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Catalytic Mechanism of the Oxidative Demethylation of 4-(Methoxymethyl)phenol by Vanillyl-Alcohol Oxidase

EVIDENCE FOR FORMATION OF A p-QUINONE METHIDE INTERMEDIATE*

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The catalytic mechanism for the oxidative demethylation of 4-(methoxymethyl)phenol by the covalent flavoprotein vanillyl-alcohol oxidase was studied. Using H₂¹⁸O, it was found that the carbonylic oxygen atom from the product 4-hydroxybenzaldehyde originates from a water molecule. Oxidation of vanillyl alcohol did not result in any incorporation of ¹⁸O.

Enzyme-monitored turnover experiments revealed that for both substrates a process involving flavin reduction is rate determining. During anaerobic reduction of vanillyl-alcohol oxidase by 4-(methoxymethyl)-phenol, a relatively stable spectral intermediate is formed. Deconvolution of its spectral characteristics showed a typical pH-independent absorption maximum at 364 nm (ε₃₆₄ nm = 46 m⁻¹ cm⁻¹). A similar transient species was observed upon anaerobic reduction by vanillyl alcohol.

The rate of flavin reduction and synchronous intermediate formation by 4-(methoxymethyl)phenol is 3.3 s⁻¹ and is fast enough to account for turnover (3.1 s⁻¹). The anaerobic decay of the intermediate was too slow (0.01 s⁻¹) to be of catalytical relevance. The reduced binary complex is rapidly reoxidized (1.5 × 10⁶ s⁻¹) and is accompanied with formation and release of product. Oxidation of free-reduced enzyme is an even faster process (3.1 × 10⁸ s⁻¹).

The kinetic data for the oxidative demethylation of 4-(methoxymethyl)phenol are in accordance with a ternary complex mechanism in which the reduction rate is rate-limiting. It is proposed that, upon reduction, a binary complex is produced composed of the p-quinone methide of 4-(methoxymethyl)phenol and reduced enzyme.

Vanillyl-alcohol oxidase (VAO,¹ EC 1.1.3.13) from Penicillium simplicissimum is a novel flavoprotein that acts on a wide range of 4-hydroxybenzyl compounds (1, 2). VAO is a homodimer, with each subunit containing 8a-(N²-histidyl)-FAD as a prosthetic group (3). During catalysis, the flavin cofactor is first reduced and subsequently reoxidized by molecular oxygen to yield hydrogen peroxide. In addition to the oxidation of aromatic alcohols also, demethylation, deamination, and hydroxylation reactions are being catalyzed as shown in Equation 1.

By its versatile catalytic potential, VAO may develop as a useful biocatalyst for applications in the fine chemical industry (4).

VAO is readily induced in P. simplicissimum by growth on veratryl alcohol (3). Although the enzyme is produced in relatively high amounts, the physiological role of the enzyme remained obscure for some time as VAO is not involved in the degradation of this aromatic alcohol. Only recently, it was found that the VAO-mediated oxidative demethylation of 4-(methoxymethyl)phenol is of metabolic relevance (5). When P. simplicissimum is grown on this phenolic methyl ether, VAO is induced and catalyzes the first step of the degradation pathway of 4-(methoxymethyl)phenol. Furthermore, analogs of 4-(methoxymethyl)phenol can easily be envisaged as physiological substrates enabling this ascomycetous fungus to cope with a wide variety of lignin decomposition products (5).

