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Covalent flavinylation enhances the oxidative power of vanillyl-alcohol oxidase

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Abstract

Vanillyl-alcohol oxidase (VAO) from *Penicillium simplicissimum* is an inducible flavoprotein that is active with a wide range of phenolic 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. Studies from His422 variants revealed that deletion of the histidyl–flavin bond does not result in any significant structural change. However, the covalent interaction increases the redox potential of the flavin, facilitating substrate oxidation. His61, located in the FAD-binding domain, is involved in the autocatalytic process of covalent flavinylation. This could be nicely demonstrated by creating the H61T mutant enzyme which binds the flavin in a non-covalently mode. Similar to the noncovalent His422 variants, H61T is 10-fold less active than wild-type VAO. From this and the similar crystal structures of apo and holo H61T it is concluded that the FAD binds to a preorganized binding site where His61 activates His422 for autocatalytic flavinylation.
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Keywords: Vanillyl-alcohol oxidase; Covalent flavoprotein; Protein stability; Phenolic compounds; Site-directed mutagenesis

1. Introduction

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is a covalent flavoprotein that is active with a wide range of phenolic compounds (Fig. 1) [1,2]. The enzyme is a homooctamer of about 510 kDa with each subunit containing two domains (Fig. 2) [3]. The larger FAD-binding domain is conserved in many other flavoproteins [4], whereas the smaller cap domain covers the isoalloxazine ring which is covalently linked via its 8α-methyl group to the N3 atom of His422 [3]. Covalent attachment of a flavin cofactor has been encountered in a variety of flavoenzymes, but the rationale for this type of protein–ligand interaction is poorly understood [5]. Recently, it was shown that covalent tethering of the flavin can prevent inactivation of the cofactor [6] or facilitate intermolecular electron transfer [7]. Besides from being beneficial for catalysis, the covalent protein–flavin interaction might also improve protein stability or be favorable for flavoenzymes that are

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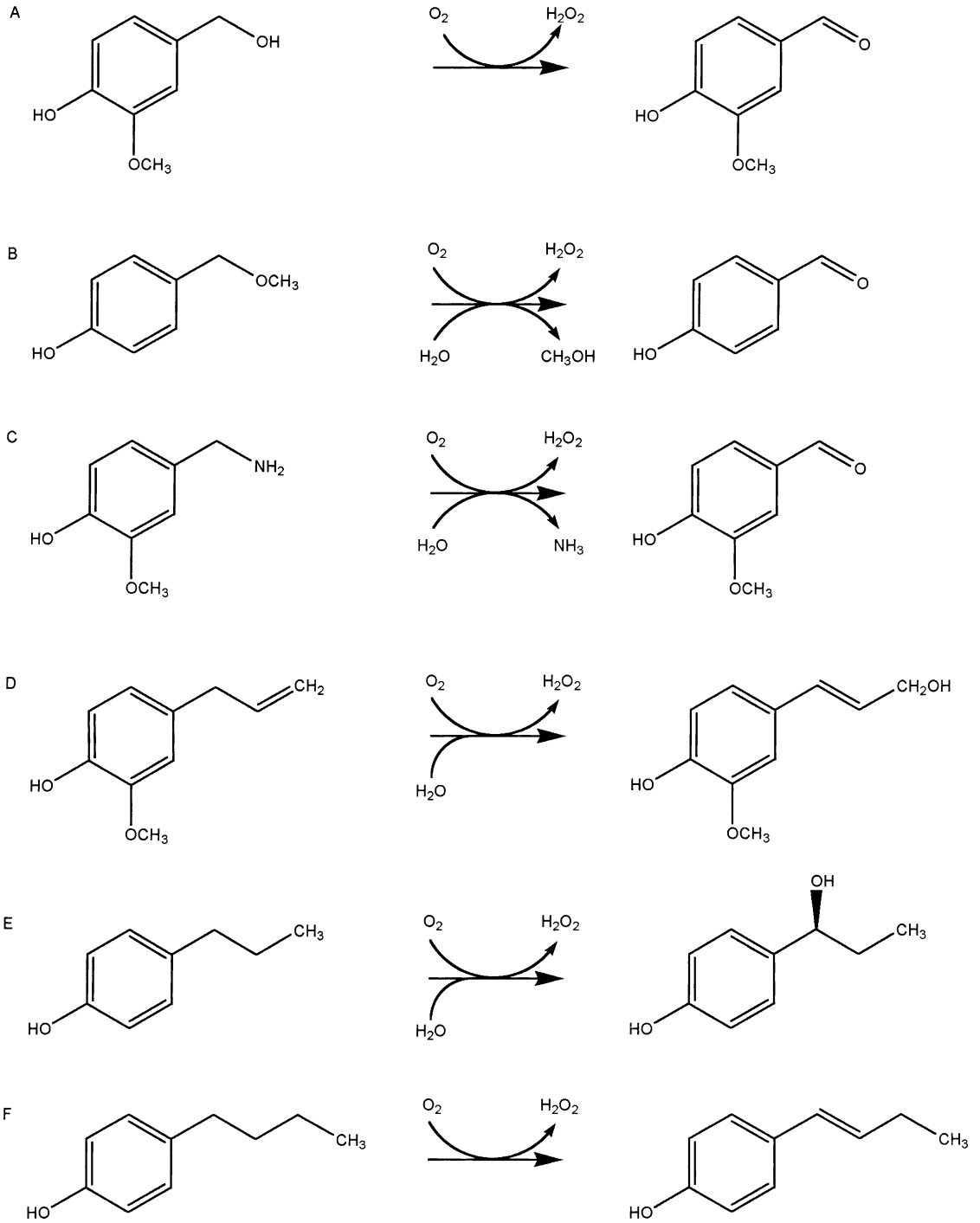

