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Kinetic and Mechanistic Studies on the Reaction of Melilotate Hydroxylase with Deuterated Melilotate†

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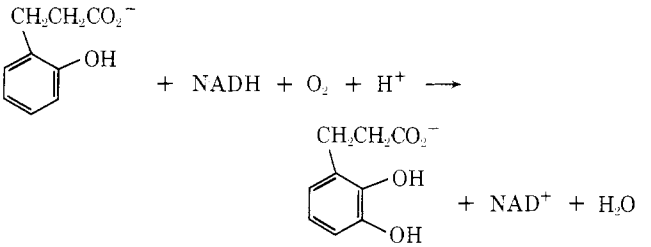
ABSTRACT: [3,5-^{2}H]Melilotate has been synthesized from melilotate by iodination followed by reductive deiodination in the presence of deuterated hydrazine. The deuterated melilotate has been employed in investigations of the reaction mechanism of melilotate hydroxylase. Stopped-flow spectrophotometry has revealed no isotope effect in the formation or decay of the oxygenated intermediate which is observed when reduced melilotate hydroxylase reacts with molecular oxygen. Steady-state analysis has corroborated this result, and in addition shows that there is no isotope effect in the reductive cycle of the enzyme mechanism. This analysis does reveal a reproducible 8% decrease in V_{max} for the enzyme when using deuterated melilotate. These observations are compatible with the thesis that the above intermediate is an oxygenated form of the reduced flavine prosthetic group and that the last step of the proposed mechanism is rapid and involves a primary isotope effect. The existence of the NIH shift mechanism has been studied using combined gas chromatography–mass spectrometry. No evidence could be obtained for intramolecular migration of deuterium during the hydroxylation reaction. However, the small amount of migration expected when phenols are hydroxylated precludes elimination of the NIH shift as a possibility.

Melilotate hydroxylase is a flavoprotein which catalyzes the conversion of melilotate (2-hydroxyphenylpropionate) to 2,3-dihydroxyphenylpropionate (Levy and Frost, 1966). In the course of a detailed kinetic study of this enzyme, an intermediate enzyme form was observed by stopped-flow spectrophotometry when the reduced enzyme was reacted with molecular oxygen in the presence of melilotate (Strickland and Massey, 1973b). The transitory nature of this intermediate precluded more than its spectrophotometric characterization at that time. It was proposed that the intermediate is a complex between reduced enzyme, oxygen, and melilotate, with a covalent bond between the flavine prosthetic group and oxygen.



There did exist a reasonable alternative explanation for the absorption spectrum observed in these experiments. Conceivably, an *o*-quinone form of the product complexed

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48104. *Received November 1, 1974.* This research was supported by Grants No. GM-11106 and GM-00187 from the U.S. Public Health Service. The mass spectral facility is supported by Grant RR-004080 from the U.S. Public Health Service.

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to the enzyme could absorb in the 400-nm region. Therefore, it was important to determine which of the two explanations was correct. One manner in which to do this was to examine the kinetics of formation and decay of the intermediate using deuterated melilotate.

In addition to answering this fundamental question, there would be other benefits from experiments using deuterated melilotate. The intramolecular migration of ring substituents during the hydroxylation of aromatic substrates, the NIH shift, is a phenomenon which has been extensively investigated by Jerina, Daly, and coworkers (Jerina and Daly, 1973). Their seminal experiments have led to the mechanistic conclusion that hydroxylations catalyzed by the rat liver microsomal system proceed via an arene oxide intermediate (Jerina et al., 1970). It is the subsequent protonation of this arene oxide followed by rearrangement which leads to the final hydroxylated product. One interesting question concerning the NIH shift is whether the arene oxide pathway is an obligatory route for aromatic hydroxylations occurring via reductive activation of molecular oxygen. Experiments with hydroxylation systems such as flavoproteins might shed some light on this question.

Finally, in previous studies on melilotate hydroxylase, there was good agreement between rapid reaction, steady-state, and equilibrium results (Strickland and Massey, 1973b). The proposed mechanism could be subjected to further experimental test by examining the steady-state behavior of the enzyme using deuterated melilotate.

