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Steady-State and Stopped-Flow Kinetic Measurements of the Primary Deuterium Isotope Effect in the Reaction Catalyzed by *p*-Cresol Methylhydroxylase†

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ABSTRACT: Steady-state kinetic studies for the reaction of the flavocytochrome *p*-cresol methylhydroxylase with the reducing substrates (S) *p*-cresol, 4-ethylphenol, and their corresponding α-deuteriated analogues are presented. The results from these experiments and those from studies involving various reoxidizing substrates support the proposed apparent ping-pong mechanism. With phenazine methosulfate (PMS) as the reoxidant for studies at pH 7.6 and 6 or 25 °C, the isotope effects on k_{cat} are lower than the intrinsic isotope effect. The values for $^{D}(k_{cat}/K_S)$ are equal to the intrinsic effect for *p*-cresol at 25 °C and for 4-ethylphenol at both 6 and 25 °C. However, the value for this steady-state parameter at 6 °C for *p*-cresol is lower than the intrinsic effect. The values for $^{D}(k_{cat}/K_{PMS})$ are nearly equal to 1.0 under all conditions. In contrast, the steady-state kinetic analysis for the isolated flavoprotein subunit of *p*-cresol methylhydroxylase involving *p*-cresol and PMS as substrates indicates that a random-binding mechanism is operating. Additionally, several of the steady-state parameters yield values for the apparent intrinsic isotope effect for the flavoprotein. The results of stopped-flow kinetic studies are also reported. At pH 7.6 the intrinsic isotope effect ($^{D}k_2$) for the reduction of the enzyme by 4-ethylphenol is 4.8–5.0 at 25 °C and 4.0 at 6 °C. This technique yields a value for $^{D}k_2$ of 7.05 at 6 °C and pH 7.6 for *p*-cresol. The combined results from the stopped-flow and steady-state kinetic experiments at pH 7.6 and 6 °C for *p*-cresol also allow the calculation of several important kinetic parameters for this enzyme. These calculations are viewed with caution, since some discrepancies develop when a comparison of the data from the two kinetic methods is made for both *p*-cresol and 4-ethylphenol. Surprisingly, the stopped-flow method could not be used to measure an isotope effect for the isolated flavoprotein subunit.

Over the past few years a variety of properties of the flavocytochrome *p*-cresol methylhydroxylase from *Pseudomonas putida* have been investigated. The three-dimensional structure of this tetrameric enzyme, which is composed of two identical flavoprotein and two identical *c*-type cytochrome subunits, is known at 6-Å resolution (Shamala et al., 1986). Additionally, the primary structure of the cytochrome subunit is available (McIntire et al., 1986), and studies are under way to determine the amino acid sequence of the flavoprotein from the corresponding DNA sequence. It is also known that FAD is covalently bound to the flavoprotein through a tyrosyl ether

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linkage, and the amino acid sequence at this site has been determined (McIntire et al., 1981).

The subunits of this enzyme are easily separated by isoelectric focusing (McIntire & Singer, 1982), with the pure flavoprotein subunit retaining only a few percent of the maximal velocity as compared to the flavocytochrome and acting via a different steady-state mechanism (McIntire et al., 1985). On reconstitution of the flavocytochrome from the subunits all the kinetic and physical properties measured are recovered (Koerber et al., 1985a,b).

Flash photolysis and continuous irradiation of the flavocytochrome in the presence of deazariboflavin indicate that the reduction of both the heme and flavin by the deazaflavin radical is diffusion controlled and that intraenzymic electron transfer occurs. Similar experiments involving the isolated cytochrome and flavoprotein subunits also established a diffusion-controlled reduction of these proteins by the deazaflavin radical and that the neutral flavin radical, which is initially formed on the flavoprotein, eventually converts to the anionic radical (Bhattacharyya et al., 1985; McIntire et al., 1985, 1986). Finally, studies involving the stereochemical course for the oxidation of 4-ethylphenol have been initiated (McIntire et al., 1984).

A previous paper (McIntire et al., 1985) presented preliminary steady-state kinetic information for the flavocytochrome, including ionic strength and pH effects and reactivity with natural and artificial electron acceptors and phenolic substrates. Available kinetic data were compatible with either a ping-pong or an ordered binding mechanism. In contrast, the flavoprotein displayed converging lines in double-reciprocal plots, indicative of either an ordered binding or random mechanism.

This paper extends these studies with the aim of further delineating the mechanisms for the flavocytochrome and the separated flavoprotein. The studies reported involve the use of various electron acceptors and the reducing substrates *p*-cresol, 4-ethylphenol, and their corresponding α-deuteriated analogues. Measurement of the primary deuterium isotope effect on various steady-state parameters is also included. The magnitude of $^{D}(k_{cat}/K_S)$, where S was either *p*-cresol or 4-ethylphenol, suggests that the intrinsic isotope effect for these substrates was being measured. To confirm that the intrinsic effect was in fact obtained by the steady-state analysis, stopped-flow kinetic measurements were conducted. Comparison of the steady-state and stopped-flow data also allowed the determination of the steps contributing to the rate-limiting properties of the enzyme.

Not only could rate constants be determined, but direct spectral observation of intermediates in the reaction were made by the stopped-flow method. This has allowed visualization of the reduction of both the heme and flavin prosthetic groups of *p*-cresol methylhydroxylase. During the course of this investigation the rapid formation of the anionic flavin radical was detected, and interestingly, there was also spectral evidence for an unidentified flavin radical formed prior to the appearance of the anionic radical when *p*-cresol-α,α,α-d_3 reacted with the enzyme.

A detailed steady-state kinetic analysis for the reaction of *p*-cresol and PMS with the pure flavoprotein subunit is also presented, and it is concluded that a random-ordered binding mechanism is obeyed. The value of the isotope effect on several of the steady-state parameters is equal to that of the apparent intrinsic isotope effect. Stopped-flow studies have also been carried out with the pure flavoprotein subunit to permit comparison with the data obtained from steady-state kinetic experiments. Curiously, no dependence was noted for the rate of reduction on the concentration or isotopic substitution, and the reaction was biphasic.

Experimental Procedures

Materials and Methods. *p*-Cresol methylhydroxylase (the A form from *Pseudomonas putida* NCIB 9869) was purified as described earlier (Keat & Hopper, 1978; Koerber et al., 1985a). *p*-Cresol (gold label), 4-ethylphenol, $LiAlH_4$, and deuterium chloride (37% solution in D_2O, 99 atom % D, gold label) were from Aldrich Chemical Co. 4-Hydroxybenzaldehyde, ethyl alcohol-*O*-d_1, (99.5+ atom % D), phenazine methosulfate (PMS),[1] phenazine ethosulfate (PES), *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, and horse heart cytochrome *c* (type VI) were purchased from Sigma Chemical Co. 2,6-Dichlorophenolindophenol (DCIP) was supplied by General Biochemicals, Inc. *p*-Anisate methyl ester was obtained from Eastman Kodak Co. Deuteriated water (99.8 atom % D) was from Stohler Isotope Chemicals, $LiAlD_4$ (99 atom % D) was a product of KOR Isotopes, and $CDCl_3$ (99.5 atom % D) and tetramethylsilane were purchased from Merck Sharp & Dohme, Canada, Ltd. Phenol reagent was from Fisher Scientific Co.

Thin-layer silica gel plates with a fluorescent indicator (HETLC-GHFL) were from Analtech, Inc. Silica gel for "flash chromatography" (40-μm particle size) was a product of J. T. Baker Chemical Co., and the flash chromatography column was obtained from Ace Glass Co. The Mono P column for "chromatofocusing" was purchased from Pharmacia Fine Chemicals, Inc.

Many of the chemicals were further purified by the procedures listed under the particular compounds, and the purity was determined by one or more of the following methods. (1) ^{1}H NMR spectra were taken on a Varian EM-360 or a Varian FT-80A spectrometer (all chemical shifts are reported in ppm relative to tetramethylsilane). (2) Mass spectra were obtained at the University of California, San Francisco. (3) High-pressure liquid chromatography (HPLC) runs were carried out on a Beckman Ultrasphere-ODS (octadecylsilane-derivatized silica gel) column, 4.6 × 250 mm, 5-μm particle size, and with monitoring of the effluent at 254 nm. The retention times *R* are reported in minutes, with a flow rate of 1 mL/min, with acetonitrile/H_2O, 1:1 (v/v), as the solvent. (4) Thin-layer chromatograms (TLC) were run on silica gel plates with a fluorescent indicator. The compounds were detected either by fluorescence quenching or by spraying with the phenol reagent/H_2O, 1:1 (v/v), followed by a spray of a saturated Na_2CO_3 solution. The solvents systems were (1) hexane/benzene, 4:1 (v/v), and (2) $CHCl_3$/methanol, 20:1 (v/v). All melting points and boiling points reported are uncorrected.

p-Cresol. *p*-Cresol showed the following: NMR ($CDCl_3$) δ 2.22 (s, 2.9, CH_3), 6.16 (s, 1.0, OH), 6.68 and 6.97 (2 d, 4.0, Ar-H); mass spectrum, *m*/*e* (relative intensity) 108 (M^+, 96), 107 (100), 91 (7.1), 90 (8.7), 79 (15.7), 78 (6.3), 77 (27.4); HPLC, *R* = 5.22 min (99.7%).

