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Substrate specificity of flavin-dependent vanillyl-alcohol oxidase from *Penicillium simplicissimum*

Evidence for the production of 4-hydroxycinnamyl alcohols from 4-allylphenols

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The substrate specificity of the flavoprotein vanillyl-alcohol oxidase from *Penicillium simplicissimum* was investigated. Vanillyl-alcohol oxidase catalyzes besides the oxidation of 4-hydroxybenzyl alcohols, the oxidative deamination of 4-hydroxybenzylamines and the oxidative demethylation of 4-(methoxy-methyl)phenols. During the conversion of vanillylamine to vanillin, a transient intermediate, most probably vanillylimine, is observed.

Vanillyl-alcohol oxidase weakly interacts with 4-hydroxyphenylglycols and a series of catecholamines. These compounds are converted to the corresponding ketones. Both enantiomers of (nor)epinephrine are substrates for vanillyl-alcohol oxidase, but the *R* isomer is preferred.

Vanillyl-alcohol oxidase is most active with chavicol and eugenol. These 4-allylphenols are converted to coumaryl alcohol and coniferyl alcohol, respectively. Isotopic labeling experiments show that the oxygen atom inserted at the Cγ atom of the side chain is derived from water. The 4-hydroxycinnamyl alcohol products and the substrate analog isoeugenol are competitive inhibitors of vanillyl alcohol oxidation.

The binding of isoeugenol to the oxidized enzyme perturbs the optical spectrum of protein-bound FAD. pH-dependent binding studies suggest that vanillyl-alcohol oxidase preferentially binds the phenolate form of isoeugenol ($pK_a < 6$, 25 °C). From this and the high pH optimum for turnover, a hydride transfer mechanism involving a *p*-quinone methide intermediate is proposed for the vanillyl-alcohol-oxidase-catalyzed conversion of 4-allylphenols.

Keywords: 4-allylphenols; aromatic alcohol oxidase; covalently bound flavin; substrate specificity; vanillin.

Several fungi produce aryl-alcohol oxidases that are involved in the biodegradation of lignin, the most abundant aromatic biopolymer (de Jong et al., 1994). The monomeric aryl-alcohol oxidases are excreted by basidiomycetes and contain non-covalently bound FAD as a prosthetic group (Guillén et al.,

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Abbreviations. Adrenalone, 3′,4′-dihydroxy-2-methylaminoacetophenone; 4-allylanisole, 4-methoxy-allylbenzene; anisyl alcohol, 4-methoxybenzyl alcohol; chavicol, 4-allylphenol; cinnamyl alcohol, 3-phenyl-2-propene-1-ol; coniferyl alcohol, 4-hydroxy-3-methoxycinnamyl alcohol; coumaryl alcohol, 4-hydroxycinnamyl alcohol; epinephrine, 3,4-dihydroxy-α-(methylaminomethyl)benzyl alcohol (adrenalin); eugenol, 4-allyl-2-methoxyphenol; homovanillyl alcohol, 4-hydroxy-3-methoxyphenethyl alcohol; isoeugenol, 2-methoxy-4-propenylphenol; isosafrole, 1,2-(methylenedioxy)-4-propenylbenzene; metanephrine, 4-hydroxy-3-methoxy-α-(methylaminomethyl)benzyl alcohol; norepinephrine, α-(aminomethyl)-3,4-dihydroxybenzyl alcohol (noradrenalin); normetanephrine, α-(aminomethyl)-4-hydroxy-3-methoxybenzyl alcohol; octopamine, α-(aminomethyl)-4-hydroxybenzyl alcohol; safrole, 4-allyl-1,2-(methylenedioxy)benzene; synephrine, 4-hydroxy-α-(methylaminomethyl)benzyl alcohol; vanillyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol; vanillylamine, 4-hydroxy-3-methoxybenzylamine; vanillin, 4-hydroxy-3-methoxybenzaldehyde; veratryl alcohol, 3,4-dimethoxybenzyl alcohol.

Enzymes. Alcohol oxidase (EC 1.1.3.13); catalase, hydrogen-peroxide oxidoreductase (EC 1.11.1.6); glutamate dehydrogenase (EC 1.4.1.3); vanillyl-alcohol oxidase (EC 1.1.3.7).

1992). By catalyzing the oxidation of the secondary metabolites veratryl alcohol (3,4-dimethoxybenzyl alcohol) and anisyl alcohol (4-methoxybenzyl alcohol), the extracellular aryl-alcohol oxidases generate hydrogen peroxide, which is an essential substrate for the ligninolytic process (de Jong et al., 1994).

In a previous report, we described a novel type of aryl-alcohol oxidase isolated from the ascomycete *Penicillium simplicissimum* (de Jong et al., 1992). This intracellular enzyme catalyzes the oxidation of vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol) to vanillin (4-hydroxy-3-methoxy benzaldehyde) and is a homooctamer with each subunit containing 8α-(N^3-histidyl)-FAD as a covalently bound prosthetic group. Urea unfolding has shown that the enzyme is also active as a dimer (van Berkel et al., 1994). Vanillyl-alcohol oxidase from *P. simplicissimum* is induced when the fungus is grown upon veratryl alcohol as the sole carbon source. As veratryl alcohol is not a substrate for the oxidase (de Jong et al., 1992) and vanillyl alcohol seems not to be an intermediate in the degradation of veratryl alcohol (de Jong et al., 1990), the physiological role of the enzyme remains to be solved.

