtained by calculating the dissociation constants at the appropriate ionic strength using Eqn (1). (B) The natural logarithm of the K_d values is plotted as a function of $f(\kappa)$ (see text) for three pH values: pH 6.2 (O), pH 7.0 (△) and pH 8.25 (O). The enzyme concentration was 5 μM and the temperature 20 °C. For further details see Materials and Methods

are neglected [16] (see below). In addition, $V(R)/kT$ generally includes hydrophobic and London-Van der Waal's interactions. These effects are not taken into account here, because they are hardly dependent on the ionic strength.

In Fig. 1B ln $K_{d(f)}$ is plotted versus $f(\kappa)$ at various pH values. The data do not necessarily yield a straight line, as expected from Eqn (1). According to Eqn (1) the slope of this line is given by:

$$\frac{d \ln K_{d(I)}}{d f(\kappa)} = \frac{q^2}{4\pi\varepsilon_0 kTR} \left\{ \frac{Z_2 P_1 \cos \theta_1}{qR} + \frac{\kappa R}{(1+\kappa R)^2} \frac{Z_1 Z_2}{\frac{1-\exp(-2\kappa R_2)}{2\kappa R_2} + \frac{\kappa R_1[1-\exp(-2\kappa R_2)]}{2(1+\kappa R_1)^2} - \frac{(1+\kappa R)\exp(-2\kappa R_2)}{1+\kappa R_1}} \right\}. \quad (2)$$

In the ionic strength range studied (0.03 – 0.13 M) the slope of each line varies less than 10 % at any pH value if pH < 7. This justifies the drawing of straight lines for pH values < 7 in Fig. 1B. At higher pH values, where the monopole-monopole term $Z_1 Z_2$ increases, the ionic strength range must be decreased to obtain a straight line. At pH 8.5 for instance the slope varies about 50 % in the ionic strength range mentioned. At pH > 7, therefore, slopes in mean values for $f(\kappa)$ were determined.

Using Eqn (2) either the net charge of the enzyme can be calculated when the dipole moment is known, or the dipole moment can be calculated when the net charge of the enzyme is known. The net charge of the enzyme was calculated from pH-dependent titration experiments (cf. Materials and Methods), using the experimentally determined isoionic point of the enzyme as the reference value of zero net charge. The value for the isoionic point agrees well with that of the isoelectric point of the enzyme, determined previously by isoelectric focussing [10]. The results are summarized in Table 1. Z_2 is calculated using a pK value of 6.1 for the 2'-phosphate of NADPH [17]. The component of the dipole moment of the enzyme in the direction of the binding site for NADPH is thus derived using Eqn (2) and the slopes obtained from plots as shown in Fig. 1B. The value of 2.5 nm for R_1 for the monomeric enzyme was deduced from the

existing low-resolution (0.25 nm) three-dimensional model of the enzyme [18]. The radius R_2 for the extended form of NADPH was estimated to be about 0.6 nm. The calculated data are shown in Fig. 2A. The pH dependence of the dipole moment of the enzyme exhibits an almost constant region in the pH range 6.4 – 7.0 (about 1.8×10^3 Debye). At lower and higher pH values the dipole moment of the enzyme gradually increases and decreases, respectively.

Once the dipole moment is known, the dissociation constant at any ionic strength can be calculated using Eqn (1). Thus we have calculated the values for $K_{d(\infty)}$, reflecting the affinity of the enzyme for NADPH when electrostatic interactions between the reactants are (almost) absent. The theoretical data are plotted in Fig. 2B (curve a). The pH dependence of the dissociation constant at infinite ionic strength reveals a different behaviour from that shown in Fig. 1A at finite ionic strengths. It should, however, be noted that at pH values < 7 an optimum is observed at about pH 6.5 and that at pH values > 7 the affinity of the enzyme for NADPH is steadily increasing.

In contrast, the dissociation constant at zero ionic strength, where the electrostatic interactions between the enzyme and its ligand are optimal, increases rapidly with decreasing pH values. The theoretical data are plotted in Fig. 2B (curve b). It should be noticed that the scales for the two theoretical curves in Fig. 2B differ by a factor of 1000. In addition the calculated values for K_d at $I = 0$ must be considered as lower limits because the linearization to solve the Poisson-Boltzmann equation leading to Eqn (1) is violated [16] (and references therein).

From Fig. 2B it is predicted that the enzyme should show a higher affinity for NADPH under alkaline conditions when electrostatic interactions are absent and a higher affinity under acidic condition when electrostatic interactions are dominant.

Table 1. *The net charge of* p-*hydroxybenzoate hydroxylase from* P. *fluorescens* (Z_1) *and NADPH* (Z_2) *at different pH values and 20 °C*

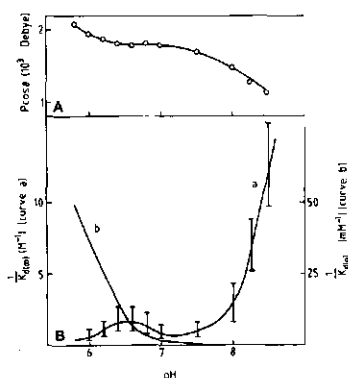| pH	Z_1	Z_2
5.8	+0.5	−3.3
6.0	0	−3.4
6.2	−1	−3.6
6.4	−2	−3.7
6.6	−3	−3.8
6.8	−3.5	−3.8
7.0	−4.0	−3.9
7.5	−5.0	−4.0
8.0	−6.0	−4.0
8.25	−6.5	−4.0
8.5	−7.0	−4.0

Fig. 2. *Component of the dipole moment of* p-*hydroxybenzoate hydroxylase in the direction of the NADPH binding site (A) and dissociation constants at infinite and zero ionic strength (B) as a function of the pH*. (A) The dipole moment is calculated from the slopes of Fig. 1B, the data in Table 1 and Eqn (2), with $R_1 = 2.5$ nm, $R_2 = 0.6$ nm and $R = 3.1$ nm. For the ionic strength an average value of 70 mM was used. (B) The dissociation constant at $I = \infty$ (curve a) ($K_{d(\infty)}$) is calculated from the data used and obtained in using Eqn (1). The dissociation constant at $I = 0$ (curve b) ($K_{d(0)}$) is calculated using the $K_{d(\infty)}$ values. The vertical bars represent the limiting values for the calculations

Under experimental conditions the net effect should be an optimum in between, which is exactly what was found (Fig. 1A).

The binding of NADPH analogues

p-Hydroxybenzoate hydroxylase was titrated with 2',5'-ADP at different pH values and constant ionic strength. The K_d values obtained are plotted in Fig. 3A together with the calculated K_d values (Eqn 1), using the data given in Table 1 and Fig. 2A. The pH dependence of Z_2 for 2',5'-ADP was calculated as described above for NADPH, using a pK value of 6.2 for both the 2' and 5' phosphate groups of ADP [17]. The experimentally determined data can be reasonably fitted when the calculated $K_{d(\infty)}$ values for NADPH binding are used. This means that the difference in the affinity of the enzyme for
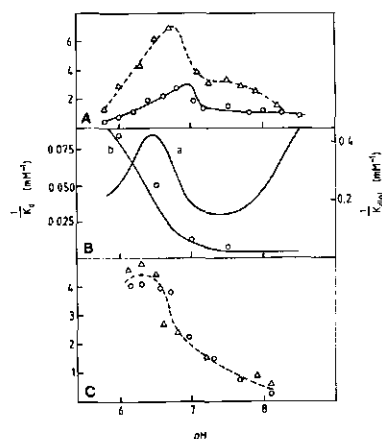

