Studies on the Oxidative Half-reaction of p-Hydroxyphenylacetate 3-Hydroxylase*

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The oxidative half-reaction of the two-protein enzyme, p-hydroxyphenylacetate 3-hydroxylase from Pseudomonas putida, has been studied by absorbance stopped-flow techniques. The formation of three flavin-oxygen intermediates, the anionic and protonated forms of the flavin hydroperoxide (intermediates I and I*) and the flavin hydroxyl (intermediate III), was observed during the course of the oxygen reaction with the reduced flavoprotein-coupling protein complex. The flavin hydroperoxide, which is formed in a second-order reaction with oxygen, is in rapid equilibrium with the aromatic substrate, p-hydroxyphenylacetate. Due to this rapid equilibrium, p-hydroxyphenylacetate effectively competes with other ligands, such as p-chlorophenylacetate and p-aminophenylacetate and proceeds through the hydroxylation pathway. Furthermore, dehydrogenation of intermediate III is subject to substrate inhibition in the presence of excess p-hydroxyphenylacetate, similar to the observations made with phenol hydroxylase. A reaction mechanism for the oxidative half-reaction in the presence of the aromatic substrate, p-hydroxyphenylacetate, is proposed.

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**Fig. 1.** Lineweaver-Burk plot of steady-state turnover of the enzyme complex in the presence of excess \( p \)-hydroxyphenylacetate (\( p \)-OHPA). Initial velocity measurements are of the flavoprotein (60 \( \mu \)mol-coupling protein (180 \( \mu \)mol) complex, pre-equilibrated with 50 \( \mu \)M \( p \)-hydroxyphenylacetate in 0.025 M phosphate buffer containing 10% glycerol at pH 7.0, when reacted with an equal volume of buffer containing 50 \( \mu \)M NADH, air-saturated oxygen (room temperature), and varying concentrations of \( p \)-hydroxyphenylacetate (0–2 \( \mu \)mol). The measurements were made at 4 °C with a stopped-flow apparatus connected to an XY-recorder. Enzyme activity was monitored by the loss of absorbance at 340 nm due to the oxidation of NADH. The enzyme is severely inhibited by excess substrate. \( eV \), enzyme concentration/velocity.

**Fig. 2.** Oxidative half-reaction of \( p \)-hydroxyphenylacetate 3-hydroxylase in the presence of excess \( p \)-hydroxyphenylacetate. Absorbance changes at 400 (panel A) and 480 nm (panel B) when reduced flavoprotein (22 \( \mu \)mol) plus coupling protein (3.4 equivalents) in the presence of 250 \( \mu \)M \( p \)-hydroxyphenylacetate was mixed with an equal volume of oxygenated buffer (final concentration of oxygen after mixing was 0.61 mM) containing 0.25–5 mM \( p \)-hydroxyphenylacetate (final concentrations of \( p \)-hydroxyphenylacetate after mixing were 250, 375, 625, 1125, and 2625 \( \mu \)mol, curves from top to bottom in the 10–50 ms range in Panel A and from left to right in panel B). Both solutions were in 0.025 \( \nu \) phosphate buffer containing 10% glycerol, at pH 7.0 and 4 °C. Panel A, inset, direct plot of the observed rate for the formation of intermediate \( I^* \), versus the concentration of \( p \)-hydroxyphenylacetate. A limiting rate of \( \approx -200 \) s\(^{-1} \) for the formation of \( I^* \), and a \( K_d \) of 1 mM for the binding of \( p \)-hydroxyphenylacetate to intermediate I were calculated. Panel B, inset, direct plot of the observed rate of formation of oxidized enzyme versus the concentration of \( p \)-hydroxyphenylacetate. A rate of \( 3.1 \) s\(^{-1} \) for the decay of free intermediate III and 0.15 s\(^{-1} \) for the decay of intermediate III bound to substrate was calculated from the graph. In both panels the traces between 1 and 4 ms represent absorbance during flow. Meaningful absorbance changes begin at approximately 4 ms. The instrument has a dead time of 3 ms between mixing and observation of absorbance changes.

