The Mechanism of Action of the Flavoprotein
Melilotate Hydroxylase/

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SUMMARY

The reaction mechanism of melilotate hydroxylase has been investigated by a variety of kinetic methods. A steady state analysis has indicated that the enzyme has a mechanism involving two ternary complexes but not a quaternary complex of the enzyme and the three substrates.

The reduction of the enzyme-melilolate complex by NADH has been studied by measuring fluorescence changes in the stopped flow apparatus. This reaction is second order and very rapid. The product of this reaction, observed by stopped-flow spectrophotometry, is believed to be a charge-transfer complex between the reduced FAD of the enzyme and NAD+. It has a broad, long wave length band centered at 750 nm and little absorption at 450 nm. This long wave length band disappears with a rate independent of the NADH concentration; this is presumably due to the dissociation of NAD+ from the complex.

A charge-transfer complex can also be formed by anaerobic titration of reduced enzyme with NAD+ or 3-acetylpyridine-NAD+. The charge-transfer band is sensitive to the nature of the pyridine nucleotide. The spectrum of the complex between reduced enzyme and NAD+ appears to be identical with the spectrum observed transiently during the reduction of the enzyme by NADH in the stopped flow apparatus.

The reaction of reduced melilolate hydroxylase with molecular oxygen in the absence of melilolate is a second order reaction. The rate of this reaction is enhanced more than 10-fold by the presence of melilolate. The reaction profile becomes more complex. This complexity is due to the formation of an intermediate, which is believed to be an adduct of molecular oxygen to the reduced FAD of the enzyme. The spectrum of this intermediate has been determined by analog computer simulation using experimentally determined rate constants for its formation and decay, as well as the known extinction coefficients for reduced and oxidized enzyme.

A reaction mechanism is proposed based on the steady state analysis. This analysis enabled the kinetic constants for the reaction to be determined. These kinetic constants have also been predicted from individually determined rate constants assuming the proposed mechanism. The values obtained by these two methods are in excellent agreement. They also correlate with the Kdis previously determined for the dissociation of melilolate from the enzyme-substrate complex.

Melilotate hydroxylase is a flavoprotein which catalyzes the conversion of melilolate (2-hydroxyphenylpropionate) to 2,3-dihydroxyphenylpropionate. The enzyme isolated from Pseudomonas has many properties in common with other bacterial flavoprotein hydroxylases. The most noticeable among these similarities is that melilolate forms a 1:1 complex with the enzyme and that the rate of reduction of this complex by NADH is approximately 106-fold faster than the reduction of free enzyme.

In the past several years, a number of kinetic investigations of flavoprotein hydroxylases has been published. The reaction of p-hydroxybenzoate hydroxylase (Pseudomonas fluorescens) with its effector 6-hydroxynicotinate has been the subject of a detailed steady state analysis (3), and this enzyme has also been studied extensively by stopped flow spectrophotometry (3-5). Likewise, p-hydroxybenzoate hydroxylase from Pseudomonas desmolytica (6) and salicylate hydroxylase from Pseudomonas putida (7) have been investigated by steady state and rapid reaction techniques. Finally White-Stevens et al. have made extensive use of stopped flow spectrophotometry to investigate salicylate hydroxylase obtained from an unidentified soil organism (8).

The present paper reports a kinetic investigation of melilolate hydroxylase by steady state and rapid reaction techniques. A reaction mechanism is postulated from a steady state analysis and corroborated by measurement of the individual rate constants in the stopped flow spectrophotometer. In addition, these rapid reaction studies have revealed the presence of two intermediates in the catalytic cycle. The first appears to be a charge-transfer complex between reduced melilolate hydroxylase and NAD+.

The second is postulated to be an oxygenated flavin intermediate composed of enzyme, melilolate, and oxygen, similar to the one proposed for p-hydroxybenzoate hydroxylase (4, 5). The reoxidation studies have further shown that the presence of melilolate increases the rate of reaction of the reduced enzyme with molecular oxygen and that its presence is necessary for the formation of the oxygenated intermediate.

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EXPERIMENTAL PROCEDURE

Materials—Melilotate and melilolate hydroxylase (2) and glucose oxidase (9) were prepared as previously described. NADH (Grade III) was purchased from Sigma; catalase (B grade) from Calbiochem; and 3-acetylpyridine-NAD from P-L Biochemicals.

