A New Procedure for Deconvolution of Inter-/Intramolecular Intrinsic Primary and α-Secondary Deuterium Isotope Effects from Enzyme Steady-State Kinetic Data

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Abstract: The A_2B_2 flavocytochrome *p*-cresol methylhydroxylase (PCMH) from *Pseudomonas putida* oxidizes 4-methylphenol (*p*-cresol) to 4-hydroxybenzyl alcohol in a process requiring scission of an α-C−H bond with concomitant reduction of covalently bound FAD in each A subunit. Values of k_{cat}/K were determined from steady-state kinetic data for the reactions of PCMH with the following substrates: 4-methylphenol, 4-($^2H_1$)-methylphenol, 4-($^2H_2$)methylphenol, and 4-($^2H_3$)methylphenol. A procedure was devised to extract the intrinsic primary deuterium and intrinsic α-secondary deuterium kinetic isotope effects from these values of k_{cat}/K. The primary effect, *P*, is 6.71 ± 0.08, and the secondary effect, *S*, is 1.013 ± 0.014. The magnitudes of these effects are discussed in terms of an early or late transition state, hydrogen tunneling, coupled motion between the leaving and remaining hydrogens of the methyl group, and a H^- expulsion mechanism versus a substrate radical mechanism versus a covalent substrate−FAD intermediate mechanism. The reaction of 4-ethylphenol with PCMH produces 4-vinylphenol and (−)-*S*-1-(4-hydroxyphenyl)ethanol (∼100% enantiomeric excess). The evidence indicates that these are formed from a common intermediate, presumably a *p*-quinone methide. From the partition ratios for the formation of the alcohol and 4-vinylphenol from 4-ethylphenol, 4-(1′,1′-$^2H_2$)-ethylphenol, and 4-(2′,2′,2′-$^2H_3$)ethylphenol, the primary isotope effect for conversion of the *p*-quinone (2′,2′,2′-$^2H_3$)methide to 4-(2′,2′,2′-$^2H_2$)vinylphenol was estimated to be about 2, and the α-secondary isotope effect for conversion of *p*-quinone (1′-$^2H_1$)methide to 1-(4-hydroxyphenyl)−(1′-$^2H_1$)ethanol was found to be inverse (=0.83), as expected for sp^2 to sp^3 hybridization change at the α-carbon. Values of k_{cat}/K were determined for 4-ethylphenol, *R,S*-(±)-4-(1′-$^2H_1$)ethylphenol (abbreviated *R,S*-D), *S*-(−)-4-(1′-$^2H_1$)ethylphenol (*S*-D), *R*-(+)-4-(1′-$^2H_1$)ethylphenol (*R*-D), and 4-(1′,1′-$^2H_2$)ethylphenol (D2). The $^{D2}(k_{cat}/K)$ value was found to be 5.1−6.1, the same as determined in an earlier study. Unexpectedly, the values for $^{R,S-D}(k_{cat}/K)$, $^{S-D}(k_{cat}/K)$, and $^{R-D}(k_{cat}/K)$ were all about the same (∼1.7), indicating that there is nearly an equal probability for *pro-R* or *pro-S* C−H bond scission. An apparent flux ratio for the *pro-S* path/*pro-R* path was estimated to be 0.78 ± 0.02. The same procedure devised to determine values for *P* and *S* for 4-methylphenol was used to determine these values for the 4-ethylphenol reaction (commitment to catalysis = 0); $P = 5.98 \pm 0.12$ and $S = 0.967 \pm 0.021$. These values are essentially the same as those determined for 4-methylphenol. Thus, the chemical mechanisms for both substrates are assumed to be similar.

Introduction

Intrinsic primary and especially secondary deuterium isotope effects have proven to be extremely useful in deciphering the exact chemical mechanisms of action for numerous enzymes.[1−3] Revelations, not to mention a great deal of theoretical and experimental work, of the past two decades have indicated that interpretation of these effects are complicated by the possible involvement of H/D tunneling and/or coupled motion of secondary H/D with the breaking of the primary H/D−C bond.[1,2] However, these complexities have not diminished the interest or importance of deuterium isotope effects.[2−4] Thus, development of new, fast, and convenient methods of extracting these effects from kinetic data is of general interest. While working with several enzymes that oxidize methyl groups of their substrates, we pondered different methods for determining the intrinsic primary and secondary effects for the transformations of these groups.

When only the trideuteriomethyl substrate is compared with unlabeled substrate, the true primary and α-secondary effects are impossible to separate, since the measured isotope effect is intramolecular in nature. The observed effect is the product of primary, *P*, and secondary effects, *S*, $KIE = PS^2$, since one deuterium is removed and two secondary deuteriums remain. The special nature of the methyl group allows for each H/D

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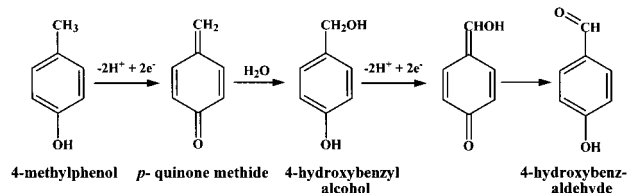
position to be spatially equivalent, assuming that there is little or no isotope effect for positioning H or D for bond scission. Under some circumstances, it might be possible to extract the intrinsic effects from steady-state kinetic constants for the unlabeled (D0), the mono- (D1), the di- (D2), and the trideuterated (D3) substrates.

For D1, D2, and D3, steady-state constants k_{cat}/K_m and k_{cat} will be affected by intra- and/or intermolecular isotope effects. With D1, some molecules will undergo scission of a C–H bond, whereas others will undergo a C–D bond scission. Therefore, the effect on the kinetic constants will be intermolecular (competition between two molecules) in nature. In contrast, for D2, some molecules will undergo C–D scission and will produce an *SP* intramolecular isotope effect, while other molecules will undergo C–H bond cleavage, for an intramolecular S^2 effect. In this case, the alteration in values of kinetic parameters will result from inter- and intramolecular effects. As mentioned earlier, the D3 rate constants will be influenced by an intramolecular effect only.

This special nature of the methyl group has been appreciated by other research groups, and methyl intra-/intermolecular effects have been measured for a number of enzymes.[5−8] However, in these cases, the isotope effects were derived by analyzing the deuterium content of extracted material by mass spectral analyses. While considering this approach, it occurred to us that, under the right circumstances, it should be possible to extract intrinsic primary and secondary deuterium isotope effects directly from an analysis of steady-state kinetic data.

It was decided to test this notion using the A form of *p*-cresol (4-methylphenol) methylhydroxylase [PCMH; *p*-cresol:(acceptor) oxidoreductase (methyl hydroxylating); EC 1.17.99.1] from *Pseudomonas putida* N.C.I.M.B. 9869. PCMH is composed of two flavoprotein subunits and two *c*-type cytochrome subunits.[9] The enzyme belongs to a subclass of flavoproteins that have FAD covalently bound at the 8α-carbon of the isoalloxazine ring.[10−12] The preferred substrate, 4-methylphenol (*p*-cresol), is thought to be oxidized by bound FAD to *p*-quinone methide, which is hydrated to give product, 4-hydroxybenzyl alcohol. This alcohol subsequently can be converted to 4-hydroxybenzaldehyde by oxidized enzyme. PCMH can convert a variety of other 4-alkylphenols to α-carbinols and, interestingly, to 4-vinylphenols. The α-carbinols derived from *n*-alkylphenols are further oxidized by PCMH to the α-carbonyl derivatives.[13,15] PCMH presents a case that should be ideal for testing the method for direct extraction of the isotope effects from steady-state kinetic data, since it was found that $^D(k_{cat}/K_m) = {^D}k_2$ for 4-($^2H_3$)-methylphenol.[14]

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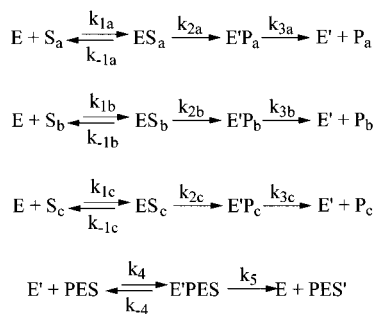
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Scheme 1

Previously, it was demonstrated that 4-ethylphenol was converted to optically active 1-(4-hydroxyphenyl)ethanol, which was composed of 66% *S*-isomer and 34% *R*-isomer.[16] Using an improved method described herein, it was demonstrated that the alcohol product of 4-ethylphenol, in fact, is >97% *S*-isomer. In another report, the intrinsic deuterium kinetic isotope effects (KIEs) were determined for 4-methylphenol/4-($^2H_3$)methylphenol (KIE = 7.05) and for 4-ethylphenol/4-(1′,1′-$^2H_2$)ethylphenol (KIE = 4.8−5.3).[14] However, because of the multiple deuterium label in the α position, the isotope effects are the product of the intrinsic primary and intrinsic α-secondary effects.

The following compounds were synthesized with the aim of determining the magnitudes of the intrinsic primary and secondary deuterium isotope effects and the stereochemistry of the reactions catalyzed by PCMH: *R*-, *S*-, and *R,S*-4-(1′-$^2H_1$)-ethylphenol; 4-(1′,1′-$^2H_2$)ethylphenol; 4-(2′,2′,2′-$^2H_3$)ethylphenol; 4-($^2H_1$)methylphenol, 4-($^2H_2$)methylphenol, and 4-($^2H_3$)-methylphenol; and *R*-, *S*-, and *R,S*-1-(4-hydroxyphenyl)ethanol and the corresponding 1′-^2H-ethyl derivatives. This treatise presents the results of studies carried out using these compounds. It will be demonstrated that the true primary and secondary intrinsic effects can be extracted from the values of the various $(k_{cat}/K_m)_{H/D}$ constants for 4-methylphenol. It will also be demonstrated, with reasonable assumptions, that these effects can also be derived from the values of $(k_{cat}/K_m)_{H/D}$ for the 4-ethylphenol derivatives, which undergo oxidation of an α-methylene carbon atom. Because the binding of 1-(4-hydroxyphenyl)ethanol is slow and rate limiting, it was not possible to determine the isotope effects from steady-state data.

Results

Intrinsic Primary and Secondary α-Deuterium Kinetic Isotope Effects for the Oxidation of 4-Methylphenol. The proposed mechanism for PCMH oxidation of 4-methylphenol is presented in Scheme 1. PCMH converts 4-methylphenol to a putative *p*-quinone methide by removal of the equivalent of two protons and two electrons. The quinone methide is subsequently attacked by water at its most nucleophilic center to produce an alcohol. A similar oxidation of the alcohol by the enzyme yields 4-hydroxbenzaldehyde. Previously, a large isotope effect (7.03 ± 0.41) on k_{cat}/K_{4-MP} was found with 4-methylphenol (4-MP) and 4-($^2H_3$)methylphenol as substrates. Stopped-flow kinetic measurements confirmed that this is the intrinsic effect.[14] However, this effect is the product of intrinsic primary and α-secondary effects. With the goal of measuring these two intrinsic effects, k_{cat}/K_{4-MP} values were determined from steady-state kinetic assays done at a fixed concentration of PES (1.0 mM phenazine ethosulfate), with varying concentrations of 4-methylphenol (D0), 4-($^2H_1$)methylphenol (D1), 4-($^2H_2$)-methylphenol (D2), or 4-($^2H_3$)methylphenol (D3). Earlier,[14] it was found that the reaction of PCMH with PES and 4-methylphenol obeyed a ping-pong mechanism; thus, the slopes of $1/v$ vs $1/[4\text{-methylphenol}]$ plots at all [PES] are parallel and will provide the true value for k_{cat}/K_{4-MP} (the slope of $1/v$ vs

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Scheme 2

1/[4-methylphenol] plots is (K_{4-MP}/k_{cat}), regardless of the [PES]. The values of the parameters Q_X (= k_{cat}/K_X) and K_X were determined for X = D0, D1, D2, or D3 by nonlinear regression analysis[13] using eq 1.[17]

$$v = \frac{k_{cat}[S]}{K_X + [S]} = \frac{(k_{cat}/K_X)[S]}{1 + [S]/K_X} = \frac{Q_X[S]}{1 + [S]/K_X} \quad (1)$$

From the values of k_{cat}/K_X, three isotope effects on this parameter were calculated. This series of experiments was done four times (Table 1). For each set of experiments, different solutions of D0, D1, D2, D3, and PCMH were used to minimize systematic error. The $^{D3}(k_{cat}/K_{4-MP})$ isotope effect (weighted average = 6.63 ± 0.08) is the same as determined earlier.[14] By direct inspection of these values, it is not possible to discern the values of the true intrinsic primary and α-secondary isotope effects.

