electronic promotion ($\pi \rightarrow \pi^*$) within the porphyrin ring. This might be the reason that we have not observed the enhancement of the ν(Fe–NO) stretch in pentacoordinated NO–heme upon Soret excitation. The excitation profile of the 592-cm^{-1} line detected by Stong et al. (1980) in nitrosyl-HbA with IHP should resolve the question of whether it is enhanced *via* a charge-transfer transition.

References

Asher, S. A., Vickery, L. E., Schuster, T. M., & Sauer, K. (1977) *Biochemistry 16*, 5849.

Cassoly, R. (1974) *C. R. Hebd. Seances Acad. Sci., Ser. D 278*, 1417.

Chottard, G., & Mansuy, D. (1977) *Biochem. Biophys. Res. Commun. 77*, 133.

Desbois, A., Lutz, M., & Banerjee, R. (1979) *Biochemistry 18*, 1510.

Felton, R. H., & Yu, N.-T. (1978) in *The Porphyrins* (Dolphin, D., Ed.) Vol. III, Chapter VIII, Academic Press, New York.

Maxwell, J. C., & Caughey, W. S. (1976) *Biochemistry 15*, 388.

Nagai, K., Hori, H., Yoshida, S., Sakamoto, H., & Morimoto, H. (1978) *Biochim. Biophys. Acta 532*, 17.

Nagai, K., Welborn, C., Dolphin, D., & Kitagawa, T. (1980) *Biochemistry 19*, 4755.

Nishikura, K., & Sugita, Y. (1976) *J. Biochem. (Tokyo) 80*, 1439.

Nozaki, Y., & Tanford, C. (1967) *Methods Enzymol. 11*, 733.

Perutz, M. F., Kilmartin, J. V., Nagai, K., Sazbo, A., & Simon, S. R. (1976) *Biochemistry 15*, 378.

Rein, H., Ristau, O., & Scheler, W. (1972) *FEBS Lett. 24*, 24.

Scheidt, W. R., Brinegar, A. C., Ferro, E. B., & Kirner, J. F. (1977) *J. Am. Chem. Soc. 99*, 7315.

Scholler, D. M., Wang, M.-Y. R., & Hoffman, B. M. (1979) *J. Biol. Chem. 254*, 4072.

Spaulding, L. D., Chang, R. C. C., Yu, N.-T., & Felton, R. H. (1975) *J. Am. Chem. Soc. 97*, 2517.

Stong, J. D., Burke, J. M., Daly, P., Wright, P., & Spiro, T. G. (1980) *J. Am. Chem. Soc. 102*, 5815.

Szabo, A., & Barron, L. D. (1975) *J. Am. Chem. Soc. 102*, 5815.

Taketa, F., Antholine, W. E., Mauk, A. G., & Libnoch, J. A. (1975) *Biochemistry 14*, 3229.

Tsubaki, M., & Yu, N.-T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3581.

Tsubaki, M., Srivastava, R. B., & Yu, N.-T. (1981a) *Biochemistry 20*, 946.

Tsubaki, M., Srivastava, R. B., & Yu, N.-T. (1981b) *Biochemistry* (preceding paper in this issue).

Wright, P. G., Stein, P., Burke, J. M., & Spiro, T. G. (1979) *J. Am. Chem. Soc. 101*, 3531.

Yu, N.-T., & Srivastava, R. B. (1980) *J. Raman Spectrosc. 9*, 166.

Yu, N.-T., & Tsubaki, M. (1980) *Biochemistry 19*, 4647.

Kinetic Isotope Effects in the Oxidation of Isotopically Labeled NAD(P)H by Bacterial Flavoprotein Monooxygenases†

Carol Cummings Ryerson, David P. Ballou, and Christopher Walsh*

ABSTRACT: Three bacterial flavoprotein monooxygenases, *p*-hydroxybenzoate hydroxylase, orcinol hydroxylase, and salicylate hydroxylase, have been examined for steady-state kinetic isotope effects with (4*R*)-[4-^{2}H]NAD(P)H and (4*R*)-[4-^{3}H]NAD(P)H. The observed isotope selections are for deuterium, ^DV = 1.8–3.5 and $^D(V/K)$ = 1.7–5.1, and for tritium, $^T(V/K)$ = 5–19. For both orcinol hydroxylase and *p*-hydroxybenzoate hydroxylase, reduction of enzyme-bound FAD by (4*R*)-[4-^{2}H]NAD(P)H in pre-steady-state assays reveals intrinsic deuterium isotope effects of 10 ± 2 on this redox step. These values are at the upper end of substrate deuterium effects seen in enzymatic reactions. Suppression of ~83% of the intrinsic isotope effects in the overall reaction rate (e.g., k_H/k_D = 10 down to ^DV = 2.5) corroborates earlier kinetic data on *p*-hydroxybenzoate hydroxylase [Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254*, 6657] and suggests that these bacterial phenolic monooxygenases balance out internal transition states such that no single barrier is fully rate limiting.

In reactions involving transformations where C–H bonds break at some stage during catalysis, substitution of deuterium or tritium for hydrogen permits mechanistic analysis based on the consequent kinetic isotope effect. Most enzymatic studies using isotopically labeled substrates have focused on steady-state kinetics and measurement of observed isotope effects on overall turnover (on V_{max} or on V_{max}/K_m).[1] The observed isotope effects can vary from full expression of the

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[1] The following definitions shall be used throughout this paper: ^DV indicates a deuterium isotope effect observed on overall turnover in V_{max}; $^D(V/K)$ represents the observed deuterium isotope effect on V_{max}/K_m, determined from comparing overall rates with deuterated and unlabeled substrate; $^T(V/K)$ represents a tritium isotope effect observed on overall turnover in V_{max}/K_m, determined in an internal competition experiment with tritiated substrate; k_H/k_D or ^Dk indicates the intrinsic or primary deuterium isotope effect; similarly, k_H/k_T is the intrinsic or primary tritium isotope effect.

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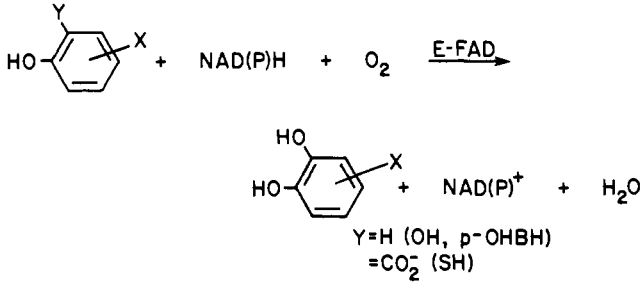
intrinsic isotope effect on the elementary C–H cleavage step(s) to complete masking of the rate differential, $^{D}V = 1$, when some other step is slow in the catalytic sequence. To measure the intrinsic isotope effect, one must usually resort to pre-steady-state kinetic studies (e.g., stopped-flow measurements) to isolate the elementary rate constant. This may also require substrate quantities of pure enzyme. Yet, to interpret any observed isotope effects on macroscopic steady-state parameters and evaluate when along the reaction coordinate the isotopically sensitive step occurs and to what degree that step controls turnover rates, one must evaluate k_H/k_D, the intrinsic isotope effect on the elementary step.