Steady State Experiments—The steady state experiments were done by two methods, both at 1–1.5°C. Experiments in which the concentration of oxygen was held constant were done with a Cary 17 recording spectrophotometer at 340 nm. All reactants except the enzyme were added to the cuvette, which was then incubated at 1°C in the sample compartment of the spectrophotometer for 5 min. The reaction was started by the addition of enzyme. The sample compartment was constantly flushed with dry air to prevent condensation of water on the optical surfaces. Those experiments in which the concentration of oxygen was varied were done with the stopped-flow apparatus of Gibson and Milnes (10). The electronics of the instrument were replaced with a solid state circuit which includes an internally calibrated, logarithmic operational amplifier. This circuit produces an output directly proportional to absorbance. The enzyme solution, air-saturated at 1°C, was placed in one syringe of the stopped flow apparatus. Buffer, containing the appropriate amount of melilolate and NADH, was equilibrated with one atmosphere of 5% oxygen (95% nitrogen), 10% oxygen (90% nitrogen), 21% oxygen (air), or 100% oxygen in a tonometer at 25°C. This solution was transferred to the stopped flow apparatus and equilibrated for >3 min at 1°C. The final concentration of oxygen was calculated from the known solubilities of oxygen in water at the appropriate temperatures. The concentration of enzyme was chosen so that the reaction took place over approximately 10 to 60 s. The concentrations of NADH, melilolate, and melilolate hydroxylase were determined spectrophotometrically using their respective extinction coefficients: NADH, 6220 M⁻¹ cm⁻¹ at 340 nm (11); melilolate, 1910 M⁻¹ cm⁻¹ at 271 cm (1); and melilolate hydroxylase, 11,300 M⁻¹ cm⁻¹ at 460 nm (2). The steady state results are expressed as turnover numbers, i.e., moles of NADH oxidized per min per mole of enzyme-bound FAD. The initial rate equations derived by the schematic method of King and Altman (12).

Stopped Flow Experiments—All stopped flow experiments were done at 1–1.5°C with the unit described above. Anaerobic techniques were as previously described (2) except that the nitrogen gas was purified by storage over Fieser’s solution (13). For stopped flow measurements at wave lengths longer than 600 nm, a Corning 3-70 glass filter was placed directly in front of the face of the photomultiplier tube to eliminate second order light from the monochromator.

The fluorescence experiments were done with the stopped flow apparatus using a fluorescence observation cell based on the design of Gibson et al. (14). The tungsten ribbon filament lamp was replaced with a 150-watt xenon arc powered by a Hewlett Packard 6267B power supply. The starting voltage for the lamp was provided by a custom-made unit. The xenon lamp was mounted on a table independent of the mixing block to reduce vibrational noise associated with the hydraulic driving mechanism. For these experiments, excitation light was monochromated at 340 nm by the normal stopped flow monochromator; emitted light was filtered by a Corning 3-70 glass filter to eliminate contributions from the exciting light.

To insure complete anaerobiosis in the stopped flow apparatus, glucose and glucose oxidase were routinely added to the tonometer containing the enzyme solution. In the tonometers containing other reactants, agitation was vigorous enough during the anaerobiosis procedure to make this unnecessary. However, in the fluorescence experiments done at low concentrations of reactants, this oxygen scrubbing system was included in both tonometers.

The tonometer used for the enzyme solution in these experiments had a ground glass joint which allowed attachment of a spectrophotometer cell. This arrangement enabled anaerobic, static spectra to be recorded of the solution in the tonometer. For reoxidation studies, the enzyme was reduced either by a 2-fold excess of NADH or by irradiation with visible light in the presence of EDTA (2). Identical results were obtained for both methods.

Anaerobic Titrations—Anaerobic titrations were done in the unit described by Foust et al. (15). Previously described techniques were used (2). NAD⁺ and AP-NAD⁺ were dissolved in 0.1 M KPi, pH 7.3, and were standardized using extinction coefficients of 17,800 M⁻¹ cm⁻¹ at 259 nm and 16,400 M⁻¹ cm⁻¹ at 260 nm, respectively (16). The enzyme solution was placed in the cuvette assembly, made anaerobic, and photoreduced in the presence of EDTA. When reduction was complete, the titration was begun by adding pyridine nucleotide.

Analog Computer Simulations—Computer simulations were done on an Applied Dynamics AD2-64PD analog computer console. The reaction scheme simulated was:

\[ \text{E-FADH}_2\text{Mel} + \text{O}_2 \rightarrow \text{E-FADH}_2\text{O}_2\text{Mel} \]

where \( E \) is melilolate hydroxylase. The appropriate values for the rate constants \( k_1 \) and \( k_2 \) are shown in Table II. Previously determined extinction coefficients for reduced and oxidized melilolate hydroxylase were used (2).

Spectrophotometric Methods—Routine enzyme assays were performed as previously described (2) with a Gilford recording spectrophotometer. Static spectra were taken with a Cary 17 recording spectrophotometer. For wave lengths longer than 700 nm, the infrared detector and multipotentiometers were used.