Experimental Procedure

Materials

Melilotate hydroxylase and melilotate were prepared as previously described (Strickland and Massey, 1973a). NADH (Grade III) was obtained from Sigma; catalase (B grade) from Calbiochem; 10% palladium on charcoal from Matheson Coleman and Bell; 99.8% deuterium oxide from Thompson-Packard; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and 99.5% dimethyl-d_6 sulfoxide were from Aldrich; bis(trimethylsilyl)trifluoroacetamide was from Regis; and silica gel N-HR/UV 254 precoated on plastic sheets was from Brinkman. Diethyl ether was freed of peroxides before use by shaking with a saturated solution of $FeSO_4$ in H_2O, drying over anhydrous $MgSO_4$, filtering, and storing over KOH. The *p*-dioxane was of scintillation quality; all other chemicals were of reagent grade.

Methods

The rapid reaction and steady-state experiments were done with a modified Gibson-Milnes stopped-flow spectrophotometer as previously described (Strickland and Massey, 1973b). Nuclear magnetic resonance (NMR) spectra were recorded with a Varian T-60 instrument.

Melilotate (R_f 0.47), 3,5-diiodomelilotate (R_f 0.61), and 2,3-dihydroxyphenylpropionate (R_f 0.36) were separated on silica gel using a benzene–ethanol–acetic acid (10:1:1) system. Spots were detected under short wavelength ultraviolet light, with iodine vapor, or by spraying with 1% $FeCl_3$ in H_2O. With the latter method, melilotate gives a purple and diiodomelilotate an orange spot.

Preparation of [3,5-^{2}H]Melilotate. Melilotate was iodinated with I_2 and KI to give 3,5-diiodomelilotate (Matsuura and Nishinaga, 1964). The product was recrystallized from ethanol–water after reduction of excess iodine with solid sodium dithionite. The final product was light yellow and had a melting point of 120–122° (reported value, 128–129°). The NMR spectrum of the product confirmed the structure, with an aromatic region (6–7 ppm) virtually identical with that of 3,5-diiodocresol (Kondo, 1965).

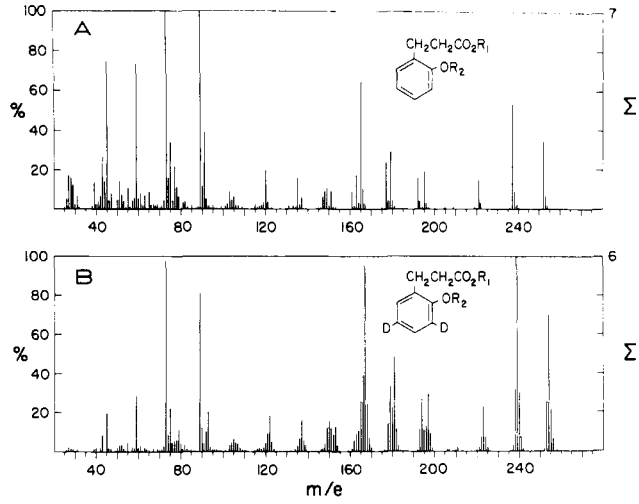


FIGURE 1: Mass spectrum of derivatized melilotate (A) and [3,5-^{2}H]melilotate (B). R_1 = $-CH_3$, R_2 = $-Si(CH_3)_3$.

Deuterated hydrazine was prepared by dissolving anhydrous hydrazine (10 g) in 99.8% ^2H_2O (90 g), and distilling water from the mixture under N_2 to leave a final volume of 25 ml. This yielded a stock solution of 40% hydrazine with an enrichment of approximately 90% in ^2H.