Fig. 1. Reactions catalyzed by VAO. (A) Oxidation of vanillyl alcohol; (B) demethylation of 4-(methoxymethyl)phenol; (C) deamination of vanillyl amine; (D) hydroxylation of eugenol; (E) hydroxylation of 4-propylphenol and (F) dehydrogenation of 4-butylphenol.

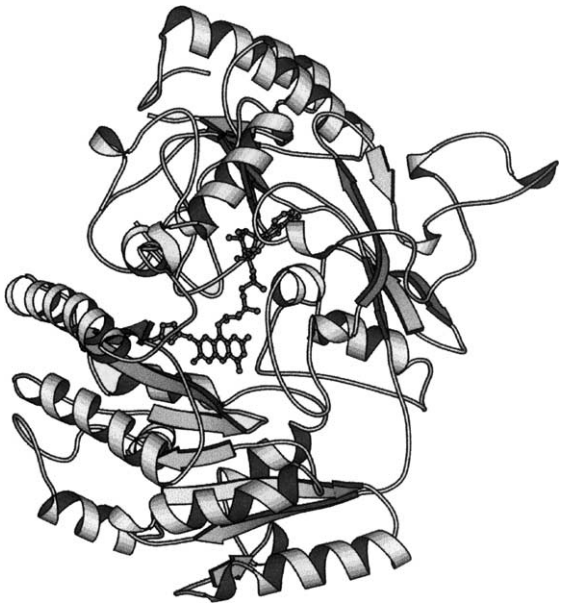

Fig. 2. Ribbon presentation of a VAO monomer [8]. The histidyl-bound FAD cofactor is shown in *ball-and-stick* model.

localized in a flavin-deficient environment [8]. Because the performance of flavoenzymes is often limited by the dissociation of the flavin cofactor under stressful conditions, tailor-made covalent flavoenzymes could be of great value for biotechnological applications. With this in mind we have addressed the role of the histidyl–flavin bond in VAO by site-directed mutagenesis [8–10]. In short, our studies have revealed that covalent flavinylation is an autocatalytic process and that the covalent interaction between the isoalloxazine ring and the protein moiety enhances the oxidative power of VAO. The knowledge gained from these studies will be valuable for the future design of biotechnologically relevant flavoenzymes.