4-Ethylphenol. The brown crystals were twice recrystallized from petroleum ether and then sublimed at 38–40 °C (0.01–0.015 mmHg), mp 42–44 °C. The compound was pure by HPLC, *R* = 7.22 min (99.7%). Spectral data: NMR (CCl_4) δ 1.20 (t, 3.0, CH_3), 2.56 (q, 2.0, CH_2), 6.88 and 6.95 (s and 2 d, 5.0, OH and Ar-H); mass spectrum, *m*/*e* (relative

[1] Abbreviations: PMS, phenazine methosulfate; PES, phenazine ethosulfate; DCIP, 2,6-dichlorophenolindophenol; ESR, electron spin resonance; FAD, flavin adenine dinucleotide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

intensity) 122 (M^+, 40), 107 (100), 77 (20).

Synthesis of p-Cresol-α,α,α-d_3. 4-Methoxybenzyl chloride-α,α-d_2 was synthesized from *p*-anisate methyl ester by the methods of Gates et al. (1974) and Baldwin et al. (1975), with $LiAlD_4$ and thionyl chloride. The 4-methoxybenzyl alcohol-α,α-d_2 was purified by flash chromatography (Still et al., 1978) and the 4-methoxybenzyl chloride-α,α-d_2 by distillation (bp 95–97 °C at 0.8–1.0 mmHg). ^1H NMR chemical shift values for these compounds were identical with the literature values (Gates et al., 1974). Eight grams of the chloride (0.0504 mol) was added dropwise over a 1-h period to a mixture of 100 mL of dry tetrahydrofuran and 3.40 g of $LiAlD_4$ (0.081 mol) while refluxing under Argon. After an additional 3.5 h of refluxing, TLC (solvent system 1) indicated that the reaction was complete.

The mixture was allowed to cool, and 5 g of $Na_3PO_4 \cdot 12H_2O$ (0.013 mol) was slowly added over a 45-min period. The thick gray slurry was dried in vacuo, 300 mL of water was added, and the mixture was extracted 3 times with 150–200 mL of $CHCl_3$. The $CHCl_3$ solution was dried over anhydrous $MgSO_4$ and the solvent removed by rotary evaporation. The yield was 6.0 g (93%) of 4-methylanisole-α,α,α-d_3 as a light yellow liquid. Six grams of this compound (0.0468 mol) and 36 mL of 49% HBr were vigorously refluxed, and after 4 h an additional 5 mL of HBr was added and refluxing was continued for 1 h. TLC (solvent system 1) indicated that little starting material was present. The mixture was poured into 150 mL of ice and neutralized with KOH. This solution was extracted 3 times with 150 mL of $CHCl_3$. The organic phases were combined and dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The yield was 4.70 g of a crude yellow liquid.

The material was distilled once at 2.2 mmHg, bp 75–77 °C, and again at 1.1 mmHg, bp 66 °C. The product was obtained as a light yellow liquid. The final step involved a simultaneous distillation–sublimation. The compound was put into a microdistillation apparatus and the distilling flask totally submersed in an oil bath at 35–40 °C. A vacuum of 0.02–0.03 mmHg was applied. A yellow liquid was collected in the receiving flask and a colorless solid crystallized throughout the inner surface of the distillation head. The liquid was identified as the starting material, and the solid was the deuteriated *p*-cresol: yield 3.25 g (0.0292 mol, 37% from starting material); NMR ($CDCl_3$) δ 6.27 (s, 1.1, OH), 7.08 (2 d, 4.0, Ar-H), 2.23 (s, D_2CH); estimated content of hydrogen in methyl group, 0.6%; mass spectrum, *m/e* (relative intensity) 111 (M^+, 100), 110 (57), 109 (47.5), 94 (3.8), 93 (6.2), 92 (4.9), 83 (5.9), 82 (12.4), 81 (6.6), 80 (5.9); HPLC, R = 5.33 min (99.4%); *p*-cresol + *p*-cresol-α,α,α-d_3, R = 5.33 min.

Synthesis of 4-Ethylphenol-α,α-d_2. This procedure is a modification of a literature method (Read & Wood, 1955). 4-Hydroxyacetophenone (5 g, 0.037 mol) was dissolved in 3.20 mL of warm ethyl alcohol-*O*-d_1 (0.054 mol), and 3.0 mL of D_2O (0.166 mol) was added. The liquid was removed in vacuo and the procedure repeated twice to affect a calculated 99.7% exchange of the phenolic proton. Zn(Hg) amalgam was made by the method of Caesar (1963), washed in a filter funnel with H_2O, ethanol, and finally ether, and dried in a desiccator under vacuum for 2 h.

4-Hydroxyacetophenone-*O*-d_1 was added to a flask containing 20 mL of ethyl alcohol-*O*-d_1 and 20 g of Zn(Hg). DCl (14 mL, 35% in D_2O) was added dropwise over a 6-h period. The mixture was stirred vigorously, and the flask was vented with a drying tube containing $CaCl_2$. TLC (solvent system 2) indicated that all the acetophenone had been reduced. The liquid was decanted from the inorganic material, which was then washed with three changes of 1–2 mL of ethyl alcohol-*O*-d_1, 4 times with 20 mL of dry benzene, and finally with four changes of 150 mL of dry benzene. The combined liquid fractions were washed twice with 150 mL of 5% Na_2CO_3. These washes were reextracted with 50 mL of benzene. The organic phases were combined and dried with anhydrous $MgSO_4$, and the solvent was removed in vacuo, leaving a dark yellow liquid (5.05 g).

"Flash chromatography" on silica gel (Still et al., 1979), with $CHCl_3$/methanol (40:1 v/v) as eluant, followed by the simultaneous distillation–sublimation described in the purification of *p*-cresol-α,α,α-d_3 yielded 3.24 g of a colorless solid: yield 71%; NMR ($CDCl_3$) δ 1.20 (s, 2.9, CH_3), 6.45 (s, 1.0, OH), 7.13 (2 d, 4.0, Ar-H), ~2 (several small peaks, –DCH–); estimated α-^1H content 1–2%; mass spectrum, *m/e* (relative intensity) 124 (M^+, 40), 109 (100), 79 (10), 78 (10); HPLC, R = 7.19 min (99.3%).

Isolation of the Pure Flavoprotein Subunits. The flavoprotein subunit of *p*-cresol methylhydroxylase was separated from the cytochrome subunit by the isoelectric focusing methods previously reported (McIntire & Singer, 1982; Koerber et al., 1985a) or by HPLC chromatofocusing on a Mono P column. The starting buffer for the chromatofocusing was 20 mM histidine, adjusted to pH 6.2 with acetic acid, and the elutant was a 1:30 dilution of the buffer mixture used for isoelectric focusing experiments (Koerber et al., 1985a). (The detailed procedures for application of chromatofocusing and the Mono P column are described in technical bulletins available from Pharmacia Fine Chemicals, Inc.) Since the cytochrome component of the enzyme has a tendency to autoreduce, both buffers contained 10 μM $K_3Fe(CN)_6$ to ensure optimal separation of the subunits.

Steady-State Kinetics. Both PMS and PES were used as reoxidizing substrate for *p*-cresol methylhydroxylase, and the reduction of these dyes was monitored by coupling to DCIP. The reduction of DCIP was followed at 600 nm (ϵ = 21 700 M^{-1} cm^{-1}). Wurster's blue, the radical of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, made by the method of Michaelis and Granick (1943), and horse heart cytochrome *c* were also used as electron acceptors. The reduction of Wurster's blue was followed at 564 nm ($\Delta\epsilon$ = 11 400 M^{-1} cm^{-1}) and that of cytochrome *c* at 590 nm ($\Delta\epsilon_{ox-red}$ = 1900 M^{-1} cm^{-1}). Assays were performed in 50 mM Tris-HCl, I = 0.05 (with NaCl), or 25 mM sodium phosphate, I = 0.075 (with NaCl), at pH 7.6 and 25 or 6 °C.

Stopped-Flow Analysis. The experiments involving the flavocytochrome were conducted with an Aminco-Morrow stopped-flow apparatus (dead time ~ 3 ms), equipped with an Aminco linear log photometer and interfaced to a Nova 2/4 microcomputer (On Line Instruments Systems, Athens, GA). For the flavoprotein, the reaction was monitored with a Hewlett-Packard 8451A diode array spectrophotometer equipped with a portable stopped-flow accessory (dead time ~ 90 ms; Hi-Tech Scientific Ltd., Salisbury, England). Spectra were taken between 320 and 470 nm, every 200 ms after mixing, for 10 s. The scan time was 25 ms per spectrum. When the slowest reaction was studied, the range was from 250 to 600 nm, the scan time was 60 ms, and spectra were taken every 5 s for 300 s.

