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Structure, function and redesign of vanillyl-alcohol oxidase

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Abstract

Vanillyl-alcohol oxidase (VAO) from *Penicillium simplicissimum* is an inducible flavoprotein involved in the biodegradation of lignin-derived aromatic compounds. The enzyme is the prototype of a newly recognized family of structurally related oxidoreductases, whose members share a conserved FAD-binding domain. The flavin cofactor in VAO is covalently linked to His422 of the cap domain. This covalent interaction increases the redox potential of the flavin, facilitating the oxidation of a wide range of phenolic substrates. Covalent flavinylation is an autocatalytic process in which His61 activates His422. Vanillyl-alcohol oxidase (VAO) catalysis involves the formation of a *p*-quinone methide product intermediate. With 4-ethylphenol as a substrate, this intermediate is stereospecifically attacked by water, yielding the *R*-enantiomer of 1-(4'-hydroxyphenyl)ethanol in high enantiomeric excess. Studies from site-directed mutants revealed that Asp170 is critically involved in substrate hydroxylation. This prompted us to create a double mutation (D170S/T457E) with the idea to relocate the putative active site base to the opposite face of the substrate-binding pocket. In this way, and for the first time, the stereospecificity of a redox enzyme was inverted by rational redesign.

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Keywords: Vanillyl-alcohol oxidase; Covalent flavoprotein; Enantioselectivity; Protein engineering; Quinone methide

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1. Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is an inducible flavoprotein that acts on a wide range of 4-hydroxybenzylic compounds. During the past few years, we have studied the structure, mechanism and biotechnological potential of this enzyme in great detail. As a mark of honor to the pioneering work of Prof. Osamu Hayaishi in the field of oxygenases and oxidases, we summarize in this paper our research on VAO and illustrate what structural features are important for VAO catalysis. For details about the redox properties and biocatalytic potential of VAO, the reader is referred to recent reviews [1–3].

Vanillyl-alcohol oxidase (VAO) was discovered in 1990 during studies on the biodegradation of aromatic compounds by the fungus *P. simplicissimum* [4]. This ascomycete, isolated from paper mill wastewater, was selected for its ability to use veratryl alcohol, a key compound in lignin biodegradation as the sole source of carbon and energy. Upon growth on veratryl alcohol, it was found that *P. simplicissimum* produces relatively large amounts of a yellow protein. Subsequent purification and characterization identified this protein as a covalent flavoenzyme that was able to oxidize vanillyl alcohol to vanillin with a concomitant reduction of molecular oxygen to hydrogen peroxide. Therefore, the enzyme was named vanillyl-alcohol oxidase [5]. Further analysis showed that VAO is an octamer composed of identical subunits of about 65 kDa. From proteolytic fragmentation of the polypeptide chain and fluorescence analysis, the flavin cofactor was identified as 8 α -(N^3)-histidyl)-FAD.

Although the expression of VAO is induced by veratryl alcohol, the enzyme is not involved in the catabolism of veratryl alcohol in *P. simplicissimum* [4]. This raised the question about the physiological significance of VAO. Subsequent growth experiments revealed two other VAO inducers, that is, anisyl alcohol and 4-(methoxymethyl)phenol [6]. The latter compound proved to be a good substrate for VAO and was rapidly degraded by the fungus. From this, it was proposed that VAO is involved in the biodegradation of *p*-cresol ethers, compounds that have never been described in literature as being present in nature.

Up to now, only one VAO analog has been reported. This enzyme from *Byssoschlamys fulva* has been purified but not extensively characterized [7]. On the other hand, under anaerobic conditions, certain *Pseudomonas* species produce a VAO analog with a tetrameric $\alpha_2\beta_2$ structure. These flavocytochromes share similar substrate specificity with VAO and are involved in the biodegradation of *p*-cresol [8], *p*-ethylphenol [9] and eugenol [10]. Instead of oxygen, these enzymes use their heme-containing subunit as the terminal electron acceptor.