Reductive deiodination of 3,5-diiodomelilotate was achieved by a modification of the procedure described by Mosby (Mosby, 1959). Deuterated hydrazine (6 ml of the 40% solution in ^2H_2O), ^2H_2O (24 ml) (final enrichment in ^2H, 98%), *p*-dioxane (20 ml), 10% palladium on charcoal (50 mg), and 3,5-diiodomelilotate (1 g) were refluxed under N_2. After 2 hr, an additional 2 ml of the deuterated hydrazine solution was added and the mixture refluxed for a further 1.5 hr. The solution was cooled, filtered, acidified to pH 2 with concentrated HCl, and the dioxane removed as the water azeotrope in a rotary evaporator at 50°. The resulting solution was extracted three times with 50-ml portions of diethyl ether; the combined extracts were dried over $MgSO_4$, filtered, and evaporated to dryness under vacuum. The yellow-brown product was treated with activated charcoal and recrystallized from toluene–cyclohexane to yield 210 mg (53% of the theory) of white, crystalline material, mp 70–72° (melilotate mp 72–75°). A mixture melting point of the product and authentic melilotate showed no depression. Thin-layer chromatography and the ultraviolet absorption spectrum further confirmed the identity of the product as melilotate. The NMR spectrum of the deuterated melilotate indicated the same pattern for the aliphatic side chain protons (2–3 ppm) as for melilotate. However, in the aromatic region, the signals due to the protons at positions 3, 4, 5, and 6 in melilotate had collapsed to a single peak centered at around 7 ppm. The integration of this peak indicated the exchange of two protons. Finally, the mass spectrum (Figure 1) of the compound indicated that it was 85% dideuteriomelilotate. This evidence, together with its unequivocal chemical synthesis, confirms the structure of the final product as [3,5-^{2}H]melilotate. The deuterated product showed no exchange of deuterium when dissolved in 0.1 *M* KP_i (pH 7.3) for 24 hr at 25°.

NIH Shift Experiment. Melilotate, protium or deuteri-

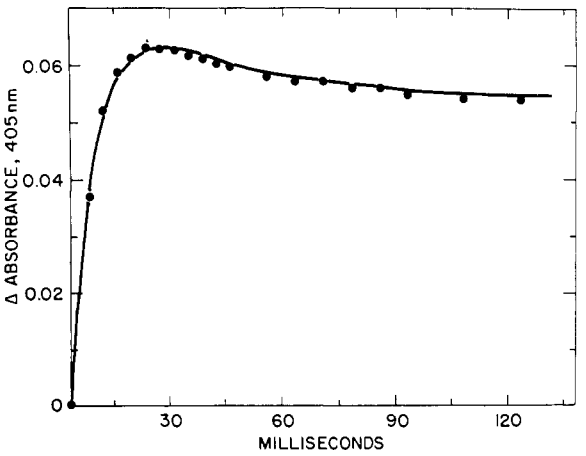


FIGURE 2: The reaction of reduced melilotate hydroxylase with molecular oxygen in the presence of melilotate (—) or [3,5-^{2}H]melilotate (●). Change in absorbance at 405 nm vs. time. Conditions: 35 μM melilotate hydroxylase was photoreduced with EDTA (see Strickland and Massey, 1973b) and rapidly mixed with an equal volume of buffer to give a final oxygen concentration of 0.625 mM. Both syringes contained 0.1 M KP_i (pH 7.3); 60 mM EDTA; and 1 mM of melilotate or [3,5-^{2}H]melilotate; temperature = 1°.

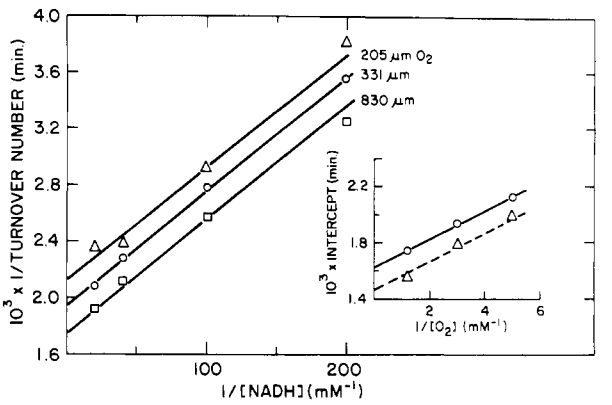


FIGURE 3: Steady-state analysis of melilotate hydroxylase using [3,5-^{2}H]melilotate as the substrate. Conditions: 0.1 M KP_i (pH 7.3); 1.0 mM [3,5-^{2}H]melilotate; temperature = 1°. A similar analysis using the protium form of melilotate gave plots of the same slope. Inset: Secondary plot of y axis intercepts vs. reciprocal oxygen concentration: protium form of melilotate (Δ - - Δ), deuterium form of melilotate (- O -).

