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Regio- and Stereospecific Conversion of 4-Alkylphenols by the Covalent Flavoprotein Vanillyl-Alcohol Oxidase

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The regio- and stereospecific conversion of prochiral 4-alkylphenols by the covalent flavoprotein vanillyl-alcohol oxidase was investigated. The enzyme was active, with 4-alkylphenols bearing aliphatic side chains of up to seven carbon atoms. Optimal catalytic efficiency occurred with 4-ethylphenol and 4-\(\alpha\)-propylphenols. These short-chain 4-alkylphenols are stereoselectively hydroxylated to the corresponding (R)-1-(4'-hydroxyphenyl)alkyl alcohols (F. P. Drijfhout, M. W. Fraaije, H. Jongejan, W. J. H. van Berkel, and M. C. R. Franssen, Biotechnol. Bioeng. 59:171–177, 1998). (S)-1-(4'-Hydroxyphenyl)ethanol was found to be 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)alkanes. 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 masses 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 stereocchemical outcome of vanillyl-alcohol oxidase-mediated conversions of 4-alkylphenols.

Vanillyl-alcohol oxidase (EC 1.1.3.38) 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 (2). The biological function of vanillyl-alcohol oxidase is unknown, but recent studies have indicated that the enzyme is involved in the biodegradation of 4-(methoxymethyl)phenol (6). Vanillyl-alcohol oxidase is a homotetramer, with each 64-kDa subunit containing an 8\(\alpha\)-(2\(\bar{\text{N}}\))-histidyl)-flavin adenine dinucleotide (FAD) as a covalently bound prosthetic group (2). The vaoA gene has been cloned (1), and the vanillyl-alcohol oxidase structure has been determined at 2.5-\(\AA\) resolution (11). These studies, together with sequence alignments, have revealed that the enzyme belongs to a novel oxidoreductase family sharing a conserved FAD binding domain (8). Vanillyl-alcohol oxidase is a versatile biocatalyst, mechanistically. It can convert a wide range of 4-hydroxybenzylic compounds by catalyzing oxidation, hydroxylation, demethylation, deamination, and desaturation reactions (3). Some of these reactions are of particular interest for biotechnological applications (16).

Based on studies with eugenol and 4-(methoxymethyl)phenol, we have proposed that vanillyl-alcohol oxidase catalysis involves the initial transfer of a hydride from the C-\(\alpha\) atom of the substrate to N-5 of the flavin cofactor (5, 7). 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).

A similar reaction mechanism has been proposed for the conversion of 4-alkylphenols by the related bacterial flavocytochrome p-cresol methyldemethylase (10, 13). Further support for the hydride transfer mechanism comes from crystallographic data (11). The vanillyl-alcohol oxidase 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 C-\(\alpha\) atom of the substrate. However, the exact function of this residue in catalysis remains to be elucidated.

Vanillyl-alcohol oxidase displays a remarkable reactivity towards short-chain 4-alkylphenols. Recent studies showed that 4-ethylphenol and 4-\(\alpha\)-propylphenols are stereospecifically converted into the corresponding 1-(4'-hydroxyphenyl)alkyl alcohols with an e.e. of 94\% for the \(R\) enantiomers. During these reactions, considerable amounts of 1-(4'-hydroxyphenyl)alkanes are formed as side products, indicating that rearrangement of the p-quinone methide intermediate competes with water addition (3). 4-Methylphenol, the parent substrate of p-cresol methyldemethylase, is a very poor substrate for vanillyl-alcohol oxidase. Crystallographic and kinetic data suggest that this is due to the stabilization of a flavin N-5 adduct (9, 11). To obtain more insight into the catalytic performance of this unusual flavoenzyme, we have addressed the reactivity and stereochemistry of vanillyl-alcohol oxidase 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.

MATERIALS AND METHODS

**Chemicals.** 4-Methylphenol, 4-ethylphenol, 4-\(\alpha\)-propylphenol, 2-methoxy-4-\(\alpha\)-propylphenol, 4-isopropylphenol, 4-n-butylphenol, 4-sec-butylphenol, 4-(2'-methylcrotyl)phenol, 5-indan, 5,6,7,8-tetrahydro-2-naphthol, vanillyl alcohol (4-hydroxy-3-methoxybenzenaldehyde), tyramine [4-(2-aminoethyl)phenol], trans-stilbene [1-(4'-hydroxy-3'-methoxyphenyl)propene], 3-(4'-hydroxyphenyl)propanol, 4-phenylphenol, and 4-benzylphenol were from Aldrich. 4-n-Pentylphenol, 4-\(\alpha\)-heptylphenol, 4-\(\alpha\)-non-
Vanillyl-alcohol oxidase was purified from *P. simplicissimum* (Oudem.) Thom. CIBS 170.90 (ATCC 90172) as described previously (5).