2. Results and discussion

2.1. Substrate specificity

Substrate specificity studies showed that VAO is active with a wide range of 4-hydroxybenzylic compounds (Fig. 1). The best substrates are eugenol and chavicol. These

Fig. 1. Reactions catalyzed by VAO. (A) Oxidation of vanillyl alcohol, (B) demethylation of 4-(methoxymethyl)phenol, (C) deamination of vanillyl amine, (D) hydroxylation of eugenol, (E) hydroxylation of 4-propylphenol, and (F) dehydrogenation of 4-butylphenol.

4-allylphenols are converted to the corresponding 4-hydroxycinnamyl alcohols [11]. Isotopic labeling experiments showed that the oxygen atom inserted at the $C\gamma$ atom of the aromatic side chain is derived from water. Binding studies with the competitive inhibitor isoeugenol indicated that VAO preferentially binds the phenolate form of aromatic substrates. From this and the high pH optimum for turnover, a hydride transfer mechanism involving a *p*-quinone methide intermediate was proposed [11].

Vanillyl-alcohol oxidase (VAO) catalyzes the enantioselective hydroxylation of short-chain 4-alkylphenols into the corresponding 1-(4'-hydroxyphenyl)alcohols, with an *ee* of 94% for the *R*-enantiomer [12]. The (*R*)-1-(4'-hydroxyphenyl)alcohol products are not further oxidized to the corresponding ketones because VAO is rather specific for (*S*)-1-(4'-hydroxyphenyl)alcohols [13]. Studies with medium-chain 4-alkylphenols showed that the efficiency of water attack is dependent on the size and/or hydrophobicity of the alkyl side chain. As a result, bulkier 4-alkylphenols are converted to the corresponding 1-(4'-hydroxyphenyl)alkenes (Fig. 1). The relative *cis*–*trans* stereochemistry of these reactions is also dependent on the nature of the alkyl side chain [13].

2.2. Catalytic mechanism

The catalytic mechanism of VAO with 4-(methoxymethyl)phenol proceeds according to the following scheme [14] (Fig. 2):

In the reductive half-reaction, the aromatic substrate is dehydrogenated to the corresponding quinone methide. This electrophilic species is stabilized in the active site of the reduced enzyme (Fig. 3). In the presence of molecular oxygen, the flavin is reoxidized and a stoichiometric amount of hydrogen peroxide is formed. Concomitantly with flavin reoxidation, the quinone methide of 4-(methoxymethyl)phenol is hydrated to the unstable hemiacetal, which decomposes rapidly to 4-hydroxybenzaldehyde and methanol.

The kinetic data of the oxidative demethylation of 4-(methoxymethyl)phenol are in accordance with a ternary complex mechanism in which the reductive half-reaction is rate-limiting in catalysis. However, the kinetic mechanism of VAO is strongly dependent on the type of aromatic substrate. With vanillyl alcohol, the quinone methide intermediate is less stable and the reaction may also follow a ping-pong mechanism [14]. Furthermore, with *p*-cresol, a covalent flavin–substrate adduct is formed which is highly stable under aerobic conditions [15].

2.3. Crystal structure

The crystal structure of VAO has been determined in the free state and in complex with several inhibitors (Table 1) [16]. The enzyme is an octamer with 42 symmetry and each

Fig. 2. Proposed reaction mechanism of VAO with 4-(methoxymethyl)phenol [14]. E-FAD_{ox}, oxidized enzyme; E-FAD_{red}, reduced enzyme.

Fig. 3. Reductive half-reaction of VAO with 4-(methoxymethyl)phenol. 7 μM VAO was anaerobically mixed with 500 μM 4-(methoxymethyl)phenol in the stopped-flow spectrophotometer at 25 $^{\circ}\text{C}$, pH 7.5 [14]. Spectral scans are shown from 5.6 to 965.6 ms with intervals of 80 ms. The absorbance at 450 nm decreases due to flavin reduction. The absorbance increase at 364 nm is ascribed to the formation of the *p*-quinone-methide of 4-(methoxymethyl)phenol.

subunit is built up by two $\alpha\beta$ domains (Fig. 4). The larger domain forms the FAD binding site and the smaller cap domain covers the isoalloxazine ring, which is covalently linked via its 8 α -methyl group to the N3 atom of His422. The active site is formed by an elongated cavity at the *si*-face of the flavin and is solvent inaccessible. Inside this cavity, the aromatic substrate is ideally positioned for hydride transfer to flavin N5. Burying of the substrate underneath the protein surface is a recurrent strategy, common to many flavoenzymes [17].