Excess substrate also severely inhibits the dehydrogenation of intermediate III (last phase) to form oxidized enzyme. As shown in Fig. 2, the dehydration of intermediate III, which is associated with a decrease in absorbance at 400 nm and a large absorbance increase at 480 nm, is slowed down in the presence of increasing concentrations of \( p \)-hydroxyphenylacetate. From the plot of the observed rate for the decay of intermediate III versus the concentration of the aromatic substrate (Fig. 2B, inset), a \( K_d \) of \( \approx -175 \) \( \mu \)mol for the binding of the aromatic substrate to intermediate III was calculated. Furthermore, extrapolation to the y axis gave a rate of \( 3.1 \) s\(^{-1} \) for the decay of free intermediate III to oxidized enzyme. Also, a rate of 0.15 s\(^{-1} \), the...
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Scheme I. Stability of the C4a-hydroxyflavin of p-hydroxyphenylacetate 3-hydroxylase in the presence of the aromatic substrate p-hydroxyphenylacetate.

Scheme II. Proposed reaction mechanism for the oxidative half-reaction of p-hydroxyphenylacetate 3-hydroxylase in the presence of p-hydroxyphenylacetate.
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TABLE I

<table>
<thead>
<tr>
<th>Compound in complex with reduced enzyme</th>
<th>Minimum scheme for each reaction from observed kinetic</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Chlorophenylacetate</td>
<td>( E_{red} - \text{CPA} \rightarrow O_2 \rightarrow \text{I} \rightarrow E_{ox} )</td>
</tr>
<tr>
<td></td>
<td>( k_{1/2} = 17 \text{ min} )</td>
</tr>
<tr>
<td></td>
<td>( 1.66 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} )</td>
</tr>
<tr>
<td>p-Aminophenylacetate</td>
<td>( E_{red} - \text{NH}<em>{2} - \text{PA} \rightarrow O_2 \rightarrow \text{I} \rightarrow E</em>{ox} )</td>
</tr>
<tr>
<td></td>
<td>( 3.4 \text{ s}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( -43 % )</td>
</tr>
<tr>
<td></td>
<td>( 0.073 \text{ s}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( 0.024 \text{ s}^{-1} )</td>
</tr>
<tr>
<td>p-Hydroxyphenylpropionate</td>
<td>( E_{red} - \text{OHPP} \rightarrow O_2 \rightarrow \text{I} \rightarrow E_{ox} )</td>
</tr>
<tr>
<td></td>
<td>( 11 \text{ s}^{-1} )</td>
</tr>
<tr>
<td></td>
<td>( -60 % )</td>
</tr>
<tr>
<td></td>
<td>( 0.09 \text{ s}^{-1} )</td>
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<tr>
<td></td>
<td>( 0.04 \text{ s}^{-1} )</td>
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very well with the experimental results.

