The kinetic mechanism of salicylate hydroxylase as studied by initial rate measurement, rapid reaction kinetics, and isotope effects

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The kinetic mechanism of Pseudomonas cepacia salicylate hydroxylase has been examined by steady state initial rate measurements, and stopped flow and equilibrium studies. Results indicate that salicylate and NADH bind to the hydroxylase randomly. The enzyme is reduced and NAD$^+$ is released. Oxygen subsequently binds to the reduced enzyme-substrate complex, leading to the production of hydroxylated product, CO$_2$, and water. Based on results of anaerobic rapid mixing experiments, the rate of enzyme reduction by NADH is enhanced 290- and 240-fold when the hydroxylase is complexed with salicylate and benzoate (a nonsubstrate effector), respectively. Salicylate enhances, whereas benzoate slightly weakens, the NADH binding to the enzyme. Primary isotope effects were observed with (4R)-[4-$^3$H]- and (4R)-[4-$^3$H]NADH but not with the (4S)-[4-$^3$H]NADH. Using varying concentrations of benzoate, the pattern of tritium isotope effect on $V_m$, $T(V/K)$, also indicates that benzoate and NADH bind to the enzyme randomly. The intrinsic isotope effects, $\delta V_K$, of (4R)-[4-$^3$H]NADH on the reduction of enzyme-salicylate and enzyme-salicylate complexes were found to be 5.57 and 5.96, respectively. The former is much repressed but the latter is only slightly so in the expression of their corresponding deuterium isotope effects on $V_m$, $\delta V$. Furthermore, values of $\delta V$ (1.69 to 5.07) show a rough correlation with the extents of uncoupling of substrate hydroxylation and H$_2$O$_2$ formation activities for a series of benzenoid effectors. These results indicate that relative to the step of enzyme reduction, the subsequent reaction(s) leading to H$_2$O$_2$ formation must be fast whereas that for substrate hydroxylation contains at least one slow step.

In the absence of salicylate, this enzyme catalyzes the decarboxylative hydroxylation of salicylate to form catechol as shown below.

\[
\text{COOH} \quad \text{OH} \quad + \text{NADH} + H^+ + O_2 \rightarrow \text{FAD}
\]

\[
\text{OH} \quad \text{OH} \quad + \text{CO}_2 + \text{NAD}^+ + \text{H}_2\text{O}
\]

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solved (9-11).

In recent years we have isolated this hydroxylase from Pseudomonas cepacia cells and investigated the nature of flavin binding under both noncatalytic and catalytic conditions (12, 13). In the present study, we have examined the kinetic mechanism of this salicylate hydroxylase by several approaches. Results of steady state and stopped-flow measurements indicate that this hydroxylase follows a reaction pathway involving, sequentially, random binding of salicylate and NADH, release of NAD+ reaction with O2, and the final release of catechol, CO2, and water. The random binding of benzoate and NADH has been independently demonstrated by studies of the tritium isotope effect of [(4R)-[4-3H]NADH].

The importance of the reduction of enzyme-substrate complexes, formed with salicylate and a number of benzenoid effectors, by NADH in relation to the overall reaction rate has also been studied by examining isotope effects of [(4R)-[4-3H]NADH] on the isolated reduction step, and on Vm/Km of the overall reaction.

**Experimental Procedures**

**General Materials**—NADH, equine liver alcohol dehydrogenase, catalase, and glucose oxidase were all obtained from Sigma. NADH was further purified as described later. Lyophilized porcine heart heart diaphorase was a product of Boehringer Mannheim Biochemicals. Salicylate and benzenoid effectors were all purchased from Aldrich. Concentrations of NADH, salicylate, and salicylate hydroxylase (monomer) were determined spectrophotometrically using extinction coefficients, in m-ccm⁻¹, of 6,220 at 340 nm, 3,400 at 296 nm, and 11,300 at 450 nm, respectively. Oxygen (zero grade) and nitrogen (high purity grade) gases were products of Union Carbide. The latter was made oxygen-free by passing through a BASF catalyst (Ace Glass Co.) column heated to 150 °C. Variations of the concentration of dissolved oxygen were achieved by equilibrating sample solutions with desired mixtures of nitrogen and oxygen obtained by the use of calibrated flowmeters (Series 180K, Union Carbide).