Previous studies have revealed some interesting mechanistic properties of VAO. A striking feature of all substrates is the necessity of a p-hydroxy group that is probably a prerequisite for binding. Moreover, a large pKᵦ shift observed upon binding of the competitive inhibitor isoeugenol indicates that substrates become deprotonated upon binding (1). For the reaction of VAO with the substrate eugenol, it was established that the oxygen atom incorporated into the formed product coniferyl alcohol is derived from water. From these results, a catalytic mechanism for the hydroxylation of eugenol was proposed which involves formation of a p-quinone methide intermediate (1). A similar catalytic mechanism has been proposed for the hydroxylation of 4-alkylphenols by the flavocytochrome, p-cresol methylhydroxylase (6). So far, no real evidence has ever been presented for the formation of p-quinone methide intermediates during flavin-mediated reactions. We have suggested that hydride transfer to the oxidized flavin cofactor following deprotonation of the substrate would be a feasible sequence of reactions leading to the formation of the labile p-quinone methide intermediate (1). Hydride transfer mechanisms have been proposed for several other flavin-dependent oxidases like meth-
an alcohol oxidase (7) and cholesterol oxidase (8). Recently, Mattevi et al. (9) provided evidence from crystallographic studies that, for d-amino acid oxidase also, a hydrier transfer is a likely event during catalysis. Because well diffracting crystals of VAO have been obtained, we aim to relate the catalytic properties of this flavoenzyme with the crystal structure in the near future (10).

In this paper, we report on the kinetic and catalytic mechanism of VAO with the physiological substrate 4-(methoxymethyl)phenol. Evidence from rapid reaction studies is presented which shows that the hydroxylation of phenolic compounds by VAO involves the formation of a p-quinone methide intermediate. Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol; R,e=OCH3, R,c=H, and R,p=OH in Equation 1) was included as a model substrate in this study to examine both an oxidative demethylation and an alcohol oxidation reaction.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—VAO was purified from P. simplicissimum as described by De Jong et al. (3) with the modification that a 200-liter fermentor was used for cultivation and that cells were disrupted using a Manton-Gaulin homogenizer. The ratio A450/A600 for the purified enzyme was 11.0. Glucose oxidase (grade II) and catalase were from Boehringer Mannheim. H2O18O (97 mol/100 mol 16O) was obtained from Campro (Veenendaal, The Netherlands). Vanillyl alcohol, vanillin (4-hydroxy-3-methoxybenzaldehyde), 4-hydroxybenzaldehyde, and 4-(methoxymethyl)phenol were purchased from Aldrich.

Analytical Methods—All experiments were performed at 25 °C in 50 mM phosphate buffer, pH 7.5, unless stated otherwise. VAO concentrations were calculated from the molar absorption coefficient of the oxidized form (ε439 nm = 12.5 mM⁻¹ cm⁻¹ (3)).

Isotope Labeling Experiments—For 18O incorporation experiments, 195 μl of H2O18O was added to 400 μl of 1.0 mM substrate solutions. After addition of VAO (25 μl, 200 μkat) and catalase (5 μl, 100 μkat), the samples were incubated for 5 min at 25 °C and subsequently twice extracted with 500 μl diethylether. After evaporation, the samples were analyzed by GC/MS. GC/MS analysis was performed on a Hewlett Packard HP 5973 MSD and HP 6890 GC equipped with an HP-5 column.

Steady-state Kinetics—Steady-state kinetic experiments were performed essentially as described earlier (1). Vanillyl alcohol and 4-(methoxymethyl)phenol activity were determined spectrophotometrically by recording the formation of vanillin (ε420 nm (pH 7.5) = 14.0 mM⁻¹ cm⁻¹) and 4-hydroxybenzaldehyde (ε340 nm (pH 7.5) = 10.0 mM⁻¹ cm⁻¹), respectively. Oxygen concentrations were varied by mixing buffers saturated with 100% nitrogen and 100% oxygen in different ratios.

