EXPLORING THE LIMITS OF VANILLYL-ALCOHOL OXIDASE

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
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in het openbaar te verdedigen
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Contents

Stellingen behorende bij het proefschrift
Exploring the limits of vanillyl-alcohol oxidase

1. Er is geen fundamentele reden waarom enzymen minder actief zouden zijn in niet-waterige media dan in waterige media.

   Klibanov, A. M. Improving enzymes by using them in organic solvents. *Nature* **409**, 241-246 (2001).

2. Om een winnaar te zijn moet een atleet niet alleen pijn accepteren, maar ook leren om ermee te leven en zelfs leren om er naar uit te kijken.

3. Net zoals een mens het 'recht' heeft om te leven heeft een mens ook het 'recht' om te sterven.

4. Tussen humaan en *Escherichia coli* methylenetetrahydrofolate reductase bestaat 30% sequentie identiteit. Daarom is voorzichtigheid geboden wanneer men de interpretatie van de kristalstructuur van het *Escherichia coli* enzym wil extrapoleren naar het humane enzym.

   Guenther, B.D. et al. The structure and properties of methylenetetrahydrofolate reductase from *Escherichia coli* suggest how folate ameliorates human hyperhomocysteinemia. *Nat. Struct. Biol.* **6**, 359-365 (1999).

5. De wereldbevolking valt te verdelen in vluchtelingen en toeristen.

   De Napolitaanse schilder Francesco Clemente.

6. Kleuren leiden alleen maar af, het gaat om de contrasten.

7. Men zou vaker bij zichzelf te rade moeten gaan in plaats van verantwoordelijkheden af te wenden naar overheidsinstanties.

8. Gezien het feit dat een verbetering van de enantiomere ratio van 1 (*e.e.* = 0) naar 1000 (*e.e.* > 99%) overeenkomt met een $\Delta\Delta G$ voor katalyse van slechts 17 $kJ*mol^{-1}$ is het niet verassend dat één of enkele aminozuurmutaties in een enzym grote effecten kunnen hebben op de stereospecificiteit.

   May, O., Nguyen, P.T. & Arnold, F.H. Inverting enantioselectivity by directed evolution of hydantoinase for improved production of L-methionine. *Nat. Biotechnol.* **18**, 317-320 (2000).
   van den Heuvel, R.H.H., Fraaije, M.W., Ferrer, M., Mattevi, A. & van Berkel, W.J.H. Inversion of stereospecificity of vanillyl-alcohol oxidase. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 9455-9460 (2000).

9. Sporten resulteert in hoge doses geestelijke en lichamelijke energie.

Chapter 1

BIOCATALYTIC PRODUCTION OF NATURAL VANILLIN AND OPTICALLY PURE COMPOUNDS

Introduction

The research described in this thesis was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Dutch Ministry of Economy Affairs. Together with six other projects, we were active in the Enzymatic Oxidation cluster. The aim within this cluster was to evaluate the potential of oxidative enzymes for the biocatalytic production of industrially relevant compounds.

In recent years, the interest for enzymatic processes has increased because of the rising demand for natural and environmentally friendly ingredients and products that have improved functionalities. The key advantage of enzymes is that they catalyze reactions specifically, often regio- and/or stereospecifically, and under mild conditions, thereby saving energy. Chemical processes are often less selective and less environmentally friendly. Another important factor that has triggered the use of enzymes for the production of valuable compounds such as pharmaceuticals, fine-chemicals, and flavors and fragrances is the enormous progress made in genetic, medium, and protein engineering. These techniques provide us with the tools to overproduce and redesign enzymes, paving the way to form large amounts of the product of interest. In this chapter, we discuss the biocatalytic methods for the production of the flavor compound vanillin (4-hydroxy-3-methoxybenzaldehyde) (**1**) and the production of optically pure aromatic compounds. Next, we introduce the enzyme vanillyl-alcohol oxidase as being a promising candidate to produce these compounds.

Production of natural vanillin

Vanillin is widely used as a flavoring compound in the food industry. Vanillin has also some potential medical applications, since in recent years, it has become apparent that vanillin is an antioxidant and may have the potential to prevent oxidative damage in mammalian cells [1,2]. Moreover, it has been reported that vanillin is a potential agent for the treatment of sickle cell anemia [3].

Natural vanillin is produced from glucovanillin (**2**) when the beans of the orchid *Vanilla planifolia* are submitted to a multi-step curing procedure [4] (Fig. 1). After this curing process, vanillin is the most abundant component of the bean at a level of about 2-3% by weight [5,6]. Other important aromatic components in the bean are 4-hydroxybenzaldehyde, 4-hydroxybenzylalcohol, and vanillic acid (4-hydroxy-3-methoxybenzoic acid). At present, only 0.4% of the world flavor market ($5*10^4$ kg/year out of $12*10^6$ kg/year) is originating from *V. planifolia* with synthetic vanillin providing the remainder [7]. Synthetic vanillin can be obtained from a number of chemical procedures using the feedstocks coniferin (glucoside of coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol), eugenol (4-allyl-2-methoxyphenol), lignin, or

guaiacol (2-methoxyphenol) [4], and is by far cheaper than the natural product from vanilla beans.

With the increasing interest in natural products alternative processes are being developed to produce natural vanillin. This is possible since the European Union legislation allows the origin of natural products deriving from living cells or their components, including incubations in which only isolated enzymes are applied [8]. The *Vanilla* plant is not ideal for biotechnological purposes, as the vanillin pathway is not very actively expressed and the plant is slowly growing [4,9]. Therefore, much effort has been put in the production of vanillin metabolites using microorganisms. Microbes can be selected for their ability to grow on a precursor of vanillin as sole energy and carbon source and the expression levels can be raised by molecular genetics.

Among the potential feedstocks suggested in literature, eugenol (**3**), the main component of clove oil, and ferulic acid (4-hydroxy-3-methoxycinnamic acid) (**4**), an abundant cell-wall material, are the most promising (for recent reviews see [4,10]). Eugenol catabolism to vanillin via ferulic acid has been characterized in *Pseudomonas* strains [11,12] and *Serratia marcescens* [13] (Fig. 1). It is interesting to mention here that the first reaction in the pathway from eugenol to ferulic acid, the hydroxylation of eugenol to coniferyl alcohol, is also catalyzed by vanillyl-alcohol oxidase from *Penicillium simplicissimum* [14].

Vanillic acid (**5**) is the major product obtained from the catabolism of ferulic acid by several microorganisms like *Pseudomonas* [15], *Streptomyces* [16], and *Rhodotorula* [17] species. Both carboxylic acids can be used as starting material for the production of natural vanillin. It has been reported that a *Nocardia* strain produces vanillin from vanillic acid [18]. Substantially higher levels of vanillin have been obtained with *Amycolatopsis* [19] and *Streptomyces setonii* [16]. Lesage-Meessen et al. [8,20] reported a two-step bioconversion process for the production of vanillin from ferulic acid. First, ferulic acid is catabolized by *Aspergillus niger* to vanillic acid. Second, vanillic acid is reduced by *Pycnoporus cinnabarinus* into vanillin (Fig. 1)

Next to microbes, isolated enzymes form an attractive alternative source for the production of natural vanillin. The pungent principal of red pepper, capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) (**6**) is a precursor of vanillin and can be easily obtained at relatively low cost. In the first step of the conversion of capsaicin to vanillin, capsaicin is enzymically or chemically hydrolyzed to form vanillylamine (4-hydroxy-3-methoxybenzylamine) (**7**) [21]. This latter compound can be easily oxidized by amine oxidases [22] and vanillyl-alcohol oxidase [23] yielding vanillin (Fig. 1).

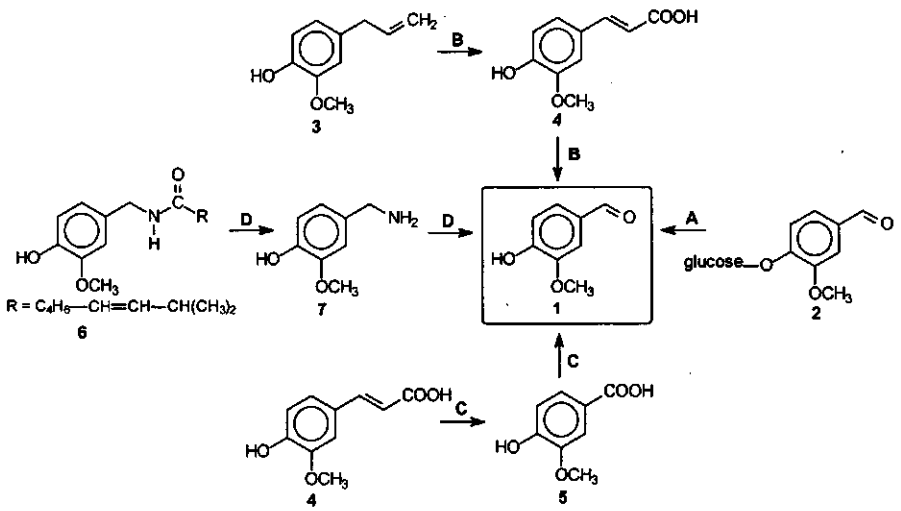

Fig. 1. Routes for the production of natural vanillin. (**A**) *Vanilla planifolia*, (**B**) catabolism of eugenol via ferulic acid, (**C**) catabolism of ferulic acid via vanillic acid, and (**D**) hydrolysis of capsaicin, followed by the oxidation of vanillylamine.

Production of optically pure compounds

Enantiospecific biological effects associated with chiral compounds are well recognized in the fine-chemical and pharmaceutical industry. For example, 50 of the 100 most widely used drugs, including iboprofen, ritalin, and barbiturates are produced as a single enantiomer to avoid negative side effects and unwanted toxicity of the inactive enantiomer [24].

There are several methods to obtain optically pure compounds. An obvious method is the use of pure enantiomers that exist in nature. These compounds can serve as feedstocks to produce new optically pure compounds. However, their use is limited as the number of useful enantiomeric pure compounds is relatively low [25]. The second method involves the resolution of a racemic mixture. In this method the relatively cheap symmetric synthesis of racemates is followed by a resolution step [26]. Kinetic resolution of a racemate is based on the difference in reaction rates of the enantiomers with a chiral catalyst. This can either be a chemical catalyst, an enzyme, or a microorganism. Asymmetric synthesis is the third method to produce enantiopure compounds. In this way, a prochiral substrate is converted chemically or enzymically to a chiral end product [27].

Enzymes are highly suitable for the production of optically pure compounds, as they have the ability to catalyze reactions regio- and/or stereospecifically. A major disadvantage, however, is that a desired enantiomer of a certain compound often

requires an expensive search for a suitable enzyme with the proper selectivity. To overcome this bottleneck much effort has been put in the development of methods to change the selectivity of enzymes.

Currently, medium engineering, site-directed mutagenesis, and directed evolution have been successfully used to alter the stereospecificity of enzymes. Examples in which the enzyme stereospecificity was successfully improved using organic solvents, include α-chymoptrypsin [28,29], lipase from *Pseudomonas* sp. [30], subtilisin Carlsberg [28,31], and lipase from *Candida rugosa* [32]. The increased stereospecificity of epoxide hydrolase upon the substitution of Tyr215 is a good example of the use of rationale mutagenesis to increase the stereospecificity of enzymes [33].

Random mutagenesis and recombination of a target gene or a family of related genes are other means to create molecular diversity and alter enzymatic properties. For example, the stereospecific hydrolysis of 2-methyldecanoic ester using lipase from *Pseudomonas aeroginosa* increased from an enantiomeric excess of 2% to >90% using directed evolution [34]. Only recently, the first example of a reversal in stereospecificity by recombinant tools was reported. Through random and saturation mutagenesis, the stereospecificity of D-hydantoinase was inverted and the reactivity was increased five-fold. This improved the production of L-methionine in a multi-enzyme process [35].

Vanillyl-alcohol oxidase

In this Ph. D. project, we have studied the catalytic potential of the enzyme vanillyl-alcohol oxidase (VAO; EC 1.1.3.38). VAO is a FAD-dependent enzyme, obtained from the ascomycete *Penicillium simplicissimum*, that converts vanillyl alcohol to vanillin [36]. *P. simplicissimum* was isolated near a paper mill in Wageningen (Parenco) and was shown to use a wide range of phenolic lignin derivatives as sole carbon and energy source [37]. When grown on veratryl alcohol, the ascomycete produces large amounts of VAO (up to 5% of total protein). Nevertheless, it appeared that the enzyme is not involved in the degradation of this aromatic alcohol. Further investigation revealed that VAO is also induced when the fungus is grown on anisyl alcohol or 4-(methoxymethyl)phenol, but that the enzyme is only involved in the biodegradation of the latter compound (Fig. 2). From this it was proposed that 4-(methoxymethyl)phenol and *p*-cresol ether analogs serve as physiological substrates for VAO [38].

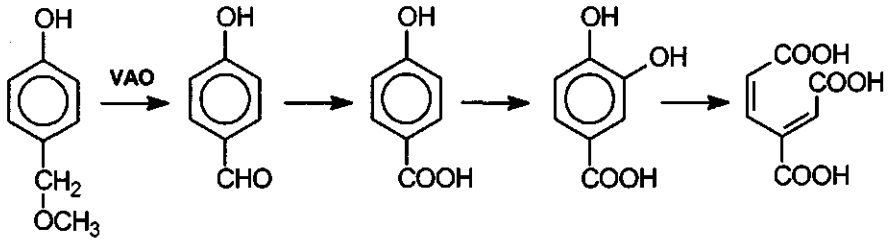

Fig. 2. Biodegradation of 4-(methoxymethyl)phenol by *P. simplicissimum*.

Substrate specificity studies have shown that the scope of reactions catalyzed by VAO is rather broad [23,39]. The enzyme converts a wide range of 4-hydroxybenzylic compounds by catalyzing oxidation, oxidative demethylation, oxidative deamination, hydroxylation, and dehydrogenation reactions (Fig. 3). The hydroxylation of eugenol, the main component of clove oil, to coniferyl alcohol is patented by Quest International [14]. Moreover, the production of vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzylalcohol (all components of vanilla flavor), 4-vinylphenol (present in wine and orange juice), and optically pure aromatic alcohols have biotechnological potential.

Catalysis of flavoprotein oxidases involves two half-reactions in which first the flavin cofactor is reduced by the substrate and subsequently the reduced flavin is reoxidized by molecular oxygen. The reaction mechanism of VAO has been studied in great detail using steady-state and stopped-flow spectrophotometric techniques [40,41]. Reaction of VAO with 4-(methoxymethyl)phenol involves the initial transfer of a hydride from the Cα-atom of the substrate to the N5-atom of the flavin, resulting in a complex between reduced enzyme and *p*-quinone methide intermediate. Next, the reduced flavin is reoxidized by molecular oxygen with the concomitant hydration of the *p*-quinone methide (Fig. 4).

A few years ago the crystal structure of free VAO and in complex with several inhibitors was elucidated [42] (Fig. 5). Since then, a number of crystal structures of VAO variants have been determined (Table 1). The *vao*-gene was cloned and expressed in *Escherichia coli* [43]. Multiple sequence alignments and structural interpretation revealed that VAO belongs to a novel family of structurally related oxidoreductases sharing a conserved flavin-binding domain [44]. To date, this family has already more than 100 members and several crystal structures are known.

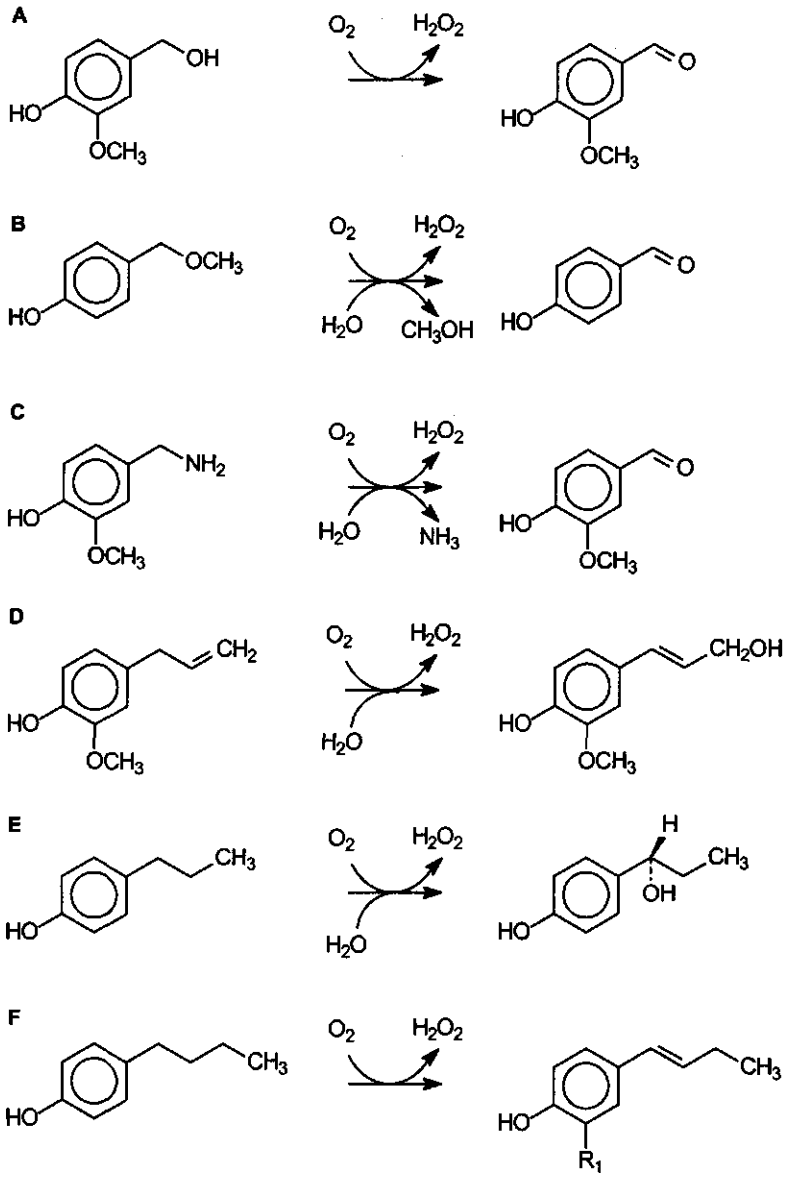

Fig. 3. Reactions catalyzed by VAO. (**A**) Oxidation of vanillyl alcohol, (**B**) oxidative demethylation of 4-(methoxymethyl)phenol, (**C**), oxidative deamination of vanillylamine, (**D**) hydroxylation of eugenol, (**E**) hydroxylation of 4-propylphenol, and (**F**) dehydrogenation of 4-butylphenol.

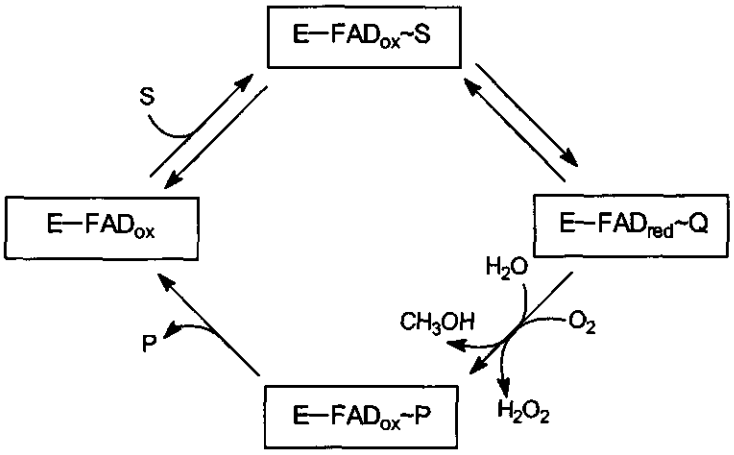

Fig. 4. Overall reaction for the oxidative demethylation of 4-(methoxymethyl)phenol by VAO. S, 4-(methoxymethyl)phenol; Q, *p*-quinone methide intermediate; P, 4-hydroxybenzaldehyde; E-FAD_{ox}, oxidized VAO; E-FAD_{red}, reduced VAO.

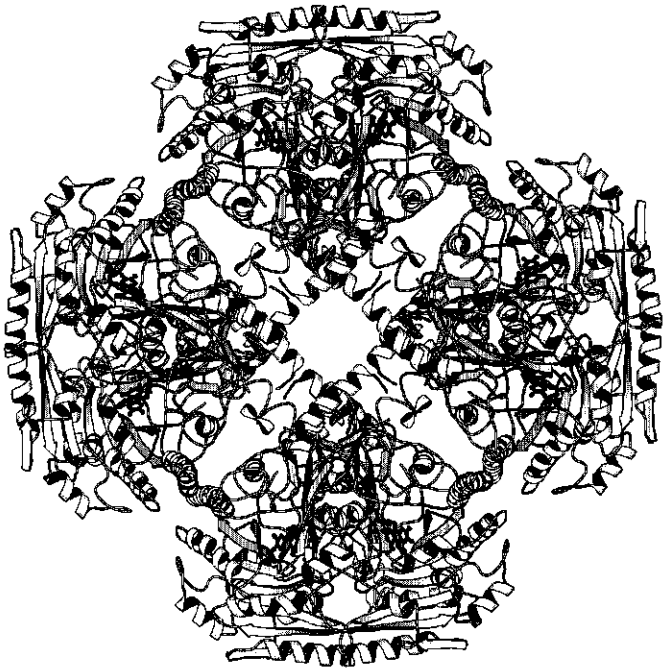

Fig. 5. Drawing of the quaternary structure of VAO. This figure was prepared with MOLSCRIPT [45].

These include the peripheral membrane respiratory flavoenzyme D-lactate dehydrogenase [46], MurB (UDP-*N*-acetylenolpyruvylglucosamine reductase), an enzyme involved in peptidoglycan biosynthesis [47], the molybdenum iron-sulfur flavoprotein carbon monoxide dehydrogenase [48], the molybdopterin iron-sulfur flavoenzyme xanthine dehydrogenase [49], and the flavocytochrome *p*-cresol methylhydroxylase (PCMH) [50].

The substrate specificity of PCMH is comparable to that of VAO, however, this enzyme uses a cytochrome and not molecular oxygen as electron acceptor. Interestingly, in PCMH the flavin is linked to the enzyme via a tyrosyl residue [51], whereas in VAO the FAD is linked via a histidine residue [36]. Moreover, the quaternary structures are different as PCMH is a heterotetramer (2 × 58,700 Da and 2 × 9,200 Da) [52] and VAO a homooctamer (8 × 63,691 Da) [43,53] (Fig. 5).

Table 1. Crystal structures of VAO variants.

VAO variant	Inhibitor bound	Resolution (Å)	PDB entry	Reference
Wild type VAO	-	2.5	1VAO	42
Wild type VAO	*p*-Cresol	2.7	1AHU	42
Wild type VAO	4-Heptenylphenol	3.3	1AHZ	42
Wild type VAO	Isoeugenol	3.1	2VAO	42
Wild type VAO	2-Nitro-*p*-cresol	3.1	1AHV	42
H422A	-	2.2	1QLT	54
H422A	Isoeugenol	2.4	1QLU	54
H61T-holo	4-(Trifluoromethyl)phenol	2.1	1E8G	55
H61T-apo	ADP	2.6	1E8H	55
H61T-apo	-	2.9	1E8F	55
D170S	Isoeugenol	2.8	1DZN	56
D170S/T457E	4-(Trifluoromethyl)phenol	2.75	1E0Y	57

Outline of this thesis

In this Ph. D. research project we aimed to enlarge the catalytic potential of the enzyme VAO for the production of industrially relevant compounds. In **Chapter 2**, we report about the VAO-mediated conversion of creosol and vanillylamine to vanillin. The principal component of red pepper capsaicin, a precursor of vanillylamine, appears to be a promising candidate for the production of natural vanillin.

Chapter 3 describes the VAO-mediated conversion of short-chain 4-alkylphenols to optically pure aromatic alcohols and the conversion of medium-chain 4-alkylphenols to *cis* or *trans* aromatic alkenes.

In **Chapter 4**, the reactivity of VAO with 4-alkylphenols in non-aqueous solutions is described. Part of this work was performed at the University of Strathclyde (Glasgow, UK) in collaboration with Prof. Dr. Peter Halling. At low water activities the efficiency of substrate hydroxylation decreased compared to the reaction performed in aqueous solution. The addition of specific monovalent anions to the medium had a similar effect.

The availability of the VAO crystal structure and the expression system in *Escherichia coli* allowed us to investigate the possibilities of tuning the reactivity of VAO with 4-alkylphenols by protein engineering. In **Chapter 5** and **6**, it was found that Asp170 is of importance for activity of VAO, the process of self-catalytic covalent flavinylation, and the efficiency of 4-alkylphenol hydroxylation. The X-ray structure of one of the VAO variants (D170S) showed that the Asp170 replacement does not induce any structural perturbation (in collaboration with dr. Andrea Mattevi (University of Pavia, Pavia, Italy)).

In **Chapter 7**, we describe the inversion of the stereospecificity of VAO by protein engineering. When Asp170 is transferred to the opposite face of the substrate-binding pocket, the stereospecificity of the enzyme is reverted. Crystallographic analysis of the D170S/T457E double mutant revealed no major structural changes compared to wild type VAO. This is the first example of the inversion of the stereospecificity of an enzyme using a rationale approach.

In **Chapter 8**, the role of the covalent protein-flavin interaction was studied by site-directed mutagenesis. The covalent flavin attachment appears to be crucial for efficient enzyme catalysis by raising the redox potential of the flavin.

In **Chapter 9**, the oligomeric structure of VAO was investigated by electrospray ionization mass spectometry as a function of pH (in collaboration with Prof. dr. Albert Heck (University of Utrecht, Utrecht)). These studies showed for the first time that the accurate determination of protein molecular masses above 1 million Da is feasible. Furthermore, these studies have yielded more insight in the subunit-subunit interaction of VAO and thus in its stability.

Chapter 2

ENZYMATIC SYNTHESIS OF NATURAL VANILLIN

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Submitted

With the increasing interest in natural vanillin two enzymatic routes for the synthesis of vanillin were developed. The flavoprotein vanillyl-alcohol oxidase (VAO) acts on a wide range of phenolic compounds and converts both creosol and vanillylamine to vanillin with high yield. The VAO-mediated conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. Catalysis is limited by the formation of an abortive complex between enzyme-bound flavin and creosol. Moreover, in the second step of the process, the conversion of vanillyl alcohol is inhibited by the competitive binding of creosol. The VAO-catalyzed conversion of vanillylamine proceeds efficiently at alkaline pH values. Vanillylamine is initially converted to a vanillylimine intermediate product, which is hydrolyzed non-enzymically to vanillin. This route to vanillin has biotechnological potential as the widely available principal of red pepper, capsaicin, can be hydrolyzed enzymically to vanillylamine.

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavor compound in food and personal products [4] and in high concentrations the anti-oxidizing properties of this compound prevent oxidative damage in mammalian cells [1,2]. By far the dominant route for vanillin production is the chemical synthesis from lignin, coniferin (glucoside of coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol)), guaiacol (2-methoxyphenol), or eugenol (4-allyl-2-methoxyphenol) [4]. Natural vanillin supplies for less than 1% of the total demand for vanillin and is produced from glucovanillin when the beans of the orchid *Vanilla planifolia* are submitted to a multi-step curing process. After this curing process, vanillin is the most abundant component of the bean at a level of about 2-3% by weight [5,6].

With the increasing interest in natural products alternative processes are being developed to produce natural vanillin. This is possible since the European Union legislation allows the origin of natural products deriving from living cells or their components, including incubations in which only isolated enzymes are applied [8]. In recent years, a large number of studies have been made on natural vanillin biosynthesis using microorganisms or isolated enzymes [7,9,10,18,58]. However, these bioconversions are not economically feasible yet.

The flavoprotein vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* [36] is a versatile biocatalyst that is of particular interest for the production of vanillin. Eugenol (4-allyl-2-methoxyphenol) is the principal constituent of clove oil and is an economically realistic feedstock. VAO catalyzes the hydroxylation of eugenol to coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) [14,23]. This aromatic alcohol can be further converted, via ferulic acid (4-hydroxy-3-methoxycinnamic acid), to vanillin by several microorganisms (for recent reviews see [4,10]).

VAO can also produce vanillin by catalyzing the oxidation of vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), the oxidative demethylation of 2-methoxy-4-(methoxymethyl)phenol, and the oxidative deamination of vanillylamine (4-hydroxy-3-methoxybenzylamine) [23]. Vanillyl alcohol, 2-methoxy-4-(methoxymethyl)phenol, and vanillylamine are not widely available in nature and thus not of direct interest for biotechnological applications. However, vanillylamine can be obtained from the abundant precursor compound capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principal of hot red pepper, by the cleavage of its amide bond [21,59] (Fig. 1A). Another potential feedstock for vanillin is creosol (2-methoxy-*p*-cresol), which is the major component in creosote obtained from heating wood or coal tar [60,61]. On the basis of the reactivity of VAO with *p*-cresol (Fig. 1B) it was anticipated that creosol is oxidized to vanillin [41].

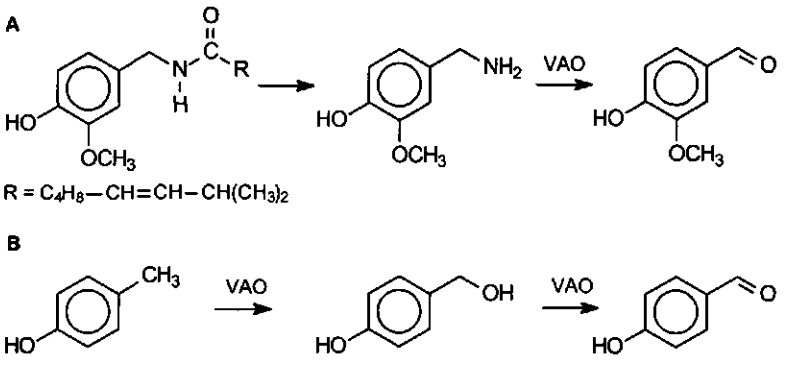

Fig. 1. Two-step enzymatic conversion of (**A**) capsaicin to vanillin and (**B**) *p*-cresol to 4-hydroxybenzaldehyde.

In this study, we have investigated the VAO-mediated synthesis of vanillin from natural feedstocks. We selected creosol, which is the major product in creosote, and vanillylamine, as this compound can be obtained from capsaicin. We found that VAO oxidizes both compounds to vanillin with high yields.

Materials and Methods

Materials. Creosol (2-methoxy-*p*-cresol), vanillylamine (4-hydroxy-3-methoxybenzylamine), vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), and vanillin (4-hydroxy-3-methoxybenzaldehyde) were obtained from Aldrich and capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) from Fluka. Penicillin G acylase from *Escherichia coli* was a kind gift from DSM/Gist, Delft, The Netherlands. Lipase B from *Candida antarctica* was obtained from Novo Nordisk, carboxypeptidase A from bovine pancreas, carboxypeptidase Y from yeast, acylase from pig kidney, and thermolysin from *Bacillus thermoproteolyticus* were purchased from Sigma. All other chemicals were obtained as described before [56,62]. Rat liver microsomes were prepared essentially as described previously [63].

Expression and purification of VAO. *E. coli* strain TG2 [64] and the plasmid pEMBL19(-) (Boehringer Mannheim) were used for expression of the *vao*A gene. Transformed *E. coli* cells were grown in Luria-Bertani medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside [43]. VAO was purified as described [43,56].

Analytical methods. VAO activity was determined at 25 °C by monitoring absorption spectral changes of aromatic products or by oxygen consumption experiments using a Clark electrode [23]. Vanillin production was measured at 340 nm (ε_{340} varies from 2.2 $mM^{-1}*cm^{-1}$ at pH 5.6 to ε_{340} = 23.3 $mM^{-1}*cm^{-1}$ at pH 10.5 with a pK_a of 7.2) and vanillylimine production at 390 nm. For enzyme-monitored-turnover experiments, air-saturated enzyme and substrate solutions were mixed, and the redox-state of the flavin prosthetic group was monitored at 439 nm using a Hewlett-Packard HP 8453 diode-array spectrophotometer [41,65]. Stopped-flow kinetics were performed with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech SU-40 spectrophotometer, essentially as described previously [40]. In anaerobic reduction experiments, glucose-containing enzyme solutions were flushed with oxygen-free argon gas and glucose oxidase was added to eliminate final traces of oxygen. Fluorescence emission spectra were recorded at 25 °C on an Aminco SPF-500C spectrofluorometer. The excitation wavelength was 360 nm [41].

HPLC experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode-array detector and a 4.6 by 150 mm Alltima C18 column (Alltech) using mixtures of methanol and water containing 1% (v/v) acetic acid. For monitoring the conversion of creosol to vanillin a methanol/water ratio of 35/65 (v/v) was used. For monitoring the conversion of capsaicin to vanillylamine elution started with a linear gradient from 8% to 13% (v/v) methanol in 20 min followed by an increase to 75% in 3 min, which was held for 20 minutes. The VAO-mediated conversion of creosol was carried out in different buffers at 25 °C. The hydrolysis of capsaicin by penicillin G acylase was performed in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C and the hydrolysis of capsaicin by rat liver microsomes was performed in 50 mM potassium phosphate buffer, pH 7.0 at 37 °C. Chemical hydrolysis of capsaicin was carried out in 10 mM or 100 mM sodium hydroxide at 95 °C. The reactions were terminated by the addition of 5% (w/v) trichloroacetic acid and subsequent centrifugation.

Results

Conversion of creosol. Table 1 summarizes the steady-state kinetic parameters of VAO with 4-(methoxymethyl)phenol, vanillyl alcohol, vanillylamine, and creosol at pH 7.5. 4-(Methoxymethyl)phenol and vanillyl alcohol are efficient substrates for VAO, whereas both vanillylamine and creosol react rather slowly with the enzyme. In contrast to the other substrates, the conversion of creosol proceeds via a two-step enzymatic process. In the first step creosol is hydroxylated by VAO to yield vanillyl alcohol and in the second step vanillyl alcohol is oxidized to yield vanillin.

Table 1. Steady-state kinetic parameters for VAO in air-saturated 50 mM potassium phosphate buffer, pH 7.5 at 25 °C.

Substrate	k'_{cat}	K'_m	k'_{cat}/K'_m
	s^{-1}	μM	$s^{-1}*mM^{-1}$
4-(methoxymethyl)phenol*	3.1	55	56.4
Vanillylamine	0.02	48	0.42
Creosol	0.07	50	1.4
Vanillyl alcohol*	3.3	160	20.6

*Data from ref. [40].

Earlier studies have shown that the reaction of VAO with 4-(methoxymethyl)phenol and vanillyl alcohol is optimal around pH 10 [23,36]. However, when the VAO-mediated conversion of creosol was studied at pH 10, nearly no activity was observed. Upon determining the pH optimum of the reaction, we found that VAO is most active with creosol at pH 7.5 (Fig. 2). This pH optimum is identical to the optimum of the VAO-mediated oxidation of *p*-cresol [41] and clearly distinct from other VAO-catalyzed oxidations [36]. It should be noted here that the pH optimum of the reaction with creosol was determined at a substrate concentration of 150 μM. At higher substrate concentrations the overall conversion rate decreased due to the competitive binding of creosol, inhibiting the conversion of the intermediate product vanillyl alcohol.