The extracellular aryl-alcohol oxidases have an acidic pH optimum thereby oxidizing a variety of non-activated or methoxylated aromatic alcohols (Sannia et al., 1991; Guillén et al., 1992). In contrast, vanillyl-alcohol oxidase from *P. simplicissimum* has a basic pH optimum and acts solely on 4-hydroxybenzyl alcohols (de Jong et al., 1992). In this report, we have studied the substrate specificity of vanillyl-alcohol oxidase to a greater extent. It is demonstrated that the enzyme acts on a wide

range of 4-hydroxybenzylic compounds. The nature of the reaction intermediates and products allows some conclusions about the reaction mechanism of vanillyl-alcohol oxidase. Some preliminary results have been presented elsewhere (van Berkel et al., 1994).

MATERIALS AND METHODS

General. Catalase, glutamate dehydrogenase, and NADPH were from Boehringer. Eugenol (4-allyl-2-methoxyphenol), isoeugenol (2-methoxy-4-propenylphenol), chavicol (4-allylphenol), safrole [4-allyl-1,2-(methylenedioxy)benzene], and isosafrole [1,2-(methylenedioxy)-4-propenylbenzene] were from Quest. Vanillin and vanillyl alcohol were products of Janssen Chimica and adrenalone (3',4'-dihydroxy-2-methylaminoacetophone) was purchased from Fluka. Alcohol oxidase, R-norepinephrine [α-(aminomethyl)-3,4-dihydroxybenzyl alcohol], R,S-epinephrine [3,4-dihydroxy-α-(methylaminomethyl)benzyl alcohol] and R-epinephrine were products of Sigma. S-norepinephrine, $H_2{}^{18}O$ (10 mol/100 mol ^{18}O) and all other chemicals were obtained from Aldrich.

P. simplicissimum (Oudem.) Thom. CBS 170.90 (ATCC 90172) was grown essentially as described earlier (de Jong et al., 1990), except that a 20-l fermentor was used for cultivation. Vanillyl-alcohol oxidase was purified to apparent homogeneity as described previously (de Jong et al., 1992). A single band, stained with Coomassie brilliant blue R250 (Serva) was observed after SDS/PAGE.

Analytical methods. Mass spectra were determined using a Hewlett Packard HP 5890 GC equipped with a 30-m DB17 column and a HP 5970 MSD, essentially as described before (de Jong et al., 1992). Samples were prepared by extraction of reaction mixtures with diethylether and injected without derivization into the GC/MS. Reaction mixtures, containing 10 μmol substrate and 0.2 U enzyme in 2 ml 50 mM glycine/NaOH, pH 10.0, were incubated at 30°C for 8 h.

For HPLC experiments, an Applied Biosystems 400 pump and a Waters 996 photodiode array detector were used. The column (100 mm×4.6 mm) filled with ChromSpher C_{18} (3 μm particles) was from Chrompack. The conversion of monoamines was analyzed by gradient elution using 0−80% methanol in 2% acetic acid.

^{1}H-NMR spectra were recorded with a Bruker AMX-500 MHz spectrometer. NMR samples were prepared as for mass spectroscopy experiments, dried after diethylether extraction, and dissolved in deuterated chloroform.

Enzyme concentrations were determined spectrophotometrically using a molar absorption coefficient $\varepsilon_{439} = 12.5 \text{ mM}^{-1} \text{ cm}^{-1}$ for protein-bound FAD (de Jong et al., 1992). The substrate specificity of vanillyl-alcohol oxidase was routinely tested by following absorption spectral changes after the addition of a catalytic amount of enzyme. Vanillyl-alcohol oxidase activity was assayed at 30°C, pH 10.0, by recording the formation of product or by following oxygen consumption using a Clark electrode (de Jong et al., 1992). Vanillin production was measured at 340 nm ($\varepsilon_{340} = 22.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The formation of 4-hydroxycinnamyl alcohols from 4-allylphenols was recorded at 325 nm [4-hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol): $\varepsilon_{325} = 7.5 \text{ mM}^{-1} \text{ cm}^{-1}$] or 310 nm [4-hydroxycinnamyl alcohol (coumaryl alcohol): $\varepsilon_{310} = 5.5 \text{ mM}^{-1} \text{ cm}^{-1}$], respectively. The production of hydrogen peroxide was determined by measuring oxygen levels before and after addition of 150 U catalase. Ammonia and methanol were enzymically determined using glutamate dehydrogenase (Bergmeyer, 1970) and alcohol oxidase (Geissler et al., 1986), respectively.