**Microorganism and Salicylate Hydroxylase—**A P. sp. 29351 cell stock was obtained from American Type Culture Collection. After single colony selection the organism was identified to be P. cepacia (12). Salicylate hydroxylase was purified from P. cepacia cells following the previously reported method (8) with slight modifications (12). This P. cepacia enzyme (12) was at first expected to be the same as that originally obtained from P. sp. 29351 (9), and both indeed showed identical molecular weight, subunit structure, and FAD content (13). However, we have later found that the P. cepacia enzyme neither exhibits any long wavelength absorption upon reduction nor binds to an acetylated amino acid effector and is being distinct from the enzyme isolated from P. sp. 29351 (9, 14). These two hydroxylases also differ in values of several isotope effects observed with NADH isotopically labeled at the 4R position (see "Results"). For over a period of four years and about 12 runs of cell growth and enzyme purification in our laboratory, the isolated P. cepacia salicylate hydroxylase samples were apparently identical in spectral, structural, and kinetic properties. However, it is uncertain whether the selected P. cepacia strain was a contaminant or a spontaneous mutant of the original P. sp. 29351 cell stock (from American Type Culture Collection). The P. sp. 29351 strain was originally derived from P. sp. 29352 apparently by spontaneous mutation (9). The P. cepacia enzyme is also different from the P. sp. 29352 enzyme (8) in extents of activity uncoupling by benzenoid effectors (see "Results").

**Preparation of Isotopically Labeled NADH—**[(4R)-[4-3H]NADH] was synthesized by incubation of NAD+ with [U-3H]ethanol (96% H3, Merck) and equine liver alcohol dehydrogenase (15). The (4S)-[4-3H]NAD+ was prepared from NAD+ by the porcine heart diaphorase-catalyzed reaction in H2O (99.8% H, Aldrich) as previously reported (15). The [(4R)-[4-3H]NADH] was similarly obtained by the enzymatic reduction of [(4R)-[4-3H]NAD+] (Amer sham) in H2O. All the isotopically labeled NADH samples, initially obtained as a barium precipitate and the commercial NADH samples were further purified on DEAE-cellulose columns (carbohydrate form pre-equilibrated with water) eluted with a 0 to 0.15 M gradient of (NH4)2CO3 at 4 °C. Fractions with A340/A260 < 2.3 were pooled and lyophilized. The purified [(4R)-[4-3H]NAD+ had a specific radioactivity of 5.3 mCi/mmol.

**Steady State Kinetics**—Activity assays were carried out at 23 °C in 25 mM KP, pH 7.6, containing 14 mM salicylate hydroxylase, benzenoid substrate (or effector), and NADH or isotopically labeled NADH. Reactions were monitored by following A340/min using a Perkin-Elmer absorption spectrophotometer 552. For initial rate measurements involving systematic variations of the concentrations of salicylate, NADH, and O2, reactions were carried out at 23 °C using a Dionex D-110 stopped flow spectrophotometer interfaced to a Northstar microcomputer (On-line Instrument Systems). One drive syringe was filled with 20 mM KP, pH 7.6, containing 14 mM salicylate hydroxylase (based on monomer concentration), 0.8 μM FAD, a designated amount of salicylate, and either 0 or 0.24 mM O2. The other drive syringe contained various concentrations of NADH and O2 in the same buffer. Upon mixing, changes in A340 were recorded for 40 to 60 s. A fresh enzyme working solution was prepared for each set of five or six repetitive determinations under the same testing conditions and results were averaged.

**Activity Uncoupling**—Using salicylate and various benzenoid effectors, the extents of uncoupling of the monoxygenase and oxidase activities were determined, using a Gilson oxigraph model 5/6, by measuring O2 consumptions and H2O2 productions in the absence and presence of catalase following the method of White-Stevens and Kamin (8).

**Anaerobic Reduction of Enzyme**—The kinetics of reduction of bound FAD by NADH or [(4R)-[4-3H]NADH] in the presence or absence of salicylate or benzoate was determined using a Dionex D-110 stopped flow spectrophotometer. Oxygen was removed from reaction solutions by 8 cycles of evacuation and re-equilibration with deoxygenated nitrogen. The final trace of oxygen was removed by including glucose (0.3 mM) and glucose oxidase (10 units/ml) in sample solutions. One drive syringe contained the NADH or [(4R)-[4-3H]NADH] solution and the other was filled with a solution of the holoenzyme with or without salicylate or benzoate. The solutions were mixed at 23 °C and reactions were followed by measuring changes in A340. For each condition tested, at least four repetitive measurements were made and averaged.