**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 by using a molar absorption coefficient, ε_{265}, of 12.5 mM^{-1} cm^{-1} for protein-bound FAD (2). Vanillyl-alcohol oxidase activity was determined by monitoring absorption spectrum changes of aromatic substrates or by oxygen consumption experiments using a Clark electrode. Vanillin production was measured at 340 nm (ε_{270} = 1.3 mM^{-1} cm^{-1}).

**Results**

**Substrate specificity.** Earlier studies revealed that vanillyl-alcohol oxidase has a relaxed substrate specificity towards 4-hydroxybenzylcyclic compounds (3, 5) (Fig. 2). 4-Ethylphenol, 4-n-propylphenol, and 2-methoxy-4-n-propylphenol are efficiently oxidized by vanillyl-alcohol oxidase (9). In this study, we investigated the substrate specificity in further detail. Medium-chain 4-alkylphenols with aliphatic side chains of up to seven carbon atoms were converted by vanillyl-alcohol oxidase. 4-Alkylphenols with longer aliphatic side chains, like 4-n-nonylphenol, did not react. Table 1 shows that straight-chain 4-alkylphenols interact tightly with vanillyl-alcohol oxidase and that the catalytic efficiency (expressed as k_{cat}/K_m) is optimal at an alkylnonylphenol length of three carbon atoms. Substituents in the aliphatic side chain significantly affected the catalytic efficiency of vanillyl-alcohol oxidase. Like 4-isopropylphenol and 4-sec-butylyphenol, frambinon [1-(4’-hydroxyphenyl)-2-butanol] was converted at a significant rate (Table 1). As 4-allylphenols are among the best substrates of vanillyl-alcohol oxidase, 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. Vanillyl-alcohol oxidase 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 alkanoates. This might be due to the high enantioselectivity of the initial hydroxylation reaction, which predominantly yields the R isomer (3). 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 vanillyl-alcohol oxidase 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 (3). This suggests that for these substrates, the same kinetic resolution mechanism is operative. Vanillyl-alcohol oxidase reacted poorly with C-β and C-γ hydroxylated 4-hydroxyphenyl alkanoates (Table 1). In line with this, the enzyme was not active with 4-(aminoalkyl)phenols including tyramine.

**Product identification.** Vanillyl-alcohol oxidase produces different amounts of 1-(4’-hydroxyphenyl)alcohols and 1-(4’-hydroxyphenyl)alkenes from short-chain 4-alkylphenols (3).
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 C\textsubscript{18} 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 and 256 nm, respectively, and minor differences in the characteristic aromatic shoulder near 290 nm (Fig. 3B). The mass spectra of the products were identical, with a molecular ion at m/z 256 (96%) and the following diagnostic fragments with more than 25% abundance: 147 (35%), 133 (100%), and 105 (37%). This fragmentation pattern is indicative of 2-(4'-hydroxyphenyl)-sec-butene (Fig. 4A). The second allyllic product contained a molecular ion at m/z (relative intensity) (M+\textsuperscript{+}) 148 (96%) and the following diagnostic fragments with more than 25% abundance: 147 (35%), 133 (100%), and 105 (37%). This fragmentation pattern is indicative of 2-(4'-hydroxyphenyl)-sec-butene (Fig. 4A). The second allyllic product contained a molecular ion at m/z (relative intensity) (M+\textsuperscript{+}) 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)propene.

Moreover, from the identical HPLC elution time and absorption spectrum of the main allyllic product and the reference compound trans-isoeugenol, it is evident that trans-isoeugenol is formed in large excess over the cis isomer (Table 3).

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+\textsuperscript{+}) 148 (96%) and the following diagnostic fragments with more than 25% abundance: 147 (35%), 133 (100%), and 105 (37%). This fragmentation pattern is indicative of 2-(4'-hydroxyphenyl)-sec-butene (Fig. 4A). The second allyllic product contained a molecular ion at m/z (relative intensity) (M+\textsuperscript{+}) 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, comparisons 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.
No alcoholic products are formed in the reaction of vanillyl-alcohol oxidase 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 C-α proton and C-β proton, respectively. This established that the aromatic butene has a cis-relative stereochemistry (Table 3). HPLC analysis of the enzymatic conversion of 4-n-pentylphenol showed a mixture of two alkenylic products. The mass spectra of the 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: 163 (71%), 145 (63%), 127 (37%), 115 (26%), 107 (100%), and 43 (47%). This points towards the formation of the C-γ 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$_2$O gave bands at $\delta$ 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). The $^1$H-NMR spectrum in D$_2$O gave bands at $\delta$ 1.85 (3H, s, methyl), 4.99 (d, $J = 2.0$ Hz, 1H, vinylic), 5.03 (d, $J = 2.1$ Hz, 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), and 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 C-α and C-β protons have a trans-relative stereochemistry.