RESULTS

Steady State Analysis—The general initial rate equation for an enzyme-catalyzed reaction involving three substrates is (17):

\[ e_{i/v_i} = \phi_A + \phi_B + \phi_C + \frac{\phi_A \phi_B}{[A][B]} + \frac{\phi_A \phi_C}{[A][C]} + \frac{\phi_B \phi_C}{[B][C]} + \frac{\phi_A \phi_B \phi_C}{[A][B][C]} \]

Initial rate equations derived for other mechanisms may be considered special cases of this general form. The kinetic parameters, \( \phi_A, \phi_B, \phi_C, \) etc., can be evaluated by plotting the reciprocal initial velocity versus the reciprocal concentration of each substrate at fixed concentrations of the other two (17).

Lineweaver-Burk plots (18) in which the concentrations of melilolate and NADH were varied at a fixed concentration of oxygen are shown in Fig. 1. The lines converge to a common point whose horizontal coordinate is \(-2.8 \times 10^4 \text{ M}^{-1}\). Two other sets of Lineweaver-Burk plots are shown in which (a) the concentrations of melilolate and oxygen were varied at a fixed

1 D. Ballou and G. S. Ford, to be published.
FIG. 1. Steady state analysis of melilotate hydroxylase varying melilotate and NADH concentrations. Conditions: 0.1 M KPi, pH 7.3; 0.41 mM O2; temperature was 1°. Lineweaver-Burk plots of primary data. Inset a, secondary plot of y-axis intercepts versus reciprocal NADH concentration. Inset b, secondary plot of slopes versus reciprocal NADH concentration. The arrow on the main figure shows the calculated $K_{diss}$ of melilotate from the enzyme.

concentration of NADH (Fig. 2), and (b) the concentrations of NADH and oxygen were varied at a fixed concentration of melilotate (Fig. 3). Both of these latter two series yielded sets of parallel lines.

The initial rate equation which fits these results has the form:

$$
\frac{1}{v} = \frac{1}{v_{max}} + \frac{[S]}{K_{M} + [S]} + \frac{[O_2]}{K_{O_2} + [O_2]} + \frac{[S][O_2]}{K_{M}[S][O_2]}
$$

A reaction scheme which agrees with this initial rate equation must include a ternary complex of enzyme, NADH, and melilolate (17). One mechanism which is consistent with this analysis, written in the shorthand notation of Cleland (19), is

$$
\begin{align*}
E & \rightarrow E \cdot \text{Mel} \\
E \cdot \text{Mel} & \rightarrow E \cdot \text{Mel} \cdot \text{NADH} \\
E \cdot \text{Mel} \cdot \text{NADH} & \rightarrow E \cdot \text{Mel} \cdot \text{NAD}^+ \\
E \cdot \text{Mel} \cdot \text{NAD}^+ & \rightarrow E \cdot \text{Mel} \cdot \text{O}_2 \\
E \cdot \text{Mel} \cdot \text{O}_2 & \rightarrow E \cdot \text{DiOH} \\
E \cdot \text{DiOH} & \rightarrow E
\end{align*}
$$

This type of reaction is called either a concerted-substitution, type II, b mechanism (17), or a Bi Uni Uni Bi Ping Pong mechanism (19). A concerted reaction of the enzyme with two of the substrates occurs which forms an altered form of the enzyme, and this altered form then reacts with the third substrate in a separate step.

The values of the kinetic constants for the reaction mechanism can be determined from secondary plots of the initial rate data (17). Plots of this nature are shown in the insets to Figs. 1, 2, and 3. The kinetic constants so determined are summarized in Table I.

For the Lineweaver-Burk plots shown in Fig. 1, the negative reciprocal of the horizontal coordinate of the point of intersection is very nearly equal to the $K_{diss}$ for the enzyme-melilotate complex. The value determined for this $K_{diss}$ from the data in Fig. 1 is $3.6 \times 10^{-4}$ M; and $K_{diss}$ measured by a spectrophotometric titration of the enzyme with melilolate is $3.8 \times 10^{-4}$ M.

![Fig. 2](image2.png) Steady state analysis of melilotate hydroxylase varying melilolate and O2 concentrations. Conditions: 0.1 M KPi, pH 7.3; 0.1 mM NADH; temperature was 1°. Lineweaver-Burk plots of primary data. Inset, secondary plot of y-axis intercepts versus reciprocal oxygen concentration.

![Fig. 3](image3.png) Steady state analysis of melilotate hydroxylase varying melilolate and NADH concentrations. Conditions: 0.1 M KPi, pH 7.3; 1.0 mM melilolate; temperature was 1°. Lineweaver-Burk plots of primary data. Inset, secondary plot of y-axis intercepts versus reciprocal oxygen concentration.

