Effect of Monovalent Anions on the Mechanism of Phenol Hydroxylase*

Kristina Detmer and Vincent Massey

From the Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

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The mechanism of phenol hydroxylase (EC 1.14.13.7) has been studied by steady state and rapid reaction kinetic techniques. Both techniques give results consistent with the Bi Uni Uni Bi ping-pong mechanism for other flavin-containing aromatic hydroxylases. The enzyme binds phenolic substrate and NADPH in that order, followed by reduction of the flavin and release of NADP+. A transient charge transfer complex between reduced enzyme and NADP+ can be detected. Molecular oxygen then reacts with the reduced enzyme-substrate complex. Two to three flavin-oxygen intermediates can be detected in the oxidative half-reaction depending on the substrate, provided monoanions are present. Oxygen transfer is complete with the formation of the second intermediate. Based on its UV absorption spectrum and on the fact that oxygen transfer has taken place, the last of these intermediates is presumably the flavin C(4a)-hydroxide.

Monovalent anions are uncompetitive inhibitors of phenol hydroxylase. The mechanistic step most affected is the dehydration of the flavin C(4a)-hydroxide to give oxidized enzyme. Chloride also kinetically stabilizes the blue flavin semiquinone of phenol hydroxylase during photoreduction. These data suggest binding of monoanions results in stabilization of a proton on the N(5) position of the flavin.

It is now apparent that flavoenzymes catalyzing the same class of reactions have many structural and mechanistic features in common (1). The flavin-containing aromatic hydroxylases are no exception. These hydroxylases have the same steady state kinetic mechanism (Bi Uni Uni Bi ping-pong (2-4)), are inhibited by monoanions (5-8), form charge transfer complexes between enzyme and pyridine nucleotide (2, 9, 10), are more rapidly reduced by pyridine nucleotides in the presence of substrate (4, 10-12), and form one or more flavin-oxygen intermediates following reaction of the reduced enzyme-substrate complex with molecular oxygen (2, 13-16). One atom of molecular oxygen is transferred to the substrate; the other is reduced to H₂O.

The oxygen atom is incorporated into a position either ortho or para to an existing hydroxyl group on the aromatic substrate, i.e. at an activated position. Four electrons are required for this reaction. Two electrons are supplied by the pyridine nucleotide; the other two are supplied by the substrate. Phenol hydroxylase catalyzes the following reaction.

\[
\begin{align*}
&\text{R} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{R} + \text{H}_2\text{O} + \text{NADP}^+ \\
&\text{OH} \\
\end{align*}
\]

The enzyme is isolated from a soil yeast, Trichosporon cutaneum (7), making phenol hydroxylase the only flavin-dependent aromatic hydroxylase readily obtainable from an eucaryotic organism. The other enzymes in this class are all derived from various procaryotes, mostly pseudomonads (17). We were interested in comparing this eucaryotic aromatic hydroxylase with the evolutionarily distant microbial aromatic hydroxylases.

We were especially interested in the number of flavin-oxygen intermediates we could detect in the oxidative half-reaction. Earlier work with p-OH-benzoate hydroxylase demonstrated the formation of three intermediates during the oxidative half-reaction (15). Since one of our operating assumptions has been that the mechanism of oxygen activation is the same for all the flavin-dependent aromatic hydroxylases, we sought to confirm the p-OH-benzoate hydroxylase observation with phenol hydroxylase.

In this paper, we show that phenol hydroxylase displays Bi Uni Uni Bi ping-pong kinetics with ordered addition of phenolic substrate and pyridine nucleotide. Reduction by pyridine nucleotide is accelerated by the binding of substrate, and a transient charge transfer complex between reduced enzyme and NADP+ can be detected. Monovalent anions are inhibitors. In their presence, three transient intermediates can be detected in the oxidative half-reaction. Monovalent anions retard, by several orders of magnitude, the conversion of the third of these intermediates to oxidized flavin. The third intermediate appears to be the flavin C(4a)-hydroxide.