um form (5 mg), NADH (28 mg), and FAD (5 mg) were dissolved in 0.1 M KP_i (pH 7.3) (200 ml). The reaction was started by adding 10 μl of melilotate hydroxylase (A_{450} = 0.64), and allowed to proceed at room temperature with constant stirring. The extent of the reaction was monitored by the loss of NADH absorption at 340 nm. After about 2 hr, the reaction was complete and the mixture was cooled to 0°, acidified to pH 1 with concentrated HCl, and extracted three times with peroxide-free diethyl ether. The combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness. The residue was transferred to a 1-dram vial with a small volume of ether and again taken to dryness.

Combined Gas Chromatography–Mass Spectrometry. Diazomethane in anhydrous ether (3 ml), generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Fieser and Fieser, 1967), was added to the residue in the 1-dram vial. After 10 min at room temperature, the solution was evaporated to dryness with a stream of nitrogen. This procedure should methylate only the carboxyl group of melilotate, but the reactivity of the hydroxyl groups as a result of their ortho orientation leads to the formation of some monomethyl ether. Distilled pyridine (100 μl) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (25 μl) were then added to the vial to form the silyl ether of any free hydroxyl groups remaining.

Combined gas chromatography–mass spectrometry, including single scanning and computer controlled repetitive scanning (mass chromatography), were carried out on an LKB Model 9000 instrument which had been interfaced to a dedicated PDP-8/I minicomputer for data acquisition and display (Sweeley et al., 1970, Laine et al., 1975). The gas chromatography inlet consisted of a silanized coiled glass column with helium carrier gas maintained at a constant flow rate of 35 ml/min. The column used was a 6 ft × 0.25 in. glass column packed with 1% SE-30 on 100/120 Supelcoport maintained isothermally at 150°. The operating conditions of the mass spectrometer were as follows: ion source temperature, 290°; molecular separator temperature, 240°; trap current, 60 μA; full accelerating voltage of 3.5 kV; and electron energy, 70 eV.

Table I: Stoichiometry of Melilotate Hydroxylase Reaction.[a]

	Protium Form	Deuterium Form
Initial melilotate	0.15 mM	0.15 mM
NADH consumed	0.14 mM	0.13 mM
% completion	93%	87%
% completion by gas chromatography[b]	96%	91%

[a] The experiment was performed as described under Experimental Procedure. [b] Obtained by comparing the amount of 2,3-dihydroxyphenylpropionate formed to the amount of melilotate remaining.

Results

Effect of [3,5-^{2}H]Melilotate on Stopped-Flow and Steady-State Kinetics. While previous studies have indicated that the intermediate formed in the reoxidation of melilotate hydroxylase is an oxygenated form of the enzyme, a quinoid form of the product was not excluded. Since the formation of this quinone would require cleavage of the C(3)–H bond, if the intermediate were the quinone its formation from [3,5-^{2}H]melilotate should show a primary isotope effect. Therefore, melilotate hydroxylase was reduced, complexed with melilotate, and reacted with molecular oxygen in the stopped-flow apparatus. It was found that substituting the deuterated substrate for the protium form had no effect at any wavelength on the rate of formation or decay of the intermediate. This result is illustrated in Figure 2 at a wavelength where the intermediate has a greater extinction coefficient than the reduced or oxidized enzyme.

Although no isotope effect was observed in the formation or decay of the oxygenated flavine intermediate, a small but reproducible isotope effect was observed in steady-state turnover as shown in Figure 3. Analysis of the results revealed that when [3,5-^{2}H]melilotate was substituted for melilotate, both the primary and secondary slopes remained the same, while the V_{max} was decreased 8%.