2. Functional role of histidyl–flavin bond

To study the functional role of the covalent histidyl–FAD bond in VAO, three His422 variants were created by site-directed mutagenesis. All three mutant proteins, H422A, H422T and H422C, contained tightly but non-covalently bound FAD [8]. Steady-state kinetics with 4-(methoxymethyl)phenol revealed that the mutant enzymes were one order of magnitude slower than wild-type VAO. In contrast to observations with some other mutated covalent flavoenzymes lacking the covalent linkage [6,11,12], the activity remained constant during prolonged incubations with substrate. This indicates that the FAD cofactor remains bound to the protein and is not inactivated during catalysis. The crystal structure of H422A clearly established the absence of the covalent histidyl–flavin bond and confirmed 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 suggested 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 [8]. Recently, this hypothesis was corroborated by studies from cholesterol oxidase from *Brevibacterium sterolicum* [13]and PCMH from *Pseudomonas putida* [14].

3. Mechanism of covalent flavinylation

From its position at the domain interface we inferred that His61, located in the FAD domain, might play a role in linking the flavin to His422 of the cap domain. To test this hypothesis, we prepared the mutant enzyme H61T [10]. This VAO variant weakly bound FAD and could be purified in its apo form. In agreement with the supposed functional role of the covalent flavin linkage, H61T reconstituted with FAD was 10-fold less active with 4-(methoxymethyl)phenol than wild-type VAO. The crystal structures of both the holo and apo form of H61T (Fig. 3) were highly similar to the structure of wild-type VAO, indicating that binding of FAD does not require major structural rearrangements. Fluorescence binding studies revealed that H61T has a similar affinity for FAD and ADP, but does not interact with FMN. These studies show that covalent flavinylation of VAO is an autocatalytic process in which His61 plays a crucial role by activating

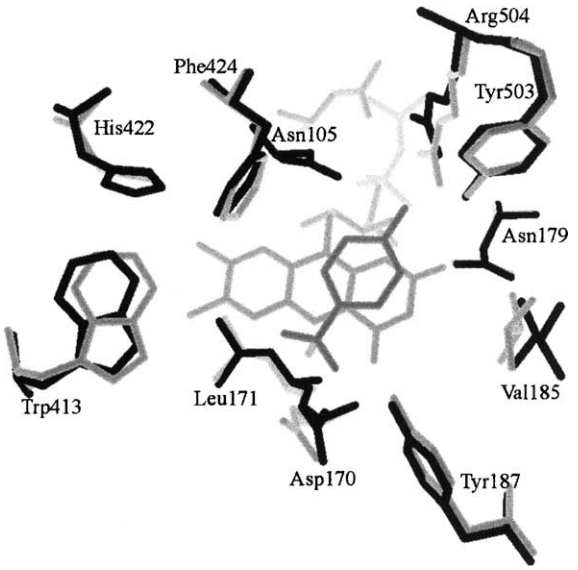

Fig. 3. Superposition of active site residues in the apo (dark) and the holo form complexed with 4-(trifluoromethyl)phenol (light) structures of the VAO H61T mutant [10].

His422. Furthermore, our studies clearly demonstrate that in VAO, the FAD binds via a typical lock-and-key approach to a preorganized binding site [10].

Asp170, located near the N5 atom of the flavin, has been proposed to act as an active site base [3]. Studies from D170E, D170S, D170A, and D170N variants indicated that Asp170 plays some role in covalent flavinylation [9]. Spectral analysis, together with the crystal structure of D170S, showed that the Asp170 replacements do not induce major structural changes. However, in D170A and D170N, respectively 50 and 100% of the flavin is non-covalently bound. This suggests that in VAO, Asp170 may act as the proton donor in the process of autocatalytic flavinylation.

4. Conclusions

VAO is a covalent flavoprotein that catalyzes transformations of industrial relevance. VAO products of biotechnological interest include vanillin, coniferyl alcohol and enantiomeric pure 1-(4′-hydroxyphenyl)-alcohols. Studies from site-directed mutants established that the covalent flavin attachment enhances the oxidative power of VAO. Moreover, covalent flavinylation was found to be an autocatalytic process. This provides interesting opportunities for the future design of covalent flavin-dependent biocatalysts from their non-covalent counterparts.

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