**Kinetic Isoenzyme Effects**—The nomenclature and notations of Northrop (16) for deuterium and tritium isotope effects are followed. For measurements of Vj and Vj/Kj, initial rate reactions were determined, by the manual steady state assay method, at 0.24 mM O2 using constant levels of benzenoid substrate (or effectors) and varying concentrations of NADH, [(4R)-[4-3H]NADH, or (4S)-[4-3H]NADH. The intrinsic kh effect was determined by stopped flow measurements of the rates of enzyme reduction by labeled and nonlabeled NADH, with results analyzed by the previously described method (see "Results") (17). For the determination of kh/Vj, effect, reactions were carried out by the manual assay method using a constant level of [(4R)-[4-3H]NADH] and designated amounts of enzyme and salicylate or benzoate. At various times, the fractional conversions of substrate to product, f, were determined by measuring changes in A340. Aliquots (40-50 μl each) were then transferred to a DEAE-cellulose column (0.5 x 3.5 cm) and washed with 1.5 ml of H2O to elute all tritiated water. The specific activity of the product formed at a designated time point, R, was calculated from the microcuries of H released (into water) and the micromoles of NADH oxidized. In all cases, data were collected for f ≥ 0.2. The T/Vj was then determined (18) as

\[
T/V_j = \frac{\log(1 - f)}{\log(1 - IR/RO)}
\]

where Rj is the specific activity of the initial [(4R)-[4-3H]NADH] substrate. Some lyophilized [(4R)-[4-3H]NADH] samples were found, using the same DEAE-cellulose column chromatographic method in the absence of enzyme, to contain trace amounts of tritiated water. Such contaminations, if present, were corrected for in calculating the amount of H released enzymatically into water.

**Results**

**Initial Rate Studies**—The initial rate equation for a three-substrate enzymatic reaction, which follows Michaelis-Menten kinetics with respect to each substrate, has been previously shown to assume the general form of

\[
V = \frac{V_{max} [S_1] [S_2] [S_3]}{K_{M1} [S_1] + K_{M2} [S_2] + K_{M3} [S_3] + K_{M12} [S_1][S_2] + K_{M13} [S_1][S_3] + K_{M23} [S_2][S_3] + K_{M123} [S_1][S_2][S_3]}
\]
$v = \frac{\phi_o + \phi_{AB} + \phi_B + \phi_C + \phi_{ABC} + \phi_{A/[B]} + \phi_{A/[C]} + \phi_{B/[C]} + \phi_{A/[B][C]}}{[A] + [B] + [C]}$  

(3)

where $\phi$ is the concentration of active center and $v$ is the observed initial rate. The concentrations of the three substrates are [A], [B], and [C]. The reciprocal of the maximum rate with unit enzyme concentration is shown as $\phi_o$ whereas $\phi_A$, $\phi_B$, etc. are functions of rate and/or dissociation constants as defined by Dalziel (19).

Initial velocities of salicylate hydroxylase were examined at various levels of the three substrates and results were analyzed based on Equation 3. When concentrations of salicylate were varied at a constant level of oxygen and several fixed concentrations of NADH, double reciprocal plots of initial rates yielded a set of linear lines that converged to a common point (Fig. 1). Using the same data, initial rates were also plotted versus varying NADH concentrations at a constant level of $O_2$ and several constant concentrations of salicylate. Such double reciprocal plots again yielded a family of converging lines. These results clearly indicate that the hydroxylase is capable of forming a ternary complex containing salicylate and NADH.

Double reciprocal plots of initial rate versus NADH concentration at a fixed concentration of salicylate and several constant levels of oxygen, however, produced a set of parallel lines (Fig. 2). Parallel lines were also obtained from double reciprocal plots of initial rates versus varying salicylate concentrations at a constant NADH concentration and several fixed levels of oxygen (Fig. 3). These results indicate a ping-pong type of reaction; subsequent to the binding of salicylate and NADH, a product is released and then $O_2$ binds to an enzyme form which is distinct from the original oxidized holoenzyme.