Absorption spectra were recorded at 25°C on an automated Aminco DW-2000 spectrophotometer. Binding studies as a function of pH were performed in 50 mM citric acid/sodium phosphate (pH 4−5), 50 mM sodium phosphate (pH 6−8), and 50 mM glycine/NaOH (pH 9−11). Dissociation constants of enzyme/inhibitor complexes were determined from flavin absorption difference spectra by titration of a known concentration of enzyme with the desired inhibitor. The ionization state of enzyme-bound isoeugenol was monitored at 320 nm by recording absorption spectra of 10 μM enzyme in the absence or presence of the inhibitor. Both sample and reference cell were titrated with known concentrations of isoeugenol, diluted in the appropriate buffer. Titration results were analyzed by non-linear least square fitting using the FORTRAN *numerical recipes* subroutine package (Press et al., 1986) of KALEIDAGRAPH (Adelbeck Software).
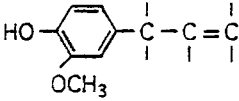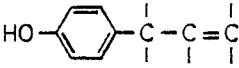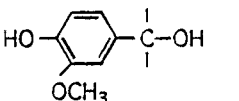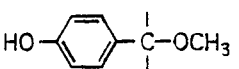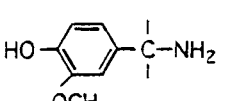
RESULTS

Substrate specificity. Initial characterization of vanillyl-alcohol oxidase from *P. simplicissimum* has revealed that the substrate specificity of this flavoenzyme is different from that of the extracellular FAD-dependent aryl-alcohol oxidases (de Jong et al., 1992). Besides catalyzing the conversion of vanillyl alcohol to vanillin, vanillyl-alcohol oxidase was reported to act solely on 4-hydroxybenzyl alcohol. This and the inhibitory effect of cinnamyl alcohol (3-phenyl-2-propene-1-ol) (de Jong et al., 1992) prompted us to investigate th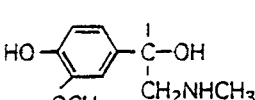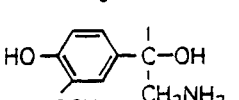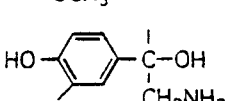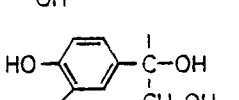e catalytic versatility of vanillyl-alcohol oxidase in more detail by introducing substituents at the Cα atom of the benzyl moiety of the substrate. Different novel types of substrates were found. In addition to the oxidation of simple 4-hydroxybenzyl alcohols, vanillyl-alcohol oxidase from *P. simplicissimum* catalyzed the conversion of α-(aminomethyl)-4-hydroxybenzyl alcohols, 4-hydroxyphenylglycols, 4-hydroxybenzylamines, 4-(methoxymethyl)phenols, and 4-allylphenols (Table 1). In agreement with earlier observations (de Jong et al., 1992), no activity or inhibition was found with homovanillyl alcohol (4-hydroxy-3-methoxyphenethyl alcohol). Furthermore, hydroxylated aromatic acids like 4-hydroxyphenylacetic acid, 4-hydroxymandelic acid, 4-hydroxyphenylglycine, 4-hydroxyphenylpyruvic acid, and tyrosine were not substrates for this flavoenzyme.

Table 1 shows that vanillyl-alcohol oxidase is specific for 4-hydroxybenzylic compounds. No activity or inhibition was observed with 4-aminobenzylamine, 4-nitrobenzyl alcohol and allylbenzene. Furthermore, substituted allylbenzenes like 4-allylanisole (4-methoxy-allylbenzene), safrole, and isosafrole were not converted by vanillyl-alcohol oxidase.

Catalytic properties. Table 1 shows the steady-state kinetic parameters of vanillyl-alcohol oxidase with some newly investigated substrates and with vanillyl alcohol as a reference. With all substrates, turnover of vanillyl-alcohol oxidase is optimal around pH 10. This suggests that the phenolate forms of the substrates may play an important role in catalysis (see also below). From Table 1, it is evident that the 4-allylphenols eugenol and chavicol are the best substrates. No activity or inhibition of vanillyl-alcohol oxidase was observed in the presence of 4-allyl-2,6-dimethoxyphenol. This suggests that the presence of an additional *ortho* methoxy substituent introduces steric constraints. From Table 1, it can also be observed that the catecholamine derivatives metanephrine [4-hydroxy-3-methoxy-α-(methylaminomethyl)benzyl alcohol], normetanephrine [α-(aminomethyl)-4-hydroxy-3-methoxybenzyl alcohol], and norepinephrine are poor substrates for vanillyl-alcohol oxidase. In analogy,

Table 1. Steady-state kinetic parameters for vanillyl-alcohol oxidase from *P. simplicissimum*. All experiments were performed at 25 °C in 50 mM glycine/NaOH, pH 10.0. Methanephrine, normethanephrine, norepinephrine, and 4-hydroxy-3-methoxyphenylglycol were tested as racemic mixtures. Norephinephrine was assayed in the presence of ascorbate (10 mM) to prevent auto-oxidation of the substrate.