NIH Shift Experiment. When melilotate, protium or deuterium form, was incubated with melilotate hydroxylase and an excess of NADH, a consumption of NADH essentially stoichiometric with the amount of melilotate added could be observed spectrophotometrically (Table I). Fur-

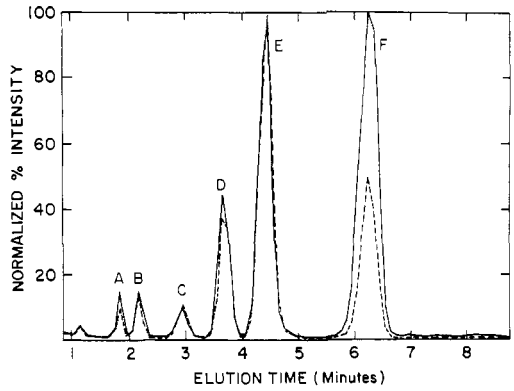


FIGURE 4: The normalized ion intensity vs. elution time for the derivatized products of the reaction of melilotate hydroxylase with melilotate (—) protium form; (- - -) deuterium form. The experimental procedure is presented under Experimental Procedure. The ion intensity refers to the total number of ions detected by the mass spectrometer at any time, and is equivalent to analysis by a flame ionization detector. See text for identification of each peak.

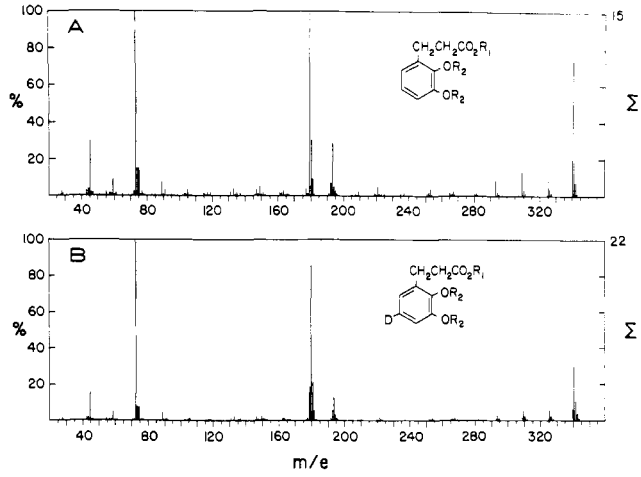
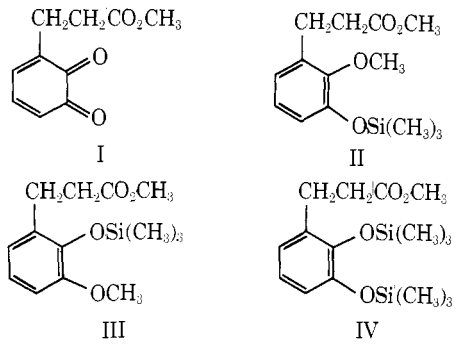


FIGURE 5: Mass spectrum of derivatized 2,3-dihydroxyphenylpropionate, formed from the reaction of melilotate hydroxylase with melilotate. The derivative depicted corresponds to peak F in Figure 4. (A) protium form; (B) deuterium form. $R_1 = -CH_3$; $R_2 = -Si(CH_3)_3$.

thermore, analysis of the resulting extracted material (see Methods) by thin-layer chromatography showed predominantly a single spot corresponding to 2,3-dihydroxyphenylpropionate, with a trace of melilotate remaining.

Analysis of the extracted material by gas chromatography after derivatization showed six major peaks (Figure 4). The identity of these peaks was investigated by mass spectrometry. Peak B is residual melilotate, and peak C appears to be the *o*-quinone form (I) of dihydroxyphenylpropionate, which arises from air oxidation during the isolation procedure. The three main peaks, D, E, and F, are due to different derivatives of dihydroxyphenylpropionate. Peaks D and E result from the two isomeric silyl and methyl ethers (II and III). The last major peak, F, is due to the disilyl ether (IV) of the dihydroxy product. Peak A, which accounts for



less than 3% of the total ions observed, remains unidentified, but it is probably not derived from melilotate since no difference in its mass spectrum was observed for the protium vs. the deuterium form. Thus, of the total ions observed, more than 95% can be accounted for by residual starting material and product.