Table 1
Crystal structures of VAO variants

VAO variant	Inhibitor bound	Resolution ($\text{\AA}$)	PDB entry	Reference
wild type	–	2.5	1VAO	[16]
wild type	<i>p</i> -cresol	2.7	1AHU	[16]
wild type	4-heptenylphenol	3.3	1AHZ	[16]
wild type	isoeugenol	3.1	2VAO	[16]
wild type	2-nitro- <i>p</i> -cresol	3.1	1AHV	[16]
H422A	–	2.2	1QLT	[26]
H422A	isoeugenol	2.4	1QLU	[26]
H61T-holo	4-(trifluoromethyl)phenol	2.1	1E8G	[29]
H61T-apo	ADP	2.6	1E8H	[29]
H61T-apo	–	2.9	1E8F	[29]
D170S	isoeugenol	2.8	1DZN	[32]
D170S/T457E	4-(trifluoromethyl)phenol	2.75	1E0Y	[36]

Fig. 4. Ribbon presentation of a VAO monomer [16]. The histidyl-bound FAD cofactor is shown in ball-and-stick model. This figure was prepared with MOLSCRIPT [37].

Several residues have been implicated from the structure to be involved in substrate binding and substrate conversion (Fig. 5). The structure of VAO linked with isoeugenol revealed that Tyr108, Tyr503 and Arg504 all interact with the 4-hydroxy moiety of the inhibitor, stabilizing its phenolate form. Another key residue is Asp170, which is located close to the N5 atom of the flavin. The negative charge of this aspartate could stabilize the protonated form of the reduced cofactor, increasing the FAD redox potential. The side chain of Asp170 is also close to the propenyl α carbon of isoeugenol. From this, we proposed that Asp170 might act as an active site base, either by activating the water involved in substrate hydroxylation or by deprotonating the *p*-quinone methide in the case of substrate dehydrogenation [16]. The crystal structure also revealed that the substrate specificity of VAO is controlled by a size exclusion mechanism. This is concluded from the fact that the active site is completely filled by the competitive inhibitor 4-(1-heptenyl)phenol.

Fig. 5. Drawing of the active site cavity of VAO with bound inhibitor isoeugenol [16]. The side chain of Asp170 is 3.5 Å from flavin N5 and the C α -atom of isoeugenol. This figure was prepared with MOLSCRIPT [37].

The reactivity of *p*-cresol towards the crystalline enzyme has been investigated by single crystal microspectrophotometry [16]. In agreement with kinetic studies performed in solution [15], it was found that a covalent adduct is formed between the *p*-cresol methyl group and the flavin N5 atom. The high stability of this adduct explains why VAO is almost not active with *p*-cresol, the physiological substrate of the related flavocytochrome *p*-cresol methylhydroxylase (PCMH).

2.4. Novel flavoprotein family

Cloning of the *vaoA* gene provided the necessary amino acid sequence information, which together with the three-dimensional structure established the basis for protein engineering studies [18]. The *vao*-cDNA nucleotide sequence revealed an open reading frame of 1680 base pairs encoding a 560-amino acid protein with a deduced mass of 62,915 excluding the covalently bound FAD. The deduced primary structure of VAO shows 31% sequence identity with the 8 α -(*O*-tyrosyl)-FAD containing a subunit of PCMH. Expression of the *vaoA* gene in *Aspergillus niger* or *Escherichia coli* resulted in active, fully covalently flavinylated enzyme, suggesting that flavinylation is an autocatalytic process. In contrast, for PCMH, it was reported that autocatalytic flavinylation only occurs after binding of the cytochrome subunit [19].