Results shown in Figs. 1–3, together, are consistent with a reaction scheme in which the hydroxylase binds salicylate and NADH to form a ternary complex. The flavin moiety of the holoenzyme is reduced and a product, namely $NAD^+$, is released. Oxygen then binds to and reacts with the reduced enzyme-salicylate species leading to the formation of water, $CO_2$, and catechol along with the regeneration of oxidized holoenzyme. Consequently, the general Equation 3 can now
be simplified to
\[ \frac{e}{v} = \phi_0 + \frac{\phi_{\text{Sal}}}{[\text{Sal}]} + \frac{\phi_{\text{NADH}}}{[\text{NADH}]} + \phi_2 + \frac{\phi_3}{[\text{Sal}][\text{NADH}]} \]  
(4)

where Sal stands for salicylate.

The intercepts and/or slopes of plots shown in Figs. 1–3 can be used for secondary plots (19) to determine values of various kinetic coefficients in Equation 4. Such secondary plots are shown as insets to Figs. 1–3 and values of the kinetic coefficients so determined are summarized in Table I.

Results obtained thus far, however, do not distinguish a random from a fixed-order binding of salicylate and NADH to the hydroxylase. This question can be resolved by comparisons of dissociation constants for the binding of salicylate (K_{\text{Sal}}) and NADH (K_{\text{NADH}}) determined by equilibrium measurements with those deduced from steady state kinetic results (2, 20). If the hydroxylase binds salicylate first and then NADH, the value of K_{\text{Sal}} deduced from steady state kinetic data should be the same as that determined by equilibrium measurement but values of K_{\text{NADH}} determined by the two methods will be significantly different. When salicylate and NADH bind to the hydroxylase in a random order, both methods lead to the determination of the same K_{\text{Sal}} and the same K_{\text{NADH}}.

In a fluorimetric titration experiment (445 nm excitation; emission measured at 520 nm) using 3.84 μM salicylate hydroxylase and various amounts of salicylate, the value of K_{\text{Sal}} was determined to be 12 μM at 23 °C and pH 7.6. Alternatively, K_{\text{Sal}} was determined, as the negative reciprocal of the horizontal coordinate for the common point of intersection of lines shown in Fig. 1, to be 12.5 μM correlating quite well with that obtained by equilibrium measurements. As mentioned above, the same data shown in Fig. 1 were also used for double reciprocal plots of initial rate versus NADH concentration at several constant levels of salicylate. The K_{\text{NADH}} was thus similarly determined to be 0.4 mM from such replots. In another experiment, the equilibrium constant K_{\text{NADH}} was determined by stopped flow measurements of the anaerobic reduction of enzyme by various excess amounts of NADH in the absence of salicylate. Based on a previously detailed method (17), results of such an experiment can be analyzed according to a scheme
\[ E \rightleftharpoons K_1 \text{NH} \rightarrow E \cdot \text{NH} \rightarrow E \cdot \text{HN} \]  
(5)
in which the total concentration of NADH is in excess of that of enzyme and a fast equilibrium exists for the binding step, i.e. k_1(NH), k_2 \gg k_3. A steady state concentration of E-NH is not required in this nonturnover system. This model predicts that (a) the enzyme reduction (monitored as ΔA_{445}) follows apparent first order kinetics with no significant initial lag and (b) the double reciprocal plot of k_{obs} versus NADH concentration is linear allowing the determination of both the binding equilibrium constant and k_3. The anaerobic reductions of salicylate hydroxylase by excess amounts of NADH indeed followed pseudo-first order kinetics with no apparent initial lag (Fig. 4, inset). The double reciprocal plot of values of k_{obs} versus NADH concentrations was linear (Fig. 4). Both the first order rate constant for enzyme reduction and the equilibrium constant K_{\text{NADH}} were then determined to be 0.68 s^{-1} and 0.45 mM, respectively. A good agreement was again observed between the value of K_{\text{NADH}} obtained as described above and that determined from steady state kinetic data. Considering all the results described in this section as a whole, random binding of salicylate and NADH to the enzyme emerges as the only pattern consistent with all the observations.