MATERIALS AND METHODS

Phenol and high performance liquid chromatography grade methanol were purchased from Mallinkrodt Chemical Works, resorcinol from J. T. Baker Chemical Co., and potassium azide from Eastman; all were used without further purification. Catechol and 1,2,4-trihydroxybenzene were purchased from Pfaltz and Bauer, NADPH type III and glucose-6-phosphate dehydrogenase type XII from Sigma, and mixtures of nitrogen and oxygen from Matheson, Coleman and Bell. Glucose-6-phosphate was purchased from Nutritional Biochemicals.

Phenol hydroxylase was isolated by a modification of the method of Neujahr and Gaal (7) as previously described (18).

Standard Experimental Conditions—Unless otherwise stated, all experiments were carried out in 50 mM KPi, pH 7.6, at 4°C.

Steady State Kinetics—Enzyme activity was measured by following the consumption of NADPH at 340 nm with a Gilford recording spectrophotometer or by monitoring oxygen consumption using a Clark Yellow Springs oxygen electrode. Oxygen concentrations were varied by bubbling with N₂/O₂ mixtures of known composition before measurement.

Product Determination and Quantitation—Products were analyzed...
using a Varian variable wavelength high performance liquid chromatograph with detection at 276 nm. Reaction mixtures were acidified, filtered, and injected onto an ultrasharp ODS (5 μm) C18 column from Altex Scientific C. Mobile phases used were 50 mM KH2PO4 containing varying quantities of methanol.

**RESULTS**

**Steady State Kinetics**—In the formalism of Dalziel (20) the general initial rate equation for an enzyme-catalyzed reaction involving three substrates is

\[
\frac{v}{e} = \frac{\phi_A}{[A]} + \frac{\phi_A}{[B]} + \frac{\phi_A}{[C]} + \frac{\phi_{ABC}}{[A][B][C]}
\]

A complete steady state analysis of such an enzyme requires that two substrates be varied at fixed concentration of the third for each pair of substrates. Plots of reciprocal velocity versus reciprocal substrate yield families of intersecting or parallel lines depending on the mechanism. The kinetic parameters \(\phi_A\), \(\phi_{BC}\), etc., can be determined from secondary or tertiary plots (20).

All steady state experiments were carried out resorcinol (1,3-dihydroxybenzene) as substrate since substrate inhibition occurs with phenol at low concentrations (7). Temperature was 25°C.

When double reciprocal plots are made of initial rates as a function of resorcinol concentration at several fixed NADPH concentrations and air saturated \(O_2\) lines which intersect on the left-hand side of the velocity axis are obtained (Fig. 1A). Replots of the slopes and intercepts are linear (Fig. 1B and C). The intersecting lines in the primary plot indicate that NADPH and resorcinol bind to enzyme species which are "reversibly connected" (21), in this case, to oxidized enzyme.

Parallel lines are obtained from double reciprocal plots of initial rate data when resorcinol and oxygen concentrations are varied at constant NADPH concentration (Fig. 2A) and when NADPH and oxygen concentrations are varied at a constant resorcinol concentration (results not shown). Replots of the intercepts are linear (Fig. 2B). A mechanism consistent with these results is shown in Scheme 1 using the notation of Cleland (22).

![Fig. 1. Initial velocity measurements of enzyme activity at varying concentrations of resorcinol and NADPH. A, reciprocal velocity versus reciprocal resorcinol concentration at 0.26 mM \(O_2\) and the following fixed concentrations of NADPH (bottom to top): 300, 200, 100, 67, and 50 μM in 50 mM KP, pH 7.6, at 25°C. The rate was measured by the consumption of oxygen, monitored with a Clark-Yellow Springs oxygen electrode, and reaction rates were calculated as mol min \(^{-1}\) mol \(^{-1}\) of enzyme. B, secondary plot of slopes from the data in A against reciprocal NADPH concentration. C, secondary plot of intercepts from the data in A against reciprocal NADPH concentration.](image1)