Substrate	Structure	K_m	k_{cat}	k_{cat}/K_m
		µM	s^{-1}	10^3 s^{-1} M^{-1}
Eugenol	HO—⟨⟩—C—C=C / OCH$_3$	4.9	13.0	2700
Chavicol	HO—⟨⟩—C—C=C	4.8	6.5	1400
Vanillyl alcohol	HO—⟨⟩—C—OH / OCH$_3$	290	5.4	19
4-(Methoxymethyl)-phenol	HO—⟨⟩—C—OCH$_3$	65	5.3	82
Vanillylamine	HO—⟨⟩—C—NH$_2$ / OCH$_3$	240	1.3	5.4
Methanephrine	HO—⟨⟩—C—OH / OCH$_3$ CH$_2$NHCH$_3$	1600	0.8	0.5
Normetanephrine	HO—⟨⟩—C—OH / OCH$_3$ CH$_2$NH$_2$	1400	0.7	0.5
Norepinephrine	HO—⟨⟩—C—OH / OH CH$_2$NH$_2$	2900	0.3	0.1
4-Hydroxy-3-methoxyphenylglycol	HO—⟨⟩—C—OH / OCH$_3$ CH$_2$OH	6000	3.6	0.6

vanillyl-alcohol oxidase slowly reacted with epinephrine, octopamine [α-(aminomethyl)-4-hydroxybenzyl alcohol], and synephrine [4-hydroxy-α-(methylaminomethyl)benzyl alcohol] ($k'_{cat} < 0.3$ s^{-1}). The kinetic parameters for these compounds were not studied in further detail.

Identification of reaction products. Vanillyl alcohol oxidase is active with 4-hydroxybenzyl alcohols substituted at the Cα atom (Table 1). The enzymic oxidation of (nor)metanephrine, (nor)-epinephrine, and 4-hydroxy-3-methoxyphenylglycol resulted in the formation of an absorption peak around 350 nm resembling the optical spectrum of vanillin. As an example, the spectral changes induced upon oxidation of normetanephrine are recorded in Fig. 1. For a more thorough product identification, the enzymic conversion of *R*-epinephrine was followed by HPLC analysis using diode array detection. The retention time and absorption spectrum of the aromatic product were identical to those of adrenalone indicating oxidation of the Cα hydroxyl group:

HO—⟨⟩—C—C—N—CH$_3$ ⟶ HO—⟨⟩—C—C—N—CH$_3$.

By oxidizing the α-hydroxy group of catecholamines, vanillyl-alcohol oxidase differs from the flavin-dependent monoamine oxidases. The latter enzymes preferably convert the catecholamines by oxidative deamination (Singer, 1991). Another interesting feature of the vanillyl-alcohol-oxidase-catalyzed conversion of catecholamines is the stereospecificity of the reaction. Experiments with pure enantiomers showed that the oxidation rate of *S*-norepinephrine (at 2.0 mM and 4.0 mM) is only 30% with respect to the oxidation rate of the *R* isomer (100%). With epinephrine, the same preference was observed: the *R* isomer was significantly faster oxidized than the *S*-isomer (tested as the racemic mixture).

Vanillyl-alcohol oxidase was also active with 4-hydroxybenzylamines (Table 1). Besides vanillylamine (4-hydroxy-3-methoxybenzylamine), 3,4-dihydroxybenzylamine was oxidized,
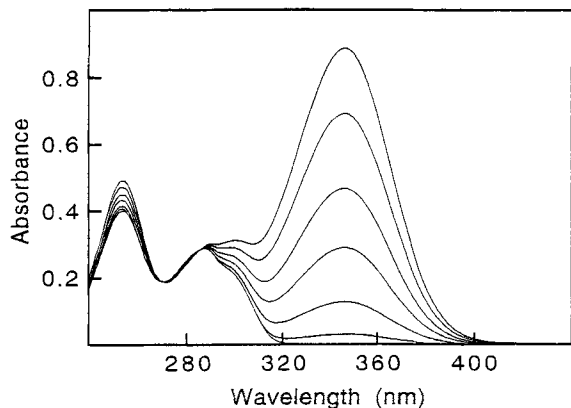
Fig. 1. Spectral changes observed upon oxidation of normeta-nephrine by vanillyl-alcohol oxidase from *P. simplicissimum*. The reaction mixture contained 0.1 mM normetanephrine in 1.0 ml 50 mM glycine/NaOH, pH 10. Spectra (from bottom to top) were recorded at t = 0, 1, 5, 15, 30, 60, and 120 min at 25°C after the addition of 0.62 nmol vanillyl-alcohol oxidase.
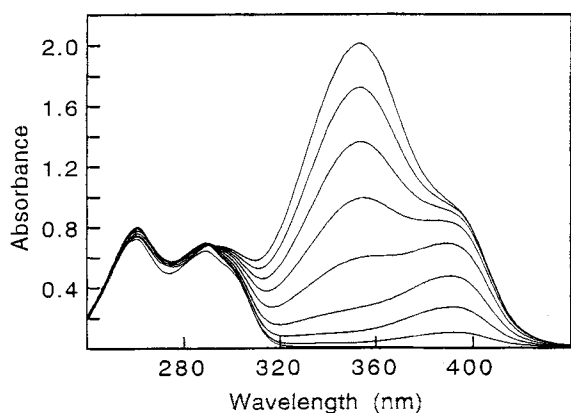

Fig. 2. Spectral changes observed upon oxidation of vanillylamine by vanillyl-alcohol oxidase from *P. simplicissimum*. The reaction mixture contained 0.2 mM vanillylamine in 1.0 ml 50 mM glycine/NaOH, pH 10. Spectra (from bottom to top) were recorded at t = 0, 1, 2, 5, 10, 15, 20, 25, and 30 min at 25°C after the addition of 0.31 nmol vanillyl-alcohol oxidase.

