Exploring the Biocatalytic Potential of Vanillyl-Alcohol Oxidase by Site-Directed Mutagenesis

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Abstract: Vanillyl-alcohol oxidase (VAO) is a flavoprotein containing a covalently bound FAD cofactor. The enzyme acts on a wide variety of 4-alkylphenols bearing aliphatic side-chains up to seven carbon atoms in length. Short-chain 4-alkylphenols are predominantly hydroxylated to (*R*)-1-(4'-hydroxyphenyl) alcohols, whereas medium-chain 4-alkylphenols are dehydrogenated to the corresponding 1-(4'-hydroxyphenyl)alkenes. In this account, we summarize our work on the structure, mechanism, and biocatalytic potential of VAO. It is shown that the efficiency of hydroxylation of 4-alkylphenols is dependent on the type of amino acid residue engineered at position 170. Furthermore, it is demonstrated that the stereospecificity of the hydroxylation reaction can be inverted by relocating the active site base to the opposite face of the substrate-binding pocket.

Keywords: enzyme catalysis; flavoprotein; site-directed mutagenesis; stereoselectivity; vanillyl-alcohol oxidase.

1 Introduction

It is well recognized that enzymatic synthesis is an attractive alternative to conventional chemical methods for the production of food ingredients, fine-chemicals, and pharmaceuticals. In addition, the use of enzymes may serve as a solution to certain intractable synthetic problems. Enzymes are fascinating catalysts that accept a wide range of compounds, catalyzing reactions with high chemo-, regio-, and stereospecificities and with high reaction rates. Moreover, enzymes catalyze their reactions often under mild conditions, such as atmospheric pressure, mild temperature, and neutral pH value, and generate few by-products.

Recent advances in enzyme catalysis have been extended to the synthesis of commodity chemicals.[1] However, the restricted availability of enzymes with the desired substrate and product selectivity limits the development of new biocatalytic processes. In the case that Nature does not provide us with an enzyme that carries out the desired reaction, we have to do a search for a suitable enzyme with the proper specificity. To overcome this bottleneck, much effort has been put in the development of methods to regulate the selectivity of enzymes, allowing us to create multi-functional enzymes. Currently, three methods are successfully used to alter the selectivity of enzymes: medium engineering,[2,3] site-directed mutagenesis,[4] and directed evolution.[5,6]

In this account, we focus on the reactivity of the flavin-dependent redox enzyme vanillyl-alcohol oxidase (VAO) with 4-alkylphenols.[7] By exploiting the recently solved three-dimensional structure[8] and mechanism of action of VAO,[9] the product and stereospecificity of the enzyme was modulated by site-directed mutagenesis. The enlarged product scope of VAO enhances its biocatalytic potential and may serve as a model for related enzymes.

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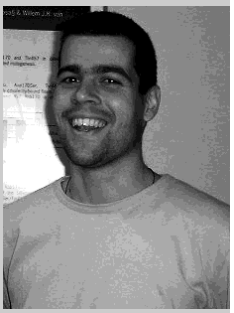

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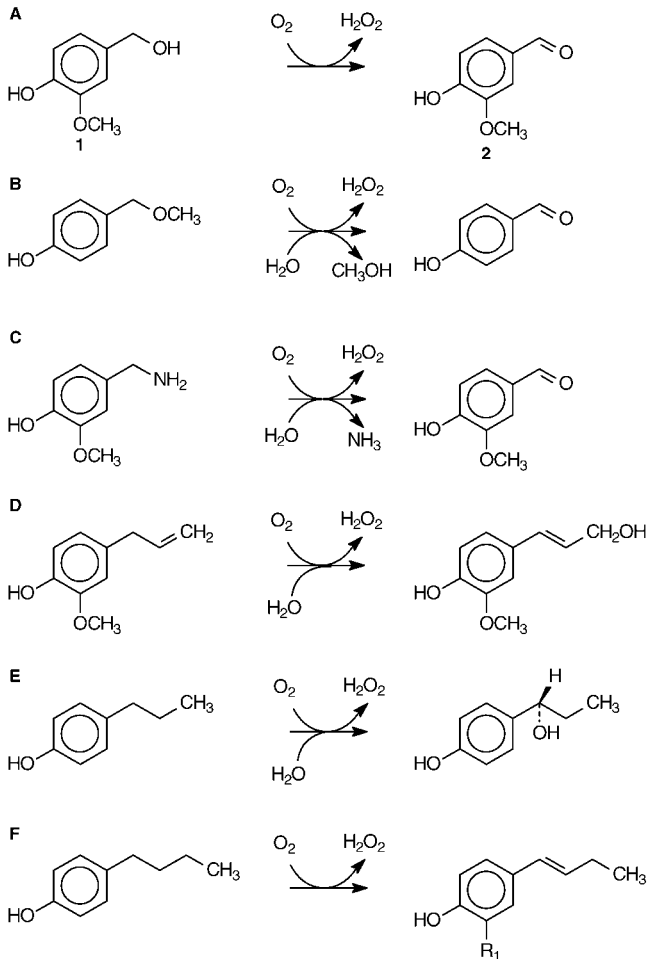
2 Vanillyl-Alcohol Oxidase