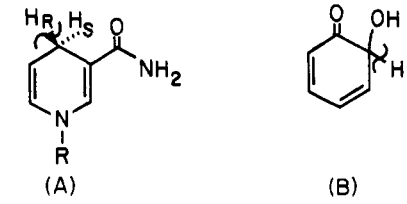
There are rather few cases in the enzymatic literature where both intrinsic and observed isotope effects on V_{max} have both been measured and the correlation analyzed. A notable example in oxidation–reduction enzymes is the study of alcohol dehydrogenase with both (4*R*)-[4-^{2}H]NADH and (1*R*)-^{2}H-labeled alcohols (Shore & Gutfreund, 1970; Cook & Cleland, 1981). In another report, the adrenal mitochondrial flavoenzyme adrenodoxin reductase, an electron transferase, was examined for observed isotope effects on V_{max} with (4*S*)-[4-^{2}H]NADPH. The intrinsic deuterium isotope effect on reduction of enzyme-bound FAD was factored out both by systematic variation of flavin redox potential until that step became fully rate determining and by direct analysis via stopped-flow studies (Light & Walsh, 1980). In that dihydronicotinamide flavin redox enzyme, $k_H/k_D = 7.4$.

In this paper we now focus on a distinct category of NAD(P)H-oxidizing flavoproteins, the bacterial aromatic ring monooxygenase class. We report data on *p*-hydroxybenzoate hydroxylase for which a plethora of mechanistic information has been collected by Massey and co-workers (Entsch et al., 1976; Husain & Massey, 1979) and can therefore be used as a paradigm and in addition data on orcinol hydroxylase (Ohta et al., 1975) and salicylate hydroxylase (White-Stevens et al., 1972; Takemori et al., 1972), two enzymes which have been much less well studied for mechanism.

The reaction type catalyzed by these flavoenzyme monooxygenases involves the four-electron reductive splitting of O_2 at the expense of a two-electron oxidation of aromatic substrate and a two-electron oxidation of either NADH or NADPH as indicated below. Substitution at "X" will vary depending on the enzyme under consideration.



At some stage during these redox reactions, at least two C–H bond cleavage steps occur: (a) breakage of the C_4–H_R bond (You et al., 1977) of the dihydronicotinamide cosubstrate (A) and (b) replacement of an aromatic C–H bond by the C–OH bond (B), to form the catecholic product. The first C–H cleavage occurs early in catalysis, during reduction of bound flavin coenzyme, and generates the flavin oxidation state competent for combination with O_2 (Massey & Flashner, 1974; Massey & Hemmerich, 1975; Walsh, 1980). The second cleavage, an aromatic C–H breakage step, may well occur late in the sequence, possibly even as a nonenzymatic aromatization of a hydroxycyclohexadienoid tautomer (B) of the product catechol (e.g., Entsch et al., 1976).



No kinetic isotope effect is expressed on overall turnover when an isotopic label is in the phenolic substrate (Entsch et al., 1976; Ryerson & Walsh, 1979), implying that the second C–H breakage step discussed above (e.g., to form B) does not play any role in limiting the overall reaction rate. Isotopic substitution in NAD(P)H, however, does result in the expression of a kinetic isotope effect on turnover (V_{max}, V_{max}/K_m). In this paper, we report pre-steady-state and steady-state kinetic isotope effect studies with (4*R*)-[4-^{2}H]NAD(P)H and (4*R*)-[4-^{3}H]NAD(P)H in reaction with the indicated three bacterial flavoenzyme monooxygenases. The role which the flavin reduction step plays as a rate-limiting step on overall catalysis is discussed.

Experimental Procedures

Materials

Pseudomonas putida 01 was a generous gift of Professor D. W. Ribbons. Purified salicylate hydroxylase was kindly supplied by Professor H. Kamin and Dr. R. Presswood. Purified *p*-hydroxybenzoate hydroxylase was a generous gift from Professor V. Massey and was prepared as described previously (Husain et al., 1978).

[1-^{3}H]Ethanol (100 mCi/mmol) and sodium boro[^{3}H]-hydride (>100 mCi/mmol) were obtained from New England Nuclear. Ethanol-d_6 (99.5% D) was obtained from Stohler Isotope Chemicals. Sodium borodeuteride was purchased from Merck & Co., Inc. All substrate solutions were prepared fresh daily.

Methods

Preparation of Isotopically Labeled NADH. The (4*R*)-[4-^{2}H]NADH and (4*R*)-[4-^{3}H]NADH samples were prepared by using yeast alcohol dehydrogenase (Oppenheimer et al., 1971). The solid samples of isotopically labeled NADH were stored desiccated at −20 °C. The average specific activities of the (4*R*)-[4-^{3}H]NADH samples were 4.0 μCi/μmol. NMR analysis (270 MHz) on the solid (4*R*)-[4-^{2}H]NADH sample confirmed greater than 95% deuteration and validated the 4*R* chirality according to the methods of Arnold & You (1978) and Oppenheimer et al. (1971).

Determination of Specific Radioactivity of (4R)-[4-^{3}H]-NADH. The specific activity of (4*R*)-[4-^{3}H]NADH was routinely determined by assay with alcohol dehydrogenase. The assay mixture[2] contained 1 mL (0.05–0.06 mM) of (4*R*)-[4-^{3}H]NADH in 20–50 mM KH_2PO_4, pH 7.0–7.5, 3 μL (140 mM) of propionaldehyde (observed $V_H/V_D \simeq 2$ with this substrate), and 5 μL (0.5 mg/mL) of alcohol dehydrogenase. The NADH consumption was monitored (at 25 °C) with a Perkin-Elmer 200 spectrometer or a Gilford 220 spectrophotometer until oxidation was complete (when $\Delta A_{340} = 0$). When oxidation of NADH was complete, a 20-μL aliquot of the

[2] Unless otherwise indicated, the components for each assay are specified as a volume to be added from a stock solution of given concentration. The concentrations of buffer salts are listed as final concentrations.

reaction mixture was removed onto a Dowex 1-Cl^- column and washed with 1 mL of water; the [^{3}H]propanol generated in the reaction will be in the eluted fraction. The specific activity was obtained from the ratio of microcuries of ^{3}H released (into the material not bound to Dowex 1-Cl^-) to micromoles of NADH oxidized. Essentially quantitative oxidation and labilization of ^{3}H were achieved by this assay. No contaminant was detected in these preparations, such as was found in the NADPH samples (below).

For studies on the increase in specific radioactivity of (4*R*)-[4-^{3}H]NADH with time during orcinol hydroxylase catalysis, "unreacted" NADH was recovered and assayed. The reaction mixture with orcinol hydroxylase contained 3 mL (0.24 mM) of (4*R*)-[4-^{3}H]NADH in 50 mM KH_2PO_4, pH 6.8 (initial specific activity 3.67 μCi/μmol), 20 μL (25 mM) of orcinol, and 25 μL ($\sim$19 μg) of enzyme. The reaction rate was monitored (at 30 °C) as NADH consumption with a Perkin-Elmer 200 spectrometer. At both 2 (34% of NADH oxidized) and 2.5 min (42% of NADH oxidized), a 1-mL aliquot was removed into 10 μL of 5 N HCl, and the aliquots were applied to columns of DE-52 (0.5 × 6.0 cm) equilibrated with water. The column was washed with 6 mL of H_2O and then 3 mL of 0.3 M NH_4HCO_3, pH 9.0. An aliquot from the peak (assayed by A_{340}) fraction was used to determine the specific activity with alcohol dehydrogenase. The elution buffer, 0.3 M NH_4HCO_3, pH 9.0, has no effect per se on alcohol dehydrogenase activity.