Fig. 3. *Dissociation constants of* p-*hydroxybenzoate-hydroxylase — NADPH-analogue complex as a function of pH*. (A) Enzyme − 2',5'-ADP complex at $I = 45$ mM, 20 °C. The enzyme concentration was 8.5 μM, in the absence (O) and in the presence (△) of *p*-hydroxybenzoate (1.25 mM). The solid curve is calculated according to the procedure mentioned in Fig. 2 (see also text). In this case Z_2 represents the charge of 2',5'-ADP, and is calculated as described in the text. (B) Theoretical curves for the pH-dependent dissociation constant of the enzyme — NADH complex at various ionic strength. The curves are calculated as mentioned in Fig. 2 (see also text), the charge for NADH (Z_2) is − 2. The theoretical curves are calculated for $I = 0$ (curve b) and $I = 86$ mM (curve a). Note that for curve b the scale on the right side of the figure is applicable. Published values (O) [4] for the enzyme from *P. desmolytica* are added to curve b to demonstrate the close agreement between our theoretical values and the experimental values obtained for the enzyme from *P. desmolytica*. (C) The pH dependence of the dissociation constant of the enzyme — t-NADPH complex at $I = 86$ mM, 20 °C, in the absence (O) and in the presence (△) of 0.5 mM *p*-hydroxybenzoate. The enzyme concentration was 10 μM

NADPH and 2',5'-ADP can be fully accounted for by the difference in charge between the two nucleotides. As also shown in Fig. 3A the enzyme-substrate complex exhibits a stronger interaction with 2',5'-ADP than the free enzyme. Although the two curves are not identical in shape, they are similar. The difference between the two curves must probably be ascribed to the fact that the enzyme undergoes a conformational change upon binding of *p*-hydroxybenzoate and that the net charge of the enzyme changes when the enzyme-substrate complex is formed.

Similar calculations were carried out for the complex between the enzyme and NADH. Fig. 3B shows the predicted dissociation constants for the enzyme-NADH complex as a function of the pH at two ionic strengths ($I = 86$ mM, curve a, and $I = 0$, curve b). Z_2 for NADH is − 2 over the entire pH range studied [17]. There is a roughly 20-fold difference between the dissociation constant of the enzyme-NADPH and that of the enzyme-NADH complex. This difference is in good agreement with published, experimentally determined values obtained from the *Pseudomonas desmolytica* enzyme [4]. However, one must be cautious in comparing the published data directly with our results because the ionic strength in the

Table 2. *Observed dissociation constants of the p-hydroxybenzoate – enzyme complex at 20 °C and calculated values using Eqn (1) and values of $K_{d(obs)}$ at $I = 20$ mM*

Z_1 and $P_2 \cos \theta_1$ values are those from Table 1 and Fig. 2A, respectively; $Z_2 = -1$

pH	$K_{d(obs)}$ at		$K_{d(calc)}$ at		
	$I = 70$ mM	$I = 20$ mM	$I = \infty$	$I = 0$	$I = 70$ mM
	μM				
6.4	37.1	24.4	305	0.02	35.8
6.9	40.2	30.0	280	0.14	40.6
7.5	45.5	37.4	270	0.48	46.1

Fig. 4. *Dissociation constant of the p-hydroxybenzoate-hydroxylase – NADPH complex as a function of ionic strength and temperature.* The titrations were performed in Mes buffer pH 6.5 at 4 °C (□), 10 °C (△) and 20 °C (○). The enzyme concentration was 10 μM. $f(\kappa)$ is a function of the ionic strength, see text. The insert shows the Van't Hoff plot used for the determination of ΔH^0 at $f(\kappa) = 0.7$

published work was not kept constant over the pH range under consideration. At any rate our data show that the enzymes from *Pseudomonas fluorescens* and *P. desmolytica* exhibit very similar properties with respect to the interaction with nucleotides. Moreover, these data also strongly indicate the validity of our approach. To demonstrate this validity published dissociation constants for the enzyme-NADH complex from *P. desmolytica* [4] have been added to the calculated curve b in Fig. 3B. The published values were normalized to our scale.

The third analogue used for binding studies was t-NADPH. The data shown in Fig. 3C were also obtained at constant ionic strength ($I = 86$ mM). When compared with values for NADPH at the same ionic strength, they reveal an approximate twofold difference. As t-NADPH and NADPH possess the same charge under identical conditions, a difference in charge cannot explain the higher K_d value of the t-NADPH –enzyme complex. Apparently the (slight) conformational difference between the pyridine moieties of the two nucleotides is of greater influence for the interaction with the enzyme than the chemical difference between NADPH and 2',5'-ADP.

Influence of p-hydroxybenzoate on the binding of NADPH analogues

The *p*-hydroxybenzoate –enzyme complex was titrated with 2',5'-ADP and t-NADPH at constant ionic strength and different pH values. The results are shown in Fig. 3A and 3C, respectively. As has been shown elsewhere for NADPH itself [1,4,19 – 21], the affinity of the enzyme for the nucleotide increases in the presence of *p*-hydroxybenzoate, although this is less pronounced in the case of t-NADPH. However, no drastic shift of the pH optimum for the nucleotide binding is found in the presence and absence of *p*-hydroxybenzoate. This is apparently in contrast to the interpretation of published results for *p*-hydroxybenzoate hydroxylase from *P. desmolytica*, where a substrate induced shift of the binding optimum from pH 5.6 to pH 7.3 was observed (Fig. 9 in [4]). This conclusion was drawn from the pH dependence of K_m values obtained in the presence of substrate or benzoate. The latter compound uncouples the monooxygenase activity of the enzyme. The conclusion drawn from K_m values of two different reactions is therefore questionable, especially as the K_m values do not represent either association or dissociation constants, but a complex number, i.e. comprising different rate constants.
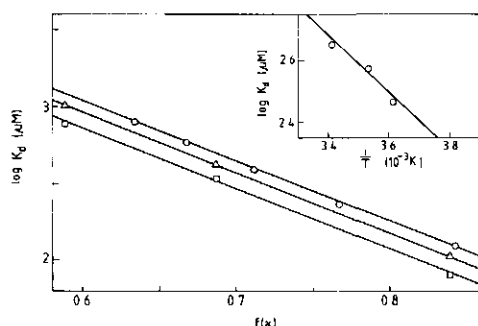
Influence of ionic strength on substrate binding

Titration of the enzyme with *p*-hydroxybenzoate at constant ionic strength (20 mM) and at different pH values, yields dissociation constants for the enzyme-substrate complex which differ only slightly between pH 6 and pH 8. This observation is in agreement with published data [22]. Assuming that *p*-hydroxybenzoate is under the influence of the same dipole moment as NADPH, the pH-dependent $K_{d(z)}$ can be calculated using Eqn (1). These $K_{d(\infty)}$ values can then be used to predict dissociation constants at other ionic strengths. In Table 2 observed K_d values are compared with those calculated using Eqn (1), and with observed K_d values at $I = 20$ mM. The results show that the dependence on ionic strength can be described using Eqn (1) in this case as well.

Temperature dependence of NADPH binding

Dissociation constants of the enzyme-NADPH complex at pH 6.5 and several ionic strengths were determined at different temperatures. When log K_d is plotted as a function of $f(\kappa)$ (Fig. 4), parallel lines are obtained. This is expected because electrostatic interactions are hardly temperature-dependent. In Eqn (1) the product εkT is independent of themperature within 0 – 40 °C owing to the decrease of ε when the temperature is increased [23]. A plot of K_d at an arbitrary ionic strength as a function of the inverse temperature was used to calculate the dissociation enthalpy of the enzyme-NADPH complex as shown in Fig. 4 (insert) for $f(\kappa) = 0.7$. A value of 17 kJ mol^{-1} for ΔH^0 was thus found.