The buffers used for the experiments were Tris-HCl, 0.05 M, I = 50 (with NaCl), or 75 mM, I = 0.075 (with NaCl), at pH 7.6 and 25 °C or sodium phosphate, 25 mM, I = 0.075 (with NaCl), at pH 7.6 or 6.5 and 6 °C. The substrate so-

lutions were diluted from 50 mM stock solutions to the desired concentrations in these buffers. The enzyme solutions were made by dissolving the lyophilized protein in the appropriate buffer. The concentration of these solutions was determined by the absorbance at 412 nm for the fully oxidized enzyme (ϵ = 143 mM^{-1} cm^{-1}). The enzyme and substrate solutions were put into separate syringes and were allowed to equilibrate for 10 min. Since the reoxidation of the reduced proteins in air-saturated buffers was slow (McIntire et al., 1981), no attempt was made to exclude oxygen from the solutions.

Nomenclature. Steady-state parameters are defined as follows: initial rates, v; catalytic constant, k_{cat}; Michaelis constant for PMS, K_{PMS}, and the phenolic substrate, K_S; inhibition constant, K_i. The units for k_{cat} and v are expressed in s^{-1} and for k_{cat}/K values in M^{-1} s^{-1}.

For the isotope effects the nomenclature of Northrop (1977) is used. For example, the deuterium isotope effect on the maximal velocity is expressed as $^{D}k_{cat}$ and is equal to the value of k_{cat} for the hydrogenated substrate divided by k_{cat} for the deuteriated substrate.

Data Processing. The data were fit by computer to the nonlinear equations, by a "log transformed weighted linear jackknife" algorithm (Oppenheimer et al., 1981), modified as described in a previous paper (McIntire et al., 1985). For the steady-state kinetic data, eq 1 was used to obtain values for

$$v = \frac{k_{cat}[S][PMS]}{[S][PMS] + K_{PMS}[S] + K_S[PMS]} \quad (1)$$

the parameter when both the phenolic substrate (S) and PMS were varied. Typically, 25–35 data points were available. The following equation was also used to derive the values for some of the parameters:

$$v = \frac{k'_{cat}[X]}{K'_X + [X]} \quad (2)$$

X represents the phenolic substrate at constant [PMS] or PMS at constant [S]. The primed parameters indicate that these represent the apparent values, unless the concentration of the constant substrate is saturating. The equation used for the analysis of the steady-state kinetics for the flavoprotein is presented in the text.

The method for calculating the rate constants for the stopped-flow data involved fitting the time-dependent absorbance changes to the sum of two exponentials for data collected at 420, 440, or 552 nm. The changes at 385 nm for *p*-cresol-α,α,α-d_3 were complicated because there were increases and decreases in absorbance, which resulted in four distinct phases. Since the two slow phases were independent from the fast phases and produced a decrease in the absorbance, the following equation was used:

$$A = A_1e^{-k_1t} + St + A_3$$

The slowest reaction produced a linear absorbance change during the time scale monitored and was fit to a straight line with slope S and intercept A_3.

The magnitude of the rate constants for the fast reactions at 385 nm dictated that the two processes be treated as irreversible coupled reactions, i.e.

$$E \xrightarrow{k_1} E' \xrightarrow{k_2} E''$$

where E is the fully oxidized enzyme, E″ is the enzyme containing the anionic flavin radical, and E′ is the enzyme with the unidentified intermediate (see Results and Discussion). As a result, the observed time-dependent absorbance change is the sum of the changes for each species. The equation describing these changes is therefore a complicated function of the extinction coefficients for the three forms of the enzyme and of the two rate constants (Capellos & Bielski, 1974). However, the total absorbance can be expressed as the sum of two exponentials:

$$A = A_1e^{-k_1t} + A_2(1 - e^{-k_2t}) + A_3$$

A_1 and A_2 are not the absorbance change for any particular species but are functions of the rate constants and extinction coefficients. The rate constants k_1 and k_2 are separated in the exponential parts of this equation (A_3 is the absorbance at t = 0 for the oxidized enzyme). The fast reactions at 385 nm were also simulated by computer with an algorithm based on the Runga–Kutta method, written by Dr. S. C. Koerber of this laboratory.

When the dependent variables had standard errors (σ), they were propagated by using the σ values as weights for the nonlinear regression analysis. Numbers calculated as averages were "weighted averages" found from (Meyer, 1975)

$$\bar{x} = \frac{\sum_i^i x_i/\sigma_i^2}{\sum_i^i 1/\sigma_i^2}$$

For the value of k_2 and K_d, calculated solely from the steady-state data, the errors were propagated by a method similar to that described by Palcic and Klinman (1983).

Results

Steady-State Kinetic Isotope Effect in the p-Cresol Methylhydroxylase Reaction. The previous analysis of the steady-state kinetics for the enzyme indicated that an apparent ping-pong mechanism is operating when either *p*-cresol or 4-ethylphenol is the substrate (McIntire et al., 1985). These studies have now been extended to include the α-deuterio analogues of these compounds. The values of steady-state parameters, derived from eq 1 and 2, and the isotope effects resulting from these are presented in Table I.

The steady-state data for *p*-cresol and *p*-cresol-α,α,α-d_3 at pH 6.5 and 6 °C produced very different results from those at pH 7.6 and 6 °C. The $1/v$ vs. $1/[PMS]$ plots were curved, and there did not appear to be a large isotope effect. A full kinetic analysis was not possible because at a very low concentration of *p*-cresol (2.68 μM) the rate was still about 70% of the maximal velocity (from $1/v$ vs. $1/[S]$ plots not shown).

One explanation of the curved lines is a switching of the mechanism from a ping-pong type to an ordered binding type at high PMS concentration. This is the explanation suggested by Mannervick (1973) for glutathione reductase, by Pabst et al. (1974) for glutathione *S*-transferase A, and by Bright and Porter (1976) for L-amino acid oxidase. Alternatively, there may be two sites for electron transfer to PMS, one defined by large values for K_M and k_{cat} and the other by low values for these parameters. Curved plots are also expected for a random binding mechanism for which the squared concentration terms in the steady-state equation are important. In any case, as the pH is lowered there appears to be a change in the mechanism.

Reoxidizing Substrates for p-Cresol Methylhydroxylase. For the reducing substrates *p*-cresol, 4-ethylphenol, 2,4-dimethylphenol, and 4-hydroxybenzyl alcohol, the k_{cat}/K_{PMS} ratios are identical (McIntire et al., 1985). This fact supports the alternative ping-pong and ordered binding mechanisms (McIntire et al., 1985). When the reoxidizing substrate is varied, the k_{cat}/K_S ratio should also be constant for these mechanisms. These values have now been determined with

Table I: Steady-State Kinetic Isotope Effects for *p*-Cresol Methylhydroxylase at 25 and 6 °C[a]

substrate (S)	k_{cat} (s^{-1})	K_S (μM)	K_{PMS} (mM)	k_{cat}/K ($\times 10^{-4}$ M^{-1} s^{-1}) S	k_{cat}/K ($\times 10^{-4}$ M^{-1} s^{-1}) PMS	$^D(k_{cat}/K)$ S	$^D(k_{cat}/K)$ PMS	$^Dk_{cat}$
				Flavocytochrome at 25 °C				
p-cresol	125 ± 8.0	14.3 ± 1.0	4.48 ± 0.41	870 ± 12	2.80 ± 0.08	7.03 ± 0.41	1.00 ± 0.03	2.59 ± 0.17
p-cresol-α,α,α-d_3	48.3 ± 0.2		1.84 ± 0.08	125 ± 2.3[b]	2.62 ± 0.05			
4-ethylphenol	70.3 ± 4.2	933 ± 67	2.59 ± 0.21	7.55 ± 0.5	2.72 ± 0.05	5.21 ± 0.20	1.10 ± 0.05	1.59 ± 0.11
4-ethylphenol-α,α-d_2	44.3 ± 0.2	314 ± 16	1.88 ± 0.10	1.41 ± 0.02	2.38 ± 0.03			
				Flavocytochrome at 6 °C				
p-cresol	43.3 ± 1.9		6.18 ± 0.43	477 ± 12[b]	0.703 ± 0.017	3.43 ± 0.15	0.925 ± 0.031	3.12 ± 0.17
p-cresol-α,α,α-d_3	13.6 ± 0.4		1.83 ± 0.08	139 ± 5.2[b]	0.760 ± 0.017			
4-ethylphenol[c]				0.300 ± 0.013	0.783 ± 0.023	3.59 ± 0.17	0.952 ± 0.054	
4-ethylphenol-α,α-d_2[c]				0.0833 ± 0.0017	0.823 ± 0.040			
				Flavoprotein Subunit[d]				
p-cresol	4.42 ± 0.37	24.6 ± 3.1	7.81 ± 0.82	18.0 ± 0.8	0.0567 ± 0.0011	7.12 ± 0.52	1.26 ± 0.02	3.35 ± 0.31
p-cresol-α,α,α-d_3	1.32 ± 0.05	52.4 ± 3.8	2.95 ± 0.14	2.53 ± 0.15	0.0449 ± 0.0008			

[a] The experiments were carried out in Tris-HCl buffer (I = 0.05) at 25 °C and in 25 mM buffer (I = 0.075) at 6 °C and pH 7.6. [b] Calculated with eq 2. K_S values were not listed because the concentration of PMS was subsaturating. [c] Only the k_{cat}/K values were calculated because both [S] and [PMS] were subsaturating. [d] The experiment was carried out at 25 °C in Tris buffer. The values for parameters were calculated from eq 9 where α = 1. The K' values were (2.13 ± 0.15) × 10^{-2} M^2 and (4.92 ± 0.44) × 10^{-2} M^2 for *p*-cresol and *p*-cresol-α,α,α-d_3, respectively.