Scheme 2 presents the model used to derive the equations necessary to extract the intrinsic primary and secondary effects. In the scheme, a substrate subscript represents one of the three methyl hydrogens/deuteriums that can be removed; that is, for D1, S_a denotes substrate that binds with one of the hydrogens positioned to be removed, S_b denotes substrate that binds with the other hydrogen positioned to be removed, and S_c denotes substrate that is positioned for removal of deuterium. While isotope effects on binding of substrate have been reported, it is unlikely that the effect will be significant for a compound with a labeled methyl group. Thus, it was assumed that there are no isotope effects for substrate binding or release.[18] Therefore, for D0, $k_{1a} = k_{1b} = k_{1c} = k_1$, $k_{2a} = k_{2b} = k_{2c} = k_2$, and $k_{3a} = k_{3b} = k_{3c} = k_3$. Since $k_{-1} > k_2$ for PCMH,[14] even if the methyl group rotates when bound at the active site of PCMH (i.e., $ES_a \rightleftarrows ES_b \rightleftarrows ES_c \rightleftarrows ES_a$), the resulting equations are unchanged (assuming no isotope effect for rotation). (This is not true if $k_{-1} \leq k_2$, i.e., if the substrate were sticky.) For D1, $k_{2a} = k_{2b} = (s)k_2$ and $k_{2c} = (p)k_2$, where k_{2a} and k_{2b} are rate constants for steps when ^1H is removed, k_{2c} is the rate constant for ^2H removal, and k_2 is the rate constant for removal of ^1H from unlabeled 4-methylphenol. As a result, the change in magnitude of the values of steady-state parameters will be due to an intermolecular isotope effect. The constants $s = 1/S$ and $p = 1/P$, where S and P are the intrinsic secondary α-deuterium and intrinsic primary deuterium isotope effects, respectively. Similarly, for D2, $k_{2a} = k_{2b} = (sp)k_2$ (^2H removed) and $k_{2c} = (s^2)k_2$ (^1H removed). Thus, the observed effect is a combination of inter- and intramolecular isotope effects. The rate constants for D3 are $k_{2a} = k_{2b} = k_{2c} = (s^2p)k_2$, purely an intermolecular effect. This analysis also assumes that the rule of geometric mean holds: $k_{HHH}/k_{DHH} = k_{HHD}/k_{DHD} = k_{HDD}/k_{DDD}$ and $k_{HHH}/k_{HHD} =$

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$k_{HDH}/k_{HDD} = k_{DHH}/k_{DHD} = k_{DDH}/k_{DDD}$; i.e., the primary effect and secondary effects are independent (the first atom in the subscript is the one removed from substrate). The rule is invalid only when there is coupled motion between the removed and remaining H/D atoms.[2] The analysis of the isotope effect could also be complicated by tunneling. As will be discussed later, it is unlikely that both tunneling and coupled motion are at work in the reaction of PCMH. The last line in Scheme 2 represents the reoxidation of reduced E (E′) by PES. Since the mechanism is of a ping-pong type,[14] this portion of the mechanism is uncoupled from the half-reaction involving 4-methylphenol, and it is unaffected by the isotopic labeling of the substrate.

The equations for k_{cat}/K_{4-MP} for D0, D1, D2 and D3 are given below (eq 2a−d), where $C = k_2/k_{-1}$ is the forward commitment to catalysis factor[15,18,19] and $X = k_1k_2/k_{-1} = k_2/K_D$. Also provided are the same equations when $C = 0$ ($k_{-1} \gg k_2$), which is the case for the PCMH reaction with 4-methylphenol.[14]

$$\left(\frac{k_{cat}}{K}\right)_{D0} = \frac{X}{1+C} = X \quad (2a)$$

$$\left(\frac{k_{cat}}{K}\right)_{D1} = \frac{X(3spC + 2s + p)}{3(1 + [s+p]C + spC^2)} = \frac{X(2s+p)}{3} \quad (2b)$$

$$\left(\frac{k_{cat}}{K}\right)_{D2} = \frac{Xs(3s^2pC + s + 2p)}{3(1 + s^2C + spC + s^3pC^2)} = \frac{X(s^2 + 2sp)}{3} \quad (2c)$$

$$\left(\frac{k_{cat}}{K}\right)_{D3} = \frac{Xs^2p}{1 + s^2pC} = Xs^2p \quad (2d)$$

(See the Experimental Procedures section for details of the method used to derive the expressions in equation sets 2−5.) With $C > 0$, it seemed that it would be possible to solve these four equations for S (the intrinsic α-secondary effect) = $1/s$, P (the intrinsic primary effect) = $1/p$, and C. Unfortunately, the solutions for each of these parameters are transcendental functions. Simulations (unpublished results) indicate that the various k_{cat}/K_{4-MP} values would need to be measured accurately and precisely to approximately 1 part in 10 000 in order to determine the values of these parameters.

The same procedure used to derive eq 2a−d was invoked to derive eq 3a−d, which corrects for ^2H incorporation of less than 100% in the labeled 4-methylphenols.

$$\left(\frac{k_{cat}}{K}\right)_{D0} = X \quad (3a)$$

$$\left(\frac{k_{cat}}{K}\right)_{D1} = \frac{X(3[1 - 0.986] + 0.986[2s + p])}{3} = \frac{X(3[1 - 0.986] + 0.986[2/S + 1/P])}{3} \quad (3b)$$

$$\left(\frac{k_{cat}}{K}\right)_{D2} = \frac{X([1 - 0.993][2s + p] + 0.993[s^2 + sp])}{3} = \frac{X([1 - 0.993][2/S + 1/P] + 0.993[1/S^2 + 2/SP])}{3} \quad (3c)$$

$$\left(\frac{k_{cat}}{K}\right)_{D3} = \frac{X([1 - 0.974][s^2 + 2sp] + 3(0.974)[s^2p])}{3} = \frac{X([1 - 0.974][1/S^2 + 2/SP] + 3(0.974)/S^2P)}{3} \quad (3d)$$

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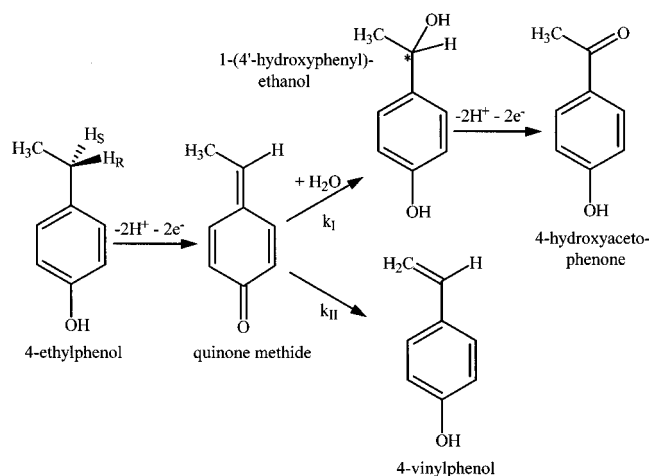
Table 1. Values of $(k_{cat}/K_S)_{Dx}$,[a] $^{Dx}(k_{cat}/K_S)$, and Intrinsic Primary (P) and Secondary (S) Deuterium Isotope Effects for 4-Methylphenol and 4-Ethylphenol

4-methylphenol	$(k_{cat}/K_S)_{Dx}$ ($\mu M^{-1} s^{-1}$)				$^{D1}(k_{cat}/K_S)$	$^{D2}(k_{cat}/K_S)$	$^{D3}(k_{cat}/K_S)$	S	P
	$(k_{cat}/K_S)_{D0}$[b]	$(k_{cat}/K_S)_{D1}$	$(k_{cat}/K_S)_{D2}$	$(k_{cat}/K_S)_{D3}$					
set 1	8.06 ± 0.20	5.71 ± 0.14	3.63 ± 0.21	1.14 ± 0.04	1.41 ± 0.05	2.22 ± 0.14	7.09 ± 0.30	0.988 ± 0.029	7.52 ± 0.35
set 2	8.06 ± 0.17	5.73 ± 0.07	3.75 ± 0.06	1.32 ± 0.01	1.41 ± 0.03	2.03 ± 0.06	6.11 ± 0.14	0.960 ± 0.037	6.44 ± 0.25
set 3	8.06 ± 0.31	5.45 ± 0.08	3.21 ± 0.03	1.19 ± 0.01	1.48 ± 0.06	2.51 ± 0.10	6.79 ± 0.27	1.039 ± 0.011	6.71 ± 0.09
set 4	8.06 ± 0.08	5.52 ± 0.12	3.63 ± 0.12	1.16 ± 0.02	1.46 ± 0.03	2.22 ± 0.08	6.95 ± 0.13	1.000 ± 0.049	7.27 ± 0.50
weighted av[c]					1.44 ± 0.02	2.18 ± 0.04	6.63 ± 0.08	1.013 ± 0.014	6.71 ± 0.08

4-ethylphenol	$(k_{cat}/K_S)_{Dx}$ ($\mu M^{-1} s^{-1}$)					$^{R-D}(k_{cat}/K_S)$	$^{S-D}(k_{cat}/K_S)$	$^{R,S-D}(k_{cat}/K_S)$	$^{D2}(k_{cat}/K_S)$	S	P	A
	$(k_{cat}/K_S)_{H2}$[b]	$(k_{cat}/K_S)_{R-D}$	$(k_{cat}/K_S)_{S-D}$	$(k_{cat}/K_S)_{R,S-D}$	$(k_{cat}/K_S)_{D2}$							
set 1	70.0 ± 2.4	44.8 ± 2.3	47.0 ± 0.8	44.1 ± 0.7	13.7 ± 0.1	1.59 ± 0.06	1.49 ± 0.06	1.56 ± 0.10	5.09 ± 0.18	0.927 ± 0.060	5.96 ± 0.16	0.84 ± 0.07
										0.894 ± 0.078	6.18 ± 0.26	1[d]
set 2	70.0 ± 1.1		46.7 ± 1.0	40.3 ± 0.3	13.0 ± 0.2	1.73 ± 0.03	1.50 ± 0.04	1.70 ± 0.07	5.36 ± 0.31	0.967 ± 0.027	6.03 ± 0.16	0.76 ± 0.03
										1.030 ± 0.121	5.61 ± 0.71	1[d]
set 3	70.0 ± 0.2	39.8 ± 0.3	45.7 ± 0.6	40.0 ± 0.3	12.4 ± 0.2	1.75 ± 0.01	1.55 ± 0.02	1.76 ± 0.01	5.62 ± 0.08	1.020 ± 0.055	5.99 ± 0.83	0.82 ± 0.14
										1.041 ± 0.062	5.86 ± 0.89	1[d]
set 4	70.0 ± 0.9	36.3 ± 0.3	46.4 ± 0.5	40.6 ± 1.2	11.5 ± 0.2	1.72 ± 0.05	1.56 ± 0.03	1.93 ± 0.03	6.10 ± 0.11	1.158 ± 0.197	5.71 ± 1.11	0.51 ± 0.28
										1.109 ± 0.216	5.97 ± 1.33	1[d]
weighted av[c]						1.74 ± 0.01	1.54 ± 0.02	1.77 ± 0.01	5.69 ± 0.06	0.967 ± 0.021	5.98 ± 0.12	0.78 ± 0.02
										0.996 ± 0.044	6.09 ± 0.23	1[d]

[a] There was an approximately 10% variability in the values of $(k_{cat}/K_S)_{Dx}$ between sets, although the relative values of these in each set were nearly identical, as reflected in the ratioed values, $^{Dx}(k_{cat}/K_S)$, in this table. However, this variability does not affect the nonlinear least-squares analysis, since only data with one set are used to calcluate S, P, and A. The variability is due to using different enzyme preparations over the span of several years. (Also note that $k_{cat}/K_S = k_2/K_D$.) [b] To correct for the variability, the values of $(k_{cat}/K_S)_{Dx}$ for a set were normalized to a constant value of $(k_{cat}/K_S)_{D0}$. [c] The weighted average was calculated from $\Sigma[^D(k_{cat}/K_S)/\sigma^2]/(\Sigma 1/\sigma^2)$ and the error from $[\Sigma 1/\sigma^2]^{-1/2}$, where σ are the errors in the table. [d] Assumed values of A.