Fig. 3. Spectral changes observed upon oxidation of eugenol by vanillyl-alcohol oxidase from *P. simplicissimum*. (A) The reaction mixture contained 0.1 mM eugenol in 1.0 ml 50 mM glycine/NaOH, pH 10. Spectra (from bottom to top) were recorded at t = 0, 0.5, 1.5, 3, 5, and 10 min at 25°C after the addition of 0.03 nmol vanillyl-alcohol oxidase. (B) Reference spectra at 25°C of 0.1 mM coniferyl alcohol recorded at pH values of 7.0 (1), 9.1 (2), 9.6 (3), 10.2 (4), and 12.0 (5).

though at a slower rate (k'_{cat} about 0.9 s^{-1}). With the latter substrate, estimation of kinetic parameters was complicated by the instability of this compound. Mass spectral analysis revealed that vanillyl-alcohol oxidase converts vanillylamine to vanillin. As expected for oxidative deamination, conversion of vanillylamine resulted in the production of stoichiometric amounts of hydrogen peroxide and ammonia. When recording the enzymic oxidation of vanillylamine spectrophotometrically, initially an absorption increase was observed around 390 nm (Fig. 2). This transient peak gradually changed into the spectrum of vanillin. The extent and rate of formation of the spectral intermediate was dependent on temperature and enzyme concentration (data not shown). These findings suggest the initial formation of vanillyl-imine, which is subsequently hydrolyzed non-enzymically:
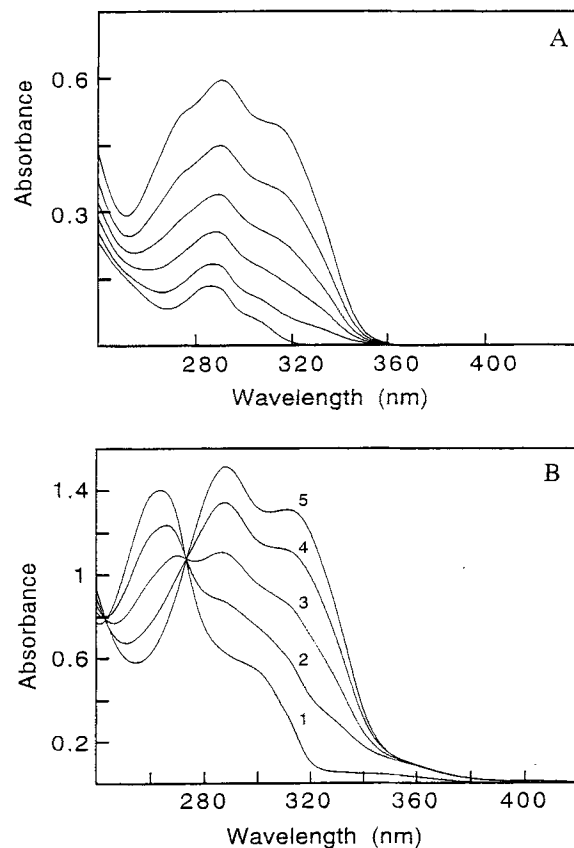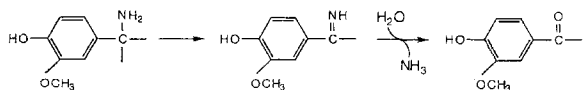

In analogy to the conversion of vanillylamine to vanillin, vanillyl-alcohol oxidase catalyzed the formation of 4-hydroxy-benzaldehyde from 4-(methoxymethyl)phenol (compare Table 1). The oxidative demethylation of 4-(methoxymethyl)phenol was evidenced by mass spectral analysis of the aromatic product ({M}$^+$ = 122). Enzymic analysis with alcohol oxidase from *Candida boidinii* revealed that the conversion of 4-(methoxy-methyl)phenol by vanillyl-alcohol oxidase involved the stoichiometric production of methanol, indicating fission of the ether bond.