Table I

<table>
<thead>
<tr>
<th>Term</th>
<th>Kinetic coefficienta</th>
<th>Value determinedb</th>
</tr>
</thead>
<tbody>
<tr>
<td>φ₀</td>
<td>1</td>
<td>9.2 \times 10⁻⁴ min</td>
</tr>
<tr>
<td>φ_{\text{Sal}}</td>
<td>K_{\text{NADH}}\phi₀</td>
<td>0.8 \times 10⁻⁹ M⁻¹·min</td>
</tr>
<tr>
<td>φ_{\text{NADH}}</td>
<td>K_{\text{Sal}}·φ₀</td>
<td>9.2 \times 10⁻¹ M⁻¹·min</td>
</tr>
<tr>
<td>φ₀</td>
<td>K_{\text{Sal}}·φ₀</td>
<td>3.1 \times 10⁻⁸ M⁻¹·min</td>
</tr>
</tbody>
</table>

a See Scheme 2 for rate constant assignments. K_{\text{Sal}}, K_{\text{NADH}}, and K_{\text{Sal}·\text{NADH}} are dissociation constants for the binding of salicylate to enzyme, salicylate to enzyme·NADH, and NADH to enzyme·salicylate, respectively.

b Determined from insets of Figs. 1−3.

![Figure 4. Anaerobic reduction of salicylate hydroxylase by NADH.](image)

Fig. 4. Anaerobic reduction of salicylate hydroxylase by NADH. Solutions containing 11 μM salicylate hydroxylase in 20 mM KP, pH 7.6, were each mixed with an equal volume of the same buffer containing various amounts of NADH in a stopped flow apparatus at 23 °C under anaerobic conditions. Reductions of enzyme were followed by monitoring changes in A_{445}, and were found, in all cases, to follow apparent first order kinetics. The observed rate constants were plotted against corresponding NADH concentrations (indicated as the levels after the mixing) in a double reciprocal form. Inset, a typical stopped flow trace of anaerobic reduction of enzyme by 0.125 mM NADH. The ΔA_{445} signal is defined as the difference of observed A_{445} at a given time point minus the A_{445} at the completion of the reduction.
The minimal kinetic mechanism of salicylate hydroxylase can now be described, following the shorthand notation of Cleland (21), as shown in Scheme 1, where E, EH₂, Sal, and Cat refer to oxidized enzyme, reduced enzyme, salicylate, and catechol, respectively.

**Tritium Isotope Effect—Effects of (4R)-[4-3H]NADH on V₅₀/K₉₀**: using either salicylate or benzoate as a cosubstrate have been investigated. At a constant salicylate level (140 μM), the value of ³(V/K) was determined (based on a total of 16 measurements, using solutions containing 3 nM enzyme, 1 μM FAD, and 52 μM (4R)-[4-2H]NADH) to be 3.36 ± 0.37. In another set of experiments, values of ³(V/K) were measured at various benzoate concentrations and results are shown in Fig. 5. Based on the analyses described previously (22, 23), ³(V/K) should be independent of benzoate concentration if this effector binds to enzyme prior to NADH. On the other hand, ³(V/K) should be reduced to unity as the benzoate concentration approaches infinity if the hydroxylase binds benzoate first and then NADH. While the observed dependence of ³(V/K) on benzoate concentration is inconsistent with either of the two ordered binding schemes indicated above, our results are in good agreement with the mechanism involving random binding of NADH and benzoate (22, 23).

Therefore, the kinetic mechanism deduced from steady state and rapid mixing measurements is further confirmed by this independent tritium isotope effect study.

**³V and ³(V/K) Effects—Initial rates were measured at 23 °C using a constant level of each benzenoid substrate (or effector) and various concentrations of NADH, (4R)-[4-2H]NADH, or (4S)-[4-2H]NADH. Lineweaver-Burk plots of initial rate versus reduced pyridine nucleotide concentration were linear in all cases. Values of ³(V/K) were determined based on such plots and results are shown in Table II, along with ³(V/K) measurements obtained as described earlier. With (4S)-[4-2H]NADH, no significant isotope effects on either V₅₀ or K₉₀ were observed using salicylate or various benzenoid effectors. Such results are consistent with the known stereospecificity of this hydroxylase (24, 25) and other microbial flavoprotein monooxygenases (24, 26) for the hydrogen (or its isotopes) at the 4R position of NAD(P)H. For salicylate and all the benzenoid effectors tested, significant isotope effects of (4R)-[4-2H]NADH on both V₅₀ and K₉₀ were observed. While the latter varies between 1.93 and 2.77, the former spans a range of 1.69 to 5.07. The levels of activity uncoupling have now been determined for the P. cepacia hydroxylase using salicylate (100%)}
substrate hydroxylation), benzoate (100% $H_2O_2$ formation), and several other benzenoid effectors. Interestingly, the observed percentages of activity uncoupling roughly correlate with the $\Delta V$ effects (Table II).