DISCUSSION

Eqn (1) has been derived under the condition that $|V(R)| < kT$ [16]. However, the validity of Eqn (1) has also been shown under conditions exceeding the kT value, as in our case where $3kT < |V(R)| < 8kT$ [24]. Only in the case of $I = 0$ are the conditions violated too much and the values of K_d at zero ionic strength must therefore be regarded as lower limits.

Strictly speaking, the interaction energy between the enzyme monopole and the NADPH dipole as well as the

dipole-dipole interaction energy should also be included in Eqn (1a). Since the dipole moment of NADPH ($P_2 < 60$ Debye) is very small compared to the dipole moment of the enzyme, these terms may be neglected [15,16].

An attempt has been made to estimate the dipole moment of the enzyme using the coordinates of the charged groups in the existing, low-resolution three-dimensional model of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* [18]. It should be kept in mind that these coordinates refer to the enzyme-substrate complex, crystallizing as a dimer. The calculation based on a monomeric molecule in the crystalline state indicates that a dipole moment indeed exists in the enzyme. The calculated dipole moment is in fair agreement with our theoretical value at pH 7.5. Moreover the dipole moment deduced from X-ray data is in the direction of the proposed binding site of NADPH [25]. It must, however, be mentioned that no structural data are yet available for the enzyme-NADPH complex. A similar calculation for the dimeric molecule suggests that the two dipole moments are aligned to parallel, resulting in an increased overall dipole moment. These results thus strongly support our theoretical approach.

In solution, the situation is more complex since the enzyme can exist in several oligomeric forms [10]. More recently we were able to isolate the enzyme as a pure dimer (unpublished results). In this study only these preparations were used, eliminating possible complicating factors. The dimeric enzyme has been studied by modern fluorescence technique (Visser, Penners, Van Berkel and Müller, unpublished results). This study showed that there is an equilibrium between monomers and dimers. The dissociation constant for the dimer is about 1 µM. It has also been found that the presence of NADPH does not influence this equilibrium. The enzyme concentration used in the titration experiments was 5 µM. Clearly, under these experimental conditions, monomeric and dimeric molecules are present in solution. This fact does not influence our conclusions since both molecules possess a dipole moment.

It is possible that the pK_a value of 6.1 for the 2'-phosphate of NADPH is somewhat lower in the encounter complex due to the positive charges of the enzyme. This has, however, little influence on the calculation of the dipole moment of the enzyme. The difference is at most 150 Debye at pH 5.8 on the assumption that the pK value of NADPH has decreased for example to 5.6. An increase of the pK value of NADPH would lead to a similar result.

The difference between NADPH binding at zero ionic strength and infinite ionic strength is striking. In the pH and ionic strength range studied, the pH dependence of the dissociation constant of the enzyme-NADPH complex is dominated by the influence of electrostatic interactions. As can be seen in Eqn (1a), the contribution of the monopole-monopole term to the electrostatic interaction energy is very small at high ionic strength and large at zero ionic strength. This causes the optimum found at pH 6.4 in the ionic strength range studied to almost disappear as the ionic strength approaches zero. The influence of the electrostatic interaction in the case of NADH binding is less than in the case of NADPH binding because of the smaller charge of NADH. This results in a curve of the pH dependence (Fig. 3B, curve a, $I = 86$ mM) which resembles in shape more the curve at infinite ionic strength (Fig. 2B, curve a) than those of NADPH at the same ionic strengths (Fig. 1 and Fig. 2, curve a).

Before complex formation of NADPH in the active site of *p*-hydroxybenzoate hydroxylase can occur, several events have to take place: formation of an encounter complex, optimal orientation of the reacting species, unfolding of NADPH and penetration of NADPH into the enzyme to form the catalytically active complex. Only the first two events are described by the electrostatic term of Eqn (1). The other events together with the actual complex formation in the active site include hydrophobic interaction, salt bridge formation and hydrogen bond formation. These effects are assumed to be independent of the ionic strength, unless competitive inhibitive ions are present. As potassium sulphate was used to attain the desired ionic strengths, no competitive inhibition with respect to NADPH is expected [12].

The overall dissociation constants determined do not yield information on possible intermediate states involved in the formation of the active complex. It must be realized, however, that the dissociation constants determined in this paper also include the possible formation of intermediate complexes, since K_d is defined as:

$$K_d = \frac{[\text{NADPH}]\,[\text{enzyme}]}{\Sigma\,[\text{NADPH-enzyme}]}$$

where Σ [NADPH-enzyme] is the sum of the concentrations of the intermediate and the final complexes.

In our case it is especially important to correlate the thermodynamic data of the formation of the enzyme-NADPH complex to the ionic strength. At pH 6.5 ΔH^0 for complex formation is found to be -17 kJ/mol, independent of the ionic strength (Fig. 4). At zero ionic strength K_d^{-1} is 10 mM^{-1} at pH 6.5 (20 °C), thus $\Delta G^0 = -2.5$ kJ/mol and ΔS^0 is 19 kJ/mol K are calculated, using the equations $\Delta G^0 = -RT \ln K_d^{-1}$ and $\Delta G^0 = \Delta H^0 - T\Delta S^0$, respectively. At infinite ionic strength K_d^{-1} is 1.5 M^{-1} at pH 6.5 (20 °C), yielding $\Delta G^0 = -1.0$ kJ/mol and $\Delta S^0 = -55$ J/mol K. It should be noticed that the only difference in these calculations is that in the former case the electrostatic forces in the formation of the complex are maximal while in the latter case these forces are ruled out. This indicates that in the absence of electrostatic interactions complex formation results in a decrease in entropy, while in the presence of such interactions complex formation results in an increase in entropy.

The dissociation constants for the enzyme $-2',5'$-ADP and the enzyme NADH complexes could be fully accounted for by considering the electrostatic interactions and using the $K_{d(\infty)}$ values as calculated for the enzyme-NADPH complex. This means that the ΔG^0 values for these complexes at infinite ionic strength are identical and that the different ΔG^0 values at finite ionic strength are merely caused by a difference in electrostatic interactions due to different charges of these molecules. This is not the case for t-NADPH where dissociation constants were higher than expected when only electrostatic interactions are considered. Apparently the chemical modification of the nicotinamide ring disfavours the events following the formation of the encounter complex. The most probable cause for the increased affinity of the enzyme for 2',5'-ADP in the presence of the substrate is a change of the conformation of the enzyme induced by the substrate (effector). Such a conformational change has been reported [2,26].

The thermodynamic data presented above cannot be compared directly with data that usually appear in the literature where the electrostatic contributions are not specified.

A dissociation constant of 0.11 mM (4 °C. pH 6.5 and 6.6, $I = 80$ mM and 140 mM) for the enzyme-NADPH complex [1,20] has been reported for the enzyme from *P. fluorescens*. The values of 0.29 mM and 0.62 mM, respectively, for these ionic strengths are deduced from Fig. 4. The values redetermined experimentally under the conditions stated in the literature, but using Mes instead of phosphate buffer, are 0.29 mM and 0.50 mM, respectively. The good agreement between our theoretical and experimental values is in line with our theory. The discrepancy between published data and our own results shows that it is of utmost importance to keep the actual ionic strength in such experiments under strict control, to allow a reliable comparison of dissociation constants obtained in different laboratories. This is especially true for systems strongly dependent on the ionic strength, such as the one reported in this paper.