Eugenol and chavicol are the best substrates for vanillyl-alcohol oxidase. Fig. 3A shows the absorption spectral changes observed during the conversion of eugenol by vanillyl-alcohol oxidase. The final spectrum obtained resembled the absorption spectrum of coniferyl alcohol as recorded at pH 10 (Fig. 3B). The actual formation of coniferyl alcohol was confirmed by ^{1}H-NMR (Rothen and Schlosser, 1991) and mass spectral analysis. The mass spectrum of the aromatic product was identical to that of authentic coniferyl alcohol; molecular ion at m/z (relative intensity) {M}$^+$ 180 (64.7%) and the following diagnostic fragments with more than 30% abundance: 137 (100%), 124 (59.0%), and 91 (46.9%). In a similar way the product of the enzymic conversion of chavicol was identified as coumaryl alcohol. This product contained the molecular ion at m/z (relative intensity) {M}$^+$ 150 (39.3%) and the following diagnostic fragments had more than 30% abundance: 107 (100%), 94 (50.9%), and 77 (30.6%).
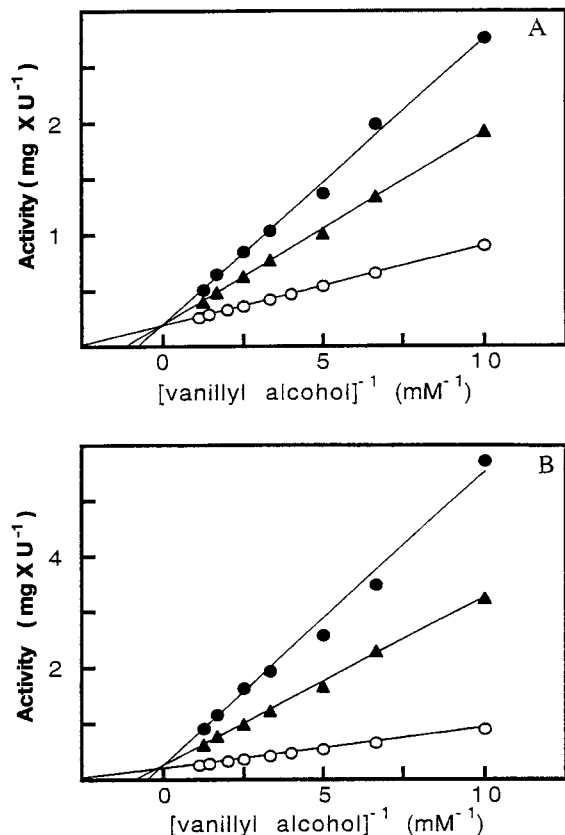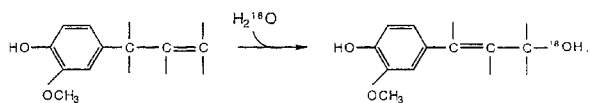
Fig. 4. Competitive inhibition of vanillyl-alcohol oxidase from *P. simplicissimum*. The activity of vanillyl-alcohol oxidase was measured by the increase of absorbance at 340 nm (25 °C, 50 mM glycine/NaOH, pH 10) with vanillyl alcohol as the variable substrate. All fits have R values >0.995. (A) The presence of the following are indicated: no inhibitor (O); 50 μM coniferyl alcohol (▲); 100 μM coniferyl alcohol (●). (B) The presence of the following are indicated: no inhibitor (O); 50 μM isoeugenol (▲); 100 μM isoeugenol (●).
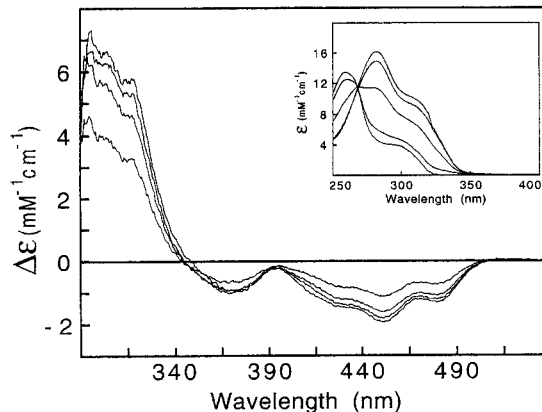

Fig. 5. Absorption difference spectra observed upon binding of isoeugenol to vanillyl-alcohol oxidase from *P. simplicissimum*. The absorption spectra were recorded at 25 °C in 50 mM sodium phosphate, pH 7.0. Both sample cell (containing 10 μM enzyme) and reference cell were titrated with isoeugenol. The curves shown are the difference spectra, the corrected enzyme spectra in the presence of 15, 30, 44, and 59 μM isoeugenol, minus the enzyme spectrum in the absence of isoeugenol. The inset shows the spectral properties of free isoeugenol as a function of pH. From bottom to top: absorption spectrum of isoeugenol at pH 5.0, 9.0, 10.0, 11.0, and 12.4, respectively.

During the enzymic conversion of eugenol to coniferyl alcohol, the consumption of oxygen was coupled to the production of stoichiometric amounts of hydrogen peroxide. This suggests that the oxygen atom incorporated in coniferyl alcohol is derived from water. This was tested by performing the enzymic conversion of eugenol in the presence of 10% $H_2^{18}O$. Mass spectral analysis of the formed product yielded a spectrum similar to the spectrum of coniferyl alcohol except for the presence of an additional peak with a mass of 182 (10% relative to {M^+} 180). This result confirms the involvement of water as a reactant in the enzymic production of coniferyl alcohol from eugenol:

Binding studies. Previously we reported that cinnamyl alcohol is a competitive inhibitor of vanillyl-alcohol oxidase (de Jong et al., 1992). Consequently, it was of interest to study the inhibitory effect of the 4-hydroxycinnamyl alcohols produced from enzymic conversion of eugenol and chavicol. Coniferyl alcohol is a strong competitive inhibitor of vanillyl alcohol oxidation ($K_i = 31 \pm 6$ μM) (Fig. 4A). The binding of coniferyl alcohol to the oxidized enzyme was confirmed by absorption difference spectroscopy. At pH 10, the binding of coniferyl alcohol resulted in small perturbations of the absorption spectrum of protein-

bound FAD (data not shown). From the absorption differences at 490 nm, a dissociation constant, $K_d = 9 \pm 2$ μM was estimated for the binary enzyme-inhibitor complex. The binding of coniferyl alcohol was fully reversible as evidenced from the absorption spectrum recorded after gel filtration of the enzyme-coniferyl alcohol complex.