Table II: Steady-State Parameters for the Flavocytochrome as a Function of the Reoxidizing Substrate[a]

reoxidant	k_{cat} (s^{-1})	K_S (μM)	K_{reox} (mM)[b]	k_{cat}/K_S ($\times 10^{-6}$ M^{-1} s^{-1})
PMS	87.8 ± 8.0	12.9 ± 1.6	3.73 ± 0.48	7.02 ± 0.50
PES	89.3 ± 11.2	15.4 ± 2.1	6.54 ± 1.03	6.08 ± 0.38
Wurster's blue	108 ± 2.0	21.0 ± 1.0	0.0558 ± 0.0027	5.47 ± 0.17
cytochrome *c*	98.5 ± 0.2	15.9 ± 0.8	0.269 ± 0.010	6.20 ± 0.18

[a] The experiments were carried out at 25 °C in 0.05 M Tris-HCl, pH 7.6 (I = 0.05), with a saturating concentration of *p*-cresol. The parameters were calculated from eq 2. [b] K_{reox} represents the K_M for the reoxidizing substrate.

Table III: Apparent Steady-State Isotope Effects for the Flavoprotein Subunit at Different Concentrations of the Nonvaried Substrate[a]

p-cresol[b] concn (μM)	*p*-cresol[b] $^D(V/K_{PMS})$	PMS[b] concn (mM)	PMS[b] $^D(V/K_S)$
4.54	3.99 ± 0.19	0.706	7.10 ± 0.29
6.81	3.59 ± 0.37	0.957	7.53 ± 0.26
11.40	2.54 ± 0.21	1.310	7.39 ± 0.21
34.10	1.91 ± 0.06	1.810	7.59 ± 0.22
1000	1.25 ± 0.03	2.520	7.13 ± 0.15
			7.31 ± 0.09[c]

[a] The experiments were carried out in 0.05 M Tris-HCl at pH 7.6 and 25 °C (I = 0.05). The V/K values for each concentration were calculated with eq 2. [b] The substrate held constant at the concentrations given. [c] The weighted average of the isotope effects for the various concentrations of PMS.

PMS, PES, Wurster's blue, and horse heart cytochrome *c* as reoxidants of the enzyme when S is *p*-cresol. The steady-state parameters are given in Table II.

Steady-State Isotope Effect in the Flavoprotein Subunit Reaction. In an earlier paper (McIntire et al., 1985) the steady-state data for the flavoprotein produced nonparallel, linear $1/v$ vs. $1/$[*p*-cresol] and $1/v$ vs. $1/$[PMS] plots, indicating that the mechanism is not of the ping-pong type. The apparent parameters at various concentration of each substrate used in this present study were calculated with eq 2, and from these the apparent isotope effects were determined (Table III). The parameters in Table III are the most telling in terms of the type of mechanism operating for the flavoprotein. Following the method of Cook and Cleland (1981), it will be shown how all but a random-order binding mechanism can be eliminated.

Stopped-Flow Experiments on the Reaction of 4-Ethylphenol and 4-Ethylphenol-α,α-d_2 with the Enzyme. Reaction of the enzyme with 4-ethylphenol at 25 or 6 °C resulted in two independent absorbance changes (Figure 1). Because the greatest change took place at 420 nm, this wavelength was

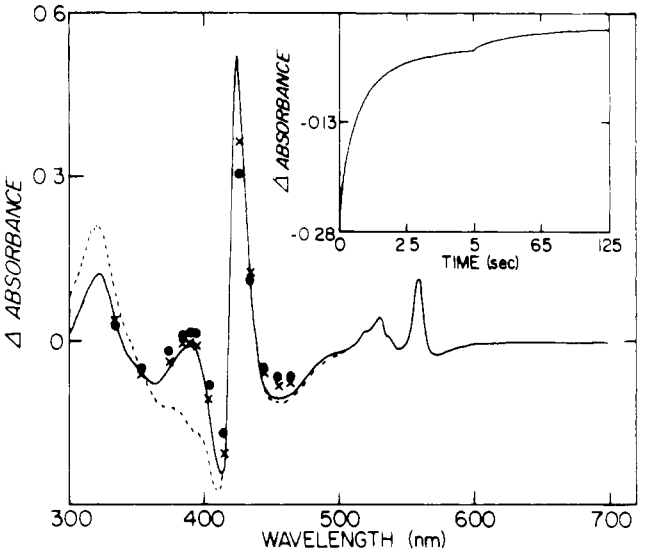


FIGURE 1: Absorbance changes at various wavelengths for the reaction of 4-ethylphenol with the enzyme in 25 mM sodium phosphate, I = 0.075, at pH 7.6 and 6 °C. The solid line is the difference spectrum (reduced–oxidized) recorded ~3 min after the addition of 254 μM 4-ethylphenol-α,α-d_2 to 6 μM enzyme. The filled circles are the ΔA values obtained for the fast phase (at 3 s) after mixing 180 μM 4-ethylphenol-α,α-d_2 with 4.51 μM enzyme. The (×) symbols are the ΔA values for the fast and slow phases combined (at 2 min). The dashed line is the difference spectrum for the fully reduced enzyme. The enzyme (7.91 μM) was treated with 3 electron equiv of sodium dithionite, whereby both the flavin and heme components were reduced. The large difference in the region around 390 nm reflects the fast formation of the anionic flavin radical. The ΔA values were normalized to correspond to 6 μM enzyme. (Inset) The plot of ΔA vs. time for the reaction of the substrate with the enzyme at 420 nm (note the change in the time scale, occurring at 5 s, which highlights the fast and slow phases). Similar data were obtained for stopped-flow experiments at 25 °C.

Table IV: Stopped-Flow Kinetic Isotope Effect Data for 4-Ethylphenol at pH 7.6[a]

temp (°C)	buffer	substrate[b]	k_2 (s^{-1})	K_d (mM)	k_2/K_d ($\times 10^{-3}$ M^{-1} s^{-1})	$^Dk_2$[c]	$^Dk_2$[d]	k_{-2} (s^{-1})	$^Dk_{-2}$
25	Tris-HCl	H	284 ± 41	2.17 ± 0.38	131 ± 4	4.90 ± 1.44	4.92 ● 0.23		
	(50 mM, I = 0.05)	D	57.9 ± 14.7	2.17 ± 0.63	26.6 ± 1.0				
	Tris-HCl	H	393 ± 71	2.77 ± 0.55	142 ± 3	5.34 ± 2.36	5.03 ± 0.31		
	(75 mM, I = 0.075)	D	68.0 ± 24.9	2.40 ± 0.99	28.3 ± 1.6				
	sodium phosphate	H	218 ± 8	1.56 ± 0.08	138 ± 2	4.94 ● 0.19	4.82 ± 0.21		
	(25 mM, I = 0.075)	D	44.2 ± 0.4	1.53 ± 0.37	2.90 ± 1.3				
6	sodium phosphate	H			21.7 ± 0.8		4.01 ± 0.17	0.603 ± 0.330	1.95 ± 0.84
	(25 mM, I = 0.075)	D			5.45 ± 0.10			0.310 ± 0.038	

[a] The concentrations of enzyme used for the experiment at 25 °C in 50 mM Tris-HCl, 75 mM Tris-HCl, and phosphate buffer were 4.25, 4.55, and 4.55 μM, respectively. The enzyme concentration at 6 °C was 4.51 μM. [b] H and D represents 4-ethylphenol and 4-ethylphenol-α,α-d_2. [c] k_{2H}/k_{2D}. [d] $(k_2/K_d)_H/(k_2/K_d)_D$.