Dissociation constants for the NADPH-apoenzyme from *P. fluorescens* have recently been reported in dependence of the ionic strength [9]; i.e. 12.5 µM and 43 µM at pH 6.5 (20 °C) for $I = 18$ mM and 63 mM, respectively. These two values can also be theoretically supported using Eqn (1), i.e. substitution of 12.5 µM, $I = 18$ mM, predicts a $K_{d(\infty)}$ of 140 mM and a K_d value of 49 µM at $I = 63$ mM. The reported values for the NADH-apoenzyme complex [9], however, could not be confirmed by a similar calculation. This indicates that additional factors are involved in the formation of the NADH-apoenzyme complex. Participation of the available FAD binding site in the complex formation cannot be excluded.

Some pH-dependent K_d values for the NADPH complex of the enzyme from *Pseudomonas desmolytica* in Mes. Hepes and phosphate buffers were recently reported [8]. Differences between values determined in Mes and phosphate buffers at pH 6 and values in Hepes and phosphate buffers at pH 8 were attributed to phosphate inhibition. However, the enzyme is only weakly inhibited by phosphate ions, the inhibition being competitive with respect to NADPH [27]. Although Shoun et al. [8] used identical buffer concentrations, the ionic strength of such solutions differ widely, which is especially the case for phosphate buffers used at different pH values. In fact the major differences of the reported data [8] between Mes and Hepes buffers, on one side, and phosphate buffers, on the other side, can be accounted for by the differences in ionic strength using Eqn (1). This strongly suggests that the same relationship holds for the two enzymes from the different sources. Furthermore, Shoun et al. [4] reported K_d values of the complex between NADPH or NADH and the enzyme from *P. desmolytica* in dependence of the pH using phosphate buffer. The published data (Fig. 9A in [4]) can in fact be ascribed to the pH dependence of the ionic strength of the phosphate buffer and the pK_a value derived from the curve in the figure can therefore not be attributed to an ionizing group of the NADPH (NADH) binding site of the enzyme but most probably represents merely the pK_a value of the phosphate buffer.

Although NADPH binding is not the rate-limiting step in the overall catalytic reaction [1,4], it has always puzzled us that the enzyme is most active in a pH region where NADPH binding is far from optimal. The results presented here indicate that the pH optimum for NADPH binding is the net result of favoured electrostatic interactions under acidic conditions and favoured non-electrostatic interactions under alkaline conditions.

Theoretically the favoured electrostatic interactions under acidic conditions also hold for the binding of $NADP^+$. It can therefore be expected that optimal activity is observed at pH values where $NADP^+$ binding is less favourable. This is indeed the case for the enzyme from *P. fluorescens* showing an optimal pH at about 8.

There is of course no reason to believe that our model is unique for *p*-hydroxybenzoate hydroxylase. An example of an enzyme showing similar behaviour with respect to the pH dependence of pyridine nucleotide binding and enzymic activity is mitochondrial malate dehydrogenase from pig heart. When the reversed reaction, i.e. the reduction of oxaloacetate is considered, optimal activity is found at pH 9 [28], while optimal NADH binding is found at pH 5.7 [28,29]. The pH dependence of NADH binding has been explained by assuming that there are different K_d values for the complexes with the enzyme possessing a protonated or a deprotonated histidine residue [29,30]. As (de)protonation involves the change of the net charge, it is possible that these different K_d values are the result of different electrostatic interactions. It is interesting to note that there is a striking difference between the pH dependence of the dissociation constant given by Raval and Wolfe [28], determined at $I = 50$ mM, and the ones given by Lodda et al. [29] and Schwerdtfeger et al. [30], determined at $I = 100$ mM.

The pH dependence of $K_{d(\pi)}$ in Fig. 2B suggests that several residues with different pK_a values are involved in actual NADPH binding. The importance of histidine residues in this binding have been reported [6,7]. Studies to identify the other residues are now in progress.

REFERENCES

1. Husain. M. & Massey. V. (1979) *J. Biol. Chem. 254*, 6657 – 6666.
2. Hosokawa. K. & Stanier. R. Y. (1966) *J. Biol. Chem. 241*, 2453 – 2460.
3. Spector. T. & Massey. V. (1972) *J. Biol. Chem. 247*, 4679 – 4687.
4. Shoun. H., Highashi. N., Beppu. T., Nakamura. S., Hiromi. K. & Arima. K. (1979) *J. Biol. Chem. 254*, 10944 – 10951.
5. Shoun. H., Beppu. T. & Arima. K. (1979) *J. Biol. Chem. 254*, 899 – 904.
6. Shoun. H. & Beppu. T. (1982) *J. Biol. Chem. 257*, 3422 – 3428.
7. Wijnands. R. A. & Müller. F. (1982) *Biochemistry 21*, 6639 – 6646.
8. Shoun. H., Arima. K. & Beppu. T. (1983) *J. Biochem. (Tokyo) 93*, 169 – 176.
9. Müller. F. & van Berkel. W. J. H. (1982) *Eur. J. Biochem. 128*, 21 – 27.
10. Müller. F., Voordouw. G., van Berkel. W. J. H., Steennis. P. J., Visser. S. & van Rooijen. P. J. (1979) *Eur. J. Biochem. 101*, 235 – 244.
11. Sillén. L. G. & Martell. A. E. (1964) in *Stability Constants of Metal-Ion Complexes*, Special Publication *17*, The Chemical Society, London.
12. Steennis. P. J., Cordes. M. M., Hilkens. J. G. H. & Müller. F. (1973) *FEBS Lett. 36*, 177 – 180.
13. Bielman. J. F. & Jung. M. J. (1971) *Eur. J. Biochem. 19*, 130 – 144.

644

14. Benesi, H. A. & Hildebrand, J. J. (1949) *J. Am. Chem. Soc. 71*, 2703−2707.
15. van Leeuwen, J. W., Mofers, F. J. M. & Veerman, E. C. J. (1981) *Biochim. Biophys. Acta 635*, 434−439.
16. van Leeuwen, J. W. (1983) *Biochim. Biophys. Acta 743*, 408−421.
17. Dawson, R. M. C., Elliott, D. C., Elliot, W. H. & Jones, K. M. (1969) *Data for Biochemical Research.* pp. 146, 148, 204, Oxford University Press, Oxford.
18. Wierenga, R. K., de Jong, R. J., Kalk, K. H., Hol, W. G. J. & Drenth, J. (1979) *J. Mol. Biol. 131*, 55−73.
19. Howell, L. G., Spector, T. & Massey, V. (1972) *J. Biol. Chem. 247*, 4340−4350.
20. Entsch, B., Ballou, D. P., Husain, M. & Massey, V. (1976) *J. Biol. Chem. 251*, 7367−7379.
21. Entsch, B., Husain, M., Ballou, D. P., Massey, V. & Walsh, C. (1980) *J. Biol. Chem. 255*, 1420−1429.
22. Shoun, H., Higashi, N., Beppu, T., Arima, K. & Nakamura, S. (1974) *FEBS Lett. 49*, 13−17.
23. Weston, R. & Schwarz, H. A. (1972) *Chemical Kinetics*, pp. 151−179, Prentice Hall Inc., Eaglewood Cliffs, NJ.
24. Lampert, M. A. & Crandall, R. S. (1979) *Chem. Phys. Lett. 68*, 473−476.
25. Wierenga, R. K., Kalk, K. H., van der Laan, J. M., Drenth, J., Hofsteenge, J., Weijer, W. J., Jekel, P. A., Beintema, J. J. Müller, F. & van Berkel, W. J. H. (1982) in *Flavins and Flavoproteins* (Massey, V. & Williams, C. H., eds) pp. 11−18, Elsevier/North-Holland, New York.
26. Yano, K., Higasi, N. & Arima, K. (1969) *Biochem. Biophys. Res. Commun. 34*, 1−7.
27. Müller, F., van Berkel, W. J. H. & Steennis, P. J. (1983) *Biochem. Int. 7*, 115−122.
28. Raval, N. R. & Wolfe, R. G. (1962) *Biochemistry 1*, 1118−1123.
29. Lodola, A., Parker, D. M., Jeck, R. & Holbrook, J. J. (1978) *Biochem. J. 173*, 597−605.
30. Schwerdtfeger, K., Woenckhaus, C., Parker, D. M. & Holbrook, J. J. (1982) *Z. Naturforsch., C:Biosci. 37*, 547−549.