Oxidative Half-reaction with p-Chlorophenylacetate—
Preliminary steady-state experiments (16) in which the substrate specificity of the enzyme was tested suggested that the flavoprotein by itself is fairly nonspecific. However, in the presence of the coupling protein, it is very specific for p-hydroxyphenylacetate. In the presence of p-chlorophenylacetate, the flavoprotein alone catalyzes the oxidation of NADH, but, when it is complexed with the coupling protein, NADH is not oxidized, and no hydroxylation of p-chlorophenylacetate is observed. Results of studies on the reductive half-reaction of the enzyme (± coupling protein) clearly demonstrated that the coupling protein had no effect on the reductive half-reaction (17). Hence, experiments were designed to study the reaction with oxygen of the flavoprotein-coupling protein complex in the presence of 1 mM p-chlorophenylacetate. The results of such an experiment are presented in Fig. 4A. A flavin-oxygen intermediate attributed to the flavin C4a-hydroperoxide (intermediate I), with absorbance maximum at 375 nm, is formed rapidly but is stabilized quite dramatically (even more than what is observed in the absence of any substrates (16)). The formation of this intermediate was linearly dependent on the oxygen concentration, and a second order rate of \( 1.66 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \), the same as that observed in the presence of p-hydroxyphenylacetate, was calculated for its formation. The decay of the flavin-oxygen intermediate to oxidized enzyme occurred with \( a t_{1/2} = 17 \text{ min} \) (Fig. 4A, inset). In these reactions ~37% of the reduced enzyme was directly converted to the oxidized form without formation of intermediates, in a reaction which has the same dependence on the oxygen concentration as that observed for the formation of hydroperoxide. This is presumably due to incomplete complex formation between the reduced flavoprotein and the coupling protein. A minimum scheme for the kinetics observed in the oxidative half-reaction with p-chlorophenylacetate as the aromatic ligand is presented in Table I. When the enzyme complexed with p-chlorophenylacetate was reacted with an equal volume of buffer containing 1 mM p-hydroxyphenylacetate, as shown in Fig. 4B, p-hydroxyphenylacetate effectively competed with p-chlorophenylacetate, and the enzyme was rapidly oxidized. Since the substrate binds slowly to the reduced enzyme and does not compete effectively with the reaction of oxygen with the reduced enzyme (17), the competition between p-hydroxyphenylacetate and p-chlorophenylacetate must occur at the level of intermediate I. This observation further illustrates that a rapid equilibrium exists for the binding of aromatic substrates to intermediate I.

Oxidative Half-reaction with p-Aminophenylacetate—In steady-state experiments with p-aminophenylacetate, although the flavoprotein component by itself catalyzed the oxidation of NADH (~70% efficiency compared with p-hydroxyphenylacetate), the rate of oxidation of NADH was slowed down in the presence of the coupling protein (~4%, compared with p-hydroxyphenylacetate). However, under these conditions, the lower rate of NADH oxidation resulted in p-aminophenylacetate being hydroxylated (~60% efficiency).

In the oxidative half-reaction of the reduced flavoprotein-coupling protein complex in the presence of 1 mM p-aminophenylacetate, at least three flavin-oxygen intermediates, attributed to intermediates I, I*, (anion and protonated forms of the flavin hydroperoxide), and III (flavin hydroxide), were formed. The formation of only the first oxygen intermediate was dependent on the oxygen concentration (Fig. 5/A), and the second order rate for the formation of intermediate I was also \( 1.66 \times 10^6 \text{ M}^{-1} \text{ s}^{-1} \). Intermediate I*, is formed at a rate of \( 3.4 \text{ s}^{-1} \). The absorbance traces at 480 nm (where only oxidized enzyme has absorbance) show that ~40% of the initial enzyme has been
converted to the oxidized form due to a bifurcation in the kinetic pathway, as shown in Table I. This bifurcation occurs in the same time frame as the formation of intermediate III, i.e. at the stage of intermediate I*, and occurs at 0.073 s⁻¹. Furthermore, since the enzyme only partially hydroxylates p-aminophenylacetate (16), it can be concluded that this bifurcation must occur at the level of the flavin-hydroperoxide, the non-productive decay of which results in oxidized flavin and H₂O₂. Thus, the second oxygen intermediate designated as intermediate I* must still represent some form of the flavin-hydroperoxide, probably the neutral (protonated) hydroperoxide, as postulated with phenol hydroxylase (10). Intermediate III, resulting from productive hydroxylation, dehydrates to oxidized enzyme at a rate of 0.024 s⁻¹ and, thus, completes the catalytic cycle. In the reaction of reduced p-hydroxybenzoate 3-hydroxylase with oxygen in the presence of p-aminobenzoate, the formation of a high extinction intermediate (intermediate II) was observed (2). Such an intermediate was not seen with p-hydroxyphenylacetate 3-hydroxylase with p-aminophenylacetate as the aromatic substrate. The spectra of the flavin oxygen intermediates derived from deconvolution of the reaction traces are presented in Fig. 6. Similar to the oxidative half-reaction in the presence of p-hydroxyphenylacetate, the flavin-oxygen intermediates have absorbance maxima in the 350-400 nm region. When the reduced protein complex equilibrated with p-aminophenylacetate was rapidly mixed with an equal volume of oxygenated buffer containing 1 mM p-hydroxyphenylacetate instead of p-aminophenylacetate, the p-aminophenyllactate initially present was displaced, as shown in the reaction traces of Fig. 5B where the oxidized enzyme returned faster than when p-hydroxyphenylacetate was absent. When the oxygenated buffer contained 5 ms p-hydroxyphenylacetate, excess substrate inhibition (at the stage of dehydration of III), similar to the experiment presented in Fig. 2, was observed (results not shown). This clearly indicates that the substrate displacement reaction occurring in the presence of p-hydroxyphenylacetate proceeds through hydroxylation.