**Effect of (4R)-[4-3H]NADH on Anaerobic Enzyme Reduction**—The kinetics of anaerobic reduction of enzyme-salicylate and enzyme-benzoate complexes by various concentrations of NADH and (4R)-[4-3H]NADH, all present in excess of the amount of enzyme species, were determined by experiments similar to that described in Fig. 4. In all cases, excellent pseudo-first order kinetics were observed with no detectable lag period. Furthermore, double reciprocal plots of $k_{obs}$ versus reduced pyridine nucleotide concentration were all linear. Following the same method described for Fig. 4, the dissociation constants for reduced pyridine nucleotide binding and the rates of the isolated step of enzyme reduction were determined (Table III). Similar to many other flavohydroxylases (1), the presence of salicylate and benzoate enhances the rate of salicylate hydroxylase reduction by NADH by 290- and 240-fold, respectively. Values of the rates of enzyme reduction by isolates of enzyme-salicylate and enzyme-benzoate species. On the other hand, the intrinsic isotope effect was only slightly repressed in the expression of $\Delta V$ using the nonsubstrate effector benzoate (as indicated by $f_v = 0.82$) but was much repressed with the hydroxylatable salicylate as a substrate ($f_v = 0.15$).

**DISCUSSION**

A mechanism depicting both the substrate hydroxylation and $H_2O_2$ formation activities of salicylate hydroxylase is proposed as shown in Scheme 2, where $E$ and $EH_2$ are oxidized and reduced enzyme, respectively; $N$ and $NH$ are NAD$^+$ and NADH, respectively; and $S$ and SOH refer to substrate (or effector) and hydroxylated product, respectively. For the substrate hydroxylation reaction, the $EH_2-S$ complex actually reacts with $O_2$ in a number of discrete steps, such as $O_2$ binding, reoxidation of bound flavin, hydroxylation (and decarboxylation) of substrate, and products release. For simplicity, these steps are collectively described by the constant $k_1$. The constants $k_2$ and $k_3$ are defined similarly.

The reductive half of the proposed mechanism for salicylate hydroxylase is well supported by our results. Initial rate measurements (Fig. 1 and corresponding replots) along with comparisons of $K_{D_{SOH}}$ and $K_{D_{S}}$ determined by different kinetic and equilibrium techniques (Figs. 1 and 4 and related results) clearly indicate a random binding pattern for salicylate and NADH. The dependence of observed $T(V/K)$ of (4R)-[4-3H]NADH on benzoate concentration also indicates a random binding of benzoate and NADH (Fig. 5). Initial rate studies further establish that NAD$^+$ is released prior to the binding of $O_2$ to the reduced enzyme-substrate complex (Figs. 2 and 3). The overall kinetic mechanism proposed for *P. cepacia* salicylate hydroxylase is very similar to that for $p$-hydroxybenzoate hydroxylase (2). Previously, an ordered sequential binding of salicylate and NADH was implied for salicylate hydroxylase obtained from *P. putida* cells (10) and proposed for that from *P. sp.* 29352 cells (11). In neither case has conclusive evidence been presented to support this ordered binding scheme. Whether the patterns of salicylate and NADH addition of these two previously studied salicylate hydroxylases are truly different from that of the *P. cepacia* hydroxylase should, at the present time, still be considered as being uncertain.

Charge transfer complexes between oxidized enzyme and reduced pyridine nucleotide and between reduced enzyme and oxidized pyridine nucleotide have been demonstrated or proposed to exist for melleitite hydroxylase (3, 4) and $p$-hydroxybenzoate hydroxylase (2, 27). Upon mixing salicylate hydroxylase-salicylate complex with NADH in our anaerobic stopped flow experiments, no 690-nm absorbing species was detected either during or after the completion of enzyme

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**Table III**

<table>
<thead>
<tr>
<th>Benzenoid Substrate</th>
<th>Dissociation Constant $K_d$</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>$f_v$</th>
</tr>
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<tbody>
<tr>
<td>NADH (4R)-[4-3H]NADH</td>
<td>0.075 mM</td>
<td>95</td>
<td>35</td>
</tr>
<tr>
<td>NADH (4R)-[4-3H]NADH</td>
<td>1.0 mM</td>
<td>161</td>
<td>27</td>
</tr>
</tbody>
</table>

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**Scheme 2**
enzymes. Furthermore, changes in $A_{400}$ accompanied the flavin reduction in the same experiments following apparent first order kinetics with no detectable lag period. Therefore, there is thus far no evidence for the existence of any charge transfer complex between $P$. cepacia salicylate hydroxylase and pyridine nucleotide.