More detailed sequence comparisons established that VAO shows sequence similarity to a large number of other flavoproteins with representatives in all kingdoms [20]. Moreover, from inspection of the three-dimensional structure of VAO, compelling evidence was obtained that the homologous sequences represent a new family of

structurally related oxidoreductases, sharing a conserved FAD-binding domain. To date, this family has already more than 100 members and several crystal structures are known. These include the peripheral membrane respiratory flavoenzyme D-lactate dehydrogenase [21], MurB (UDP-*N*-acetylenolpyruvylglucosamine reductase), an enzyme involved in peptidoglycan biosynthesis [22], the molybdenum iron–sulfur flavoprotein carbon monoxide dehydrogenase [23], the molybdopterin iron–sulfur flavoenzyme xanthine dehydrogenase [24] and the flavocytochrome PCMH [25].

From the multiple-sequence alignment, several VAO-related enzymes were found to harbor a covalently bound FAD. Furthermore, a surprisingly large number of VAO-related proteins with unknown functions appeared to contain a conserved histidine, putatively involved in covalent flavin binding. Thus, unlike the well-known Rossmann fold, this newly identified FAD-binding fold apparently favors covalent flavinylation [20].

2.5. Functional role and mechanism of covalent flavinylation

To study the functional role of the covalent histidyl–FAD bond in VAO, three His422 variants were prepared, which all contained tightly but non-covalently bound FAD [26]. Steady-state kinetics with 4-(methoxymethyl)phenol revealed that all three mutants (H422A, H422T and H422C) were one order of magnitude slower than the wild-type VAO. The crystal structure of H422A (Table 1) established that the decreased activity of this mutant is not caused by structural perturbations. Stopped-flow kinetics showed that the only significant change in the catalytic cycle of the H422A variant is a marked decrease in the rate of enzyme reduction. Further characterization revealed that the deletion of the histidyl–FAD bond decreases the midpoint redox potential from +55 mV (wild-type VAO) to –65 mV (H422A). From this and the fact that covalent flavoenzymes generally display a relatively high redox potential, we postulated that the covalent interaction between the isoalloxazine ring and the protein moiety in specific flavoenzymes might have evolved as a way to contribute to the enhancement of their oxidative power [26]. Recently, this hypothesis was corroborated by studies on cholesterol oxidase from *Brevibacterium sterolicum* [27] and PCMH from *Pseudomonas putida* [28].

From the crystal structure of wild-type VAO, it was deduced that His61, located in the FAD domain, might play a role in linking the flavin to His422 of the cap domain. To study the mechanism of covalent flavinylation in more detail, we prepared the designed mutant His61Thr [29]. In the mutant enzyme, the covalent His422–flavin linkage is not formed, while the enzyme is still able to bind FAD and perform catalysis. In agreement with the supposed functional role of the covalent flavin linkage, H61T was 10-fold less active with 4-(methoxymethyl)phenol than wild-type VAO. The crystal structures of both the holo and apo forms of H61T (Table 1) were highly similar to the structure of wild-type VAO, indicating that binding of FAD does not require major structural rearrangements. Interestingly, the H61T mutant displayed a similar affinity for FAD and ADP, but did not interact with FMN. Soaking of apo enzyme crystals with substrate analogs did not result in ligand binding, indicating that the positioning of the isoalloxazine ring system is a prerequisite for substrate binding. This is different from the situation in, for example, *p*-hydroxybenzoate hydroxylase [30,31] and demonstrates that in VAO, the FAD binds via a typical lock-and-key approach to a binding site that has already been organized [29].

Furthermore, our studies from site-directed mutants show that covalent flavinylation of VAO is an autocatalytic process in which His61 plays a crucial role by activating His422.

2.6. Functional role of Asp170

Asp170, located near the N5-atom of the flavin, has been proposed to act as an active site base. To study the functional role of this acidic residue in more detail, we addressed the properties of four Asp170 variants, D170E, D170S, D170A, and D170N [32]. Spectral analysis, together with the crystal structure of D170S (Table 1), indicated that the Asp170 replacements do not induce major structural changes. However, in D170A and D170N, respectively, 50% and 100% of the flavin are non-covalently bound, suggesting that Asp170 is involved in the process of autocatalytic flavinylation. As self-catalytic covalent flavinylation is thought to occur via initial flavin tautomerization, followed by donation of a proton to the iminoquinone methide form of the flavin [33], Asp170 may act as the proton donor in this process.