Stopped-flow Kinetics—Stopped-flow kinetics were carried out with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech M300 monochromator diode-array detector (Salisbury, United Kingdom). Spectral scans were collected each 10 ms. For accurate estimation of rate constants, single wavelength kinetic traces were recorded at 439 nm using a Hi-Tech SU-40 spectrophotometer. In anaerobic experiments, solutions were flushed with argon and contained glucose (10 mM) and glucose oxidase (10 mM), the samples were flushed with argon and contained glucose (10 mM) and glucose oxidase (0.1 μkat) to ensure anaerobic conditions. To determine the maximal rate of enzyme reduction by 4-(methoxymethyl)phenol and vanillyl alcohol, apparent rates were determined at five different substrate concentrations. To obtain accurate estimates of reduction rate constants observed during anaerobic reduction by vanillyl-alcohol, measurements were also performed at 355 and 393 nm. Deconvolution analysis of spectral data was performed using the Specfit Global Analysis program Version 2.10 (Spectrum Software Assn., Chapel Hill, NC).

RESULTS

Isotope Labeling Experiments—In a previous report, we already identified the products formed from 4-(methoxymethyl)phenol and vanillyl alcohol as their corresponding aldehydes (1). In this study, H2O18O was used to test the involvement of water in the VAO-mediated conversion of 4-(methoxymethyl)phenol and vanillyl alcohol. Substrate solutions (containing 30% H2O18O (w/w)) were incubated for 5 min in the presence of a catalytic amount of enzyme. Blank reactions with the aromatic products 4-hydroxybenzaldehyde and vanillin revealed that, under these conditions, less than 5% of the carboxylic oxygen atoms had exchanged with H2O18O. For the VAO-mediated conversion of 4-(methoxymethyl)phenol, it was found that the aromatic product 4-hydroxybenzaldehyde was fully hydroxylated by action of water (97 ± 4%) (Fig. 1). The incorporation of 18O confirms an earlier finding (1) that conversion of 4-(methoxymethyl)phenol by VAO results in cleavage of the methoxyl group as methanol. The VAO catalyzed oxidation of vanillyl alcohol to vanillin did not result in significant 18O incorporation (Fig. 1). This shows that with this aliphatic substrate water is not involved in the enzymatic reaction.

Steady-state Kinetics—By measuring the VAO activity upon varying the concentration of oxygen at different 4-(methoxymethyl)phenol concentrations, a set of parallel Lineweaver-Burk plots was obtained. This suggests that for this reaction a ping-pong mechanism may be operative. Parallel line kinetics can, however, also occur in some limited cases of a ternary complex mechanism where some specific rate constants are
methyl)phenol, the substrate concentrations (500 due to the low solubility of vanillyl alcohol and 4-(methoxy-
high concentration of substrate. It should be noted here that
was aerobically mixed in the stopped-flow apparatus with a
polar character of the benzylic moiety compared with 4-(me-
from secondary plots using several substrate concentrations (25, 50,
slow than those of the oxidative part of the catalytic cycle. This suggests that processes involving flavin reduction are
4-(methoxymethyl)phenol (1).

\[ \text{Table I} \]

| Constant          | Experimental | Calculated |
|-------------------|--------------|------------|
| \( k_{1} \)       | 48 \( \mu \)M |            |
| \( k_{2} \) (= \( k_{\text{cat}} \)) | 3.3 s\(^{-1}\) |            |
| \( k_{3} \)       | 0           | -0         |
| \( k_{4} \)       | 1.5 \( \times \) \( 10^6 \) M\(^{-1}\) s\(^{-1}\) | 1.4 \( \times \) \( 10^6 \) M\(^{-1}\) s\(^{-1}\) |
| \( k_{l} \)       | 50 s\(^{-1}\) |            |
| \( k_{o} \)       | 0.01 s\(^{-1}\) |            |
| \( k_{r} \)       | 3.1 \( \times \) \( 10^6 \) M\(^{-1}\) s\(^{-1}\) | 3.1 s\(^{-1}\) |
| \( K_{m(S)} \)    | 55 \( \mu \)M | 45 \( \mu \)M |
| \( K_{m(O)} \)    | 24 \( \mu \)M | 21 \( \mu \)M |