Table 1 shows that the catalytic efficiency of VAO with creosol at pH 7.5 is 15-fold lower compared to the efficiency with vanillyl alcohol. This suggests that the slow hydroxylation of creosol limits the synthesis of vanillin. Therefore, we studied the initial enzymatic conversion of creosol to vanillyl alcohol in more detail. This reaction can be described by two half-reactions. First, the enzyme-bound flavin cofactor is reduced by the substrate, forming a complex between the reduced enzyme and the *p*-quinone methide intermediate of creosol (Eqn. 1). In the second step, the reduced enzyme is reoxidized by molecular oxygen and water attacks the intermediate product, forming vanillyl alcohol (Eqn. 2).

$$\text{Eox} + \text{creosol} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Eox}\sim\text{creosol} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Ered}\sim\text{quinone methide} \qquad \text{(Eqn. 1)}$$

$$\text{Ered}\sim\text{quinone methide} + O_2 + H_2O \xrightarrow{k_3} \text{Eox} + \text{vanillyl alcohol} + H_2O_2 \qquad \text{(Eqn. 2)}$$

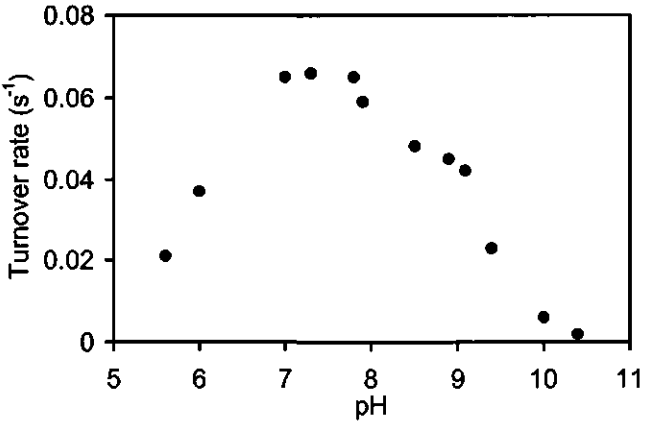

Fig. 2. pH dependence of the reaction of VAO with creosol. The activity measurements were performed in 50 mM succinate/sodium hydroxide (pH 5.6-6.9), 50 mM potassium phosphate (pH 6.1-7.8), 50 mM Tris/sulfuric acid (pH 7.9-8.9), or 50 mM glycine/sodium hydroxide (pH 9.1-10.4) at 25 °C using 150 μM creosol and 0.5 μM VAO.

In most VAO-catalyzed reactions studied so far, the reductive half-reaction is limiting catalysis [40,41]. When creosol and VAO were mixed in the stopped-flow spectrophotometer under anaerobic conditions, a monophasic decrease in absorbance at 439 nm was observed, indicative for the reduction of enzyme-bound flavin. The rate of reduction was dependent on the concentration of creosol with a dissociation constant K_d = 159 ± 34 μM at pH 7.5 (Fig. 3). At low substrate concentrations the reduction rate reached a finite value of 0.16 s^{-1}, suggesting that the reduction is a reversible process in which k_2 = 0.16 ± 0.01 s^{-1} [41,66]. The forward reduction rate (k_2) was calculated to be 0.30 ± 0.02 s^{-1}, which is 4-fold higher than the turnover rate of VAO with this substrate. This strongly indicates that the reductive half-reaction, in analogy to the VAO-mediated conversion of *p*-cresol, only partially limits catalysis [41].

When VAO was mixed with creosol under aerobic conditions the redox-state of the flavin cofactor could be followed spectrophotometrically at 439 nm. We found that the enzyme-bound flavin was mainly in the reduced state during turnover at pH 7.5 (66%), again indicating that the reductive half-reaction does not limit the turnover rate. Moreover, upon excitation at 360 nm of the aerobic complex between creosol and VAO a strong fluorescence emission with a maximum at 470 nm was observed. In line with earlier results this suggests the formation of an abortive covalent flavin-substrate adduct, which is stabilized under aerobic conditions [41].

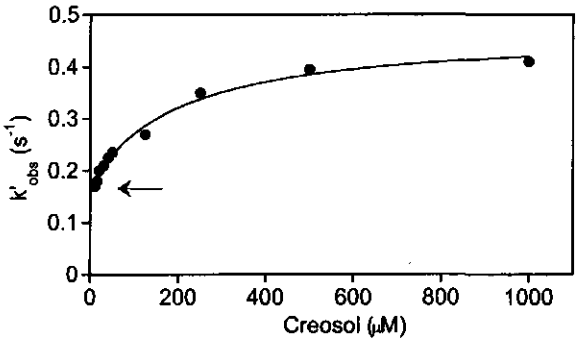

Fig. 3. Observed reduction rates of VAO with varying concentrations of creosol. VAO (2.5 μM) was mixed anaerobically with creosol (25-1000 μM) in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C. Flavin reduction was followed at 439 nm. The arrow indicates the value found for the reverse reaction (k_{-2}).

As mentioned before, the VAO-mediated turnover of creosol at pH 10 was 10-fold slower than at pH 7.5 (Fig. 2). When VAO and creosol were mixed at pH 10 under aerobic conditions, the flavin was nearly completely in the reduced state (95%). Moreover, the fluorescence emission spectrum upon excitation at 360 nm became stronger at pH 10 compared to pH 7.5. These results clearly support that the decreased turnover rate of creosol at high pH values is caused by the increased stability of the abortive creosol-flavin adduct.

When the VAO-mediated conversion of creosol was followed by HPLC only low concentrations of vanillyl alcohol were detected at pH 7.5 and 10 (Fig. 4). This is in agreement with the slow conversion of creosol relative to vanillyl alcohol. Both at pH 7.5 and 10 the final yield of vanillin was 100% when the reaction was performed at a starting substrate concentration of 100 μM as determined by HPLC and spectral analysis.

Vanillylamine production. Capsaicin, the pungent principle of red pepper, is a cheap feedstock for the production of vanillylamine. In agreement with earlier reports [21,59], we observed that capsaicin can be hydrolyzed by enzymes from liver to vanillylamine. In our experiments capsaicin was converted by rat liver microsomes to vanillylamine at a rate of $1.2*10^{-3}$ $mM*min^{-1}*mg^{-1}$ protein. Potentially, the amide bond in capsaicin can also be hydrolyzed by other hydrolases. Therefore, several commercially available enzymes were tested for their capacity to hydrolyze capsaicin. Among these enzymes (see materials and methods) only penicillin G acylase was able to hydrolyze capsaicin at a rate of $3.5*10^{-6}$ $mM*min^{-1}*mg^{-1}$. Vanillylamine was

detected as the single aromatic product. For comparison, when 5 mM capsaicin was incubated in 100 mM sodium hydroxide at 95 °C the chemical hydrolysis proceeded with a pseudo first-order rate of $2.0*10^{-3}$ min^{-1}, whereas in 10 mM sodium hydroxide at 95 °C no hydrolysis occurred.

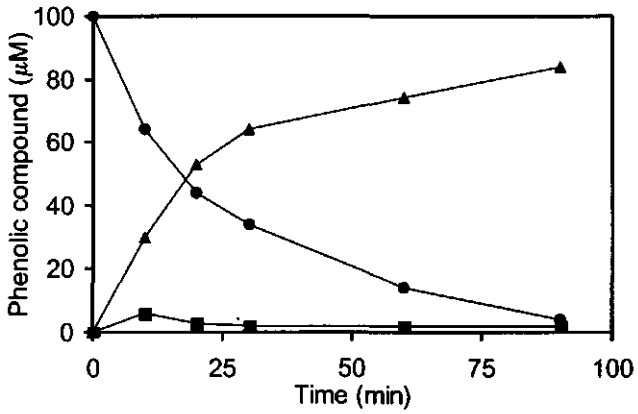

Fig. 4. HPLC analysis of the VAO-mediated conversion of creosol. The reaction mixture contained 100 μM creosol and 1 μM VAO in 50 mM potassium phosphate buffer, pH 7.5, and was incubated at 25 °C. The concentrations of creosol (●), vanillyl alcohol (■), and vanillin (▲) were determined from reference solutions.

Conversion of vanillylamine. Next to creosol, vanillylamine obtained from capsaicin is a natural precursor of vanillin. The VAO-catalyzed conversion of vanillylamine involves the initial formation of vanillylimine. Subsequently, this intermediate product is hydrolyzed non-enzymically to vanillin [23]. When the conversion of vanillylamine by VAO was studied at pH 7.5 the turnover rate of the enzyme was very low (Table 1). This prompted us to study the pH dependence of the enzymatic reaction by oxygen consumption experiments as well as spectral analysis. With both methods we found similar pH-dependent turnover rates (Fig. 5). The rate of the VAO-mediated conversion of vanillylamine increased dramatically when the pH of the reaction medium was raised above pH 9. Again, this pH profile is clearly distinct from other VAO-mediated reactions [23,36]. Due to the protein instability it was not possible to measure reliable turnover rates at pH values above 10.5. When VAO and vanillylamine were mixed aerobically the redox-state of the flavin cofactor could be followed spectrophotometrically. It was found that the enzyme is mainly in the oxidized state during turnover at both pH 7.5 (80%) and pH 9.7 (>90%), strongly indicating that, in contrast to the VAO-mediated conversion of creosol, the reductive half-reaction limits the turnover rate.

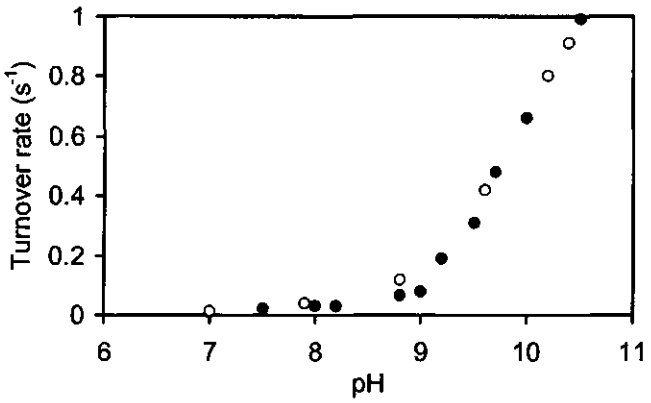

Fig. 5. pH dependence of the reaction of VAO with vanillylamine. VAO activity was monitored either by spectral analysis of vanillin production (●) or oxygen consumption (o). The reactions were performed in 50 mM potassium phosphate (pH 7.0-7.8), 50 mM Tris/sulfuric acid (pH 7.8-8.9), or 50 mM glycine/sodium hydroxide (pH 9.0-10.5) at 25 °C using 1 mM vanillylamine and 1 μM VAO for spectral studies or 4 μM VAO for oxygen consumption experiments.

When the enzymatic deamination of vanillylamine was monitored spectrophotometrically, the initial formation of the intermediate product vanillylimine and its subsequent conversion to vanillin could be observed at 390 nm and 340 nm, respectively (Fig. 6). The concentration of vanillylimine during catalysis was calculated using the molar absorption coefficient of vanillin (ε_{340} = 22.9 $mM^{-1}*cm^{-1}$ at pH 9.0) and the absorbance changes in time due to vanillylimine and vanillin formation. The transient accumulation of relatively low amounts of vanillylimine is in line with the observation that the enzymatic formation of vanillylimine is the rate-limiting step in catalysis. As for the VAO-mediated conversion of creosol, the yield of vanillin production reached nearly 100% (see inset Fig. 6).

Discussion

In the present paper we have addressed the catalytic potential of VAO for the production of natural vanillin. A potential attractive feedstock for the enzymatic formation of vanillin is creosol, which can be obtained from creosote [60,61]. The VAO-mediated conversion of creosol to vanillin reaches a yield of 100%, but the conversion rate is rather low due to the formation of a non-reactive covalent adduct between creosol and the flavin prosthetic group of VAO.

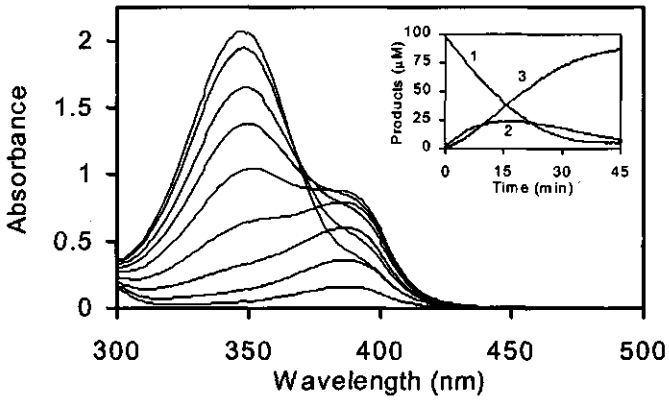

Fig. 6. Enzymatic conversion of vanillylamine via vanillylimine to vanillin. The reaction mixture contained 50 mM glycine/sodium hydroxide buffer, pH 9.0, 100 μM vanillylamine, and 0.5 μM VAO and was incubated at 25 °C . Spectra were taken at regular time intervals after the addition of VAO. The inset shows the estimated concentrations of (1) vanillylamine, (2) vanillylimine, and (3) vanillin, respectively.

The covalent adduct slowly decomposes to vanillyl alcohol, thereby determining the overall turnover rate of VAO (Fig. 7). The flavin-creosol adduct is more stable at basic pH values, hence shifting the pH optimum of VAO, which is normally around pH 10, to pH 7.5 [36,40]. Moreover, the competitive binding of creosol inhibited the conversion of the intermediate product vanillyl alcohol to vanillin. As a consequence, the optimal conditions for the VAO-catalyzed conversion of creosol were found to be at pH 7.5 and a substrate concentration of 150 μM.

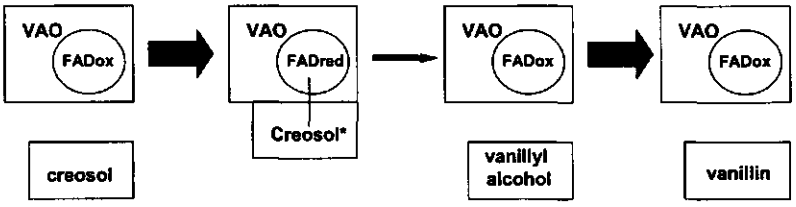

Fig. 7. Schematic drawing of the VAO-mediated conversion of creosol to vanillin.

A second potential attractive feedstock for the enzymatic production of vanillin is capsaicin. This pungent principal of red pepper can be easily obtained at low cost and can be hydrolyzed enzymically to vanillylamine by a carboxylesterase from liver. This esterase has been found in liver of chicken, hog, cow, and rat [59] and the rat liver

enzyme has been purified and characterized to some extent [21]. Capsaicin is not hydrolyzed by trypsin, peptidase, and aminoacylase [59]. Here, we found that capsaicin was extremely slowly hydrolyzed by penicillin G acylase from *E. coli*. The rate of this enzymatic hydrolysis reaction might be increased by performing the reaction in organic solvents, as the solubility of capsaicin will be enhanced.

Vanillylamine reacted slowly with VAO at neutral pH values, but was efficiently converted between pH 9 and 10.5 with a high vanillin yield. This strong increase in VAO activity around pH 9 is unusual. As the rate-limiting step in the conversion of vanillylamine to vanillin is associated with the formation of the vanillylimine intermediate, the pH dependence of the reaction might be related to the binding of vanillylamine. For other VAO substrates, it is thought that the initial formation of the quinone methide intermediate (Eqn. 1) is facilitated by the preferential binding of the phenolate form of the substrate [42]. For example, when the substrate analog isoeugenol (2-methoxy-4-propenylphenol) binds to VAO the pK_a of the phenol decreases from 9.8 to 5.0 [23]. Possibly, binding of vanillylamine does not stimulate substrate deprotonation. Alternatively, the pH effect might be related to the preferred binding of the phenolate form of vanillylamine.

In conclusion, this paper presents two novel enzymatic routes for the biocatalytic production of natural vanillin. The bi-enzymatic process from capsaicin to vanillin is most promising from a biotechnological point of view as capsaicin is a widely available compound. To make this process more feasible, the first enzyme in the reaction sequence, a carboxylesterase, needs to be characterized in detail and overexpressed in a suitable host. The VAO-catalyzed deamination of vanillylamine is efficient, does not need any external cofactors, and uses molecular oxygen as clean and mild oxidant. Moreover, the enzyme can be easily obtained in large amounts [43]. When the two enzymes can be combined in a single reaction mixture, one can design a one-pot reactor to produce natural vanillin.

Acknowledgements

We thank Maurice Franssen (Wageningen University) for fruitful discussions and the gift of lipase B from *Candida antarctica* and Marlou van Iersel (Wageningen University) for the gift of rat liver microsomes. We also thank Niek Bastiaensen and Rigoberto Gonzales for technical assistance. This work was performed within the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Dutch Ministry of Economy Affairs (project IKA 96005).

Chapter 3

REGIO- AND STEREOSPECIFIC CONVERSION OF 4-ALKYLPHENOLS BY THE COVALENT FLAVOPROTEIN VANILLYL-ALCOHOL OXIDASE

Robert H.H. van den Heuvel, Marco W. Fraaije, Colja Laane, and Willem J.H. van Berkel

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The regio- and stereospecific conversion of prochiral 4-alkylphenols by the covalent flavoprotein vanillyl-alcohol oxidase (VAO) was investigated. The enzyme was active with 4-alkylphenols bearing aliphatic side chains up to seven carbon atoms. Optimal catalytic efficiency occurred with 4-ethylphenol and 4-n-propylphenols. These short-chain 4-alkylphenols are stereoselectively hydroxylated to the corresponding (*R*)-1-(4'-hydroxyphenyl)alcohols [39]. (*S*)-1-(4'-hydroxyphenyl)ethanol was a far better substrate than (*R*)-1-(4'-hydroxyphenyl)ethanol, explaining why during the enzymatic conversion of 4-ethylphenol nearly no 4-hydroxyacetophenone is formed. Medium-chain 4-alkylphenols were exclusively converted by vanillyl-alcohol oxidase to the corresponding 1-(4'-hydroxyphenyl)alkenes. The relative *cis-trans* stereochemistry of these reactions was strongly dependent on the nature of the alkyl side chain. The enzymatic conversion of 4-*sec*-butylphenol resulted in two (4'-hydroxyphenyl)-*sec*-butene isomers with identical mass but different fragmentation patterns. We conclude that the water accessibility of the enzyme active site and the orientation of the hydrophobic alkyl side chain of the substrate are of major importance in determining the regiospecific and stereochemical outcome of the VAO-mediated conversions of 4-alkylphenols.

Introduction

Vanillyl-alcohol oxidase (EC 1.1.3.38; VAO) is a flavoprotein from *Penicillium simplicissimum* that originally was shown to catalyze the oxidation of vanillyl alcohol to vanillin with the simultaneous reduction of molecular oxygen to hydrogen peroxide [36]. The biological function of VAO is unknown, but recent studies have indicated that the enzyme is involved in the biodegradation of 4-(methoxymethyl)phenol [38]. VAO is a homooctamer with each 64 kDa subunit containing a 8α-(N^3-histidyl)-FAD as covalently bound prosthetic group [36]. The *vao*A gene has been cloned [43] and the VAO structure has been solved at 2.5 Å resolution [42]. These studies, together with sequence alignments, have revealed that the enzyme belongs to a novel oxidoreductase family sharing a conserved FAD binding domain [44]. VAO is a versatile biocatalyst, mechanistically. It can convert a wide range of 4-hydroxybenzylic compounds by catalyzing oxidation, hydroxylation, demethylation, deamination and desaturation reactions [23,39]. Some of these reactions are of particular interest for biotechnological applications [14].

Based on studies with eugenol (4-allyl-2-methoxyphenol) and 4-(methoxymethyl)phenol, we have proposed that VAO catalysis involves the initial transfer of a hydride from the Cα-atom of the substrate to N5 of the flavin cofactor [23,40]. Formation of the resulting *p*-quinone methide intermediate is facilitated by substrate deprotonation upon binding. The *p*-quinone methide product intermediate subsequently reacts with water in the enzyme active site to form the final aromatic product.

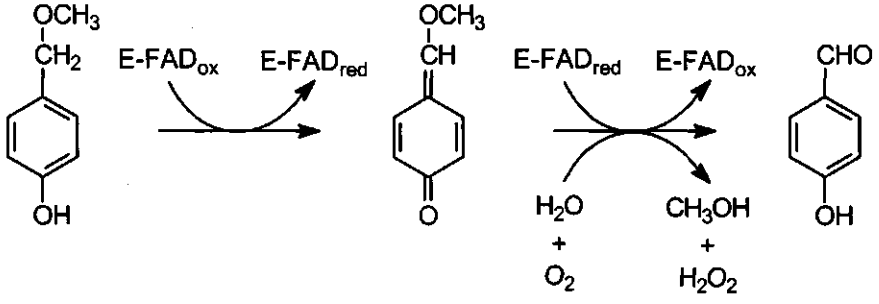


Fig. 1. Proposed reaction mechanism of VAO with 4-(methoxymethyl)-phenol.

A similar reaction mechanism has been proposed for the conversion of 4-alkylphenols by the related bacterial flavocytochrome *p*-cresol methylhydroxylase [67,68]. Further support for the hydride transfer mechanism comes from crystallographic data [42]. The VAO structure has revealed that the active site is located in the interior of the protein and contains an anionic binding pocket facilitating substrate deprotonation.

His422 was identified as the residue, which covalently links the flavin cofactor. The crystal structure also revealed that the side chain of Asp170 is located close to the Cα-atom of the substrate. However, the exact function of this residue in catalysis remains to be elucidated.

VAO displays a remarkable reactivity towards short-chain 4-alkylphenols. Recent studies showed that 4-ethylphenol and 4-n-propylphenols are stereospecifically converted into the corresponding 1-(4'-hydroxyphenyl)alcohols with an *e.e.* of 94% for the *R*-enantiomers. During these reactions, considerable amounts of 1-(4'-hydroxyphenyl)alkenes are formed as side products, indicating that rearrangement of the *p*-quinone methide intermediate competes with water addition [39]. 4-Methylphenol, the parent substrate of *p*-cresol methylhydroxylase, is a very poor substrate for VAO. Crystallographic and kinetic data suggest that this is due to the stabilization of a flavin N5 adduct [41,42]. To obtain more insight into the catalytic performance of this unusual flavoenzyme, we have addressed the reactivity and stereochemistry of VAO with medium-chain 4-alkylphenols, bicyclic phenols and 4-hydroxyphenyl alcohols. The results of this study are discussed in relation to the recently determined crystal structure.

Material and Methods

Chemicals. 4-Methylphenol, 4-ethylphenol, 4-n-propylphenol, 2-methoxy-4-n-propylphenol, 4-isopropylphenol, 4-n-butylphenol, 4-*sec*-butylphenol, 4-(3'-methylcrotyl)phenol, 5-indanol, 5,6,7,8-tetrahydro-2-naphthol, vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), vanillin (4-hydroxy-3-methoxybenzaldehyde), tyramine (4-(2-aminoethyl)phenol), *trans*-isoeugenol (2-methoxy-4-propenylphenol), 3-(4'-hydroxyphenyl)propanol, 4-phenylphenol and 4-benzylphenol were from Aldrich. 4-n-Pentylphenol, 4-n-heptylphenol, 4-n-nonylphenol, 4-vinylphenol, and 2-(4'-hydroxyphenyl)ethanol were obtained from Lancaster. Frambinon (1-(4'-hydroxyphenyl)-2-butanone) was a product from Quest Int.

4-(3'-Methylcrotyl)phenol (75% pure based on GC/MS analysis) was purified to apparent homogeneity by HPLC using a Lichrospher RP8 reverse phase column. Racemic 1-(4'-hydroxyphenyl)ethanol and racemic 1-(4'-hydroxyphenyl)propanol were synthesized by Drijfhout et al. [39]. (*R*)-1-(4'-hydroxyphenyl)ethanol was obtained from the enzymatic conversion of 4-ethylphenol [39].

Enzyme purification. VAO was purified from *P. simplicissimum* (Oudem.) Thom. CBS 170.90 (ATCC 90172) as described previously [23].

Analytical methods. All experiments were performed in air-saturated 50 mM potassium phosphate buffer, pH 7.5 at 25 °C, unless stated otherwise. Enzyme

concentrations were measured spectrophotometrically using a molar absorption coefficient of ε_{439} = 12.5 $mM^{-1}*cm^{-1}$ for protein-bound FAD [36]. VAO activity was determined by following absorption spectral changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode. Vanillin production was measured at 340 nm (ε_{340} = 15.0 $mM^{-1}*cm^{-1}$). Formation of 4-vinylphenol and 1-(4'-hydroxyphenyl)ethanol from 4-ethylphenol was measured at 255 nm (ε_{255} = 14.3 $mM^{-1}*cm^{-1}$) and 270 nm (ε_{270} = 1.2 $mM^{-1}*cm^{-1}$), respectively. Formation of 1-(4'-hydroxyphenyl)propanol was monitored at 270 nm (ε_{270} = 1.3 $mM^{-1}*cm^{-1}$). Absorption spectra were recorded on a SLM Aminco DW-2000 spectrophotometer. Dissociation constants of enzyme-inhibitor complexes were determined from flavin absorption perturbation difference spectra by titration of a known concentration of enzyme with the inhibitor.

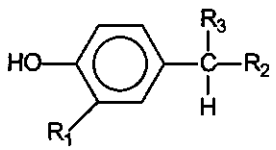
Product identification. HPLC experiments were performed using an Applied Biosystems 400 pump equipped with a Waters 996 photodiode-array detector. Enzyme reaction products were separated with a 4.6 by 150-mm Lichrospher RP8 column and isocratic methanol-water mixtures containing 1% (v/v) acetic acid as the eluent. The methanol-water ratio depended on the type of aromatic compounds to be separated. For resolution of *cis* and *trans* isomers a 4.6 by 100 mm Microspher C18 column was used applying similar conditions as for the Lichrospher RP8 column. Relative yields of aromatic products were determined using the molar absorption coefficients of 4-vinylphenol (ε_{265} = 13.0 $mM^{-1}*cm^{-1}$), 1-(4'-hydroxyphenyl)ethanol (ε_{265} = 0.9 $mM^{-1}*cm^{-1}$), 1-(4'-hydroxyphenyl)propanol (ε_{265} = 1.0 $mM^{-1}*cm^{-1}$), 4-hydroxy-acetophenone (ε_{265} = 12.5 $mM^{-1}*cm^{-1}$), and 4-hydroxypropiophenone (ε_{265} = 12.9 $mM^{-1}*cm^{-1}$) in the appropriate HPLC solvent.

GC-MS analysis was performed on a Hewlett-Packard HP 6090 gas chromatograph and a HP 5973 mass spectrometer equipped with a HP-5 column. Reaction mixtures, containing 0.5-1.0 mM aromatic substrate and 0.2-0.8 μM enzyme, were incubated at 25 °C until the reaction was ceased. Samples prepared by extracting the reaction mixtures with two volumes diethylether were injected without derivatization. The temperature program was 5 min isothermal at 50 °C, followed by an increase to 240 °C at 7 °C min^{-1}. Relative yields were calculated from the integration of the total ion current peak areas.

^{1}H-NMR spectra were recorded on a Bruker AMX-400 MHz spectrometer at 24 °C. Samples were prepared by freeze-drying the isolated products obtained from HPLC separations and dissolving the products in CD_3OD or D_2O.

Results

Substrate specificity. Earlier studies have revealed that VAO has a relaxed substrate specificity towards 4-hydroxybenzylic compounds [23,39]:



R_1: H, OH, OCH_3

R_2: H, CH_2NHCH_3, CH_2NH_2, CH_2OH

R_3: H, CH_2, C_2H_5, C_2H_3, OH, NH_2, OCH_3

Fig. 2. Substrate specificity of VAO from *P. simplicissmum*.

4-Ethylphenol, 4-n-propylphenol, and 2-methoxy-4-n-propylphenol are efficiently oxidized by VAO [41]. In this study we investigated the substrate specificity in further detail. Medium-chain 4-alkylphenols with aliphatic side chains up to seven carbon atoms were converted by VAO. 4-Alkylphenols with longer aliphatic side chains, like 4-n-nonylphenol, did not react. Table 1 shows that straight-chain 4-alkylphenols tightly interact with VAO and that the catalytic efficiency (expressed as k_{cat}/K_m) is optimal at an alkyl chain length of three carbon atoms. Substituents in the aliphatic side chain significantly affected the catalytic efficiency of VAO. Besides from 4-isopropylphenol and 4-*sec*-butylphenol, frambinon (1-(4'-hydroxyphenyl)-2-butanone) was converted at a significant rate (Table 1). As 4-allylphenols are among the best substrates of VAO [23], it was of interest to study the reactivity of 4-(3'-methylcrotyl)phenol. Table 1 shows that this branched-chain 4-allylphenol is indeed a good substrate. VAO was also active with bicyclic phenols. Both 5,6,7,8-tetrahydro-2-naphthol and 5-indanol were readily converted (Table 1). In contrast, no activity was found with 4-benzylphenol.

In an earlier study, we reported that 1-(4'-hydroxyphenyl)alcohols, produced from the enzymatic conversion of short-chain 4-alkylphenols, are not readily oxidized to the corresponding alkanones. This might be due to the high enantioselectivity of the initial hydroxylation reaction which predominantly yields the *R*-isomer [39]. Therefore, it was of interest to study the enzymatic conversion of pure enantiomers. Table 1 shows that (*S*)-1-(4'-hydroxyphenyl)ethanol is a far better substrate than (*R*)-1-(4'-hydroxyphenyl)ethanol. This explains why in the reaction of VAO with 4-ethylphenol, hardly any 1-(4'-hydroxyphenyl)acetophenone is formed. For the enzymatic conversion of 4-n-propylphenol and 2-methoxy-4-n-propylphenol, the *R*-isomer of the corresponding aromatic alcohol was identified as the main product as well [39]. This suggests that for these substrates the same kinetic resolution mechanism is operative. VAO reacted poorly with Cβ and Cγ hydroxylated 4-hydroxyphenyl alcohols (Table

1). In line with this, the enzyme was not active with 4-(aminoalkyl)phenols including tyramine.

Table 1. Steady-state kinetic parameters for VAO from *P. simplicissimum*.

Substrate*	K_m	k_{cat}	k_{cat}/K_m
	μM	s^{-1}	$s^{-1}*mM^{-1}$
4-Ethylphenol†	9	2.5	280
4-n-Propylphenol†	4	4.2	1050
2-Methoxy-4-n-propylphenol†	6	4.9	820
4-Isopropylphenol	16	1.3	81
4-*sec*-Butylphenol	72	0.5	7
4-n-Butylphenol	2	1.2	600
4-n-Pentylphenol	8	0.3	38
4-n-Heptylphenol	42	<0.001	<0.02
4-(3'-Methylcrotyl)phenol	65	1.4	21
1-(4'-Hydroxyphenyl)-2-butanone	128	0.3	2
5-Indanol	77	0.5	7
5,6,7,8-Tetrahydro-2-naphthol	94	0.7	7
(*R,S*)-1-(4'-Hydroxyphenyl)propanol	30	3.0	100
(*R*)-1-(4'-Hydroxyphenyl)ethanol	222	0.7	3
(*S*)-1-(4'-Hydroxyphenyl)ethanol	26	4.4	170
2-(4'-Hydroxyphenyl)ethanol	100	0.004	0.04
3-(4'-Hydroxyphenyl)propanol	8	0.1	13

*Standard errors of kinetic parameters were less than 10% except for 4-n-heptylphenol and 2-(4'-hydroxyphenyl)ethanol which had a standard error of about 25%.
†Data from ref. [39].

Product identification. VAO produces different amounts of 1-(4'-hydroxyphenyl)alcohols and 1-(4'-hydroxyphenyl)alkenes from short-chain 4-alkylphenols [39]. HPLC analysis of the enzymatic conversion of 4-n-propylphenol showed that the relative yield of 1-(4'-hydroxyphenyl)propene was 32% (Table 2). Further examination of this fraction on a Microspher C18 column revealed two peaks with comparable peak areas near the expected elution time of the aromatic alkene (Fig. 3A). The two products showed slightly different absorption characteristics with maxima at 251 nm and 256 nm, respectively, and minor differences in the characteristic aromatic alkene shoulder near 290 nm (Fig. 3B). The mass spectra of

both products were identical: a molecular ion at m/z (relative intensity) $(M)^+$ 134 (100%) and the following diagnostic fragments with more than 25% abundance: 132 (77%) and 107 (30%). These results indicate that a mixture of *cis-trans* isomers of 1-(4'-hydroxyphenyl)propene is formed during the VAO-mediated conversion of 4-n-propylphenol (Table 3). The VAO catalyzed conversion of 2-methoxy-4-n-propylphenol predominantly yielded the alcoholic product (Table 2). Moreover, from the identical HPLC elution time and absorption spectrum of the main alkenylic product and the reference compound *trans*-isoeugenol it is evident that *trans*-isoeugenol is formed in large excess over the *cis* isomer (Table 3).

Table 2. Relative yields of products formed form the conversion of 4-alkylphenols by VAO from *P. simplicissimum*.

Substrate*	4-Hydroxy-phenyl alcohol (%)	4-Hydroxy-phenyl alkanone (%)	4-Hydroxy-phenyl alkenes (%)
4-Ethylphenol†	76	<1	24
4-n-Propylphenol†	68	<1	32
2-Methoxy-4-n-propylphenol†	90	<1	10
4-Isopropylphenol	20	<1	80
4-*sec*-Butylphenol	26	0	74
4-n-Butylphenol	0	0	100
4-n-Pentylphenol	0	0	100
4-n-Heptylphenol	0	0	100
4-(3'-Methylcrotyl)phenol	40	0	60
5-Indanol	16	60	24
5,6,7,8-Tetrahydro-2-naphthol	4	2	94

*Relative yields were determined at saturating substrate concentrations.
†Data from ref. [39].

HPLC analysis of the enzymatic conversion of 4-*sec*-butylphenol revealed a low yield of the alcoholic product (Table 2). In addition to 2-(4'-hydroxyphenyl)-*sec*-butanol, two aromatic butenes were produced. However, these two products were not *cis-trans* isomers because their mass spectra showed different fragmentation patterns. The first eluted aromatic butene contained a molecular ion at m/z (relative intensity) $(M)^+$ 148 (96%) and the following diagnostic fragments with more than 25% abundance: 147 (35%), 133 (100%), and 105 (37%). This fragmentation pattern is indicative for 2-(4'-hydroxyphenyl)-*sec*-butene (Fig. 4A). The second alkenylic product contained a

molecular ion at m/z (relative intensity) $(M)^+$ 148 (100%) and the following diagnostic fragments with more than 25% abundance: 147 (26%), 133 (76%), 119 (88%), and 91 (38%). This points to the production of 1-(4'-hydroxyphenyl)-*sec*-butene (Fig. 4B). Furthermore, comparison of the absorption spectrum of 2-(4'-hydroxyphenyl)-*sec*-butene with identified *cis-trans* isomers of medium-chain 1-(4'-hydroxyphenyl)alkenes (see below) indicate that only *cis*-2-(4'-hydroxyphenyl)-*sec*-butene is formed.

Table 3. Relative yields of *cis* and *trans* isomers of 4-hydroxyphenyl alkenes formed from the conversion of 4-alkylphenols by VAO from *P. simplicissimum*.

Substrate	*Cis*-alkene	*Trans*-alkene
	%	%
4-n-Propylphenol	45	55
2-Methoxy-4-n-propylphenol	<1	>99
4-(3'-Methylcrotyl)phenol	0	100
4-*sec*-Butylphenol	100	0
4-n-Butylphenol	93	7
4-n-Pentylphenol	60	40
4-n-Heptylphenol	50	50

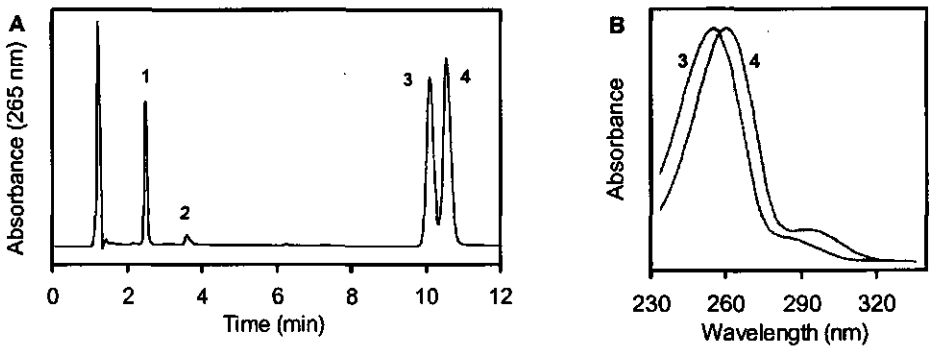


Fig. 3. (**A**) HPLC analysis of aromatic products formed from the conversion of 4-n-propylphenol by VAO from *P. simplicissimum*. (1) alcohol, (2) ketone, (3) *cis*-alkene, and (4) *trans*-alkene, The elution solvent was methanol-water-acetic acid (50:50:1) and (**B**) absorption spectrum of (3) *cis*-alkene and (4) *trans*-alkene.