Scheme 3

Figure 1. Stereochemical analysis of racemic, diacetylated 1-(4-hydroxyphenyl)ethanol (top chromatogram) and the diacetylated alcohol formed on enzymic oxidation of 4-ethylphenol (lower chromatogram). The samples were chromatographed on a Pirkle type-1-A HPLC column: *n*-hexane/2-propanol, 97:3; flow rate, 1 mL/min; 254 nm detection. The peak at 4.7 min in the lower chromatogram is due to trace 4-ethylphenol.

The numbers 0.986, 0.993, and 0.974, respectively, are the fractions of α-d_1 in the D1 compound, α-d_2 in the D2 compound, and α-d_3 in the D3 compound. Pairs of these k_{cat}/K_{4-MP} equations can be solved for S and P; however, rather than solve for the parameters in this manner, we determined the values for S and P by adapting a nonlinear least-squares analysis program to solve the four equations simultaneously[20] for each separate set of k_{cat}/K_{4-MP} values given in Table 1. This resulted in four values of S and P, which are also provided in Table 1. The final values of S and P were determined by weighted averaging of the four values of each (see footnote to Table 1). The final values are $S = 1.013 \pm 0.014$ and $P = 6.71 \pm 0.08$.

If oxidation of 4-methylphenol proceeds via a hydride elimination mechanism, then *p*-quinone methide will form in the same step in which the α-C$-$H bond is broken. Also, if the reaction has a late transition state and if this mechanism is really operating, then on progressing from the ground state to the transition state, there might be a change in the force constants for the C$-$H bonds at the 2, 3, 5, and 6 positions, and oxidation of 4-methyl(2,3,5,6-^{2}H$_4$)phenol would show an isotope effect. The measured k_{cat}/K_{4-MP} isotope effect for 4-methyl(2,3,5,6-^{2}H$_4$)phenol was determined to be 1.001 ± 0.022.

Reaction of 4-Ethylphenol with PCMH. Unlike the oxidation of 4-methylphenol by PCMH, the reaction of the enzyme with 4-ethylphenol is more complex. First, in contrast to an early observation,[16] oxidation of 4-ethylphenol produces not only 1-(4-hydroxyphenyl)ethanol but also 4-vinylphenol.[21] Additionally, there are three distinct and potentially stereoselective steps in the oxidation of 4-ethylphenol to 4-hydroxyacetophenone (Scheme 3): (1) removal of the *pro-R* or *pro-S* hydrogen from 4-ethylphenol; (2) addition of water to the *re* or *si* side of the *p*-quinone methide; and (3) oxidation of *R*- or *S*-1-(4-hydroxyphenyl)ethanol. In an early study,[16] it was determined that PCMH oxidation of 4-ethylphenol produces 66% *S*-1-(4-hydroxyphenyl)ethanol and 34% of the *R*-isomer [32% enantiomeric excess (ee) of the *S*-isomer]. While millimolar amounts of the alcohol were formed from 3$-$4 mM 4-ethylphenol, only micromolar amounts of the alcohol were converted to 4-hydroxyacetophenone. Thus, the low enantiomeric excess of the *S*-isomer cannot be the result of preferential oxidation of the *S*-alcohol to ketone by PCMH. In the previous work, the alcohol was generated in a reaction mixture that contained PCMH, 4-ethylphenol, PMS (phenazine methosulfate) to reoxidize the
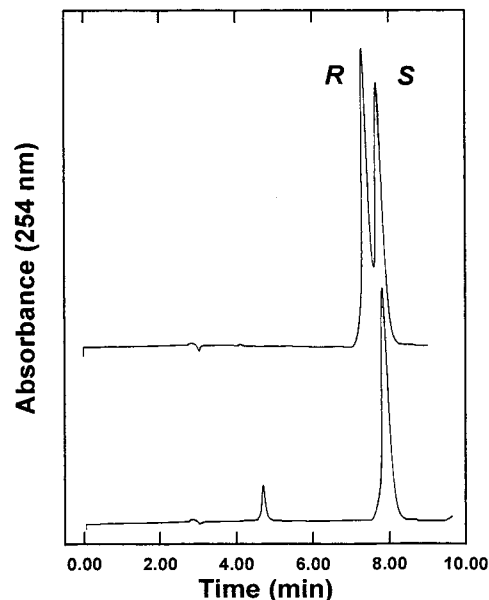
enzyme, KCN to minimize enzyme inhibition by PMS, and catalase. The reaction was carried out at pH 9.5, with shaking in air for 2 h in order to maximize the concentration of dissolved O_2, a necessity for oxidation of reduced PMS. Catalase destroys H_2O_2 formed in the oxidation of reduced PMS.[16] Unfortunately, the resulting 1-(4-hydroxyphenyl)ethanol required extensive purification to remove the other components of the mixture and base-catalyzed breakdown products of PMS.[21] This and the high pH of the reaction mixture caused concern about the stereochemical outcome of the experiment; therefore, a new method was devised to recycle oxidized enzyme at pH 7.6. The new procedure required far fewer manipulations to obtain pure alcohol formed by enzymic oxidation of 4-ethylphenol. For this method, substrate-reduced PCMH was oxidized by horse heart cytochrome *c* at low ionic strength, and the resulting reduced cytochrome was reoxidized by bovine cytochrome *c* oxidase. The reaction was stirred in air, and reduced cytochrome oxidase was oxidized by conversion of O_2 to H_2O. After the reaction was quenched with $(NH_4)_2SO_4$, protein was removed by ultrafiltration, and 4-ethylphenol and its reaction products were isolated by HPLC. In early experiments, the 1-(4-hydroxphenyl)ethanol obtained in this manner was acetylated in order to separate the *R*- and *S*-isomers on a Pirkle column (Figure 1).[22,23] Several repeat experiments gave similar results. It was estimated that the product alcohol was >97% *S*-isomer (> 94% ee). In more recent experiments, underivatized alcohol samples were chromatographed on a Chiracel column[24,25] (data not shown; see Figure 5 for separation of *R*- and *S*-isomers on the Chiracel column), and the results were the same as for the Pirkle column analyses.

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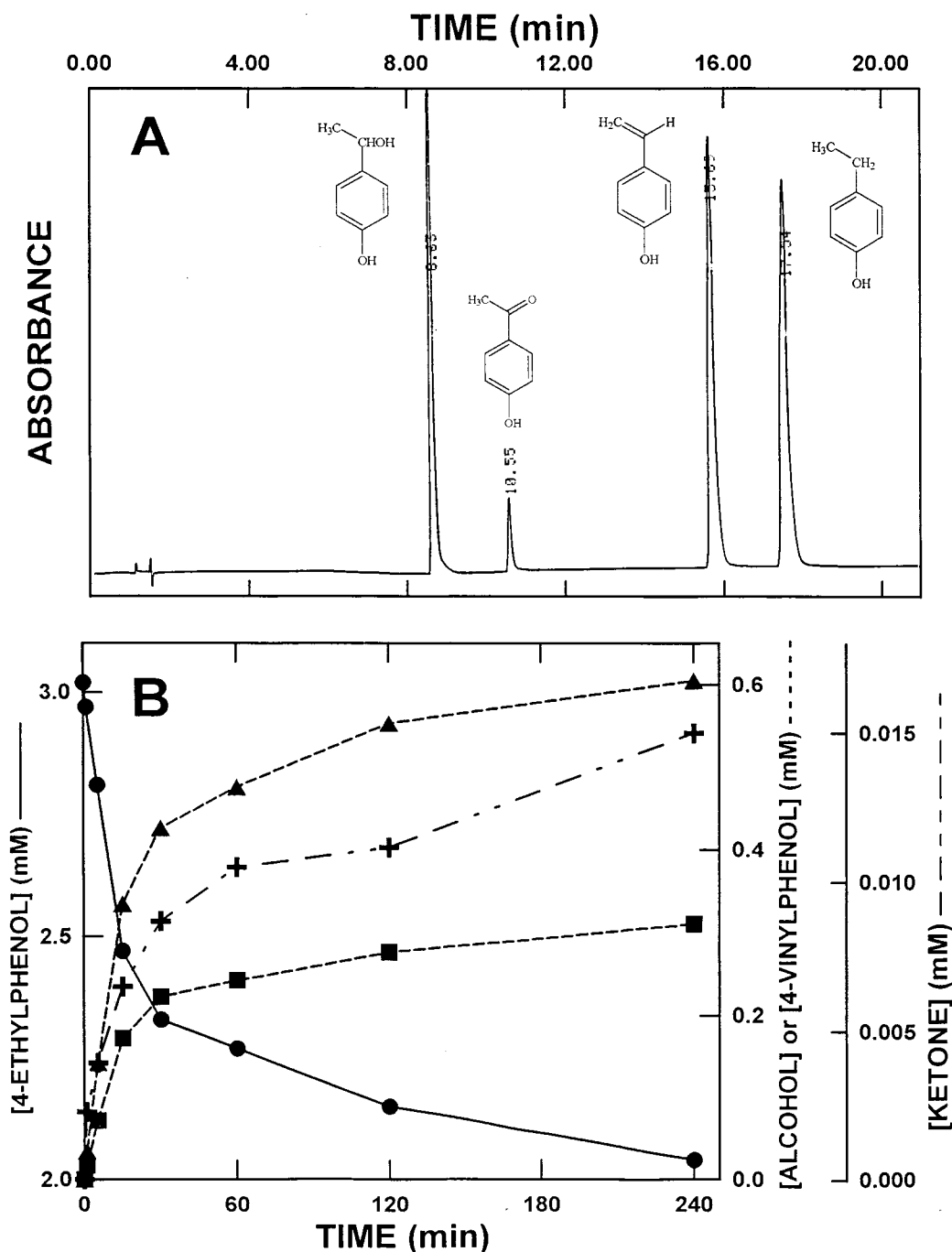
Figure 2. (A) HPLC separation of 4-ethylphenol and its enzymic oxidation products. The reaction of 4-ethylphenol (3 mM) with PCMH was carried out in 10 mM Tris-HCl, pH 7.6, $I = 0.01$, in the presence of the reoxidizing substrate, horse heart cytochrome c. The various phenolic compounds were isolated as described in the Experimental Procedures section. Shown is a chromatogram of a sample of the reaction mixture that had incubated at room temperature for 8 h. A Hypersil ODS, 5-μm, 0.46- $\times$ 25-cm column was used: 10% (v/v) MeOH/H$_2$O for 3 min, then from 10 to 40% (v/v) MeOH/H$_2$O in 12 min; 2 mL/min flow rate; 280 nm detection; 20 μL injected. (B) Time course for oxidation of 4-ethylphenol by PCMH (see above for conditions). At various times over a 240-min period, samples were removed and processed as described in the Experimental Procedures section. HPLC analyses of small aliquots for each time-point sample were done as described above. Concentrations were determined by integrating the peak areas and relating these to the areas of the corresponding peaks of HPLC runs of 20-μL samples containing known concentrations of 4-ethylphenol, 4-vinylphenol, 1-(4-hydroxyphenyl)ethanol, and 4-hydroxyacetophenone. Plots: 4-ethylphenol, ● (left axis); 1-(4-hydroxyphenyl)-ethanol, ▲ (close right axis; label, ALCOHOL); 4-vinylphenol, ■ (close right axis); 4-hydroxyacetophenone, + (far right axis; label, KETONE).

In addition to 1-(4-hydroxyphenyl)ethanol and 4-hydroxac-etophenone, a third product of enzymic oxidation of 4-ethylphe-nol was detected by reversed-phase HPLC (Figure 2A).[21] The third product was shown to be 4-vinylphenol. After purification, the material was methylated with diazomethane and shown to have the same UV−visible spectrum and reversed-phase HPLC properties as commercial 4-vinylanisole.[21] Mass spectral analy-ses confirmed that 4-vinylphenol had been produced (vide infra).