\(^{a} k_{1} = k_{cat}/K_{m(S)}.\)
\(^{b} k_{2} \) calculated from \( k_{cat} = k_{1} \cdot k_{2} / (k_{3} + k_{4}).\)
\(^{c} K_{m(S)} = k_{1} / (k_{1} + k_{2})k_{4}/(k_{3} + k_{4}).\)
\(^{d} K_{m(O)} = k_{1} / (h_{1} + h_{2})k_{3}/(k_{2} + k_{4}).\)

relatively small (12, 13). Fig. 2 shows a secondary plot of the extrapolated turnover rates at saturating oxygen concentrations versus the concentration of 4-(methoxymethyl)phenol. From this, the steady-state kinetic parameters with 4-(methoxymethyl)phenol could be calculated (Table I). The steady-state kinetic parameters for vanillyl alcohol were similarly determined (again showing series of parallel secondary plots) and were in the same range as for 4-(methoxymethyl)phenol (\( k_{cat} = 3.3 \) s\(^{-1}\), \( K_{m(S)} = 160 \) \( \mu \)M, \( K_{m(O)} = 28 \) \( \mu \)M). The relatively high \( K_{m} \) value for vanillyl alcohol might result from the more polar character of the benzylic moiety compared with 4-(methoxymethyl)phenol (1).

By measuring the redox state of the flavin cofactor during catalysis (enzyme-monitored turnover), information can be obtained about the rate-limiting step (11). For this, the enzyme was aerobically mixed in the stopped-flow apparatus with a high concentration of substrate. It should be noted here that due to the low solubility of vanillyl alcohol and 4-(methoxymethyl)phenol, the substrate concentrations (500 \( \mu \)M) were not fully saturating. During turnover, the absorbance at 439 nm was monitored to detect the amount of oxidized enzyme present. Fig. 3 shows that with both substrates most of the enzyme is in the oxidized state during turnover. The fraction of oxidized enzyme for both substrates was almost identical, 0.86 for 4-(methoxymethyl)phenol and 0.91 for vanillyl alcohol (Fig. 3). This suggests that processes involving flavin reduction are slower than those of the oxidative part of the catalytic cycle.

**Reductive Half-reaction**—To study the reductive half-reaction of VAO, the oxidized enzyme was mixed with substrate in

\[ \begin{align*}
\text{Enzyme-monitored turnover of VAO with 4-(methoxymethyl)phenol and vanillyl alcohol. The enzyme (7.5 \mu M) was reacted in the stopped-flow instrument with 500 \mu M 4-(methoxymethyl)phenol (trace A) and vanillyl alcohol (trace B). During the reaction, the absorbance at 439 nm was monitored. Trace C was the result of mixing the enzyme with buffer.}
\end{align*} \]

\[ \begin{align*}
\text{FIG. 2. Double-reciprocal plot of steady-state kinetic measurements (○) and anaerobic reductive half-reaction with 4-(methoxymethyl)phenol (●). For the steady-state kinetic data, the turnover rates at saturating oxygen concentrations were determined from secondary plots using several substrate concentrations (25, 50, 100, and 500 \mu M) with varying oxygen concentrations.}
\end{align*} \]

\[ \begin{align*}
\text{FIG. 3. Spectral changes observed upon anaerobic mixing of VAO (7.0 \mu M) with 4-(methoxymethyl)phenol (500 \mu M, 25 °C, pH 7.5). Original spectral scans are shown from 5.6 to 965.6 ms with intervals of 80 ms. The inset shows spectra obtained after deconvolution of the original spectral scans where spectrum A represents the initial spectrum of oxidized enzyme and spectrum B shows the formed spectral intermediate. The data were fit with a one-exponential function (A→B), resulting in a rate of 3.0 s\(^{-1}\).}
\end{align*} \]