No alcoholic products are formed in the reaction of VAO with aromatic substrates having an alkyl side chain of at least four carbon atoms (Table 2). With 4-n-butylphenol mostly one 1-(4'-hydroxyphenyl)butene isomer was formed. The ^{1}H-NMR spectrum of this compound showed vinylic bands at 5.52 ppm (dt, J = 11.5 Hz, 7.0 Hz, 1H) and 6.30 ppm (d, J = 11.5 Hz, 1H) for the Cβ-proton and Cα-proton, respectively. This established that the aromatic butene has a *cis*-relative stereochemistry (Table 3).

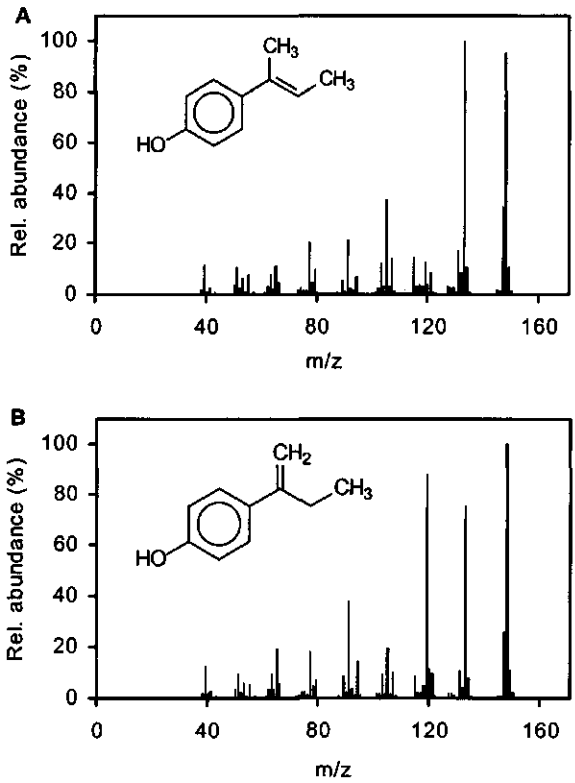


Fig. 4. Mass spectral analysis of aromatic butenes formed from the conversion of 4-*sec*-butylphenol by VAO from *P. simplicissimum*. (**A**) *Cis*-2-(4'-hydroxyphenyl)-*sec*-butene and (**B**) 1-(4'-hydroxyphenyl)-*sec*-butene.

HPLC analysis of the enzymatic conversion of 4-n-pentylphenol showed a mixture of two alkenylic products. The mass spectra of both compounds were identical with a molecular ion at m/z (relative intensity) $(M)^+$ 162 (30%) and the following diagnostic fragments with more than 25% abundance: 133 (100%) and 105 (27%). The two products had slightly different absorption characteristics with respect to the absorption maximum and the aromatic alkene shoulder (not shown). These results point to the

formation of a mixture of *cis*- and *trans*-1-(4'-hydroxyphenyl)pentene. ^{1}H-NMR analysis revealed differences between the two compounds concerning the coupling constants of the vinylic protons: *trans* isomer 6.03 ppm (dt, J = 15.8 Hz, 7.1 Hz, 1H) and 6.27 ppm (d, J = 15.8 Hz, 1H) and *cis* isomer 5.49 ppm (dt, J = 11.2 Hz, 7.1 Hz, 1H) and 6.29 ppm (d, J = 11.2 Hz, 1H) (Fig. 5) In order to rule out the possible *cis-trans* isomerization in the enzyme active site, the isolated isomers of 1-(4'-hydroxyphenyl)pentene were incubated with VAO in 50 mM potassium phosphate buffer, pH 7.5. HPLC analysis clearly demonstrated that the configuration of the *cis* and *trans* isomers of 1-(4'-hydroxyphenyl)pentene did not change with time. In analogy to the reaction of 4-n-pentylphenol, enzymatic conversion of 4-n-heptylphenol resulted in the formation of equal amounts of the *cis* and *trans* isomers of 1-(4'-hydroxyphenyl)heptene (Table 3).

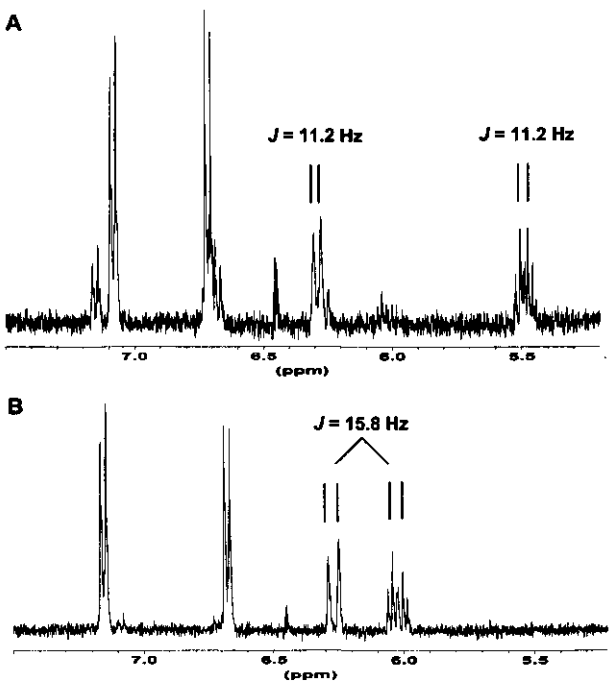


Fig. 5. Expanded ^{1}H NMR spectra of aromatic products formed from the conversion of 4-n-pentylphenol by VAO from *P. simplicissimum*. (**A**) *Cis*-1-(4'-hydroxyphenyl)pentene and (**B**) *trans*-1-(4'-hydroxyphenyl)pentene.

Conversion of eugenol by VAO results in the stoichiometric formation of coniferyl alcohol [23]. Interestingly, enzymatic oxidation of 4-(3'-methylcrotyl)phenol resulted in

the formation of two aromatic products (Table 2). The mass spectrum of the most polar product contained a molecular ion at m/z (relative intensity) $(M)^+$ 178 (24%) and the following diagnostic fragments with more than 25% abundance: 163 (71%), 145 (63%), 127 (37%), 115 (26%), 107 (100%), and 43 (47%). This points towards the formation of the Cγ hydroxylated product 4-(3'-methyl-1'-butene-3'-ol)phenol (Fig. 6A). Further evidence for the structure of the product was obtained by ^{1}H-NMR analysis. The ^{1}H-NMR spectrum in D_2O gave bands at: δ 1.80 (s, 6H, methyl), 6.20, (d, J = 16.4 Hz, 1H, vinylic), 6.43, (d, J = 16.4 Hz, 1H, vinylic), 6.77, (d, J = 8.4 Hz, 2H, aromatic), and 7.28 (d, J = 8.4 Hz, 2H, aromatic). The coupling constants of the vinylic protons also established that the product has a *trans*-relative stereochemistry. Besides this alcoholic compound a second more hydrophobic product was formed with a molecular ion at m/z (relative intensity) $(M)^+$ 160 (46%) and the following diagnostic fragments with more than 25% abundance: 145 (100%), 127 (44%), and 115 (35%) (Fig. 6B).

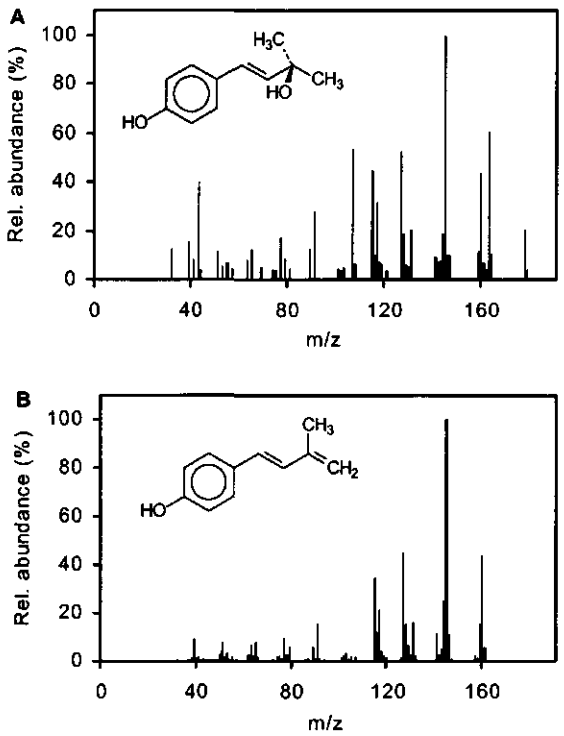


Fig. 6. Mass spectral analyis of aromatic products formed from the conversion of 4-(3'-methylcrotyl)phenol by VAO from *P. simplicissimum*. (**A**) 4-(3'-Methyl-1'-butene-3'-ol)phenol and (**B**) 4-(3'-methylbutadiene)phenol.

The ^{1}H-NMR spectrum in D_2O gave bands at: δ 1.85, 3H (s, methyl), 4.99, (d, J = 2.0, 1H, vinylic), 5.03 (d, J = 2.1, 1H, vinylic), 6.52, (d, J = 16.6 Hz, 1H, vinylic), 6.78, (d, J = 8.5 Hz, 2H, aromatic), 6.81, (d, J = 16.6 Hz, 1H, vinylic), 7.34, (d, J = 8.5 Hz, 2H, aromatic). The above data are consistent with the second product being 4-(3'-methylbutadiene)phenol. Furthermore, the coupling constants of the vinylic protons established that the Cα and Cβ protons have a *trans*-relative stereochemistry.

Substantial differences in the ratio of aromatic products were observed when VAO was incubated with bicyclic phenols. With 5-indanol, the initial alcoholic product was readily further oxidized to the alkanone and only small amounts of the alkenylic product were formed (Table 2). However, with 5,6,7,8-tetrahydro-2-naphthol, dehydrogenation of the putative *p*-quinone methide product intermediate was clearly favoured over water addition (Table 2). Because the bicyclic products were rather unstable, no attempt was made to study their identity in further detail.

Product inhibition. The enzymatic conversion of 4-alkylphenols revealed product inhibition, particularly with medium-chain 4-alkylphenols. This is in agreement with the earlier observation that the alkenylic compounds coniferyl alcohol and isoeugenol are strong competitive inhibitors of VAO [23]. 4-Vinylphenol, one of the products formed from the enzymatic conversion of 4-ethylphenol [39], appeared to be a very strong competitive inhibitor for VAO (K_i = 3 ± 1 μM; Fig. 7). A similar inhibition during the conversion of 4-ethylphenol was reported for *p*-cresol methylhydroxylase [68]. No inhibition constants of other aromatic alkenes were determined. However, the product inhibition observed during the enzymatic conversion of medium-chain 4-alkylphenols suggests that 4-alkenylphenols tightly interact with the enzyme. This is supported by the crystal structures of VAO in complex with isoeugenol and 1-(4'-hydroxyphenyl)heptene [42].

Discussion

In this study we have described the reactivity of the covalent flavoprotein VAO with medium-chain 4-alkylphenols, bicyclic phenols and 4-hydroxyphenyl alcohols. The enzyme was active with 4-alkylphenols bearing aliphatic side chains up to seven carbon atoms. This agrees perfectly with structural data which showed that the active site cavity of VAO is completely filled upon binding of the inhibitor 1-(4'-hydroxyphenyl)heptene [42].

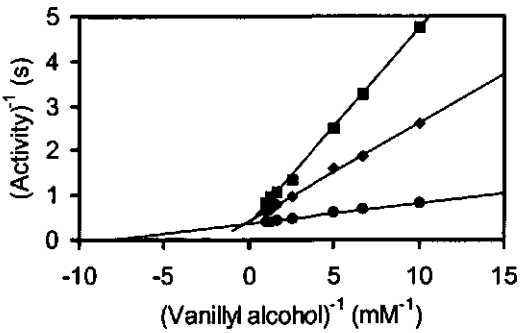


Fig. 7. Competitive inhibition of VAO by 4-vinylphenol. The enzymatic conversion of vanillyl-alcohol at pH 7.5, 25 °C was measured by the increase of absorbance at 340 nm. The presence of the following are indicated: (●) no inhibitor, (◆) 10 μM 4-vinylphenol, and (■) 20 μM 4-vinylphenol.

During the conversion of short-chain 4-alkylphenols by VAO, (*R*)-1-(4'-hydroxyphenyl)alcohols are formed as major products [39]. In this paper clear evidence is provided that the low yield of 1-(4'-hydroxyphenyl)alkanones is due to a preferred stereospecific oxidation of (*S*)-1-(4'-hydroxyphenyl)alcohols (Fig. 8). The low catalytic efficiency for the conversion of the *R*-isomers suggests that the binding of 1-(4'-hydroxyphenyl)alcohols is energetically unfavored when the Cα-hydroxyl group of the substrate faces the flavin ring. Interestingly, differences in the stereospecificity of oxidation of 1-(4'-hydroxyphenyl)alcohols were reported for the related flavocytochromes 4-ethylphenol methylenehydroxylase [69] and *p*-cresol methylhydroxylase [70].

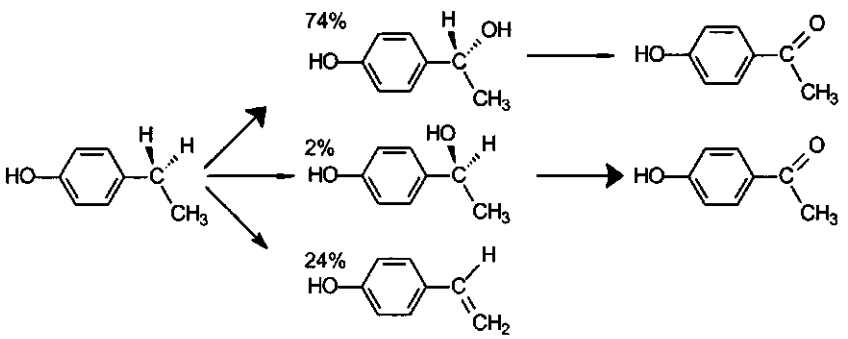


Fig. 8. Reaction pathway for the conversion of 4-ethylphenol by VAO.

Besides catalyzing stereoselective hydroxylation reactions, VAO is regioselective as well. Whereas 4-alkylphenols are exclusively hydroxylated at the Cα-atom, 4-allylphenols, like eugenol, are hydroxylated at the Cγ-atom [23]. These differences in regioselective hydroxylation suggest that the site of water attack is dependent on the delocalization of charge in the enzyme-bound *p*-quinone methide product intermediate. As noted before [42], Asp170 might activate the water, thereby acting as an active site base. Interestingly, the eugenol derivative 4-(3'-methylcrotyl)phenol is not exclusively hydroxylated at the Cγ-position. With this 4-allylphenol, dehydrogenation at the Cδ-position occurs to a significant extent.

In contrast to short-chain 4-alkylphenols, medium-chain 4-alkylphenols are exclusively converted by VAO to the corresponding 1-(4'-hydroxyphenyl)alkenes. This points to rearrangement of the *p*-quinone methide product intermediate and suggests that the efficiency of water addition to this highly reactive electrophilic species is dependent on the water accessibility of the enzyme active site. The presence of reduced glutathione during turnover of VAO with medium-chain 4-alkylphenols did not influence the stoichiometric formation of the alkenylic products, indicating that rearrangement of the *p*-quinone methide intermediate occurs in the enzyme active site. Based on the crystal structures of VAO-inhibitor complexes [42], we assume that no significant conformational changes are induced upon binding of the more bulky 4-alkylphenols. Moreover, the crystallographic data show that the carboxylate oxygen atoms of Asp170 are located at about 3.5 Å from the Cβ atom of the inhibitors [42]. Therefore, rearrangement of the *p*-quinone methide intermediate might be induced by proton abstraction by Asp170, again acting as an active site base.

In this paper we have demonstrated that VAO dehydrogenates medium-chain 4-alkylphenols stereospecifically. This suggests that the *p*-quinone methide intermediates formed with these substrates are rigidly bound in a specific orientation in the enzyme active site. This *cis-trans* stereospecificity is not unique among flavoenzymes. For example, acyl-CoA dehydrogenases introduce a *trans* double bond between C2 and C3 of their CoA substrates [71], whereas glycolate oxidase shows specificity for *re* hydrogen abstraction when prochiral glycollate is used as a substrate [72]. In contrast to these enzymes, the relative *cis-trans* stereochemistry of VAO is strongly dependent on the nature of the alkyl side chain of the substrate. However, no correlation was found between the stereochemical preference and bulkiness or length of the 4-alkylphenol side chains.

In summary, the results presented here show that VAO is active with a wide range of 4-alkylphenols. Short-chain 4-alkylphenols are mainly hydroxylated to aromatic alcohols, whereas medium-chain 4-alkylphenols are exclusively dehydrogenated to aromatic alkenes. We conclude that the regio- and stereospecificity of the VAO-mediated reactions is mainly determined by i) the intrinsic reactivity of the enzyme-

bound *p*-quinone methide intermediate, ii) the water accessibility of the enzyme active site, and iii) the orientation of the hydrophobic alkyl side chain of the substrate.

Acknowledgements

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Chapter 4

TUNING THE REACTIVITY OF ENZYME-BOUND QUINONE METHIDES BY MEDIUM ENGINEERING

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Submitted

Electrophilic *p*-quinone methide intermediates of 4-alkylphenols are toxic to living cells as they can form covalent adducts with DNA and proteins. The flavoenzyme vanillyl-alcohol oxidase is able to stabilize these quinone methides in the enzyme active site, thereby preventing deleterious effects *in vivo*. It is shown for the first time that the hydration of a *p*-quinone methide intermediate in the enzyme can be effectively inhibited by decreasing the water activity of the reaction medium or by the addition of the monovalent anions chloride, bromide, or thiocyanate.

Introduction

The cytotoxic effect of 4-alkylphenols is often linked to the formation of electrophilic quinone methide intermediates. These reactive metabolites can form adducts with cellular nucleophiles like DNA and proteins via Michael addition reactions [73-75]. With this in mind, it is intriguing that certain flavoenzyme-mediated conversions of 4-alkylphenols proceed via the stabilization of protein-bound *p*-quinone methide intermediates [40,67]. As the release of these species out of the active site would result in deleterious effects *in vivo*, efficient processing by the enzyme is a prerequisite. Until now, little is known about how these enzymes deal with the processing of these reactive intermediates. Here, we report on the reactivity of vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) with 4-propylphenol. It is shown for the first time that the hydration of a *p*-quinone methide intermediate in the enzyme active site can be effectively inhibited by decreasing the water activity of the reaction medium or by the addition of specific monovalent anions. As a result, the product spectrum of VAO can be tuned by medium engineering.

VAO is a flavoenzyme from the ascomycete *Penicillium simplicissimum* containing an 8α-(N^3-histidyl)-FAD as covalently bound prosthetic group [54]. The catalytic mechanism of VAO with 4-alkylphenols involves the initial transfer of a hydride from the substrate (**1**) to the flavin, resulting in the formation of a complex between the reduced enzyme and the *p*-quinone methide intermediate (**2**) (Fig. 1) [40]. Next, the reduced enzyme is reoxidized by molecular oxygen and the enzyme-bound intermediate is attacked in a stereospecific manner by water forming the (*R*)-1-(4'-hydroxyphenyl)-alcohol (*e.e.* = 94%) (**3**) or rearranged in a competing reaction to the 1-(4'-hydroxyphenyl)-alkene (**4**) [39].

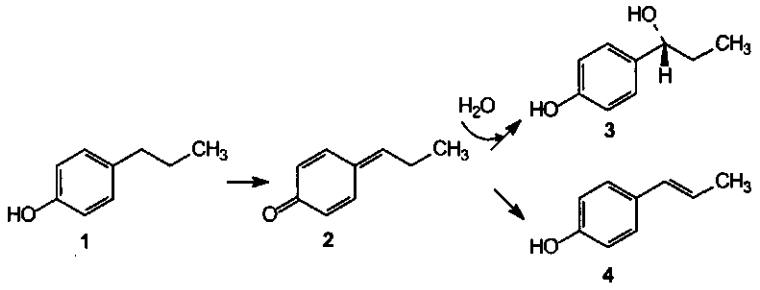

Fig. 1. Reaction scheme for the conversion of 4-propylphenol by VAO.

Studies with a wide range of substrate analogs have shown that the efficiency of alcohol formation and the ratio between the *cis* and *trans* forms of the alkene products are affected by the type of 4-alkylphenol [62]. The outcome of the enzymatic conversion of 4-alkylphenols is of biotechnological relevance because the alkene products can

serve as flavors, whereas the enantiomerically pure alcohols are suited as building blocks for the synthesis of complex chiral compounds [14,28].

Crystallographic studies of VAO in complex with substrate analogs have provided insight in the binding mode of 4-alkylphenols [42,55]. Recent studies from protein engineering have shown that Asp170, located in the proximity of the flavin N5-atom (3.6 Å) and the substrate Cα-atom (3.0 Å) [42], is involved in regulating the water attack on the *p*-quinone methide intermediate [56,76]. Moreover, by relocating the acidic residue to the opposite face of the substrate (Asp170Ser/Thr457Glu double mutation), the stereospecificity of the enzyme could be inverted [57]. It has been well established that besides protein engineering, medium engineering is an alternative approach of tuning the enzyme selectivity [77-79]. Examples in which the product pattern was successfully changed include lipase from *Candida rugosa* [32], lipase from *Pseudomonas* sp. [30], α-chymotrypsin [28,29], and subtilisin Carlsberg [28,31]. On the basis of this concept developed for hydrolytic enzymes, we aimed to obtain more insight in the water accessibility and reactivity of the *p*-quinone methide intermediate in the catalytic center of VAO.

Materials and Methods

Enzyme. Wild type VAO was overexpressed and purified as described before [43,56].

Analytical methods. In organic solvents: 0.32 mg VAO in 50 mM potassium phosphate buffer, pH 7.5, was freeze-dried in a 3 ml glass vial for 12 hours. The reaction was started by adding 1 ml of 4-propylphenol (10 mM) dissolved in the appropriate solvent at a preset water activity. The reaction mixture was then incubated at 20 °C with constant reciprocal shaking (200 min^{-1}). 200 µl samples were taken at regular time intervals and analyzed by HPLC (Gilson 715 system) equipped with a reversed phase 4.6 by 250 mm HiChrom HIRBP column. In all reaction media, except for the acetonitrile media between a_w = 0.99 and 0.86, VAO was present as a suspension. The water activity in acetonitrile was set by adding water [80] and in toluene by adding salt hydrates [81].

Product selectivity in the presence of monovalent anions: 0.13 mg VAO in 50 mM potassium phosphate buffer, pH 7.5 was mixed with 1 mM 4-propylphenol and varying concentrations of the anions (4-500 mM) in 50 mM potassium phosphate buffer, pH 7.5 in a total volume of 1 ml and 25 °C. 200 µl samples were taken at regular time intervals and analyzed by HPLC.

Dissociation constants of enzyme-inhibitor complexes were determined from flavin perturbation spectra by titrating a known concentration of enzyme with the inhibitor using an Aminco DW-2000 spectrophotometer in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C. The inhibition of VAO by monovalent anions was

measured by using vanillyl alcohol as the variable substrate. The data were treated according to Lineweaver and Burk.

The stereospecificity of hydroxylation of 4-propylphenol by VAO was measured by HPLC using an Applied Biosystems pump equipped with a 4.6 by 250 mm Chiralcel OB column (Daicel Chemical Industries). In all experiments an *e.e.* of 95 ± 1% for the *R*-enantiomer of the 1-(4'-hydroxyphenyl)-alcohol product was found.

Results and Discussion

When using low water media, we found that the activity of lyophilized VAO with 4-propylphenol was highly dependent on the water activity (a_w) of the reaction medium, but not on the polarity of the organic solvent. In both toluene and acetonitrile, the activity of VAO at $a_w = 0.8$ was about one order of magnitude lower compared to that in aqueous medium ($k' = 0.3\ s^{-1}$ vs. $k' = 4.2\ s^{-1}$) [41]. Upon lowering a_w, the turnover rate of VAO decreased and the enzyme became nearly inactive at a_w = 0.49. In an aqueous medium ($a_w = 1$), VAO converts 4-propylphenol mainly into (*R*)-1-(4'-hydroxyphenyl)propanol and low but equal amounts of *cis*- and *trans*-1-(4'-hydroxyphenyl)propene (Fig. 2).

When the VAO-catalyzed conversion was performed in an organic solvent the concentration of alcohol product decreased and the concentration of the *cis*-alkene product, but not the *trans*-alkene product, increased. This change in selectivity occurred in both toluene and acetonitrile and was dependent on a_w (Fig. 2A and 2B). These results show that the availability of water for the enzyme determines the reactivity of the enzyme-bound *p*-quinone methide and that the hydration and rearrangement of this intermediate species are competitive processes.

As the *cis/trans* alkene product ratio is dependent on the type of 4-alkylphenol, the different behavior of *cis*- and *trans*-alkene formation is not likely to be explained by the higher energy of the *cis*-conformation [62]. With 4-propylphenol as a substrate, the *p*-quinone methide intermediate can take up three different conformations (Fig. 3). **5** Has the methyl attached to the Cα-atom already in the *trans* position. This conformation can only proceed to the *trans*-alkene product. The two other conformations (**6**, **7**) have the methyl group attached to Cα in the *cis* position and are likely to give only the *cis* product. These latter two quinone methide conformations are subject to competitive rearrangement or hydration at Cα. However, we can not exclude an alternative mechanism in which there is only a single quinone methide intermediate, and attack by water competes with removal of just one of the two protons attached to the Cβ. But it is difficult to see how selection between these protons could control the alkene geometry produced, from any of the possible intermediate conformations (Fig. 3).

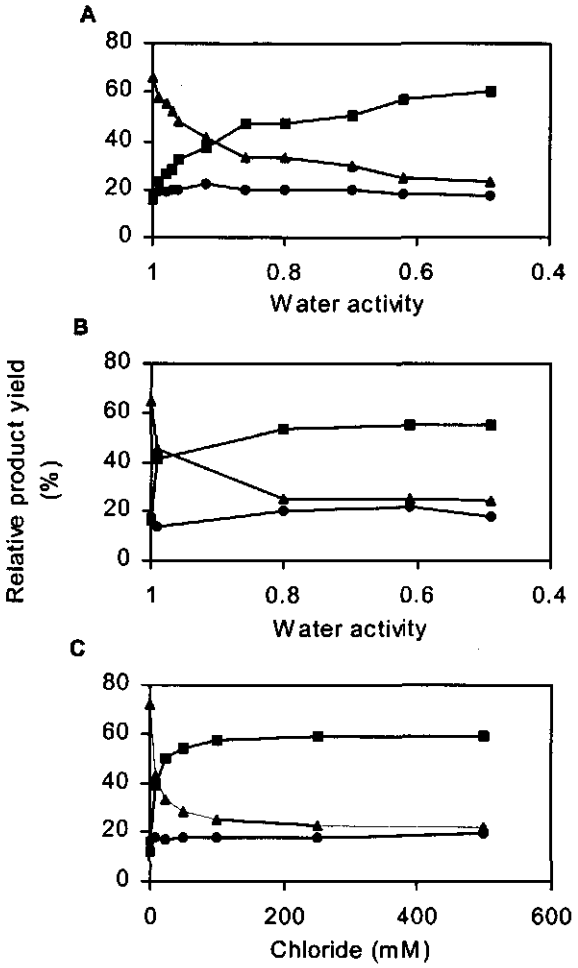

Fig. 2. Conversion of 10 mM 4-propylphenol by 0.32 mg VAO at 25 °C in the organic solvents (**A**) acetonitrile and (**B**) toluene, and (**C**) conversion of 1 mM 4-propylphenol by 0.13 mg VAO in 50 mM potassium phosphate buffer pH 7.5 at 25 °C in the presence of sodium chloride. The concentration of the products (▲) (*R*)-1-(4'-hydroxyphenyl)propanol, (■) *cis*-1-(4'-hydroxyphenyl)propene, and (●) *trans*-1-(4'-hydroxyphenyl)propene were determined by HPLC.

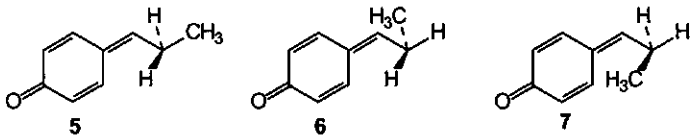

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Fig. 3. Three possible conformations of the enzyme-bound *p*-quinone methide intermediate .

A second medium engineering-based approach of tuning the reactivity of the *p*-quinone methide in the catalytic center of VAO was the addition of specific monovalent anions to the reaction medium. Previous X-ray data have established that a chloride ion binds at the *re* face of the flavin cofactor, whereas the aromatic substrate binds at the *si* face of the flavin [42]. The binding of chloride, and also bromide and thiocyanate, near the flavin was confirmed by flavin absorption difference spectroscopy (Table 1), and inhibition studies with these anions revealed non-competitive inhibition. In contrast, fluoride, sulfate, and iodide ions did not perturb the optical properties of enzyme-bound flavin nor inhibit enzyme activity.

When the enzymatic conversion of 4-propylphenol was performed in aqueous medium in the presence of chloride, bromide, or thiocyanate, the efficiency of *p*-quinone methide hydration decreased and the efficiency of *cis*-rearrangement increased (Figure 1C). Thus, the addition of specific anions and a decreased water activity have similar outcomes. The effects on hydrophobic interactions are not likely to explain the anion effect, since, according to the Hofmeister series, iodide would be expected to have a larger effect than chloride and bromide. On the basis of the crystallographic data [42] and the anion binding studies it seems likely that the decreased hydration efficiency of the *p*-quinone methide in the presence of chloride, bromide, or thiocyanate is a consequence of their specific binding near the enzyme active site. This binding does not completely block the hydration reaction, suggesting that the anions do not fully protect the *p*-quinone methide from water attack.

Table 1. Dissociation constants of VAO-inhibitor complexes and non-competitive inhibition constants of monovalent anions in 50 mM potassium phosphate buffer, pH 7.5 and 25 °C.

	K_d (mM)	K_i (mM)
Chloride	130 ± 10	120 ± 15
Bromide	93 ± 9	102 ± 10
Thiocyanate	36 ± 3	70 ± 11

The low water activity of the medium and the presence of monovalent anions did not change the *R*-stereospecificity of the hydration reaction. This is in accordance with the idea that Asp170 tunes the stereospecificity of VAO by activating the water molecule attacking the quinone methide [57].

In conclusion, we have shown in this paper that the reactivity of enzyme-bound quinone methides can be regulated by varying the composition of the reaction medium. In VAO, the use of low water activity organic media or specific monovalent anions decrease the water accessibility of the enzyme active site, thereby influencing the competition between substrate hydroxylation and substrate dehydrogenation. Moreover, under the applied conditions, no quinone methide dissociation occurs, preventing the formation of racemic mixtures or unwanted by-products.

Acknowledgements

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Chapter 5

DIRECTION OF THE REACTIVITY OF VANILLYL-ALCOHOL OXIDASE WITH 4-ALKYLPHENOLS

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The covalent flavoprotein vanillyl-alcohol oxidase (VAO) predominantly converts short-chain 4-alkylphenols, like 4-ethylphenol, to (*R*)-1-(4'-hydroxyphenyl)alcohols and medium-chain 4-alkylphenols, like 4-butylphenol, to 1-(4'-hydroxyphenyl)alkenes. Crystallographic studies have indicated that the active site residue Asp170 is involved in determining the efficiency of substrate hydroxylation. To test this hypothesis, we have addressed the reactivity of Asp170 variants with 4-alkylphenols. The substrate preference of D170E was similar to wild type VAO. However, D170S was most active with branched-chain 4-alkylphenols. The hydroxylation efficiency of the Asp170 variants was dependent on the bulkiness of the newly introduced side chain. The Glu170 mutation favored the production of alkenes, whereas the Ser170 mutation stimulated the formation of alcohols.

Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is a flavin containing oxidoreductase involved in the biodegradation of 4-(methoxymethyl)phenol [36,38]. The enzyme is a homooctamer of 509 kDa with each subunit comprising two domains [42,82]. The cap domain covers the active site, whereas the larger domain creates a binding site for the ADP part of the FAD prosthetic group. The flavin is covalently linked to His422 of the cap domain [42]. The phenolic substrate is bound almost parallel to the flavin ring and its hydroxyl group is hydrogen bonded to three basic residues (Tyr108, Arg503, and Arg504) [42].

VAO is active with a wide range of phenolic substrates, including 4-alkylphenols [23,39,62]. The catalytic cycle of VAO consists of two half-reactions [40,41]. In the reductive half-reaction, the flavin is reduced by the substrate with the concomitant formation of a *p*-quinone methide intermediate. In the oxidative half-reaction, the flavin is reoxidized and the protein-bound quinone methide either reacts with water to yield the (*R*)-enantiomer of the alcohol, or is rearranged to yield the alkene [39,41] (Fig. 1)

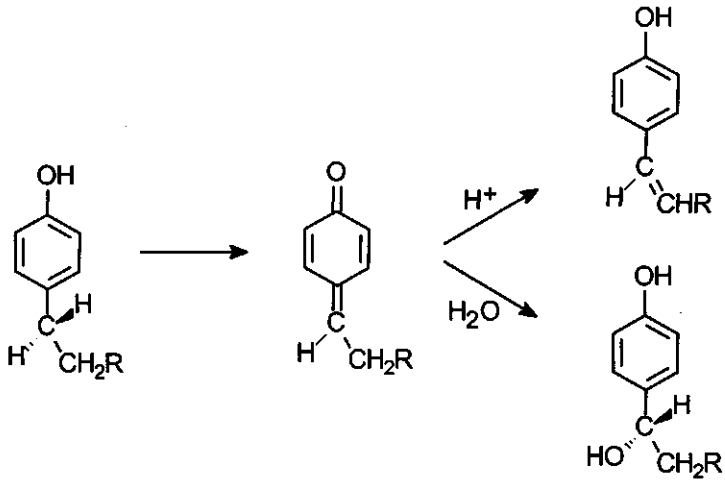

Fig. 1. Conversion of 4-alkylphenols by VAO.

Short-chain 4-alkylphenols are mainly hydroxylated to (*R*)-1-(4'-hydroxyphenyl)alcohols and medium-chain 4-alkylphenols are dehydrogenated to 1-(4'-hydroxyphenyl)alkenes [62]. Unlike the related flavocytochrome *p*-cresol methylhydroxylase (PCMH) [70], VAO is nearly inactive with *p*-cresol. Kinetic studies showed that this is due to the formation of an air-stable *p*-cresol-FAD-N5 adduct [41]. Crystallographic data have shown that the catalytic centers of PCMH and VAO are conserved except for the arrangement of the acidic residues [42,50]. VAO contains a single aspartate (Asp170) near the methylene group of the substrate [42], whereas PCMH

contains two glutamates (Glu380 and Glu427) at opposite faces of bound substrate [50]. Recent studies have demonstrated that in VAO, Asp170 is crucial for keeping the high redox potential of the FAD cofactor. This high redox potential is important for efficient substrate oxidation and for stabilization of the complex between the reduced enzyme and the *p*-quinone methide intermediate of 4-(methoxymethyl)phenol [56]. Moreover, from site-directed mutagenesis it was established that the arrangement of acidic residues in the active site cavity tunes the stereospecificity of hydroxylation of 4-ethylphenol [57].