The substrate isomer isoeugenol was another potent competitive inhibitor of vanillyl-alcohol oxidase. Fig. 4B shows the inhibition pattern obtained with vanillyl alcohol as the variable substrate. From the data of Fig. 4B an inhibition constant of $K_i = 13 \pm 5$ μM at pH 10 was estimated. Tight binding of isoeugenol to the oxidized enzyme was confirmed by difference spectroscopy. At pH 7.0, isoeugenol reversibly perturbed the flavin absorption spectrum of vanillyl-alcohol oxidase with a maximal hypochromic shift around 450 nm (Fig. 5). Binding followed simple binary complex formation with a dissociation constant of $K_d = 24 \pm 4$ μM and a maximum value of about -1.8 mM^{-1} cm^{-1} for the molar difference absorption coefficient at 450 nm. The difference spectra recorded in Fig. 5 also show that binding of isoeugenol to vanillyl-alcohol oxidase at pH 7.0 results in a large increase in absorbance around 320 nm. The shape and intensity of this peak, together with the minimal absorbance of the enzyme in this region (de Jong et al., 1992), are consistent with the formation of the phenolate form of the inhibitor (compare inset Fig. 5). Since the pK_a of isoeugenol free in solution is about 9.8 (inset Fig. 5), this suggests that the binding of isoeugenol to the oxidized enzyme perturbs the phenolic pK_a of the inhibitor. This was studied in more detail by titration of the enzyme with isoeugenol at various pH values. These experiments revealed that the shape and intensity of the flavin difference spectrum (340−500 nm, Fig. 5) are nearly pH independent. From the absorption changes at 450 nm and over the entire pH interval studied, the binding of isoeugenol was described by simple binary complex formation, yielding dissociation constants ranging from $K_d = 40 \pm 13$ μM at pH 4 to $K_d = 15 \pm 3$ μM at pH 10 (Fig. 6A). In contrast to the flavin difference spectrum, the intensity of the difference absorbance around 320 nm strongly changed with pH and in accordance with the dissociation constants reported above. Fig. 6B shows the pH dependence of the molar absorption differences at 320 nm at saturating
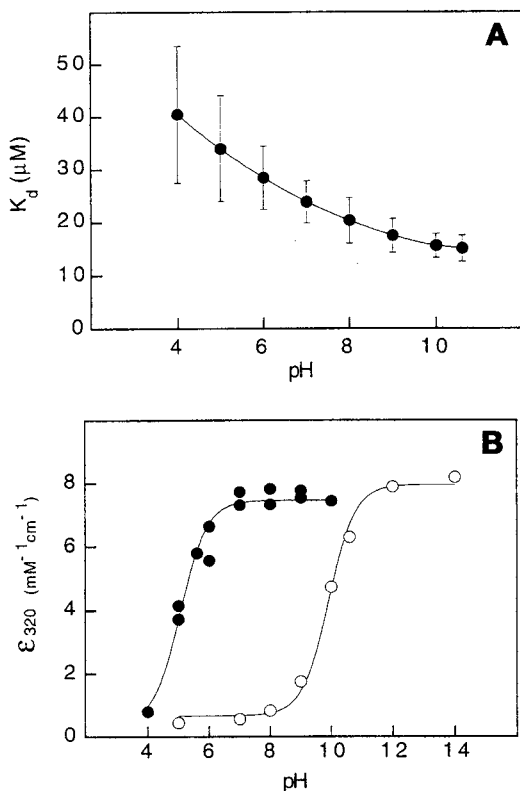
Fig. 6. The pH dependence of the dissociation constant and ionization state of the binary complex between vanillyl-alcohol oxidase and isoeugenol. 10 μM enzyme was titrated with isoeugenol at 25 °C. Absorption changes were monitored as described in the legend of Fig. 5. For buffers used and other experimental details, see the Materials and Methods section. (A) pH dependence of the dissociation constant of the complex between vanillyl-alcohol oxidase and isoeugenol. (B) The effect of pH on the molar absorption coefficient at 320 nm: at saturating concentrations of isoeugenol with vanillyl-alcohol oxidase (●); with free isoeugenol (○).

concentrations of isoeugenol. An apparent pK_a value of 5.0 was estimated from the experimental data with a maximum difference absorption coefficient of $\Delta\varepsilon_{max} = 7.5\ \text{mM}^{-1}\ \text{cm}^{-1}$ at 320 nm. The low pK_a observed for the enzyme-isoeugenol complex suggests that binding of substrates to vanillyl-alcohol oxidase involves the interaction between an acidic amino acid side chain and the phenolic moiety of the substrate.

DISCUSSION

The results presented in this study show that vanillyl-alcohol oxidase from *P. simplicissimum* is specific for 4-hydroxybenzylic compounds. This clearly discriminates the enzyme from the extracellular aryl-alcohol oxidases isolated from basidiomycetes (Guillén et al., 1992). Vanillyl-alcohol oxidase catalyzes besides the oxidation of (complex) 4-hydroxybenzyl alcohols, the oxidative demethylation of 4-(methoxymethyl)phenols, the oxidative deamination of 4-hydroxybenzylamines, and the oxidative hydration of 4-allylphenols. The enzymic conversion of eugenol is of special industrial interest in view of the production of natural flavour compounds (van Berkel et al., 1993).