\[ \begin{align*}
\text{FIG. 4. Double-reciprocal plot of steady-state kinetic measurements (○) and anaerobic reductive half-reaction with 4-(methoxymethyl)phenol (●). For the steady-state kinetic data, the turnover rates at saturating oxygen concentrations were determined from secondary plots using several substrate concentrations (25, 50, 100, and 500 \mu M) with varying oxygen concentrations.}
\end{align*} \]
absorbance around 335 nm, which was even more pronounced at higher pH due to deprotonation of the phenolic group (pKa of 4-hydroxybenzaldehyde = 7.6). The rate of aldehyde formation under anaerobic conditions was estimated to be 0.01 s⁻¹, which is too slow to be of catalytical relevance. The results obtained from the anaerobic reduction of VAO with 4-(methoxymethyl)phenol are consistent with the following Equation 2,

\[ E_{ox} + S \rightleftharpoons E_{red} \rightleftharpoons E_{red} - Q \rightleftharpoons E_{red} + P \]  

(Eq. 2)

where Q represents the high absorbance intermediate.

Reduction of VAO by vanillyl alcohol showed biphase absorption traces at 439 nm. Diode array analysis revealed that during reduction an intermediate spectrum is formed with a typical absorption maximum at 362 nm (Fig. 5, A and B). As can be seen from Fig. 5A, this intermediate decayed rapidly to yield fully reduced enzyme. For accurate kinetic data, rate constants of both reductive phases were determined at various substrate concentrations by monitoring the reaction at isosbestic points (393 and 355 nm, see Fig. 5B). It was found that the first rapid phase was an order of magnitude faster compared with the slow phase (k_red₁ = 24 s⁻¹, K_d = 270 μM compared with k_red₂ = 3.5 s⁻¹, K_d = 150 μM; Fig. 6). Using a consecutive irreversible reaction model in which the first reaction corresponds to the fast process, spectral deconvolution produced well defined spectra for the initial, intermediate, and final components (Fig. 5B, traces A, B, and C). As can be seen from Fig. 5B the distinctive absorption maximum of the intermediate spectrum formed in the first phase showed some resemblance with the intermediate spectrum formed by anaerobic reduction of VAO with 4-(methoxymethyl)phenol (see Fig. 4 inset). This analogy was confirmed by performing the anaerobic reduction experiments with vanillyl alcohol at different pH values (Fig. 5B). As with 4-(methoxymethyl)phenol, no significant effect of pH on the observed reduction rates was observed. Furthermore, these studies again showed that in contrast to the transient intermediate spectrum, the final spectrum is pH-dependent. This indicates that the final product vanillin is formed only in the second step as the spectral properties of the final spectrum agree nicely with formation of vanillin (pKₐ = 7.5, λ_max = 345 nm). Furthermore, in the case of vanillyl alcohol, the flavin apparently is only partially reduced when the high absorbance intermediate is formed as the absorbance at 439 nm is relatively high after the first reductive phase (Fig. 5B).

Oxidative Half-reaction—To measure the rate of reoxidation, reduced VAO was mixed with molecular oxygen in the stopped-flow spectrophotometer, and the increase of the flavin absorbance at 439 nm was monitored. Reoxidation of free-reduced VAO was a monophasic reaction resulting in formation of fully oxidized enzyme. By varying the concentration of oxygen, it was found that reoxidation of free-reduced VAO is a fast molecular process (3.1 × 10⁷ M⁻¹ s⁻¹) as has been found for other flavoprotein oxidases (14).

\[ E_{red}(\cdot L) + O_2 \rightleftharpoons E_{ox}(\cdot L) + H_2O_2 \]  

(Eq. 3)

For flavoprotein oxidases, the rate of enzyme reoxidation can significantly be influenced by bound ligands (L in Equation 3) (15). For instance, in the case of D-amino acid oxidase from yeast, it was reported that reoxidation of free-reduced enzyme is not of catalytical significance as it is significantly slower.
than the turnover rate. Only the rate for reoxidation of the reduced enzyme product complex was high enough to account for the observed turnover rate, which is in agreement with the proposed ternary complex mechanism (16). When the reoxidation rate of reduced VAO was studied in the presence of vanillyl alcohol or its product vanillin, the rates of flavin reoxidation were significantly lower as compared with free-reduced enzyme (1.4 × 10^5 and 1.1 × 10^5 M^−1 s^−1, respectively).