In this paper we have investigated the role of Asp170 in directing the reactivity of VAO with 4-alkylphenols. For this purpose, we selected D170E and D170S as both these mutants contain covalently-bound FAD and are active with 4-(methoxymethyl)phenol [56]. Moreover, crystallographic analysis has revealed that the Asp170Ser replacement does not induce significant conformational changes compared to wild type enzyme [42,56]. Our studies clearly reveal that the replacement of Asp170 by Glu or Ser changes the efficiency of substrate hydroxylation of VAO in an opposite direction.

Materials and Methods

Site-directed mutagenesis and enzyme purification. *Escherichia coli* strain TG2 [64] and the plasmid pEMBL19(-) (Boehringer Mannheim) were used for expression of the *vao*A gene. All other chemicals and materials were as described previously [56,62].

pBC14 (D170E) and pBC15 (D170S) were constructed from pBC11 (wild type VAO) as reported before [56]. Transformed *E. coli* cells were grown in Luria-Bertani medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside [43]. The VAO mutant proteins were purified as described [43,56].

Analytical methods. All experiments were performed in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C unless stated otherwise. HPLC experiments were conducted with an Applied Biosystems pump equipped with a Waters 996 photodiode-array detector and a 3.9 by 100 mm or 3.9 by 150 mm Waters Novapak C18 column, essentially as described earlier [62]. GC/MS analysis was performed on a Hewlett-Packard HP 6090 gas chromatograph equipped with a HP 5973 mass spectrometer and a HP-5 column [62]. Fluorescence emission spectra were recorded on an Aminco SPF-500C spectrofluorometer. The excitation wavelength was 360 nm [41].

VAO activity was routinely assayed by following absorption spectral changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode [62]. Formation of 4-hydroxybenzaldehyde was measured at 340 nm (ε_{340} = 10.0 $mM^{-1}*cm^{-1}$) and formation of 4-vinylphenol was monitored at 255 nm (ε_{255} = 14.3

$mM^{-1}*cm^{-1}$). The turnover rates of extremely slow enzymatic reactions (rate lower than 0.003 s^{-1}) were determined by HPLC. Enzyme-monitored-turnover experiments were performed as described before [41] using a Hewlett-Packard HP 8453 diode-array spectrophotometer.

Results

Catalytic properties. Table 1 summarizes the steady-state kinetic parameters of wild type VAO, D170E, and D170S with several 4-alkylphenols. With all these substrates, the turnover rate of D170E was about one order of magnitude lower than that of wild type enzyme and the Michaelis constant was increased up to five-fold. The substitution of Asp170 by Ser had a more severe effect on catalysis. All straight-chain 4-alkylphenols were extremely slowly converted, whereas branched-chain 4-alkylphenols were relatively good substrates for D170S. As found for wild type VAO, both mutant enzymes were nearly inactive with *p*-cresol [41].

Table 1. Steady-state kinetic parameters for wild type VAO, D170E, and D170S in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C.

Substrate*	Wild type†		D170E		D170S	
	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
	μM	s^{-1}	μM	s^{-1}	μM	s^{-1}
p-Cresol	n.d.	0.005	n.d.	0.0002	n.d.	n.d.
4-Ethylphenol	9	2.5	48	0.17	n.d.	0.0001
4-n-Propylphenol	4	4.2	10	0.26	n.d.	0.0002
4-Isopropylphenol	16	1.3	88	0.13	26	0.18
4-n-Butylphenol	2	1.2	6	0.12	n.d.	0.0001
4-*sec*-Butylphenol	72	0.5	75	0.05	62	0.09

*Standard errors of kinetic parameters are less than 10%.

†Data from ref. [62].

n.d., not determined.

When D170S was mixed aerobically with *p*-cresol, 4-ethylphenol or 4-propylphenol the flavin was nearly completely in the reduced state during turnover (95%, 95%, and 89%, respectively), suggesting that the reductive half-reaction does not limit the turnover rate [65]. Moreover, upon excitation at 360 nm, the aerobic complexes between D170S and short-chain 4-alkylphenols displayed a stable fluorescence emission with a maximum at 460 nm (Fig. 2), indicative for the formation of a covalent flavin N5 adduct with the substrate [41,42]. Similar flavin adducts

have been reported for lactate oxidase [83,84] and nitroalkane oxidase [85]. When D170S was incubated with 4-isopropylphenol or 4-*sec*-butylphenol, the flavin was mainly in the oxidized state during turnover (79% and 82%, respectively). In accordance with this, the fluorescence emission of 4-isopropylphenol-mixed D170S showed a maximum at 530 nm, indicative for oxidized flavin. A similar flavin fluorescence was observed when oxidized D170S was incubated with 4-vinylphenol or 1-(4'-hydroxyphenyl)ethanol. In contrast, uncomplexed D170S displayed almost no flavin fluorescence (Fig. 2). This suggests that the fluorescence emission at 530 nm represents the complex between the oxidized enzyme and the aromatic product. The D170E mutant displayed a similar enzyme-monitored-turnover behavior as wild type VAO. Thus, only upon mixing the enzyme with *p*-cresol the flavin was mainly in the reduced stated during turnover [41].

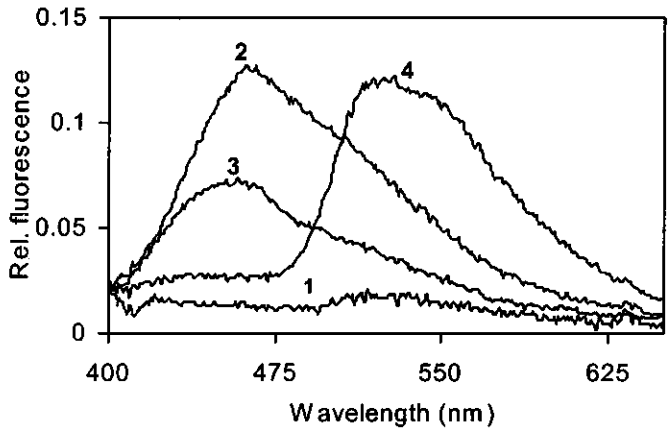

Fig. 2. Fluorescence emission properties of free D170S and after mixing with 4-alkylphenols. The excitation wavelength was 360 nm. 9 μM D170S in 50 mM potassium phosphate, pH 7.5 at 25 °C (1), after mixing with 500 μM *p*-cresol (2), 4-ethylphenol (3), and 4-isopropylphenol (4).

Conversion of 4-alkylphenols. Replacement of Asp170 by Glu and Ser considerably changed the product pattern of the VAO catalyzed reactions with 4-alkylphenols (Table 2). Like wild type enzyme [39,62], D170S was highly specific for the hydroxylation of short-chain alkylphenols. Previous studies have shown that both wild type VAO and D170S are selective for the production of the (*R*)-enantiomer of 4-ethylphenol [57]. In contrast, the hydroxylation reaction in D170E was nearly completely blocked. As a result, this mutant converted short-chain alkylphenols to the corresponding alkenes.

With medium-chain and branched-chain alkylphenols, the change in product pattern was even more pronounced. Unlike wild type enzyme [62], D170S was highly specific for the hydroxylation of these compounds. However, D170E resembled wild type enzyme and mainly produced aromatic alkenes.

Table 2. Conversion of 4-alkylphenols by wild type VAO, D170E, and D170S in 50 mM potassium phosphate buffer, pH 7.5 at 25 °C.

Substrate*	Wild type†		D170E		D170S	
	Product (%)					
	Alcohol	Alkene	Alcohol	Alkene	Alcohol	Alkene
4-Ethylphenol	76	24	8	92	92	8
4-Propylphenol	68	32	7	93	96	4
4-Isopropylphenol	20	80	8	92	80	20
4-Butylphenol	1	99	0	100	82	18
4-sec-Butylphenol	26	74	1	99	78	22

*Standard errors of relative product yields are less than 10%.
†Data from ref. [62].

Discussion

The active site cavity of VAO contains a single acidic residue, Asp170, which side chain is close to flavin N5 and the reactive Cα-atom of the substrate (Fig. 3).
Recent studies from site-directed mutants have shown that this residue is important for catalysis by raising the redox potential of the flavin and stabilizing the enzyme-bound *p*-quinone methide of 4-(methoxymethyl)phenol [56]. Furthermore, Asp170 appeared to be important for the stereospecificity of hydroxylation of 4-ethylphenol [57]. This brought up the question whether the replacement of Asp170 will also influence the efficiency of substrate hydroxylation.

The D170E variant had a similar substrate preference as wild type VAO, but the catalytic efficiency (k_{cat}/K_m) was considerably decreased. The low turnover rate is in line with the reduced redox potential of the mutant (E_m = +11 mV) compared to wild type VAO (E_m = +55 mV) [56]. In contrast to the wild type enzyme, D170E converted both short-chain and medium-chain 4-alkylphenols almost exclusively into the corresponding alkenes. The inability of D170E to hydroxylate these substrates suggests that the bulkiness of the side chain of Glu170 limits the accessibility of water to the planar quinone methide intermediate.

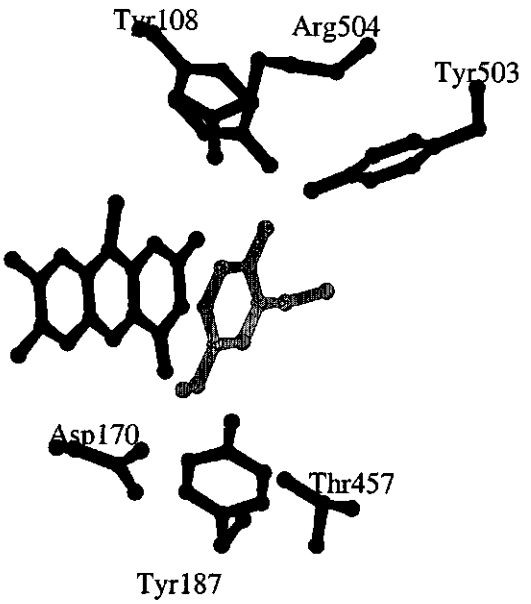

Fig. 3. Drawing of the active site cavity of VAO in complex with isoeugenol. Asp170 is positioned at 3.5 Å from flavin N5 and the Cα-atom of isoeugenol. This figure was prepared with MOLSCRIPT [45].

D170S had a drastically changed substrate preference compared to wild type VAO. This mutant was nearly inactive with straight-chain 4-alkylphenols due to the formation of air-stable covalent flavin N5 adducts. With wild type VAO, such an adduct is only observed with *p*-cresol [41]. The crystal structure of isoeugenol-complexed D170S does not give a rationale for the easy formation of flavin adducts since there are no significant structural differences compared to wild type VAO [42,56]. We have proposed before that in wild type VAO, Asp170 might be involved in covalent adduct formation by abstracting a proton from flavin N5, thereby facilitating the nucleophilic attack of the *p*-quinone methide intermediate. However, the formation of flavin N5 adducts in D170S suggests that the flavin N5-atom can also be activated by a water molecule (Fig. 4). D170S was rather active with branched-chain 4-alkylphenols. This indicates that with these substrates, adduct formation may well be prevented by steric contraints. In this respect it is interesting to note that the flavin redox potential of D170S is only –91 mV [56].

The D170S variant was more specific for the hydroxylation of 4-alkylphenols than wild type VAO. This supports an earlier observation that the hydration of the *p*-quinone methide intermediate can also occur in the absence of an active site base [56].

Furthermore, it suggests that the quinone methides of 4-alkylphenols react rapidly with unactivated water, which is in line with the reactivity of analogous quinone methides [74,86].

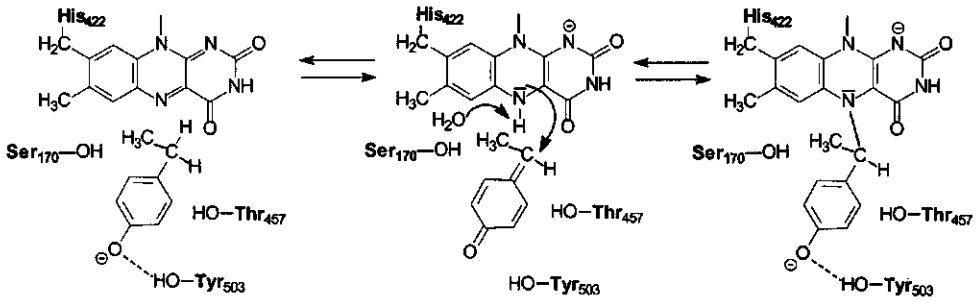

Fig. 4. Schematic drawing of the proposed mechanism for covalent adduct formation in D170S.

The differences in the efficiency of substrate hydroxylation between wild type VAO, D170S, and D170E show that the bulkiness of the side chain of residue 170 determines the outcome of the VAO catalyzed reaction. The small side chain of Ser170 increases the accessibility of water to the *p*-quinone methide intermediate in the active site and, therefore, the hydroxylation efficiency. This increased accessibility of water to the quinone methide in D170S might also explain the decreased stereospecificity of hydroxylation of 4-ethylphenol [57]. On the other hand, the large side chain of Glu170 clearly prevents the attack of water to the quinone methide. These results reinforce the idea that a single amino acid substitution can be sufficient to change the enzyme selectivity [87,88].

Acknowledgements

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Chapter 6

ASP170 IS CRUCIAL FOR THE REDOX PROPERTIES OF VANILLYL-ALCOHOL OXIDASE

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Vanillyl-alcohol oxidase (VAO) is a flavoprotein containing a covalent flavin that catalyzes the oxidation of 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde. The reaction proceeds through the formation of a *p*-quinone methide intermediate after which water addition takes place. Asp170, located near the N5-atom of the flavin, has been proposed to act as an active site base. To test this hypothesis, we have addressed the properties of D170E, D170S, D170A, and D170N variants. Spectral and fluorescence analysis, together with the crystal structure of D170S, suggests that the Asp170 replacements do not induce major structural changes. However, in D170A and D170N, respectively 50% and 100% of the flavin is noncovalently bound. Kinetic characterization of the VAO variants revealed that Asp170 is required for catalysis. D170E is 50-fold less active and the other Asp170 variants are about 10^3-fold less active than wild type enzyme. Impaired catalysis of the Asp170 variants is caused by slow flavin reduction. Furthermore, the mutant proteins have lost the capability of forming a stable complex between reduced enzyme and the *p*-quinone methide intermediate. The redox midpoint potentials in D170E (+6 mV) and D170S (-91 mV) are considerably decreased compared to wild type VAO (+55 mV). This supports the idea that Asp170 interacts with the protonated N5-atom of the reduced cofactor, thus increasing the FAD redox potential. Taken together, we conclude that Asp170 is involved in the process of autocatalytic flavinylation and is crucial for efficient redox catalysis.

Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is a homooctameric covalent flavoenzyme involved in the biodegradation of lignin-derived aromatic compounds [38]. The enzyme is the prototype of a novel family of structurally related oxidoreductases sharing a conserved FAD-binding domain [44]. VAO oxidizes its physiological substrate 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde with the concomitant reduction of molecular oxygen to hydrogen peroxide [23,40]. The enzymatic reaction is initiated by the transfer of a hydride equivalent from the Cα-atom of the substrate to flavin N5. The resulting binary complex between the reduced enzyme and the *p*-quinone methide intermediate then reacts with molecular oxygen, reoxidizing the FAD. The *p*-quinone methide product subsequently reacts with water in the enzyme active site to generate the final products 4-hydroxybenzaldehyde and methanol [40] (Fig. 1). A similar reaction mechanism has been proposed for the structurally related flavocytochrome *p*-cresol methylhydroxylase [67,70].

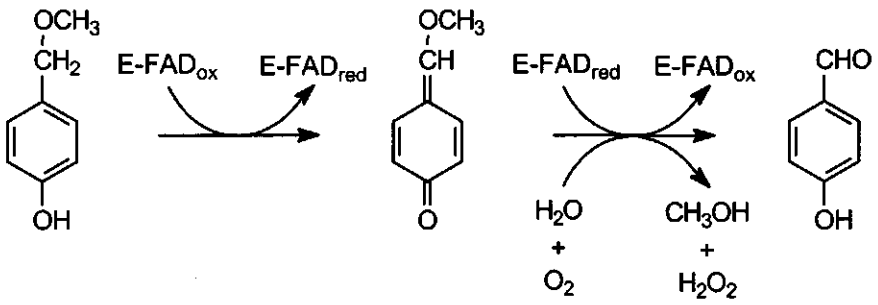

Fig. 1. Oxidative demethylation of 4-(methoxymethyl)phenol as catalyzed by VAO.

Recently, we determined the three-dimensional structures of native VAO and several enzyme-ligand complexes [42]. Each VAO 64 kDa monomer consists of two domains: the cap domain covers the active site whereas the larger domain creates a binding site for the ADP-ribityl part of the FAD cofactor. The C8α-atom of the isoalloxazine ring of the FAD is covalently linked to the Nε3-atom of His422 of the cap domain. A detailed study towards the noncovalent H422A mutant has shown that the covalent flavin linkage is not essential for tight binding of the flavin cofactor [54]. However, the covalent bond raises the redox potential of the flavin, thereby increasing the rate of substrate oxidation. The structure of VAO in complex with 4-(1-heptenyl)phenol has revealed that the shape of the active site cavity controls the substrate specificity by providing a size-exclusion mechanism. Inside the cavity, the aromatic substrate is ideally positioned for hydride transfer to flavin N5. Substrate oxidation is facilitated by ionization of the phenol, as induced by hydrogen bonding

with the side chains of Tyr108, Tyr503, and Arg504. Another interesting residue in the VAO active site is Asp170. The carboxylic moiety of this residue is close to flavin N5 (3.5 Å) and Arg398 (3.1 Å) and likely to be deprotonated. The presence of an acidic residue in the vicinity of flavin N5 is intriguing, since in most flavin-dependent oxidoreductases of known structure the N5-atom contacts a hydrogen bond donor rather than an acceptor [89]. The side chain of Asp170 is positioned in a way that, during catalysis, it might interact with the protonated N5-atom of the reduced cofactor. Moreover, we have proposed that Asp170 might act as an active site base, activating the water involved in substrate conversion [42].

In this paper, we have addressed the functional importance of Asp170 in VAO through the kinetic, spectroscopic and crystallographic analysis of D70E, D170S, D170A, and D170N It is shown that the Asp170 replacements do not introduce major structural changes, but have a profound effect on the redox properties of the enzyme. In addition, our studies indicate that Asp170 is involved in covalently tethering the flavin prosthetic group.

Materials and Methods

Chemicals, bacterial strains, and plasmids. *Escherichia coli* strain DH5αF' [90] and the plasmids pUCBM20 (Boehringer Mannheim) and pGEM-5Zf(+) (Promega) were used for cloning throughout, whereas *E. coli* strain TG2 [64] and the plasmid pEMBL19(-) (Boehringer Mannheim) were used for expression of the *vao*A gene. Oligonucleotides, T4 DNA ligase, restriction enzymes, isopropyl β-D-thiogalactopyranoside, yeast extract, and tryptone extract were from Life Technologies. Forward M13 and reverse M13 sequencing primers were from Pharmacia Biotech. Pwo DNA polymerase, dNTP's, and glucose oxidase (grade II) were purchased from Boehringer Mannheim and Super Taq DNA polymerase from HT Biotechnology. Ampicillin, guanidinium/hydrochloride, benzyl viologen, methyl viologen, safranin O, xanthine, and buttermilk xanthine oxidase (grade III) were from Sigma. Sodium dodecyl sulfate (SDS) was from BDH Chemicals. Thionin was purchased from Kodak and methylene blue from Across Chimica. 4-(Methoxymethyl)phenol, eugenol (4-allyl-2-methoxyphenol) isoeugenol (2-methoxy-4-propenylphenol), vanillyl alcohol, and phenosafranin were obtained from Aldrich. All other chemicals were from Merck and of the purest grade available.

Site-directed mutagenesis. The *vao*A-cDNA has been cloned into pEMBL19(-) to give pIM3972 [43]. In order to simplify the site-directed mutagenesis procedure, a *Sal*I restriction site was created in the *vao*A gene by a silent mutation at position 882. The *Nco*I-*Nsi*I fragment of pIM3972 was ligated into pGEM-5Zf(+) and the resulting construct was used as starting frame for polymerase chain reaction-based mutagenesis.

The polymerase chain reaction was performed with the oligonucleotide 5'-CAAGCCGTCGACATTATTCGTCC-3' where C denotes the mutated base. The mutated *Nco*I-*Nsi*I fragment was ligated into pIM3972 yielding pBC11. Successful mutagenesis was confirmed by plasmid sequencing. For the replacement of Asp170, the *Pst*I-*Sal*I fragment of pBC11 was ligated into pUCBM20. This construct was used for polymerase chain reaction-based mutagenesis with the oligonucleotide 5'-CTTGATGTACCGXXXCTTGGTGGCGGT-3' (where XXX denotes the replacement of GAT for GAG (Asp170Glu), TCT Asp170Ser), GCT (Asp170Ala), and AAC (Asp170Asn), respectively). The mutated *Pst*I-*Sal*I fragments were ligated into pBC11 yielding pBC12 (D170N), pBC14 (D170E), pBC15 (D170S), and pBC16 (D170A). Again, successful mutagenesis was confirmed by plasmid sequencing.

Enzyme purification. Transformed *E. coli* cells were grown in Luria Bertani-medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside, essentially as described before [43]. The VAO variants were purified as described previously [43], with the following modifications. After phenyl-Sepharose chromatography, the enzyme was transferred into 20 mM potassium phosphate buffer pH 7.2 and loaded onto a hydroxyapatite column equilibrated with 20 mM potassium phosphate buffer pH 7.2. After washing, the enzyme was eluted with a linear gradient of 20-1000 mM potassium phosphate buffer pH 7.2. Next, the enzyme was transferred into 50 mM potassium phosphate buffer and concentrated by ultrafiltration. To obtain highly pure Asp170 variants, a Superdex 200 PG 50/1000 gel filtration column running in 50 mM potassium phosphate buffer pH 7.2 was introduced as final chromatography step. Enzyme purity was checked by SDS-polyacrylamide gel electophoresis and by analytical gel filtration using a Superdex 200 HR 10/30 column.

Analytical methods. All experiments were performed in air-saturated 50 mM potassium phosphate buffer pH 7.5 at 25 °C unless stated otherwise. Molar absorption coefficients for protein bound flavin were determined in 100 mM sodium phosphate buffer pH 7.0 by unfolding the enzyme with 0.5% (w/v) SDS or 5 M guanidinium/hydrochloride [36]. Absorption spectra were recorded using a Hewlett-Packard HP 8453 diode-array spectrophotometer or an Aminco DW-2000 double beam spectrophotometer. The fraction of covalently bound FAD in the VAO variants was determined by the following procedure: first, the protein was treated with 5% (w/v) ice-cold trichloroacetic acid. After centrifugation, the protein precipitate was dissolved in 100 mM potassium phosphate buffer pH 7.5 containing 6 M guanidinium/hydrochloride. Next, the absorption spectral properties of supernatant and redissolved precipitate were compared with that of native enzyme. SDS-

polyacrylamide gel electrophoresis was carried out in 12.5% slab gels as described [91]. Coomassie Brilliant Blue R-250 was used for protein staining. Before protein staining, gels were incubated in 5% (v/v) acetic acid for fluorescence detection of covalently bound FAD in VAO [38]. Dissociation constants of enzyme-inhibitor complexes were determined from flavin absorption perturbation spectra by titration of the enzyme with a known concentration of inhibitor [23]. The dissociation constants of enzyme-sulfite complexes were determined from recording optical spectra as a function of sulfite concentration. Circular dichroism spectra were measured at 20 °C on a Jasco J-715 spectropolarimeter in a 1 cm path length cuvette. HPLC experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode-array detector and a 3.9 by 100 mm Waters Novapak C18 column, essentially as described earlier [62].

Redox potential determinations. The redox potentials of the VAO variants were determined in 50 mM potassium phosphate buffer pH 7.5 at 25 °C by the xanthine/xanthine oxidase method of Massey [92], essentially as described before [54]. Equal concentrations of VAO and reference dye (7-10 μM), benzyl viologen or methyl viologen (2 μM) and xanthine (400 μM) were made anaerobic by flushing with oxygen-free argon. The reduction of VAO and reference dye was initiated by adding a catalytic amount of xanthine oxidase. To ensure equilibration between the oxidized and reduced species of enzyme and reference dye the concentration of xanthine oxidase must be sufficiently low. Typically, the reduction of VAO and dye lasted 1-2 hours. The dyes used were thionin (E_m = +60 mV), methylene blue (E_m = +11 mV), FAD (E_m = -219 mV), phenosafranin (E_m = -266 mV), and safranin O (E_m = -280 mV). The oxidation-reduction potentials of the VAO variants were calculated according to the method of Clark [93]:

$$E_h(\text{dye}) = E_m(\text{dye}) + (59/n_{dye}) \times \log(\text{dye}_{ox}/\text{dye}_{red})$$

$$E_h(\text{E}) = E_m(\text{E}) + (59/n_E) \times \log(\text{E}_{ox}/\text{E}_{red})$$

$$\text{At equilibrium } E_h(\text{dye}) = E_h(\text{E})$$

Kinetic studies. All kinetic experiments were performed in 50 mM potassium phosphate buffer pH 7.5 at 25 °C unless stated otherwise. VAO activity was routinely determined by following absorption spectral changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode [62]. For enzyme-monitored-turnover experiments, air-saturated enzyme and substrate were mixed, and the redox-state of the FAD cofactor was measured [65]. Stopped-flow kinetics were performed essentially as described [40]. All concentrations mentioned in the context of stopped-

flow experiments are those after mixing. In anaerobic reduction experiments, glucose-containing enzyme solutions were flushed with oxygen-free argon. Glucose oxidase was added to eliminate final traces of oxygen. Reductive half-reactions were followed with a Hi-Tech M300 monochromator diode-array detector (Salisbury, UK). Deconvolution of spectral data was done with the Specfit Global Analysis program version 2.10 (Spectrum Software Associates, Chapel Hill, NC, USA). Rate constants of single wavelength kinetic traces were recorded using a Hi-Tech SU-40 spectrophotometer. The detection wavelength differed depending on the VAO mutant examined. For the generation of two-electron reduced enzyme, argon-flushed oxidized enzyme was treated with a small excess of argon-flushed dithionite. Reoxidation was measured by monitoring the redox-state of the flavin after mixing the reduced enzyme with varying concentrations of molecular oxygen.

Crystallization, data collection, and structure determination. Crystals of D170S VAO were grown using the hanging-drop vapor diffusion method, in conditions essentially identical to those for wild type VAO [42]. Briefly, protein solutions containing 10 mg protein/ml in 50 mM potassium phosphate buffer pH 7.5 were equilibrated against a reservoir solution containing 50 mM sodium acetate/hydrochloride pH 5.1 and 5% (w/v) polyethylene glycol (PEG) 4000. For data collection, the crystals were soaked in a solution containing 1 mM isoeugenol for 12 hours. Diffraction data were measured from a single crystal at the ID14-EH3 beam line of the European Synchrotron Radiation Facility (Grenoble, France) using a MarCCD detector at 100 K. Prior to data collection, the crystal was exposed for a few seconds to a cryoprotecting solution containing 20% (v/v) PEG 400, 20% (v/v) glycerol, 10% (w/v) PEG 4000, 10 mM isoeugenol, and 50 mM sodium acetate/hydrochloride pH 5.1. The data were processed using MOSFLM [94] and programs of the CCP4 package [95]. The mutant crystals belong to space group I4 with unit cell parameters $a = b = 131.33$, $c = 134.66$ Å and are isomorphous to the wild type crystals. The mutant structure was refined using the maximum likelihood refinement program REFMAC [96]. A bulk solvent correction was applied using the programs of the CCP4 package whereas the positions of ordered solvent molecules were located using ARP [97]. The progress of the refinement was monitored by means of R_{free}. Electron density maps were visually inspected using the program O [98]. The isoeugenol atoms were well defined in the electron density map, except for the terminal carbon atom of the propenyl substituent. This probably reflects the presence in the soaking solution of a mixture of *cis*- and *trans*-isomers, which both bind to the enzyme. Analysis of the refined structure revealed that 85.1% of the amino acid residues of the isoeugenol-complexed D170S structure fall in the most favored regions of the Ramachandran plot whereas none fall in the disallowed regions as defined by

PROCHECK [99]. Data collection and refinement statistics for the structure are displayed in Table 3. The atomic coordinates and structure factors have been deposited in the Macromolecular Structure Database of the European Bioinformatics Institute (EBI), Hinxton, UK (PDB ID code 1DZN).

Results

General properties. The Asp170 variants D170E, D170S, D170A, and D170N were purified from *E. coli* in about the same yield as wild type VAO [43]. Analytical gel filtration revealed that all VAO variants were mainly present in the octameric form. This hydrodynamic behaviour is similar to wild type enzyme [53].

Wild type VAO binds the FAD cofactor covalently via His422 [42]. When D170E was precipitated with 5% (w/v) ice-cold trichloroacetic acid, no flavin was present in the supernatant, indicating that all FAD is covalently bound. With D170S only a trace amount (3%) of free FAD was found. However, trichloroacetic acid precipitation of D170A and D170N showed that in these mutants, respectively 50% and 100% of the flavin cofactor was noncovalently bound. These data were confirmed by SDS-polyacrylamide gel electrophoresis using fluorescence analysis. The unstained SDS gel in 5% (v/v) acetic acid showed that D170E and D170S were as fluorescent as wild type enzyme. However, the fluorescent band of D170A was less intense and D170N was not fluorescent.

Spectral properties. The flavin spectral properties of the oxidized VAO variants are summarized in Table 1. The absorption characteristics of wild type enzyme and D170E are similar. However, D170S, D170A, and D170N display significantly different spectral properties (Fig. 2A).

Table 1. Flavin spectral properties of wild type VAO and Asp170 variants in the oxidized form in 50 mM potassium phosphate buffer pH 7.5 at 25 °C.

	λ_{max}		ε_{ox}		
VAO	nm		$mM^{-1}*cm^{-1}$		
	I	II	ε_I	ε_{II}	$\varepsilon_I/\varepsilon_{II}$
Wild type	356	439	13.6	12.5	1.09
D170E	351	437	13.9	12.7	1.09
D170S	345	443	19.7	11.9	1.66
D170A	348	441	16.6	13.3	1.25
D170N	344	441	27.3	13.1	2.08

Unfolding the D170S mutant with 0.5% (w/v) SDS or 5 M guanidnium/hydrochloride changed the absorbance spectrum of the covalently bound flavin to that of oxidized FAD [36]. Upon this treatment, the absorbance at 450 nm remained nearly constant, revealing that the enzyme was isolated in the oxidized form. In agreement with this, native D170S displayed no fluorescence upon excitation at 360 nm, indicating that the enzyme was not isolated as a flavin N5 adduct [41,83]. The flavin in D170S was not present in the flavin iminoquinone methide form as the cofactor was covalently attached to the enzyme.

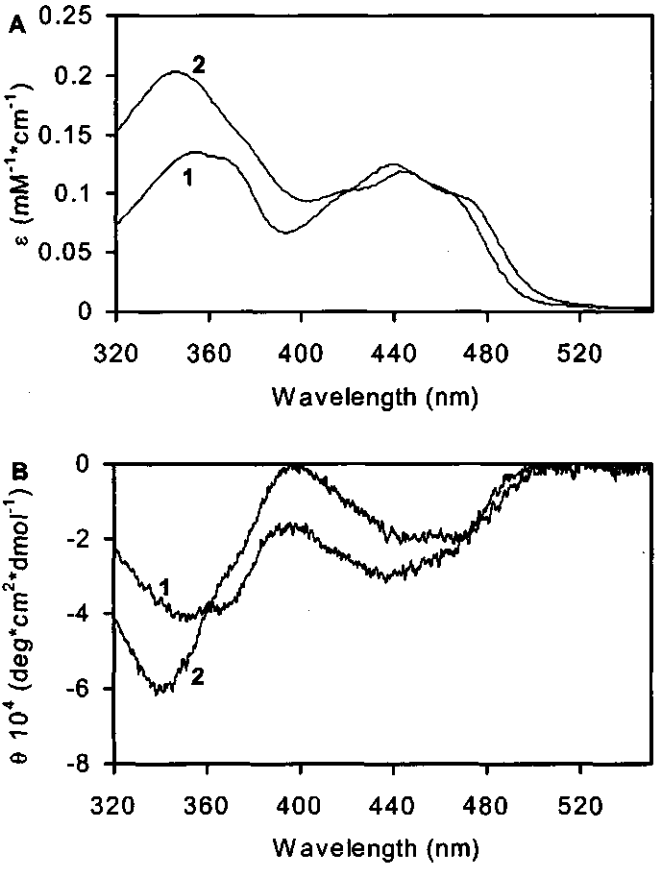

Fig. 2. Spectral properties of Asp170 variants of VAO. (**A**) Absorption and (**B**) circular dichroism spectra of (1) oxidized wild type VAO and (2) D170S in 50 mM potassium phosphate buffer pH 7.5 at 25 °C.

Moreover, the absorption characteristics of native D170S did not change after a single turnover experiment or after incubation with the substrate analog isoeugenol and subsequent elution over Biogel P6. On the basis of these results, we conclude that the unusual spectral features of D170S, D170A, and D170N are an intrinsic property of the mutant protein and the result of a changed flavin microenvironment.

Circular dichroism spectra can yield valuable information about the flavin microenvironment [100]. The flavin circular dichroism spectra of the four VAO variants were mirror images of the absorbance spectra (Fig. 2B). Thus, wild type VAO and D170E showed similar circular dichroism spectra, whereas D170S, D170A, and D170N displayed strong negative peaks in the near-UV region. These results corroborate that the Asp170 replacements introduce a change in the flavin microenvironment.

Flavoprotein oxidases generally form flavin N5 adducts with sulfite [101]. The absorbance spectra of these complexes resemble those of the reduced enzymes. However, wild type VAO does not react with sulfite [36] and we have suggested that this is due to the presence of Asp170 in the active site [42]. Binding studies with D170E and D170S showed that only the latter enzyme interacts with sulfite. In the presence of 20 mM sulfite, bleaching of the flavin in D170S was completed after 60 min and the reactivity did not significantly change with pH. From titration experiments, a dissociation constant for the D170S-sulfite complex, K_d = 3 ± 1 mM at pH 7.5 was estimated. These results are in line with the proposal that a negatively charged residue at position 170 in VAO prohibits the reaction between flavin N5 and sulfite through electrostatic repulsion.

Replacement of Asp170 by Glu, Ser, and Ala did not strongly affect the binding of the competitive inhibitor isoeugenol. The dissociation constants of the isoeugenol-enzyme complexes were 13 ± 4 μM, 18 ± 5 μM, 15 ± 10 μM, and 6 ± 2 μM for wild type VAO, D170E, D170S, and D170A, respectively, as determined by flavin perturbation difference spectroscopy [23]. At pH 7.5, binding of isoeugenol to the VAO variants resulted in a strong increase in absorbance at 320 nm. This absorbance increase is due to the formation of the phenolate form of the inhibitor [23]. As the pK_a of isoeugenol free in solution is 9.8, binding to wild type VAO, D170S, D170E, and D170A decreases the pK_a value of isoeugenol significantly. These data indicate that Asp170 is not directly involved in substrate activation. D170N does not strongly interact with isoeugenol. This is concluded from the fact that no significant absorbance changes were observed in titration experiments.

Catalytic properties. Wild type VAO oxidizes the physiological substrate 4-(methoxymethyl)phenol with a k_{cat} of 3.1 s^{-1} [40]. In contrast, turnover of D170E with 4-(methoxymethyl)phenol was rather slow and the other three mutant enzymes were

nearly inactive. A similar behavior was observed with the substrates eugenol and vanillyl alcohol (Table 2) In the following and for obvious reasons, the catalytic properties of D170E and D170S were studied in further detail.