The time course for formation of the various products from enzymic oxidation of 4-ethylphenol is shown in Figure 2B. For the reaction of PCMH with 4-(2-propyl)phenol or 4-(n-propyl)-phenol, the corresponding α-carbinols and α-alkene formed, but not the α-carbonyls (it is not possible for 4-(2-propyl)phenol to form the carbonyl). On enzymic oxidation of 5-indanol and 6-hydroxytetralin, corresponding α-carbinols, α-carbonyls, and α-alkenes formed.[21]

Two observations are apparent from Figure 2B. First, 4-ethylphenol is not completely consumed in the reaction. Even after 24 h, the relative amounts of each component are little changed from those measured at 240 min in Figure 2A. Second, the ratio of [4-vinylphenol] and [1-(4-hydroxyphenyl)ethanol] formed on oxidation of 4-ethylphenol is the same at each time point. To understand the first observation, assays were carried out with an oxygen electrode. In an assay with cytochrome *c* oxidase, 40 μM horse heart cytochrome *c*, 1.34 mM 4-ethylphenol, and 14 μg/mL of PCMH, the initial rate was 2.5 μmol of O_2 min^{-1} mg^{-1}, and the reaction leveled off after about 45 min, when $\sim$0.20 μmol of O_2/mL was consumed (at 25 °C, air-saturated buffer contains 0.26 μmol/mL of O_2[26]). Inclusion of 0.1 mM 1-(4-hydroxyphenyl)ethanol or 4-hydroxyacetophenone had little effect on the initial rate or the leveling off. However, inclusion of 0.5 mM 4-vinylphenol reduced the initial rate dramatically. Preincubation of PCMH with 4-ethylphenol, 1-(4-hydroxyphenyl)ethanol, or 4-hydroxyacetophenone made no difference. PCMH was not irreversibly inhibited because addition of saturating [4-methylphenol] to the mixture, after O_2 consumption ceased, gave a return of O_2 consumption at a rate slightly lower than that seen when only 4-methylphenol was present as substrate. It was concluded that 4-vinylphenol was acting as a good competitive inhibitor for the 4-ethylphenol (K_{4-EP} = 2.6 mM) reaction but a poor inhibitor for the 4-methylphenol (K_{4-MP} = 16 μM) reaction; i.e., it was assumed that, for 4-vinylphenol, 16 μM < K_i = K_D < 2.6 mM. Subsequent steady-state kinetic experiments proved that 4-vinylphenol was a competitive inhibitor for 4-ethylphenol. The K_i value was determined to be 94.3 ± 7.2 μM.

With regard to the second observation from Figure 2B (the constant ratio [1-(4-hydroxyphenyl)ethanol]/[4-vinylphenol] at all time points), two explanations are can be advanced. (1) Two independent, competing pathways lead from 4-ethylphenol to 4-vinylphenol and 1-(4-hydroxyphenyl)ethanol, respectively. In this case, ratio of the rates (partition ratio) of formation of the two products will be $v_a/v_b = \{(k_{cat}/K)_a[S_a]\}/\{(k_{cat}/K)_b[S_b]\}$, even when 4-vinylphenol product inhibition is occurring.[27] Since S_a and S_b are both 4-ethylphenol, then $v_a/v_b = (k_{cat}/K)_a/(k_{cat}/K)_b$. Thus, the partition ratio is independent of [4-ethylphenol]. (2) 4-Vinylphenol and 1-(4-hydroxyphenyl)ethanol are formed from a common intermediate, i.e., the *p*-quinone methide in Scheme 3. For this situation, the partition ratio is k_I/k_{II} (Scheme 3), which is also independent of [4-ethylphenol].

Ratios of [1-(4-hydroxyphenyl)ethanol]/[4-vinylphenol] were determined from HPLC experiments at various times in the reaction of 4-ethylphenol with PCMH (see Figure 2), and then these were averaged for each experiment. For 3.23, 1.62, 0.642, and 0.10 mM initial concentrations of 4-ethylphenol, averaged ratios, PR1, were 2.11 ± 0.08, 1.96 ± 0.04, 1.96 ± 0.05, and 2.05 ± 0.09, all equivalent within experimental error. These results support either the parallel[1] pathway or the branched-[2] pathway mechanism. In a similar manner, the partition ratios for 4-(1′,1′-^{2}H$_2$)ethylphenol and 4-(2′,2′,2′-^{2}H$_3$)ethylphenol were determined to be 2.53 ± 0.05 (PR2) and 4.05 ± 0.13 (PR3), respectively. If the parallel pathway mechanism were operating, and the mechanism in each path were similar, it might be expected that the ratios for 4-ethylphenol, 4-(1′,1′-^{2}H$_2$)-ethylphenol, and 4-(2′,2′,2′-^{2}H$_3$)ethylphenol would be similar; i.e., the isotope effects in each path are similar and would cancel

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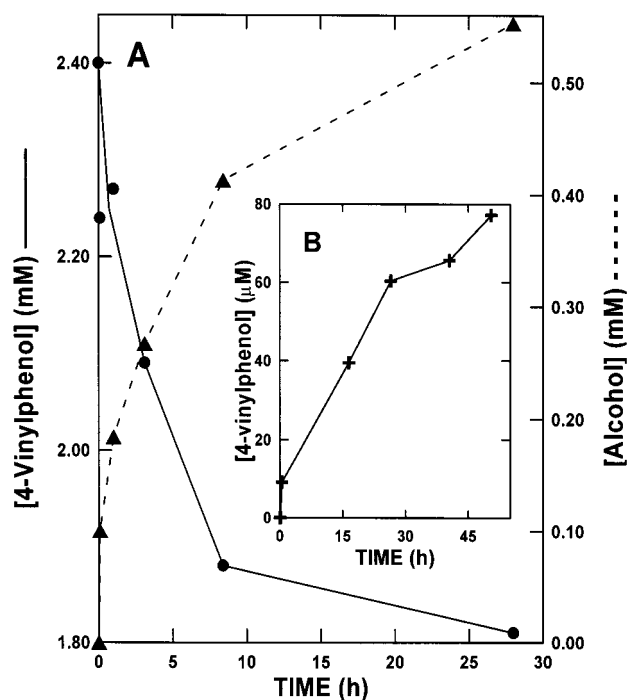

Figure 3. (A) Time course for the anaerobic reaction of 2.4 mM 4-vinylphenol with reduced PCMH (see the Experimental Procedures section for details). The plot for the change in [4-vinylphenol] is denoted by ● (left axis), and the plot for the change in [1-(4-hydroxyphenyl)-ethanol] is indicated by ▲ (right axis; label, Alcohol). (B, Inset) Time courses for the anaerobic reaction of 1-(4-hydroxyphenyl)ethanol with reduced PCMH. The plot displays the increase in [4-vinylphenol]. The change in [1-(4-hydroxyphenyl)ethanol] was too small to be measured reliably.

in the partition ratio. However, this would not be the case if the two products formed from a common intermediate (step II in Scheme 3).

To help distinguish between the two mechanisms, PCMH was anaerobically incubated with 4-vinylphenol or 1-(4-hydroxy-phenyl)ethanol. Figure 3A shows the results of an experiment where 2.4 mM 4-vinylphenol was incubated with reduced PCMH. It is apparent that a very slow conversion of 4-vi-nylphenol to 1-(4-hydroxyphenyl)ethanol occurred. No such conversion of 4-vinylphenol resulted when PCMH was absent, or when oxidized PCMH was present. The second control reaction was done under aerobic conditions in the presence of cytochrome *c* and cytochrome *c* oxidase so that any reduced PCMH would be quickly reoxidized.

Figure 3B presents the results of an experiment in which 3.4 mM 1-(4-hydroxyphenyl)ethanol is incubated with reduced PCMH. In this case, there is a very slow formation of very low amounts of 4-vinylphenol. 4-Vinylphenol was not formed when PCMH was excluded. These observations could be rationalized with the parallel pathway mechanism only if 4-vinylphenol and 1-(4-hydroxyphenyl)ethanol can be converted to 4-ethylphenol by reduced PCMH. In the reaction mixture containing only 1-(4-hydroxyphenyl)ethanol at *t* = 0, reoxidation of reduced PCMH by this compound would produce 4-ethylphenol. Since 1-(4-hydroxyphenyl)ethanol is present in such large excess, all of the newly oxidized PCMH would be reduced by the alcohol, forming 4-hydroxyacetophenone. Thus, it is expected that equimolar amounts of 4-ethylphenol and 4-hydroxyacetophe-none and very low levels of 4-vinylphenol would be seen. Additionally, slow reoxidation of reduced enzyme by trace oxygen did not take place to an appreciable extent, because

oxidized enzyme formed in this manner would be reduced by excess 1-(4-hydroxyphenyl)ethanol to form 4-hydroxyacetophenone. Almost no 4-hydroxyacetophenone, and no 4-ethylphenol, was detected in the reaction mixture after 50 h. It is concluded that 4-vinylphenol and 1-(4-hydroxyphenyl)ethanol interconvert through the putative *p*-quinone methide intermediate. This provides prima facie evidence that these two compounds are formed by enzymic oxidation of 4-ethylphenol via the branched pathway mechanism; i.e., 4-ethylphenol is converted to the *p*-quinone methide, which can either be hydrated to form 1-(4-hydroxyphenyl)ethanol or tautomerized to 4-vinylphenol (Scheme 3).

Deuterium Isotope Effects in the Reaction of 4-Ethylphenol with PCMH. Using the mechanism in Scheme 3 and making some assumptions, it is possible to extract intrinsic deuterium isotope effects on steps I and II in the scheme. With 4-(1′,1′-^{2}H$_2$)ethylphenol as substrate, the *p*-quinone methide intermediate will be α-deuterated. The rate of conversion of this intermediate to 4-(1′-^{2}H$_1$)vinylphenol ($k_{II\alpha-d}$) should be similar to the that for α-hydrogenated material since the α-carbon sp^2 hybridization is maintain in the conversion (i.e., $k_{II\alpha-d} \approx k_{II\alpha-h}$). On the other hand, conversion of *p*-quinone methide to 1-(4-hydroxyphenyl)ethanol involves a change from sp^2 to sp^3 hybridization at the α-carbon and should result in an inverse α-deuterium isotope effect (i.e., $k_{I\alpha-d} > k_{I\alpha-h}$). The values for the pertinent partition ratios are PR1 = $k_{I\alpha-h}/k_{II\alpha-h}$ = 2.11 ± 0.08 and PR2 = $k_{I\alpha-d}/k_{II\alpha-d}$ = 2.53 ± 0.05 (see previous section). Thus, $k_{I\alpha-h}/k_{I\alpha-d} = {}^Dk_{I\alpha} \approx$ PR1/PR2 = 0.83 ± 0.04. This is the intrinsic secondary α-deuterium isotope effect for step I.

When 4-(2′,2′,2′-^{2}H$_3$)ethylphenol is the substrate, the β-methyl group of the *p*-quinone methide is fully deuterated. For step II, a large primary isotope is expected because a β-H/D is lost. On the other hand, in step I, the rate would be decreased for the deuterated intermediate due to a secondary β-deuterium isotope effect. The secondary effect would result from hyperconjugation of the deuterons with an α-carbonium center.[28] It is expected that the electron-releasing ability of the *p*-hydroxyl group would minimize the carbonium ion character of the α-carbon, thus minimizing the isotope effect. This argument has been used to explain the β-deuterium isotope effects observed in the solvolysis of para-substituted 1-phenylethyl chlorides.[29] The isotope effect decreased with the electron-releasing ability of the aromatic substituent, with 1-(4-methoxyphenyl)(2,2,2-^{2}H$_3$)ethyl chloride having the lowest effect (^Dk = 1.133). The low value was explained by a decreased "conjugative demand on the β-CH bond" due to the resonance contribution of structure 1.[29]
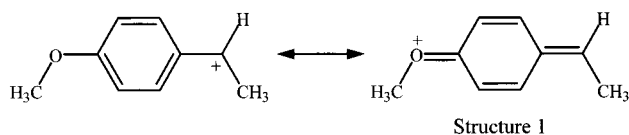
Structure 1