The mass spectra of the compounds corresponding to peaks C, D, E, and F, i.e., the compounds I, II, III, and IV which are all derived from dihydroxyphenylpropionate, showed a major molecular ion of one mass unit greater for the deuterated sample than for the protium sample. For example, the mass spectrum of the disilyl ether (IV, peak F) is shown in Figure 5. As can be seen, the major molecular ion is at 340 for the protium sample and 341 for the deuterium sample, and the fragmentation patterns are identical. The amount of mass 342 seen in the deuterium sample can be accounted for quantitatively by the natural abundance of

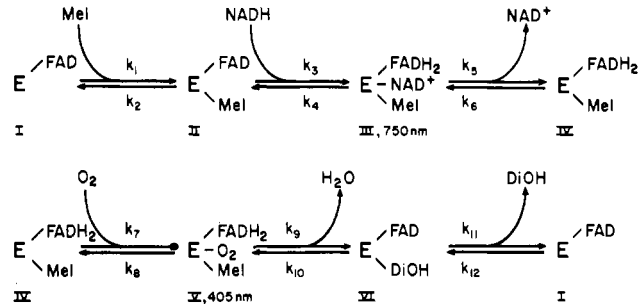


FIGURE 6: Reaction mechanism for melilotate hydroxylase (Strickland and Massey, 1973b). Species V is the proposed oxygenated intermediate. k_{11} is arbitrarily formalized as release of the dihydroxy product, but as discussed in the text probably includes an enzyme-assisted cleavage of the C(3)-H bond.

the heavy isotopes of carbon, hydrogen, and silicon (Beynon, 1960), and the small amount of trideuteriomelilotate present in the starting material (see Figure 1, *m/e* 255 in deuterium sample). Since there is no unaccounted for mass at position 342 (extra mass would be indicative of migration of deuterium, i.e., the NIH shift), it can be concluded that such migration does not occur, within the limits of detection of this experiment (<0.5%). Analysis of the mass spectra of peaks C, D, or E yields the same result.

Discussion

Previous studies have demonstrated that the reaction sequence of melilotate hydroxylase can be described as in Figure 6. The rapid reaction results presented above using [3,5-^{2}H]melilotate further indicate that the intermediate (V, Figure 6) detected in the reoxidation of the reduced enzyme is due to an oxygenated form of the reduced flavine chromophore. The lack of an isotope effect in the formation (k_7) or decay (k_9) of the intermediate indicates that the C(3)-H bond remains intact until the intermediate disappears, and thus rules out a quinoid form of the product as the absorbing species responsible for this intermediate spectrum. Of course, the absence of an isotope effect limits the interpretation that can be placed on these experiments, but as shown below, the steady-state results also support the proposition that the C(3)-H bond is cleaved subsequent to the disappearance of the intermediate.

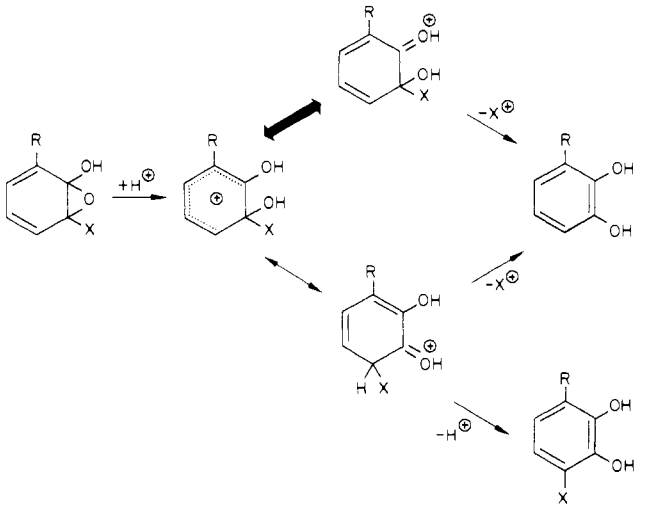


FIGURE 7: Possible intermediates in an arene oxide hydroxylation pathway (adapted from Jerina and Daly, 1973). Upper pathway shows resonance stabilization of cationoid intermediate without migration and hence no retention of substituent X. Lower pathway shows migration of X (possible retention).