R. A. Wijnands, J. van der Zee, J. W. van Leeuwen, W. J. H. van Berkel, and F. Müller*,
Laboratorium voor Biochemie, Landbouwhogeschool,
De Dreijen 11, NL-6703-BC Wageningen, The Netherlands

---

* To whom all correspondence should be sent.

## Chapter 6   Flavin mobility in chemically modified p-hydroxybenzoate hydroxylase

INTRODUCTION

Time resolved fluorescence studies were carried out on FAD bound
to chemically modified p-hydroxybenzoate hydroxylase. If a short, plane-
polarized light pulse is used for the excitation and if linearly polar-
rized components of the emission are detected, information on the mobi-
lity of the flavin can be obtained. The mobility of a system is corre-
lated to its anisotropy A, which relates the intensities of polarized
light emitted in planes parallel and perpendicular to the plane of po-
larization of the excitation light pulse. For a totally rigid system
with identical absorption and emission transition moments A = 0.40.
When during the lifetime of the excited state the molecules can tumble
fast enough to randomize their orientation, the emitted light is com-
pletely depolarized and A = 0. The rate of the exponential decay is
characterized by the rotational correlation time constant φ. For more
detailed information on this subject, the reader is referred to [1,2].
For the purpose of this study it is sufficient to know that the average
time constant τ of the fluorescence decay is related to the dynamic
quenching of the fluorescence, the quantum yield together with τ are
related to static and dynamic quenching and the time constant of the
exponential anisotropy decay is a measure of the mobility of the flavin.

MATERIALS AND METHODS

Seven samples were prepared freshly for the time dependent flu-
orescence experiments.
1. *Native enzyme*: 1 μM p-hydroxybenzoate hydroxylase in 20 mM Mes
pH 6.2, I = 25 mM.
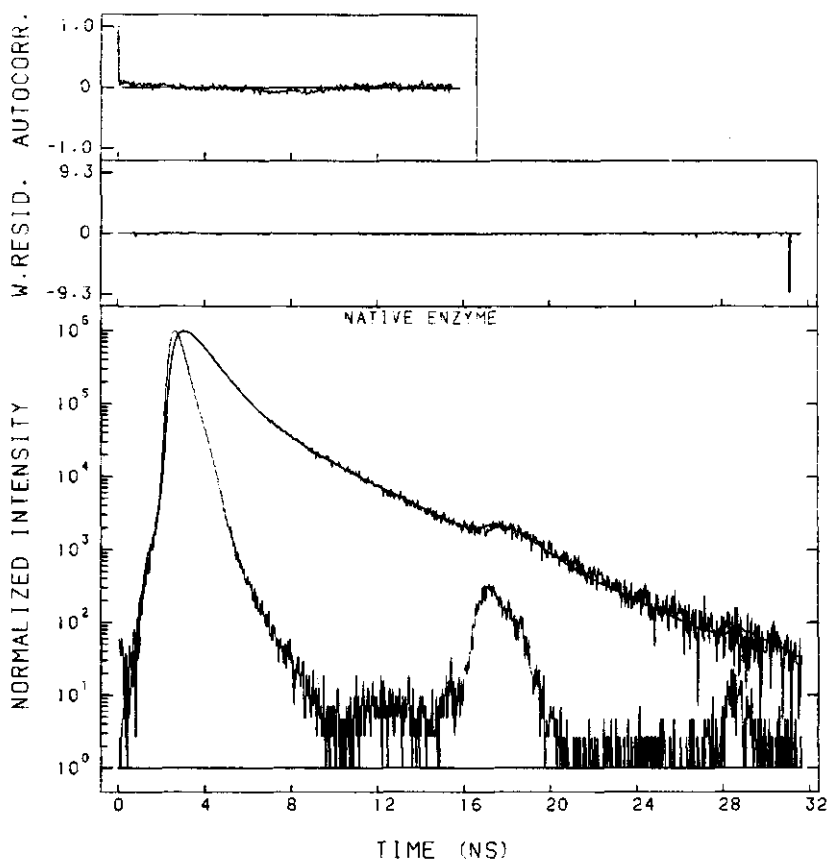
Fig. 1. Fluorescence decay of FAD bound to native p-hydroxybenzoate hydroxylase

1 μM p-Hydroxybenzoate hydroxylase in 20 mM Mes, pH 6.2, at 8°C. Shown are the laser pulses as measured by the detection system, the experimental fluorescence response and the fluorescence decay calculated according to a triple-exponential decay model using the parameters given in Table 1. On top of these curves the deviation function, defined as the difference between the experimental and the calculated fluorescence divided by the square root of the experimental fluorescence and the autocorrelation function, defined in [4] are presented. The time equivalence was 0.030896 ns per channel and 1000 channels out of 1024 were used in the analysis.

2. *Phenylglyoxal modified enzyme (8 min)*: A 5 μM solution of p-hydroxy-benzoate hydroxylase in 40 mM Hepps, pH 8, was modified by 10 mM phenyl-glyoxal at 30°C until the enzyme activity was zero (8 min).
The reaction was stopped by passing the mixture over a biogel P6-DG column equilibrated with 20 mM Mes, pH 6.2. The final enzyme concentra-tion was 1 μM.

3. *Phenylglyoxal modified enzyme (25 min)*: The procedure was identical with that of 2, except that the modification was continued until the fluorescence of the protein-bound flavin had reached its maximum (25 min).

4. *Butanedione modified enzyme (borate)*: A solution of 5 μM enzyme in 50 mM borate buffer, pH 8, was modified by 10 mM 2,3-butanedione at 30°C until the fluorescence had reached its maximum (20 min).
The reaction was stopped as described under 2 and the final enzyme con-centration was brought to 1 μM.

5. *Butanedione modified enzyme (Hepps)*: The procedure was identical with that of 4, except that the modification was performed in 40 mM Hepps, pH 8.

6. *Carbethoxylated enzyme*: A 20 μM solution of the enzyme in 40 mM Hepps, pH 8, was modified by 1 mM diethylpyrocarbonate at 20°C for 3 min. The reaction was stopped as described under 2 and the enzyme concentration was brought to 1 μM.

7. *Azocoupled enzyme*: A 4 μM solution of the enzyme in 40 mM Hepes, pH 7, was modified by addition of four portions of 1.5 mM p-diazoben-zoate at 20 min intervals. After 70 min the residual activity was 15% and the reaction was stopped as described under 2. The enzyme concen-tration was brought to 1 μM.

The fluorescence measurements were performed under the same con-ditions as described earlier [3]. The temperature was 8°C. The methods to calculate the values of τ and φ were described previously [3]. The second order average lifetimes $\bar{\tau}$:

Table 1 Effect of chemical modification on the parameters describing fluorescence and anisotropy decay of p-hydroxybenzoate hydroxylase[a].