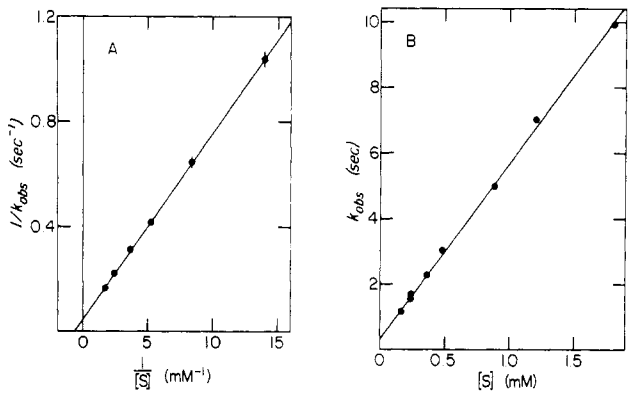


FIGURE 2: Plots of the stopped-flow data: (A) $1/k_{obsd}$ vs. $1/[4$-ethylphenol] at 25 °C; (B) k_{obsd} vs. [4-ethylphenol-α,α-d_2] at 6 °C. The buffer was 25 mM sodium phosphate, I = 0.075, at pH 7.6.

selected to monitor the reaction. It was found that the rate constant for the fast reaction at 25 °C was dependent on the concentration of the substrates and was best described by the following mechanism and equation:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'{\cdot}P \xrightarrow{k_P} E' + P \qquad (3)$$

$$k_{obsd} = \frac{k_2[S]}{[S] + k_{-1}/k_1} = \frac{k_2[S]}{[S] + K_d} \qquad (4)$$

The equation from which eq 4 was derived is

$$k_{obsd} = \frac{k_1[S](k_2 + k_{-2}) + k_{-1}k_{-2}}{k_1[S] + k_{-1} + k_2} \qquad (5)$$

and eq 5 was obtained from eq 3 by assuming the steady-state approximation to be valid (Strickland et al., 1975). E'·P is the form of the enzyme that produces the large increase at 420 nm (cytochrome reduction). If it is assumed that $k_{-1} \gg k_2$ and $k_{-2} = 0$, i.e., that there is fast equilibration between E + S and E·S and the reduction is irreversible, then eq 5 reduces to eq 4. The irreversibility of this step is supported by the reductive titration of the enzyme, in that all the electron equivalents from the substrate end up in the enzyme (McIntire et al., 1985). The step defined by k_P does not contribute to the absorbance of the fast reaction. Equation 4 can be rewritten in reciprocal form, and a graph of $1/k_{obsd}$ vs. $1/[S]$ will yield a slope of K_d/k_2 and an intercept of $1/k_2$ (Figure 2A). The parameters from a computer fit of the data to eq 4 are listed in Table IV.

The result obtained with the buffers in Table IV differ somewhat quantitatively. These data indicate that there is an ionic strength and/or buffer effect for the experiments carried out in the Tris buffers. A specific buffer effect appears to be involved, since the values from the experiments in the phosphate buffer are lower than those derived from the studies in the Tris buffers.

The K_d value for the substrates was independent of the isotopic substitution. As a result, the ratio k_2/K_d for each substrate can be used to calculate the isotope effect on k_2. Equation 4 can be rearranged to produce

$$k_{obsd} = \frac{[S]k_2/K_d}{[S]/K_d + 1} = \frac{K'[S]}{[S]/K_d + 1}$$

The data can now be fit to this equation with K' and K_d as the parameters. This analysis leads to a considerable reduction in the standard errors of the values for $^Dk_2 = K'_H/K'_D$ (Table IV).

The change in absorbance vs. time at 6 °C and pH 7.6 also produced two phases as at 25 °C (Figure 1). The change was slower and the plots of $1/k_{obsd}$ vs. $1/[S]$ were curved, and only when k_{obsd} was plotted vs. [S] for the two phenols did straight lines result (see Figure 2B). The mechanism given by eq 3 is consistent with these data, as is eq 5 under certain limiting conditions. If $k_{-1} \gg k_2$ and $K_d > [S]$, then

$$k_{obsd} = \frac{k_2[S]}{K_d} + k_{-2} = K'[S] + k_{-2} \qquad (6)$$

Evidently, the second step is reversible at this temperature, although k_{-2} is small (Table IV). Further, the values for the deuteriated and nondeuteriated substrates may be equal within the errors given. The hydrogen and deuterium that is removed initially in step 2 must be lost; otherwise, a large isotope effect should be seen for k_{-2} as well as for k_2 (Klinman, 1978).

The value for the rate constant for the slow step (see inset to Figure 1) at 6 °C was 0.0369 ± 0.0038 s^{-1} (average of three runs) and was independent of the substrate or its concentration. This value was 2–3 times slower than for the process at 25 °C.

Stopped-Flow Experiments Involving p-Cresol and p-Cresol-α,α,α-d_3. The reaction of *p*-cresol with the flavocytochrome was too fast to measure at 25 °C; so all the experiments were carried out at 6 °C. Unlike the reaction with 4-ethylphenol, the absorbance changes measured were independent of the substrate concentration. At pH 7.6 the reaction again produced two phases at 420 nm, indicating that the biphasic nature of the reduction is a property of the enzyme itself, and not of any given substrate. As with the results generated in the 4-ethylphenol experiments, the change in absorbance at 420 nm in the first phase (phase I in Table V) was about 10 times greater than the change in the next phase as this wavelength (phase III in Table V). Also, the rate constant for the slow phase was about the same as that for 4-ethylphenol.

Important differences in the experiments involving 4-ethylphenol and *p*-cresol surfaced when the reaction was monitored at various wavelengths. Between 400 and 450 nm and at 552 nm, a biphasic behavior was observed for both *p*-cresol and *p*-cresol-α,α,α-d_3. On the other hand, *p*-cresol-

Table V: Stopped-Flow Results for *p*-Cresol at 6 °C[a]

pH	substrate	k_I (s^{-1})[b]	k_{II} (s^{-1})[c]	k_{III} (s^{-1})[b]	$k_{IV}\Delta A_{IV}$ ($\times 10^4$ s^{-1})[c]
6.5	*p*-cresol	185 ± 3.0	7.60 ± 0.19	0.143 ± 0.040	−1.84 ± 0.44
	p-cresol-α,α,α-d_3	33.9 ± 0.3	7.44 ± 0.54	0.124 ± 0.008	−2.77 ± 0.11
7.6	*p*-cresol	194 ± 2		0.053 ± 0.002	
	p-cresol-α,α,α-d_3	27.5 ± 0.2	12.3 ± 1.0	0.046 ± 0.001	−1.88 ± 0.06

[a] The buffer used was sodium phosphate, 25 mM, I = 0.075. The subscripts I–IV represent the first, second, third, and fourth phases, respectively. [b] The weighted average of three to eight values obtained from data collected at 385, 420, 440 and 552 nm, involving 0.070–2.5 mM substrate and 4.4–19 μM enzyme. [c] The reactions described by these rate constants were only observed at 385 nm. The substrate concentration was 2.5 mM, and the enzyme concentration was 19 μM.

α,α,α-d_3 produced the absorbance changes at 385 nm and pH 7.6 shown in Figure 3. The parameters resulting from these absorbance changes are listed in Table V. The data indicate that the fastest phase at 385 nm corresponds to the fastest phase seen at 420 and 440 nm. At 385 nm, this phase was very fast when *p*-cresol was the reductant, and only three phases were produced. The second phase has no counterpart in any other spectral region. The third phase corresponds to the slow changes at 440 and 420 nm, and the last linear phase again is not seen elsewhere in the spectrum.

With the hope of slowing the reaction, experiments were carried out at pH 6.5 and 6 °C. As is obvious from the results in Table V, the fast reaction did not slow down but had nearly the same rate constant as found at pH 7.6. All the absorbance changes monitored were again independent of the substrate and enzyme concentrations, and the spectral changes were qualitatively the same as at pH 7.6.

Comparison of the Steady-State and Stopped-Flow Data for the Flavocytochrome. As mentioned in the previous paper (McIntire et al., 1985), two alternative mechanisms were proposed to explain the steady-state data and are given by Schemes I and II. In these schemes, O represents the oxidant PMS, and the primes indicate that these species are reduced. Scheme I is a ping-pong mechanism, and the ordered binding mechanism given by Scheme II produces the same steady-state equation (in terms of the k values) when certain assumptions are made, i.e., k_{-2}, k_{-3}, and k_{-5} = 0. This results in the expression ${}^{D}k_{cat} = ({}^{D}k_2 + R)/(1 + R)$, and only if ${}^{D}k_2 > R$ will ${}^{D}k_{cat} = {}^{D}k_2$, which is not the case when 4-ethylphenol was the substrate. From the parameters obtained at 25 °C, a value for R of 6.37 ± 1.85 is calculated.

Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'{\cdot}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E' \underset{k_{-4}}{\overset{k_4[O]}{\rightleftharpoons}} E'{\cdot}O \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E + O'$$

(P above k_3)

Scheme II

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E'{\cdot}P \underset{k_{-4}}{\overset{k_4[O]}{\rightleftharpoons}} E'{\cdot}P{\cdot}O \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E{\cdot}P \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + P$$

(O′ above k_5)

For 4-ethylphenol, the situation that best describes k_{obsd} as a function of [S] at 25 °C requires $k_{-1} \gg k_2$. Since k_{cat}/K_S is equal to $k_1k_2/(k_{-1} + k_2)$, it will reduce to $k_1k_2/k_{-1} = k_2/K_d$. Tables I and IV indicate that the value for k_2/K_d is about twice the value for k_{cat}/K_S at this temperature. At 6 °C the relationship $k_{-1} \gg k_2$ was also assumed to be valid in order to interpret the data. Now, however, $k_{-2} \neq 0$, and the expression for k_{cat}/K_S changes to

$$\frac{k_1k_2k_3}{k_2k_3 + k_{-1}(k_{-2} + k_3)}$$

and k_{cat}/K_S is no longer equal to but must be less than k_2/K_d, as was found.