The results of the steady-state experiments complement nicely the observations made by stopped-flow spectrophotometry. The fact that the slopes of the primary and secondary plots (Figure 3) are the same for the protium and deuterium forms of melilotate indicates that the rate constants in the reductive cycle of the reaction mechanism (k_1–k_6), as well as the formation (k_7) and decay (k_9) of the oxygenated intermediate, involve no isotope effect.

The small change in V_{max} may be explained with reference to previous results. For the reaction mechanism of this enzyme (Strickland and Massey, 1973b), V_{max} is determined by three rate constants (Figure 6):

$$\frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}} = \frac{1}{V_{max}}$$

In previous work, to obtain reasonable agreement between measured values of k_5, k_9, and V_{max}, the value for k_{11} had to be assumed to be large with respect to k_5 and k_9, making its reciprocal negligible in the above expression (Strickland and Massey, 1973b). A value for k_{11} of 4×10^5 min^{-1}, about 30 times as large as k_5 (1.3×10^3 min^{-1}) would satisfy this requirement. It should be noted that an average primary isotope effect of sevenfold on k_{11} would then change V_{max} about 8%. The point is, that if k_{11} is large with respect to k_5 and k_9, and there is evidence that it is, then a primary isotope effect on k_{11} would be manifest as a small change in V_{max}. Previously k_{11} was arbitrarily formalized as involving the release of the aromatic product from the oxidized enzyme as seen in Figure 6. As discussed above, it is now reasonable to postulate that this term (k_{11}) contains an enzyme-assisted step involving breakage of the C(3)–^{1}H/^{2}H bond, which would be followed by release of the product from the enzyme. The situation for the enzyme would then be not unlike many electrophilic aromatic substitutions previously studied, where the aromatization step is so fast as to preclude observation of a normal primary isotope effect for the overall reaction (March, 1968; Zollinger, 1964). Thus, as stated, these results support the interpretation of the rapid reaction data. Nevertheless, since this analysis is based on such a small change in V_{max}, it must remain tentative.

Evidence has now been accumulated that hydroxylation reactions which show intramolecular migration of ring substituents involve an arene oxide (epoxide) intermediate (Jerina et al., 1970). It has been postulated that such an intermediate arises from attack of oxene on the aromatic ring system (Jerina and Daly, 1973). Different forms of activated oxygen which are capable of hydroxylation have been considered in general (Jerina and Daly, 1973), and for flavoprotein catalyzed reactions in particular (Strickland, 1972). In both cases, oxene has been proposed as an attractive candidate. In view of recent work on squalene epoxidase (Yamamoto and Bloch, 1970 and Tai and Bloch, 1972), these ideas take on special significance for flavoprotein hydroxylases. Squalene epoxidase is apparently a flavoprotein, and one which catalyzes the epoxidation of squalene to 2,3-oxidosqualene. Thus, it appears that flavoproteins can form epoxides, which makes it increasingly tempting to suppose that flavoprotein catalyzed hydroxylations may proceed through this type of an intermediate, possibly formed from oxene insertion.

The experiments designed to test the above possibility with melilotate hydroxylase showed no evidence of deuterium migration which is characteristic of such a mechanism. However, it has been duly noted that substituents on phenols generally show little or no migration during aromatic hydroxylations with systems known to give the NIH shift with other substrates (Daly et al., 1967). This is due to the fact that the presumed cationoid intermediate is stabilized effectively through resonance without migration of a substituent (heavy arrow, Figure 7). Nevertheless, small amounts of migration, 1–5%, have been observed during hydroxylations of phenols (Daly et al., 1967). So, since even migration to a small extent would provide strong evidence that melilotate hydroxylase catalyzes the formation of an arene oxide, it was important to fully explore this possibility. The complete lack of migration which has been demonstrated for melilotate hydroxylase is thus not incontrovertible evidence that the NIH shift mechanism does not occur.