![Fig. 2. Initial velocity measurements of enzyme activity at varying concentrations of resorcinol and oxygen. A, reciprocal velocity versus reciprocal resorcinol concentration at 500 μM NADPH and the following fixed concentrations of oxygen (top to bottom): 61, 120, 260, and 610 μM. B, secondary plot of the intercepts versus reciprocal oxygen concentration. Conditions as in Fig. 1.](image2)

According to this mechanism, a complex is formed by enzyme, resorcinol, and NADPH leading to the reduction of the flavin and the subsequent release of NADP\(^+\). Oxygen then adds to the reduced enzyme-resorcinol complex to generate a second ternary complex. Enzyme-bound oxygen is activated and reacts with resorcinol. Product release completes the catalytic cycle. The reaction of oxygen with the reduced enzyme-substrate complex is essentially irreversible. Reactions of this type are called either concerted substitution, type IIb (20), or Bi Uni Uni Bi ping-pong (21). Similar kinetic mechanisms have also been found for salicylate hydroxylase (4), melleitotase hydroxylase (2), and \(p\)-hydroxybenzoate hydroxylase (3, 23). The kinetic constants derived from the secondary plots are \(V_{max} = 840\) min \(^{-1}\), \(K_{resorcinol} = 50\) μM, \(K_{NADPH} = 210\) μM, and \(K_{Ox} = 66\) μM.

The steady state data are consistent with either random or ordered addition of the first two substrates. However, it is possible to distinguish between random and ordered binding by comparing the dissociation constants for the enzyme-resorcinol and enzyme-NADPH complexes determined directly and the constants determined from the initial rate data.

Since the direct measurements of the dissociation constants were done at 4°C, an initial rate study using variable resorcinol and NADPH at constant oxygen was repeated at that temperature. The buffer was equilibrated with air at 4°C, and the concentration of oxygen was presumed to be sufficient to
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saturate the enzyme. When the data were plotted as reciprocal velocity versus reciprocal NADPH concentration, a series of lines which intersected in the third quadrant was obtained. The negative reciprocal of the horizontal coordinates at the intersection point was 150 μM. This value would be equal to the constant for the dissociation of NADPH from the enzyme-NADPH complex either if the binding of NADPH and resorcinol were random (22) or if it were ordered with NADPH binding first (24). The dissociation constant of the NADPH-enzyme complex was determined as described in a following section to be greatly in excess of 150 μM. These results thus indicate that resorcinol binds first. This conclusion was checked by comparing the $K_d$ for the enzyme-resorcinol complex determined by difference titration, 50 μM (data not shown), with the value derived from the intersection of the lines in the reciprocal velocity versus reciprocal resorcinol concentration plot, 20 μM. The agreement, to within a factor of three, indicates the preferred order of binding is resorcinol followed by NADPH.

An apparent $V_{max}$ of 100 min$^{-1}$ was determined by a replot of the intercepts from the initial rate experiment at 4 °C.

Reductive Half-reaction—When a phenolic substrate is present, phenol hydroxylase reduction by NADPH is greatly facilitated. Fig. 3 shows the effect of NADPH concentration on the rate of anaerobic phenol hydroxylase reduction in the absence (Fig. 3A) and presence (Fig. 3B) of substrate. Under the experimental conditions, the concentration of NADPH is in large excess, and reduction proceeds by a monophasic first-order reaction in the absence of substrate. Plotting the observed rates as a function of NADPH concentration (Fig. 3A) shows the reaction to be second order with a rate constant of 1.25 M$^{-1}$ s$^{-1}$. The $K_d$ for NADPH binding to the enzyme in the absence of substrate can be estimated to be in excess of 4 mM, inconsistent with the $K_d$ of 150 μM expected if NADPH binds first or if binding is random.