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a flavoprotein from the ascomycetous fungus *Penicillium simplicissimum* that catalyzes the oxygen-dependent conversion of vanillyl alcohol (3-methoxy-4-hydroxybenzyl alcohol) (**1**) to vanillin (3-methoxy-4-hydoxybenzaldehyde) (**2**)[10] (Scheme 1). The physiological function of VAO is unknown, but induction experiments have suggested that the enzyme is involved in the biodegradation of *p*-cresol ethers such as 4-(methoxymethyl)phenol.[11] Substrate specificity studies have shown that the scope of reactions catalyzed by VAO is rather broad.[7,12] The enzyme converts a wide range of phenolic compounds (including aromatic alcohols, ethers, amines, allylphenols, and alkylphenols) by catalyzing oxidation, demethylation, deamination, hydroxylation, and dehydrogenation reactions (Scheme 1). The conversion of eugenol (4-allyl-2-methoxyphenol), the main component of clove oil, to coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) has been patented by Quest Int.[13] The production of vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzyl alcohol (all components of vanilla flavor), 4-vinylphenol (present in wine and orange juice), and optically pure aromatic alcohols has biotechnological potential. Recently, it was found that natural vanillin can be synthesized from capsaicin using VAO.[14] In this two-enzyme system, capsaicin from red pepper is first hydrolyzed by a carboxylesterase from liver to vanillylamine.[15,16,17] The biocatalytic potential of VAO is also of interest for the production of synthons for pharmaceuticals such as, for



Scheme 1. Reactions catalyzed by VAO. (**A**) Oxidation of 3-methoxy-4-hydroxybenzyl alcohol, (**B**) demethylation of 4-(methoxymethyl)phenol, (**C**) deamination of vanillylamine, (**D**) hydroxylation of eugenol, (**E**) hydroxylation of 4-propylphenol, and (**F**) dehydrogenation of 4-butylphenol.

instance, *N*-methyl-D-aspartate receptor antagonists like ifenprodil[18] and nylidrin[19] and analogues.[20]

An interesting feature of VAO for biotechnological applications is that the flavin prosthetic group is covalently bound. The precise function of this covalent linkage is unknown, but it has been suggested that the covalent anchoring of the prosthetic group prevents flavin dissociation, thereby improving protein stability and resistance against proteolysis.[21] Moreover, we recently obtained evidence that the covalent linkage between the C8α atom of the isoalloxazine ring of the flavin and the N3 atom of His422 raises the redox potential of the FAD cofactor, thereby increasing the oxidative power of the enzyme.[22]

The reaction mechanism of VAO has been studied in great detail using spectroscopic techniques.[9,23] Catalysis 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. Reaction of VAO with 4-propylphenol (**3**) involves the initial transfer of a hydride from the Cα-atom of the substrate to the N5-atom of the flavin, resulting in the formation of a complex between reduced enzyme and *p*-quinone methide intermediate (**4**). Next, the reduced flavin is reoxidized by molecular oxygen with the formation of hydrogen peroxide. Concomitantly, the protein-bound quinone methide is attacked in a stereospecific manner by water to yield the (*R*)-enantiomer of 1-(4'-hydroxphenyl)propanol (**5**) (70% of total product) or rearranges in a competing reaction to yield 1-(4'-hydroxyphenyl)propene (**6**) (30% of total product) (Scheme 2).[23,24]