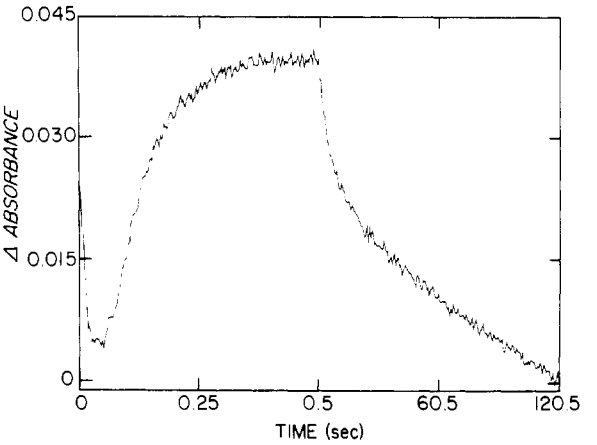


FIGURE 3: Absorbance changes at 385 nm for the reaction of 180 μM *p*-cresol-α,α,α-d_3 and 15.6 μM enzyme in 25 mM sodium phosphate, I = 0.075, at pH 7.6 and 6 °C. Note the change in the time scale, occurring at 0.5 s, which highlights the fast and slow absorbance changes.

A recent paper by Palcic and Klinman (1983) described a procedure whereby the rate constant for the isotopically sensitive step and the dissociation constant for the substrate reaction with plasma amine oxidase may be calculated from steady-state data alone, with the prerequisite that the value for the intrinsic isotope effect be known. The mechanism they presented is identical with that proposed for *p*-cresol methylhydroxylase (Scheme I). The necessary equations for this mechanism are

$$k_2 = \frac{k_{cat}({}^{D}k_2 - 1)}{{}^{D}k_{cat} - 1} \quad (7)$$

$$K_d = \frac{k_{cat}[{}^{D}(k_{cat}/K_S) - 1]}{(k_{cat}/K_S)({}^{D}k_{cat} - 1)} \quad (8)$$

Since ${}^{D}(k_{cat}/K_S) = {}^{D}k_2$ for 4-ethylphenol, the value derived for k_2 at 25 °C from eq 7 is 260 ± 50 s^{-1}, in good agreement with the stopped-flow value of 284 ± 41 s^{-1}. The value for K_d from eq 8 (6.65 ± 1.17 mM) is considerably larger than that found by the stopped-flow technique (2.17 ± 0.38 mM).

The isotope effect for k_{cat}/K_S for *p*-cresol at 25 °C and pH 7.6 was 7.03 ± 0.22, which is equal to ${}^{D}k_2$ at 6 °C and pH 7.6 (7.05 ± 0.22; stopped-flow result). The ${}^{D}(k_{cat}/K_S)$ value at 6 °C and pH 7.6, however, was lower by about half (3.43 ± 0.15). Assuming k_{-2} = 0 in eq 3 and that k_P has no effect on the stopped-flow kinetics at 6 °C, then only if $k_1[S] \gg k_{-1} + k_2$ is the observed rate constant independent of [S], and eq 5 yields ${}^{D}k_{obsd} = {}^{D}k_2$. Since ${}^{D}(k_{cat}/K_S) = ({}^{D}k_2 + k_2/k_{-1})/(1 + k_2/k_{-1})$ and if k_2 and k_{-1} have similar values, they will, however, contribute to the steady-state isotope effect, and ${}^{D}(k_{cat}/K_S) < {}^{D}k_2$. Table VI summarizes all the kinetic parameters that can be determined from the steady-state and the stopped-flow experiments for the *p*-cresol reaction at 6 °C.

Taking advantage of eq 7 and 8 and assuming ${}^{D}(k_{cat}/K_S) = {}^{D}k_2$, the values for k_2 and K_d at 25 °C, calculated exclusively

Table VI: Parameters Calculated from the Stopped-Flow and Steady-State Data for the Oxidation of *p*-Cresol at pH 7.6 and 6 °C[a]

substrate	k_{cat}/K_S ($\times 10^{-6}$ M^{-1} s^{-1})	C^a	k_{-1} (s^{-1})[b]	k_1 ($\times 10^{-6}$ M^{-1} s^{-1})[c]	K_d (μM)
p-cresol	4.77 ± 0.12	1.49 ± 0.52	130 ± 45	8.0 ± 1.3	16 ± 3
p-cresol-α,α,α-d_3	1.39 ± 0.05			7.5 ± 1.0	17 ± 4

[a] Calculated from $^D(k_{cat}/K_S) = (^Dk_2 + C)/(1 + C)$. [b] Calculated from $C = k_2/k_{-1}$. [c] Calculated from $k_{cat}/K_S = k_2k_1/(k_2 + k_{-1})$.

from the steady-state information, are 237 ± 24 s^{-1} and 54.3 ± 4.7 μM, respectively. Similarly, for 6 °C, these parameters have values of 62 ± 5 s^{-1} and 10.4 ± 1.1 μM. In contrast to the results for 4-ethylphenol, at 6 °C k_2 is lower than and K_d is about the same as the values calculated with both the stopped-flow and steady-state data (Table VI).

Stopped-Flow Experiment Involving p-Cresol and the Flavoprotein Subunit of p-Cresol Methylhydroxylase. The traditional stopped-flow method indicated that the reaction with *p*-cresol and *p*-cresol-α,α,α-d_3 was slow and biphasic when followed at 440 nm. The slow reaction allowed rapid-scan monitoring of the reaction, and again, the reaction was biphasic from 330 to 470 nm. The absorbance changes for each phase were approximately equal, and virtually no difference in the rate constants for the first (k = 0.9 s^{-1}) and the second phases (k = 0.14 s^{-1}) was found at either 0.35 or 70 mM *p*-cresol or *p*-cresol-α,α,α-d_3. These results were unexpected after the steady-state kinetic analysis, from which an apparent intrinsic isotope effect of 7 was determined. It appears, therefore, that a step prior to the reduction of the flavin is rate limiting. Further, no evidence was seen for the formation of a flavin radical in these reactions.

Discussion

Interpretation of the Kinetic Data for the Flavocytochrome. The initial steady-state kinetic work led to the conclusion that the most likely mechanism for *p*-cresol methylhydroxylase was of an apparent ping-pong type (McIntire et al., 1985). The bases for this conclusion were (1) the family of $1/v$ vs. $1/[S]$ and $1/v$ vs. $1/[PMS]$ plots were parallel for S = *p*-cresol or 4-ethylphenol and (2) the $1/v$ vs. $1/[PMS]$ plots for various phenolic substrates were also parallel; i.e., k_{cat}/K_{PMS} values for *p*-cresol, 4-ethylphenol, 2,4-dimethylphenol, and 4-hydroxybenzyl alcohol were identical. Similar experiments resulted in k_{cat}/K_S values that did not vary substantially when PMS, PES, Wurster's blue, or cytochrome *c* was the reoxidizing substrate (Table III). This is explained by the equation $k_{cat}/K_S = k_1k_2/(k_{-1} + k_2)$, which is not a function of any rate constants for the reaction of the enzyme with these reoxidants (see Schemes I and II). These results support either of the two proposed mechanisms but, unfortunately, cannot distinguish between them. The expression for k_{cat}/K_{PMS} is $k_4k_5/(k_{-4} + k_5)$ for the mechanisms given by Schemes I and II when $k_2 = 0$. The stopped-flow analysis indicates that $k_{-2} \neq 0$ for 4-ethylphenol at 6 °C. This does not change the form of k_{cat}/K_{PMS} for Scheme I but for Scheme II becomes

$$\frac{k_2k_4k_5}{(k_{-2} + k_2)(k_{-4} + k_5)}$$

and as a result

$$^D(k_{cat}/K_{PMS}) = \frac{^DK_{eq} + K_{eq}}{1 + K_{eq}}$$

where $K_{eq} = k_2/k_{-2}$. Since the range of values for the isotope effect of an equilibrium constant is 0.9–1.2 (Klinman, 1978), the $^D(k_{cat}/K_{PMS})$ ratio is still expected to be close to 1. Inspection of Table I reveals the k_{cat}/K_{PMS} isotope effect is in fact nearly equal to 1 for *p*-cresol and 4-ethylphenol. Additionally, when $k_{-2} \neq 0$ for the mechanism in Scheme II, eq 9 must be used ($K' = K_iK_{PMS}$, $K_i = k_{-1}k_{-2}/(k_{-2} + k_2) \neq 0$, and $\alpha = 1$).

$$v = \frac{k_{cat}[S][PMS]}{\alpha K' + \alpha K_{PMS}[S] + \alpha K_S[PMS] + [S][PMS]} \quad (9)$$

The equation for $^Dk_{cat}$ when $k_{-2} = 0$ is

$$\frac{^Dk_2 + R}{1 + R}$$

and when $k_{-2} \neq 0$, the appropriate equation is

$$\frac{^Dk_2 + R + {}^DK_{eq}C_r}{1 + R + C_r}$$

where $R = k_2/k_3 + k_2/k_5$ and $C_r = k_{-2}/k_3$ (R being the "ratio of catalysis" and C_r being the "reverse commitment to catalysis factor"; Northrop, 1977). When the values for R and/or C_r are similar to that for $^Dk_2$, then the value for $^Dk_{cat}$ will be depressed from this intrinsic isotope effect. This seems to be the case for *p*-cresol methylhydroxylase (Table I).