Preparation of Isotopically Labeled DL-*Malate.* The preparation of DL-[2-^{2}H]malate and DL-[2-^{3}H]malate was carried out essentially according to Lowenstein (1963). The isolation of the malate samples from the reaction mixture was achieved by column chromatography (9.4 mL, 1 × 12 cm) with AG 1-X8 anion-exchange resin. Those column fractions containing malate were located by TLC [Whatman No. 1 paper, solvent system: butanol/acetic acid/water (120:30:50)], combined, concentrated in vacuo, and lyophilized. The malate was visualized by UV light after being sprayed with an acridine solution (0.1% in 95% ethanol).

Preparation of Isotopically Labeled NADPH. Samples of (4*R*)-[4-^{2}H]NADPH were prepared in incubations containing 5 mL of 33 mM Tris-HCl and 0.3 mM EDTA,[3] pH 8.0, 10–15 mg of $NADP^+$, 300 μL (0.38 M) of DL-[2-^{2}H]malate, 100 μL (0.2 M) of $MgSO_4$, and 200 μL (66 μg) of malic enzyme. The reaction was monitored by watching the appearance of NADPH at λ 340 nm. When $A_{260}/A_{340} < 3$, the reaction mixture was loaded onto a DE-52 column (6-mL volume, 1.5 × 3.7 cm) equilibrated with H_2O. The column was washed with 20 mL each of 0, 0.1, 0.2, 0.25, and 0.3 M NH_4HCO_3. Those fractions with $A_{260}/A_{340} < 2.3$ were combined and lyophilized. The white powdery nicotinamide samples were stored desiccated at −20 °C. NMR analysis (250 MHz) on a sample of the (4*R*)-[4-^{2}H]NADPH confirmed greater than 95% deuteration and validated the 4*R* chirality (Oppenheimer et al., 1978).

Control samples of NADPH were prepared by using DL-[2-^{1}H]malate (prepared exactly as the deuterio samples). This (4*R*)-[4-^{1}H]NADPH was found to be only $\sim$86% as active as the NADPH available from Sigma, in steady-state turnover assays with *p*-hydroxybenzoate hydroxylase. In single turnover experiments, using stopped-flow techniques (Entsch et al., 1976), the (4*R*)-[4-^{1}H]NADPH was 2.5–3-fold slower than Sigma NADPH (see Table VI). However, both (4*R*)-[4-^{1}H]- and (4*R*)-[4-^{2}H]NADPH have the same activity when assayed with adrenodoxin reductase, which removes the hydrogen from the 4*S* locus of the nicotinamide (Light & Walsh, 1980). This suggests that the inhibition of activity is present in both [^{1}H]- and [^{2}H]NADPH samples. The inhibitor(s) is (are) believed to be generated during isolation of the nicotinamide from the malic enzyme incubation mixture but was found to be present only in NADPH preparations and not in NADH preparations. All kinetic isotope experiments were carried out by using the (4*R*)-[4-^{1}H]NADPH as a control.

The samples of (4*R*)-[4-^{3}H]NADPH were prepared in the same manner, except 300 μL (0.26 M) of DL-[2-^{3}H]malate was used in place of the deuterated substrate. The DE-52 column was set up as described above and washed with 125 mL of H_2O and 50 mL of 0.1 M NH_4HCO_3 until the counts per minute of ^{3}H in each fraction (3 mL) fell below background ($\sim$1000 cpm). Nicotinamide was then eluted as before.

Determination of Specific Activity of (4R)-[4-^{3}H]NADPH. In an assay with *p*-hydroxybenzoate hydroxylase (enzyme assay conditions delineated below), it was determined that 97% of the NADPH (in a solution of 0.5 OD_{340}) was oxidizable. It was assumed for specific activity calculations that all (97%) the tritium associated with the NADPH was in the 4*R* locus. The specific activity of this sample of (4*R*)-[4-^{3}H]NADPH was calculated to be 14.8 μCi/μmol. No tritium was released in the absence of enzyme.

Preparation of Orcinol Hydroxylase. Pseudomonas putida 01 cells were grown at 30 °C on mineral salts medium, and orcinol hydroxylase was prepared from the cells essentially according to Ohta et al. (1975). Typically, the yield from 18 L of cell culture ($\sim$30 g wet weight) was sonicated with a Branson sonifier 350. Phenylmethanesulfonyl fluoride was added to the soluble fraction which was then loaded onto a DE-52 column (2 × 41 cm) equilibrated with 20 mM phosphate, pH 6.8, containing 0.3% mercaptoethanol (buffer A). The protein eluted with a gradient of 0.1–0.5 M KCl in the same buffer. The pale yellow fractions possessing orcinol hydroxylase activity were concentrated and then fractionated on a Sephadex G-100 column (1.5 × 60 cm) in buffer A. The final hydroxylapatite column used by Ohta et al. (1975) was omitted.

Steady-State Kinetic Assays with Orcinol Hydroxylase. All assays were performed at 30 °C. Orcinol hydroxylase activity was routinely assayed by following oxygen consumption with an oxygen electrode. Activity can also be monitored by NADH consumption with no observed difference in kinetic constants. A typical assay mixture contained the following: 400 μL (0.24 mM) of NADH in 50 mM KH_2PO_4 buffer, pH 6.8, and, as a substrate, 5 μL (25 mM) of orcinol, or 8 μL (182 mM) of resorcinol, or 5 μL (191 mM) of *m*-cresol, 5 μL (23 mg/mL) of catalase, and 5 μL (0.017 mM) of orcinol hydroxylase. Atmospheric oxygen concentration (0.24 mM) was present in all assays unless otherwise specified. The average specific activity of the enzyme was 37 μmol min^{-1} (mg of protein)$^{-1}$ extrapolated to infinite concentrations of orcinol and NADH, and O_2.

In tritium kinetic isotope experiments, 15- or 20-μL aliquots were removed from the assay mixture (described above) onto columns of Dowex 1-Cl^- (0.5 × 6.0 cm) every 15 or 20 s (a total of five time points were obtained). The $^3HO^1H$ was eluted (with H_2O) and counted. Linear rates of tritium release and constant ratios of $^T(V/K)$ were obtained in each experiment. Tritium experiments were conducted under conditions

[3] Abbreviations: OH, orcinol hydroxylase; *p*-OHBH, *p*-hydroxybenzoate hydroxylase; 2,4-DOHB, 2,4-dihydroxybenzoate; 3,4-DOHB, 3,4-dihydroxybenzoate; *p*-OHB, *p*-hydroxybenzoate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

where 2–20% of the added NADH (limiting substrate) was consumed. At least four such determinations were averaged to obtain $^{T}(V/K)$ values.

