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Vanillyl-alcohol oxidase, a tasteful biocatalyst

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Abstract

The covalent flavoenzyme vanillyl-alcohol oxidase (VAO) is a versatile biocatalyst. It converts a wide range of phenolic compounds by catalysing oxidation, deamination, demethylation, dehydrogenation and hydroxylation reactions. The production of natural vanillin, 4-hydroxybenzaldehyde, coniferyl alcohol and enantiomeric pure phenol derivatives is of interest for biotechnological applications. The hydroxylation of 4-alkylphenols is highly stereospecific for the (R)-isomer, whereas dehydrogenation of these substrates is specific for the *cis*- or *trans*-isomer. On the basis of crystallographic data, we suggest that the stereospecificity is related to the active site residue Asp170. Another important feature of VAO is the covalent flavin attachment. Studies from site-directed mutants suggest that the covalent flavin–protein interaction improves the catalytic performance as well as the long-term stability of VAO. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) is a flavoprotein from the ascomycete *Penicillium simplicissimum*, which originally was shown to catalyse the oxidation of vanillyl alcohol to the flavour compound vanillin [1]. This relatively stable enzyme is a homooctamer with each 64-kDa monomer containing a flavin adenine dinucleotide (FAD) as covalently bound prosthetic group.

The biological function of VAO in the fungus is not fully understood. On the basis of induction experiments, we have proposed that 4-(methoxymethyl)phenol represents the physiological substrate of the enzyme [2]. The catalytic mechanism of VAO with 4-(methoxymethyl)phenol has been studied in great detail [3]. The reaction cycle with this substrate involves the initial transfer of a hydride from the Cα-atom of the substrate to the flavin N5-atom. The formed binary complex between reduced enzyme and the *p*-quinone methide product of 4-(methoxymethyl)phenol then reacts with molecular oxygen, regenerating the oxidised flavin. Subsequently, the *p*-quinone methide product reacts with water in the enzyme active site to form the final products 4-hydroxybenzaldehyde and methanol (Fig. 1).

Besides the oxidation of vanillyl alcohol and the demethylation of 4-(methoxymethyl)phenol, the enzyme also performs deamination, hydroxylation and

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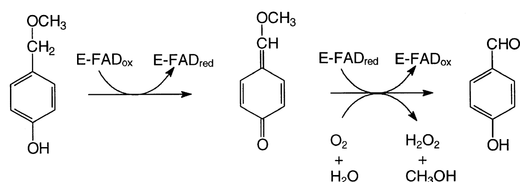

Fig. 1. Reaction mechanism of VAO with 4-(methoxymethyl)phenol.

dehydrogenation reactions [4,5]. Because of this versatile catalytic power, VAO may develop as a useful biocatalyst for biotechnological applications. In this paper, we address some features of the enzyme, which are of industrial relevance. Special emphasis is given to the structural properties of the enzyme active site, the production of natural flavouring compounds and the stereospecific conversion of prochiral substrates.

2. Structural properties

Sequence alignments using the primary structure of VAO [6] revealed that the enzyme can be considered as the prototype of a novel family of structurally related oxidoreductases sharing a conserved FAD-binding domain [7]. This family of flavoproteins is spread throughout all life forms and is involved in diverse metabolic pathways.

The crystal structure of VAO has been solved in the native state and in complex with several aromatic ligands [8]. The VAO momoner consists of two domains, with the cap domain covering the active site and the larger domain creating a binding site for the FAD cofactor. The FAD is covalently bound to His422 of the cap domain. The active site cavity of VAO is located in the interior of the protein and is completely solvent inaccessible (Fig. 2). Inside the cavity, Tyr108, Tyr503 and Arg504 form an anionic-binding pocket, stabilising the phenolate form of the substrate. The carboxylic side chain of Asp170 is located close to the flavin N5-atom and Arg398 and is likely deprotonated. We have suggested that Asp170 facilitates catalysis by acting as an active

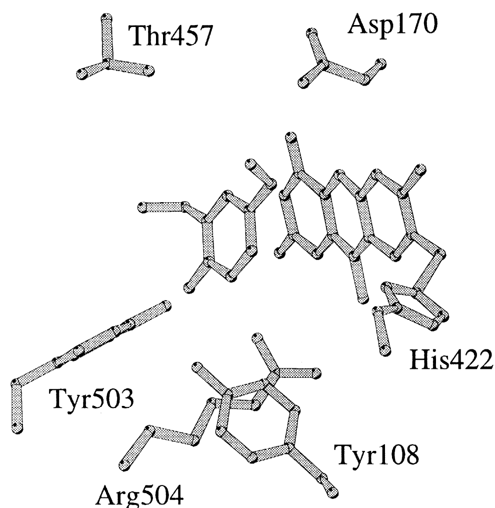

Fig. 2. Active site of VAO with bound isoeugenol. The figure was prepared with MOLSCRIPT [19].

site base and/or by stabilising the reduced form of the flavin cofactor [8].