Sample[b]	F[c]	Fluorescence Lifetime (ns)							Anisotropy (ns)
		α_1	τ_1	α_2	τ_2	α_3	τ_3	$\bar{\tau}$	φ
Native enzyme	1.0	0.67	0.10	0.28	1.1	0.05	4.1	2.1	4.6
Phenylglyoxal modified enzyme (8 min)	2.0	0.35	0.16	0.48	1.3	0.17	3.6	2.4	25
Phenylglyoxal modified enzyme (25 min)	2.7	0.41	0.11	0.42	1.3	0.17	3.8	2.5	24
Butanedione modified enzyme (borate)	1.3	0.49	0.09	0.41	1.4	0.10	3.3	2.0	18
Butanedione modified enzyme (Hepps)	2.0	0.55	0.10	0.40	1.1	0.05	2.9	1.4	29
Carbethoxylated enzyme	2.5	0.27	0.20	0.38	2.5	0.35	4.5	3.6	0.55 7.7[d]
Azocoupled enzyme		0.70	0.07	0.23	1.3	0.06	3.4	2.0	35

a. All at 8°C, λ_{exc} = 458 nm, λ_{em} = 531 nm; see text for explanation of symbols.

b. 1 μM solutions in 20 mM Mes pH 6.2.

c. Relative fluorescence quantum yield.

d. Two rotational correlation times needed to fit data. Their relative weights are 0.45 and 0.55 respectively.

$$\bar{\tau} = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2 + \alpha_3\tau_3^2}{\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3}$$

were used to calculate φ.

RESULTS AND DISCUSSION

The fluorescence decay of p-hydroxybenzoate hydroxylase in 20 mM Mes, pH 6, at 8^OC is shown in Fig. 1. A triple exponential function had to be used to fit the data [3]. Similar plots were made for all of the samples mentioned under Materials and Methods and the values of the lifetime components τ together with their relative contributions α are given in Table 1. The table also includes the second order average lifetimes $\bar{\tau}$. The heterogeneity of the lifetime has been attributed to dynamic quenching of the flavin fluorescence induced by rapidly moving amino acids in the vicinity of the flavin [3]. The analysis into three lifetimes is a minimum hypothesis. A method to determine the distribution over a large number of fixed τ values will be published in the near future (A.J.W.G. Visser, personal communication).

When the average lifetimes are compared it can be seen that the butanedione modified enzyme (in the absence of borate) shows the largest fluorescence lifetime and that the carbethoxylated enzyme shows the shortest fluorescence lifetime. Little variation is found in the other samples. Similar tendencies are found for the relative quantum yields.

More interesting information can be derived from the anisotropy decay measurements. The rotation correlation times of the seven samples are listed in Table 1 and can be divided into three classes. The first class consists of the native enzyme only, with a φ value of 4.6 ns; the second class consists of the carbethoxylated enzyme only, with two rotational correlation times of 0.55 ns and 7.7 ns, and the third class consists of all the other modified species with single φ
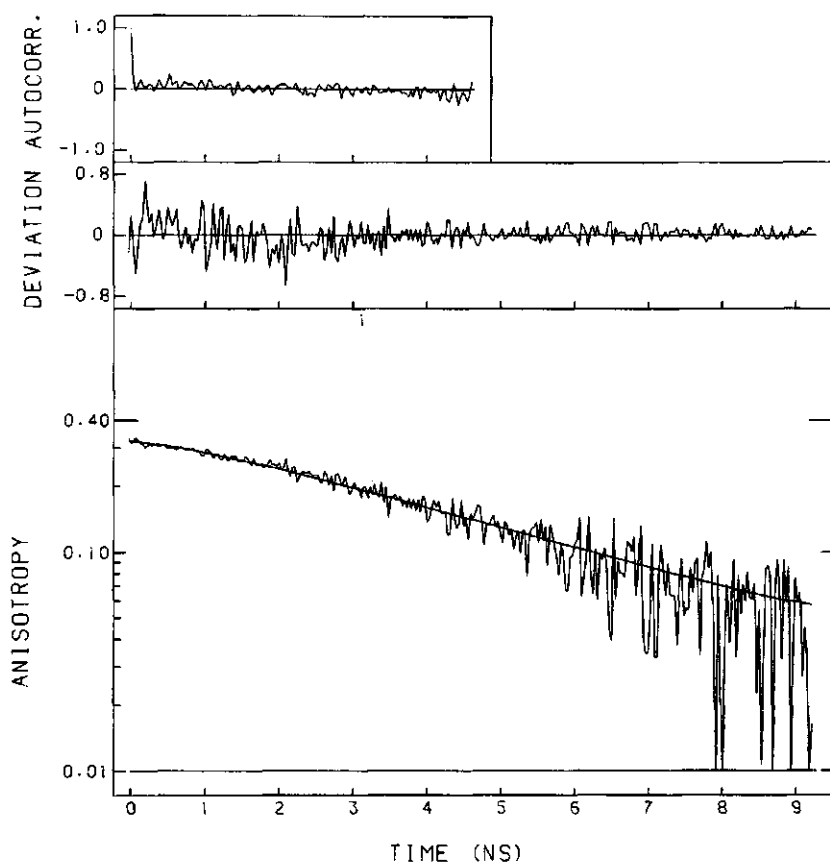
Fig. 2. Anisotropy decay of native p-hydroxybenzoate hydroxylase
1 μM p-Hydroxybenzoate hydroxylase in 20 mM Mes, pH 6.2, at 8°C. Shown
are the experimental anisotropy and the anisotropy calculated accord-
ing to a single-exponential decay model using a φ value of 4.6 ns. On
top of these curves the deviation function defined as the difference
between the experimental and the calculated anisotropy divided by the
variance of the experimental anisotropy and the autocorrelation func-
tion, defined in [4] are presented. The time equivalence was 0.030896
ns per channel and 300 channels out of 1024 were used in the analysis.

values ranging from 18 to 35 ns.

It has been published previously that p-hydroxybenzoate hydroxylase can appear in several oligomeric forms [5]. For these experiments the dimeric form was taken as the starting material but the dimer is known to dissociate, depending on the concentration of the enzyme and the temperature [5]. The rotational correlation times of the dimeric and monomeric forms of the native enzyme were calculated to be 35 ns and 3 ns, respectively [3].

From the value of 4.6 ns for the rotational correlation time of the native enzyme in this study it can be concluded that in 20 mM Mes, pH 6.2, at $8^{O}C$ and at a concentration of 1 μM, the enzyme exists mainly in the monomeric form. It can therefore also be concluded that the dissociation constant of the dimer under the conditions used in this study is much smaller than the 1 μM published previously. Using the values of the rotational correlation times of the pure dimeric and monomeric forms given above, it can be calculated that roughly 10% is in the dimeric form, which means that the dissociation constant is about 0.02 μM. The discrepancy must be attributed to the difference in buffer and ionic strength.