| Term       | From steady state | From stopped flow |
|------------|-------------------|-------------------|
| $K_m$ (Mel) | 1.3 μM            | 4.8 μM            |
| $K_m$ (NADH) | 4.7 μM            | 43 μM             |
| $K_m$ (O2)  | 50 μM             | 600 min⁻¹         |

a Calculated from Figs. 1, 2, and 3 (17).
b Calculated from Table III as described in text.
prior to NADH in an obligatory order mechanism (20) or if melilotate and NADH bind in random order to the enzyme (21). The possibility of a random addition of the first two substrates can be ruled out by replotting the data of Fig. 1 as reciprocal turnover number versus reciprocal NADH concentration at several melilotate concentrations. These plots will of course also give a set of converging lines. If the mechanism is truly random order for the first two substrates, however, the negative reciprocal of the horizontal coordinate of the point of intersection in this plot should now be equal to the \( K_{\text{dis}} \) for the NADH-enzyme complex (21). The value determined from a replotted data of Fig. 1 as described is \( 6.3 \times 10^{-4} \) M. This is not in agreement with the measured \( K_{\text{dis}} \) for the enzyme-melilotate complex, \( 3.8 \times 10^{-4} \) M (2). Thus, it appears that the substrate addition occurs by an obligatory order mechanism, with melilotate binding first.

Reduction of Melilolate Hydroxylase by NADH—The reduction of melilolate hydroxylase by NADH is a relatively slow process in the absence of melilolate, having an extrapolated rate constant of \( 1.4 \text{ min}^{-1} \) at \( 1^\circ \) (2). In the presence of melilolate, the reduction is extremely rapid and was impossible to follow in the stopped flow spectrophotometer at the concentrations normally employed. However, by lowering the concentrations of the reactants and observing changes in fluorescence rather than absorbance, it was possible to study this reaction in two ways. In the first experiments, NADH and melilolate hydroxylase were reacted at equal concentration in the presence of melilolate. The disappearance of the NADH fluorescence obeys the kinetics for an irreversible, second order reaction as shown in Fig. 4A. The second order rate constant determined for this process is \( 1.4 \times 10^{8} \text{ M}^{-1} \text{ min}^{-1} \). In these experiments, the possibility existed that the disappearance of the NADH fluorescence was associated with NADH binding to the enzyme, rather than the actual oxidation-reduction reaction. Therefore, an experiment using concentrations of the two reactants identical with those described for Fig. 4A was performed, and the decrease in absorbance at 340 nm and 450 nm was observed. The changes in absorbance at these two wave lengths are identical in rate and order with the changes in fluorescence. Thus, this fluorescence decrease is associated with the oxidation of NADH and the reduction of the enzyme flavin.

Another series of experiments, also using the stopped flow fluorescence technique, was performed in which the NADH concentration was held constant and the enzyme concentration varied. The enzyme concentration was always at least five times as large as the NADH concentration (pseudo-first order conditions). The enzyme was used as the reactant in excess to avoid the background fluorescence that would be associated with excess NADH. A plot of the reciprocal of the observed first order rate constant versus the reciprocal of the enzyme concentration (22, 23) was linear and passed through the origin. This is further evidence that the reaction is second order. These experiments yielded a rate constant of \( 1.4 \times 10^{8} \text{ M}^{-1} \text{ min}^{-1} \), identical with the value determined under second order conditions (Fig. 4A).

In order to correlate the stopped flow results with the steady state results (see “Discussion”), it was necessary to evaluate the magnitude of the rate constant for the reverse reaction. A convenient graphical method for this purpose is as follows.

Consider a reversible, second order reaction:

\[
A + B \xrightarrow{k_1} C \quad \text{(5)}
\]

where \( B \gg A, A, C \text{ (pseudo-first order approximation)} \)

\[
\text{if } B >> A, A \xrightarrow{k_1'} C \quad \text{(6)}
\]

where \( k_1' \cong k_1 [B] \)

If one assumes that the concentration of \( C \) at the beginning of the reaction is zero, the rate equation can be easily integrated and simplified by introducing the equilibrium condition (24). The resulting integrated rate equation is:

\[
\ln (A - A_0) = -(k_1' + k_2)t + \ln (A_0 - A_t) \quad \text{(8)}
\]

where \( A_0 \) is the initial concentration of \( A \), \( A \) is the concentration at any time \( t \), and \( A_t \) is the concentration at equilibrium. Thus, the approach to equilibrium is a first order process, and the observed rate constant from a plot of \( \ln A \) versus \( t \) will be the sum of the rate constants for the forward and reverse reactions.

\[
k_{\text{obs}} = k_1' + k_2 \quad \text{(9)}
\]

or \( k_{\text{obs}} = k_1[B] + k_2 \quad \text{(10)}
\]

Therefore, a plot of \( k_{\text{obs}} \) versus \( [B] \) should be linear with a slope of \( k_1 \) and a \( y \) intercept of \( k_2 \). Such a plot for the reverse experiments performed under pseudo-first order conditions is shown in Fig. 4B. The least squares regression analysis estimate of the \( y \) intercept of this plot is \(-0.20 \pm 0.28 \) (95% confidence interval, Reference 25). Thus, within the limits of error in these experiments, the rate constant for the reverse reaction can be considered zero (\( k_4 \), Fig. 10).