(Compare structure 1 with the *p*-quinone methide structure in Scheme 3.) Since the conjugative effect of a *p*-hydroxyl group is greater than that of a *p*-methoxy group, the β-deuterium isotope effect for the *p*-quinone methide intermediate in the PCMH reaction could be lower than 1.13. If we assume that
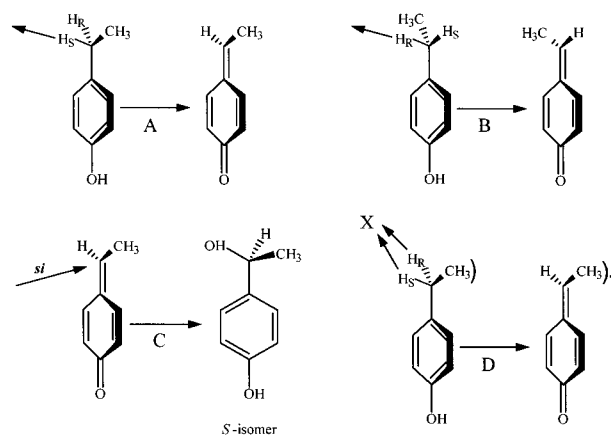
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Figure 4. Possible stereo-orientations of 4-ethylphenol at the active site of PCMH. Hypothetical reactions A and B indicate precise orientations of the C−H bond being cleaved: Reaction A, *pro-S* C−H bond cleaved; reaction B, *pro-R* C−H bond cleaved. Reaction C depicts the direction of attack by H$_2$O/HO$^-$ at the *si* side of PCMH-bound *p*-quinone methide intermediate, to produce the correct product, *S*-1-(4-hydroxyphenyl)ethanol. Reaction D is a cartoon of an orientation where the CH$_3$ group is more or less fixed in the active site; i.e., CH$_3$). In this orientation, there is a nearly equal probability that the *pro-R* or *pro-S* C−H bond will be cleaved. X represents the group to which the hydrogen is transferred, presumably FAD.

the β-deuterium isotope effect for step I in Scheme 3 is ∼1 (i.e., $k_{I\beta-d} \approx k_{I\beta-h}$), then the primary effect for step II is PR3/PR1 = $(k_{I\beta-d}/k_{II\beta-d})/(k_{I\beta-h}/k_{II\beta-h}) = k_{II\beta-h}/k_{II\beta-d} = {}^Dk_{II\beta} = 1.92$ ± 0.10. In contrast, if we assume a value of 1.3 as a maximum of the secondary β-effect (the maximum measured for solvolysis of substituted 1-phenylethyl chlorides),[29] then $^Dk_{II\beta} \approx 1.92/(1.3) \approx 1.5$.

In a prior study, the intrinsic isotope effects for the oxidation of 4-(1′,1′-^{2}H$_2$)ethylphenol by PCMH were found to be 5.21 ± 0.20 [=$^D(k_{cat}/K_m)$, steady-state kinetic parameters] and 4.94 ± 0.19 (=^Dk from stopped-flow kinetic studies).[14] However, as with 4-methylphenol, this effect results from participation of the intrinsic primary and intrinsic secondary α-deuterium effects. To isolate these two effects, the following compounds were used as substrates in steady-state kinetic experiments: 4-ethylphenol, *R*-(+)-4-(1′-^{2}H$_1$)ethylphenol, *S*-(−)-4-(1′-^{2}H$_1$)ethylphenol, *R,S*-(±)-4-(1′-^{2}H$_1$)ethylphenol, and 4-(1′,1′-^{2}H$_2$)ethylphenol. The kinetic measurements were done by varying [4-ethylphenol] and using 0.592 mM PES in 50 mM Tris-HCl, pH 7.6, I = 0.05 at 25 °C. Steady-state kinetic parameters were measured in four independent experiments. To minimize systematic errors, different substrate and PCMH solutions were used in each set of experiments. Since the 4-ethylphenol/PES reaction obeyed ping-pong-type kinetic behavior, accurate measurements of k_{cat}/K_{4-EP} at a single [PES] were obtained, and $^D(k_{cat}/K_{4-EP}) = {}^Dk_{intrinsic}$ because the commitment to catalysis factor, *C*, is zero.[14] The isotope effects for the four experiments are provided in Table 1. Surprisingly, all the α-monodeuterated ethylphenols, regardless of stereochemical nature, gave nearly identical isotope effects. This can only be rationalized by assuming that H/D in the *R*- and *S*-positions have an almost equal chance of being removed; that is, the reaction is not very stereoselective. If the H/D being removed at the α-position must be precisely positioned, then two binding orientations for 4-ethylphenol can be envisioned as depicted in A and B in Figure 4. Both orientations would have a nearly equal probability of existing. The resulting *p*-quinone methides for each would have opposite orientations; however, this conflicts with the fact that >97%

Scheme 4
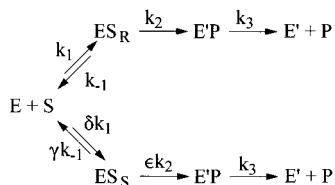
of S-1-(4-hydroxyphenyl)ethanol is formed; i.e., H_2O/HO^- always attacks at the *si* face of the α-carbon of the *p*-quinone methide in the active site of PCMH (reaction C, Figure 4). It is proposed that the CH_3 group is fixed, and the $C-H_R$ and $C-H_S$ bonds are cleaved with nearly equal propensity, resulting in transfer of either H to the same group in PCMH (reaction D, Figure 4).

Scheme 4 presents the mechanism that was invoked to derive equations for k_{cat}/K for 4-(1′,1′-$^2H_2$)ethylphenol, R-4-(1′-$^2H_1$)-ethylphenol, S-4-(1′-$^2H_1$)ethylphenol, and R,S-4-(1′-$^2H_1$)ethyl-phenol. The scheme presents two paths, one proceeding with removal of the *pro-R* hydrogen (ES_R) and the other with removal of the *pro-S* hydrogen (ES_S). It is assumed there is no isotope effect on binding. The most general situation presumes dif-ferential binding for 4-ethylphenol when either the *R* or *S* C−H bond is cleaved. The difference in binding and dissociation for the two branches is provided by δ and γ. It is also assumed that the rates of cleavage of the two C−H bonds are different (k_2 vs ϵk_2). E′P represents an equivalent (or nearly equivalent) enzyme/*p*-quinone methide species for each path. From the scheme, the following expressions were derived for k_{cat}/K for 4-ethylphenol (H2), 4-(1′,1′-$^2H_2$)ethylphenol (D2), R-4-(1′-$^2H_1$)-ethylphenol (R), S-4-(1′-$^2H_1$)ethylphenol (S), and R,S-4-(1′-$^2H_1$)-ethylphenol (RS), and assuming $C = k_2/k_{-1} = 0$:[14]

$$\left(\frac{k_{cat}}{K}\right)_{H2} = \frac{X(1 + (\epsilon/\gamma)C + A(1 + C))}{(1 + (\epsilon/\gamma)C)(1 + C)} = X(1 + A) \quad (4a)$$

$$\left(\frac{k_{cat}}{K}\right)_{R-D} = \frac{X(p + (\epsilon/\gamma)spC + A(s + spC))}{(1 + (\epsilon/\gamma)sC)(1 + pC)} = X(p + sA) \quad (4b)$$

$$\left(\frac{k_{cat}}{K}\right)_{S-D} = \frac{X(p + (\epsilon/\gamma)spC + A(p + spC))}{(1 + (\epsilon/\gamma)pC)(1 + sC)} = X(s + pA) \quad (4c)$$

$$\left(\frac{k_{cat}}{K}\right)_{R,S-D} = \{COMPLEX\} = \frac{X(1 + A)(p + s)}{2} \quad (4d)$$

$$\left(\frac{k_{cat}}{K}\right)_{D2} = \frac{spX(1 + (\epsilon/\gamma)spC + A(1 + spC))}{(1 + (\epsilon/\gamma)spC)(1 + spC)} = spX(1 + A) \quad (4e)$$

In these equations, the parameter A ($=\delta\epsilon/\gamma$) is a partition ratio, which is equal to $(k_2/K_D)_S/(k_2/K_D)_R = (k_{cat}/K)_S/(k_{cat}/K)_R$, since $k_2/K_D = k_{cat}/K$ for 4-ethylphenol, and $p = 1/P$ and $s = 1/S$, where P and S are the intrinsic primary deuterium isotope effect and the secondary α-deuterium isotope effect, respectively. Further, it is assumed that the ground states and transition states for the two pathways are essentially the same; thus, for both paths, values of S are the same, and values of P are the same. These equations can be modified to incorporate corrections for less than 100% deuterium content and stereochemical purity ("S-isomer" is assumed to 100% S, and "R-isomer" is assumed to be 83% R, based on the optical rotation experiments; see the Experimental Procedures section):

$$\left(\frac{k_{cat}}{K}\right)_{H2} = X(1 + A) \quad (5a)$$

$$\left(\frac{k_{cat}}{K}\right)_{R-D} = X\left(1 + 0.967\left[\frac{1}{S} - 1 + 0.83\left(\frac{1}{P} - \frac{1}{S}\right)\right] + A\left[1 + 0.967\left(\frac{1}{P} - 1 + 0.83\left[\frac{1}{S} - \frac{1}{P}\right]\right)\right]\right) \quad (5b)$$

$$\left(\frac{k_{cat}}{K}\right)_{S-D} = X\left(1 + 0.983\left[\frac{1}{S} - 1\right] + A\left[1 + 0.983\left(\frac{1}{P} - 1\right)\right]\right) \quad (5c)$$

$$\left(\frac{k_{cat}}{K}\right)_{R,S-D} = \frac{X(1 + A)(0.971[1/S + 1/P - 2] + 2)}{2} \quad (5d)$$

$$\left(\frac{k_{cat}}{K}\right)_{D2} = \frac{X(1 + A)([1 - 0.965][1/S + 1/P] + 2(0.965)(1/SP))}{2} \quad (5e)$$

(See the Experimental Procedures section for the method used to derive these equations.) In these equations, 0.967, 0.983, 0.971, and 0.965, respectively, are the fractions of the d_1 derivative in *R*-D, *S*-D, and *R,S*-D and the fraction of the d_2 derivative in D2. In eq 5b, 0.83 is the fraction of the *R*-D in the "*R*-isomer". These five k_{cat}/K values were measured in four separate experiments (Table 1). For each set, the values of S, P, and A were determined using the same nonlinear regression analysis used for the 4-methylphenol case. The final values of these parameters are the weighted averages of the four values given in Table 1. These average values are $S = 0.967 \pm 0.021$, $P = 5.98 \pm 0.12$, and $A = 0.78 \pm 0.02$. The analysis was redone assuming $A = 1$. In this case, the weighted average values of S and P are 0.996 ± 0.044 and 6.09 ± 0.23, respectively.

In the above analysis, it was assumed that the values of S were identical and the values of P were identical for both branches in Scheme 4. Alternatively, it can be assumed that the values of S are different and the values of P are different. Nonlinear least-squares analyses with the appropriate equations failed to converge to a minimum sum-of-squared residuals, regardless of the initial estimates of the parameters.