As reduction by 4-(methoxymethyl)phenol resulted in a relatively stable reduced enzyme-intermediate complex (E_{red} − Q), the rate of reoxidation of this complex was measured as well. Therefore, after reducing VAO by 4-(methoxymethyl)phenol, the reduced complex was mixed in the stopped-flow apparatus with oxygen to measure the rate of formation of oxidized enzyme. It was found that the complex readily reacted with oxygen in a fast monophasic process reoxidizing the flavin with simultaneous product formation as evidenced by the increase of absorbance at 335 nm. By varying the oxygen concentration, the second order rate constant for reoxidation of the reduced enzyme intermediate complex was estimated to be 1.5 × 10^5 M^−1 s^−1. Similar values for the rate of reoxidation have been reported for other flavin-dependent oxidases (15–18). The apparent turnover rate with 4-(methoxymethyl)phenol during steady-state conditions is much slower as the rate of reoxidation. Evidently, reoxidation is not determining the rate of catalysis in case of 4-(methoxymethyl)phenol, which is in agreement with the enzyme monitored turnover results.

**DISCUSSION**

The experiments described here represent the first study on the kinetic mechanism of VAO catalyzed reactions. Furthermore, evidence is presented for the participation of p-quinone methides in the catalytic mechanism of VAO. Previously, we proposed a reaction mechanism for the conversion of eugenol by VAO, which included formation of a p-quinone methide intermediate (1). Addition of water to this putative electrophilic intermediate would result in the formation of the product coumarin alcohol. From isotopic labeling experiments, we have demonstrated in the present study the involvement of water during the VAO catalyzed demethylation of 4-(methoxymethyl)phenol. VAO-mediated demethylation of this physiological substrate resulted in the introduction of an oxygen atom originating from a water molecule. This indicates that during catalysis the substrate is activated, after which water can attack the C−atom. In contrast, with vanillyl alcohol, no oxygen atom derived from water is introduced into the aromatic product vanillin.

The rapid reaction data presented in this paper showed the formation of intermediate reduced enzyme complexes during the reductive half-reaction. Anaerobic reduction of VAO by 4-(methoxymethyl)phenol revealed the formation of an eminently stable intermediate with typical spectral properties (ε_{304 nm} = 46 M^−1 cm^−1). The spectral characteristics of the intermediate binary complex did not resemble any known flavoprotein oxidase complex. Intermediate complexes formed during catalysis of, for example, lactate monooxygenase (19) and d-amino acid oxidase (20) have specific absorbances above 500 nm and are due to a charge transfer interaction between the reduced enzyme and product. The formation of a flavin adduct as intermediate in the reaction of VAO with 4-(methoxymethyl)phenol is rather unlikely as the spectral properties of the intermediate complex do not resemble any known flavin adduct spectrum (21). However, the spectral properties of the formed intermediate generated during the anaerobic reaction of VAO with 4-(methoxymethyl)phenol closely resembled reported spectra of several p-quinone methides of structural analogs of the phenolic substrate (λ_{max} = 360 nm, ε = 40 M^−1 cm^−1) as obtained by chemical synthesis or flash photolysis of the corresponding phenols (22–26). However, spectra of the p-quinone methides of vanillyl alcohol and 4-(methoxymethyl)phenol have never been described. These compounds are highly unstable because of the lack of an electron donating group to stabilize the electrophilic methide carbon atom. The data presented here for VAO-mediated conversion of 4-(methoxymethyl)phenol are all consistent with formation of a p-quinone methide intermediate, which subsequently will react with water. The p-quinone methide formed from this substrate is highly stabilized in the active site as long as the enzyme remains reduced. This suggests that the active site of the reduced enzyme intermediate complex is solvent inaccessible. Upon reoxidation of the flavin, the p-quinone methide intermediate rapidly reacts with water, indicating that during this process local structural changes occur leading to a more solvent accessible active site. The p-quinone methide is hydrated to form the unstable hemiacetal product of 4-(methoxymethyl)phenol, which decomposes rapidly to give 4-hydroxybenzaldehyde (Equation 4). The presence of reduced glutathione during turnover of 4-(methoxymethyl)phenol did not influence the stoichiometric formation of the aldehyde product, indicating that hydration of the formed p-