When the reduction of melilolate hydroxylase by NADH is studied by the usual stopped flow spectrophotometric techniques, \( 90\% \) of the absorption at 450 nm associated with oxidized enzyme is lost in the 3-ms dead time of the instrument. However, a new absorption band at longer wave lengths appears during the dead time and then disappears more slowly. The spectrum of the intermediate which is formed in the first 3 ms after mixing was determined as described previously (4) and is

![Fig. 4. A, the anaerobic reduction of melilolate hydroxylase by equimolar NADH. Spectral change of the reaction at 450 nm and 340 nm is provided.](http://www.jbc.org/)

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Fig. 5. Spectral intermediate formed during the anaerobic reduction of melilotate hydroxylase by NADH. Conditions: 38 μM melilotate hydroxylase was rapidly mixed with an equal concentration of 500 μM NADH. Both syringes contained 0.1 M KPi, pH 7.3; 1 mM melilotate. Temperature was 1°. - - - , initial oxidized spectrum; - - - , spectrum obtained immediately after mixing (3 ms); - - - - , final reduced spectrum. Absorbance is for a path length of 2 cm. Inset, changes in the absorbance at 750 nm with time in the presence and absence of oxygen, other conditions as above. , anaerobic reduction; , catalytic turnover, in the presence of an initial concentration of 120 μM O2.

Fig. 6. Anaerobic spectral titration of reduced melilotate hydroxylase with NAD+. Conditions: 0.1 M KPi, pH 7.3; 60 mM EDTA; 1 μg per ml of catalase; 1 mM melilotate; 35 μM melilotate hydroxylase. Temperature was 1°. The enzyme was photoreduced (2) in the absence of melilotate, then melilotate was tipped into the solution from a side arm and the titration begun. The inset to Fig. 5 shows two similar experiments in which the long wavelength absorption band was observed. In the presence of oxygen, the intermediate is fully formed and decays initially (until a steady state level is reached) at the same rate as anaerobically. This implies that during catalysis oxygen reacts with the reduced enzyme after the dissociation of NAD+ has already occurred.

Charge-Transfer Interaction between Reduced Melilotate Hydroxylase and NAD+.—If the long wave length band observed in the stopped flow apparatus is due to a charge-transfer interaction as postulated, it should be possible to form this species in static experiments. Indeed, when an anaerobic solution of reduced melilotate hydroxylase (produced by EDTA-light irradiation) is titrated with NAD+, a new absorption band does appear (Fig. 6). The similarity of the spectrum of this species to that observed in the stopped flow apparatus (Fig. 5) argues strongly...
dependence on oxygen concentration and has a second order slight decrease at 405 nm is difficult to analyze due to the small oxygen concentration. The first order rate constant for this observed followed by a process whose rate is independent of the second order fashion in the absence of melilotate (Fig. 7A). No in the presence of EDTA is reoxidized by molecular oxygen in a droxylase which has been reduced by NADH or by irradiation not determined, but from the failure to approach full formation tored at a range of wave lengths between calculated second order rate constant is 9.7 x 10^5 evidence for any intermediate is seen when the reaction is moni-

A complex similar in nature to the above is formed when AP-NAD^+ had a higher oxidation-reduction potential than NAD^+ (9). The broad, long wave length band in this case is centered at about 630 nm. The dissociation constant of this pyridine nucleotide from the reduced enzyme was determined for the dissociation of NAD^+ from the reduced enzyme-melilotate complex is 1.45ildebrand (26). The Zieldisss determined for the dissociation of ions.

The spectrum of this intermediate is difficult to obtain from the data for two reasons. (a) The rate constants for the formation and decay of the intermediate differ only by a factor of eight (maximum observed difference, at 0.625 mM O_2). Therefore, at the optimum time, only 75% of the enzyme exists as the intermediate, and the observed spectrum is a mixture of species. (b) The stopped flow apparatus is not absolutely anaerobic, and the formation of small amounts of oxidized enzyme while the reduced enzyme is transferred to the syringes is inevitable.

It is possible, however, to obtain the spectrum of the intermediate by another method. For the reoxidation process described above, the intermediate formed is essentially isosbestic with the oxidized enzyme at 415 nm. Therefore, the rate constant for the formation of the intermediate from reduced enzyme can be obtained from observations at this wave length (1.6 x 10^7 M^-1 min^-1). The rate constant for the decay of the intermediate to oxidized enzyme can likewise be determined from observations at 490 nm, a region where the reduced enzyme is isosbestic with the intermediate (1400 min^-1).

The known parameters of the reoxidation reaction are thus the absorption spectrum of the initial and final enzyme, and the rate constants for both the formation and decay of the intermediate. (At different oxygen concentrations, it is necessary to change the rate constant for the formation of the intermediate; Fig. 7B). By analog computer simulation, it is then possible to obtain an accurate representation of the absorption spectrum of the intermediate. The assumed extinction coefficient of the intermediate at each wave length was varied until the computer simulation matched the actual display observed on the oscilloscope of the stopped-flow apparatus. An excellent fit of the simulated data to the real data could be obtained at all wave lengths by varying the extinction coefficient of the intermediate.