In the presence of 1 mM resorcinol, the reduction rate shows saturation with a $k_{max}$ of 16 s$^{-1}$ and a $K_d$ of 67 μM at pH 7.6. This corresponds to an apparent acceleration of 700-fold at 100 μM NADPH. The alteration of $K_d$ in the presence of substrate further confirms the assignment of binding order. Much slower reduction by pyridine nucleotides in the absence of substrate is a common feature of the flavin-dependent aromatic hydroxylases, which can be viewed as a control to prevent the unproductive consumption of reducing equivalents.

In the presence of resorcinol, reduction of the enzyme by NADPH appears to be monophasic when observed at 450 nm (Fig. 4). The reaction obeys first order kinetics. At 550 nm, however, two phases are detected, indicating the formation and decay of a transient intermediate. Assuming an extinction coefficient of 1000 M$^{-1}$ cm$^{-1}$, taken from the reduced enzyme-NAD$^+$ charge transfer complex seen with melilotate hydroxylase (2), only about 25% of the expected absorbance was observed with the highest concentration of NADPH used. This is consistent with the rate of decay of the intermediate being somewhat faster than its formation. This intermediate is probably a charge transfer complex between NADP$^+$ and reduced enzyme. The decay of the long wavelength absorbance represents release of NADP$^+$ from the enzyme. A scheme consistent with the data is as follows:

![Scheme 2](image)

Similar schemes have been proposed to describe the reduction of p-OH-benzoate hydroxylase (3) and melilotate hydroxylase (2). Although not proof that NADP$^+$ release precedes reaction with oxygen, the ready dissociation of NADP$^+$ observed is consistent with the proposed mechanism. NAD$^+$ release appears to precede oxygen reaction during turnover of melilotate hydroxylase (2).

Oxidative Half-reaction—When reduced enzyme complexed with resorcinol is mixed with oxygenated buffer in a stopped flow spectrophotometer, spectral changes consistent with the formation of two intermediates are seen (Fig. 5). In the 350- to 375-nm region are two well separated phases of increasing absorbance, followed by a phase of decreasing absorbance. At 380 and 390 nm (Fig. 5A), there is one increasing absorbance phase followed by two phases of decreasing absorbance. The rate of formation of the fast phase is dependent on oxygen concentration with a second order rate constant of 1.5 x 10$^6$
M⁻¹ s⁻¹ (results not shown). These results indicate that there are intermediates formed during the oxidative half-reaction. In the presence of monovalent anions, an additional phase can be detected, to be discussed below.

The return of oxidized flavin absorbance is biphasic (Fig. 5B). The first phase is complete after 80 ms, and reoxidation is complete after 40 s. The first phase may represent the reaction with oxygen of a portion of the reduced enzyme not complexed with substrate. When reduced enzyme in the absence of substrate is mixed with oxygen, the rate of the return of flavin absorbance is dependent on the oxygen concentration with an approximate second-order rate constant of 1.5 × 10⁸ M⁻¹ s⁻¹.

**Binding of Chloride to Oxidized Enzyme**—When monovalent anions bind to oxidized phenol hydroxylase, the spectrum of the flavin is perturbed (25). Fig. 6 is a difference titration of enzyme with NaCl. Analysis of the spectral changes by the method of Benesi and Hildebrand (26), shown in the inset, yields a $K_a$ of 130 mM. This is 15-fold greater than the $K_i$ determined from the steady state analysis described below.