[Image 323x399 to 548x485]

**Equation 4**
However, analysis of the substrate-dependent reduction rates revealed that the reversible step of reduction must be very small (k₁ < 0.5 s⁻¹) and is too small to explain the relatively high amount of oxidized enzyme present after the first phase of flavin reduction. Furthermore, enzyme-monitored turnover experiments showed that the enzyme is mainly in the oxidized state (91%) during steady-state turnover. As a consequence, with vanillyl alcohol, a reductive step is limiting the turnover rate also.

From the rapid reaction kinetic parameters obtained in this study, it can be concluded that VAO catalyzes the oxidative demethylation of 4-(methoxymethyl)phenol via a ternary complex mechanism. Also, the parallel lines pattern of Lineweaver-Burk plots found for the steady-state kinetics are in accordance with a ternary complex mechanism as k₂ is negligibly small and k₃ is relatively large (Table I). Because k₅ is very small, only a small portion of enzyme will react via a ping-pong mechanism as represented by the left cycle in Scheme I. From single turnover experiments, it could be deduced that the rate of flavin reduction, i.e. formation of the binary complex (k₂ = 3.3 s⁻¹), is by far the rate determining step in catalysis (k₅cat = 3.1 s⁻¹). The enzyme-monitored turnover results are also consistent with the proposed kinetic mechanism. When the formation of the Michaelis-Menten complex is a relatively fast process (at infinite substrate concentrations), the ratio of enzyme in the oxidized state during steady-state can be calculated by the following.

\[
\frac{E_{\text{cat}}}{E_{\text{oxid}}} = \frac{(1/k_5) + (1/k_1)}{(1/k_2) + (1/k_3) + (1/k_4)} \quad (\text{Eq. 5})
\]

For 4-(methoxymethyl)phenol, the calculated ratio is 0.92 (k₁ = 3.9 s⁻¹ at 500 μM), which compares quite well with the experimental obtained value of 0.86. This indicates that the mechanism-based calculated rate of product release (k₉ = 50 s⁻¹) is a reasonable approximation.

Taken together, the kinetic data are consistent with a ternary complex mechanism including (right cycle of Scheme I) 1) formation of a Michaelis-Menten complex, 2) flavin reduction and synchronous formation of the reduced enzyme intermediate complex, 3) reoxidation of the reduced enzyme complex by molecular oxygen with the concomitant conversion of the intermediate to form the final product, and 4) product release completing the catalytic cycle. Although a ternary complex mechanism is operative with 4-(methoxymethyl)phenol, with vanillyl alcohol, the reaction may also follow a ping-pong mechanism (represented by the left cycle in Scheme I). With this substrate, the binary reduced enzyme intermediate complex readily decomposes to form product without prior reoxidation of the flavin. In both cases, the rate of substrate-mediated flavin reduction is mainly limited by the overall rate of catalysis.

From the results presented in this study, it can be concluded that VAO efficiently converts the transient formed p-quinone methides. Quinone methides are highly electrophilic compounds and are thought to be involved in several toxicological processes. Studies have shown that formation of analogous quinone methides can result in (cyto)toxic effects by forming covalent bonds with cellular nucleophiles like proteins or DNA (25, 28). Preliminary release of the reactive product intermediate formed by VAO would also result in spontaneous reactions with water or other nucleophiles and could, therefore, in vitro, elicit possible deleterious effects. Clearly, efficient hydration of the p-quinone methide in the active site of the enzyme is a prerequisite for the microorganism to exclude potential toxic effects.

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