Steady-State Kinetic Assays with Salicylate Hydroxylase. Assays were carried out at 27 °C. Reaction mixtures contained 500 μL of 20 mM phosphate buffer, pH 7.62, and 1 mM EDTA, 500 μL (0.24 mM) of NADH in the same buffer, 5 μL of substrate [(19.95 mM) salicylate or (1.6 M) benzoate], and 2 μL of salicylate hydroxylase [0.032 mM enzyme, specific activity 1.98 μmol min^{-1} (mg of protein)$^{-1}$]. Despite the low specific activity, this enzyme was found to be fully coupled with salicylate. Enzyme activity was monitored by NADH consumption on a Perkin-Elmer 200 spectrometer or a Gilford 220 spectrometer. Tritium assays [$^{T}(V/K)$ measurements] were carried out as described above for orcinol hydroxylase.

Steady-State Kinetic Assays with p-Hydroxybenzoate Hydroxylase. Assays were carried out at 25 °C. The standard assay system contained 1 mL (0.24 mM) of NADPH in 33 mM Tris-HCl and 0.3 mM EDTA, pH 8.0, 10 μL (33 mM) of *p*-hydroxybenzoate, or 10 μL (130 mM) of 2,4-dihydroxybenzoate, or 10 μL (33 mM) of 3,4-dihydroxybenzoate, 4 μL (0.85 mM) of FAD, and 2 μL (0.21 mM) of *p*-hydroxybenzoate hydroxylase.

Where indicated, assays were carried out in 50 mM potassium phosphate and 20 mM EDTA, pH 6.55, at both 25 and 4 °C; concentrations of substrates, cosubstrate, and enzyme remain the same. The enzyme activity was monitored by NADPH consumption on a Perkin-Elmer 200 spectrometer. Tritium assays were carried out as described above for orcinol hydroxylase.

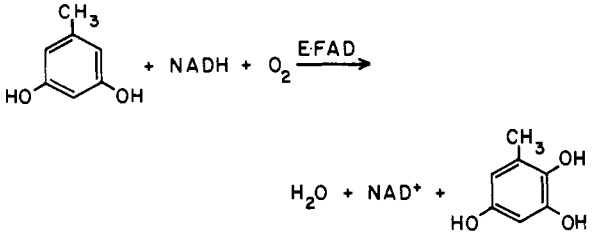
Stopped-Flow Experiments. Stopped-flow experiments (pre-steady-state kinetic assays) were performed on an anaerobic stopped-flow spectrophotometer as described in Beaty & Ballou (1981). Assay mixtures contained (as final concentrations) NADH [or (4*R*)-[4-^{2}H]NADH] (0.07–0.44 mM) in 50 mM KH_2PO_4 buffer, pH 6.8, 0.125 mM orcinol, and 0.021 mM orcinol hydroxylase. Orcinol hydroxylase activity was monitored by the reduction of the flavin chromophore at λ 450 nm. Experiments were carried out under anaerobic conditions and at 30 °C unless otherwise specified.

Other reaction mixtures contained NADPH [or (4*R*)-[4-^{2}H]NADPH] (0.04–0.53 mM) in 50 mM potassium phosphate, pH 6.6, 4 °C, 10 mM EDTA, 0.40 mM *p*-hydroxybenzoate, and 0.0058 mM *p*-hydroxybenzoate hydroxylase. To maintain anaerobic conditions, 0.01 mM protocatechuic acid and 30 nM protocatechuic hydroxylase (prepared by Dr. C. Bull at the University of Michigan) were added to the assays.

Results

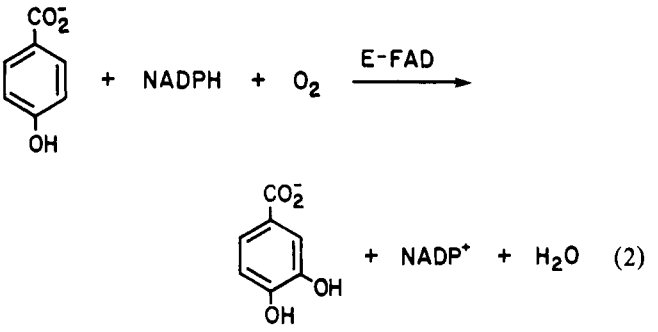
The chemical reactions catalyzed by the three bacterial flavin monooxygenases discussed in this paper are depicted in eq 1–3. In addition to those substrates indicated, the following partially coupled substrates were included in these kinetic studies: with orcinol hydroxylase, resorcinol (66% coupled)[4] and *m*-cresol (59% coupled); with *p*-hydroxybenzoate hydroxylase, 2,4-dihydroxybenzoate (2,4-DOHB, 100% coupled) and 3,4-dihydroxybenzoate (3,4-DOHB, fully uncoupled); with salicylate hydroxylase, benzoate (fully uncoupled).

orcinol hydroxylase



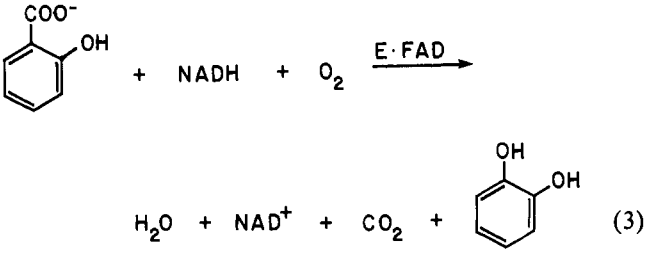
(1)

p-hydroxybenzoate hydroxylase



(2)

salicylate hydroxylase



(3)

The steady-state kinetic mechanism for all three of these flavin monooxygenases (C. C. Ryerson and C. Walsh, unpublished results; Husain & Massey, 1979; White-Stevens et al., 1972; Takemori et al., 1972) appears to be bi-bi ping-pong and is summarized here in shorthand notation (Cleland, 1963) using orcinol hydroxylase as an example (see Chart I).

The order of addition of the first two substrates has been unarguably determined by careful studies of Massey and colleagues in the case of *p*-hydroxybenzoate hydroxylase only, where the mechanism is random ordered (Husain & Massey, 1979; Entsch et al., 1976).

Steady-State Isotope Effects with (4R)-[4-^{2}H]NAD(P)H and (4R)-[4-^{3}H]NAD(P)H. The steady-state kinetic isotope effects on the overall reaction catalyzed by orcinol hydroxylase, *p*-hydroxybenzoate hydroxylase, and salicylate hydroxylase with (4*R*)-[4-^{2}H]- and (4*R*)-[4-^{3}H]NAD(P)H have been determined and are collected in Tables I–III. The ^{1}H rate and ^{2}H rate were monitored spectrophotometrically at 340 nm and are V_{max} isotope effects. The rate of reaction of ^{3}H-labeled molecules was assayed by the appearance of tritium as $^{3}HO^{1}H$; these are, by definition, isotope effects on V_{max}/K_m (Northrop, 1975). The deuterium isotope effects on V_{max}/K_m are the following: for *p*-hydroxybenzoate hydroxylase with substrate *p*-hydroxybenzoate, 3.5 ± 0.5; for orcinol hydroxylase with substrate orcinol, 2.6 ± 0.2; for salicylate hydroxylase with substrate salicylate, 1.83 ± 0.1. These values were determined from the ratio of slopes of reciprocal plots generated by systematic variation of concentrations of [^{1}H]NAD(P)H and then of [^{2}H]NAD(P)H (Cleland, 1977). The observed deuterium

[4] These product stoichiometries are reflected by the ratio of $O_2 \rightarrow H_2O$ (concomitant with hydroxylation) during NADH oxidation compared to $O_2 \rightarrow H_2O_2$ (when no hydroxylation occurs). Other authors have referred to these types of substrates as effectors which uncouple nicotinamide oxidation from substrate oxygenation. If a substrate is 50% coupled, for example, for each mole of O_2 consumed, 0.5 mol of substrate is converted to 0.5 mol of hydroxylated product with 0.5 mol of H_2O produced; in addition, 0.5 mol of H_2O_2 is produced.