Table 2. Kinetic parameters of wild type VAO and Asp170 variants in air-saturated 50 mM potassium phosphate buffer pH 7.5 at 25 °C

Substrate	Wild type		D170E		D170S	
	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
	μM	s^{-1}	μM	s^{-1}	μM	s^{-1}
4-(Methoxymethyl)phenol	55*	3.1*	113	0.07	n.d.	0.004
Vanillyl alcohol	160*	3.3*	340	1.3	n.d.	0.004
Eugenol	4	17.5	2	0.34	2	0.01

Substrate	D170A		D170N	
	K_m	k_{cat}	K_m	k_{cat}
	μM	s^{-1}	μM	s^{-1}
4-(Methoxymethyl)phenol	n.d.	0.001	n.d.	0.002
Vanillyl alcohol	n.d.	n.d.	n.d.	n.d.
Eugenol	n.d.	n.d.	n.d.	n.d.

*Data from ref. [40].

n.d., not determined.

By measuring the redox-state of the FAD cofactor during turnover, information can be obtained about the rate-limiting step in catalysis [65]. When oxidized D170E and D170S were mixed with excess 4-(methoxymethyl)phenol in the stopped-flow spectrophotometer, the fraction of oxidized enzyme during the steady-state phase of the reaction was 0.90 and 0.75, respectively. This indicates that, similar to wild type VAO, a step in the reductive half reaction is limiting the rate of overall catalysis [40].

The reductive half-reaction of wild type VAO with 4-(methoxymethyl)phenol is described by the following equation:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}{-}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{red}{-}Q \xrightarrow{k_3} E_{red} + P$$

Under anaerobic conditions, wild type VAO is reduced by 4-(methoxymethyl)phenol in a single irreversible step (k_2 = 3.3 s^{-1}) with the concomitant formation of a stable complex between the *p*-quinone methide of the product and the reduced enzyme. This

complex displays a typical absorbance maximum at 364 nm (ε_{364} = 46 $mM^{-1}*cm^{-1}$) and its decay is too slow to be of catalytic importance (k_3 = 0.01 s^{-1}) [40]. When the rate of anaerobic flavin reduction of D170E was determined by stopped-flow spectroscopy as a function of the substrate concentration, the reduction appeared to be a biphasic process when monitored at 437 nm and 364 nm. During this process the enzyme became fully reduced. The first relatively rapid phase was virtually independent of the substrate concentration (k_{obs1} = 0.70 ± 0.04 s^{-1}) (Fig. 3A). The second phase was an order of magnitude slower than the first phase and was dependent on the 4-(methoxymethyl)phenol concentration (k_{obs2} = 0.07 ± 0.01 s^{-1} at saturating substrate conditions).

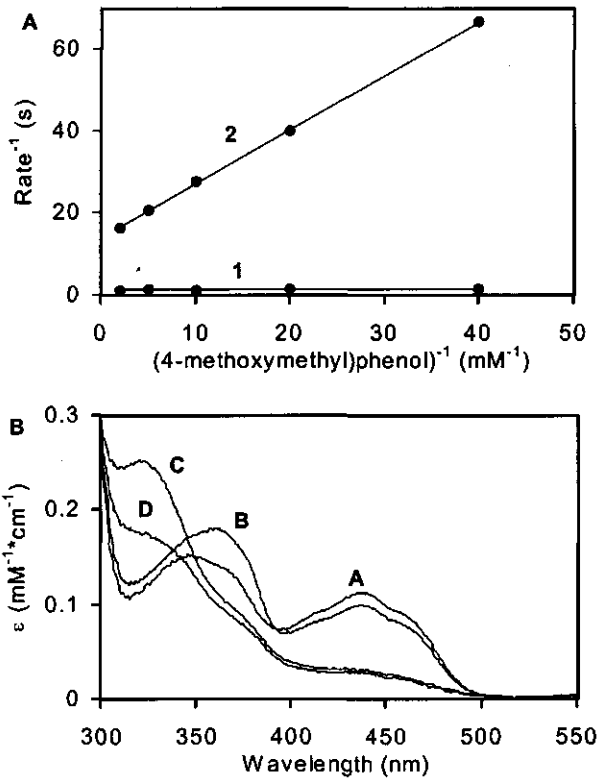

Fig. 3. Reductive half-reaction of D170E in 50 mM potassium phosphate buffer pH 7.5 at 25 °C. (**A**) Double reciprocal plots of the two reduction rates observed during the anaerobic reductive half-reaction of 2.5 μM D170E with 4-(methoxymethylphenol) at 439 nm. (1) k_{obs1} and (2) k_{obs2} (**B**) Spectra obtained from deconvolution of spectral changes caused by the anaerobic reduction of 5 μM D170E by 500 μM 4-(methoxymethyl)phenol. The data were fitted with the consecutive model A→B→C. When the reaction was performed at pH 6.5 only the final spectrum (D) changed.

Diode-array spectral analysis of the anaerobic flavin reduction in D170E revealed the formation of an intermediate spectrum with an absorbance maximum at 364 nm during the first phase of reduction (k_{obs1}) (Fig. 3B). We estimated a molar absorption coefficient of $\varepsilon_{364} = 50 \pm 15$ $mM^{-1}*cm^{-1}$, taking into account that the enzyme was only partially reduced in the first phase of reduction (15%). pH-dependent analysis of the reaction showed that the spectral properties of the intermediate did not change between pH 6.5 and 8.0. These data suggest that the intermediate spectrum reflects the *p*-quinone methide intermediate-reduced enzyme complex. The final spectrum upon reduction was pH-dependent and showed an absorbance maximum at 335 nm. This points to the formation of 4-hydroxybenzaldehyde during the second phase of reduction (k_{obs2}) and shows that the reductive half-reaction of D170E can be defined by the same equation as described for wild type enzyme. However, it should be stressed here that with wild type enzyme, the reduced enzyme-*p*-quinone methide intermediate complex is highly stabilized under anaerobic conditions.

The first phase in the reductive half-reaction of D170E leads to an equilibrium in which 15% of the flavin is in the reduced state. This suggests that the first step in flavin reduction by 4-(methoxymethyl)phenol is reversible ($k_{-2} > 0$). By measuring the fraction of oxidized enzyme present at the end of the first reductive step and the apparent reduction rate at saturating conditions ($k_{app} = k_2 + k_{-2}$) we could estimate k_2 and k_{-2} ($k_{-2}/(k_2 + k_{-2})) = (E_{ox}/E_{total})$ [41,66]. The calculated reduction rate (k_2) and the rate of the reverse reaction (k_{-2}) were 0.1 s^{-1} and 0.6 s^{-1}, respectively. Reversible reduction was also observed for the reaction of wild type VAO with short-chain 4-alkylphenols [41] and gives a rational for the apparent substrate concentration independent reduction in the first phase. The reversible reduction of the flavin in D170E is followed by a substrate concentration dependent second step in the reductive half-reaction, which reflects the decomposition of the *p*-quinone methide intermediate-reduced enzyme complex (k_3). This substrate dependent phenomenon was also reported for D-amino acid oxidase in case of k_{-2} being an important term in the reductive half-reaction [102,103].

Anaerobic reduction of D170S by 4-(methoxymethyl)phenol was an extremely slow process ($k_{obs} = 0.005 \pm 0.002$ s^{-1}) and its rate was in the same range as the turnover rate ($k_{cat} = 0.004$ s^{-1}). During this process, the flavin became almost completely reduced with the concomitant formation of the product 4-hydroxybenzaldehyde (Fig. 4). Using diode-array analysis, we could not detect the formation of the *p*-quinone methide intermediate-reduced enzyme complex, indicating that with this mutant enzyme the complex is not stabilized. Similar to D170S, reduction of D170A and D170N was extremely slow and no *p*-quinone methide intermediate product was observed.

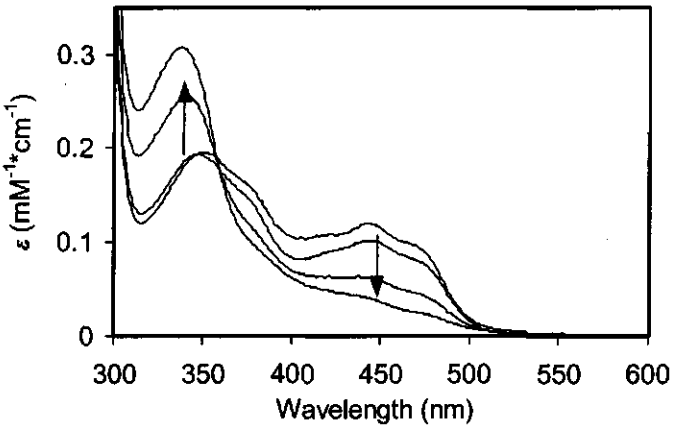

Fig. 4. Reductive half-reaction of D170S. Spectral changes observed upon anaerobic mixing of 10 μM D170S with 500 μM 4-(methoxymethyl)phenol in 50 mM potassium phosphate buffer pH 7.5 at 25 °C. Spectra were taken after 0, 1, 12, and 29 min. The arrows indicate the direction of the absorbance changes.

Reoxidation of free reduced wild type VAO is a fast bimolecular process ($3.1*10^5$ $M^{-1}*s^{-1}$) [40]. Analysis of the reoxidation of the VAO variants with varying concentrations of molecular oxygen showed that the mutants also reoxidized in a monophasic reaction. However, the reoxidation rates of D170E and D170S were significantly lower as compared to wild type enzyme ($1.2*10^5$ $M^{-1}*s^{-1}$ and $3.2*10^4$ $M^{-1}*s^{-1}$, respectively). Moreover, the reduced D170A and D170N variants reoxidized with the same rate as D170S in a monophasic fashion.

Redox potentials. Since the side chain of Asp170 is close to flavin N5, it is conceivable that this residue influences the redox potential of the flavin. Upon reduction of wild type VAO with the xanthine/xanthine oxidase system in the presence of thionin (E_m = +60 mV) as reference dye, the enzyme and thionin reduced simultaneously via a single two-electron reduction process (Fig. 5). From plotting $\log(E_{ox}/E_{red})$ versus $\log(dye_{ox}/dye_{red})$ according to the method of Minnaert [104] a redox potential of +55 ± 2 mV was estimated. When D170E was reduced in the presence of methylene blue (E_m = +11 mV), we also observed a two-electron reduction with a midpoint redox potential of +6 ± 5 mV (not shown).

In contrast to wild type VAO and D170E, the xanthine/xanthine oxidase-mediated reduction of D170S initially gave rise to the formation of a one-electron reduced flavin semiquinone anion with a typical absorbance maximum at 385 nm [36] (Fig. 6). This red flavin semiquinone was formed for more than 95% during reduction,

indicating that the redox potentials of the two couples (oxidized/semiquinone and semiquinone/hydroquinone) are separated by at least 200 mV [93]. The one-electron redox potentials for both couples were determined using thionin (E_m = +60 mV) and phenosafranin (E_m = -266 mV), respectively. The log(E_{ox}/E_{red}) versus log(dye_{ox}/dye_{red}) plots for the two one-electron reductions revealed slopes of 1.81 and 1.21, whereas both plots should give two-unit slopes [104], indicating that there might be a kinetic barrier for reduction of the FAD in D170S. We verified the data of the second one-electron reduction using the reference dyes FAD (E_m = -219 mV) and safranin O (E_m = -280 mV). These reduction experiments resulted in similar redox potential values. The redox potentials of the two couples were +76 ± 10 mV and -257 ± 8 mV leading to a midpoint redox potential for D170S of -91 ± 9 mV. These results establish that Asp170 in VAO is essential for stabilization of the two-electron reduced form of the FAD.

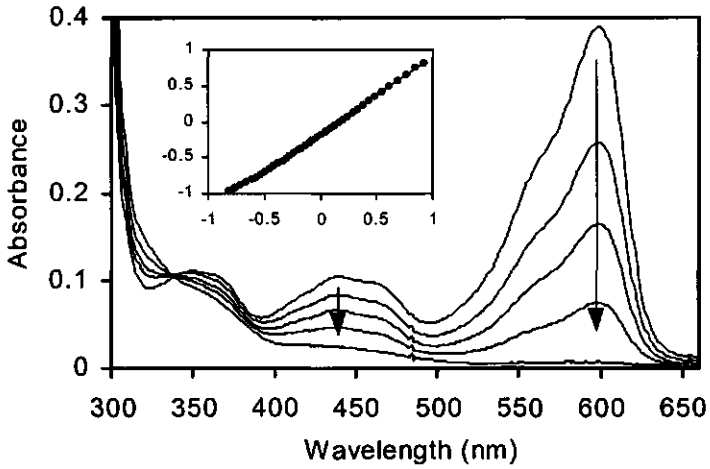

Fig. 5. Determination of the redox potential of wild type VAO. 9 μM VAO in 50 mM potassium phosphate buffer pH 7.5 at 25 °C was reduced in the presence of 9 μM thionin by the xanthine/xanthine oxidase system. The reduction was finished after 100 min. The arrows indicate the direction of the absorbance changes. The inset shows the log(E_{ox}/E_{red}) (measured at 439 nm after correction for thionin) versus log(dye_{ox}/dye_{red}) (measured at 600 nm) plot.

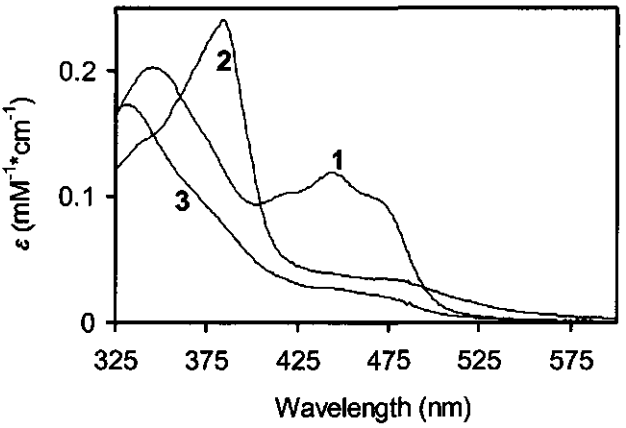

Fig. 6. Spectral properties of D170S upon reduction with the xanthine/xanthine oxidase system. D170S in 50 mM potassium phosphate buffer pH 7.5 at 25 °C was reduced by the xanthine/xanthine oxidase system under anaerobic conditions. (1) Oxidized enzyme, (2) semiquinone form, and (3) hydroquinone form.

Structural properties of D170S. Crystals of D170S in complex with isoeugenol diffracted to 2.8 Å resolution (Table 3). The structure of this mutant enzyme-inhibitor complex is highly similar to that of the corresponding complex of wild type enzyme [42]. The root mean square deviation for the two structures for all Cα-atoms is 0.30 Å. The active site of D170S is virtually identical to wild type enzyme with the side chain of Ser170 pointing in the same direction as the side chain of Asp170 in wild type VAO (Fig. 7). The Oγ-atom of Ser170 is engaged in two weak hydrogen bonds with NH1 of Arg398 (3.2 Å) and O4 of the flavin (3.3 Å) whereas the distance from the cofactor N5 is 4.0 Å. The isoeugenol inhibitor binds in the same position as in wild type enzyme. The only noticeable difference concerns the orientation of the propenyl substituent, which is directed towards Ser170 (Fig. 7). From these crystallographic data we conclude that the Asp170Ser replacement does not induce significant protein structural changes which could explain the low reactivity of D170S.

Table 3. Data collection and refinement statistics for D170S in complex with isoeugenol.

Measurement	Value
Resolution, Å	20-2.8
Observed reflections	23,315
Unique reflections	16,730
Completeness of data, %*	83.7 (83.8)
Multiplicity*	2.4 (2.0)
Intensities, I/σ*	2.5 (1.9)
R_{sym}, %*,†	15.8 (32.3)
Cell dimensions, Å	$a = b = 131.33$, $c = 134.66$
R_{factor}, %	22.6
R_{free} , %	29.1
Number of ligand atoms	22 (isoeugenol)
r.m.s.d. for ideal value‡	
Bond lengths, Å	0.013
Bond angles, °	2.9
Trigonal atoms, Å	0.023
Planar groups, Å	0.011
Ramachandran plot**	85.1/14.9/0

*The values relating to the highest resolution shell (2.9-2.8 Å) are given in brackets.

†$R_{sym} = \Sigma|I_j - \langle I_j \rangle| / \Sigma \langle I_j \rangle$, where I_j is the intensity of an observation of reflection j and $\langle I_j \rangle$ is the average intensity for reflection j.

‡The root mean square deviations (r.m.s.d.) were calculated using the program REFMAC [96].

**Percentage of residues in most favored, allowed and disallowed regions of the Ramachandran plot as checked with the program PROCHECK [99].

Discussion

VAO is the prototype of a novel family of structurally related flavoproteins [44]. On the basis of crystallographic data [42], we have proposed that Asp170, located near the flavin N5-atom, is a key residue for VAO catalysis. The presence of an acidic residue near flavin N5 is rather unusual in flavoproteins and prompted us to address the role of Asp170 in VAO via site-directed mutagenesis.

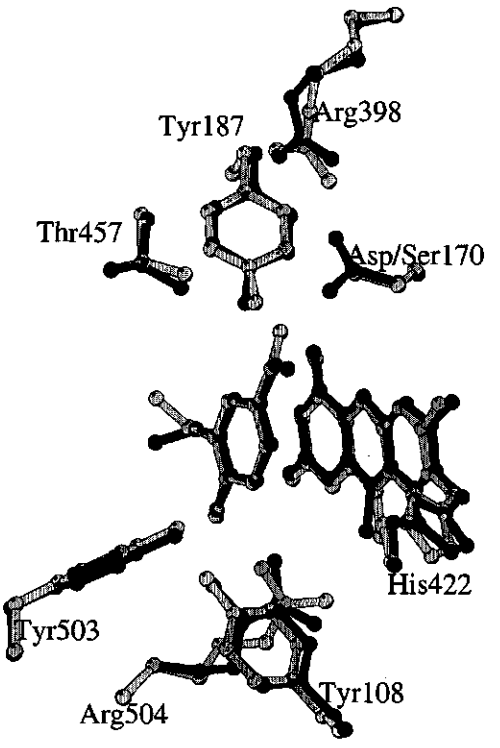

Fig. 7. Drawing of the superposition of wild type VAO (black bonds) and D170S (grey bonds) in complex with isoeugenol. The side chain of Asp170 in wild type enzyme is 3.5 Å from flavin N5 and the side chain of Ser170 in D170S is 4.0 Å from flavin N5. This figure was prepared with MOLSCRIPT [45].

The conservative replacement of Asp170 by Glu did not lead to structural changes in the active site. This is concluded from the flavin spectral properties of D170E, including absorption and circular dichroism data and inhibitor binding. However, the D170E replacement significantly reduced the rate of conversion of aromatic substrates. Replacement of Asp170 by Ser, Asn, and Ala induced severe changes in the flavin spectral properties and almost completely blocked enzyme activity. The crystal structure of D170S showed that the atypical spectral characteristics of this mutant enzyme are not caused by significant structural changes. At present, we have no clear explanation for the unusual high absorbance of the oxidized flavin in D170S, D170A, and D170N around 345 nm. Nevertheless, the crystal structure of D170S and additional spectroscopic studies showed that this high absorbance is not caused by binding of a compound in the active site or a chemical modification of the flavin. Another interesting feature of D170S is the formation of a flavin-sulfite complex. This

finding strongly supports that unlike many other flavoprotein oxidases, VAO does not interact with sulfite because of the electrostatic repulsion of sulfite by the acidic side chain at position 170. To our best knowledge, this is the first direct evidence that a charged residue near flavin N5 is of importance for the reaction with sulfite.

Charged residues located near the isoalloxazine ring of protein-bound flavin can have a major impact on the redox potential of the flavin and thus its reactivity [105-107]. In general, the presence of a positive charge near the N1-C2 locus stabilizes the anionic reduced form of the flavin cofactor [108]. This positive charge (Arg504 in VAO) also influences flavin N5, however, this influence is more subtle as N5 is protonated in the reduced form. In most flavin-dependent oxidoreductases a main chain nitrogen is hydrogen-bonded to flavin N5 [89], limiting the possibilities for rational mutagenesis. The presence in VAO of an acidic residue near flavin N5 offers the unique opportunity to evaluate the role of N5 interactions for flavin catalysis. The present study clearly shows that a negative charge near flavin N5 drastically increases the redox potential of the flavin cofactor, suggesting that the ionized carboxylic moiety of Asp170 counterbalances the protonated flavin N5. This shift in redox potential is reflected in the low oxidative power of the mutant enzymes. Apart form this direct effect on catalysis, Asp170 is also indirectly involved in modulating the redox potential of the enzyme as this residue is of importance for the covalent attachment of the flavin to His422. Recently, we have shown that the covalent histidyl-flavin bond increases the redox potential of VAO by 110 mV, thereby increasing the rate of substrate-mediated enzyme reduction by a factor of ten [54].

The precise mechanism for covalent flavinylation is still unclear, but all the evidence presently available suggests that this process is autocatalytic [54,109,110]. The proposed linkage mechanism involves the initial removal of a proton from the 8α position of the flavin, yielding the electrophilic iminoquinone methide form [111]. In the following step, the reduced covalent cofactor is formed through donation of a proton by an acidic side chain or a water molecule [112]. Our studies show that covalent flavinylation of VAO is strongly dependent on the identity of residue 170, which is located more than 8 Å from the target residue of flavinylation, His422. In agreement with the proposed linkage mechanism, D170E might retain the ability to donate a proton to N5, thus promoting covalent bond formation, whereas D170N is unable to act as a proton donor, preventing formation of the covalent bond. The partial flavinylation of D170A and the almost fully covalent binding of the flavin in D170S suggest that in these mutants, water might substitute for Asp170 in activating the flavin. However, it can not be excluded that the impairment of covalent bond formation in D170A and D170N is caused by steric constraints.

Detailed kinetic studies of D170E with 4-(methoxymethyl)phenol revealed that with this substrate, and similar to wild type VAO, a step in the reductive half reaction

is rate-limiting in overall catalysis. However, the 4-(methoxymethyl)phenol-mediated reduction of D170E appeared to be highly reversible, whereas reduction of the flavin in wild type enzyme is irreversible [40]. Under anaerobic conditions, the complex between the reduced D170E and the *p*-quinone methide intermediate is not as stabilized as in wild type enzyme and reacts with water to form the final products 4-hydroxybenzaldehyde and methanol. On the basis of these observations, we propose that the introduction of Glu170 changes the electrostatic interactions between the side chain of residue 170 and flavin N5 such that the complex between the reduced enzyme and the *p*-quinone methide intermediate is less stabilized (Fig. 8). The 4-(methoxymethyl)phenol-mediated flavin reduction in D170S was extremely slow. As a result, the mechanism for oxidative demethylation of 4-(methoxymethyl)phenol by D170S may also follow a ping-pong mechanism, whereas wild type enzyme reacts through a true ternary complex mechanism.

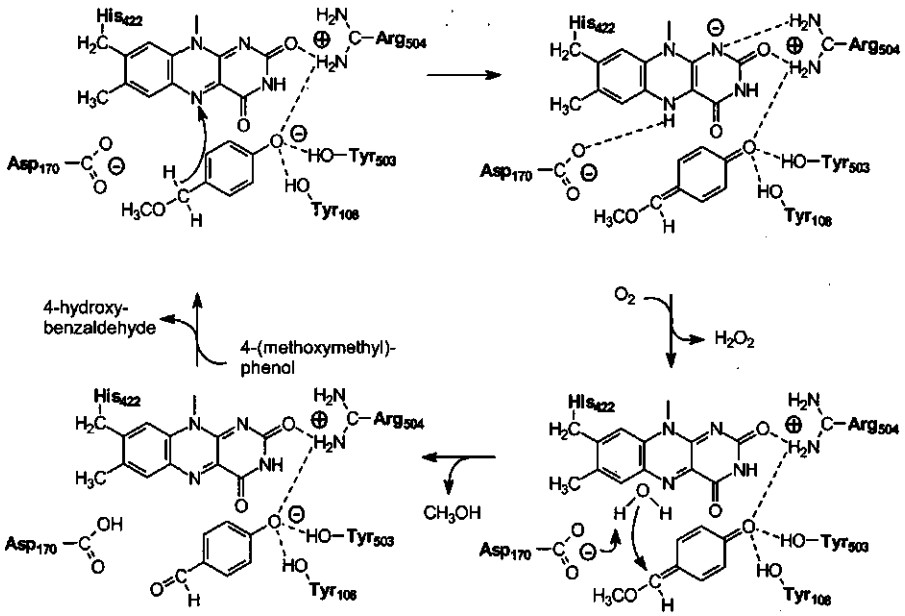

Fig. 8. Proposed role of Asp170 in the VAO-mediated conversion of 4-(methoxymethyl)phenol [40,42,50].

We have proposed before that Asp170 might act as an active site base by activating the water involved in hydration of the *p*-quinone methide intermediate (Fig. 8) [42]. However, D170S, D170N, and D170A also efficiently hydrate the *p*-quinone methide intermediate, as 4-(methoxymethyl)phenol is quantitatively converted to 4-hydroxybenzaldehyde. Like wild type enzyme [40], the formation of

4-hydroxybenzaldehyde by the mutant enzymes is not influenced in the presence of reduced glutathione, indicating that the *p*-quinone methide intermediate reacts with water in the enzyme active site. Earlier studies have shown that quinone methides are highly electrophilic and toxic as they can form covalent adducts with proteins and DNA [74,75]. This strongly suggests that the *p*-quinone methide of 4-(methoxmethyl)phenol reacts readily with water in the active site of the VAO mutants, forming the final products 4-hydroxybenzaldehyde and methanol. These findings, together with the observed similarity between the wild type and D170S structures, suggest that the low redox potential is the main factor limiting the catalytic activity of the D170S mutant.

In conclusion, the present study has clearly unraveled a dual role for Asp170 in VAO. This active site residue is involved in both catalysis and covalent flavinylation. Furthermore, we have obtained insight into the mechanisms of fine-tuning of flavin redox properties and reactivity. Interestingly, the structurally related covalent flavocytochrome *p*-cresol methylhydroxylase contains a Glu (Glu380) at the position of Asp170 in VAO [50,113]. Possibly, Glu380 in *p*-cresol methylhydroxylase fulfills a similar role as Asp170 in VAO, resulting in efficient covalent flavinylation and redox catalysis.

Acknowledgements

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Chapter 7

INVERSION OF STEREOSPECIFICITY OF VANILLYL-ALCOHOL OXIDASE

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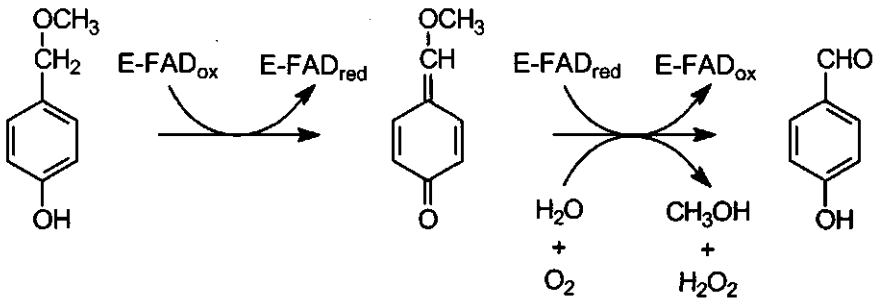
Vanillyl-alcohol oxidase (VAO) is the prototype of a newly recognized family of structurally related oxidoreductases sharing a conserved FAD binding domain. The active site of VAO is formed by a cavity where the enzyme is able to catalyze many reactions with phenolic substrates. Among these reactions is the stereospecific hydroxylation of 4-ethylphenol forming (*R*)-1-(4'-hydroxyphenyl)ethanol. During this conversion, Asp170 is probably critical for the hydration of the initially formed *p*-quinone methide intermediate. By site-directed mutagenesis, the putative active site base has been relocated to the opposite face of the active site cavity. In this way, a change in stereospecificity has been achieved. Like native VAO, the single mutants T457E, D170A, and D170S preferentially converted 4-ethylphenol to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol. The double mutants D170A/T457E and D170S/T457E exhibited an inverted stereospecificity with 4-ethylphenol. Particularly D170S/T457E was strongly (*S*)-selective with an enantiomeric excess of 80%. The crystal structure of D170S/T457E in complex with trifluoromethylphenol showed a highly conserved mode of ligand binding and revealed that the distinctive catalytic properties of this mutant are not caused by major structural changes.

Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a flavoenzyme from *Penicillium simplicissimum* containing a covalently bound FAD. The enzyme is the prototype of a newly recognized family of structurally related oxidoreductases sharing a conserved FAD binding domain [44]. Prominent members of this family include MurB (UDP-*N*-acetylenolpyruvylglucosamine reductase) [47], the molybdenum enzyme carbon monoxide dehydrogenase [48], and the flavocytochrome *p*-cresol methylhydroxylase (PCMH) [50]. VAO is active with a wide range of phenolic compounds [23,62]. Conversion of the physiological substrate 4-(methoxymethyl)phenol by VAO involves the initial transfer of a hydride from the substrate to the flavin, resulting in the formation of a complex between the two-electron reduced enzyme and the *p*-quinone methide product intermediate. Next, the reduced flavin is reoxidized by molecular oxygen with the concomitant hydration of the *p*-quinone methide [40]:



The crystal structure of VAO represents the first crystal structure of a flavoenzyme with a histidyl-bound flavin [42]. Each VAO monomer comprises two domains with the cap domain covering the active site cavity and the larger domain forming the binding site of the FAD prosthetic group. All present evidence suggests that the covalent anchoring of the flavin is a self-catalytic process [109]. From the properties of His422 variants it was established that the covalent interdomain interaction with His422 serves to increase the flavin redox potential, an essential feature for efficient redox catalysis [54]. Another important residue in the enzyme active site is Asp170. The side chain of this acidic residue is only 3.6 Å from flavin N5 and the reactive methylene group of the phenolic substrate [42]. From studies on Asp170 variants, we recently showed that Asp170 is important for maintaining the high redox potential of the enzyme and thus its reactivity [56]. Furthermore, these studies revealed that Asp170 assists in covalent flavinylation, possibly by donating a proton to flavin N5 [56]. It has been suggested that in the related flavocytochrome PCMH, Glu380 fulfills a similar role during the flavinylation process [50,56,112].

VAO and PCMH have a similar substrate specificity [39,62,114] and the active site architecture of both enzymes is highly conserved [42,50]. However, VAO preferentially converts 4-ethylphenol and 4-propylphenol to the corresponding (*R*)-1-(4'-hydroxyphenyl)alcohols [39], whereas PCMH favors the production of the (*S*)-enantiomers [68,70]. The structural determinants involved in the stereochemistry of VAO and PCMH are unknown. It has been proposed that Asp170 in VAO [42,56] and the equivalent Glu380 in PCMH [50] might activate the water involved in substrate conversion. However, in PCMH another acidic residue (Glu427) is situated near the reactive carbon of bound *p*-cresol at the opposite face of the substrate [50]. In VAO, this position is occupied by Thr457 (Fig. 1), raising the possibility that this structural variation plays a role in the opposite stereospecificity of water attack.

To investigate this hypothesis and the function of Asp170 in the active site of VAO in further detail, we have generated the single mutant T457E and the double mutants D170A/T457E and D70S/T457E. Here, we present the biochemical and structural characterization of the first VAO variant with an inverted stereospecificity. Furthermore, evidence is provided that Glu457 may substitute for Asp170 in promoting the covalent tethering of the flavin ring.

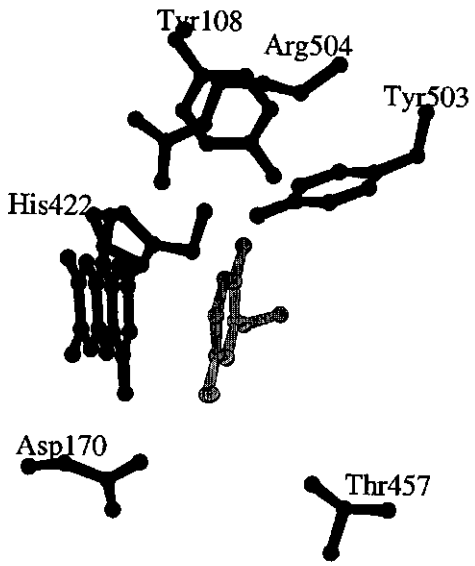


Fig. 1. Drawing of the active site cavity of isoeugenol-complexed VAO. The side chains of Asp170 and Thr457 in VAO align with Glu380 and Glu427 in PCMH, respectively. This figure was prepared with MOLSCRIPT [45].

Materials and Methods

Materials. *Escherichia coli* strain DH5αF' [90] and the plasmid pUCBM20 (Boehringer Mannheim) were used for cloning throughout, whereas *E. coli* strain TG2 [64] and the plasmid pEMBL19(-) (Boehringer Mannheim) were used for expression of the *vao*A gene. Yeast extract and tryptone peptone extract was from Difco. Racemic 1-(4'-hydroxyphenyl)ethanol was synthesized as described [39] and (*R*)-1-(4'-hydroxyphenyl)ethanol was obtained from the enzymatic conversion of 4-ethylphenol. All other chemicals were purchased as reported earlier [56,62].

Site-directed mutagenesis and enzyme purification. pBC11 (wild type), pBC15 (D170S), and pBC16 (D170A) were constructed as described before [56]. The *Sal*I-*Kpn*I fragment of pBC11 was ligated in pUCBM20 and used for PCR-based mutagenesis with the oligonucleotide 5'-GATTTTATCGGCGAGTTCACAGT-CGGTATG-3' (where GAG denotes the replacement of Thr by Glu). The mutated *Sal*I-*Kpn*I fragment was ligated into pBC11, pBC15, and pBC16 yielding pBC22 (T457E), pBC24 (D170S/T457E), and pBC23 (D170A/T457E), respectively. Successful mutagenesis was confirmed by plasmid sequencing. Transformed *E. coli* cells were grown in Luria-Bertani medium supplemented with 75 μg/ml ampicillin and 0.25 mM isopropyl β-D-thiogalactopyranoside [43]. The VAO mutant proteins were purified according to a previously established procedure [56]. Enzyme purity was checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and by analytical gel filtration using a Superdex 200 HR 10/30 column.

Analytical methods. All experiments were performed in air-saturated 50 mM potassium phosphate buffer pH 7.5 at 25 °C, unless stated otherwise. Molar absorption coefficients for protein-bound flavin and the fraction of covalently bound flavin were determined as described previously [56]. SDS-polyacrylamide gel electrophoresis was carried out in 12.5% slab gels essentially as reported earlier [38,91]. The redox potentials of the VAO mutants were determined by the xanthine/xanthine oxidase method [92], essentially as described before [54,56].

HPLC experiments were performed with an Applied Biosystems pump equipped with a Waters 996 photodiode-array detector and a 3.9 by 150 mm Waters Novapak C18 column, essentially as described earlier [62]. GC analysis was performed on a Varian Star 3400 CX gas chromatograph with a 50 m capillary Chrompack CP-cyclodextrin column (0.25 mm internal diameter and 0.25 μM film thickness) having CP-cyclodextrin-B-2,3,6-M-19 as the chiral stationary phase. The temperature program started at 80 °C, followed by an increase with 7 °C min^{-1} to 160 °C which was held for 30 min [39].

VAO activity was determined by following absorption spectral changes of aromatic substrates or by measuring the rate of product formation by HPLC [56,62]. Rapid reaction stopped-flow kinetics were conducted with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech M300 monochromator diode-array detector (Salisbury, UK), essentially as described [40]. Deconvolution of spectral data was performed using the Specfit Global Analysis program version 2.10 (Spectrum Software Associates, Chapel Hill, N.C., USA).