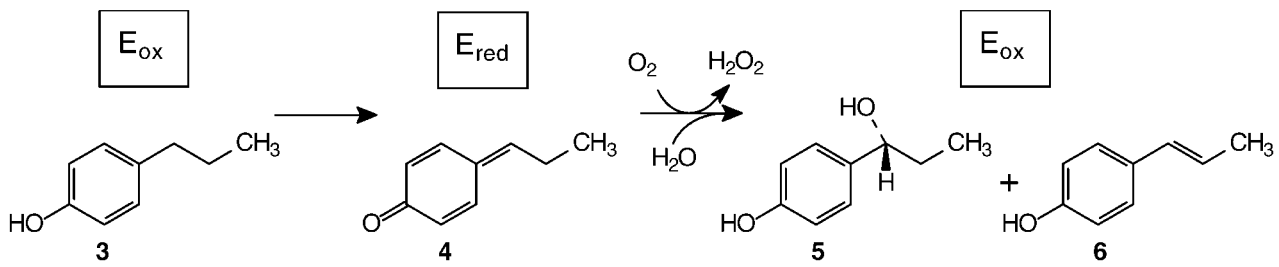
Studies with a wide range of 4-alkylphenols have shown that the ratio between the alcohol and alkene product depends on the length and bulkiness of the alkyl side-chain.[7] Short-chain 4-alkylphenols, like 4-ethylphenol and 4-propylphenol, are mainly converted to (*R*)-1-(4'-hydroxyphenyl) alcohols, whereas medium-chain 4-alkylphenols, like 4-butylphenol, are converted to 1-(4'-hydroxyphenyl)alkenes. From this it was proposed that the different outcome of products formed from short-chain and medium-chain 4-alkylphenols is due to variations in the intrinsic reactivity of the enzyme-bound *p*-quinone methide intermediate, the water accessibility of the enzyme active site, and the orientation of the alkyl side-chain of the substrate.[7]

3 Structural Properties of Vanillyl-Alcohol Oxidase

Rational redesign of the product specificity of an enzyme requires a detailed understanding of its three-dimensional structure and mechanism of action. A few years ago, the crystal structures of free VAO and in complex with several inhibitors were elucidated.[8] Moreover, from structure-based sequence alignments it was revealed that VAO is a member of a novel family of oxidoreductases, whose members share a conserved flavin-binding domain.[25] To date, this family has already more than 100 members and several crystal structures are known.[26,27,28,29]

Each VAO monomer contains a covalently bound FAD cofactor and comprises two domains: residues 1 – 270 and 500 – 560 form the FAD binding domain, while residues 271 – 499 form the cap domain. The active site cavity is deeply buried within the protein at the interface between the two domains. This cavity is solvent inaccessible and the shape is that of a closed elongated cavity with a total volume of about 200 Å^3. Crystal soaking experiments revealed that the cavity is completely filled when 1-(4'-heptenyl)phenol is bound. This is in line with the fact that no catalytic activity is observed with 4-alkylphenols when the aliphatic side-chain is longer than seven carbon atoms.[7] Thus, the substrate preference of VAO seems to be dictated by a size-exclusion mechanism. Inside the active site cavity, the aromatic substrate is ideally positioned for hydride transfer to flavin N5.

The structural features determining the reactivity of VAO are not completely understood, but several residues are likely to play a direct role. Substrate oxidation is facilitated by ionization of the phenolic moiety of the substrate induced by hydrogen bonding to Tyr108, Tyr503, and Arg504 (Figure 1). Another key residue is Asp170, which is located close to the N5-atom of the flavin (3.6 Å) and the reactive methylene



Scheme 2. Reaction scheme for the conversion of 4-propylphenol by VAO. The redox state of VAO during catalysis is depicted on the top. E_{ox}, oxidized enzyme; E_{red}, reduced enzyme; **3**, 4-propylphenol; **4**, *p*-quinone methide of 4-propylphenol; **5**, 1-(4'-hydroxyphenyl)propanol; **6**, 1-(4'-hydroxyphenyl)propene.