Chart I

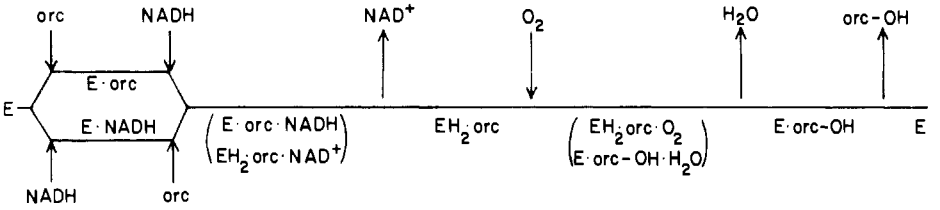


Table I: Steady-State Kinetic Isotope Effects with Orcinol Hydroxylase[a]

	substrate					
	orcinol		resorcinol		*m*-cresol	
cosubstrate	$^{D}(V)$	$^{T}(V/K)$	$^{D}(V)$	$^{T}(V/K)$	$^{D}(V)$	$^{T}(V/K)$
(4*R*)-[4-^{2}H]NADH	2.4 ± 0.25		4.0 ± 0.23		3.4 ± 0.10	
(4*R*)-[4-^{3}H]NADH		10.2 ± 0.32		10.4 ± 1.5		8.85 ± 0.69

[a] Assay mixtures are described under Methods. The deuterium and tritium isotope effect values shown here are an average of at least four determinations.

Table II: Steady-State Kinetic Isotope Effects with *p*-Hydroxybenzoate Hydroxylase[a]

	substrate					
	p-OHB		2,4-DOHB		3,4-DOHB	
cosubstrate	$^{D}(V)$	$^{T}(V/K)$	$^{D}(V)$	$^{T}(V/K)$	$^{D}(V)$	$^{T}(V/K)$
(4*R*)-[4-^{2}H]NADPH	2.58 ± 0.09 2.45 ± 0.47[b] 2.52 ± 0.20[c]		2.47 ± 0.11		5.10 ± 0.20	
(4*R*)-[4-^{3}H]NADPH		13.8 ± 0.3		19.4 ± 2.0		ND[d]

[a] Assays were carried out as described under Methods. The $^{D}(V)$ values are an average of at least four determinations. The $^{T}(V/K)$ values are an average of three determinations. [b] This value for $^{D}(V)$ was determined at 4 °C and pH 8.0; other assay conditions are described under Methods. [c] This value was determined at 4 °C in an assay mixture which contained 50 mM KH_2PO_4 buffer, pH 6.55, 20 mM *p*-OHB, and enzyme. [d] Not determined. The rate of tritium release into H_2O was not sufficiently above background to be measured by the techniques described under Methods.

Table III: Steady-State Kinetic Isotope Effects with Salicylate Hydroxylase[a]

	substrate			
	salicylate		benzoate	
cosubstrate	$^{D}(V)$	$^{T}(V/K)$	$^{D}(V)$	$^{T}(V/K)$
(4*R*)-[4-^{2}H]NADH	1.74 ± 0.08		3.24 ± 0.34	
(4*R*)-[4-^{3}H]NADH		5.1 ± 0.07		11.9 ± 0.53

[a] The $^{D}(V)$ values are each an average of six determinations. The $^{T}(V/K)$ values are each an average of four determinations. Assays were carried out as described under Methods.

isotope effects on V_{max}/K_m are very near the respective values of the V_{max} isotope effects, which span the range ^{D}V = 1.74–5.10. It is noted that V_{max} and V_{max}/K_m are different kinetic parameters, each made up of different groups of rate constants; deuterium isotope effects can be measured on both parameters, whereas tritium isotope effects can only be measured on V/K (Northrop, 1975). It may be only fortuitous that $^{D}V \simeq {}^{D}(V/K)$. The observed tritium isotope effects for these three enzymes are in the range $^{T}(V/K)$ = 5.1–19.4.

The isotope effect data show that the three flavoproteins examined maintain 4*R* chirality in dihydronicotinamide oxidation, in agreement with previous findings for orcinol hydroxylase (Ryerson & Walsh, 1979) and salicylate hydroxylase (You et al., 1977). We can also conclude that reduction of enzyme-bound flavin is at least partially rate limiting on overall turnover in all three cases. It is apparent, however, that flavin reduction is not solely rate determining and some other step(s) (further along the reaction coordinate) is (are) also limiting the overall reaction rate.

Since the observed $^{T}(V/K)$ tritium isotope effects of 10–19 are quite large but not unprecedented, in enzymatic catalysis,

Table IV: Specific Radioactivity of (4*R*)-[4-^{3}H]NADH[a]

reaction time (min)	expt][b] (μCi/μmol)	predicted[c] (μCi/μmol)	calcd error[d]
0	3.57	3.57	0
2.0	5.68	5.22	8.4
2.5	6.62	5.95	10

[a] At the indicated times, the (4*R*)-[4-^{3}H]NADH was recovered from an assay mixture initially containing 3 mL (0.24 mM) of (4*R*)-[4-^{3}H]NADH, 20 μL (50 mM) of orcinol, and 25 μL (7.9 μM) of orcinol hydroxylase. NADH consumption was monitored spectrophotometrically. Details are delineated under Methods. [b] These specific activity values were determined by assay with alcohol dehydrogenase. [c] These numbers were calculated by using $^{T}(V/K)$ = 10.2. [d] These numbers were calculated from the differences between the experimental and predicted values for specific activity.

the results were checked carefully. The tritium discriminations in Tables I–III were obtained by measure of tritium released into water as a function of time. In parallel, since (4*R*)-[4-^{3}H]NAD(P)H molecules experience a 10-fold (or greater)

Table V: Relative Rates in *p*-Hydroxybenzoate Hydroxylase Catalysis and in Orcinol Hydroxylase Catalysis[a]

cosubstrate	overall turnover (V_{max}) (s^{-1})	flavin reduction (s^{-1})	product release (s^{-1})	intrinsic isotope effect
	A. *p*-Hydroxybenzoate Hydroxylase Catalysis[b]			
NADPH	7.68	46–50[c] (13.3)[d]	14.5[c]	
(4*R*)-[4-^{2}H]NADPH	3.2	1.28[d]	14.5	10.4 ± 2[e]
(4*R*)-[4-^{3}H]NADPH	0.56[f]	0.46[g]	14.5	29.1 ± 2.9[h]
	B. Orcinol Hydroxylase Catalysis[i]			
NADPH	14.0	182	≥14.0[j]	
(4*R*)-[4-^{2}H]NADPH	5.8	18.2	≥14.0	10 ± 2[k]
(4*R*)-[4-^{3}H]NADPH	1.5	6.6[l]	≥14.0	27.5 ± 2.9[m]