3. Product spectrum

VAO readily oxidises a wide range of *para*-substituted phenols into interesting flavouring compounds (Fig. 3) [4]. VAO efficiently demethylates its physiological substrate 4-(methoxymethyl)phenol into 4-hydroxybenzaldehyde, which is next to vanillin the most important constituent of vanilla. Vanillin on the other hand, is formed by the VAO-mediated

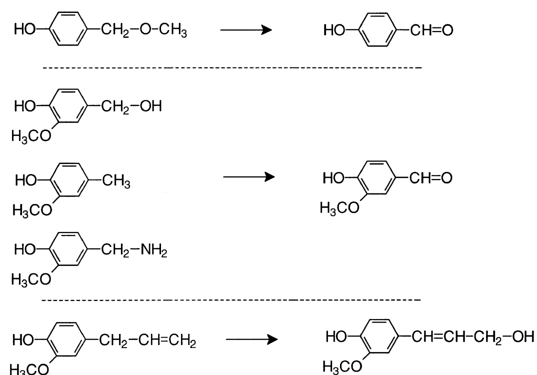

Fig. 3. Formation of flavouring compounds by VAO.

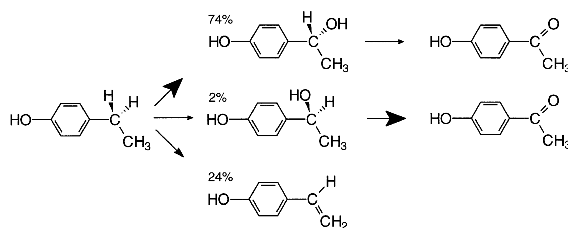

Fig. 4. Reaction of VAO with 4-ethylphenol.

conversion of vanillyl amine, vanillyl alcohol and creosol. In addition, VAO stoichiometrically hydroxylates eugenol, the main component of clove, to coniferyl alcohol [9].

VAO also displays a remarkable reactivity towards 4-alkylphenols, bearing aliphatic side chains of up to seven carbon atoms. Optimal catalytic efficiency occurs with 4-ethylphenols and 4-*n*-propylphenols. Short-chain 4-alkylphenols are mainly hydroxylated at the Cα-position to 1-(4′-hydroxyphenyl)alcohols, whereas medium-chain 4-alkylphenols are dehydrogenated to 1-(4′-hydroxyphenyl)alkenes [5]. Preliminary results indicate that the dehydrogenation of 4-alkylphenols is promoted in the presence of monovalent anions and by a low water content. The hydroxylation of 4-alkylphenols is highly stereospecific for the *R*-isomer with an *e.e.* of 94% [10]. Furthermore, VAO oxidises the *S*-isomers of 1-(4′-hydroxyphenyl)alcohols far more efficient than the *R*-isomers, yielding highly pure (*R*)-1-(4′-hydroxyphenyl)alcohols (Fig. 4) [5]. In addition to stereospecific hydroxylation, and depending on the type of alkyl side chain, VAO dehydrogenates medium-chain 4-alkylphenols stereospecifically into *cis*- or *trans*-1-(4′-hydroxyphenyl)alkenes [5]. Again, these aromatic alkenes are flavouring compounds. A well-known example is 4-vinylphenol, which, among others, is present in beer and wine [11].

4. Covalent flavin binding

The rationale for the covalent binding of the flavin prosthetic group in certain flavoproteins is unclear as is the mechanism by which the covalent bond is formed [12]. It has been suggested that covalent binding might prevent inactivation of the cofactor [13], improve protein stability and resistance against proteolysis, and increase the reactivity of the flavin [14,15]. We have started to address the mechanism of covalent flavin binding in VAO by a protein engineering approach [16,17]. More insight into this mechanism may lead to the design of covalent flavoproteins, which originally contained a dissociable flavin cofactor [18].

In a first approach, we selectively changed the active site residue Asp170 into Glu, Asn and Ser [16]. Characterisation of these Asp170 mutant proteins established that Asp170 is crucial for efficient redox catalysis and is involved in activating the flavin ring for covalent attachment. Furthermore, these studies revealed that the generation of stable flavin-dissociable VAO mutants is feasible. His422, the target residue of covalent flavinylation of VAO, was selectively replaced by Ala, Thr and Cys [17]. All three mutant enzymes contain noncovalently tightly bound FAD, showing that covalent flavinylation is not essential for efficient FAD binding. Moreover, the crystal structure of His422Ala revealed that deletion of the covalent protein–flavin interaction does not result in any significant structural perturbation. Kinetic studies showed that all His422 mutants are poorly active due to a strongly decreased rate of flavin reduction. Furthermore, slow flavin reduction is linked to a strong decrease in the redox potential of protein-bound FAD. From this, we conclude that formation of a histidyl-flavin bond in specific flavoenzymes might have evolved as a way to contribute to the enhancement of their oxidation power [17].

Besides improving the catalytic performance of VAO, the covalent attachment of the flavin might also be important for the (operational) stability of the enzyme. Preliminary experiments showed that the long-term stability of wild-type VAO is considerably higher than that of the mutant enzymes. This suggests that covalent tethering of the flavin is indeed advantageous for practical applications.

5. Conclusions

VAO can be considered as the protype of a novel family of oxidoreductases sharing a conserved FAD-

binding fold. The enzyme is a relatively stable covalent flavoprotein, which produces a wide range of interesting flavouring compounds and enantiomeric pure phenol derivatives. Studies from site-directed mutants have revealed that covalent flavin attachment improves the catalytic performance as well as long-term stability of VAO. Presently, we are investigating the role of several active site residues in enzyme catalysis. These studies are of utmost importance for the future design of VAO variants with alternative substrate specificities.

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