Substantial differences in the ratios of aromatic products were observed when vanillyl-alcohol oxidase 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 favored 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 vanillyl-alcohol oxidase (5). 4-Vinylphenol, one of the products formed from the enzymatic conversion of 4-ethylphenol (3), appeared to be a very strong competitive inhibitor for vanillyl-alcohol oxidase ($K_i \approx 3 \pm 1 \mu M$ [Fig. 7]). Similar inhibition during the conversion of 4-ethylphenol was reported for p-cresol methylhydroxylase (13). 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 interact tightly with the enzyme. This is supported by the crystal structures of vanillyl-alcohol oxidase in complex with isoeugenol and 1-(4'-hydroxyphenyl)heptene (11).

**DISCUSSION**

In this study, we have described the reactivity of the covalent flavoprotein vanillyl-alcohol oxidase with medium-chain 4-alkylphenols, bicyclic phenols, and 4-hydroxyphenyl alcohols. The enzyme was active, with 4-alkylphenols bearing aliphatic side chains of up to seven carbon atoms. This agrees perfectly with structural data which showed that the active-site cavity of vanillyl-alcohol oxidase is completely filled upon binding of the inhibitor 1-(4'-hydroxyphenyl)heptene (11).

During the conversion of short-chain 4-alkylphenols by vanillyl-alcohol oxidase, (R)-1-(4'-hydroxyphenyl)alkanones are formed as major products (3). 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)alkanes (Fig. 8). The low catalytic efficiency of the conversion of the R isomers suggests that the binding of 1-(4'-hydroxyphenyl)alkanones is energetically unfavored when the C-α hydroxyl group of the substrate faces the flavin ring. Interestingly, differences in the stereospecificities of oxidation of 1-(4'-hydroxyphenyl)alkanones were reported for the related flavocytochromes 4-ethylphenol methylenehydroxylase (15) and p-cresol methylhydroxylase (12).

Besides catalyzing stereoselective hydroxylation reactions, vanillyl-alcohol oxidase is regioselective as well. Whereas 4-alkylphenols are exclusively hydroxylated at the C-α atom, 4-alkenylphenols, like eugenol, are hydroxylated at the C-γ atom (5). These differences in regioselective hydroxylation suggest that the site of water attack is dependent on the delocalization of

![FIG. 6. Mass-spectral analysis of aromatic products formed from the conversion of 4-(3'-methylcrotyl)phenol by vanillyl-alcohol oxidase from P. simplicissimum. (A) 4-(3'-Methyl-1'-butene-3'-ol)phenol. (B) 4-(3'-Methylbutadiene)-phenol.](image)

![FIG. 7. Competitive inhibition of vanillyl-alcohol oxidase by 4-vinylphenol.](image)

![FIG. 8. Reaction pathway for the conversion of 4-ethylphenol by vanillyl-alcohol oxidase.](image)
charge in the enzyme-bound p-quinone methide product intermediate. As noted before (11), Asp170 might activate the water, thereby acting as an active-site base. Interestingly, the eugenol derivative 4-(3′-methylcrotolyl)phenol is not exclusively hydroxylated at the C-γ position. With this 4-allylphenol, dehydrogenation at the C-δ position occurs to a significant extent.

In contrast to short-chain 4-alkylphenols, medium-chain 4-alkylphenols are exclusively converted by vanillyl-alcohol oxidase 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 vanillyl-alcohol oxidase with medium-chain 4-alkylphenols did not influence the stoichiometric formation of the alkenic products, indicating that rearrangement of the p-quinone methide intermediate occurs in the enzyme active site. Based on the crystal structures of vanillyl-alcohol oxidase-inhibitor complexes (11), 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 C-δ atom of the inhibitors (11). 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 vanillyl-alcohol oxidase 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-coenzyme A dehydrogenases introduce a cis double bond between C-2 and C-3 of their coenzyme A substrates (14), whereas glycolate oxidase shows specificity for re hydrogen abstraction when prochiral glycolate is used as a substrate (4). In contrast to these enzymes, the relative cis-trans stereochirality of vanillyl-alcohol oxidase is strongly dependent on the nature of the alkyl side chain of the substrate. However, no correlation was found between the stereochemical preference and the bulkiness or length of the 4-alkylphenol side chains.

In summary, the results presented here show that vanillyl-alcohol oxidase 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 vanillyl-alcohol oxidase-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.

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