Oxidation of 1-(4-Hydroxyphenyl)ethanol by PCMH. As previously reported, PCMH can oxidize 1-(4-hydroxyphenyl)-ethanol to 4-hydroxyacetophenone.[13,15,21] Incubation of PCMH with racemic 1-(4-hydroxyphenyl)ethanol, and analysis of the chemical (reversed-phase HPLC) and stereochemical (HPLC analysis of the remaining alcohol with a Pirkle column) nature of the reaction mixture as a function of time, indicated that the *S*-isomer was preferentially oxidized to the *R*-isomer.[21] More recently, this reaction was monitored using the Chiracel OB column rather than the Pirkle column. The results of the analysis are shown in Figure 5. Figure 5A shows the separation of the *R*- and *S*-isomers that is achieved on the Chiracel column. Figure 5B present the progress curves for the reaction. Notice that the total [1-(4-hydroxyphenyl)ethanol] and [*R*-1-(4-hydroxyphenyl)-ethanol] converge after prolonged reaction time. Also shown in Figure 5B is a plot of ee (%) as a function of time. The data in this figure were analyzed using the equation $T = R^n/R_0^{n-1} + R$ (see the Experimental Procedures section for derivation), where T is the concentration of the total remaining alcohol, R is the concentration of the remaining *R*-isomer, R_0 is the initial *R*-isomer concentration, and $n = (k_{cat}/K_m)_S/(k_{cat}/K_m)_R$, the ratio of k_{cat}/K_m for the *S*- and *R*-isomers. The parameter n can be thought of as a partition or selectivity ratio for the isomers. This
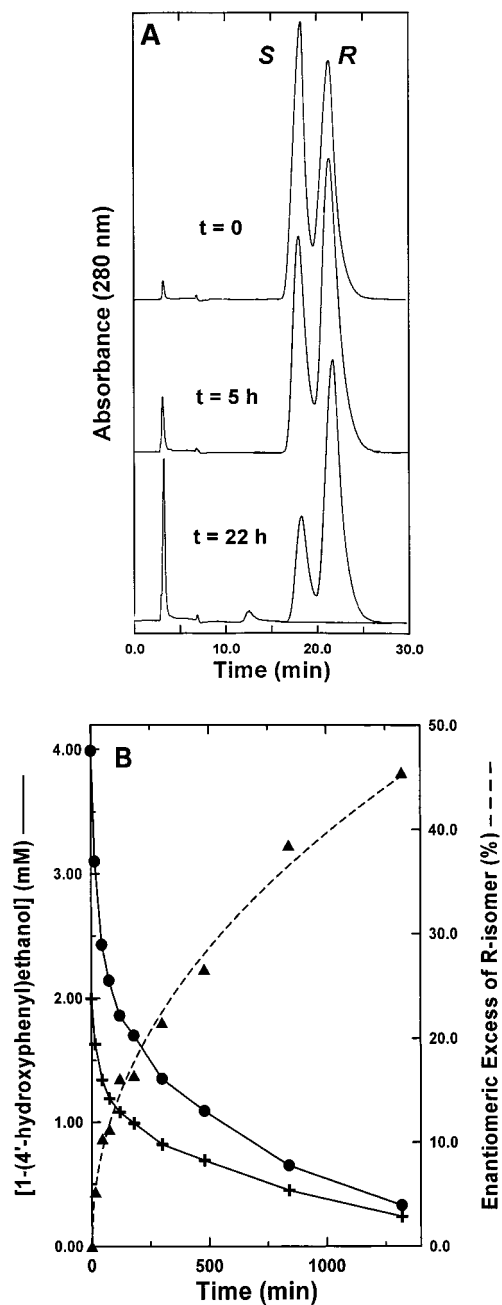
Figure 5. (A) HPLC separation of the remaining *R*- and *S*-1-(4-hydroxyphenyl)ethanol during a reaction started by mixing racemic alcohol and PCMH. Chromatography of each 5-μL sample was accomplished on a Chiracel OB column with 1 mL/min flow rate, using 92:8 (v/v) *n*-hexane/2-propanol, and 280 nm detection. Shown are chromatograms of the alcohol extracted from the reaction mixture at *t* = 0, 5, and 22 h (top to bottom), respectively. The increases in magnitude of artifact peaks at retention times 3.5 and 12.8 min are a result of increased detector sensitivity, which was required since progressively less material was extracted as the reaction proceeded. Graph B presents the time course for the reaction. The solid lines represent the remaining concentrations of *R*- and *S*-1-(4-hydroxyphenyl)ethanol (●) and of *R*-1-(4-hydroxyphenyl)ethanol (+), and the dashed line represents the remaining % enantiomeric excess (ee) of the *R*-isomer (▲).

equation was fit by nonlinear regression with *T* as the dependent variable, *R* as the independent variable, and R_0 as constant. The analysis provided *n* = 1.50 ± 0.01. This value indicates that, when the *R*-isomer and *S*-isomer are presented to PCMH in equimolar concentrations, the *S*-isomer is oxidized 1.5 times

faster than the *R*-isomer. Data obtained using the Pirkle column provided values of *n* = 1.57 ± 0.02 and 1.45 ± 0.03 for the oxidation of initially racemic 1-(4-hydroxyphenyl)ethanol and 1.39 ± 0.04 for oxidation of initially racemic 1-(4-hydroxyphenyl)(1-$^2H_1$)ethanol. Since binding of these alcohols to PCMH was very slow and rate limiting, it was not possible to determine the isotope effects from steady-state data.

Discussion

Herein we describe a new, quick, and convenient method for extracting intrinsic primary and intrinsic α-secondary deuterium isotope effects from k_{cat}/K values determined from steady-state kinetic measurements using unlabeled and mono-, di-, and trideuteriomethyl analogues of 4-methylphenol, or using unlabeled and mono- and dideuteriomethylene derivatives of 4-ethylphenol. These analyses can only be done if the commitment to catalysis factor, *C* (=k_2/k_{-1}) is very small compared to the intrinsic isotope effects. To determine the effect a finite commitment to catalysis would have on the values of *P* and *S*, calculations were carried out with assumed, small values of *C*. The equations in equation set 4 (4-ethylphenol) were written to include a constant value of *C* from 0 to 0.4. For each value of *C*, nonlinear least-squares analysis of the equations in this set was carried out as if the assumption *C* = 0 were valid. This analysis provided new values of *S*, *P*, and *A*. The results showed that apparent values of *P* and *S* increased by about 3−4% as *C* increased from 0 to 0.2 (data not shown). Earlier, it was reported that the $^D(k_{cat}/K)$ value for 4-($^2H_3$)methylphenol and the value $^Dk_2$ (the intrinsic effect) determined by stopped-flow measurements were identical.[14] As a result, we believe *C* is very small, and likely smaller than 0.2. However, even a value of *C* as high as 0.2 would not significantly alter conclusions drawn from an interpretation of the values of *S* and *P*. A similar analysis was done for 4-methylphenol isotope effects, and the conclusion concerning the impact of *C* is the same.

4-Methylphenol Oxidation by PCMH. Analysis of the steady-state kinetic data provided values of the intrinsic primary (*P*) and intrinsic secondary (*S*) α-deuterium isotope effects for 4-methylphenol: *P* = 6.71 ± 0.08 and *S* = 1.013 ± 0.014. Ours is not the first report of the concurrent determination of primary and α-secondary deuterium isotope effects. Intra-/intermolecular isotope effect studies of the oxidation of methyl groups of toluene[5,6] and *n*-octane by cytochrome P-450,[7] and oxidation of the methyl group of 4-methylanisole by chloroperoxidase,[8] have also provided primary and α-secondary kinetic isotope effects. For these studies, products were isolated, derivatized, and analyzed by mass spectrometry. In contrast, the method reported herein involved direct spectrophotometric steady-state assays. The procedure requires neither extraction nor derivatization and makes use of a good recording UV−visible spectrophotometer of the type available in biochemistry laboratories, and individual assays can be done in a few minutes. To our knowledge, ours is the first study to concurrently determine *S* and *P* via a direct steady-state kinetic method involving inter-/intramolecularly labeled substrates.

The value of *P* for 4-methylphenol is in the range expected for a semiclassical primary isotope effect, which suggests, but does not prove, that tunneling is absent during H/D transfer.[2,28,30,31] Significant tunneling could inflate the value of

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the primary effect out of the semiclassical range: $^D k > 7-10$. In the absence of tunneling, the large primary effect also suggests a symmetrical transition state.[31] An α-secondary effect of ~1 implies that significant tunneling, accompanied by coupled motion for the leaving and remaining H/D, does not take place. Significant coupled motion plus tunneling would increase the secondary effect and would decrease the primary effect.[2] Interestingly, the calculated semiclassical primary isotope effect for the reaction $C-CH_2CH_2Cl + HO^- \rightarrow CCH=CH_2 + Cl^- + H_2O$ increased from 5.03 to 7.07 when tunneling was included for a symmetrical transition state,[32] whereas the α-secondary effect changed little (from 1.048 to 1.053). As progressively more coupled motion was added to the model in the absence of tunneling, the semiclassical primary effect decreased significantly. The corresponding effect with tunneling varied somewhat but tended to increase. On the other hand, the semiclassical α-secondary effect did not change much as coupled motion increased in the absence of tunneling, but it increased significantly for the tunneling model as coupling increased (from 1.03 to 1.27).[32] For PCMH, several scenarios present themselves: (1) a symmetrical transition state with modest tunneling but no coupled motion; (2) a transition state with coupled motion without tunneling; and (3) a mechanism with an early transition state without coupled motion but with significant tunneling.

One way to detect H/D tunneling is to measure S and P at various temperatures. The Arrhenius equations for the H- and D-labeled substrates are $\ln(k_H) = \ln(A_H) + E_{aH}/(RT)$ and $\ln(k_D) = \ln(A_D) + E_{aD}/(RT)$, where A is the Arrhenius prefactor and E_a is the activation energy. Without tunneling, $A_H = A_D$, and plots of $\ln(k_H)$ vs $1/T$ and $\ln(k_D)$ vs $1/T$ should intersect the ordinate at the same point.[2] Unfortunately, the useful temperature range for PCMH is very narrow. While $^{D3}(k_{cat}/K_{4-MP})$ (7.03 ± 0.41) is apparently equal to the intrinsic effect ($^{D3}k_2$ at 25 °C and pH 7.6), at 6 °C, $^{D3}(k_{cat}/K_{4-MP}) = 3.43 \pm 0.15$ is considerably lower than the measured intrinsic effect (7.05 ± 0.22) at this temperature.[14] At 6 °C, $C = k_2/k_{-1} = 1.49$ is considerably greater than 1. Additionally, PCMH is unstable above 35 °C. Therefore, the feasible temperature range for k_{cat}/K_{4-MP} measurements would be 20–35 °C, a range that is too narrow for long extrapolation to $1/T = 0$ for $\ln(k)$ vs $1/T$ plots. Another way to determine if tunneling is present requires measurement of $(k_{cat}/K_{4-MP})_H/(k_{cat}/K_{4-MP})_T$ and $(k_{cat}/K_{4-MP})_D/(k_{cat}/K_{4-MP})_T$ (subscript T represents tritium).[2] Obviously, it is not practical (or very safe) to carry out spectrophotometric steady-state assays with tritium-labeled substrates.

The original hypothetical mechanism for PCMH required heterolytic cleavage of a C−H bond of the methyl group, with H$^-$ as the leaving group. This mechanism was formulated because the *p*-hydroxy group would facilitate H$^-$ expulsion, particularly if 4-methylphenol were bound in the phenolate form. The negative charge could be destabilized by other negative charges of PCMH in the vicinity of phenolate oxygen, or by the charged oxygen localized in a hydrophobic environment. Either or both of these conditions would force electron density on the phenolate oxygen into the benzene ring of 4-methylphenol, with concomitant weakening of the methyl C−H bond, thus facilitating H$^-$ elimination (structure 2). This would result in formation of the *p*-quinone methide (see Scheme 1). In this mechanism, there would be a change from sp^3 to sp^2 hybridization at the benzylic carbon. Equilibrium isotope effects for analogous E2 eliminations indicate that a maximal secondary α-deuterium isotope effect of 1.12–1.20 is expected (values

Structure 2
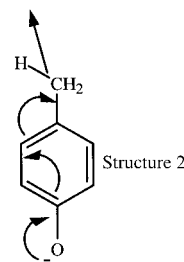
for a late transition state).[29,33] Further, secondary α-deuterium kinetic isotope effects of 1.13–1.30 were reported for solvolysis of meta- and para-substituted 1-phenylethyl chlorides. The lack of significant variability of the effect with substitution suggested that the "tightness of binding of the α-deuterium in the transition state is independent of the electrophilicity of the resulting carbonium ion". Further, "...the α-deuterium effect is nearly constant even though the amount of transition state double bond character must increase appreciably between 1-phenylethyl chloride and 1-(4-methoxyphenyl)ethyl chloride"[29] (see structure 1). If the proposed mechanism is operating for PCMH, a small secondary α-deuterium effect could indicate an early transition state; however, the magnitude of the primary effect suggests a rather symmetrical transition state, although, as mentioned earlier, an early transition state with tunneling could result in a large primary and small secondary effect.

A mechanism involving proton transfer followed by electron transfer is ruled out because of the high pK_a of the α-C−H. The pK_a of the methyl group of toluene is 28–35[34] or 54 (in acetonitrile), and the pK_a of *p*-methylanisole is 55 (in acetonitrile).[35] The resonance effect of the *p*-OH/O$^-$ would raise the pK_a.

Alternative mechanisms involve substrate-based radical formation. In one version, H$^•$ is generated by homolytic cleavage of an α-C−H bond of the methyl group, leaving an sp^3 carbon-centered radical. (While carbon-centered radicals can have either sp^3 or sp^2 hybridization,[36] for the substrates used in this study, this is not very feasible, unless the enzyme environs at the active site enforce an sp^3 structure.) Little change in hybridization would result in a low intrinsic α-secondary deuterium isotope effect.