Examples of this agreement are shown in Fig. 8. The calculated absorption spectrum of the intermediate has a maximum at 405 nm and an extinction coefficient at that wave length of 8500 M^-1 cm^-1 (Fig. 9).
The steady state results reported in this paper suggest that melilotate hydroxylase has the reaction mechanism shown in Fig. 10. This mechanism involves two different ternary complexes but not a quaternary complex of the enzyme and its three substrates. Similar mechanisms have been proposed for other flavoprotein hydroxylases (4, 6–8).

There is an alternate mechanism which would fit the initial rate data. However, this mechanism is inconsistent with other known facts about the enzyme. Thus, Dalziel’s concerted-substitution II, c mechanism (Cleland’s Bi Bi Uni Uni Ping Pong mechanism) would have both products from the first ternary complex released before the addition of the third substrate. This clearly cannot be the case with melilotate hydroxylase. Melilotate must be bound in a ternary complex with oxygen since the source of the oxygen atom in the incorporated hydroxyl group is molecular oxygen (1).

Previously, the order of substrate addition has been investigated for p-hydroxybenzoate hydroxylase. Nakamura et al. (6) reported that the addition of p-hydroxybenzoate and NADPH to this enzyme is ordered, with the aromatic substrate binding first. However, this conclusion was based on the fact that the enzyme-p-hydroxybenzoate complex is reduced much more rapidly than the free enzyme, which is not conclusive evidence. For example, Howell et al. (3) have shown by steady state analysis that with the nonsubstrate effector 6-hydroxynicotinamide, which does enhance the rate of reduction of the enzyme by NADPH, the addition of the effector and pyridine nucleotide is random. The present work documents that with melilotate hydroxylase the substrate melilotate binds first in an ordered addition process.

The reduction of melilotate hydroxylase by NADH ($k_i$) appears to be effectively a second order, irreversible reaction. Rigorously speaking, a complex of oxidized enzyme and NADH must exist.

$$FAD + NADH \rightleftharpoons E-NADH \rightleftharpoons E-NAD^+ \quad (11)$$

However, if the collapse of this oxidized enzyme complex to the reduced enzyme complex is very rapid compared to its formation, an over-all second order process will be observed. The complex of oxidized enzyme and NADH will then be kinetically invisible.

The magnitude of the reverse rate constant $k_d$ (Fig. 10) was zero within the limits of detection reported here. In any case, it is clear from the 95% confidence interval of the $y$ intercept of Fig. 4B that $k_d$ can be no larger than approximately 30 to 40 min$^{-1}$.

The intermediate species observed during the reduction of melilolate hydroxylase by NADH (Fig. 5) is certainly a complex between reduced enzyme and NAD$^+$. This conclusion is based on the observed absorption at 450 nm and on the fact that an identical species is formed upon anaerobically mixing reduced enzyme and NAD$^+$ (Fig. 6). To prove that this interaction is of the charge-transfer type, several conditions must be met. First and foremost, the complex must have an absorption band not present in either the donor (FADH$_2$) or acceptor (NAD$^+$) molecule (28). The very broad absorption band centered at 750 nm for this complex obviously satisfies this requirement. Another good test for charge-transfer bands is that they should be visible for the residence time of the complex (29).
be sensitive to substituents on the donor and acceptor molecules (28). The absorption band for the complex between reduced enzyme and AP-NAD$^+$ has a wave length maximum of 630 nm, a shift of 120 nm. At first glance, the fact that AP-NAD$^+$ shifts the absorption band to the blue may appear contradictory to the hypothesis. The reduction potential (30°, pH 7.0) for the NAD$^+$ system is -290 mvolts (29); for the AP-NAD$^+$ system, it is -248 mvolts (27). Due to its more positive reduction potential, one might expect the charge-transfer interaction between AP-NAD$^+$ and reduced flavin to be facilitated and the new absorption band to have a maximum at a longer wave length than for NAD$^+$. However, the origin of the charge-transfer band must be kept in mind. For weak charge-transfer interactions, the absorption arises from a transition which is effectively a one-electron transfer from the donor to the acceptor molecule (30). Therefore, the energy required for this transition will be sensitive not only to the reduction potentials of the donor and acceptor but also to the geometry of binding. It may be in this case that AP-NAD$^+$ is bound to the enzyme in a less favorable conformation than NAD$^+$ for partial electron transfer to occur. Thus, even though AP NAD$^+$ has a more favorable reduction potential for the process, more energy may be required. The crucial fact is that the nature of the new absorption band is sensitive in some manner to changes in the acceptor molecule. It should be noted that the spectrum reported here for the reduced enzyme-NAD$^+$ complex is very similar to the spectrum of a complex between reduced lipoyl dehydrogenase and NAD$, in which evidence for a charge-transfer interaction was also obtained (31).