[a] The data presented in this table clearly demonstrate that upon isotopic substitution in NAD(P)H, the flavin reduction step becomes more rate limiting on overall turnover relative to release of hydroxylated product. In both cases, product release is the slowest step in the reaction sequence with [^{1}H]NAD(P)H. [b] The assay system used here included 50 mM KP_i and 10 mM EDTA, pH 6.6, at 4 °C. Other details are delineated under Methods. This analysis was done only with *p*-OHB as substrate. [c] This value was determined by Husain & Massey (1979) under the same conditions stated above. [d] This is the value calculated from Figure 1a and is low due to the presence of some contaminant in the NADPH preparation (see Methods). [e] This is the value for the intrinsic deuterium isotope effect (k_H/k_D) calculated from Figure 1a. [f] This number is calculated by using $^T(V/K)$ = 13.8 (Table II) and the measured value for V_{max} with [^{1}H]NADPH. [g] This number is calculated by using k_H/k_T = 29.1 and the measured rate constant for flavin reduction with [^{1}H]NADPH. Note that this is the rate calculated for ^{3}H-labeled molecules only. [h] Given k_H/k_D = 10.4 ± 2, this value for k_H/k_T was calculated from the Swain equation $(k_H/k_D)^{1.44} = k_H/k_T$ (Swain et al., 1958). [i] Assay mixtures are as described under Methods. This analysis was done only with orcinol as substrate. [j] The rate constant for product release has not been experimentally determined for this enzyme system. However, it is consistent with the kinetic isotope effect studies presented here that product release is the slowest step in the reaction sequence. Product release can only be as slow as turnover (14.0 s^{-1}) and is most likely somewhat faster. [k] This is the value for k_H/k_D as calculated from Figure 1b. [l] This number is calculated by using k_H/k_T = 27.5 and the measured rate constant for flavin reduction with [^{1}H]NADH. Note that this is the rate calculated for ^{3}H-labeled molecules only. [m] This is the value for k_H/k_T as calculated by using k_H/k_D = 10 ± 2 in the Swain equation $(k_H/k_D)^{1.44} = k_H/k_T$ (Swain et al., 1958).

kinetic discrimination, the specific radioactivity of the residual NAD(P)H molecules in the population should rise predictably during the course of the oxidation reaction. To test this, orcinol hydroxylase incubations were allowed to proceed for 2 (34% conversion) and 2.5 min (42% conversion), and the (4*R*)-[4-^{3}H]NADH was reisolated by DEAE-cellulose (DE-52) column chromatography; its specific radioactivity was redetermined by assay with alcohol dehydrogenase. The results are shown in Table IV along with the values of specific radioactivities calculated for a proposed $^T(V/K)$ = 10.2, as observed in the ^{3}HO^{1}H experiments with orcinol hydroxylase (Table I). There is reasonable agreement and clear indication that the specific radioactivity of the remaining (4*R*)-[4-^{3}H]NADH molecules is rising as a consequence of isotopic discrimination during enzyme oxidation. Verification that all the tritium present in the [^{3}H]NADH samples was in the 4*R* locus was obtained by quantitative transfer of tritium to propionaldehyde on incubation with yeast alcohol dehydrogenase (4*R* specific). As a further control, there was no loss of tritium from (4*R*)-[4-^{3}H]NADH in incubations in the absence of enzyme.

Pre-Steady-State Isotope Effects with (4R)-[4-^{2}H]NAD(P)H. As described under Discussion, one can calculate a deuterium isotope effect on a single catalytic step in a reaction sequence such as flavin reduction (Northrop, 1975) by using experimentally determined $^D(V/K)$ and $^T(V/K)$. The observed deuterium isotope effect on overall rate (^DV = 2.6 or $^D(V/K)$ = 3.5) is much lower than the calculated value (^Dk = 24) for k_H/k_D and indicates that the intrinsic isotope effect is masked by some slower step which occurs later in the reaction sequence.

In an effort to determine the exact magnitude of the intrinsic deuterium isotope effect during flavin reduction by dihydronicotinamide, anaerobic single turnover experiments were performed with substrate quantities of either orcinol hydroxylase or *p*-hydroxybenzoate hydroxylase. These assays could not be carried out with salicylate hydroxylase due to the short supply of enzyme. Figure 1 gives graphical representations of the pre-steady-state kinetic data. The kinetic constants for flavin reduction and overall turnover (using rate constants for product release obtained by Husain & Massey (1979) for *p*-hydroxybenzoate hydroxylase) are compared in Table V.

The dissociation constant (Figure 1a) calculated for the binding of both [^{1}H]- and [^{2}H]NADPH to *p*-hydroxybenzoate hydroxylase is K_D = 0.17 mM, which is in good agreement with the value for [^{1}H]NADPH determined from steady-state assays, K_D = 0.12 mM (Husain & Massey, 1979). The rate constant for flavin reduction (reciprocal of the intercept on the *y* axis) with (4*R*)-[4-^{1}H]NADPH is k_{red} = 13.3 s^{-1} and with (4*R*)-[4-^{2}H]NADPH is k_{red} = 1.28 s^{-1}, directly establishing an intrinsic isotope effect of k_H/k_D = 10.4 ± 2.

The pre-steady-state kinetic results seen with orcinol hydroxylase (Figure 1b, Table VB) also reveal a very large primary deuterium isotope effect for E–FAD reduction by [^{2}H]NADH. The rate constant for enzyme–FAD reduction with (4*R*)-[4-^{1}H]NADH is k_{red} = 182 s^{-1} and with (4*R*)-[4-^{2}H]NADH is k_{red} = 18.2 s^{-1}, establishing an intrinsic deuterium isotope effect on the flavin reduction step of k_H/k_D = 10 ± 2. The dissociation constant for both [^{1}H]- and [^{2}H]-NADH is K_D = 0.5 mM, slightly larger than the K_D value (0.17 mM) calculated in steady-state kinetic assays (data not shown).

Discussion

An intrinsic deuterium isotope effect in an enzymatic reaction can be estimated by the method of Northrop (1975), eq 4, which depends on the fact that the intrinsic tritium

$$\frac{^D(V/K)}{^T(V/K)} = \frac{^Dk - 1}{(^Dk)^{1.44} - 1} \quad (4)$$

isotope effects are the 1.44 power of the deuterium ones (Swain et al., 1958). However, the values that were measured for $^D(V/K)$ and $^T(V/K)$ (Tables I–III) do not accurately predict the value for ^Dk, which was determined in pre-steady-state experiments[5] (Figure 1). This experimental difference implies for the enzymatic catalysis examined herein that $^D(V/K)$ and

[5] For example, if the values measured for $^D(V/K)$, 3.5, and $^T(V/K)$, 13.8, in *p*-hydroxybenzoate hydroxylase catalysis are used in eq 4, a value of ^Dk = 24 is predicted.