The $^D(k_{cat}/K_S)$ ratio for the phenolic substrate, when $k_{-2} = 0$, is

$$\frac{^Dk_2 + C_f}{1 + C_f} \quad (10)$$

and is equal to

$$\frac{^Dk_2 + C_f + {}^DK_{eq}C_r}{1 + C_f + C_r}$$

when $k_{-2} \neq 0$, where C_f ($=k_{-1}/k_2$) is the "forward commitment factor" (Northrop, 1977). The values for $^D(k_{cat}/K_S)$ at 25 °C are 5 for 4-ethylphenol and 7 for *p*-cresol. We have confirmed that these are in fact the intrinsic isotope effects for the enzymic reaction by stopped-flow kinetic measurements, which indicates that the commitment factors are of little consequence. At 6 °C and pH 7.6, the $^D(k_{cat}/K_S)$ value for *p*-cresol is lower than the intrinsic isotope effect, whereas the value for 4-ethylphenol at this temperature is equal to the maximum as measured by the stopped-flow technique. A detailed comparison of the steady-state and pre-steady-state kinetics is offered under Results.

The intrinsic isotope effect for 4-ethylphenol at 6 °C is less than the effect at 25 °C (4.02 vs. 4.8–5.0). An expression of the kinetic isotope effect (KIE) is

$$KIE = A_H/A_De^{-[\Delta E_a]/RT}$$

where A_H and A_D are the frequency factors and $[\Delta E_a]$ is the difference in the activation energy for the hydrogenated and deuteriated substances, and is a function of the real normal mode vibrational frequencies of the ground state and the transition state (Buddenbaum & Shiner, 1977). According to this equation the isotope effect should *increase*, not decrease, as temperature is lowered. A change in structure of the transition state would explain the decrease.

Since there is more than one α-deuterium atom in the labeled substrates, a secondary effect may contribute to the overall effect measured. The magnitude of this secondary

α-deuterium isotope effect depends on the transition state in the enzymic reaction, and the true primary effect could be lower than those reported here.

For all substrates there is a fast equilibration in the E + S $\rightleftharpoons$ ES step. The rate-limiting step in the reaction of the enzyme with 4-ethylphenol appears to be the cleavage of the C–^{1}H or C–D bond and results in the reduction of the flavin. This is followed by a very rapid reduction of the heme with concomitant formation of the anionic flavin radical (the "red" radical). During the reaction of *p*-cresol, the formation of the red radical lags behind (k = 7.6 s^{-1} at pH 6.5) the appearance of the reduced heme (k = 194 s^{-1} for *p*-cresol and 27.5 s^{-1} for *p*-cresol-α,α,α-d_3). The evidence that the increase at 385 nm is a result of the formation of the red radical comes from an analysis of the adsorption and ESR spectra of the enzyme and of the flavoprotein subunit during reductive titrations of these proteins (McIntire et al., 1981, 1985; Ackrell et al., 1982).

Formation of a flavin radical must occur with the same rate as the reduction of the cytochrome, because each substrate molecule oxidized supplies two electrons to the enzyme. If one electron is transferred to the cytochrome, the other must remain associated with the flavin. This requires that the radical initially formed on reduction of the enzyme (the *decrease* at 385 nm) have a lower absorbance than does the red radical. Several possibilities may be envisaged to explain the observation. First, the neutral flavin radical may be formed when the heme is reduced, and a slower deprotonation would result in the progressive appearance of the red radical. The neutral flavin radical thus formed will absorb at 650 nm (McIntire et al., 1981; Müller et al., 1972) without interference from heme absorbance. When the reaction was monitored at this wavelength, no changes were observed; thus, it seems unlikely that the neutral radical is an intermediate.

A second possibility involves a complex between the product *p*-hydroxybenzyl alcohol-α,α-d_2 and the red radical. A charge-transfer complex is known to exist between the flavin bound to D-amino acid oxidase and both its substrate and its product (Yagi et al., 1962). Another possibility is that a covalent intermediate is formed between the radical of *p*-cresol methylhydroxylase and the product. Such adducts usually involve the N(5) or C(4a) positions of the flavin (Walsh, 1981). These last two proposals allow the prediction that initially the normal radical absorbance will be suppressed at 385 nm and, when the product is released, full expression of the absorbance will manifest itself.

A computer simulation of the absorbances changes taking place at 385 nm for *p*-cresol-α,α,α-d_3 was carried out with a Runga–Kutta algorithm. It was necessary to supply the program with the rate constants and extinction coefficients for the protein-bound oxidized heme and flavin and for the reduced heme. The latter were supplied by the corresponding spectra of the pure isolated flavin and cytochrome subunits. This seemed appropriate since an earlier study indicated that the spectrum of the flavocytochrome is virtually identical with the sum of the spectra of the isolated subunits (Koerber et al., 1985b). By trial and error, the best fit to the data produced an extinction coefficient at 385 nm of 26 900 M^{-1} cm^{-1} for the unidentified flavin radical formed initially and 30 600 M^{-1} cm^{-1} for the red radical, which developed more slowly. The values of these coefficients are similar to that estimated for the red radical formed during the sodium dithionite titration of the flavoprotein subunit (McIntire et al., 1985). The results of the simulation are depicted in Figure 4.

In all the stopped-flow traces recorded, a slow reaction was also observed (an increase at 420 and 552 nm and a decrease

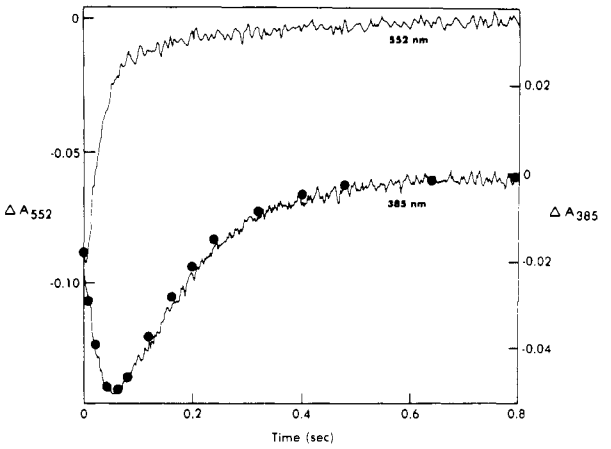


FIGURE 4: Stopped-flow time course at 385 and 552 nm for the reduction of 19 μM enzyme by 2.51 mM *p*-cresol-α,α,α-d_3 in 25 mM sodium phosphate, I = 0.075, at pH 6.5 and 6 °C. The absorbance change at 552 nm is predominately due to the reduction of the heme. The circles represent the value obtained from the computer simulation described in the text.

at 385 and 440 nm). Perhaps this is a reflection of a slow conformational change accompanying reduction of the enzyme, a result of a slow release of the product from the reduced enzyme (k_P in eq 3), or ~10% of the enzyme existing in a form with decreased activity.

At 385 nm a very slow linear decrease in absorbance occurred (Figure 3). From reductive titration data for the enzyme and the flavoprotein subunit (McIntire et al., 1981, 1985), a very slow disproportionation of the red radical was noted. This process took place over a 10–20-min period after the addition of the reductant and caused a decrease in the absorbance at 385 nm that was of the right magnitude to explain the change in the stopped-flow traces.

The values for the intrinsic isotope effect derived by the steady-state and the stopped-flow methods were in excellent agreement. However, there were discrepancies for other parameters measured by these techniques. The tentative conclusion is that the dyes PMS and DCIP used to monitor the steady-state oxidation of the phenols were the cause of these differences. The dye PMS is known to cause various problems in some enzyme kinetic studies. In the case of *p*-cresol methylhydroxylase as well as other enzymes, there is severe substrate inhibition by this oxidant at concentrations on the order of the K_{PMS} values (McIntire et al., 1985; Rendina & Singer, 1974; Singer & Kearney, 1957; unpublished observations). Molecular oxygen can reoxidize the reduced PMS formed in the enzymic reaction, forming H_2O_2, which will oxidize sulfhydryl groups of the proteins. PMS has also been found to interact directly with the sulfhydryl groups in proteins (Massey, 1959). Potassium cyanide is often included in the assays to protect the –SH groups (Singer & Kearney, 1957; Singer, 1974). Steady-state assays of *p*-cresol methylhydroxylase involving PMS seemed unaffected by KCN or partial anaerobiosis.