The only known aromatic substrates which are hydroxylated by flavoproteins are phenols, and the search for the NIH shift in these systems would therefore suffer from the same ambiguities as presented above. As a possible exception, the reaction of *p*-hydroxybenzoate hydroxylase with benzoate was earlier reported to yield small amounts of *m*-hydroxybenzoate (Spector and Massey, 1972). This reaction has been reinvestigated in detail, and the product was found to be chromatographically very similar to but not identical with *m*-hydroxybenzoate.[1] The exact chemical nature of this product is still unknown.

Acknowledgment

We are indebted to Dr. Charles C. Sweeley for providing the facilities for the gas chromatography–mass spectrometry, as well as for valuable advice. In addition, we are grateful to Dr. Mark A. Bieber and Mr. Jack Harten for their generous assistance with the mass analysis.

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The Mechanism of the Bond Forming Events in Pyridine Nucleotide Linked Oxidoreductases. Studies with Epoxide Inhibitors of Lactic Dehydrogenase and β-Hydroxybutyrate Dehydrogenase†

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ABSTRACT: 2,3-Epoxybutyrate and 2,3-epoxypropionate act as effective competitive inhibitors of pig heart lactic dehydrogenase. $K_I$app for both inhibitors was pH dependent and varied according to the general equation $K_I\text{app} = K_I(1 + K_a/H^+)$ which may be predicted if the binding of the epoxide to the E-NADH complex involves a compulsory protonation step. Values of $K_{I(\text{epoxybutyrate})}$, $K_{I(\text{epoxypropionate})}$ and pK_a were estimated as 150 μM, 860 μM, and 6.8, respectively. The formation of an E-NADH epoxide inhibitor complex was followed directly by fluorescence measurements. Both epoxybutyrate and epoxypropionate enhanced fluorescence of the E-NADH complex and caused a 20-nm blue shift in the maximum emission wavelength. The dissociation constants measured by fluorescence titration for both epoxides increased as the pH was raised reflecting a decreased affinity for the E-NADH complex. 2,3-Epoxybutyrate was also shown to inhibit β-hydroxybutyrate dehydrogenase by a mechanism which is consistent with compulsory protonation prior to addition of the epoxide. These results are discussed in terms of a general mechanism for the bond forming events in pyridine nucleotide linked oxidoreductases.

Pyridine nucleotide oxidoreductases have long been the subject of extensive mechanistic studies and in particular a considerable amount of kinetic data have been obtained concerning the formation and dissociation of binary and ternary complexes (Holbrook and Gutfreund, 1973). Despite a detailed understanding of these phenomena for a number of oxidoreductases, little information is available on the precise nature of events involved in the transformation of substrate to product. Although the classical work of Westheimer and Vennesland (Fisher et al., 1953; Loewus et al., 1953) established that there was a direct hydride transfer between substrate and coenzyme, with the concomitant involvement of a proton to maintain stoichiometry, the precise mechanism of activation of the substrate in the ternary complex has not been determined. Studies on the pyridine nucleotide linked reductions of certain carbon–carbon double bonds (Wilton et al., 1966, 1968, Watkinson et al., 1971a,b; Akhtar et al., 1972) established that the orientation of addition of the hydride ion and proton was consistent with a Markovnikov mechanism where substrate activation is achieved by initial protonation to give a carbonium ion intermediate which is subsequently neutralized by hydride transfer (Scheme I). This mechanism of substrate activation by initial protonation has also been proposed in the case of pyridine nucleotide linked carbonyl oxidoreductases (Akhtar and Wilton, 1970, 1973; Akhtar et al., 1972).

In order to evaluate the role of enzyme mediated sub-

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