Kinetic characterization of the VAO variants revealed that Asp170 is required for catalysis [32]. D170E is 50-fold less active and the other Asp170 variants are about 1000-fold less active than the wild-type VAO. Anaerobic stopped-flow experiments showed that 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 the reduced enzyme and the *p*-quinone methide intermediate. The redox potentials of the covalently FAD-containing mutants D170E (+6 mV) and D170S (−91 mV) are considerably lower than that of wild-type VAO (+55 mV). This supports the idea that Asp170 interacts with the protonated N5-atom of the reduced cofactor, thereby increasing the FAD redox potential.

Product analysis showed that the Asp170 substitutions have profound effects on the hydroxylation efficiency of 4-alkylphenols [34]. D170A and D170S favored the formation of 1-(4'-hydroxyphenyl)alcohols, whereas the D170E mutant favored the formation of alkenes. This suggests that the hydration efficiency of the *p*-quinone methide with these substrates is regulated by the bulkiness of the side-chain of residue 170 and not by its ionic character. It also suggests that Asp170 is not essential for substrate hydroxylation and that the electrophilic enzyme-bound *p*-quinone methide may also react with unactivated water.

2.7. Inversion of stereospecificity

Vanillyl-alcohol oxidase (VAO) and PCMH have similar substrate specificity and the active site architecture of both enzymes is highly conserved [16,25]. However, VAO preferentially converts short-chain 4-alkylphenols to the corresponding (*R*)-1-(4'-hydroxyphenyl)alcohols, whereas PCMH favors the production of the (*S*)-enantiomers [35]. From this, we rationalized that the opposite stereospecificity might be related to the position of the acidic residues in the active site. Glu380 in PCMH is the equivalent of Asp170 in VAO. However, in PCMH, another acidic residue (Glu427) is located at the opposite face of the substrate, which is not present in VAO (Thr457) (Fig. 5). Therefore, we relocated the putative active site base in VAO (Asp170) to the opposite face of the active site cavity

(Glu457) and investigated the double mutants D170A/T457E and D170S/T457E as well as the single mutants D170A, D170S and T457E for their enantioselectivity [36].

The crystal structure of D170S/T457E in complex with trifluoromethylphenol (Table 1) 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. With the exception of D170A, all of the mutants contained fully covalently bound FAD, suggesting that Glu457 can replace Asp170 in the process of covalent flavinylation. The midpoint redox potentials of T457E, D170A/T457E and D170S/T457E were +20 mV, +22 mV and +31 mV, respectively. These values are somewhat lower than that for the wild-type enzyme, but considerably higher than that for D170S ($E_m = -91$ mV). Replacement of T457 by Glu slightly decreased the catalytic efficiency with 4-(methoxymethyl)phenol. The double mutants were rather poor catalysts, confirming that Asp170 is crucial for VAO catalysis. Nevertheless, the double mutants were considerably more active than D170A and D170S [36].

Product analysis showed that the single mutants D170A and D170S preferentially hydroxylated 4-ethylphenol to the (*R*)-enantiomer of 1-(4'-hydroxyphenyl)ethanol (Fig. 6). The lower (*R*)-specificity compared to the wild-type VAO 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. The double mutants D170A/T457E and D170S/T457E exhibited an inverted stereospecificity with 4-ethylphenol. Especially D170S/T457E was 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 C α -atom of the substrate analog trifluor-

Fig. 6. Stereospecificity of hydroxylation of 4-ethylphenol by wild-type VAO and Asp170/Thr457 mutants [36]. (*R*)-1-(4'-hydroxyphenyl)ethanol (black) and (*S*)-1-(4'-hydroxyphenyl)ethanol (white).

omethylphenol. 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, was highly specific for the (*R*)-enantiomer of the alcohol (Fig. 6). 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.

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