All modifications of class 3 have the same effect on the rotational correlation times of the modified enzyme. Considering the above this means that either the flavin has become less mobile within the enzyme or there has been a shift of the dissociation constant. Another possibility is of course a combination of these two phenomena. A shift of the dissociation constant of the dimer must be the most important effect as complete immobilization of the flavin within the monomer would yield a rotational correlation time of 15 ns [3]. Stabilization of the dimeric form of D-amino acid oxidase by complexation with the inhibitor benzoate has been reported [6]. Moreover modification of the active site is usually also accompanied by modification of amino acid residues on the surface of the protein, which could have a more direct effect on the interaction between the monomers. On the other hand, modification of amino acid residues on the surface of the protein may also change
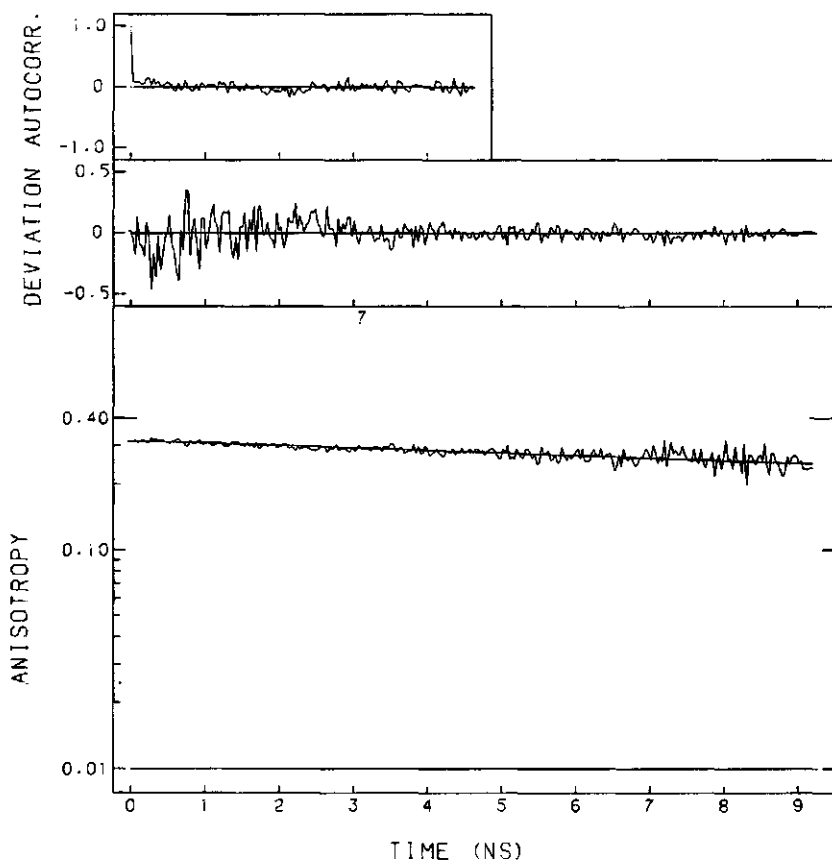
Fig. 3. Anisotropy decay of azocoupled p-hydroxybenzoate hydroxylase
All conditions and additional information can be found under Fig. 3.
In this case however a φ value of 35 ns was used to calculate the ani-
sotropy.

the degree of hydration of the enzyme surface, which also gives rise to changes in rotational correlation time.

Immobilization of the flavin within the enzyme is also possible as our depolarization data of the native enzyme-bound flavin indicate that the flavin possesses considerable rotational freedom. A discussion on the significance of this rotational freedom has been presented [3,7]. The incorporation of additional (bulky) groups into the active site could very well influence the mobility of the protein-bound flavin.

In the above, the interpretation of the rotational correlation times has been done according to the second model given by Visser *et al.* where φ is considered as a composite correlation time characteristic for both monomer and dimer rotations [3]. The anisotropy decay of carbethoxylated enzyme-bound flavin is double exponential and the larger φ value of 7.7 ns is considered to be of the same nature. As described above this could mean a small shift in the dissociation constant of the dimer but also a small degree of immobilization of the flavin compared to the native enzyme. The short correlation time of 0.55 ns could be characteristic for either the motion of FAD within the protein, or the motion of a small domain containing FAD with respect to the protein.
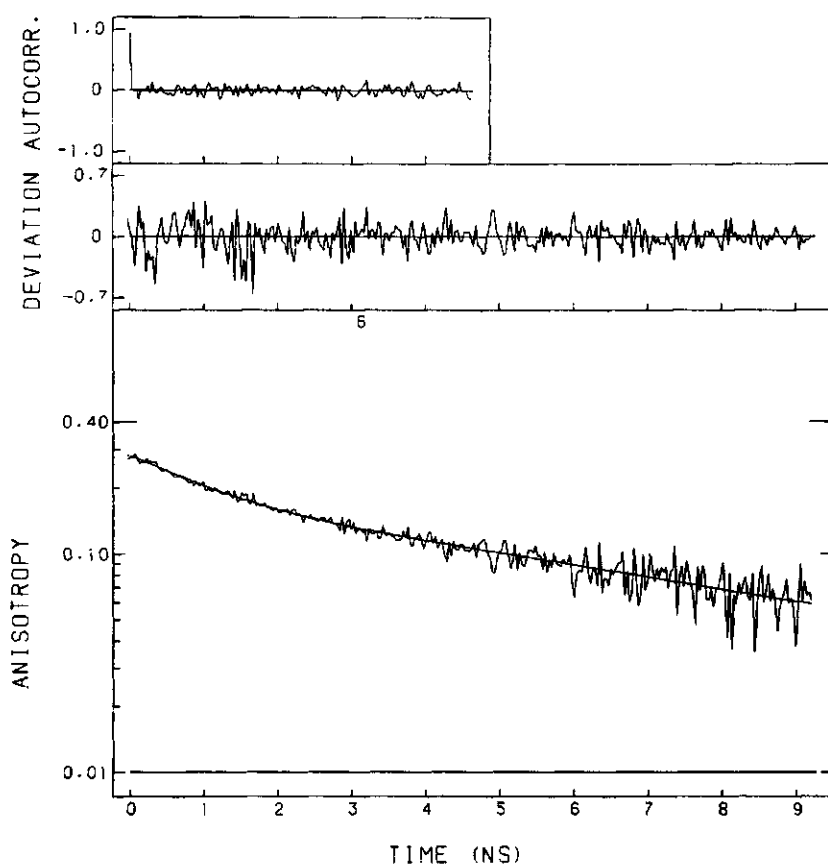
ACKNOWLEDGEMENTS

Fig. 4. Anisotropy decay of carbethoxylated p-hydroxybenzoate hydroxylase

All conditions and additional information can be found under Fig. 3.
In this case the anisotropy was calculated according to a double-exponential decay model using φ values of 0.55 ns (45%) and 7.7 ns (55%).

REFERENCES

[1] Cantor, C.R. and Schimmel, P.R. (1980) Biophysical Chemistry part 2, Techniques for the study of biological structure and function, pp. 433-465, W.H. Freeman and Company, San Francisco.

[2] Cundall, R.B. and Dale, R.E. (eds.) (1983) Time-resolved fluorescence spectroscopy in biochemistry and biology, Plenum Press, New York.

[3] Visser, A.J.W.G., Penners, G.N.H., Van Berkel, W.J.H. and Müller, F. (1984) Eur.J.Biochem., in press.

[4] Grinvald, A. and Steinberg, I.Z. (1974) Anal.Biochem. *59*, 583-598.

[5] Müller, F., Voordouw, G., Van Berkel, W.J.H., Steennis, P.J., Visser, S. and Van Rooijen, P.J. (1979) Eur.J.Biochem. *101*, 234-244.

[6] Yagi, K., Tanaka, F., Nakashima, N. and Yoshihara, K. (1983) J.Biol.Chem. *258*, 3799-3802.

[7] Visser, A.J.W.G., Penners, G.N.H. and Müller, F. (1983) in Mobility and Recognition in Cell Biology (Sund, H. and Veeger, C., eds.) pp. 137-154, Walter de Gruyter, Berlin.

Summary

Chemical modification was used to examine the role of some amino acid residues in the binding of the substrates to the enzyme p-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. Ionic strength dependent binding studies were used to investigate the role of the protein as a whole in the complex formations. In the general introduction the present knowledge on p-hydroxybenzoate hydroxylase and on chemical modifications of amino acid residues in general is reviewed. Whereas most studies on this enzyme were done on the prosthetic group FAD, it was our aim to acquire more insight into the role of the surrounding protein.