**Effect of Anions on the Steady State Kinetics**—Monovalent anions inhibit phenol hydroxylase (25, 27). If reciprocal velocity is plotted against reciprocal NADPH concentration at constant phenol (167 μM) and oxygen (256 μM) concentration in the presence of azide (Fig. 7A) and chloride (not shown) at 25°C, a series of parallel lines is obtained indicating monovalent anions inhibit enzyme turnover uncompetitively with NADPH. Replots of the intercepts yield a $K_i$ for azide of 0.5 mM (Fig. 7B) and a $K_i$ for chloride of 8.6 mM. Assuming that inhibition during turnover by monovalent anions is the result of formation of a reversible dead-end complex with the enzyme, then $K_i = K_d$ (22). Although monovalent anions can bind to the oxidized form of the enzyme, the uncompetitive inhibition pattern seen indicates that monovalent anions inhibit the enzyme at a stage of the catalytic cycle which follows the binding of substrate and pyridine nucleotide (22). We do not know if the anion binding site apparent during turnover is different from the site on the oxidized enzyme or if the same site alters its affinity for anion during turnover.

**Effect of Chloride on the Photoreduction of Phenol Hydroxylase**—When phenol hydroxylase is partially reduced by deaerated buffer was added to the enzyme in the reference cuvette. —, enzyme versus enzyme; — -- 21, -- 40, -- 59, and -- 114 mM NaCl. Temperature, 25°C. Inset, Benesi-Hildebrand plot of the absorbance change. The $K_d$ for chloride binding was calculated from the slope divided by the intercept.

**Oxidative Half-reaction in the Presence of Monovalent Anions**—When reduced enzyme complexed with resorcinol is mixed with oxygenated buffer containing 0.5 mM KCl in a stopped flow spectrophotometer, four phases can be detected (Fig. 9) indicating that three sequential intermediates (I, II, and III) are formed before oxidized flavin reappears. At 350 nm three phases are seen. The first phase, the formation of intermediate I, is complete after 80 ms. Intermediate II for-
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**Stoichiometry of 1,2,4-trihydroxybenzene formation under steady state conditions**

Table I shows that under steady state assay conditions, consumption of NADPH by catalytic quantities of enzyme was monitored at 340 nm under standard conditions at 4 °C. When the reaction was complete, substrate consumption and product formation were measured by high performance liquid chromatography as described under “Materials and Methods.”

<table>
<thead>
<tr>
<th>[NADPH]</th>
<th>[Resorcinol]</th>
<th>[Resorcinol]</th>
<th>%</th>
<th>[1,2,4-Trihydroxybenzene]</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 μM</td>
<td>95 μM</td>
<td>71 μM</td>
<td>96%</td>
<td>19 μM</td>
<td>76%</td>
</tr>
<tr>
<td>50 μM</td>
<td>95 μM</td>
<td>60 μM</td>
<td>70%</td>
<td>44 μM</td>
<td>88%</td>
</tr>
<tr>
<td>50 μM</td>
<td>95 μM</td>
<td>58 μM</td>
<td>74%</td>
<td>44 μM</td>
<td>88%</td>
</tr>
<tr>
<td>75 μM</td>
<td>95 μM</td>
<td>31 μM</td>
<td>85%</td>
<td>52 μM</td>
<td>69%</td>
</tr>
<tr>
<td>100 μM</td>
<td>190 μM</td>
<td>110 μM</td>
<td>80%</td>
<td>62 μM</td>
<td>62%</td>
</tr>
</tbody>
</table>

* The low yield of product at the higher NADPH concentrations is probably a result of oxidation of the product.

**Time course of product formation in the oxidative half-reaction**

The reaction mixture was prepared by mixing substrate and enzyme in a rapid quench device at 4 °C and quenching the oxidative half-reaction at selected intervals in order to monitor over a period of about an hour. The formation of 1,2,4-trihydroxybenzene, as the amount of catechol formed after reoxidation is complete, is summarized in Table II. Two seconds after mixing with oxygen, when phenol is the substrate, intermediate III is completely formed, and almost no oxidized flavin absorbance is formed. (cf. Fig. 5B; 80 ms time scale).