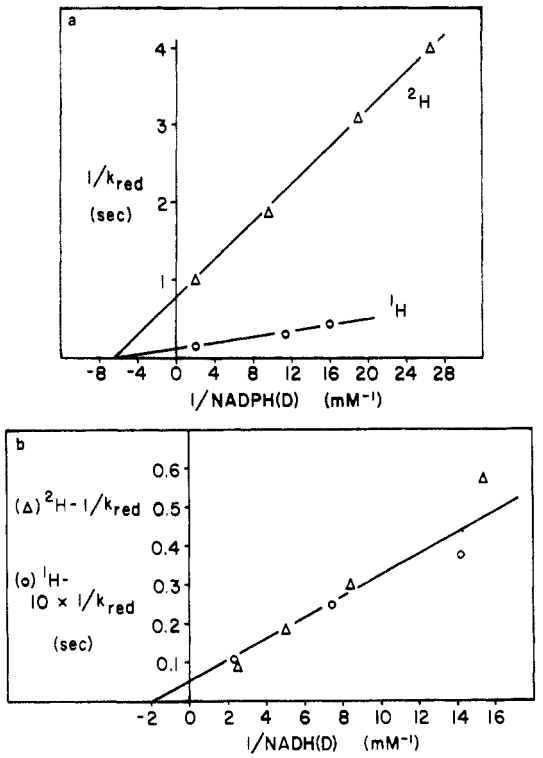


FIGURE 1: (a) Anaerobic reduction of *p*-hydroxybenzoate hydroxylase. Anaerobic reduction of *p*-hydroxybenzoate hydroxylase by NADPH and (4*R*)-[4-^{2}H]NADPH in the presence of *p*-OHB. Assay conditions are described under Methods. From the above data, $k_H/k_D = 10.4 \pm 2$. The dissociation constant for both [^{1}H]- and [^{2}H]NADPH is $K_D = 0.17$ mM. (b) Anaerobic reduction of orcinol hydroxylase. Assay conditions are described under Methods. Reduction by NADH is indicated by (O) and (4*R*)-[^{2}H]NADH by (Δ). We took the liberty of drawing the line shown for the following reasons: (a) the dissociation constant for both nicotinamides is expected to be the same; i.e., a simple ^{1}H to ^{2}H exchange should not change NADH binding; (b) the largest error is expected to be experienced at low concentrations of NADH. Readers are referred to Massey & Flashner (1974) for the original derivation for the treatment of data in this manner. From the above data $k_H/k_D = 10 \pm 2$. The dissociation constant for both nicotinamides is $K_D = 0.5$ mM.

Table VI: Range of Values for Kinetic Parameters[a]

	p-OHBH	OH
$a = k_3/k_2$	$0 \rightarrow 1.195$	$0 \rightarrow 1.88$
$b = k_4/k_5$	$0 \rightarrow 1.195$	$0 \rightarrow 1.88$
$c = k_6/k_7$	$1.31 \rightarrow$ any no.	$2.32 \rightarrow$ any no.
d (see eq 7)	$2.5 \rightarrow 3.7$	$4.19 \rightarrow 6.07$

[a] These values were calculated from eq 8-10 by first allowing a to become very small and approach zero and then solving for b, c, and d. Values for the following expressions (which are merely convenient forms which develop from the algebra) were then determined: $a + b$, $d + b$, bc, and $d - a$. By use of these expressions, b is allowed to become small and approach zero, thus giving values for a, c, and d. A definite range can be established for a, b, and d, but c can become any value while bc remains constant.

$^T(V/K)$ do not involve identical steps in the reaction sequence [see Cleland (1981) for further discussion].

One can also point out that the observed deuterium isotope effect, $^D(V/K)$, is considerably less than the experimentally determined intrinsic deuterium isotope effect on enzyme–flavin reduction, ^Dk (Table V), indicating that flavin reduction is not the sole rate-limiting step on overall catalysis with any of the three enzymes. Actually, in the case of *p*-hydroxybenzoate hydroxylase [where rate constants for most of the catalytic steps have been delineated (Husain & Massey, 1979)], upon isotopic substitution in the reduced pyridine nucleotide, the flavin reduction step becomes slower than that of product release (the slowest step in turnover with [^{1}H]NADPH) (Table V).

The failure of the deuterium and tritium isotope effects on V/K to obey eq 4 requires the following model, which is slightly more complicated than has been previously presented[6] (Schopfer & Massey, 1979; Husain & Massey, 1979):

$$\underset{A}{E\cdot FAD\text{-}S} + NAD(P)H \overset{K_{D,NAD(P)H}}{\rightleftharpoons} \underset{B}{E\cdot FAD\text{-}S\text{-}NAD(P)H} \overset{k_1}{\underset{k_2}{\rightleftharpoons}}$$

$$\underset{C}{(E\cdot FAD\text{-}S\text{-}NAD(P)H)^*} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \underset{D}{(E\cdot FADH_2\text{-}S\text{-}NAD(P))^*} \overset{k_5}{\underset{k_6}{\rightleftharpoons}}$$

$$\underset{E}{E\cdot FADH_2\text{-}S\text{-}NAD(P)} \overset{k_7}{\underset{NAD(P)}{\rightleftharpoons}} \underset{F}{E\cdot FADH_2\text{-}S} \overset{k_9}{\underset{O_2}{\rightarrow}} E\cdot FAD + S\text{-}OH + H_2O$$

The reaction intermediates (C and D) involved in the actual electron transfer at k_3 may be charge-transfer complexes between enzyme-bound flavin and nicotinamide. Evidence has been forwarded both in enzymatic (Schopfer & Massey, 1979; Howell et al., 1972) and in nonenzymatic chemical model systems (Bruice et al., 1971; Brustlein & Bruice, 1972; Porter et al., 1973) for the involvement of such a species prior to electron transfer; this species has been characterized spectrally as a charge-transfer complex between reduced pyridine nucleotide and oxidized flavin. The involvement of a charge-transfer complex between reduced flavin and oxidized pyridine nucleotide (such as complex D) generated subsequent to electron transfer (k_3) has been demonstrated in the catalytic sequence of other dihydronicotinamide-oxidizing flavoenzymes including lipoamide dehydrogenase (Matthews et al., 1979).

The steady-state results presented in Tables I–III indicate that the rate of flavin reduction (k_3) is sensitive to isotopic substitution in NAD(P)H. Keeping in mind that we are using 100% deuterated NADPH, in the above model the first irreversible step for the chemical reaction is release of NAD(P) (k_7) (these are initial rates and [NADP] ~ 0), and thus when comparing overall rates with deuterated and unlabeled NAD(P)H

$$V/K = \frac{k_1k_3E_t/k_2}{K_{D,NAD(P)H}(1 + k_3/k_2 + (k_4/k_5)(1 + k_6/k_7))} \quad (5)$$

However, the overall tritium isotope effect was measured by monitoring labilization of tritium into water. If hydrogen can exchange rapidly with solvent in (E·FADH$_2$–S–NAD(P))* (complex D), constituting an irreversible step for tritium release to water, then for an internal competition experiment with tritiated NAD(P)H, the equation for V/K does not involve the constants k_6 and k_7:

$$V/K = \frac{k_1k_3E_t/k_2}{K_{D,NAD(P)H}(1 + k_3/k_2 + k_4/k_5)} \quad (6)$$

Since the rate equations for $^D(V/K)$ and $^T(V/K)$ are not the same, one would not expect eq 4 to apply. The reaction between E·FADH$_2$–S and O$_2$ can be represented by a net rate constant, k_9, since no step in that portion of the reaction sequence is solely rate limiting (as is suggested by the lack of a deuterium isotope effect with deuterated aromatic substrates). For this model the value of V is

$$V = \frac{k_1k_3E_t/(k_1 + k_2)}{1 + d + (k_4/k_5)(1 + k_6/k_7)} \quad (7)$$

[6] The mechanistic models which have been reported for the bacterial flavoprotein monooxygenases (e.g., Schopfer & Massey, 1979; Husain & Massey, 1979) have not explicitly utilized the involvement of steps indicated by k_1 and k_5.