Diethylpyrocarbonate was used to modify histidine residues. Four out of nine histidine residues were modified, which was accompanied by total inactivation of the enzyme. Two of these residues appeared to be essential for the enzyme activity and to be involved in the binding of NADPH. The rate of inactivation increased with increasing pH. From this pH dependence the pK value of two cooperatively ionizing histidine residues was determined. At pH>7 tyrosine residues appeared to be carbethoxylated, accompanied by a loss of substrate binding capacity.

The modification of arginine residues is described in chapter 3. Out of the three inactivating reagents used, one appeared to react with two arginine residues which are important for NADPH and substrate binding. A model was postulated to account for the results. The other two reagents reacted with different arginine residues, which was explained by the differences in charge and hydrophobicity. The complexity of arginine modification is due to the large number (38) of arginine residues present in the enzyme.

In chapter 4 the modification of tyrosine residues is described. Some additional experiments using diethylpyrocarbonate were carried out, but more attention is given to the modification by the substrate analogue p-diazobenzoate. Although the identification experiments

did not yield the desired quantitative result, it could be established that a reaction with tyrosine-201 was responsible for the loss of substrate binding capacity. It was concluded that tyrosine-385 is also essential and that tyrosine-222 is non-essential.

In chapter 5 the protein as a whole is considered. The ionic strength dependence of NADPH binding to the enzyme is explained using a model in which the enzyme is considered as both a monopole and a dipole. The dipole moment of the enzyme in the direction of the NADPH binding site was calculated and compared with a dipole moment estimated on the basis of charge distribution as revealed by the three-dimensional model. The apparent contradiction between the pH dependences of NADPH binding and enzyme activity are also discussed.

Chapter 6 finally is dedicated to the results of time resolved flavin fluorescence measurements on enzyme molecules modified by various reagents. The rotation correlation times determined were compared to obtain information on the mobility of the flavin within the enzyme. The differences between the rotation correlation times could be accounted for by a shift of the dissociation constant of the dimeric form of p-hydroxybenzoate hydroxylase.

Samenvatting

Met behulp van chemische modificatie werd gekeken naar de rol van enige aminozuur residuen bij de binding van substraat en coenzyme aan p-hydroxybenzoaat hydroxylase uit *Pseudomonas fluorescens* en met behulp van ionsterkte afhankelijke bindingsstudies werd gekeken naar de rol van het eiwit in zijn geheel bij deze bindingen. In de algemene inleiding wordt aandacht besteed aan resultaten van vijftien jaar studie aan bovengenoemd enzym door verschillende groepen enerzijds en aan het gebruik van chemische modificatie van aminozuren in eiwitten in het algemeen anderzijds. Terwijl de meeste studies aan p-hydroxybenzoaat hydroxylase gericht zijn op de prosthetische groep FAD, was het onze bedoeling m.b.v. chemische modificatie meer informatie te krijgen over de rol van het eiwit daar omheen.

In hoofdstuk 2 wordt ingegaan op de rol van histidine. De modificatie werd uitgevoerd met diethylpyrocarbonaat. Vier van de negen histidine residuen bleken te reageren, wat gepaard ging met totale inactivatie. Twee hiervan bleken essentieel voor de enzymactiviteit en betrokken te zijn bij de binding van NADPH. De inactivatiesnelheid was sterk afhankelijk van de pH en nam toe bij toenemende pH. Uit de pH afhankelijkheid van de snelheidsconstante kon de pK waarde van twee coöperatief ioniserende histidine residuen worden bepaald. Bij pH>7 bleken er ook tyrosine residuen gecarbethoxyleerd te worden en ging de substraat bindingscapaciteit verloren.

De modificatie van arginine residuen wordt beschreven in hoofdstuk 3. Van de drie gebruikte inactiverende reagentia bleek er één met twee arginine residuen te reageren die van belang zijn voor de binding van NADPH en substraat. Een model werd opgesteld om de resultaten te verklaren. De andere twee reagentia bleken met andere residuen te reageren, wat verklaard is met verschillen in lading en hydrofobiciteit. De complexiteit van de arginine modificatie is te wijten aan het grote aantal arginines dat in p-hydroxybenzoaat hydroxylase aanwezig is (38).

96

In hoofdstuk 4 wordt de modificatie van tyrosine residuen beschreven. Voortbordurend op de resultaten van hoofdstuk 2 werden er nog enkele proeven met diethylpyrocarbonaat gedaan en daarnaast werd er gemodificeerd met het substraat analoog p-diazobenzoaat. Hoewel de identificatie proeven net als bij de arginine modificatie niet de gewenste kwantitatieve resultaten gaven, kon toch worden vastgesteld dat een reactie met tyrosine-201 verantwoordelijk was voor het verlies aan substraat-bindingscapaciteit. Geconcludeerd werd dat van de andere twee tyrosine residuen in het actieve centrum tyrosine-385 ook essentieel is terwijl tyrosine-222 niet essentieel is.

In hoofdstuk 5 wordt naar het eiwit in zijn geheel gekeken. De ionsterkte-afhankelijkheid van NADPH binding aan het enzym wordt verklaard met behulp van een model waarbij het enzym als zowel monopool als dipool een rol speelt. Het dipoolmoment in de richting van de NADPH-bindingsplaats werd berekend en vergeleken met een op basis van ladingsverdeling zeer ruw geschat dipoolmoment. Verder wordt er ingegaan op de schijnbaar tegenstrijdige pH-afhankelijkheden van NADPH-binding en enzymactiviteit.

Hoofdstuk 6 tenslotte, is gewijd aan de resultaten van tijdsafhankelijke fluorescentie-metingen aan gemodificeerd enzym-gebonden flavine. De hieruit bepaald rotatie correlatietijden werden vergeleken, om meer te kunnen zeggen over de mobiliteit van de flavine binnen het eiwit. De verschillen in de gevonden rotatie correlatietijden konden verklaard worden met een verschuiving van het dissociatie-evenwicht van de dimere vorm van p-hydroxybenzoaat hydroxylase.

Curriculum vitae

Robert Adriaan Wijnands, geboren op 24 december 1954 te Leiden. Na de lagere school gedeeltelijk in Nederland en gedeelteijk in Zambia te hebben doorlopen, begon ik in 1968 onder het bezielde toezicht van de broeders van het instituut „St. Louis" te Oudenbosch aan mijn middelbare schoolopleiding. Het diploma atheneum B behaalde ik aan het Thomas More College te Oudenbosch in 1974. In datzelfde jaar begon ik met de scheikunde studie aan de Rijksuniversiteit te Leiden. Het kandidaatsexamen S-2 (scheikunde en biologie, met wiskunde en natuurkunde) legde ik in januari 1977 af. De studie voor het doctoraalexamen zette ik voort onder leiding van prof.dr. L. Bosch met als hoofdvak biochemie bij dr. J. van Duin, als bijvak organische chemie bij prof.dr. J. van Boom en dr. J. den Hartog en als derde richting spectroscopische methoden bij prof.dr. C. Altona. In deze periode haalde ik ook de onderwijsbevoegdheid in de scheikunde. Het doctoraalexamen legde ik op 29 augustus 1980 af. Drie dagen later was ik als wetenschappelijk ambtenaar in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.) om aan dit proefschrift te werken bij prof.dr. F. Müller aan de vakgroep biochemie van de Landbouwhogeschool te Wageningen. Op 1 september 1984 eindigde dit dienstverband met Z.W.O. wegens beeindiging van de subsidie van mijn aandeel in dit onderzoek en sinds 1 oktober 1984 ben ik werkzaam bij het Instituut Kindergeneeskunde van de Erasmus Universiteit Rotterdam.