**Determination of the Oxygen Transfer Step—** By acid quenching the oxidative half-reaction at selected intervals in the presence of chloroform, the amount of product formed at a given time could be measured. The results for phenol and resorcinol are summarized in Table II. Two seconds after mixing with oxygen, when phenol is the substrate, intermediate III is completely formed, and almost no oxidized enzyme is formed. The amount of catechol formed at 2 s is the same as the amount of catechol formed after reoxidation is complete, over an hour later, indicating that product formation precedes or is concomitant with intermediate III formation. When resorcinol is the substrate, the amount of product is the same at 1.2 s when intermediate II is completely formed and at 20 s when intermediate III is completely formed. These results indicate that oxygen transfer takes place with the formation of intermediate II, as was previously observed with p-hydroxybenzoate hydroxylase (15). The rather low yield of product with resorcinol may be attributed to a combination...
of incomplete saturation of the reduced enzyme with substrate and some uncoupling of oxidation of enzyme-substrate complex from oxygen transfer to substrate.

**Generation of Intermediate III in Situ**—Since the conversion of intermediate III to oxidized enzyme becomes rate limiting in the presence of monovalent anions, it is possible to trap essentially all the enzyme as intermediate III by use of an NADPH-generating system. Fig. 10 shows phenol hydroxylase in the presence of azide, glucose-6-phosphate, and phenol; and 10 min after the addition of catalytic quantities of glucose-6-phosphate dehydrogenase and NADPH. The spectrum of intermediate III thus obtained has an absorbance maximum at 375 nm and an extinction coefficient of 9.9 mM\(^{-1}\) cm\(^{-1}\). Intermediate III exhibits an intense greenish fluorescence with an emission maximum at 505 nm (Fig. 10, inset). The emission intensity is 40% that of a lumiflavin standard of comparable concentration.

The absorbance spectrum of intermediate III is similar to the spectra of enzymes containing N(5)-ethyl, C(4a)-hydroxy model flavins (29) and to calculated spectra of transient intermediates detected in other flavin aromatic hydroxylases (15, 16). Oxidation of luciferase, a nonaromatic flavin-dependent monoxygenase generates an intermediate with similar absorbance (30) and fluorescence emission (31) spectra. This intermediate was shown to be the flavin C(4a)-hydroperoxide by \(^{13}C\) NMR (32). Since oxygen transfer has taken place with phenol hydroxylase by the time intermediate III is formed, intermediate III must logically be the flavin C(4a)-hydroxide.

**Expanded Mechanism**—Putting the information from the rapid reaction experiments together with the steady state kinetic data results in the expanded mechanism written in Scheme 3.

Oxidized phenol hydroxylase binds first to the phenolic substrate and then to NADPH. The presence of substrate facilitates the reduction of the enzyme by NADPH in part by decreasing the \(K_d\) for NADPH binding. The alteration in the enzyme's affinity for NADPH suggests that a conformational change may take place on binding substrate to the enzyme. Good evidence for such a conformational change has been obtained from x-ray diffraction studies of the p-OH-benzoate hydroxylase-substrate complex. The substrate is bound to the enzyme in such a way as to require a conformational change on binding (33). Since the reduction of the enzyme by NADPH in the absence of substrate appears second order at the concentrations of NADPH used, it cannot be determined from these data if the rate of reduction is altered as well when substrate is present.

Once the ternary complex of enzyme, substrate, and NADPH forms, electrons are transferred to the flavin, and the oxidized pyridine nucleotide dissociates. Since the ratio of product formed to NADPH consumed is less than 1, the mechanism must bifurcate into productive and unproductive paths at some point. It is consistent with the stopped flow experiments, which show a rapidly reoxidized fraction of the enzyme, to assign the unproductive path as arising from the dissociation of substrate from the reduced enzyme-substrate complex. The reduced enzyme minus the substrate then reacts with oxygen to form oxidized enzyme and \(H\_2O\_2\). In the productive path the reduced enzyme-substrate complex reacts with oxygen essentially irreversibly. Three intermediates are formed. However, for all three intermediates to be detected required is the presence of a monovalent anion such as chloride or azide. Product release and formation of oxidized flavin complete the catalytic cycle.