According to Scheme 2, the fraction of activity uncoupling is related to the relative magnitude of $k_1$ and $k_2$. We have observed a rough positive correlation between the extent of activity uncoupling and the size of $D\nu$ for a number of benzenoid effectors (Table II). Our findings indicate that the step(s) leading from reduced enzyme to $H_2O_2$ formation must be fast, in comparison with the isotopically sensitive step of enzyme reduction, whereas one or more than one slow step exists in the substrate hydroxylation pathway collectively described by $k_1$. This is further substantiated by the observation that the intrinsic $D\rho$ effect on the enzyme reduction step is much better expressed in the $D\nu$ for benzooxate, which exhibits 100% $H_2O_2$ formation activity, than for salicylate, which leads exclusively to substrate hydroxylation (see values of $f_1$ in Table III).

Deuterium isotope effect of $(4R)$-[(4-$^2$H)]pyridine nucleotide on the rate of enzyme reduction and $V_m$ have been previously reported, respectively, for melilotate hydroxylase (4) and, by us, for $P$. cepacia salicylate hydroxylase (25). Recently, Ryerson et al. (27) have reported a thorough study on $p$-hydroxybenzoate and orcinol hydroxylases and, to a lesser extent, on $P$. sp. 29351 salicylate hydroxylase with respect to deuterium and triton isotope effects of reduced pyridine nucleotides isotopically labeled at the $4R$ position. The observed values of $T(V/K)$ for both $p$-hydroxybenzoate and orcinol hydroxylases were significantly larger than those calculated from $D(V/K)$ and $Dk$ according to the following relationship.

$$\frac{D(V/K)}{T(V/K)} = \frac{Dk - 1}{Tk - 1} = \frac{1}{(Tk)^{1.44} - 1}$$

They pointed out that the first irreversible step for the chemical reaction catalyzed by $p$-hydroxybenzoate hydroxylase is the release of NADP+. Furthermore, the $T(V/K)$ was determined by monitoring the incorporation of tritium into water. If the tritium in the reduced flavin moiety of $E$. FADH$_2$-S$^-$NADP$^+$ rapidly exchanges with the $H$ medium, this will constitute an irreversible step of tritium release into water prior to the step of NADP$^+$ release. The similar rapid exchange of deuterium with water, however, did not constitute any irreversible step because $D(V/K)$ was determined by following the oxidation of reduced pyridine nucleotide rather than deuterium release. Consequently, $D(V/K)$ and $T(V/K)$ have different kinetic expressions rendering the observed $T(V/K)$ larger than that calculated according to Equation 6 (27). We believe that another factor should also be considered. In the reduction of enzyme-substrate complex by $(4R)$-[(4-$^2$H)] NAD(P)H, the forward reaction is associated with an isotope effect, but the isotope effect on the reverse reaction will be abolished if the deuterium in the bound FADH$_2$ rapidly exchanges with solvent. Consequently the deuterium equilibrium isotope effect will be $>1$, and Equation 6 cannot be validated (18, 28).

We have previously demonstrated the bound FADH$_2$ cofactor of $P$. cepacia salicylate hydroxylase, formed during catalysis, is exposed to medium (12) and will most probably exchange hydrogen with water. However, the value of 3.30 for $T(V/K)$ calculated using Equation 6, $D\rho = 5.57$, and $D(V/K) = 1.97$ (measured at $140 \mu M$ salicylate) corresponds quite well with a $T(V/K)$ of 3.36 observed at the same salicylate concentration. Similarly, the value of 4.04 for $T(V/K)$ calculated using $Dk = 5.96$ and $T(V/K) = 2.55$ (measured at 30 mM benzooxate) again is in good agreement with $T(V/K)$ of 3.56 at 30 mM benzooxate determined by the secondary plot method of Kliman et al. (22) using results shown in Fig. 5. The good correlations between calculated and observed $T(V/K)$ values in both cases can be accounted for simply by assuming an irreversible step for the reduction of enzyme-substrate complex by NADH. This will eliminate problems associated with different equations for $D(V/K)$ and $T(V/K)$ and the equilibrium isotope effect as described earlier. The application of Equation 6 can then be justified even when the reduced enzyme exchanges hydrogen with water rapidly.

REFERENCES