**Oxidative Half-reaction with p-Hydroxyphenylpropionate**

The oxidative half-reaction of the reduced flavoprotein-coupling protein complex in the presence of the aromatic substrate, p-hydroxyphenylpropionate, was similar to the reaction in the presence of p-aminophenylacetate. Three flavin-oxygen intermediates were formed in the oxygen reaction, which can be attributed to the anionic and protonated forms of the flavin hydroperoxide (intermediates I and I*) and the flavin hydroxide (intermediate III). 36% of the initial enzyme was reoxidized due to a bifurcation at the stage of intermediate I*, similar to the oxygen reaction in the presence of p-aminophenylacetate. The kinetics of the oxidative half-reaction in the presence of p-hydroxyphenylpropionate are summarized in Table I.

**DISCUSSION**

In this paper, we present data on the reaction of the reduced flavoprotein-coupling protein complex with oxygen in the presence of different aromatic substrates. This enzyme complex behaves as a typical hydroxylase, sharing several similarities with the other aromatic hydroxylases such as p-hydroxyphenylacetate 3-hydroxylase (2-5), phenol hydroxylase (7-10), melilotate hydroxylase (11), and anthranilate hydroxylase (6). The half-reaction with oxygen of enzyme in complex with the aromatic substrate fits a sequence of three intermediates in the catalytic cycle, resulting in hydroxylation of the substrate.

Intermediate I is formed directly in a second-order reaction of enzyme-bound reduced flavin with oxygen, and, under some conditions, can decay to H₂O₂ and the oxidized enzyme. This intermediate is formed both in the presence and the absence of the aromatic substrate at the same rate of 1.66 × 10⁴ s⁻¹, and because of its direct rate of dependence on the concentration of oxygen, it is identified as the flavin C4a-hydroperoxide. The fact that its rate of formation is independent of the presence or absence of substrate suggests that the binding of substrates occurs primarily to intermediate I rather than to the reduced enzyme. Furthermore, the binding of the aromatic substrate to the reduced enzyme is slow and does not compete effectively with the reaction of oxygen for the reduced enzyme (17). While the slow binding of substrate to reduced enzyme has also been reported for p-hydroxybenzoate hydroxylase (2) and phenol hydroxylase (10), these enzymes do not stabilize the flavin hydroperoxide in the absence of substrate and do not readily exchange bound ligand at the level of this intermediate.

Most flavin-dependent aromatic hydroxylases show a relatively narrow substrate specificity. Several substrate analogs can act as effectors, stimulating the rate of reduction of the enzyme-bound flavin without resulting in substrate hydroxylation. A few substrate analogs, however, can serve as substrates and also become hydroxylated at a position ortho to the hydroxyl group (20). The deprotonation of the 4-hydroxy group of the substrate facilitates electrophilic attack by the C4a-hydroperoxide (20-22). Based on frontier orbital theory and molecular orbital calculations, Vervoort et al. (20) provide insight into the reason underlying activation of the aromatic substrate.
p-chlorophenylacetate (a good effector for NADH oxidation) is not hydroxylated. This result is consistent with the fact that comes reactive for electrophilic attack (not because of the ad-

frontier π electrons become located in the C-3 position, which makes this position more reactive for electrophilic attack (20)). This would explain why certain substrates such as 4-fluorobenzoate and benzoate are not hydroxylated by 4-hydroxy-

debenzoate 3-hydroxylase, whereas p-hydroxybenzoate, p-amo-

benzoate, and 2,4-dihydroxybenzoate are hydroxylated (20).