The rate of formation of the first of the intermediates in the oxidative half-reaction is directly dependent on the oxygen concentration. In analogy with similar enzymes, this species is presumably a flavin hydroperoxide. Oxygen is transferred to substrate as intermediate I converts to intermediate II. Intermediate II converts to intermediate III and then to oxidized enzyme. Product release probably takes place with

**TABLE III**

<table>
<thead>
<tr>
<th>Step</th>
<th>(-Cl)</th>
<th>(+Cl) (0.25 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(h_0/h_1)</td>
<td>20 (\mu)M</td>
<td>ND</td>
</tr>
<tr>
<td>(k_0/k_3)</td>
<td>67 (\mu) M</td>
<td>ND</td>
</tr>
<tr>
<td>(k_3)</td>
<td>16 (s)(^{-1})</td>
<td>ND</td>
</tr>
<tr>
<td>(k_7)</td>
<td>(\geq 16) (s)(^{-1})</td>
<td>ND</td>
</tr>
<tr>
<td>(k_9)</td>
<td>1.5 (\times 10^6) (M)(^{-1}) (s)(^{-1})</td>
<td>(1.2 \times 10^6) (M)(^{-1}) (s)(^{-1})</td>
</tr>
<tr>
<td>(k_{10})</td>
<td>(-1.5) (\times 10^6) (M)(^{-1}) (s)(^{-1})</td>
<td>ND</td>
</tr>
<tr>
<td>(k_{14})</td>
<td>8 (s)(^{-1})</td>
<td>2 (s)(^{-1})</td>
</tr>
<tr>
<td>(k_{13})</td>
<td>0.33 (s)(^{-1})</td>
<td>0.22 (s)(^{-1})</td>
</tr>
<tr>
<td>(k_{12})</td>
<td>(\geq 0.3) (s)(^{-1})</td>
<td>3 (\times 10^{-2}) (s)(^{-1})</td>
</tr>
<tr>
<td>(k_{17})</td>
<td>ND(^e)</td>
<td>ND</td>
</tr>
<tr>
<td>(k_6)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>(k_5)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^e\) ND, not determined.
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the formation of oxidized flavin.

The rate constants for the various steps are summarized in Table III. In the absence of chloride, $k_{12}$ must be very much greater than $k_{13}$ since intermediate III is not formed in detectable quantities. In the presence of chloride, $k_{12}$ is much less than $k_{13}$. Similar experiments in the presence of azide show that azide retards the conversion of intermediate III to oxidized enzyme even more than does chloride. The inhibition of this step caused by monovalent anions is consistent with the steady state inhibition kinetics which predict that the step in the mechanism affected would occur after the binding of both substrate and NADPH.

Reaction of reduced enzyme complexed with phenol in the presence of either chloride or azide reveals two flavin-oxygen intermediates (34). Again the return of oxidized flavin is retarded.

The appearance of three intermediates in the oxidative half-reaction when resorcinol is the substrate when only two are seen when phenol is the substrate parallels the case of p-hydroxybenzoate hydroxylase. This aromatic hydroxylase forms two detectable intermediates in the oxidative half-reaction when p-hydroxybenzoate is the substrate and three intermediates when 2,4-dihydroxybenzoate is the substrate (15). Apparently, in the case of both phenol and p-hydroxybenzoate, intermediate II breaks down much faster than it is formed and so is kinetically invisible. To be consistent with the nomenclature established for p-hydroxybenzoate hydroxylase, the intermediates seen with phenol hydroxylase in the presence of phenol have been labeled I and III.

**DISCUSSION**

It is significant that three intermediates are observable in the oxidative half-reaction of phenol hydroxylase in the presence of monovalent anions, when resorcinol is the substrate. Until now, of the other flavin-dependent aromatic hydroxylases, only p-OH-benzoate hydroxylase has shown more than two intermediates. Based primarily on their UV-visible absorption spectra, intermediates I and III have earlier been identified as flavin C(4a)-hydroperoxides and -hydroxides, respectively (34). The structure of intermediate II is still unknown. In a subsequent paper we will present spectra of intermediates I, II, and III obtained with a variety of substrates.