The reoxidation studies show that melilotate affects the rate of reoxidation of melilotate hydroxylase as well as the rate of reduction (2). In the presence of melilolate, the rate of reaction of the reduced enzyme with oxygen was increased by more than 10-fold. Furthermore, in the absence of melilolate, no intermediate was seen during the reoxidation.

The intermediate observed during the reaction of reduced enzyme with oxygen in the presence of melilolate is particularly interesting. This is the third case in which such an intermediate has been found for a flavoprotein hydroxylase (4, 5). The spectrum of the intermediate in the present work was obtained by analog simulation. This spectrum can only be as accurate as the rate constants and extinction coefficients also used in the simulation. All of these values are known to at least 10%.

Both of the previous observations of an intermediate in the reoxidation of a reduced flavoprotein hydroxylase were with $p$-hydroxybenzoate hydroxylase (4, 5). In one case, using 2,4-dihydroxybenzoate as the substrate (4), the intermediate had a spectrum somewhat similar to the one reported here but had much higher extinction coefficients (e.g. at 410 nm, e $\sim$ 13,000 M$^{-1}$ cm$^{-1}$). With $p$-hydroxybenzoate as the substrate (5), the intermediate had a spectrum with a maximum at 380 nm and an extinction coefficient of about 8,000 M$^{-1}$ cm$^{-1}$. The spectrum of the intermediate using 2,4-dihydroxybenzoate must be considered the better determination. In that case, the relative rates of formation and decay of the intermediate allowed essentially its quantitative production for observation in the stopped flow apparatus.

The question as to the molecular structure of the intermediate remains unanswered. The fact that the rate of formation of the intermediate is directly dependent on the oxygen concentration while its decay is independent implies that it is some type of oxygen adduct. The possibility that it is an oxygen complex with the substrate cannot be excluded. However, perhaps the most likely candidate is an adduct of molecular oxygen to reduced flavin. There are three positions on the isoalloxazine ring system where this adduct might occur.

The positions C-4a and C-10a have previously been proposed as likely sites of oxygen attack (32). In fact, the spectrum of the intermediate reported here resembles that of a proposed C-10a methoxy adduct to 10-pentamethyl-1,5-dihydro-isoalloxazine (33). The C-4a adduct might also be considered an attractive hypothesis in this case for several reasons. (a) Previously observed C-4a compounds have similar extinction coefficients to that of the intermediate reported here, although their absorption maxima are usually below 380 nm (34, 35). (b) C-10a addition to a reduced flavin blocks an amidine center, whereas C-4a addition blocks an azomethine center. In general, the C-4a addition might be considered more favorable (36). (c) Song$^3$ has recently predicted from molecular orbital calculations that the C-4a oxygen adduct should have an absorption maximum close to 400 nm, compared to 445 nm for the C-10a species.

In addition to these two positions, N-5 must also be considered a possibility. From theoretical considerations, it appears that N-5 is at least equally susceptible to attack by molecular oxygen (considered as an electrophile or radical) as C-4a and C-10a. Unfortunately, the unavailability of model flavins with an oxygen function at position N-5 prohibits further evaluation of this possibility.

The postulated reaction mechanism for melilotate hydroxylase is shown in Fig. 10, and the corresponding rate constants are tabulated in Table II. The mechanism shown is the one pre-}

![Image](http://www.jbc.org/)

**Table II**

| Step | Rate constant | Dissociation constant |
|------|---------------|-----------------------|
| $k_1$ | $5.7 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_1/k_2 = 3.8 \times 10^4$ M$^{-1}$ |
| $k_2$ | $2.2 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_3/k_2 = 3.2 \times 10^4$ M$^{-1}$ |
| $k_3$ | $1.4 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_4/k_3 = 1.45 \times 10^4$ M$^{-1}$ |
| $k_4$ | $1.3 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_5/k_4 = 3.8 \times 10^4$ M$^{-1}$ |
| $k_5$ | $9.0 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_6/k_5 = 4.5 \times 10^4$ M$^{-1}$ |
| $k_6$ | $1.6 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_7/k_6 = 4.5 \times 10^4$ M$^{-1}$ |
| $k_7$ | $1.4 \times 10^4$ M$^{-1}$ min$^{-1}$ | $k_8/k_7 = 4.5 \times 10^4$ M$^{-1}$ |
| $k_8$, $k_{11}$, $k_{12}$ | | |

$^* $ See Fig. 10 for identification of each step. Except where noted, each rate constant was measured directly in the stopped flow spectrophotometer. Temperature = 1°C.

$^b $ Calculated from the kinetic constant $\phi_{4a}$, obtained from the steady state analysis.

$^c $ Calculated from $k_4$ and $k_5/k_4$.

$^d $ Within the limits of detection in these experiments.