Another variation of a radical mechanism assumes rapid electron abstraction from −OH or −O$^-$ of 4-methylphenol to leave a "stable" phenoxy radical. This is followed by slow explusion of H$^•$ from the methyl group (Scheme 5). This radical mechanism is untenable if the observation is valid that electron transfer from FAD to heme in PCMH is much faster than any bond-breaking steps. The first electron removed (step 1, Scheme 5) will convert FAD to semiquinone radical, but, presumably, the electron will very rapidly transfer to heme. As a result, the rate of heme reduction will not be coupled to α-C−H/D bond breakage and will not be sensitive to isotopic substitution. This is contrary to the observation that the rate of heme reduction in PCMH, apparently, fully reflects the isotope effect.[14] Thus, if this radical mechanism is operating, homolytic cleavage of the α-C−H/D bond must occur before electron transfer from FAD$^{•-}$ to heme and be rate limiting.

The driving force for removal of a single electron from substrate by FAD is not apparent. Electron transfer from FAD$^{•-}$

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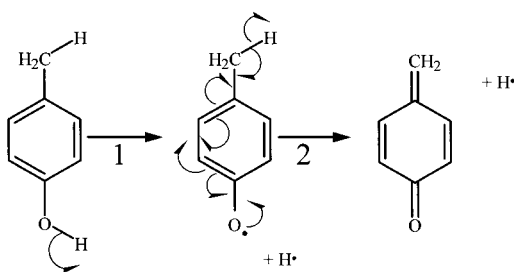
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Scheme 5

to heme in the absence of substrate is quite fast, at 200 s^{-1}.[37] However, the electron tunnels through 13 σ bonds and "jumps" 2.96 Å in its journey from flavin to the heme iron of the cytochrome subunit of PCMH.[38] As a result, there must exist a large thermodynamic driving force for electron transfer from flavin to heme. Therefore, the one-electron $E_7°$ for FAD bound to PCMH is expected to be much lower than +248 mV, the potential for the heme.[39] On the other hand, the one-electron reduction potential at pH 7.0 for the 4-methylphenol radical is +870 mV.[40] Thus, it seems unlikely that FAD could extract a single electron from the substrate. In addition, as discussed elsewhere,[41] protein-bound ground-state flavin does not have the reactivity for H$^{\bullet}$ abstraction. Such an abstraction could be accomplished by another reactive group, X, on the enzyme, which has a homolytic X–H bond dissociation energy greater than that for 4-methylphenol (86 kcal/mol, gas phase).[40] This group could be an amino acid-based radical. However, there is absolutely no evidence for this type radical in PCMH (e.g., electron spin resonance measurements). Finally, the 4-methylphenol dissociation energy quoted above is for the O–H bond of 4-methylphenol. It is expected that the α-C–H homolytic bond dissociation energy will be larger.

Last, mechanisms involving a covalent FAD/substrate were considered. For one such mechanism (Figure 6A), rather than direct formation of *p*-quinone methide, the negative charge on the para oxygen of the substrate is forced into to the benzene ring, which enforces a direct and rapid nucleophilic attack on FAD by the ring carbon at the 4-position of 4-methylphenol. The attack can be at the 4a-position of the isoalloxazine ring (see Figure 6) or the N5-position, resulting in a cyclohexadienone–FAD intermediate. This step is followed by slow, base-catalyzed abstraction of substrate α-proton, with concerted scission of the cyclohexadienone–FAD bond. This reaction yields the *p*-quinone methide intermediate and two-electron-reduced FAD. Note that, in this mechanism, the rate-determining heterolytic α-C–H/D bond cleavage would be reflected in the rapid reduction of heme by two-electron-reduced FAD. A variation of this mechanism involves rapid attack of FAD by the phenolate oxygen (Figure 6B), followed by slow α-proton abstraction, concerted oxygen–FAD bond cleavage, which results in free *p*-quinone methide.

4-Ethylphenol Oxidation by PCMH. For this reaction, a large intrinsic primary deuterium isotope effect was found (5.98 ± 0.12, or 6.09 ± 0.23 when *A* was set equal to 1), again suggesting a rather symmetrical transition state. The intrinsic
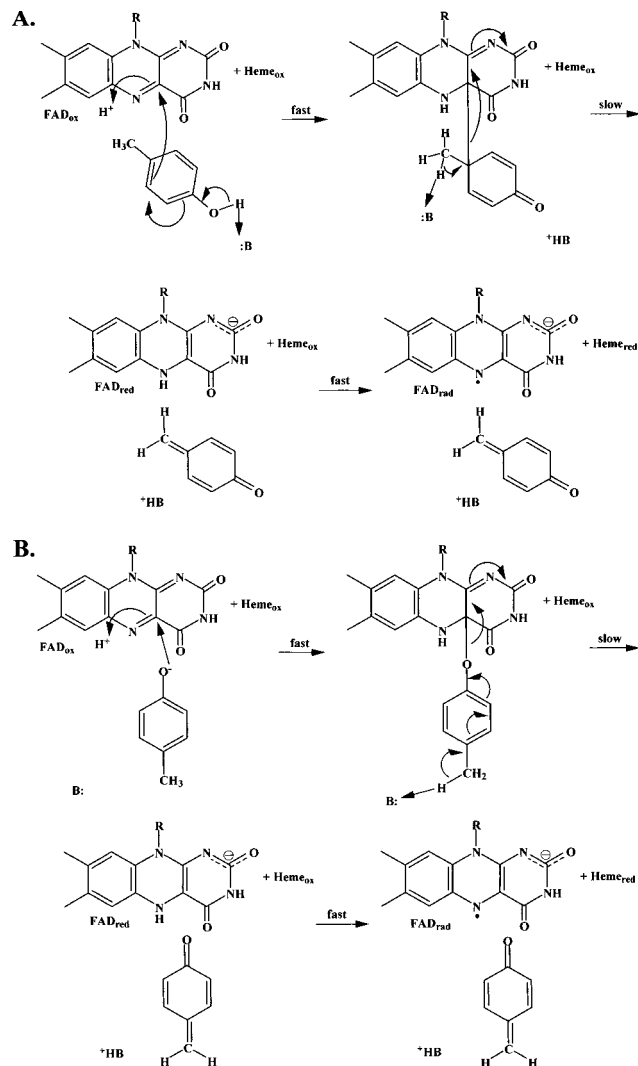
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Figure 6. Possible mechanisms for oxidation of 4-methylphenol involving covalent flavin–substrate intermediates.

secondary α-deuterium isotope effect was 0.967 ± 0.021, or 0.996 ± 0.044 when *A* was set equal to 1. Because the primary and secondary effects are similar to those found for the 4-methylphenol/PCMH reaction, mechanistic arguments similar to those presented for 4-methylphenol can be advanced.

In our analysis of the steady-state kinetic results, it was assumed that values of *P* for both branches of the reaction in Scheme 4 were the same, and this was also true for the values of *S* for both branches. Additionally, it was assumed that each branch has different rate constants for substrate binding and dissociation and catalysis. With these assumptions, it was possible to extract the *P* and *S* from the data. It was impossible to determine values of *P* and *S*, assuming these values are different for the two paths of Scheme 4, regardless of any assumption concerning the relative rates of substrate binding and dissociation, and catalysis. It is possible that the true intrinsic primary and secondary effects are somewhat different for each path.

It is interesting that the stereochemistry of the products of each branch are identical because ~100% of (−)-*S*-1-(4-hydroxyphenyl)ethanol is formed; i.e., the *p*-quinone methide is always attacked by H$_2$O/HO$^-$ on the *si* side of the α-carbon. This suggests that the transition states for both paths of Scheme 4 are the same or nearly so and suggests that the binding of 4-ethylphenol is essentially the same for both paths (see Figure

4, reaction D). If this is true, then there is a nearly equal probability for removal of the *pro-R* or *pro-S* hydrogen of 4-ethylphenol (*A*, the flux ratio for the *S*-isomer/*R*-isomer paths = 0.78).

It may be more accurate to think of the PCMH chemical mechanism in terms of a nonspecific α-hydrogen "explosion" from the substrate, rather than a specific hydrogen "abstraction". If the phenolate forms of 4-methylphenol and 4-ethylphenol bind to PCMH, then the negative charge on the substrate oxygen would be destabilized if its immediate environment is hydrophobic and/or there is a strong unfavorable electrostatic interaction. The unfavorable interaction would be minimized by forcing the negative charge into the benzene ring of substrate, thereby increasing its quinonoid character and necessarily weakening an α-C—H bond. Eventually, there is an indiscriminant explosion of H⁻, and the closest electrophilic center [e.g., the N5-position of enzyme-bound FAD] would be the recipient of H⁻. In fact, as the C—H bond weakens, the reaction will be facilitated by increasing interaction between the increasing $\delta-$ on the hydrogen and the $\delta+$ of an electrophilic center of the flavin.

PCMH optimally oxidizes 4-methylphenol; therefore, it can be envisioned that binding of 4-ethylphenol in the active site of the enzyme involves less than optimal steric interactions. This is reflected in K_D values for 4-methylphenol (16 ± 3 μM) and for 4-ethylphenol (2.17 ± 0.38 mM). Possible "steric strain" could be relieved in the 4-ethylphenol transition state, and the relief could be greater in reactions with α-secondary C—D. This greater relief is due to the shorter effective length of the C—D bond relative to the C—H bond and leads to lowering of *S*.[42] Similarly, it can be argued that steric interactions allow deuterated 4-ethylphenol slightly closer approach to FAD and/ or other catalytic groups than the nondeuterated form in a highly structured, crowded active site. This would result in slightly more favorable interaction(s) in the isotopically sensitive step. If this is true, then the underlying assumption that there are no isotope effects for binding may be incorrect. The magnitudes of errors for K_d values measured by stopped-flow for 4-ethylphenol[14] precludes the determination of small isotope effects for binding.

Experimental Procedures

Materials. 4-Methylphenol (99+%, gold label), 4-ethylphenol (97%), 4-hydroxyactophenone (99%), 4-methoxybenzyl alcohol (98%), 4-vinylanisole (97%), deuterium chloride (37% solution in D₂O, 99 atom % D, gold label), and LiAlH₄ were from Aldrich Chemical Co. (St. Louis, MO). Other materials and sources were as follow: 4-anisate methyl ester, Eastman Kodak Co. (Rochester, NY); ²H₂O (99.8 atom % ²H), Stohler/KOR Stable Isotopes (Cambridge, MA); LiAl²H₄ (99 atom % ²H), KOR Isotopes (Cambridge, MA); di(²H₃)methyl sulfoxide (99.5 atom % ²H), Diaprep, Inc. (Atlanta, GA); 4-methyl(2,3,5,6-²H₄)-phenol (98.6 atom % ²H), tetramethylsilane, and C²HCl₃ (99.8 atom % ²H), Merck & Co. (Rahway, NJ); horse heart cytochrome *c* (type VI), CH₃CH₂O²H (99.5+ atom % ²H), CH₃O²H (99.5+ atom % ²H), phenazine methosulfate (PMS), and phenazine ethosulfate (PES), Sigma Chemical Co. (St. Louis, MO); sodium 2,6-dichlorophenol indophenol (DCIP), General Biochemicals (Chagrin Falls, OH). Solvents for high-pressure liquid chromatography (HPLC) were HPLC grade. All other chemical were of reagent grade.

Analytical Methods. Substrates purities were checked by (A) ¹H NMR using a Varian EM-360 spectrometer at room temperature (chemical shifts, δ, in ppm relative to tetramethylsilane); (B) HPLC

using a 5-μm Ultrasphere-ODS (octadecylsilyl-derivatized silica gel) 0.46- × 25-cm column (column 1) (Beckman Instruments, Inc., Fullerton, CA), a Spherex 3-μm, C-18, 0.46- × 7.5-cm column (column 2), or a Hypersil 3-μm, ODS, 0.46- × 7.5-cm column (column 3) (Phenomenex, Torrance, CA). A Beckman Instruments, Inc. model 332 gradient liquid chromatography system and a Kratos Analytical Instrument Spectroflow 757 detector (Ramsey, NJ) were used. Unless otherwise stated, the flow rate was 1 mL/min, and effluents were monitored at 254 or 280 nm; and (C) mass spectral analyses done at the Mass Spectrometry Facility at the University of California, San Francisco. All reported melting points and boiling points are uncorrected.

Purifications. The A form of PCMH was isolated from *P. putida*, N.C.I.M.B. 9869 by a published method.[43] Cytochrome *c* oxidase was purified from beef heart mitochondria to the red/green split stage.[44]

4-Ethylphenol was purified as described earlier.[14] It was pure as judged by NMR and HPLC, t_R = 7.22 min (100%, column 1, H₂O/ CH₃CN, 1:1, v/v) and 1.84 min (100%, column 3, H₂O/CH₃CN, 3:2, v/v). Mass spectrum: *m/z* (relative intensities) 122 (M⁺, 40) 107 (100), 77 (20).