Data from the present study show that the major inhibitory effect of monovalent anions is on the rate of dehydration of the flavin C(4a)-hydroxide to give oxidized enzyme. We, therefore, must question the proposal of Neujahr (25, 27) that monovalent anions inhibit phenol hydroxylation by disrupting the attachment of FAD to the apoprotein leading to its dissociation. This proposal arises from the observation that anions cause perturbation of the enzyme spectrum. Certainly prolonged exposure to high salt concentrations will remove FAD from the protein; however, several experimental results are inconsistent with the proposal that the kinetically relevant inhibitory effect of monovalent anions is the result of induced flavin dissociation.

First, the $K_d$ for chloride binding to the oxidized enzyme, which Neujahr interprets to be disruption of flavin binding, exceeds by an order of magnitude the $K_d$ for chloride inhibition. Second, the oxidative half-reaction studies show that the FAD is still attached to protein; without the protein, reduced FAD does not form three detectable intermediates upon reaction with oxygen. Third, the observation that it is possible to trap intermediate III using an NADPH-generating system in the presence of phenol and azide shows that the enzyme in the presence of 0.2 M KN$_3$ is capable of binding substrate, of binding and being reduced by NADPH, and of reacting with molecular oxygen and transferring an oxygen atom to substrate. Such catalytic competence argues that interference with FAD binding to the apoprotein is minimal.

We propose instead that binding of monovalent anions results in stabilization of a proton on the N(5) position of the flavin. Stabilization could arise either from a conformational change which brings a base into the N(5) region or from coordination of the anion with the N(5) proton directly, as illustrated in Scheme 4.

**Scheme 4**

This hypothesis is strengthened by the observation that addition of chloride stabilizes the blue semiquinone of phenol hydroxylase. The blue flavin semiquinone can be stabilized by fixing an alkyl group or a proton at N(5) (35). Flavoproteins that stabilize the blue semiquinone are thought to stabilize the N(5) proton by means of a hydrogen bridge to a base on the enzyme (36) as has been shown in the crystal structure of the semiquinone form of flavodoxin of *Megasphera elsdonii* (37). Stabilization of a proton on the N(5) position could also explain the effect of monovalent anions on the rate of the dehydration of the flavin C(4a)-hydroxide. As addition of nucleophiles to the C(4a) position is acid catalyzed (38), by the law of microscopic reversibility removal of the N(5) proton is thought to facilitate the loss of hydroxide at the C(4a) position. If a base forms a hydrogen bond with the proton at N(5), the proton becomes inaccessible to abstraction by OH$^-$. If chloride is bound in the N(5) region, electrostatic repulsion could interfere with abstraction of the N(5) proton.

Monovalent anions have been used with p-OH-benzoate hydroxylase and melilotate hydroxylase to increase the kinetic separation of the intermediates in the oxidative half-reaction. As with phenol hydroxylase, the rate of conversion of the p-OH-benzoate hydroxylase C(4a)-hydroxide is retarded by about 2 orders of magnitude in the presence of azide (15). The effect of iodide on melilotate hydroxylase, while not as dramatic, is consistent (16).

Although the above data are suggestive of a common inhibitory mechanism, such a conclusion would be premature. The steady state kinetic patterns of anion inhibition are not consistent; monovalent anions are competitive inhibitors of NADPH with p-OH-benzoate hydroxylase (39), noncompetitive inhibitors with melilolate hydroxylase (8), and uncompetitive inhibitors of phenol hydroxylase. The differences in the steady state patterns indicate anions may exert effects at more than one stage in the catalytic cycle; the relative magnitude of these effects determines the type of inhibition seen.

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**REFERENCES**

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