where

$$d = \left[\frac{k_1 k_3}{k_1 + k_2}\right]\left[\frac{1}{k_1} + \frac{1}{k_5}\left(1 + \frac{k_6}{k_7}\right) + \frac{1}{k_7} + \frac{1}{k_9}\right]$$

In the pre-steady-state studies we are directly observing flavin reduction, k_3. If we neglect any equilibrium isotope effects on the various steps in the reductive half-reaction [the overall equilibrium isotope effect for transfer of deuterium from NADH to water is only 0.98 (Cleland, 1980) and can be neglected, while the fractionation factor of the presumably protonated flavin in (E·$FADH_2$–S–NAD(P))* and E·$FADH_2$–S–NAD(P) is unknown], the isotope effects seen in the present study are given by

$$^{D}(V/K) = \frac{k_{3H}/k_{3D} + a + b(1 + c)}{1 + a + b(1 + c)} \quad (8)$$

$$^{T}(V/K) = \frac{k_{3H}/k_{3T} + a + b}{1 + a + b} \quad (9)$$

$$^{D}V = \frac{k_{3H}/k_{3D} + d + b(1 + c)}{1 + d + b(1 + c)} \quad (10)$$

where $(V_H/V_D)_{\text{pre-steady-state}} = k_{3H}/k_{3D}$, $a = k_3/k_2$, $b = k_4/k_5$, and $c = k_6/k_7$; for d see eq 7. The ranges calculated for a–d for both *p*-hydroxybenzoate hydroxylase and orcinol hydroxylase are presented in Table VI. The parameters a, b, and c represent partition ratios for reaction intermediates C, D, and E, respectively. The determination of all of the specific rate constants for k_2–k_7 would allow calculation of the commitment factors for this portion of the catalytic system (Cleland, 1981; Schimerlik et al., 1977). It is likely that the slowest step in the sequence is k_7 (Husain & Massey, 1979). This coupled with the observation that $k_6/k_7 > 1$ under all circumstances (Table VI) would predict no appreciable forward commitment for the system through k_7.

In summary, this discussion presents a model which involves two isomerization steps (postulated to be formation of charge-transfer complexes). The first charge-transfer complex has been previously reported by Schopfer & Massey (1979) in melilotate hydroxylase (another bacterial flavoprotein monooxygenase) catalysis and by Howell et al. (1972) in *p*-hydroxybenzoate hydroxylase catalysis. The failure of the kinetic isotope effects measured on the flavin reduction reaction to comply with eq 4 necessitates the second isomerization step. In addition, Schopfer & Massey (1979) reported a lack of correspondence between a computer simulation and experimental observation in their mechanism for melilotate hydroxylase and suggested that an additional intermediate might be required. A second isomerization step as in the model above might solve their problem.

The electron-transfer step or flavin reduction step (k_3) is not the sole rate-determining step on turnover, however, since the deuterium isotope effect (k_H/k_D) on that step is masked in the overall deuterium isotope effect $^{D}(V/K)$ (see Table V). Partial, but not complete, suppression of intrinsic k_H/k_D values of 10 down to ^{D}V of 2–4 in these monooxygenases conforms to the idea (Albery & Knowles, 1977) that these enzymes balance transition state heights so that no single step is severely rate limiting. The practical consequence is that one must obtain a k_H/k_D on the elementary step itself, i.e., the intrinsic isotope effect, in addition to the observed expression of that isotope effect on overall turnover (in V_{max} or V_{max}/K_m) if one is to use the results for direct mechanistic interpretation about that step in the catalytic sequence.

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References

Albery, W. J., & Knowles, J. R. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 285.
Arnold, L. J., & You, K. (1978) *Methods Enzymol. 54*, 223.
Beaty, N. B., & Ballou, D. P. (1981) *J. Biol. Chem. 256*, 4611, 4619.
Bruice, T. C., Main, L., Smith, S., & Bruice, P. Y. (1971) *J. Am. Chem. Soc. 93*, 7327.
Brustlein, M., & Bruice, T. (1972) *J. Am. Chem. Soc. 94*, 6548.
Cleland, W. (1963) *Biochim. Biophys. Acta 67*, 104.
Cleland, W. (1977) in *Isotope Effects on Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) p 153, University Park Press, Baltimore, MD.
Cleland, W. (1980) *Methods Enzymol. 64*, 104.
Cleland, W. (1981) *Methods Enzymol.* (in press).
Cook, P., & Cleland, W. (1981) *Biochemistry 20*, 1797, 1805.
Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251*, 2550.
Howell, L. G., Spector, T., & Massey, V. (1972) *J. Biol. Chem. 247*, 4340.
Husain, M., & Massey, V. (1979) *J. Biol. Chem. 254*, 6657.
Husain, M., Schopfer, L. M., & Massey, V. (1978) *Methods Enzymol. 53D*, 543.
Light, D., & Walsh, C. (1980) *J. Biol. Chem. 255*, 4264.
Lowenstein, J. M. (1963) *Methods Enzymol. 6*, 871.
Massey, V., & Flashner, M. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) p 245, Academic Press, New York.
Massey, V., & Hemmerich, P. (1975) *Enzymes, 3rd Ed. 12*, 191.
Matthews, R. G., Ballou, D. P., & Williams, C. H., Jr. (1979) *J. Biol. Chem. 254*, 4974.
Northrop, D. B. (1975) *Biochemistry 14*, 2644.
Ohta, Y., Higgins, D. J., & Ribbons, D. W. (1975) *J. Biol. Chem. 250*, 3814.
Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200.
Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1978) *Biochemistry 17*, 2613.
Porter, D., Blakenhorn, G., & Ingraham, L. (1973) *Biochem. Biophys. Res. Commun. 52*, 447.
Ryerson, C. C., & Walsh, C. (1979) *J. Biol. Chem. 254*, 4349.
Schimerlik, M., Grimshaw, C., & Cleland, W. (1977) *Biochemistry 16*, 571.
Schopfer, L., & Massey, V. (1979) *J. Biol. Chem. 254*, 10634.
Shore, A. J., & Gutfreund, H. (1970) *Biochemistry 9*, 4655.
Swain, C. G., Stiver, E. C., Reuwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885.
Takemori, S., Nakamura, M., Suzuki, K., Katagiri, M., & Nakamura, T. (1972) *Biochim. Biophys. Acta 284*, 382.
Walsh, C. (1980) *Acc. Chem. Res. 13*, 148.
White-Stevens, R. H., Kamin, H., & Gibson, Q. H. (1972) *J. Biol. Chem. 247*, 2371.
You, K., Arnold, L. J., & Kaplan, N. O. (1977) *Arch. Biochem. Biophys. 180*, 550.