Crystallographic analysis of mutant D170S/T457E. Crystals of the double mutant D170S/T457E were grown using the hanging-drop vapor diffusion method, in conditions similar to those for wild type VAO [42]. Briefly, protein solutions containing 10 mg protein/ml in 50 mM potassium phosphate buffer pH 7.5 were equilibrated against a reservoir solution containing 100 mM sodium acetate/hydrochloride pH 4.6 and 3% (w/v) polyethylene glycol (PEG) 4000. Binding of the inhibitor trifluoromethylphenol was achieved by soaking the crystals for 5 hours in a solution containing 10 mM of the substrate analog. Diffraction data were measured from a single crystal at the X11 beam line of the European Molecular Biology Laboratory/Deutsches Elektronen Synchrotron (Hamburg, Germany) using a MarCCD detector at 100 K. Prior to data collection, the crystal was exposed for a few seconds to a cryoprotecting solution containing 20% (v/v) PEG 400, 10% (w/v) PEG 4000, and 50 mM sodium acetate/hydrochloride pH 4.6. The data were processed using MOSFLM [94] and programs of the CCP4 package [95]. The double mutant crystals are isomorphous to those of wild type enzyme and belong to space group I4 with unit cell parameters $a = b = 131.28$, $c = 134.36$ Å. Crystallographic refinement was performed with the REFMAC program [96]. A bulk solvent correction was applied using the programs of the CCP4 package whereas the positions of ordered solvent molecules were located using ARP [97]. The free *R*-factor was used to monitor the progress of the refinement. Electron density maps were visually inspected using the program O [98]. Table 2 gives a summary of the final refinement statistics. The atomic coordinates and structure factors have been deposited in the Macromolecular Structure Database of the European Bioinformatics Institute (EBI), Hinxton, UK (PDB ID code 1E0Y).

Results

Enzyme characterization. The purified VAO mutants T457E, D170A/T457E, and D170S/T457E were bright yellow and their near UV absorption properties were virtually identical to wild type enzyme [36]. Fluorescence analysis of unstained SDS gels showed that the mutant enzymes were as fluorescent as wild type VAO. This, and the fact that the mutants did not release any flavin upon trichloroacetic acid treatment [36], indicated that all flavin was covalently bound. Moreover, analytical gel filtration

revealed that the mutants were mainly octameric with only a small fraction in the dimeric form. This oligomerization behavior is similar to wild type VAO [53,82].

The redox potentials of the VAO mutants were measured by the xanthine/xanthine oxidase system [92] by using the reference dyes thionin (E_m = +60 mV) and methylene blue (E_m = +11 mV). As found for wild type enzyme [54], all three mutants were reduced in a single two-electron step with no formation of any semiquinone (Fig. 2). Upon plotting $\log(E_{ox}/E_{red})$ versus $\log(dye_{ox}/dye_{red})$ [93,104], midpoint redox potentials of +20 ± 1 mV, +22 ± 2 mV and +31 ± 2 mV were estimated for T457E, D170A/T457E, and D170S/T457E, respectively. These values are lower than for wild type VAO (E_m = +55 mV) [54], but considerably higher than the value reported for D170S (E_m = -91 mV) [56].

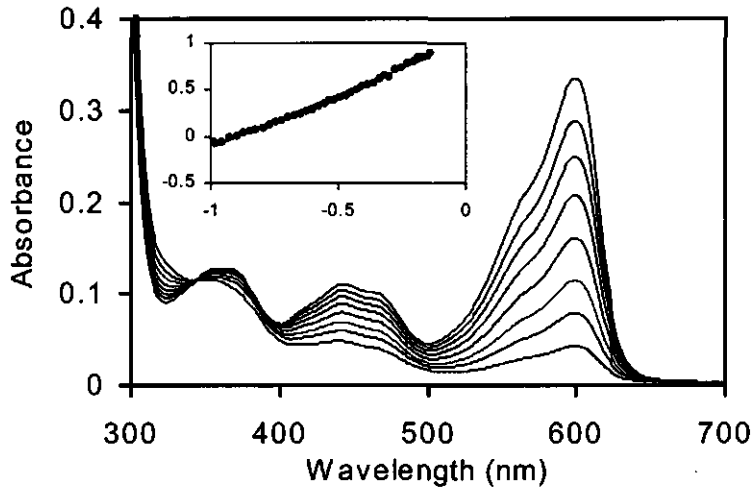


Fig. 2. Anaerobic reduction of 8 μM D170S/T457E by the xanthine/xanthine oxidase system in the presence of 8 μM thionin in 50 mM potassium phosphate buffer pH 7.5 at 25 °C. Spectra were taken at regular time intervals (10 min) and full reduction was achieved after 95 min. The inset shows the $\log(E_{ox}/E_{red})$ (measured at 440 nm after correction for thionin) versus $\log(dye_{ox}/dye_{red})$ (measured at 600 nm) plot.

Catalytic properties. VAO converts 4-(methoxymethyl)phenol with a maximum turnover rate of 3.1 s^{-1} and a K_m value of 55 μM [40]. Replacement of Thr457 by Glu decreased the catalytic efficiency (k_{cat}/K_m) with this substrate by about a factor of 7 (Table 1). The double mutants D170A/T457E and D170S/T457E were rather poor catalysts (Table 1). Nevertheless, these mutants were considerably more active with 4-(methoxymethyl)phenol than D170A and D170S [56].

Previous studies have shown that the turnover rate of VAO with 4-(methoxymethyl)phenol is mainly determined by the rate of flavin reduction.

Moreover, rapid kinetics have established that in the absence of oxygen, the reduced enzyme forms a rather stable complex with the *p*-quinone methide intermediate of the substrate [40]:

$$E_{ox} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}\text{–}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_{red}\text{–}Q$$

In analogy to wild type enzyme, anaerobic reduction of T457E by 4-(methoxymethyl)phenol followed monoexponential kinetics resulting in fully reduced enzyme when monitored at 440 nm. The maximum reduction rate of 1.2 s^{-1} approached the maximum turnover rate (Table 1), indicating that flavin reduction mainly determines the rate of overall catalysis. Diode-array spectral analysis of the anaerobic reduction of T457E with 4-(methoxymethyl)phenol revealed the formation of a strong absorption band with a maximum at 364 nm, representing the binary complex between the reduced enzyme and the *p*-quinone methide product intermediate. As in wild type VAO [56], the decomposition of this complex, forming 4-hydroxybenzaldehyde, was very slow (k_{obs} = 0.01 s^{-1}).

The rate of anaerobic reduction of D170A/T457E and D170S/T457E by 4-(methoxymethyl)phenol was 0.02 s^{-1} and 0.03 s^{-1}, respectively. Again, these values are in the same range as the corresponding turnover rates (Table 1). With both double mutants, the flavin became fully reduced in a monophasic process with the concomitant formation of 4-hydroxybenzaldehyde. In constrast to T457E and wild type VAO, no stabilization of the *p*-quinone methide-reduced enzyme complex was observed. A similar behavior was recently observed for D170A and D170S [56].

Stereochemistry of the mutant enzymes. Wild type VAO hydroxylates 4-ethylphenol stereospecifically to (*R*)-1-(4'-hydroxyphenyl)ethanol with an enantiomeric excess of 94% [39]. To probe the role of Asp170 and Thr457 in this reaction, we studied the conversion of 4-ethylphenol by the single mutants T457E, D170A, and D170S and by the double mutants D170A/T457E and D170S/T457E. The turnover rates of the mutant enzymes with 4-ethylphenol were about two orders of magnitude lower than with wild-type VAO (not shown). Nevertheless, all mutants catalyzed the formation of 1-(4'-hydroxyphenyl)ethanol in high yield. As found for native VAO [39], only minor amounts of the side product 1-(4'-hydroxyphenyl)ethene were formed. T457E showed a high preference for the formation of the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol (Fig. 3A).

Table 1. Kinetic parameters of wild type VAO and Asp170/Thr457 mutants with 4-(methoxymethyl)phenol in air-saturated 50 mM potassium phosphate buffer pH 7.5 at 25 °C.

Parameter	Wild type*	D170S†	D170A†
k_{cat} (s^{-1})	3.1	0.004	0.001
K_m (μM)	55	n.d.	n.d.
k_{red} (s^{-1})‡	3.3	0.005	0.002

Parameter	T457E	D170S/T457E	D170A/T457E
k_{cat} (s^{-1})	1.3	0.05	0.02
K_m (μM)	168	238	65
k_{red} (s^{-1})‡	1.2	0.03	0.02

*Data from ref. [40].

†Data from ref. [56].

‡k_{red} was measured at a substrate concentration of 500 μM.

n.d., not determined.

D170A and D170S were less (*R*)-selective with enantiomeric excess values of 26% and 50%, respectively. The double mutants exhibited an opposite stereospecificity with 4-ethylphenol. Especially D170S/T457E showed a strong preference for the (*S*)-enantiomer with an enantiomeric excess of 80% (Fig. 3B).

Structure of D170S/T457E. The crystal structure of D170S/T457E in complex with the substrate analog trifluoromethylphenol was determined at 2.75 Å resolution (Table 2). The inhibitor was clearly visible in the electron density map of one of the two crystallographically independent subunits (subunit B). Its orientation in the proximity of the flavin ring was nearly identical to the orientation in several previously determined enzyme-inihibitor complex structures [42,54,56]. Superposition of the structures of trifluoromethylphenol-complexed D170S/T457E and isoeugenol-complexed wild type VAO [42] showed that the two amino acid replacements did not cause any significant conformational change, which is reflected in a root mean square deviation for all Cα-atoms of only 0.23 Å. The active site cavity of D170S/T457E was highly similar to that of wild type enzyme [42] (Fig. 4) and D170S [56]. The Oγ-atom of Ser170 in D170S/T457E points towards flavin N5 at a distance of 3.7 Å from the isoalloxazine ring.

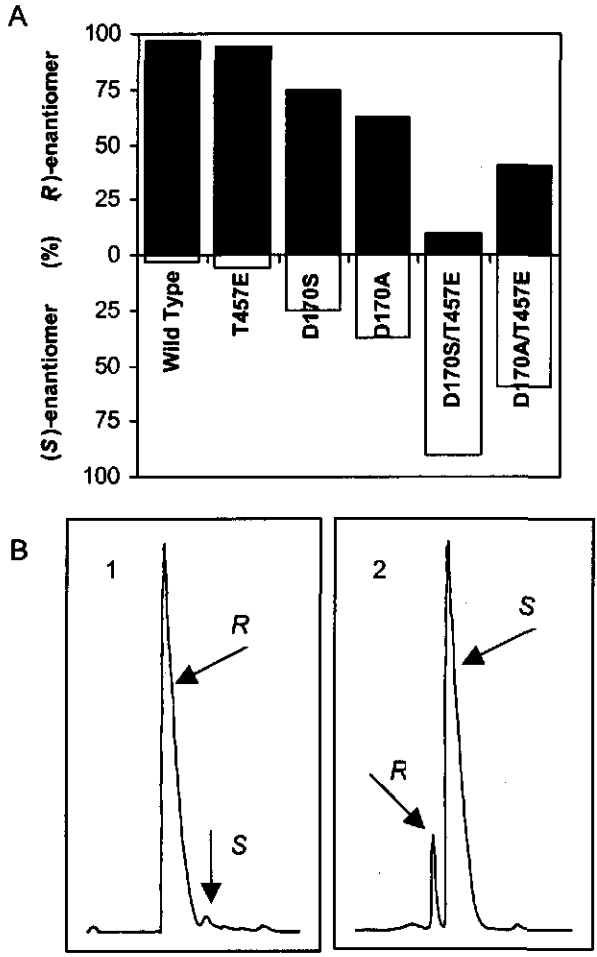


Fig. 3. (**A**) Stereospecificity of hydroxylation of 4-ethylphenol by wild type VAO and Asp170/Thr457 mutants in 50 mM potassium phosphate buffer pH 7.5 at 25 °C. (*R*)-1-(4'-hydroxyphenyl)ethanol (black) and (*S*)-1-(4'-hydroxyphenyl)ethanol (white). (**B**) Expanded chiral GC separation pattern of the hydroxylation of 4-ethylphenol by (1) wild type VAO and (2) D170S/T457E.

Compared to Ser170, the newly introduced Glu457 is positioned at the opposite side of the substrate-binding cavity. Its side chain points towards the Cα-atom of bound trifluoromethylphenol resulting in a distance between the carboxylic moiety of Glu457 and the Cα-atom of the inhibitor of 3.5 Å. In wild type VAO, Thr457 points also towards this Cα-atom, however, the distance between the inhibitor Cα and Oγ of Thr457 is 6.2 Å [42].

Table 2. Data collection and refinement statistics for T457E/D170S VAO in complex with trifluoromethylphenol.

Measurement	Value
Resolution, Å	20-2.75
Observed reflections	69,063
Unique reflections	28,740
Completeness of data, %*	97.3 (95.0)
Multiplicity*	2.4 (2.2)
Intensities, I/σ*	7.4 (2.1)
R_{sym}, %*,†	11.0 (37.1)
Cell dimensions, Å	$a = b = 131.28$, $c = 134.36$
R_{factor}, %	22.5
R_{free}, %	28.7
Number of ligand atoms	11 (trifluoromethylphenol)
r.m.s.d. for ideal value‡	
Bond lengths, Å	0.009
Bond angles, °	2.5
Trigonal atoms, Å	0.019
Planar groups, Å	0.001

*The values relating to the highest resolution shell (2.90-2.75 Å) are given in brackets.

†$R_{sym} = \Sigma|I_j - \langle I_j \rangle| / \Sigma \langle I_j \rangle$, where I_j is the intensity of an observation of reflection j and $\langle I_j \rangle$ is the average intensity for reflection j.

‡The root mean square deviations (r.m.s.d.) were calculated using the program REFMAC [96].

Discussion

The active site topology of VAO is highly similar to that of the flavocytochrome PCMH [42,50]. Nevertheless, subtle structural differences between both flavoenzymes exist which are likely of relevance with respect to the mechanism and specificity of catalysis. One striking difference is the arrangement of acidic residues (Fig. 1). The active site of VAO contains a single aspartate (Asp170) which side chain is close to flavin N5 and the reactive Cα-atom of the substrate [42]. In contrast, PCMH harbors two glutamates at opposite faces of the substrate [50]. From this we rationalized that the different arrangement of acidic residues might explain the opposite stereochemistry in the reaction with 4-alkylphenols [39,68,70].

The spectral and hydrodynamic properties of T457E, D170A/T457E, and D170S/T457E were similar to wild type VAO. Moreover, the crystal structure of D170S/T457E established that the introduction of an acidic side chain at position 457 does not result in any significant structural perturbation. In all three mutants, and different from D170A and D170S [56], the flavin prosthetic group was fully covalently bound.

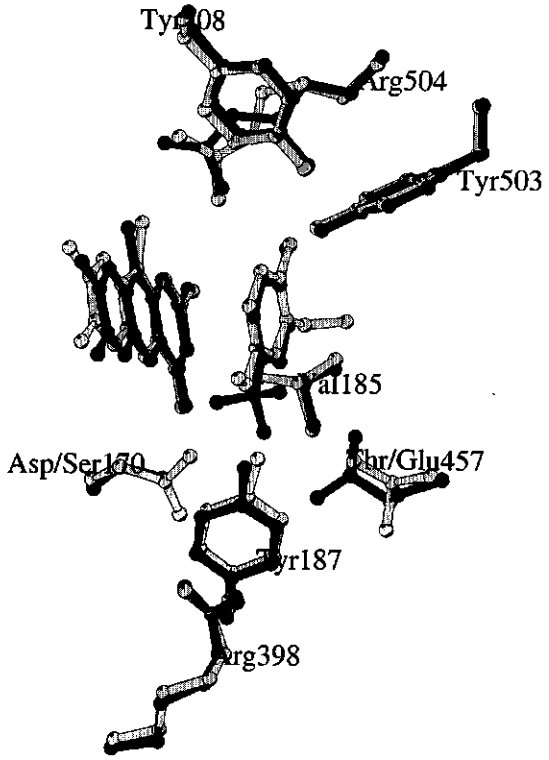


Fig. 4. Drawing comparing the active site cavities of trifluoromethylphenol-complexed D170S/T457E (black) and isoeugenol-complexed wild type VAO (grey). This figure was prepared with MOLSCRIPT [45].

Previous studies have indicated that the covalent interaction between His422 and the isoalloxazine ring markedly increases the redox potential of the flavin cofactor [54]. The redox potentials of D170A/T457E and D170S/T457E were only slightly lower than that of wild type VAO, supporting the proposal that an acidic residue in the active site cavity is required for maintaining the high redox potential [56]. Self-catalytic covalent flavinylation is thought to occur via initial flavin tautomerization [111,115], followed by donation of a proton to the iminoquinone methide form of the flavin [112].

In VAO, Asp170 is envisaged to act as the proton donor in this process [56]. As D170A/T457E and D170S/T457E contained fully covalently bound flavin, this indicates that Glu457 can replace Asp170 in the process of covalent flavinylation. The structure of D170S/T457E showed that the carboxylic moiety of Glu457 is about 7.0 Å from flavin N5. This suggests that Glu457 can not donate a proton directly to the flavin and that the proton might be transferred via a water molecule as observed in sarcosine oxidase [112], rather than by direct donation from Glu457.

The single mutant T457E was rather active with 4-(methoxymethylphenol) and fully capable of stabilizing the complex between the reduced enzyme and the *p*-quinone methide intermediate. From this and the relatively high redox potential of T457E, we conclude that the Thr457Glu replacement has no profound effects on the electrostatic interactions in the enzyme active site. The double mutants reacted more slowly with 4-(methoxymethylphenol) and did not stabilize the quinone methide. This strongly supports our earlier proposal that Asp170 is essential for efficient redox catalysis by forming a hydrogen bond with the protonated N5-atom of the reduced flavin [56].

Wild type VAO preferentially converts 4-ethylphenol to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol [39]. The high yield of this asymmetric compound results from the stereospecific attack of a water molecule to the *p*-quinone methide intermediate in the active site. The D170A/T457E and D170S/T457E double mutants exhibit an inverted stereospecificity with 4-ethylphenol. This (*S*)-selectivity is likely caused by the attack of a water molecule from the other side of the substrate. Another possibility for the inverted stereospecificity is that the substrate is bound in a different orientation. The crystal structure of D170S/T457E in complex with trifluoromethylphenol reveals that this possibility is rather unlikely. In the double mutant, the carboxylic moiety of Glu457 is positioned at the opposite face of the substrate analog and only 3.5 Å from the Cα-atom. This indicates that in the double mutants, Glu457 directs the stereospecific water attack to the planar quinone methide intermediate, presumably by acting as an active site base (Fig. 5). In this respect it is important to note that the single mutant T457E exhibits a strong preference for the formation of the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol. This suggests that in this particular mutant, Asp170 favorably competes with Glu457 for the site of water attack. The (*R*)-selectivity of T457E is in marked contrast with the stereochemical properties of PCMH. Like T457E, PCMH contains two acidic residues at opposite faces of the substrate binding site [50] but hydroxylates 4-ethylphenol preferably to (*S*)-1-(4'-hydroxyphenyl)ethanol [68,70]. Further mutagenesis studies are needed to establish whether the opposite stereospecificity of PCMH and T457E are related to the different type of acidic side chains (i.e Glu380 and Asp170, respectively). Nevertheless, the above data show that subtle variations in active site topology

determine the outcome of the enzyme stereospecificity and reinforce the idea that inversion of enantioselectivity can be generally carried out without major structural reconstruction [35]. This is also in line with studies from medium-chain acyl-CoA dehydrogenase, where it was demonstrated that a functional glutamate involved in substrate dehydrogenation can be relocated in the enzyme active site by a double amino acid substitution [116,117].

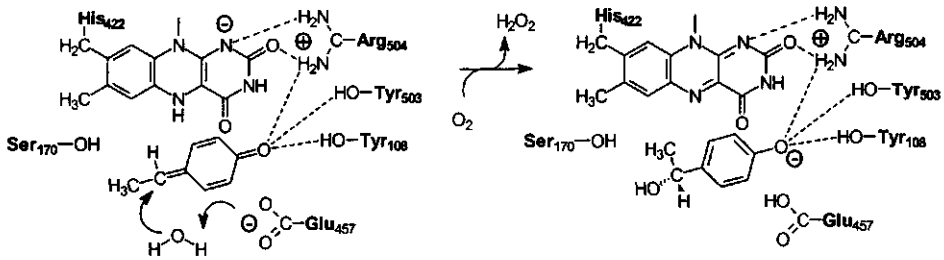


Fig. 5. Schematic drawing of the hydration of the planar *p*-quinone methide intermediate of 4-ethylphenol in D170S/T457E. We propose that the Glu457-activated water attacks the quinone methide, yielding the (*S*)-enantiomer of the alcohol.

The results presented here underline the important role of acidic residues for the function of VAO. In summary, Glu457 can replace Asp170 with respect to covalent flavin attachment, but not with respect to efficient redox catalysis. The stereospecificity of VAO depends on the position of the acidic residue in the enzyme active site. With Asp170 present, VAO is highly stereospecific for the production of (*R*)-alcohols. However, the enzyme becomes (*S*)-selective when the acidic residue is relocated to the opposite face of the substrate-binding site.

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Chapter 8

COVALENT FLAVINYLATION IS ESSENTIAL FOR EFFICIENT REDOX CATALYSIS IN VANILLYL-ALCOHOL OXIDASE

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By mutating the target residue of covalent flavinylation in vanillyl-alcohol oxidase (VAO) the functional role of the histidyl-FAD bond was studied. Three His422 mutants (H422A, H422T and H422C) were purified which all contained noncovalently bound FAD. As expected, the missing covalent bond resulted in drastic changes in the flavin absorbance spectra. It was found that the FAD cofactor is tightly bound to the mutant enzymes indicating that covalent flavinylation is not a prerequisite for efficient FAD binding. Gel filtration experiments showed that the hydrodynamic properties of the mutants were similar to wild type enzyme resulting in an equilibrium of octamers and dimers. Steady-state kinetics revealed that the mutants have retained enzyme activity although the turnover rates have decreased by one order of magnitude. Stopped-flow analysis showed that the H422A mutant is still able to form a stable binary complex of reduced enzyme and a quinone methide product intermediate, a crucial step during VAO mediated catalysis. The only significant change in the catalytic cycle of the H422A mutant is a marked decrease in reduction rate. Redox potentials of both wild type and H422A VAO have been determined. During reduction of H422A a large portion of the neutral flavin semiquinone is observed. Using suitable reference dyes, the redox potentials for the two one-electron couples have been determined: -17 mV and -113 mV. Reduction of wild type enzyme did not result in any formation of flavin semiquinone and revealed a remarkably high redox potential of +55 mV. The marked decrease in redox potential caused by the missing covalent histidyl-FAD bond is reflected in the reduced rate of substrate mediated flavin reduction limiting the turnover rate.

Elucidation of the crystal structure of the H422A mutant in the unliganded and isoeugenol bound states, established that deletion of the histidyl-FAD bond did not result in any significant structural changes. These results clearly indicate that covalent interaction of the isoalloxazine ring with the protein moiety can markedly increase the redox potential of the flavin cofactor thereby facilitating redox catalysis. Thus, formation of a histidyl-FAD bond in specific flavoenzymes might have evolved as a way to contribute to the enhancement of their oxidative power.

Introduction

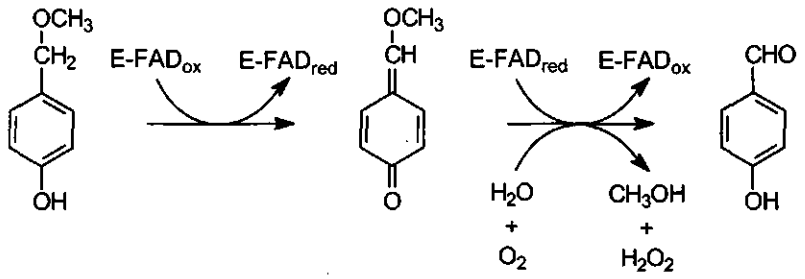
Until now, several hundred flavin-containing enzymes have been described. Most of these enzymes contain a dissociable FAD or FMN cofactor. However, it has been shown that in several cases the flavin is covalently linked to an amino acid of the polypeptide chain. In fact, in humans 10% of the cellular FAD is covalently bound to enzymes like e.g. succinate dehydrogenase and monoamine oxidase [118]. Within the group of covalent flavoproteins, 5 different types of covalent flavinylation have been identified. Except for a few examples of cysteinyl or tyrosyl linked flavins, tethering to a histidine is by far the most favored binding mode as it has been observed in about 20 isolated flavoenzymes (see for a recent review [109]).

Although the first covalent flavoprotein, succinate dehydrogenase, was already identified in 1955 [119] the rationale for covalent flavinylation is still unresolved. Only recently, a clear influence of the covalent bond on the reactivity of the cofactor has been observed in trimethylamine dehydrogenase. Unlike the wild type enzyme, mutants of trimethylamine dehydrogenase containing dissociable FMN [120,121] are inactivated by hydroxylation of the cofactor. Apparently, covalent tethering of the cofactor can prevent inactivation of the cofactor. Another striking role of the covalent flavin bond has been suggested for *p*-cresol methylhydroxylase. Inspection of the crystal structure of this flavocytochrome indicates that the tyrosyl-FAD bond facilitates electron transfer from the reduced FAD to the heme of the cytochrome *c* subunit [122]. It has also been shown that introduction of a covalent bond at the 8α-position of the isoalloxazine ring can result in an increase of the redox potential [123-125]. A reduced oxidative activity of mutants of succinate dehydrogenase and fumarate dehydrogenase was rationalized by a decrease of the flavin redox potential due to the missing covalent bond [126-128].

Although the above-mentioned results indicate that covalent flavinylation might be advantageous for catalysis it should be mentioned that several covalent flavoenzymes have homologous flavin-dissociable counterparts that display similar enzyme activities [85,129,130]. Therefore, the rationale for this atypical protein modification might not be uniform and also other factors may have attributed to the formation and conservation of covalent flavoproteins throughout evolution. For example, covalent binding of flavins can be favourable for flavoenzymes that are localized in a flavin deficient environment. In this respect it is noteworthy to mention that almost all eucaryotic covalent flavoproteins have been found to be compartmentalized [131]. Therefore, the motive for covalent flavinylation might also reside in the physiological function and environment of these flavoenzymes. Further, covalent flavinylation can also be of structural benefit. Except for cofactor saturation of the active site the

introduction of a covalent flavin-protein bond may well result in an improved protein stability.

In this study, we have adressed the function of covalent flavinylation in vanillyl-alcohol oxidase (VAO) which is a covalent flavoprotein containing 8α-(N^3-histidyl)-FAD [36]. VAO is a fungal enzyme of 560 residues that can efficiently oxidize a broad range of phenolic compounds [23,62]. Kinetic analysis of the conversion of the physiological substrate 4-(methoxymethyl)phenol has revealed that the enzyme operates in a ternary complex mechanism [40]:



During the first half-reaction the substrate reduces the flavin cofactor resulting in the formation of a stable binary complex of reduced enzyme and a *p*-quinone methide intermediate of the substrate. Subsequent reoxidation of the cofactor by molecular oxygen completes the catalytic cycle yielding the final products and oxidized enzyme.

Recently, we have solved the crystal structure of VAO [42] representing the first crystal structure of a flavoenzyme with a histidyl bound FAD. The VAO monomer comprises two domains with the larger domain forming a FAD binding module while the cap domain, containing the histidine linking the FAD, covers the active site (Fig. 1). By sequence homology analysis and inspection of the crystal structure of VAO, we have identified a novel flavoprotein family sharing a conserved FAD binding domain [44]. Members of this family that have been characterized catalyze a variety of oxidation/reduction reactions and include several well-studied covalent flavoproteins: e.g. 6-hydroxy-D-nicotine oxidase [132], *p*-cresol methylhydroxylase [122] and L-gulono-γ-lactone oxidase [133]. Interestingly, in about 35% of the sequences of these VAO homologs a conserved histidine residue is found which is predicted to be flavinylated [44]. Apparently, covalent flavinylation within this flavoprotein family is a relatively frequent event indicating that the conserved topology is particularly suited for covalent flavinylation or that this flavoprotein family originates from an ancestral covalent flavoprotein.

Fig. 1. Ribbon representation of a VAO monomer. The histidyl-bound FAD cofactor is shown in ball-and-stick model. This figure was prepared with MOLSCRIPT [45].

To study the effect of covalent flavinylation on the structural and kinetic properties of VAO, we have mutated the target residue for flavinylation, His422, thereby preventing formation of the histidyl-FAD bond. Here we present the crystal structure of the H422A mutant containing a dissociable FAD. Furthermore, the effects of the covalent bond deletion on the kinetic and redox properties of VAO are discussed.

Materials and Methods

Materials. *Escherichia coli* strain DH5αF' [90] and the plasmids pUCBM20 (Boehringer Mannheim) and pGEM-5Zf(+) (Promega) were used for cloning whereas *E. coli* strain TG2 [64] and the plasmid pEMBL19(-) (Boehringer) were used for expression of the *vao*A gene. T4 DNA ligase, restriction enzymes, isopropyl-β-D-thiogalactopyranoside, yeast extract and tryptone extract were from Life Technologies. *Pwo* DNA polymerase and dNTP's were purchased from Boehringer Mannheim and Super *Taq* DNA polymerase from HT Biotechnology. Ampicillin and

sodium dodecyl sulfate (SDS) were from BDH Chemicals. Forward M13 and reverse M13 sequencing primers were from Pharmacia Biotech. Oligonucleotides were synthesized by Life Technologies.

Glucose oxidase was from Boehringer, benzyl viologen, indigo disulfonate, indigo tetrasulfonate, methylene blue, xanthine and xanthine oxidase were from Sigma. Resorufin, isoeugenol (2-methoxy-4-propenylphenol) and 4-(methoxymethyl)phenol were products from Aldrich and thionin was from Kodak. All other chemicals were from Merck and of the purest grade available.

Mutagenesis. To simplify the site-directed mutagenesis procedure a *Sal*I restriction site was created by a silent mutation at position 882 in the original expression plasmid pIM3972 [43]. For this, the *Nco*I-*Nsi*I fragment of pIM3972 was ligated into pGEM-5Zf(+). Subsequently, the silent mutation was introduced by PCR-based mutagenesis using the oligonucleotide 5'-CAAGCCGTCGACATTATTCGTCC-3' (C denotes the mutated base). The mutated *Nco*I-*Nsi*I fragment was ligated into pIM3972. The resulting pBC11 plasmid was used for PCR-based mutagenesis. For the H422 replacements, the *Sal*I-*Kpn*I fragment of pBC11 was ligated into pUCBM20. This construct was used for PCR-based mutagenesis with the oligonucleotide 5'-CCCTAATGGTGCGXXTCTGTTCTTCTCTCC-3' (XX denotes the replacement for GC (His422Ala), AC (His422Thr) and TG (His422Cys), respectively). The mutated *Sal*I-*Kpn*I fragments were ligated into pBC11 yielding pBC20 (H422A), pBC21 (H422T) and pBC22 (H422C). Successful mutagenesis was confirmed by plasmid sequencing.

Analytical methods. Mutant proteins were expressed and purified as described for wild type VAO [43]. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [91]. Protein staining of the gels was achieved using Coomassie Brilliant Blue R-250 while histidyl-FAD fluorescence was detected as described earlier [38]. Analytical gel filtration was performed on a Superdex 200 HR 10/30 column using a Äkta system (Pharmacia) under conditions described elsewhere [53].

All analytical experiments were performed at 25°C in 50 mM potassium phosphate buffer, pH 7.5. Flavin absorption spectra were recorded on an automated Aminco DW-2000 spectrophotometer. Flavin fluorescence emission spectra (excitation at 450 nm) were obtained using a Aminco SPF-500 fluorimeter. Redox potentials were determined by the method described by Massey [92]. For this, a cuvette containing enzyme (2-12 μM), benzyl viologen (2.0 μM), redox dye (2-10 μM) and xanthine (400 μM) was made anaerobic by flushing with argon after which 0.2-0.5 nM xanthine oxidase was added (total volume was 750 μl). To maintain anaerobic

conditions during the reductive titration the headspace of the septum-closed cuvette was continuously flushed with argon. During the xanthine oxidase mediated reduction (lasting typically 1-2 h) spectra were recorded automatically every 30 seconds using a Hewlett-Packard HP 8453 diode-array spectrophotometer.

Steady-state kinetic experiments were performed as described earlier [23]. Stopped-flow kinetics were carried out with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech M300 monochromator diode-array detector (Salisbury, UK) as described previously [40]. Deconvolution of spectral data was performed using the Specfit Global Analysis program version 2.10 (Spectrum Software Ass., Chapel Hill, N.C., USA).

Crystallization, data collection, and processing. Bright yellow crystals of all H422 mutants were obtained using the hanging-drop vapor diffusion method at similar conditions (5% (w/v) PEG (polyethylene glycol) 4000, 100 mM sodium acetate/hydrochloride, pH 5.1) that were used for crystallizing wild type VAO [42]. Crystallization of H422A resulted in the most regularly shaped crystals. The crystal structure of H422A was determined in the absence and presence of the substrate analog isoeugenol using crystals of about 0.2 x 0.2 x 0.3 mm^3. Binding of isoeugenol was achieved by soaking the crystal for 3 hours in a solution containing 1 mM of the inhibitor.

Datasets were collected at the X-ray diffraction beam line of ELETTRA (Trieste, Italy) at 100K. Before freezing the crystals were briefly transferred to a solution containing 20% (w/v) PEG 400, 5% (w/v) PEG 4000, 20% (v/v) glycerol and 100 mM acetate/hydrochloride, pH 5.1. A complete dataset was collected at 100 K using a single crystal. The data were processed using MOSFLM (written by A.G.W. Leslie). Crystals of the H422A mutant were isomorphous to those of wild type enzyme and they belong to space group I4. Crystallographic refinement was performed with the REFMAC program [96]. A bulk solvent correction was applied using programs of the CCP4 program package [95]. Model building was carried out using the program O [98] whereas positions of ordered water were identified using the ARP program [97]. The free *R*-factor was calculated employing the same reflections used for the free R-factor calculations in the refinement of the wild type structure [97]. The free *R*-factor was used to monitor the progress of refinement. Table 2 gives a summary of the final refinement statistics. The atomic coordinates and structure factors for the H422A structures have been deposited in the Macromolecular Structure Database of the European Bioinformatics Institute (EBI), Hinxton, UK (PDB ID codes for native and isoeugenol-complexed H422A are 1QLT and 1QLU, respectively).

Results

Purification and general characterization. The three His422 mutant enzymes were purified using the same protocol as has been developed for wild type VAO resulting in bright yellow protein preparations. SDS-polyacrylamide gel electrophoresis combined with protein staining showed that all three mutant preparations contained protein with a molecular weight similar to that of wild type VAO. Further, fluorescence analysis of the same gel revealed that the His422 mutants do not contain covalent histidyl-FAD as, in contrast to the wild type enzyme, no flavin fluorescence could be observed. This finding was confirmed by precipitating the mutant enzymes with 5% (v/v) trichloroacetic acid resulting in a yellow supernatant containing FAD and a colorless pellet of aggregated protein. Treating wild type VAO in the same way resulted in a colorless supernatant while a yellow pellet is formed [36].

Analytical gel filtration experiments showed that the His422 mutants are mainly in the octameric form with a small portion being present as a dimer. By simultaneously monitoring the absorbance at 280 nm and 450 nm the presence of FAD in both oligomeric forms could be demonstrated. In both the octameric and dimeric state the ratio of protein (280 nm) absorbance and flavin (450 nm) absorbance was identical. These results indicate that the purified mutants display a similar oligomerization behavior with respect to wild type VAO [53].