The type of oxidation reaction catalyzed by vanillyl-alcohol oxidase is determined by the substituents at the Cα atom of the aromatic substrate. The introduction of a bulky substituent in the 4-hydroxybenzyl alcohols results in relatively low but signifi-

cant oxidation rates. The high K_m values and the partial enantioselective conversion of at least two of these compounds suggest some steric limitations. It is evident, however, that additional data on the individual reaction steps are needed to determine which parameters dictate the rate of substrate oxidation.

The spectral intermediate observed during the oxidative deamination of vanillylamine is tentatively assigned to vanillylimine (or its benzoquinonimine derivative). Imine intermediates have been observed with other flavoprotein oxidases, like, for example, D-aminoacid oxidase (Curti et al., 1992), monoamine oxidase (Singer, 1991), and pyridoxamine-5-phosphate oxidase (Kwok and Churchich, 1992). The detection of a spectral intermediate in the reaction with vanillylamine suggests that the relatively low turnover rate with this compound (Table 1) may be due to slow dissociation of the reduced enzyme-vanillylimine complex. Kinetic studies on the various half-reactions are needed to settle this point.

Vanillyl-alcohol oxidase is most active with 4-allylphenols. This and the high pH optimum for turnover suggest that the allylic side chain facilitates oxidation by the ability to form stable quinoid intermediates due to resonance effects involving the phenolate form. The preferred binding of the phenolate form of substrates is supported by the binding properties of the competitive inhibitor isoeugenol. The enzyme presumably plays a crucial role in stimulating the formation of a quinoid intermediate in which negative charge is localized at the Cα atom. The formation of the 4-hydroxycinnamyl alcohol products suggests that the initial oxidation of the Cα-H bond results in delocalization and stabilization of charge at the Cγ atom. From the above considerations, hydration at the Cγ atom is most simply explained by a mechanism involving a *p*-quinone methide intermediate species, generated by the initial transfer of a hydride equivalent to the flavin cofactor:
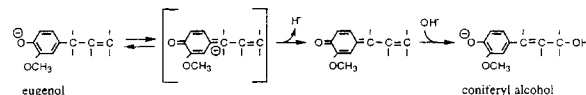
The high pH optimum and specificity for 4-hydroxybenzylic compounds suggest that the chemical mechanism depicted above is also operative in the other type of oxidation reactions catalyzed by vanillyl-alcohol oxidase. For instance, the conversion of 4-(methoxymethyl)phenol to 4-hydroxybenzaldehyde results in the production of stoichiometric amounts of methanol, indicative for the nucleophilic attack of water. However, additional data are needed to exclude a radical mechanism as proposed for flavin-dependent oxidation reactions of substrates in which the C-H bond to be oxidized is not activated (Williams et al., 1977; Silverman, 1984; Sherry and Abeles, 1985; Geissler et al., 1986).

From the specificity for 4-hydroxybenzylic compounds and from a mechanistic point of view, vanillyl-alcohol oxidase from *P. simplicissimum* has some resemblance to the periplasmic 8α-(O-tyrosyl)-FAD-containing 4-alkylphenol methylhydroxylases from *Pseudomonas putida* (Hopper and Taylor, 1977; McIntire et al., 1981; Reeve et al., 1989). These $\alpha_2\beta_2$ flavocytochromes convert their substrates by subsequent dehydrogenation and hydration but cannot act with oxygen as electron acceptor. Instead, the electrons are transferred one at a time from the flavin to a heme on a separate subunit (Mathews et al., 1991). The 4-alkylphenol methylhydroxylases are most active with 4-alkylphenols but also convert 4-hydroxybenzyl alcohol and eugenol (Reeve et al., 1989). Unfortunately, with these enzymes no attempt was made to identify the product of the conversion of eugenol.

From the present study, the question arises of whether the term vanillyl-alcohol oxidase is appropriate as it does not cover

the catalytic versatility of this fungal flavoenzyme. According to the substrate specificity and catalytic efficiency, one might argue that a more appropriate name would be 4-allylphenol oxidase. It is doubtful, however, whether the antiseptic 4-allylphenols are physiological substrates of the enzyme. Nevertheless, the *in vitro* formation of the monolignols, coniferyl alcohol and coumaryl alcohol, by this flavoenzyme is noteworthy. Plants are thought to be the only organisms to produce these precursors for the biosynthesis of lignans or the lignin polymer (Paré et al., 1994). In this respect, it is interesting to note that vanillyl-alcohol oxidase is not able to oxidize 4-allyl-2,6-dimethoxyphenol to form the third lignin building block, sinapyl alcohol (4-hydroxy-3,5-dimethoxycinnamyl alcohol).

Vanillyl-alcohol oxidase from *P. simplicissimum* is an inducible intracellular octameric flavoprotein oxidase. These features may indicate that this oxidase is a peroxisomal enzyme like the alcohol oxidases from methylotrophic yeasts (Borst, 1989; Müller et al., 1992). Similar octameric alcohol oxidases, acting on primary alcohols, were recently isolated from several fungi (Danneel et al., 1994). Most eukaryotic covalent flavoproteins studied so far are localized in mitochondria (Mihalik and McGuinness, 1991). Ultrastructural localization of vanillyl-alcohol oxidase will hopefully shed more light on the physiological role of this 8α-(N^3-histidyl)-FAD-containing flavoenzyme.

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