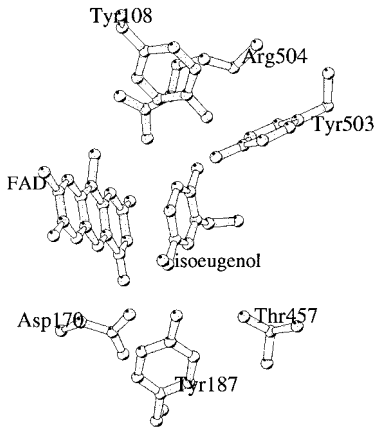


Figure 1. Drawing of the active site cavity of VAO with bound inhibitor isoeugenol. This figure was prepared with MOLSCRIPT.[39]

group of the substrate (3.0 Å). The location of this acidic residue is intriguing as in most flavin-dependent oxidoreductases of known structure the N5-atom contacts a hydrogen bond donor rather than an acceptor.[30,31] During catalysis Asp170 might interact with the protonated N5-atom of the reduced flavin. Moreover, it has been proposed that Asp170 might act as an active site base, activating the water molecule involved in substrate hydroxylation.[8] At the opposite face of the substrate-binding pocket the side-chain of Thr457 points to the methylene moiety of the aromatic substrate as well (Figure 1). However, this residue is far away from the flavin and seems not directly involved in catalysis.

4 Catalytic and Structural Properties of Asp170 Variants

To investigate the role of Asp170 in VAO catalysis in more detail, four Asp170 variants, D170A, D170E, D170N, and D170S were expressed in *Escherichia coli* and purified to homogeneity. Like in wild-type enzyme, the FAD in D170E and D170S was fully covalently bound. However, in D170A and D170N, only 50% and 0%, respectively, of the flavin was covalently attached to the protein.[32] The Asp170 replacements strongly affect the rate of turnover of the enzyme. With vanillyl alcohol, eugenol, and 4-(methoxymethyl)phenol as substrates, D170E was 5- to 100-fold less active than wild-type VAO. The other Asp170 variants are even much slower with these substrates and more than 1,000-fold less active than wild-type VAO.[32] Anaerobic reduction experiments showed that, like wild-type VAO, the reductive half-reaction limits the turnover rate of the mutant enzymes. Further characterization revealed that the impaired activity of the Asp170 variants is at least partly due to lowered redox potentials of the flavin cofactor.[32] The conservative Asp170Glu substitution lowers the flavin redox potential by 50 mV, whereas the Asp170Ser substitution lowers the potential by 145 mV. The redox potential of the flavin is a measure for its reactivity and, therefore, for the oxidative power of the enzyme.

The altered catalytic properties of the Asp170 variants are not caused by any structural rearrangements. This is concluded from their spectral and hydrodynamic properties and from the fact that the crystal structure of D170S in complex with the aromatic inhibitor isoeugenol is nearly identical to that of wild-type VAO.[32] Thus, the properties of the Asp170 variants indicate that Asp170 is involved in the process of autocatalytic covalent flavinylation and is crucial for efficient redox catalysis.

5 Efficiency of Hydroxylation of 4-Alkylphenols

To gain more insight in the role of Asp170 in regulating the water reactivity of the enzyme-bound *p*-quinone methide, we investigated the catalytic properties of the Asp170 variants with 4-alkylphenols.[33] Product analysis showed that the substitution of Asp170 by Ser or Glu has profound effects on the hydroxylation efficiency of 4-alkylphenols. Interestingly, D170S favors the hydration of the *p*-quinone methide intermediate, whereas the D170E mutant favors the formation of alkenes (Table 1). When the same experiments were performed with the D170A variant it was found that its product spectrum is similar to that of D170S. This suggests that the efficiency of hydroxylation of 4-alkylphenols is regulated by the bulkiness of the side-chain of residue 170 and not by its ionic character. The observation that D170S efficiently catalyzes the hydration of the *p*-quinone methide also suggests that the electrophilic enzyme-bound *p*-quinone methide reacts readily with unactivated water. This is in line with previous studies with analogous *p*-quinone methide species.[34,35] In conclusion, these studies show that Asp170 plays a crucial role in determining the product specificity of the VAO-mediated reaction with 4-alkylphenols.

Table 1. Conversion of 4-alkylphenols by wild-type VAO, D170E, and D170S.