Spectral properties. The ratio of absorbance between 280 nm and 439 nm for all mutants was 11.5, which is similar to the value reported for wild type VAO [36]. Further, the flavin absorption spectra of all three mutants were virtually identical with maxima at 390 nm and 439 nm (Fig. 2). When comparing the flavin spectra of the mutants and wild type enzyme it can be clearly seen that the spectral characteristics at lower wavelengths have drastically changed while the spectral characteristics at around 439 nm are hardly affected by the mutation. These results are in line with the fact that the mutants do not form a covalent FAD bond as 8α-substituted histidyl-FAD can be identified by a typical hypsochromic shift of the near-UV absorbance maximum with respect to unmodified FAD [134]. Except for histidyl-FAD also cysteinyl-bound FAD has been observed in several covalent flavoproteins [109]. The observation that the H422C mutant does not contain any detectable covalent FAD indicates that formation of the covalent histidyl-FAD linkage in VAO is a very specific mechanism. Further, although the mutations prevent covalent linkage of FAD, all mutants contain tightly bound FAD as they were purified in the holo form. Attempts to generate apo enzyme using established methods [135] failed. The noncovalent His422 mutants displayed similar flavin fluorescence properties when

compared with wild type VAO. When exciting at 450 nm emission fluorescence spectra were observed with maxima at 535 nm. Similar to wild type VAO the flavin fluorescence quantum yields were very low indicating that the covalent histidyl-FAD bond is not a major factor in flavin fluorescence quenching in VAO. From these experiments it can also be concluded that the His422 mutants have a high affinity for FAD as no significant amounts of free FAD could be detected. These results indicate that the covalent histidyl-FAD bond is not essential for FAD binding.

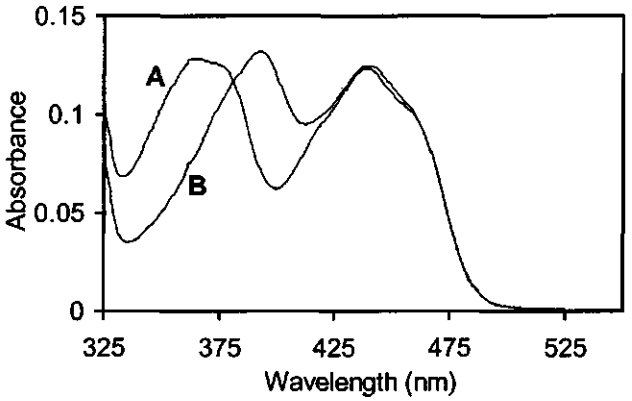


Fig. 2. Flavin absorption spectra of 10 μM (A) wild type VAO and (B) the H422A mutant in 50 mM potassium phosphate, pH 7.5.

Kinetic characterization. To examine the effect of the mutations on enzyme kinetics the steady-state kinetic parameters were determined using 4-(methoxymethyl)phenol as substrate (Table 1). It was revealed that all purified mutants display significant enzyme activity. The k_{cat} values of the three mutants have decreased by about one order of magnitude while the K_m values are significantly lower with respect to wild type enzyme. In contrast to observations with some other mutated covalent flavoenzymes [109,136,137], the activity remained constant during prolonged incubations (10 min) with substrate. This indicates that the FAD cofactor remains bound to the enzyme and is not inactivated during catalysis. As all three mutants showed similar spectral and steady-state kinetic properties we decided to perform a more detailed study on the H422A mutant.

The apparent increased affinity for phenolic ligands was studied in some more detail by determining the dissociation constant for the competitive inhibitor isoeugenol. Titration experiments with the H422A mutant revealed that the dissociation constant for isoeugenol was also significantly lower when compared with the corresponding K_d value for wild type enzyme [23]: 4.6 μM vs. 22 μM.

Table 1. Kinetic constants for the reaction of the His422 mutants and wild type VAO with 4-(methoxymethyl)phenol (pH 7.5, 25°C).

Parameter	Wild type*	H422A	H422C	H422T
K_m (μM)	55	34	37	41
k_{cat} (s^{-1})	3.1	0.27	0.32	0.28
K_d (μM)	48	18	n.d.	n.d.
k_{red} (s^{-1})	3.3	0.30	n.d.	n.d.
k_{ox} ($M^{-1}*s^{-1}$)	$1.5*10^5$	$2.5*10^5$	n.d.	n.d.

*data from ref. [40].

n.d, not determined.

To identify the rate-limiting step in catalysis by the H422A mutant, stopped-flow experiments were performed. For this, the kinetics of the individual half-reactions were studied. The reductive half-reaction of H422A appeared to be a monophasic process as has been found for wild type VAO (Fig. 3). Anaerobic reduction by 4-(methoxymethyl)-phenol resulted in fully reduced enzyme as monitored by the absorbance decrease at 439 nm. This indicates that, as in wild type enzyme, the substrate induced reduction of the flavin cofactor is an irreversible process [41]. The maximal reduction rate of 0.30 s^{-1} approaches the maximal turnover rate indicating that the rate of turnover is mainly determined by the rate of flavin reduction. This finding was confirmed by an enzyme-monitored turnover experiment, which showed that, during turnover, more than 90% of H422A is in the oxidized state. In line with the decreased K_m value, it was found that the dissociation constant for 4-(methoxymethyl)phenol is also relatively low (Table 1). Furthermore, by using diode-array detection it could be shown that upon anaerobic reduction with 4-(methoxymethyl)phenol H422A is able to form and stabilize a binary complex between reduced enzyme and the *p*-quinone methide product intermediate. Decay of this complex, resulting in formation of 4-hydroxybenzaldehyde, was only observed when the anaerobic reduction reaction is monitored for a relatively long period of time (>10 s) (see inset Fig. 3). Apparently, the mutant enzyme has still retained the remarkable ability to shield the formed quinone methide intermediate from solvent [40]. Upon reoxidation of the reduced complex by molecular oxygen the quinone methide intermediate was efficiently hydrated as has been found for the wild type enzyme. Analysis of the oxidative half-reaction showed that the rate of reoxidation of the binary complex is also similar to that of the wild type enzyme (Table 1). From these kinetic measurements it can be concluded that except for a decrease in the rate of

flavin reduction, the catalytic mechanism by which substrate is converted has essentially been conserved in the H422A mutant.

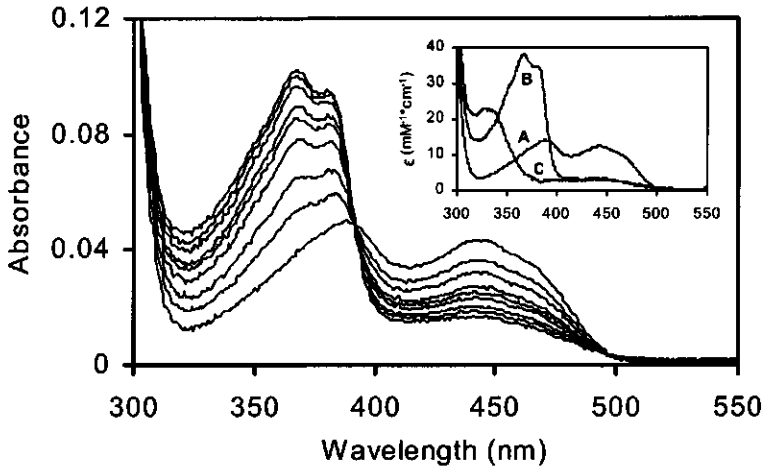


Fig. 3. Spectral changes observed upon anaerobic reaction of H422A (4.0 μM) with 4-(methoxymethyl)phenol (500 μM, 25°C, pH 7.5). Original spectra are shown from 5.6 ms to 10 s with intervals of 1 s. The inset shows normalized spectra obtained after deconvolution of the original 96 spectral scans ranging from 5.6 ms to 20 s where A represents the initial spectrum, spectrum B the intermediate species and spectrum C the finally obtained spectrum at indefinite time. The data were fit with a consecutive irreversible model (A→B→C).

Redox potential determination. For both wild type VAO and the H422A mutant the flavin redox potential was determined using the method described by Massey [92] (see materials and methods). When the xanthine oxidase mediated reduction of the mutant was monitored in the absence of a reference dye, the initial formation of a one electron reduced flavin species was observed (Fig. 4). Based on the typical absorbance properties in the 500 to 600 nm region the radical intermediate could be identified as the blue neutral flavin semiquinone [138,139]. Previously, it has been observed that in wild type VAO the red anionic flavin radical is transiently formed upon light induced reduction [36]. Apparently, the His422Ala mutation results in an increase of the pK_a of the one electron reduced form of the flavin cofactor in VAO. Such a pK_a shift of the flavin semiquinone has also been observed in flash photolysis studies on 8α-histidyl flavins [140]. From estimation of the maximal amount of flavin radical formed during the reduction process (70-75%) (see inset Fig. 4), it can be deduced that the redox potentials for the oxidized/semiquinone couple (E_1) and the

semiquinone/hydroquinone couple (E_2) are separated by about 85 ±7 mV [93]. By using methylene blue (+11 mV) and indigo disulfonate (-118 mV) as reference dyes, the two redox potentials could be accurately determined. The redox potential difference with respect to the dye could be calculated by plotting the log(ox/red) ratio of the enzyme versus the corresponding log (ox/red) ratio of the dye [104]. The slopes for the two respective plots were 0.52 and 0.55 approaching the theoretical value of 0.5 indicating that equilibrium between the redox components during the reduction experiment was established. The two redox potentials E_1 and E_2 were -17 mV and -113 mV, respectively. These values are in line with the above mentioned estimated separation of the two redox potentials. Furthermore, the data obtained by reducing the mutant in the presence of resorufin (-51 mV) also corroborated with these results. Reduction of this dye only occurred after formation of flavin semiquinone while full reduction of the flavin only started when most of the dye was already reduced.

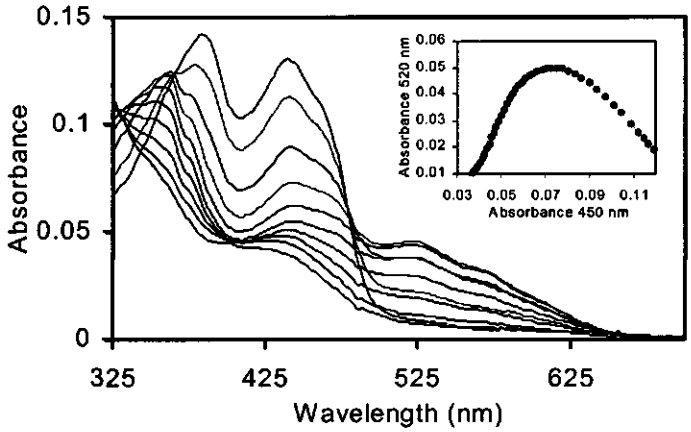


Fig. 4. Spectral changes during the anaerobic xanthine oxidase mediated reduction of 12.0 μM H422A. Spectra were obtained at regular time intervals (12 min) while full reduction was achieved after 120 min. Inset: by plotting the absorbance at 520 nm versus the absorbance at 450 nm the amount of transiently formed flavin semiquinone can be estimated (data are shown with 3 min intervals).

The midpoint redox potential (E_m) of H422A is -65 mV ($E_m = (E_1+E_2)/2$, see [93]). The E_m of wild type VAO could be similarly determined by using thionin (+60 mV) as reference dye. In contrast with H422A, reduction of wild type VAO did not result in any observable formation of a radical species. Plotting the data according to Minnaert [104] resulted in a slope of 0.97, which is close to the theoretical value of 1.0 for a two-electron reduction process, and a midpoint redox potential of +55 mV. The E_m for

wild type enzyme (+55 mV) is markedly higher when compared with the E_m for the H422A mutant (-65 mV). This indicates that the histidyl-FAD covalent bond together with specific noncovalent cofactor-protein interactions results in an exceptional high E_m, which is about 250 mV higher than the E_m of free FAD [92]. To our best knowledge only one other example of a flavoenzyme exhibiting an equally high E_m (+55 mV) has been reported, thiamine oxidase [141]. Interestingly, this bacterial enzyme also contains a histidyl-bound FAD.

Structure of H422A. The crystal structure of unliganded H422A was determined at 2.2 Å (Table 2). The resolution obtained for the mutant structure is an improvement when compared to the originally determined structure of wild type VAO (2.5 Å) [42]. The X-ray analysis clearly shows that there is no covalent bond between the FAD cofactor and any residue of the polypeptide chain. A comparison between the mutant and the wild type models showed that the His422Ala replacement did not cause any large conformational change. Superposition of the two structures produced a r.m.s. deviation for all Cα atoms of only 0.27 Å. Particularly, as in the wild type structure, the flavin ring is fully planar and, except for the covalent bond, all other interactions with the isoalloxazine ring are conserved. The position of the Cα atom of Ala422 did not change significantly, resulting in a distance between the C8 methyl group of the flavin and the Cβ atom of Ala422 of 5.3 Å. Further, all active site residues appear to have essentially retained their positions (Fig. 5) and, as in the wild type enzyme structure, an acetate molecule is found in the active site. The largest variations concern residues 410-417, which interact with the edge of the dimethylbenzene moiety of the flavin. The largest movement is that of Cδ1 of Ile 414 (1.3 Å), though all other atomic shifts in these residues do not exceed 1.0 Å. Movements of these atoms accommodate for the missing side chain of His422. Furthermore, the imidazole ring of His61 rotates by about 50° pointing to the C8α-methyl group of the flavin ring.

The crystal structure of the H422A mutant in complex with isoeugenol was determined at 2.4 Å. The crystal soaked with isoeugenol shows clear density for the substrate analog isoeugenol in proximity of the flavin ring. The two structures of the H422A mutant are virtually identical as indicated by a r.m.s. deviation of 0.13 Å for all Cα atoms. The deletion of the covalent histidyl-FAD bond does not result in any evident structural perturbations upon ligand binding which could explain the reduced enzyme activity of the H422A mutant. Further, the H422A-isoeugenol complex structure revealed an apparent identical binding mode for the phenolic inhibitor when compared to the wild type inhibitor complex structure [42].

Table 2. Summary of crystallographic analysis of H422A.

Measurement	H422A	H422A with isoeugenol
Resolution, Å	20-2.2	20-2.4
Observed reflections	285,553	216,054
Unique reflections	55,414	43,343
Completeness of data, %*	93.1 (78.8)	94.8 (88.4)
Multiplicity*	2.1 (1.8)	2.1 (1.9)
Intensities, I/σ*	6.0 (2.8)	4.9 (2.5)
R_{sym}, %*	8.9 (22.6)	11.9 (32.1)
Cell dimensions, Å	$a = b = 129.66$, $c = 132.30$	$a = b = 129.84$, $c = 133.90$
R_{factor}, %	21.0	21.9
R_{free}, % (2000 reflections)	26.4	27.3
Number of protein atoms	8692	8692
Number of water atoms	373	257
Number of FAD atoms	106	106
Number of ligand atoms	8 (acetate)	22 (isoeugenol)
r.m.s.d. from ideality†		
Bond lengths, Å	0.013	0.012
Bond angles, °	2.3	2.3
Trigonal groups, Å	0.023	0.022
Planar groups, Å	0.011	0.011
Ramachandran plot, %‡	88.3 / 11.6 / 0.1 / 0	89.0 / 10.8 / 0.2 / 0

*The values relating to the highest resolution shell are given in brackets.

†The r.m.s. deviations (r.m.s.d.) were calculated using the program REFMAC [96].

‡Percentage of residues in most favored, allowed, generously allowed and disallowed regions of the Ramachandran plot as checked with the program PROCHECK [99].

Discussion

The results presented in this paper demonstrate that covalent flavinylation is not a prerequisite for efficient FAD binding in VAO. All three His422 mutants were purified in the holo form while no FAD dissociation could be detected in any of the performed experiments and no influence on the hydrodynamic properties was observed.

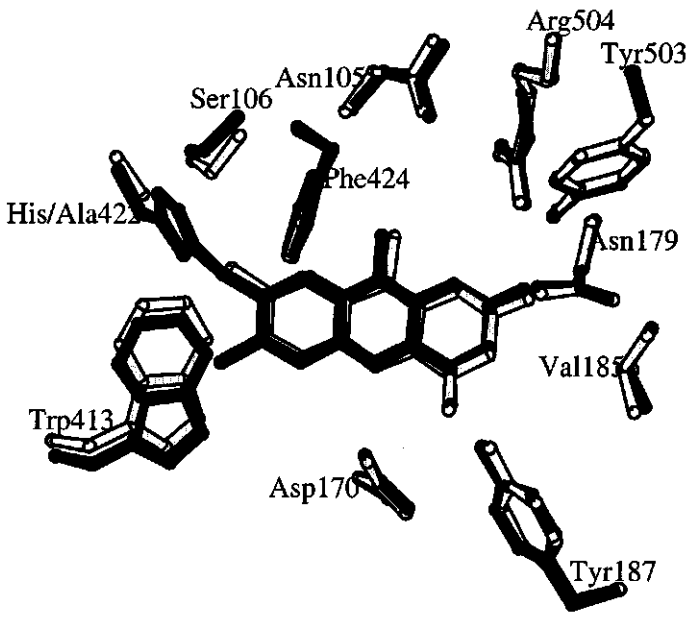


Fig. 5. Superposition of active site residues in the unliganded H422A (white) and wild type VAO structures (black). The figure was prepared with MOLSCRIPT [45].

These results are perfectly in line with the determined crystal structure of the H422A mutant. Analysis of the 3D structure of this noncovalent FAD containing VAO variant revealed that except for the replacement of the histidine no significant structural perturbations can be observed with respect to the wild type VAO structure [42]. From a thorough comparison of the mutant structure with that of wild type VAO it can be concluded that the two structures are virtually identical as shown by a r.m.s. deviation for all Cα atoms of 0.27 Å. Inspection of the active site also showed that the active site architecture has been fully conserved. This was confirmed by the crystal structure of the H422A variant in complex with the substrate analog isoeugenol. The binding of this phenolic ligand was identical to the binding mode in wild type enzyme [42]. Except for the deletion of the covalent histidyl-FAD bond, no significant change in cofactor-protein interactions could be observed. This clearly indicates that the covalent FAD linkage does not necessarily induce a specific structural feature or is a prerequisite for proper protein folding. Evidently, the noncovalent interactions of the FAD binding domain in VAO are competent in tight binding of the cofactor.

Steady-state kinetic analysis showed that with respect to wild type enzyme the turnover rate for all His422 mutants has markedly decreased (Table 1). The stopped-

flow technique was used to identify the reaction step that limits the turnover rate. Using the H422A mutant, it was found that the rate of flavin reduction was drastically reduced while the rate and nature of the other kinetic processes are almost unaffected by the mutation. Also enzyme-monitored turnover experiments with the H422A mutant indicated that the kinetic mechanism is similar to that of wild type VAO [40]. Spectral analysis of the reductive half-reaction also showed that the mutant is still able to stabilize the *p*-quinone methide product intermediate. This is indicative for a conserved active site architecture, which was confirmed by the crystal structure. As the active site architecture is fully conserved the decrease in the flavin reduction rate directly reflects an apparent decrease in flavin reactivity. Nevertheless, these results also clearly show that the covalent histidyl-FAD bond in VAO is not a prerequisite for catalysis as the His422 mutants still display appreciable activity.

Determination of the redox potential of several flavin derivatives has revealed that covalent aminoacyl modifications at the 8α-position of the isoalloxazine ring can result in an increase of the redox potential by 50-60 mV [123,125]. Furthermore, studies on 8α-*N*-imidazolylflavins have shown that ionization of the imidazole substituent can have significant effects on the flavin redox potential [142]. In the H422A mutant a markedly lower midpoint redox potential was found when compared with wild type VAO (-65 mV vs. +55 mV). As mentioned above, analysis of the H422A structure shows that the decrease in midpoint redox potential is not caused by any evident structural changes but is merely caused by the covalent bond deletion. Apparently, the drastic reduced reactivity of the flavin is directly reflected in a decreased substrate mediated flavin reduction rate. Further, opposite to wild type VAO, the H422A mutant displays a significant stabilization of the one electron reduced state. This indicates that the covalent histidyl-FAD linkage and the resulting interaction with the protein environment modulates the redox properties of the flavin cofactor in such a way that it can efficiently be reduced by a direct two electron transfer mechanism at a relatively high potential. As suggested by Parsonage et al. [143] this may well reflect the ability of the enzyme to be tuned to accept simultaneously two electrons from the reducing substrate during catalysis which is in agreement with the proposed hydride transfer mechanism [40].

Mutagenesis of the target residue for flavinylation and subsequent kinetic characterization of the resulting mutant protein has previously been performed with only a few other covalent flavoenzymes: 6-hydroxy-D-nicotine oxidase [136], monoamine oxidase A [137], fumarate reductase [126], succinate dehydrogenase [128], and trimethylamine dehydrogenase [120]. With most of these noncovalent mutant proteins enzyme activity was retained to some extent. Only for succinate dehydrogenase it was found that the noncovalent variants had lost the capacity of oxidizing succinate. In the

case of fumarate reductase and succinate dehydrogenase the decrease in oxidation rate of these mutant enzymes was tentatively assigned to the redox properties of the flavin cofactor [128]. However, no comparative redox potentials were determined to support this hypothesis. Nevertheless, it is striking to notice that, when comparing flavoenzymes of which the redox potential has been determined, flavoproteins displaying a relatively high redox potential often contain covalent FAD or FMN. For example, the covalent flavoenzymes succinate dehydrogenase [144] (-3 mV), trimethylamine dehydrogenase [145] (+40 mV), thiamine oxidase [141] (+55 mV) and vanillyl-alcohol oxidase (+55 mV) all have redox potentials near the upper limit of redox potentials that have been determined for flavoprotein oxidoreductases [146]. Apparently, an increase of the flavin redox potential by covalent flavinylation is a widely used mechanism to enhance the oxidative power of specific flavoproteins.

Recent studies on trimethylamine dehydrogenase have indicated that covalent bond formation may play a major role in preventing inactivation of the enzyme by flavin modification [109]. Trimethylamine dehydrogenase mutants, which are unable to form the 6-*S*-cysteinyl-FMN bond, readily are inactivated by hydroxylation of the C6 of the flavin ring. It was proposed that this protective effect might also be the rationale for the occurence of 8α-methyl flavinylated enzymes. However, from the results described in this study it can be concluded that this proposed self-protecting function of covalent flavinylation is not generally valid. Spectral analysis of the VAO mutants following extensive incubations with or without substrate did not result in any detection of modified FAD. Further, in all experiments performed with the mutant enzymes no appreciable inactivation could be observed. A plausible reason for the chemical stability of the isoalloxazine ring in VAO comes from inspection of the crystal structure. In both the mutant structure and that of wild type VAO the benzyl moiety of the cofactor is found to be protected from solvent, preventing flavin hydroxylation reactions. Apparently, the susceptibility of flavin modification within the active site of flavoenzymes is highly dependent on the active site architecture. This might also hint to the fact that the evolutionary pressure towards covalent flavinylation is not necessarily driven by a single specific motive but can be multifold. However, the recently discovered flavoprotein family for which VAO is the prototype shows a relative high frequency of covalent flavinylation via a histidyl bond [44]. Strikingly, all characterized VAO homologs containing a histidyl bound FAD represent oxidases while all noncovalent VAO homologs represent dehydrogenases. This suggests that a relatively high redox potential caused by covalent flavinylation directs the enzyme to accept oxygen as electron acceptor as other physiological electron acceptors, e.g. NAD^+, have relatively low redox potentials.

In conclusion, this report results clearly shows that the covalent interaction of the isoalloxazine ring with the protein moiety can markedly increase the redox potential of the flavin cofactor. This increase in redox potential facilitates redox catalysis by VAO. From this, it is tempting to conclude that formation of a histidyl-FAD bond in specific flavoenzymes has evolved as a way to contribute to the enhancement of their oxidative power. Moreover, the markedly high redox potential of VAO is a good illustration of the wide range of redox potentials achieved in flavin dependent oxidases, ranging from -367 mV in nitroalkane oxidase [147] to +55 mV in VAO.

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Chapter 9

DETECTION OF INTACT MEGADALTON PROTEIN ASSEMBLIES OF VANILLYL-ALCOHOL OXIDASE BY MASS SPECTROMETRY

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Well-resolved ion signals of intact large protein assemblies, with molecular masses extending above one million Dalton, have been detected and mass analyzed using electrospray ionization mass spectrometry, with an uncertainty in mass of less than 0.2%. The mass spectral data seem to reflect known solution-phase behavior of the studied protein assembly and have therefore been directly used to probe the protein assembly topology and stability as a function of ionic strength and pH.

Introduction

With the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) [148] and electrospray ionization (ESI) [149] the detectable mass range of biomacromolecular systems by mass spectrometry has been immensely extended [150,151]. Additionally, the relatively gentle phase transfer from solution to the gas-phase, realized by in particular electrospray ionization, has enabled the intact detection by mass spectrometry of larger multi-protein assemblies [152-157]. Several recent examples have revealed that biologically relevant parameters concerning these multi-protein complexes may nowadays be investigated by mass spectrometry, examples including protein complex topology, protein-protein interactions and protein complex stability [158-163]. Although, it has been demonstrated previously that large biomacromolecules (e.g. DNA, viruses, protein-complexes) may be ionized by MALDI or ESI, extraction of useful information from such data is often hampered by the limited mass resolution achieved in the spectra partly caused by the heterogeneity of the sample. In this study unprecedented mass spectrometric results are presented on very large protein complexes of the flavoprotein vanillyl-alcohol oxidase (VAO) from *Penicillium simplicissimum*.

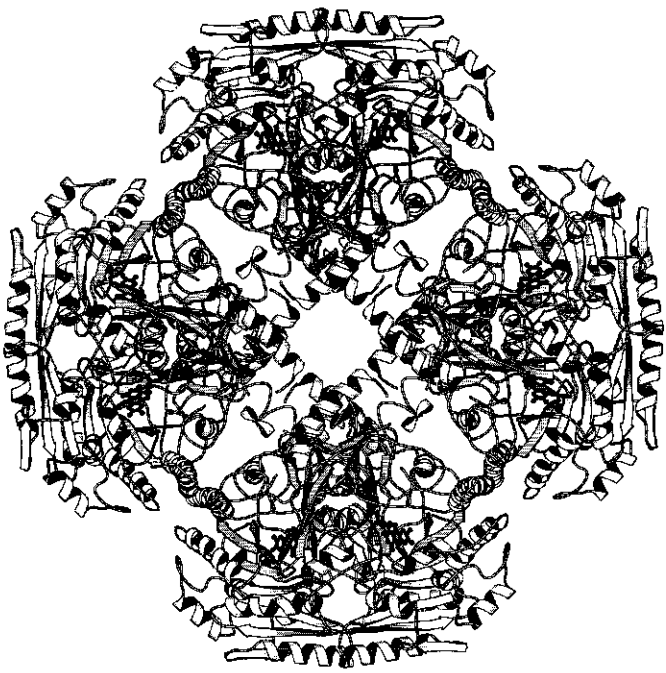

Fig. 1. Quaternary structure of VAO. Ribbon diagram of the eight subunits forming the VAO octamer, viewed along the fourfold axis. The covalently bound FAD is shown in black in ball-and-stick representation.

VAO (EC 1.1.3.38) catalyzes the oxidation of a wide variety of phenolic compounds, using 8α-(N^3-histidyl)-FAD as a covalently bound prosthetic group [36,40,62]. A striking and beneficial feature of VAO is that the enzyme is able to produce the flavor compound vanillin from four different substrates [40]. VAO is the prototype of a novel family of widely distributed oxidoreductases sharing a conserved FAD-binding domain [42,44]. The VAO monomer is built up of 560 amino acids, exhibiting a calculated average mass of 63,691 Da, including the covalently linked FAD [43]. The recently determined crystal structure of VAO [42] has confirmed earlier biochemical data [36] that at neutral pH the enzyme is predominantly an octamer. The octamer can be described as a tetramer of dimers in which each dimer is stabilized by extensive inter-subunit contacts (see also Fig. 1). The dimer-dimer interaction is much weaker which is in agreement with the observation that in solution, the octamer is in equilibrium with (fully active) dimers [53]. Moreover, VAO is rapidly inactivated by mercurial agents at low ionic strength and it has been proposed that subunit dissociation accounts for the observed loss of activity [53].

Materials and Methods

Recombinant VAO was purified from *Escherichia coli* TG2 cells, essentially as described previously [43]. VAO enzyme concentrations were determined spectrophotometrically using a molar absorption coefficient ε_{439} = 12.5 mM^{-1} cm^{-1} for protein-bound FAD [36]. Aqueous 40 mM ammonium acetate solutions (pH = 6.8) of VAO at a physiologically relevant monomer concentration of 28 μM were used for the electrospray ionization studies (*i.e.* the VAO concentration in *P. simplicissimum* is in the μM range). For the protein assembly stability studies the pH of the ammonium acetate solution was adjusted by adding small amounts of formic acid or ammonium hydroxide. Samples were introduced into the quadrupole-time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK) using the "Z-spray" nanoflow electrospray source using in-house pulled and gold-coated borosilicate needles. Pressure conditions in the Q-TOF mass spectrometer and electrospray voltages were optimised for optimal transmission of the larger multimer protein assemblies [164]. It was observed that optimal transmission of the larger multimer protein assemblies could be obtained when the pressure in the quadrupole analyzer and hexapole collision cell regions were substantially increased from their standard operation values. The pressures were increased by leaking dry nitrogen into the source region. Typical conditions were, needle voltage 1,700 V, cone voltage 80 V, quadrupole pressure $1*10^{-4}$ mbar (standard $5*10^{-6}$ mbar), TOF analyzer pressure $1*10^{-6}$ mbar (standard $3*10^{-7}$ mbar). The standard mass range scanned was 700-20,000 Thompson (Th). Spectra were recorded in the positive ion mode. In experiments on the denatured VAO protein, dissolved in a buffer of pH = 2.2, also monomer ions could be detected. From

these spectra the determined average mass of the monomer was 63,567 ± 10 Da, which revealed that the N-terminal methionine of VAO had been cleaved off during the purification process. The measured masses of the VAO protein assemblies should therefore be and are indeed multimers of 63,567 ± 10 Da.

Results and Discussion

Fig. 2 displays an overview of the nanoflow ESI spectrum of VAO when sprayed from an aqueous 40 mM ammonium acetate solution (pH = 6.8) at a VAO monomer concentration of 28 μM. In electrospray ionization of proteins multiple charged ions (multiple protonated) are usually formed. Each individual species is therefore detected at various different mass to charge (m/z) ratios. When the different charge-state signals are well resolved, as is the case for the data shown in Fig. 2, the number of charges and the mass of the species may be easily derived. The interpretation of the spectrum shown in Fig. 2 is thus quite straightforward as all the different species and charge-states are well resolved. Several different protein assemblies were identified in the ESI spectrum of VAO, each displaying relatively very narrow charge-state distributions. The mass-to-charge (m/z) values of the most abundant ions are summarized in Table 1. The most abundant species in the spectrum of Fig. 2 was found to be the VAO octamer, displaying charge-states between approximately 52 and 45, centered around m/z = 10,000 Th. The molecular mass of this species as determined from the spectrum in Fig. 2 was 508,543 ± 150 Da. The second abundant species appears to be the VAO 16-mer for which a molecular weight of 1,017,180 ± 600 Da was calculated. These 16-mers also displayed a narrow charge-state distribution ranging from 75 to 68, and were detected in the mass spectrum around 14,000 Th. Additionally, a set of broader peaks centered around m/z = 6,000 Th and a set of more narrow peaks centered around 4,000 Th were detected, which both originate from dimeric VAO species, for which a mass of 127,134 ± 30 Da was calculated. Smaller signals centered around m/z = 17,000 Th originate from 24-mer protein assemblies of the VAO enzyme for which a mass of 1,525,600 ± 1,000 Da was obtained. Also these 24-mer ions displayed a narrow charge-state distribution ranging from approximately 87 to 93. In order to illustrate the attainable mass resolution for these very large protein complexes, a zoomed in part of the mass spectrum displaying the region of the 16-mer and 24-mer is presented in Fig. 3. The width (at half height) of each of the ion signals of the 24-mer was approximately 30 Th. This renders to a width in the calculated mass of the 24-mer protein assembly of approximately 2,500 Da on an overall mass exceeding 1,500,000 Da, i.e. approximately 0.13%. Merely natural abundance isotope broadening of molecules of a mass of one million leads to a natural line width (FWHM) of approximately 100 Da. The broader linewidths

observed here may be due to small molecule adducts and/or incomplete desolvation during the electrospray process, a phenomenon discussed previously [156].

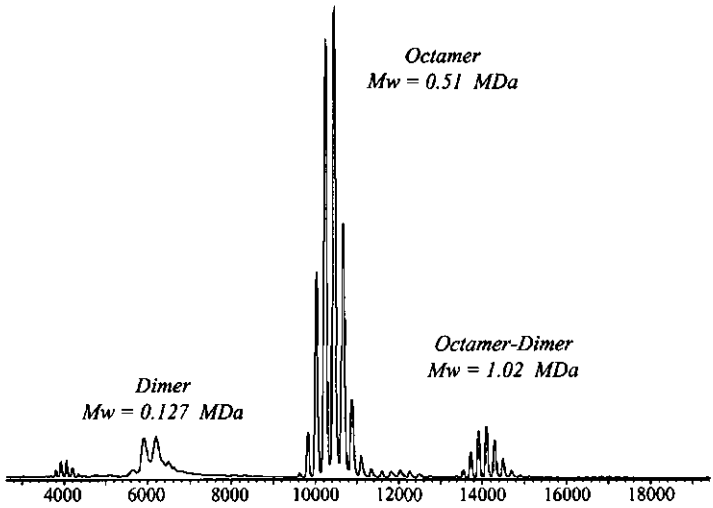

Fig. 2. Electrospray ionization mass spectrum of VAO sprayed from an aqueous 40 mM ammonium acetate solution (pH = 6.8) at a VAO monomer concentration of 28 μM.

If it is assumed that the ESI spectra reflect the abundance of the species in solution, the spectrum shown in Fig. 2 reveals that the octamer is the most stable protein assembly at a pH of 6.8 and an ammonium acetate concentration of 40 mM. However, the 16-mer is also significantly present under these conditions. Interestingly, the 16-mer was not observed in gel filtration experiments [53], suggesting that the interaction between octamers is rather weak. We speculate that the origin of the two sets of signals obtained for the dimeric species have to be rationalized differently. The broad dimer signals appearing around m/z = 6,000 probably originate from gas-phase dissociative processes of the octamer, occuring inside the mass spectrometer. This hypothesis is underlined by the fact that their abundance slightly increased with increased cone voltages and gas pressure inside the quadrupole. The lower abundant set of narrower dimeric signals appearing around m/z = 4,000 most likely originates from residual dimeric species in solution.

To investigate the stability of the protein assembly as a function of ionic strength, spectra were also recorded for VAO at a 28 μM monomer concentration but then from solutions containing 20, 10, and 5 mM ammonium acetate (all pH = 6.8). It was observed (not shown) that the ESI spectra obtained from all these different solutions were within experimental uncertainty identical to the one shown in Fig. 2. Therefore,

it seems that this range of ammonium acetate concentrations does not significantly affect the protein assembly stability.

Table 1. M/z values of the ion signals observed in the ESI spectrum of VAO as displayed in Fig. 2. Charge-states and average masses of the protein assemblies were calculated assuming that ionization occurred via multiple protonation.