Of the four potential substrates considered (p-hydroxybenzoate, p-aminophenylacetate, p-hydroxyphenylpropionate, and p-chlorophenylacetate), only p-chlorophenylacetate is not hydroxylated. This result is consistent with the fact that flavoprotein hydroxylases hydroxylate a benzene ring ortho or para to a phenolic group, i.e. in positions activated toward electrophilic attack. However, in p-chlorophenylacetate (as the halogen substitution is more electron-negative than a phenolic group) diminished electron density in the ring results, with reduced reactivity toward electrophilic attack at the C-3 position of the benzene nucleus, and, thus, it is not hydroxylated.

Intermediate I is further stabilized in the presence of p-chlorophenylacetate (t_{1/2} = 17 min); however, in the additional presence of a hydroxylatable substrate, such as p-hydroxyphenylacetate, the decay of intermediate I proceeds through the hydroxylation pathway. These results indicate that intermediate I is in rapid equilibrium with the aromatic substrate. Such observations were not reported for p-hydroxybenzoate hydroxylase and phenol hydroxylase (possibly because intermediate I, formed in these enzymes has a short half-life, whereas in p-hydroxyphenylacetate 3-hydroxylase, due to the stabilization of the tydroperoxide, p-hydroxyphenylacetate has sufficient time to compete with alternate substrates, such as p-chlorophenylacetate and proceed through hydroxylation).

These results are also consistent with the fact that in p-hydroxyphenylacetate 3-hydroxylase, the aromatic substrate exerts considerable control in the oxidative half-reaction. The futile utilization of reducing equivalents in the presence of p-chlorophenylacetate (a good effector for NADH oxidation) is prevented due to the further stabilization of intermediate I. This result illustrates the importance of the presence of rapid equilibrium binding of p-hydroxyphenylacetate to intermediate I. In the absence of such an equilibrium, simply stabilizing an intermediate would only diminish the rate of oxidation of reduced pyridine nucleotide, and this oxidation would still be nonproductive.

The formation of a high extinction intermediate (intermedi-
te II), was not observed with p-hydroxybenzoate hydroxylase in the presence of p-hydroxybenzoate; however, such an inter-

mediate was observed in the presence of p-aminobenzoate (2). In p-hydroxyphenylacetate 3-hydroxylase, no evidence for the formation of a high extinction intermediate was obtained even in the presence of p-aminophenylacetate. This suggests that either such an intermediate is not formed or, if it is formed, it decays very fast and hence is not observable.

Several flavoprotein hydroxylases are known to be severely inhibited by excess substrate (9, 10). This inhibition seems to be primarily due to the stabilization of the C4α-hydroxyflavin by the binding of substrate (10). p-Hydroxyphenylacetate 3-hydroxylase also shows excess substrate inhibition by stabilization of this intermediate. Furthermore, our results indicate that the dehydration of the C4α-hydroxyflavin is the rate-de-
termining step in catalysis, as the turnover number is compa-
rable with its rate of dehydration. At this point we can only speculate on the physiological relevance for the inhibition ob-

served with excess substrate. It may be a mode of enzyme regulation wherein the substrate is fed into the catabolic path-

way slowly. If we consider the metabolism of p-hydroxyphenyl-

cetate, the most expensive reaction for the bacterial cell is the hydroxylation reaction, and, thus, it would seem to be the most logical step that would need to be subjected to metabolic control. It is well documented that in flavoprotein hydroxylases, reduction of the enzyme-bound flavin occurs only in the presence of the aromatic substrate; however, it may become neces-
sary to set a limit on the hydroxylation reaction as the reaction requires utilization of energy. The decay of the hydroxyflavin to yield oxidized enzyme is the rate-determining step in catalysis presumably because the hydroxyflavin is comparatively chemi-

cally inert. Thus, it is conceivable that the imposition of a control on the rate of dehydration of the hydroxyflavin would be easier than on preceding steps because this intermediate is less transient than other intermediates in the hydroxylation path-

way.

REFERENCES

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