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

6 Stereospecificity of Hydroxylation

VAO converts short-chain 4-alkylphenols preferentially to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl) alcohol with an enantiomeric excess of 95%.[24] However, the related flavocytochrome *p*-cresol methylhydroxylase (PCMH) favors the production of the (*S*)-enantiomer of 1-(4'-hydroxyphenyl) alcohol (ee > 97%).[36] As VAO and PCMH catalyze similar reactions[7,24,37] and share similar folds,[8,27] we rationalized that the opposite stereospecificity of these enzymes is related to the position of the acidic residues in the active site. Asp170 in VAO and the equivalent Glu380 in PCMH might activate the water involved in hydrating the *p*-quinone methide.[27,32] However, in PCMH a second acidic residue is located at the opposite face of the substrate (Glu427), which is not present in VAO (Thr457) (Figure 1). Therefore, the VAO mutants D170A, D170S, T457E, D170A/T457E, and D170S/T457E were investigated for their enantioselectivity. On the basis of the crystal structure of the double mutant D170S/T457E it was established that the introduction of an acidic residue at position 457 in VAO does not result in any significant structural perturbation.[38]

The single mutants D170A and D170S preferentially hydroxylated 4-ethylphenol to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol, although the specificity is lower than in wild-type enzyme (Figure 2). This might be a consequence of the increased accessibility of water to the *p*-quinone methide, which would be in agreement with the high efficiency of hydroxylation of D170A and D170S.[33] The double mutants D170A/T457E and D170S/T457E exhibited an inverted stereospecificity with 4-ethylphenol. Especially D170S/T457E is specific for the (*S*)-enantiomer of the aromatic alcohol (ee = 80%). This is likely to be caused by the attack of a water molecule from the opposite face of the substrate. The crystal structure of D170S/T457E revealed that the side-chain of Glu457 is only 3.5 Å from the Cα-atom of the substrate analogue trifluoromethylphenol. This indicates that, in the double mutants, Glu457 directs the stereospecific attack of water to the *p*-quinone methide intermediate, presumably by acting as an active site base. The T457E mutant, on the other hand, is specific for the (*R*)-enantiomer of the alcohol. This suggests that, in this particular mutant, Asp170 favorably competes with Glu457 for the site of water attack. The selectivity of T457E is in marked contrast with the stereochemical properties of PCMH. This shows that subtle variations in the active site cavity of both enzymes are sufficient to invert the stereospecificity.

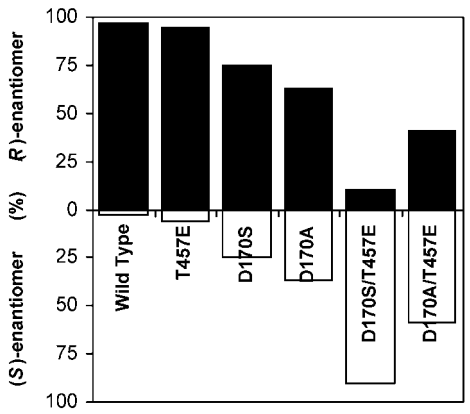


Figure 2. Stereospecificity of hydroxylation of 4-ethylphenol by wild type VAO and Asp170/Thr457 mutants. (*R*)-1-(4'-hydroxyphenyl)ethanol (black) and (*S*)-1-(4'-hydroxyphenyl)ethanol (white).

7 Conclusion

VAO catalyzes the conversion of a wide variety of *p*-substituted phenols. Some of the products formed are of interest for the food, fine-chemical, or pharmaceutical industry. In recent years a detailed insight in the mechanism of action of VAO was obtained from structural and mechanistic studies and from protein engineering. In this account, we demonstrate that Asp170 in the enzyme active site is a key residue for VAO catalysis: i) Asp170 is involved in autocatalytic flavinylation, which increases the oxidative power of the enzyme, ii) Asp170 is involved in determining the efficiency of substrate hydroxylation, and iii) Asp170 determines the stereospecificity of hydroxylation. Single replacements of Asp170 lower the activity of the enzyme, but allow the production of 1-(4'-hydroxyphenyl)alkenes or 1-(4'-hydroxyphenyl) alcohols in pure form. Double mutations at position 170 and 457 allow the inversion of the stereospecificity of hydroxylation. The relocation of the active site base in VAO to the opposite face of the substrate binding pocket is the first example of a rational redesign of the stereospecificity of a redox enzyme.

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