Observed m/z (Th)	Charge	Mass (Da)	Observed m/z (Th)	Charge	Mass (Da)
	2-mer			**8-mer**	
3,633.4	35	127,132	9,248.6	55	508,618
3,740.2	34	127,132	9,420.0	54	508,625
3,853.7	33	127,138	9,594.4	53	508,448
3,974.0	32	127,135	9,780.4	52	508,526
4,102.2	31	127,136	9,971.6	51	508,499
Average mass	2-mer	127,134	Average mass	8-mer	508543
	16-mer			**24-mer**	
13,400	76	1,018,324	16,232	94	1,525,714
13,563	75	1,017,128	16,408	93	1,525,851
13,754	74	1,017,700	16,588	92	1,526,004
13,944	73	1,017,810	16,762	91	1,525,251
14,134	72	1,017,569	16,954	90	1,525,770
14,325	71	1,016,976	17,146	89	1,525,905
14,522	70	1,016,463	17,334	88	1,525,304
14,722	69	1,015,742	17,531	87	1,525,110
14,956	68	1,016,940	17,738	86	1,525,382
Average mass	16-mer	1,017,183	Average mass	24-mer	1,525,588

To investigate the stability of the protein assembly as a function of pH, spectra were recorded for VAO at a 28 μM monomer concentration in 40 mM ammonium acetate solutions at a pH of 7.7, 6.8, 5.8, and 5.1, respectively. The spectra obtained at pH 7.7 (not shown) were qualitatively identical to those obtained at pH = 6.8 (see Fig. 2). However, at pH = 5.8 and 5.1 the smaller protein assemblies became relatively more abundant. By integrating the signal intensities of the different protein assemblies the relative abundance of the ions was deduced. Fig. 4 shows the relative abundance of the monomer, dimer, tetramer, octamer and 16-mer ions in the mass spectra obtained

at these different pH conditions. Data on the 24-mer and other detected VAO complexes (such as the VAO 6-mer and 12-mer) were omitted from Fig. 4 as their relative abundance was at all investigated pH's less than a few percent. As illustrated by Fig. 4, the mass spectra taken at different pH reveal that the larger protein assemblies are most stable in a pH range between 6.8 and 7.7. At lower pH values the stabilty of the larger complexes is diminished in favor of the dimeric VAO complexes. The detection of intense tetramer ion signals but no notable hexamer signals seems to indicate that the dissociation of the VAO octamer proceeds most likely via an intermediate tetramer.

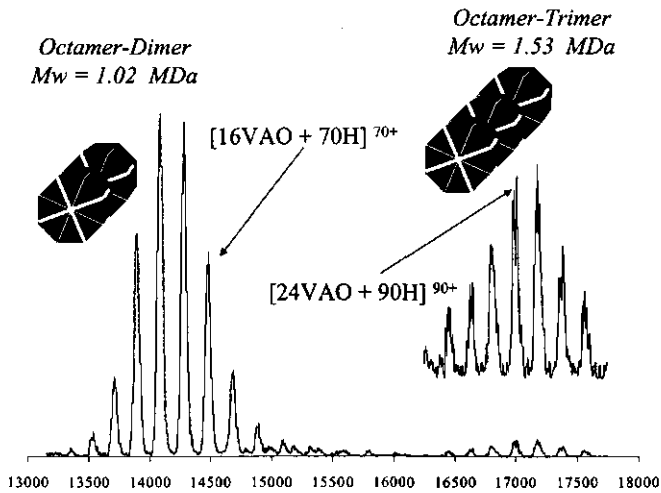

Fig. 3. Zoomed in part of the electrospray ionization mass spectrum of VAO, displayed in Fig. 2, showing the charge-state distributions of the 16-mer protein assembly (Mw = 1,017,100 ± 600 Da) and the 24-mer (Mw = 1,525,600 ± 1,000 Da). Peak widths were determined as full width at medium height (FWHM).

The mass spectrometric results reported here are in close agreement with reported data on the hydrodynamic stability of the VAO complex. The most stable form of the enzyme at neutral pH is indeed the octamer, which may be described as a tetramer of dimers (Fig. 1). In solution the octamer is in equilibrium with (fully active) dimers [53]. However, the higher oligomerization assemblies of VAO observed in the present mass spectra have not been reported before, which most likely indicates that the octamer-octamer interaction is substantial weaker than the octamer interactions.

In summary, using electrospray ionization, biologically important, intact large protein VAO assemblies, with molecular masses extending over one million Dalton, were detected and mass analyzed. The acquired mass spectra are pleasingly clean and

uncomplicated, with the different charge-states of the protein complexes well-resolved, making the interpretation straightforward. Masses of several different megaDalton protein assemblies could be measured with an uncertainty of less than 0.2%. The present data reveal that electrospray ionization mass spectrometry may play a meaningful role in studies on the topology and stability of larger protein assemblies.

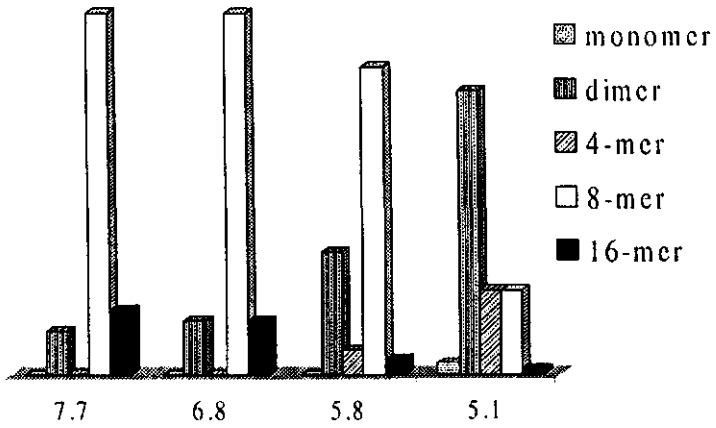

Fig. 4. Bar representation of the detected abundance of oligomer protein assembly ions as a function of the pH of the electrospray solutions.

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Summary

Vanillin is the world's principal flavoring compound, extensively used in food and personal products. The curing process of vanilla beans is labor-intensive and the *Vanilla* plant only grows in a few territories over the world, making synthetically produced vanillin far cheaper than natural vanillin. Nowadays, only 0.5% of the total market is met by extraction of *Vanilla* beans. The remaining 99.5% of the vanillin produced is of synthetic origin. However, with the increasing interest in natural products alternative methods are being developed to produce natural vanillin from sources other than *Vanilla planifolia*. Cell cultures, microorganisms, and isolated enzymes form potentially alternative sources for the production of vanillin from natural feedstock.

The Ph. D. project described here was initiated in the framework of the Innovation Oriented Research Program (IOP) Catalysis of the Dutch Ministry of Economy Affairs. In the Enzymatic Oxidation cluster of this research program the catalytic potential of oxidative enzymes for the production of valuable compounds was investigated. Enzymes are an almost unlimited source for the production of these compounds as they can produce natural products and are often highly regio- and/or stereospecific. Furthermore, biocatalytic (enzymatic) processes are in general environmentally friendlier than chemical processes. In this project, we aimed to enlarge the catalytic potential of the flavin-containing enzyme vanillyl-alcohol oxidase (VAO). To that end, the VAO-mediated production of natural vanillin and optically pure aromatic alcohols was addressed. Two different methods were used to direct the reactions to the most favorable product. In the first method we controlled the reaction medium and in the second method we introduced a few subtle changes in the enzyme. For these studies insight in the protein-flavin and protein-protein interactions were of crucial importance.

Enzymatic production of natural vanillin and optically pure alcohols

Chapter 2 describes the VAO-catalyzed conversion of creosol and vanillylamine to vanillin. The enzymatic conversion of creosol proceeds via a two-step process in which the initially formed vanillyl alcohol is further oxidized to vanillin. The production of vanillin is not optimal due to the competitive binding of creosol and

vanillyl alcohol in the enzyme active site and the fact that creosol forms a non-reactive covalent adduct with the flavin cofactor.

The oxidation of vanillylamine to vanillin proceeds readily at pH 10. However, as vanillylamine is too expensive for industrial use, we searched for a natural precursor compound. Capsaicin from red pepper is rather cheap and can be hydrolyzed enzymically to vanillylamine by a carboxylesterase from liver or chemically at basic pH values. Therefore, the use of capsaicin as feedstock for the production of vanillin is very promising.

VAO is active with a wide range of 4-alkylphenols bearing aliphatic side chains up to seven carbon atoms. In **Chapter 3**, we describe the enzymatic conversion of short-chain 4-alkylphenols to optically pure aromatic alcohols and the conversion of medium-chain 4-alkylphenols to aromatic alkenes (Fig. 1). The VAO-mediated hydroxylation of 4-alkylphenols is highly stereospecific (enantiomeric excess = 94%), and the enantiomeric excess of the *R*-product is even increased by the VAO-mediated oxidation of the (*S*)-isomer of the alcohol. The enzymatic dehydrogenation of medium-chain 4-alkylphenols is also stereospecific, suggesting that the *p*-quinone methide intermediate products are bound in a fixed orientation in the enzyme active site. Some medium-chain 4-alkylphenols are dehydrogenated to the *cis*-isomer and others to the *trans*-isomer of the alkene product. Thus, the specificity of the VAO-mediated conversions is dictated by the intrinsic reactivity, water accessibility, and orientation of the enzyme-bound *p*-quinone methide intermediate.

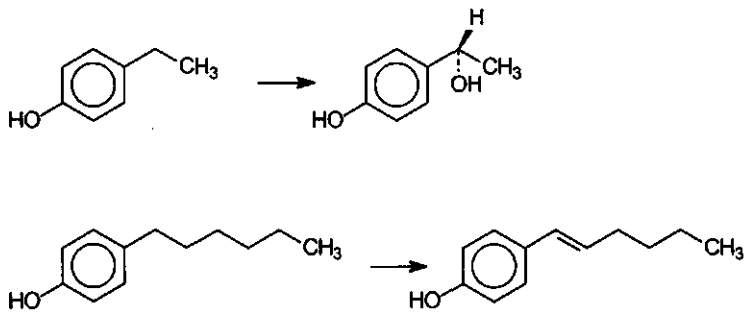

Fig. 1. Conversion of short-chain and medium-chain 4-alkylphenols by VAO

Tuning the product specificity

In the following chapters (4-7), we studied the possibilities to direct the VAO-mediated conversion of 4-alkylphenols into the most favorable direction using two different strategies. First, we varied the medium in which the reaction was performed and second, we modified the protein by rationale mutagenesis. In **Chapter 4**, we investigated the reactivity of VAO with 4-alkylphenols in the hydrophobic solvent toluene and the hydrophilic solvent acetonitrile. In both solvents the efficiency of substrate hydroxylation decreased compared to aqueous conditions. This effect on the hydroxylation efficiency was dependent on the water activity, but independent on the solvent used. This shows that the availability of water determines the efficiency of the hydroxylation reaction. A similar result was obtained by the addition of the monovalent anions chloride, bromide, or thiocyanate. The binding of these ions near the flavin prosthetic group inhibited the attack of water to the enzyme-bound quinone methide, providing a similar effect as lowering the water activity.

Protein engineering of VAO by site-directed mutagenesis proved to be another method to tune the reactivity of VAO with 4-alkylphenols (**Chapter 5** and **6**). The catalytic center of VAO harbors an acidic residue (Asp170), which is located in the proximity of the flavin N5-atom (3.6 Å) and the substrate Cα-atom (3.0 Å) (Fig. 2).

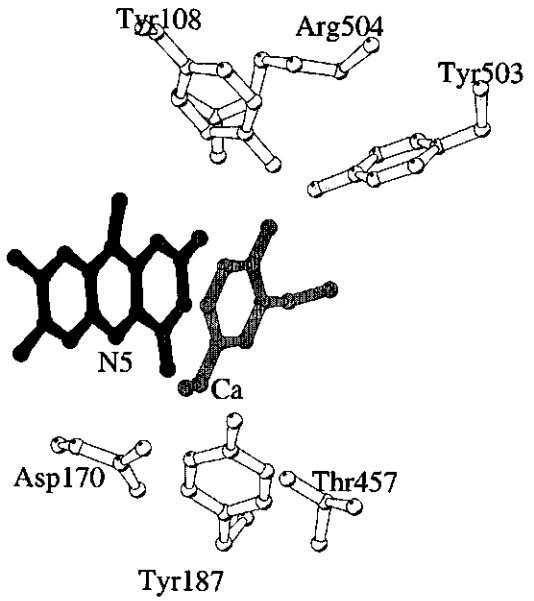

Fig. 2. Schematic representation of the active site cavity of VAO.

The location of this residue is intriguing as in most flavin-dependent oxidoreductases of known structure the flavin N5-atom contacts a hydrogen bond donor rather than an acceptor. Asp170 appeared to be crucial for the activity of VAO, the efficiency of hydroxylation of 4-alkylphenols, and the covalent binding of the flavin. Studies from site-directed mutagenesis and protein crystallography showed that Asp170 raises the oxidative power of the flavin cofactor and, therefore, the activity of the enzyme. Replacement of Asp170 by Ser or Ala resulted in a better hydroxylation efficiency of VAO, whereas the Asp170Glu replacement decreased the hydroxylation efficiency. These changes in product specificity are caused by steric effects. The small side chains of Ser170 and Ala170 increase the accessibility of water to the enzyme-bound *p*-quinone methide intermediate, whereas the more bulky side chain of Glu170 protects the quinone methide from water attack.

In **Chapter 7**, we describe the inversion of the stereospecificity of VAO by protein redesign. The active site residue Asp170, involved in water activation, was transferred to the opposite face of the substrate binding pocket (Thr457Glu mutation) (Fig. 2). As a result, the double mutants D170S/T457E and D170A/T457E hydroxylated 4-ethylphenol to the inverse enantiomer of the aromatic alcohol. This change in stereospecificity is caused by the activation of a water molecule, attacking the *p*-quinone methide, positioned at the opposite face of the substrate compared to wild type VAO. Crystallographic data confirmed that the distinctive properties of the redesigned mutants are caused by the selective mutations and not by structural changes within the protein. This is the first example of the inversion of the stereospecificity of an enzyme using a rationale redesign strategy.

Rationale of covalent flavin binding

The reason of covalent flavin binding in flavoenzymes is still a matter of debate. It has been suggested that the covalent interaction might a) increase the protein stability, b) enhance the enzyme activity, c) prevent flavin dissociation, and d) improve the resistance against proteolysis.

In **Chapter 8**, the role of the covalent protein-flavin interaction was studied by changing the residue to which the flavin is linked. The non-covalent VAO mutant H422A firmly binds the FAD cofactor, but the activity of the enzyme is decreased ten-fold. The lower enzymatic activity is not caused by structural changes but can be fully attributed to the decreased redox potential of the flavin cofactor. Thus, the covalent flavin bond is essential for the high oxidative power of the enzyme.

Oligomeric structure of VAO

At neutral pH, VAO predominantly forms homooctamers. The crystal structure of VAO has revealed that the octamer can be described as a tetramer of dimers in which each dimer is stabilized by extensive intersubunit interactions. Because some dimers are present at neutral pH and low ionic strength, it was of interest to study the stability of the protein assembly as a function of pH by electrospray ionization mass spectrometry (**Chapter 9**). At low pH values, the octamer-dimer equilibrium shifts to the dimeric form, whereas at neutral pH the enzyme is mainly present in the octameric form. Interestingly, also higher oligimerization assemblies of VAO were observed, indicating that weak interactions between the octamers exist. This information about the oligomeric structure of VAO is very useful for further studies, directed towards the stability of VAO under operational conditions. It is the first time that the mass of such a large molecule (larger than 1 million Da) is determined using this technique.

Conclusions

This research project was performed within the framework of the Enzymatic Oxidation cluster of the Innovation Oriented Research Program (IOP) Catalysis, funded by the Ministry of Economy Affairs. The aim of this cluster was to develop processes for the production of pharmaceuticals, fine-chemicals, flavors, and fragrances using oxidative enzymes, like heme peroxidases, vanadium peroxidases, and flavin-dependent oxidases.

In this thesis work, we focussed on the catalytic potential of the flavoprotein vanillyl-alcohol oxidase (VAO). VAO is active with a wide range of phenolic compounds and can produce a variety of industrially relevant products like vanillin and optically pure aromatic alcohols. We have demonstrated that the reactivity and selectivity of VAO can be modulated by medium engineering and protein engineering.

The principal component of red pepper, capsaicin, proved to be a promising candidate to produce natural vanillin using a bi-enzyme system, consisting of VAO and a hydrolase. By combining these two enzymes a one-pot conversion from capsaicin to vanillin can be realized. This production method yields natural vanillin, which is more valuable than synthetic vanillin. Moreover, the enzymatic production has, in general, environmental advantages compared to the traditional synthetic vanillin production.

VAO produces optically pure aromatic alcohols from 4-alkylphenols. The efficiency of substrate hydroxylation can be tuned by varying the availability of water in the catalytic center or by substituting a single amino acid residue (Asp170) in the enzyme. Furthermore, we were able to invert the stereospecificity of VAO by relocation of the active site base. This demonstrates that protein engineering is a powerful tool to introduce new enzyme characteristics. A major goal for further

research would be to enlarge the substrate scope of VAO and to improve the catalytic performance of VAO variants. Interesting target compounds are creosol and capsaicin, as being precursors of vanillin, and epinephrine analogs. Here, random mutagenesis and/or gene shuffling are attractive approaches, since the required changes in VAO are not easy to predict.

VAO is active over a wide pH range, but the protein assembly falls apart under extreme conditions. For possible future applications of the enzyme it is important to study the relationship between the conformational stability and oligomeric structure of VAO. In this aspect, the influence of the covalent flavin linkage is of importance as well. Interesting options to obtain a protein with improved stability properties would be gene shuffling between VAO and a homolog from a thermophilic organism or a combination of directed evolution methods.

Samenvatting

Vanilline is één van de meest gebruikte geur- en smaakstoffen in voedingsmiddelen (onder andere zuivelproducten en zoetwaren) en cosmeticaproducten. Vanilline heeft ook potentiële medische toepassingen, omdat het de laatste jaren duidelijk is geworden dat vanilline een antioxidant is die oxidatieve schade in dierlijke cellen kan voorkomen. Bovendien is er gerapporteerd dat het mogelijk is om sikkelcel bloedarmoede te behandelen met vanilline.

Natuurlijk vanilline uit de bonen van de orchidee *Vanilla planifolia* wordt gevormd uit een precursor molecuul (glucovanilline) wanneer de bonen een aantal bewerkingen ondergaan. Naast vanilline worden er in de bonen ook veel andere stoffen aangemaakt, waaronder 4-hydroxybenzaldehyde, 4-hydroxybenzylalcohol en vanillezuur. Het complex van deze smaak- en geurstoffen noemen we vanille (bekend van de vanillestokjes). Een groot nadeel van deze productiemethode is het beperkte gebied waarin de *Vanilla* orchidee groeit (Madagascar is de belangrijkste producent) en de arbeidsintensieve bewerkingsmethode die nodig is om vanille te maken. Synthetisch vanilline is veel goedkoper dan natuurlijk vanille en wordt daarom veel meer geproduceerd (meer dan 99% van de totale wereldproductie). Deze procedure is echter milieuvervuilend en het vanilline is niet-natuurlijk.

Vanwege de toenemende belangstelling voor natuurlijke producten zijn er alternatieve processen ontwikkeld om natuurlijk vanilline te maken. Hierbij worden planten, microorganismen of gezuiverde eiwitten ingezet om vanilline te vormen uit natuurlijke grondstoffen. Deze processen zijn nu nog erg duur omdat de efficiëntie nog niet hoog genoeg is. Echter, doordat de milieubelasting in het algemeen lager is en het vanilline natuurlijk is er wel duidelijke belangstelling voor deze productiemethoden.

Dit AIO-onderzoek is gestart in het kader van het Innovatiegericht Onderzoeksprogramma (IOP) Katalyse van het Ministerie van Economische Zaken. In de Enzymatische Oxidatie cluster, waar dit project deel van uitmaakte, is de mogelijkheid onderzocht om enzymen te gebruiken voor het maken voor industrieel waardevolle producten. Enzymen zijn minuscule machientjes (eiwitten) met een bepaalde taak, zoals bijvoorbeeld het maken van vanilline. Enzymen zijn zo krachtig omdat ze de omgeving van substraten zodanig kunnen veranderen dat omzetting plaats kan vinden. Hiervoor is in elk enzym een actief centrum aanwezig waar de reactie

plaatsvindt. Dit proefschrift beschrijft het onderzoek naar de productie van waardevolle producten (bijvoorbeeld vanilline en aromatische alcoholen) met behulp van het enzym vanillyl-alcohol oxidase (VAO). We hebben twee benaderingen gebruikt om de VAO-gekatalyseerde reacties in de gewenste richting te sturen. De eerste benadering is de verandering van het reactiemedium en de tweede benadering is de verandering van het eiwit op enkele specifieke plaatsen. Voor deze studies was het belangrijk om de structurele eigenschappen van het eiwit in detail te bestuderen.

Enzymatische productie van natuurlijk vanilline en optisch actieve verbindingen

In **Hoofdstuk 1** wordt een algemene inleiding gegeven over de productie van natuurlijk vanilline en aromatische alcoholen. Verder worden een aantal belangrijke eigenschappen van VAO beschreven. VAO is een eiwit dat een zeer breed scala aan aromatische stoffen kan omzetten. De aromatische alcoholen die daarbij worden gemaakt zijn optisch zuiver. Veel natuurlijke stoffen zijn optisch actief en komen voor in twee spiegelbeelden die door chemici links- en rechtsdraaiend worden genoemd. Hoewel de links- en de rechtsdraaiende varianten van moleculen chemisch en fysisch vrijwel dezelfde eigenschappen hebben kunnen ze biologisch een heel andere werking hebben. VAO produceert alleen het rechtsdraaiende aromatische alcohol (Fig. 1).

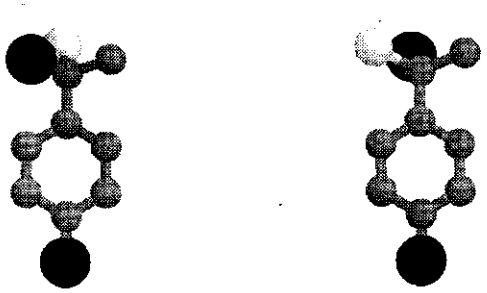

Fig. 1. De links- en rechtsdraaiende variant van een aromatisch alcohol dat wordt gevormd door VAO.

Veel andere enzymen maken ook optisch zuivere verbindingen. Dit is een groot voordeel ten opzichte van chemische omzettingen die vaak beide optische verbindingen opleveren (een racemisch mengsel). Optisch zuivere verbindingen zijn belangrijk voor de pharmaceutische en fijn-chemicaliën industrie. Maar liefst 50 van de 100 meest gebruikte medicijnen, zoals ibuprofen en ritalin, worden optisch zuiver geproduceerd om eventuele negatieve bijwerkingen en toxiciteit te voorkomen.

Hoofdstuk 2 beschrijft de VAO-gekatalyseerde omzetting van enkele natuurlijke substraten tot vanilline. We hebben gevonden dat capsaicine (de actieve component in rode peper) door middel van basische hydrolyse of het gebruik van een hydrolase (een enzym) eenvoudig kan worden omgezet in een stof die vervolgens door VAO wordt omgezet in vanilline. Op deze manier kunnen we uit de goedkope natuurlijke grondstof rode peper natuurlijk vanilline maken. Mogelijk heeft deze productiemethode een commerciële toekomst.

VAO reageert met een groot aantal aromatische verbindingen, waaronder 4-alkylphenolen. In **Hoofdstuk 3** beschrijven we de omzetting van 4-alkylphenolen tot optisch zuivere alcoholen of alkenen. 4-Alkylphenolen met een korte zijketen worden specifiek omgezet in optisch zuivere rechtsdraaiende alcoholen en 4-alkylphenolen met een lange staart worden specifiek omgezet tot aromatische alkenen (Fig. 2). Bij de omzetting van 4-alkylphenolen valt een watermolecuul zeer specifiek aan op een tussenproduct van het substraat, waardoor alleen het rechtsdraaiende alcohol wordt gemaakt. Deze selectiviteit wordt nog vergroot doordat het linksdraaiende alcohol een goed substraat is voor VAO en verder wordt omgezet. De specificiteit van VAO wordt veroorzaakt door de intrinsieke reactiviteit, de watertoegankelijkheid en de oriëntatie van het tussenproduct.

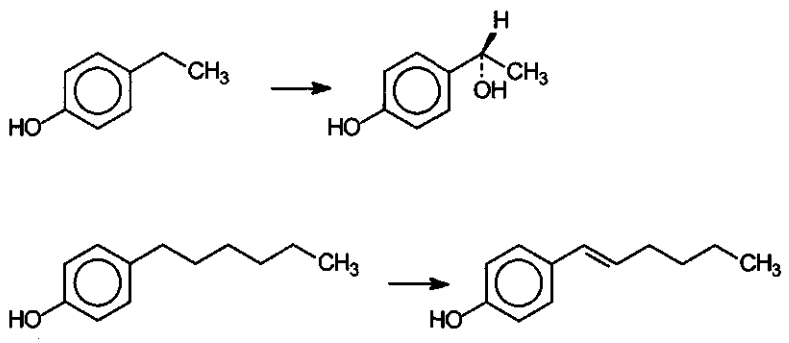

Fig. 2. Omzetting van 4-alkylphenolen met korte en lange zijketen door VAO.

Sturing van de specificiteit

In **Hoofdstuk 4** is de mogelijkheid onderzocht om de omzetting van 4-alkylphenolen doelgericht te sturen in de richting van het meest waardevolle product door middel van de verandering van het reactiemedium. Bij deze experimenten hebben we de reacties laten plaatsvinden in organische oplosmiddelen. In vergelijking met waterig milieu blijkt dat de wateraanval nu veel minder efficiënt verloopt (er wordt dus minder alcohol gevormd). Dit effect is afhankelijk van de hoeveelheid water die beschikbaar is voor het enzym en onafhankelijk van het soort organisch oplosmiddel. Een tweede

methode, gebaseerd op de verandering van het reactiemedium, is de toevoeging van zouten die binden in het eiwit vlakbij het actief centrum. Deze binding van zouten heeft hetzelfde netto effect als organische oplosmiddelen.

Als tweede benadering hebben we het eiwit een beetje veranderd (**Hoofdstukken 5, 6** en **7**). Door de verandering van één specifiek aminozuurresidu (Asp170) in het actief centrum van VAO kan de reactiviteit van het enzym met water worden gereguleerd. Dit residu blijkt ook van groot belang te zijn voor de snelheid van handelen van het enzym. Wanneer we naast Asp170 nog een tweede aminozuur (Thr457) veranderen wordt de optische activiteit van VAO omgedraaid. Het water molecuul dat betrokken is bij de reactie valt nu de andere kant van het substraat aan. Dit resulteert in het spiegelbeeldproduct. Het is de eerste keer dat een redox enzym door middel van deze methode zo wordt veranderd dat zij het spiegelbeeld van zijn originele product maakt.

Flavine hulpstof in VAO

Veel enzymen hebben een hulpstof nodig om producten te maken. VAO heeft een flavine hulpstof die vast zit aan het eiwit (covalent gebonden). Dit is niet algemeen want in de meeste flavine afhankelijke enzymen zit de hulpstof wel stevig maar niet covalent gebonden aan het eiwit. De reden voor deze verankering is niet geheel duidelijk. Er is voorgesteld dat dit belangrijk zou kunnen zijn voor de stabiliteit, de reactiviteit, het vasthouden van de hulpstof en de resistentie tegen eiwitafbraak.

Wij hebben het nut van de covalente flavinebinding onderzocht door het aminozuur waaraan het flavine vast zit te vervangen (**Hoofdstuk 8**). Hierdoor wordt een eiwit verkregen dat de hulpstof wel bindt, maar niet verankert. De eigenschappen van deze VAO-variant geven aan dat de covalente verankering aan het eiwit nodig is om de reactiviteit van de hulpfactor in het enzym te verhogen. De niet-covalente variant is daardoor veel minder goed in staat om VAO substraten om te zetten.

Structuur van VAO

Onder normale condities is VAO opgebouwd uit acht identieke eenheden (octameer). In **Hoofdstuk 9** hebben we met behulp van massaspectrometrie (een methode om gewichten van kleine moleculen, zoals eiwitten, nauwkeurig te bepalen) de oligomere structuur van VAO bepaald bij verschillende pH waarden. Wanneer het milieu zuur is, valt het eiwit gedeeltelijk uit elkaar waardoor er vier stukken van ieder twee eenheden (dimeer) worden gevormd. Met deze geavanceerde weegmethode is ook vastgesteld dat VAO kan bestaan uit tweelingen en drielingen van het octameer. Het is de eerste keer dat het gewicht van zo'n groot eiwit (meer dan één miljoen dalton) zo nauwkeurig is bepaald.

Conclusies

In dit onderzoeksproject zijn de katalytische mogelijkheden van VAO onderzocht. VAO reageert met een groot aantal stoffen en kan een aantal interessante verbindingen maken, zoals vanilline en optisch zuivere aromatische alcoholen. De bereiding van natuurlijk vanilline uit capsaicine biedt interessante mogelijkheden. De hydrolyse van capsaicine tot de stof die VAO kan verwerken moet nog worden geoptimaliseerd. De VAO-gekatalyseerde omzetting tot vanilline verloopt zeer goed. Deze methode levert natuurlijk vanilline op en heeft in het algemeen milieuvoordelen ten opzichte van de conventionele synthetische productie van vanilline.

VAO kan optisch zuivere aromatische alcoholen maken. De reactiviteit van VAO met water is te beïnvloeden door de hoeveelheid water te variëren, door het toevoegen van bepaalde zouten of door het eiwit op één specifieke plaats te veranderen. Ook kunnen we de optische activiteit van de gemaakte stoffen omkeren door enkele subtiele wijzigingen aan te brengen in het eiwit. Een belangrijk doel voor de toekomst is om VAO zodanig te veranderen dat we ook andere industrieel relevante stoffen kunnen maken. Waarschijnlijk is gerichte evolutie hiervoor de beste methode, aangezien de benodigde veranderingen in VAO lastig voorspelbaar zijn.

VAO is actief over een breed pH traject maar valt uit elkaar onder extreme condities. Voor eventuele toepassingen van het enzym is het van belang om meer inzicht te krijgen in de relatie tussen de oligomere structuur van het eiwit en de conformationele stabiliteit. Hierbij is ook de invloed van de covalent gebonden flavine hulpstof van belang. Interessante mogelijkheden om VAO varianten te verkrijgen met een verhoogde stabiliteit zijn het mengen van het gen van VAO met het gen van een VAO homoloog uit een thermofiel organisme of het toepassen van versnelde evolutie methoden.

Nawoord

Toen ik in 1991 begon aan mijn studie levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen had ik niet gedacht dat biochemie voor mij zo belangrijk zou worden in de daaropvolgende jaren. Dit tekende zich voor het eerst af toen ik een afstudeervak voor mijn doctoraalstudie ging doen aan het Laboratorium voor Biochemie bij Frank en Willem aan dat beroemde gele eiwit. Tijdens dit afstudeervak werd mijn interesse voor het doen van onderzoek gewekt. Tijdens mijn latere afstudeervakken en stage werd, mede dankzij het enthousiasme van Ronald, Jaap, Sybe, Ian en Christian, mijn interesse voor onderzoek verder versterkt. Over de AIO positie aan dat andere gele eiwit bij Willem en Colja hoefde ik dan ook niet lang na te denken.

Ik kwam terecht in een aangename omgeving. Michel werd mijn kamergenoot en ik begon proeven te doen in lab 5. Dankzij Willem en Marco nam mijn kennis van vanillyl-alcohol oxidase en flavine-bevattende eiwitten snel toe. Willem voelde ook altijd perfect aan wanneer ik wel of niet gestuurd moest worden. Colja zorgde er voor dat mijn project gestroomlijnd verliep.

Na het publiceren van het eerste artikel ging het snel. Het gen werd gekloneerd door Jacques en Nanne en ik begon mutanten te maken. De resultaten van de daaropvolgende biochemische studies waren veelbelovend, dus moesten er eiwitkristallen gemaakt worden. Hiermee begon een zeer prettige en vruchtbare samenwerking met Andrea en Marco in Pavia. De maand in Italië was zeer nuttig voor mij en ik maakte voor het eerst kennis met een Synchrotron.

Rond diezelfde periode ging ik ook een maand naar de Universiteit van Strathclyde in Glasgow om het effect van oplosmiddelen op vanillyl-alcohol oxidase uit te zoeken. Ik kwam terecht in een onbekende maar aangename onderzoekswereld, dankzij vooral Rein, Peter en Johann. Na deze twee buitenlandse 'stages' werd ik opgeschrikt door het vertrek van mijn kamergenoot en ik bemerkte al snel dat ik het 'bakkie leut' vroeg in de ochtend zeer miste. In het begin van het daaropvolgende jaar moesten we het Transitorium onverwacht verlaten vanwege de tijdens werkzaamheden vrijgekomen asbest. Drie maanden heb ik toen in relatieve rust artikelen kunnen schrijven in het Provisorium.

Net voor en net na de asbest periode ontdekten we dat we de stereospecificiteit van vanillyl-alcohol oxidase konden omdraaien. Voor mij is dit het mooiste resultaat wat

beschreven is in dit proefschrift. Na publicatie van dit belangwekkende artikel werden we aangenaam verrast door berichten in de Volkskrant en het Wb. Toen het jaar 2000 vorderde en ik me realiseerde dat mijn AIO periode bij biochemie bijna ten einde was gekomen verschoof mijn werk zich grotendeels naar de computer.

De laatste maanden waren druk maar aangenaam en hebben geresulteerd in het proefschrift dat nu voor u ligt. Nu sta ik op het punt om na tien jaar Wageningen te verlaten voor een nieuw avontuur in Italië. Willem, Marco, Michel, Colja, Andrea, Mariëlle, Yves, Maarten, Eyke, Ruchira, Miriam, Agnieszka, Niek, Rigoberto, Nanne, Jacques, Miew-Woen, Laura, Rein, Johann, Peter, Boris, Sybe, Ronald, Jaap, Frank, Ian, Christian, Willem, Henry, Eelco, Maurits, alle Pallas '67 leden, Danielle, pap en mam, Monique, Martijn en Carla wil ik bedanken voor hun directe of indirecte bijdrage bij het tot stand komen van dit proefschrift. Danielle, ik weet zeker dat we het samen zullen gaan maken.

Robert

Curriculum vitae

Robert Henricus Hubertus van den Heuvel was born in Weert, The Netherlands on July 31st 1972. In 1991 he graduated from grammar school at the Philips van Horne Scholengemeenschap in Weert (H.A.V.O. and V.W.O. degree). Subsequently, he started his studies in Food Technology at Wageningen Agricultural University (nowadays Wageningen University). For his Master's degree he did five months research in the Laboratory of Biochemistry at Wageningen University (dr. Frank van der Bolt and dr. Willem van Berkel) and three months in the Laboratory of Molecular Genetics of Industrial Microorganisms at Wageningen University (dr. Ronald de Vries and dr. Jaap Visser). Thereafter, he spent five months at Massey University in Palmerston North (New Zealand) for a research project (dr. Ian Maddox and dr. Christian Schuster). Finally, he carried out a five months research project in the Laboratory of Industrial Microbiology at Wageningen University (dr. Sybe Hartmans). In 1996 he graduated cum laude.

In September 1996 he started the Ph. D. project that is described in this thesis in the Laboratory of Biochemistry at Wageningen University (dr. Willem van Berkel and prof. dr. Colja Laane). This research was performed within the framework of the Innovation Oriented Research Project (IOP) Catalysis, funded by the Dutch Ministry of Economy Affairs. Some parts of the work were carried out at the University of Strathclyde in Glasgow, Scotland (prof dr. Peter Halling) and Università degli Studi di Pavia in Pavia, Italy (dr. Andrea Mattevi).

After his graduation, he will start a post-doc project in the laboratory of dr. Andrea Mattevi in Pavia, Italy (Dipartimento di Genetica e Microbiologia, Università degli studi di Pavia). This project will be conducted in collaboration with dr. Maria Antonietta Vanoni (Dipartimento di Fisiologia e Biochimica Generali, Università degli Studi di Milano). The project aims to reveal the mechanism of action of glutamate synthase using protein crystallography and is funded by an individual fellowship of the European Union in the framework of the 'Human Potential' program.

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