PMS is also very photosensitive and alkali sensitive. Visible light converts PMS to 1-keto-5-methyl-1,5-dihydrophenazine (pyocyanine), and the alkaline reaction will produce 2-keto-10-methyl-2,10-dihydro- and 5-methyl-5,10-dihydrophenazine (Swan & Felton, 1957). These can then be oxidized by DCIP to 1- and 2-hydroxy-10-methylphenazine and PMS, respectively. During the assays PMS was protected from light so little or no pyocyanine was present. A blank rate (the rate in the absence of the enzyme) increased as the pH was raised. This blank rate results from the reduction of DCIP by the

dihydrophenazines produced by the alkaline reaction. It is concluded that the oxidation product of the 2-keto derivative was accumulating during the assays, but it is not known whether this would have any effect on the results.

Specific effects of PMS and DCIP on other enzymes have been found. For example, the K_M values for PMS determined in the assay of α-glycerophosphate dehydrogenase with PMS and DCIP was different from that obtained with horse heart cytochrome *c* as the electron acceptor for PMS (Bednarz & Salach, 1973). In other studies it was found that DCIP alone can alter the results from kinetic studies (Bednarz & Salach, 1973; Sanadi, 1969).

Ideally the steady-state experiments should be done with the natural electron acceptor. The natural acceptor is often not known for a particular oxidoreductase, and hence, dyes must be used as reoxidants. Until recently this had been the situation for *p*-cresol methylhydroxylase; thus, a dye assay was used in this study and others involving this enzyme (McIntire et al., 1985, 1986; McIntire, 1983). An azurin has recently been isolated from the same organism used for isolation of *p*-cresol methylhydroxylase, and preliminary studies suggest that this copper protein is the electron acceptor in vivo (Causer et al., 1984; Hopper et al., 1985).

Steady-State Isotope Effects in the Flavoprotein Subunit Reaction. In contrast to the ping-pong type kinetic behavior of the flavocytochrome, the flavoprotein produces converging lines in double-reciprocal plots (McIntire et al., 1985). An ordered binding and a random binding mechanism are both consistent with this observation. A recent analysis of these type mechanisms using isotopically labeled substrates has been presented by Cook and Cleland (1981). The basic mechanism and equations they considered are given in Scheme III with some modifications and in terms of S and PMS. The expression for the $^D(k_{cat}/K)$ values is given by $(^Dk_5 + C_f)/(1 + C_f)$. When [*p*-cresol] is varied at constant [PMS]

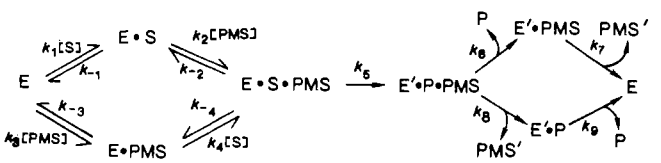
$$C_f = k_5/[k_{-4} + k_{-1}k_{-2}/(k_{-1} + k_2[PMS])] \quad (11)$$

and when [PMS] is varied at constant [S]

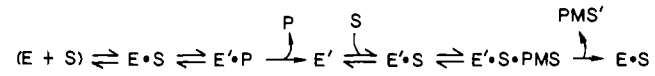
$$C_f = k_5/[k_{-2} + k_{-3}k_{-4}/(k_{-3} + k_4[S])] \quad (12)$$

According to the analysis described by Cook and Cleland (1981), the resulting expressions for $^D(k_{cat}/K_x)$ as a function of the microscopic rate at the extreme levels of the nonvaried substrate are used to derive the mechanism. Inspection of the results for the flavoprotein in Table III indicates that $^D(k_{cat}/K_S)$ is $\sim$7 at all levels of PMS, indicating that C_f, as defined by eq 11, is less than $^Dk_5$; therefore, this commitment factor can be set equal to zero. On the other hand, $^D(k_{cat}/K_{PMS})$ varies from 1.25 $\pm$ 0.03 at high [*p*-cresol] to a high value at low [*p*-cresol]. On the basis of these observations, all but the partial equilibrium random mechanism, where k_{-1} is large relative to k_2[PMS], or a steady-state random mechanism can be eliminated. From the commitment factors for these mechanisms, $k_5/k_{-2} > {}^Dk_5 > k_5/(k_{-2} + k_{-4})$, and it can be concluded that $k_{-4} > k_5 > k_{-2}$. This indicates that in the ternary complex of these mechanisms PMS is a "sticky" substrate, whereas *p*-cresol is not. From the values for $^D(k_{cat}/K_{PMS})_{S\to\infty}$ and for $^Dk_5$, a value for k_5/k_{-2} was determined to be 24.2 $\pm$ 2.7.

Scheme III



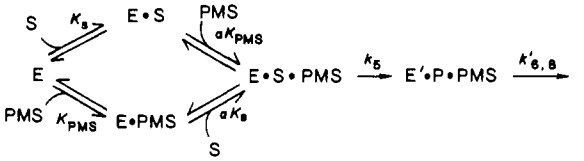
A complimentary approach may be taken for analyzing the mechanism for the flavoprotein with the available information. This approach focuses on the $\alpha K'$ term in eq 9 and allows the consideration of other mechanisms in addition to those described by Cook and Cleland (1981) and given by Scheme III. First, the ordered mechanism given by Scheme II (where $K' = K_iK_{PMS}$ and $\alpha = 1$ in eq 9) reduces to a ping-pong type mechanism when $k_{-2} = 0$, i.e., $K_i = 0$ [see earlier discussion and McIntire et al. (1985)]. Another order binding mechanism for which $K_i = 0$ involves S binding to the reduced enzyme before it can be reoxidized by PMS is



Other ordered binding mechanisms, regardless of which substrate binds first, produce a value for ^DK_i equal to 1. Even when the isotopically sensitive step is reversible, $^DK_i \sim 1$. (In these cases the effect is a function of the equilibrium constant for the sensitive step, and $^DK_{eq} \sim 1$.) In fact, the computer analysis yielded a ^DK_i value of 0.163 $\pm$ 0.024, and as a result, an ordered binding mechanism cannot be considered as a viable alternative.

For the rapid equilibrium random mechanism $K' = K_SK_{PMS}$ and α is defined in Scheme IV, for which K_S and K_{PMS} are dissociation constants. The computer fit produced an isotope effect for $\alpha = 2.88$. The isotope effect for α should be $\sim$1 because this parameter is only involved in binding equilibria, and the effect can be no larger than a few percent. The mechanism is eliminated on this basis.

Scheme IV



When the partial rapid equilibrium or steady-state random mechanisms are considered, $\alpha = 1$, and the K' term is a complicated function of the microscopic rate constants given in Scheme III. The expression for the isotope effect on this parameter $^D(K')$ is also complicated, and is certainly a function of the isotopically sensitive step. However, the isotope effect $^D(k_{cat}/K')$ was determined to be

$$\frac{{}^Dk_5 + k_5/(k_{-2} + k_4)}{1 + k_5/(k_{-2} + k_4)}$$

Accordingly, the following isotope effects should be equal if the random mechanisms mentioned earlier are operating: $^D(k_{cat}/K')$, $^D(k_{cat}/K_S)_{PMS\to\infty}$, $^D(k_{cat}/K_S)_{PMS\to 0}$, and $^D(k_{cat}/K_{PMS})_{S\to 0}$. The respective values are 7.71 $\pm$ 1.62, 7.10 $\pm$ 0.29, 7.13 $\pm$ 0.15, and 3.99 $\pm$ 0.19 at 4.54 μM *p*-cresol and apparently approaching 7 as [*p*-cresol] $\to$ 0. By this line of reasoning, the conclusion reached earlier is substantiated. The values for the steady-state parameters derived for these mechanisms are presented in Table I.

Due to the fact that the mechanism for this protein is random, the $1/v$ vs. $1/[S]$ or $1/[PMS]$ plots should in theory be nonlinear. The PMS concentration is varied over only a 3.5-fold concentration range (see Table III) because at concentrations above $\sim$3 mM there is severe substrate inhibition (McIntire et al., 1985; McIntire, 1983) and at lower concentrations the rates are extremely slow. It is not surprising then that $1/v$ vs. $1/[PMS]$ plots are linear over such a limited range. The concentration for *p*-cresol, however, is varied over a 200-fold range, and $1/v$ vs. $1/[S]$ plots are also linear. In

many cases (particularly partial rapid equilibriummechanisms) the curvature is imperceptible (Cook & Cleland, 1981; Segel, 1975; Rudolph & Fromm, 1971; Cleland & Wratton, 1969).

Unexpectedly, the stopped-flow studies did not produce an isotope effect for the reduction of the isolated flavoprotein subunit by *p*-cresol. The reaction was independent of the concentration and deuterium content of this substrate. In contrast, the steady-state experiments provided both excellent data and an apparent intrinsic isotope effect of 7, the same value for the intrinsic effect obtained for the flavocytochrome. It is interesting that the kinetic studies of the flavoprotein, using the dyes PMS and DCIP, proved more definitive. The random steady-state mechanism indicates that PMS binds to the flavoprotein, eventually leading to a ternary complex, before oxidation of *p*-cresol occurs. It is possible that the presence of these dyes, as in the case of the flavocytochrome, alters the mechanism of this protein as well.

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