$^f $ Calculated from $k_4$ and $k_5/k_4$.

$^g $ From static titration experiments, $k_5/k_4$ from Reference 2; $k_5/k_4$, this paper.

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dicated on the basis of the steady state analysis. Although this analysis appears conclusive, one must be cautious in assigning a mechanism on the basis of initial rate data alone. Correlation between a variety of kinetic methods is much firmer evidence for a postulated mechanism.

A comparison of the kinetic constants calculated from steady state and stopped flow results is shown in Table III. The term \( \phi_0 \) is the least meaningful of these comparisons, since the magnitude of \( k_{11} \) is not known. In Table III, \( k_{11} \) has been assumed to be large enough to make its reciprocal negligible in the expression for \( \phi_0 \). With this assumption the agreement between the two methods is good. It should be noted that \( k_{11} \) is the release of one of the products from the oxidized enzyme, a reaction that could quite conceivably be fast enough to fit the above requirement. For the calculation of \( \phi_{NADH} \), \( k_3 \) and \( k_4 \) were measured directly with the stopped-flow technique. Since \( k_4 \) is known to be near zero, however, the expression for \( \phi_{NADH} \) simply becomes \( 1/k_3 \). The calculation of \( \phi_{ADH} \) is analogous to that for \( \phi_{NADH} \), and in this case \( k_1 \) and \( k_2 \) were measured directly. Using similar arguments as those advanced for the estimation of \( k_{11} \) it is possible to assign a value of zero to \( k_6 \), so that \( \phi_{NAD} \) is equal to \( 1/k_6 \). The agreement between \( \phi_{NADH} \) and \( \phi_{ADH} \) obtained by the two methods is excellent (Table III). It is important to note that this agreement is not sensitive to the absolute value of the estimated rate constants \( k_3 \) and \( k_4 \). For example, if \( k_4 \) was actually 40 min\(^{-1} \) (a highly unlikely possibility in light of the data), the calculated value for \( \phi_{NAD} \) would change only 3%. It should also be emphasized that the kinetic constants \( \phi_0, \phi_{NADH}, \) etc., are related to the more conventional kinetic constants listed in Table I (17). Thus, \( V_{max} = 1/\phi_0 \), \( K_{M(NADH)} = \phi_{NAD}/\phi_0 \), etc. When these conversions are performed (Table I) the agreement between the steady state and stopped flow results is equally as striking as that shown in Table III.

Accepting the proposed mechanism as valid allows determination of \( k_{11} \), which was not possible by the methods described here. Since \( \phi_{NADH} \) is equal to \( 1/k_1 \), \( k_1 \) should equal \( 5.7 \times 10^4 \) M\(^{-1}\) min\(^{-1} \). Considering that this is for the reaction at \( 15^\circ \), the rate constant is similar in magnitude to those determined for the binding of salicylate to salicylate hydroxylase. White-Stevens et al. reported a value of \( 5.4 \times 10^4 \) M\(^{-1}\) min\(^{-1} \) at \( 20^\circ \) (8), and Takemori et al. reported \( 1.1 \times 10^5 M^{-1} \) min\(^{-1} \) at \( 25^\circ \) (7).

Finally, the kinetic analysis reported here clearly demonstrates the usefulness of probing an enzyme mechanism with a variety of kinetic techniques. For melilolate hydroxylase, analysis of the reaction mechanism by steady state, rapid reaction, and titration methods gives complementary results. This is taken as extremely strong evidence that the proposed mechanism is correct.

TABLE III

| Term          | Kinetic equivalent* | From steady state** | From stopped flow*** | From titration  |
|---------------|---------------------|---------------------|----------------------|----------------|
| \( \phi_0 \)  | \( 1/k_1 + 1/k_6 + 1/k_{11} \) | \( 1.36 \times 10^2 \) min | \( 1.48 \times 10^3 \) min |                |
| \( \phi_{NADH} \) | \( 1/k_1 \) | \( 1.95 \times 10^4 \) | \( 7.1 \times 10^4 \) m min |                |
| \( \phi_{NAD} \)  | \( (k_2 + k_7)/k_8 \) | \( 6.4 \times 10^3 \) m min | \( 6.3 \times 10^4 \) m min |                |
| \( \phi_{NADH-Mel} \) | \( k_2/k_1 \) (K_{diss} for Mel) | \( 3.1 \times 10^4 \) M | \( 3.8 \times 10^4 \) M* |                |
| \( \phi_{ADH} \)  | \( k_3/k_4 \) (K_{diss} for Mel) | \( 3.6 \times 10^4 \) M | \(                  \) |                |

* Assuming the mechanism shown in Fig. 10.
** Calculated from the data of Figs. 1, 2, and 3.
*** Calculated using the rate constants listed in Table II.
**** Calculated from the data of Fig. 1 (see text).
***** From a titration of melilotate hydroxylase with melilotate (2).

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