4-Methylphenol and 4-methyl(2,3,5,6-²H₄)phenol were purified by subliming twice under 0.1 mmHg at 20−32 °C. Mass spectral analysis of deuterium content for 4-methyl(2,3,5,6-²H₄)phenol: 94.7% ²H₄, 4.8% ²H₃, 0.4% ²H₂, and 0.2% ²H₁, which translated to a total of 98.5 atom % ²H. Both samples were deemed pure by HPLC, *R* = 5.2 min (100%, column 1, H₂O/CH₃CN, 1:1, v/v) and 1.33 min for 4-methylphenol (100%, column 3, H₂O/CH₃CN, 3:2, v/v). For 4-methylphenol:[14] NMR (C ²HCl₃) δ 2.2 (s, 2.9, CH₃), 6.16 (s, 1.0, OH), 6.7 and 7.0 (2d, 4.0, Ar—H); mass spectrum, *m/z* (relative intensities) 108 (M⁺, 96), 107 (100), 91 (7.1), 90 (8.7), 79 (15.7), 78 (6.3), 77 (27.4).

Purifications of other substrates are described with the syntheses presented in the following sections. Syntheses of 4-(²H₃)methylanisole, 4-(²H₂)methylanisole, and 4-(²H₁)methylanisole, similar to those used in the syntheses of 4-(²H₃)methylphenol, 4-(²H₂)methylphenol, and 4-(²H₁)methylphenol,[14] are also described elsewhere.[8]

4-(²H₃)Methylphenol and 4-(1′,1′-²H₂)ethylphenol. The syntheses are described elsewhere.[14] The samples were purified by twice subliming. The samples were pure by HPLC analyses: 4-(²H₃)-methylphenol, *R* = 5.33 min (100%, column 1, H₂O/CH₃CN, 1:1, v/v) and t_R = 1.29 min (100%, column 3, H₂O/CH₃CN, 3:2, v/v); 4-(1,1-²H₂)ethylphenol, *R* = 7.19 min (100%, column 1, H₂O/CH₃CN, 1:1), t_R = 4.82 min (100%, column 2, H₂O/CH₃CN, 7:3, v/v) and 1.88 min (100%, column 3, H₂O/CH₃CN, 3:2, v/v). NMR: 4-(²H₃)methylphenol (C²HCl₃), δ 6.27 (s, 1.1, OH), 7.08 (2d, 4.0, Ar-H), 2.23 (s, ²H₂CH), estimated methyl hydrogen content, 0.6%; 4-(1′,1′-²H₂)ethylphenol (C²-HCl₃), δ 1.20 (s, 2.9, CH₃), 6.45 (s, 1.0, OH), 7.13 (dd, 4.0 Ar-H), ∼2 (multiplet, -²HCH-), estimated methylene hydrogen content, 1−2%. Mass spectra: *m/z* (relative intensities), 4-(²H₃)methylphenol, 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); 4-(1′,1′-²H₂)ethylphenol, 124 (M⁺, 40), 109 (100), 79 (10), 78 (10). Mass spectral analysis of deuterium content: 4-(²H₃)methylphenol, 97.4% ²H₃, 2.3% ²H₂, 0.1% ²H, and 0.2% ¹H₃, for a total of 98.9 atom % ²H; 4-(1′,1′-²H₂)ethylphenol, 96.5% ²H₂, 3.3% ²H₁, and 0.2% ¹H₃, which translated to a total of 98.2 atom % ²H.

4-(²H₂)Methylphenol. 4-Methoxy(1′,1′-²H₂)benzyl chloride was synthesized, as reported earlier,[14] by first reducing 4-anisate methyl ester with LiAl²H₄ and then chlorinating the resulting 4-methoxy(1′,1′-²H₂)benzyl alcohol with thionyl chloride. The identity and purity of the alcohol and chloride were determined by NMR. 4-(²H₂)Methylanisole was prepared by slow addition of 8.0 g (50.4 mmol) of the chloride to a solution of 3.4 g of LiAlH₄ in 100 mL of dry tetrahydrofuran over a period of 1 h. The reaction mixture was refluxed under dry Ar during the addition period, and refluxing continued for another 3.4 h. Thin-layer chromatography of a quenched aliquot of the reaction mixture

(42) Van Hook, W. A. *Isotope Effects in Chemical Reactions*; Collins, C. J., Bowman, N. S., Eds.; Van Nostrand Reinhold: New York, 1979; Chapter 1, pp 1−89.

(43) Koerber, S. C.; McIntire, W. S.; Bohmont, C.; Singer, T. P. *Biochemistry* **1985**, *24*, 5276−5280.

(44) Wharton, D. C.; Tzagloff, A. *Methods Enzymol.* **1967, *10*,** 245−250.

indicated that the reaction had gone to completion. With stirring, 5 g of $Na_3PO_4\cdot12H_2O$ was very slowly added to the cooled mixture over a 45-min period. The mixture was stirred overnight at room temperature, then the thick gray slurry was dried in a rotary evaporator at 25 °C and 20 mmHg, and finally 300 mL of H_2O was added. The aqueous phase was extracted three times with 150–200 mL of $CHCl_3$. The $CHCl_3$ solution was dried over anhydrous $MgSO_4$ and filtered, and solvent was removed under reduced pressure. Yield: 6.0 g (48.4 mmol, 96.0%) of a light yellow liquid.

The methyl ether of 4-($^2H_2$)methylanisole was cleaved with HBr (49% in water) as described earlier for the synthesis of 4-($^2H_3$)-methylphenol.[14] From 5.3 g of the anisole, 2.5 g of 4-($^2H_2$)methylphenol and 2.4 g of 4-($^2H_2$)methylanisole were recovered by the following procedure. The HBr solution was cooled, made alkaline (pH 12–14) with NaOH, and extracted three times with 200 mL of $CHCl_3$ (extract 1). The aqueous phase was adjusted to pH 7 with HCl, and the extraction procedure was repeated (extract 2). Extract 1 was back-extracted three times with H_2O and adjusted to pH 12 with NaOH, the aqueous phase was neutralized with HCl and re-extracted three times with $CHCl_3$ (extract 3), and the combined organic phase was dried as before. Final extract 1 contained 4-($^2H_2$)methylanisole and trace amounts of 4-($^2H_2$)-methylphenol. Combined extracts 2 and 3 contained crude 4-($^2H_2$)-methylphenol. 4-($^2H_2$)Methylphenol was purified in the same manner as 4-($^2H_3$)methylphenol.[14] NMR (C^2HCl_3): δ 2.17 (m, 1.0, C^2H_2H), 6.02 (s, 1.2, OH), 6.70 and 6.96 (dd, 4.0, Ar-H). HPLC: t_R = 1.28 min (100%, column 3, H_2O/CH_3CN, 3:2, v/v). The material was further purified by sublimation as described above for 4-methylphenol. Mass spectral analysis of deuterium content: 99.3% $^2H_2$ and 0.7% $^2H_1$, for a total of 99.7 atom % ^2H.

4-($^2H_1$)Methylphenol. Eight grams (0.051 mol) of the 4-methoxy-benzyl chloride was reduced with $LiAl^2H_4$, as described above for the synthesis of 4-($^2H_2$)methoxyanisole using $LiAlH_4$. The yield was nearly quantitative. Pure 4-($^2H_1$)methylphenol was obtained from 4-($^2H_1$)-methylanisole as described in the previous section for the synthesis of 4-($^2H_2$)methylphenol. NMR (C^2HCl_3): δ 2.20 (t, 2.0, C^2HH_2), 6.11 (s, 1.3, OH), 6.68 and 6.96 (2d, 4.0, Ar-H). HPLC: R = 1.31 min (100%, column 3, H_2O/CH_3CN, 3:2, v/v). The material was further purified by sublimation as described earlier for 4-methylphenol. Mass spectral analysis of deuterium content: 98.6% ^2H.

4-(2′,2′,2′-$^2H_3$)Ethylphenol. The 2′-hydrogens of 4-hydroxyac-etophenone were exchanged by acid-catalyzed keto–enol tautomer-ization. Six grams of 4-hydroxyacetophenone (0.176 mol exchangeable hydrogens; three from the 2′-carbon, one from the phenolic hydroxyl group) was dissolved in 10 mL of CH_3O^2H, 4 mL of ^2H_2O, and 200 μL of 35% ^2HCl in ^2H_2O and incubated at 40–50 °C for 16 h, protected from atmospheric moisture. The liquid was removed by evaporation under reduced pressure, and the process was repeated four more times. This was followed by two more exchange steps using 10 mL of CH_3-CH_2O^2H and 0.4 mL of 35% ^2HCl in ^2H_2O. To remove all traces of ^2HCl, the sample was dissolved in 5 mL of $CH_3CH_2O^2H$, and the solvent immediately evaporated. This step was repeated. The remaining solid was then dissolved in 20 mL of CH_3CH_2OH to exchange deuterons at the phenolic position with protons. The solvent was evaporated immediately, and the procedure was repeated. The solid was completely dried in a vacuum desiccator over P_2O_5. NMR analysis of the resulting 4-hydroxy(2′,2′,2′-$^2H_3$)acetophenone [di($^2H_3$)methyl sulfoxide]: δ 2.46 (s, C^2H_2H), 6.90 and 7.86 (dd, 4.0, Ar-H), 10.32 (s, 0.9, OH); estimated content of ^1H in β-position, 1.6%.

Four grams of 4-hydroxy(2′,2′,2′-$^2H_3$)acetophenone (0.0288 mol) was reduced via the Clemmensen reaction in CH_3CH_2OH over Zn(Hg) amalgam using HCl.[14] When the reaction was complete, 100 mL of water was added, followed by addition of concentrated NaOH to neutralize the mixture. The mixture was extracted three times with $CHCl_3$, the combined organic phase dried with $MgSO_4$, and the solvent removed under reduced pressure. The resulting viscous liquid was purified by simultaneous distillation/sublimation.[14] This afforded 3.13 g of 4-ethylphenol-β-d_3 as a white solid (25 mmol, 87% yield). HPLC: t_R = 1.84 min (column 3, H_2O/CH_3CN, 3:2, v/v). The material

was further purified by sublimation as described for 4-ethylphenol. Mass spectral analysis of deuterium content in the β-position: 95.7% $^2H_3$, 4.1% $^2H_2$, and 0.2% $^2H_1$, which corresponds to 98.5 atom % ^2H.

4-Vinylphenol. Its synthesis was accomplished by published pro-cedures.[45,46] The material was pure as judged by HPLC (column 1; t_R = 10.6 min; $MeOH/H_2O$, 1:9 (v/v) for 3 min then to 4:6 (v/v) in 3 min; 1 mL/min flow rate) and NMR in di($^2H_3$)methyl sulfoxide.[46] It had the same UV spectrum as commercial 4-vinylanisole in pure MeOH.

R,S-($\pm$)-1-(4-Hydroxyphenyl)ethanol. Ten grams of 4-hydroxy-acetophenone (73.5 mmol) was dissolved in 100 mL of H_2O/50 mL of ethanol. With stirring, 5 g of solid $NaBH_4$ was slowly added over a 20-min period to control foaming. After all of the $NaBH_4$ was added, the mixture was stirred until all bubbling ceased ($\sim$1 h). The pH was adjusted to 6–7 with H_3PO_4, and the mixture extracted three times with 200 mL of water-saturated ethyl acetate. The solvent was removed under reduced pressure. One hundred milliliters of dry ethyl acetate was added to the residue, the mixture heated to boiling, and ethanol added to dissolve all the solid. The solution was allowed to cool slowly to room temperature, then to 0 °C, and finally at −20 °C. The resulting crystals were filtered and washed with −20 °C ethyl acetate. The yield of dried crystals was 3.9 g (28.3 mmol, 38.5%).

Other Substrates. The syntheses and purifications are reported elsewhere.[46,47] For R-(+)-1-(4-hydroxyphenyl)ethanol (all [α] values, reported in degrees, were measured at 20 °C; C = 5.00, in methanol), [α]$_D$ = +47.5, [α]$_{578}$ = +49.7, [α]$_{546}$ = + 56.9, [α]$_{436}$ = +101.0, [α]$_{365}$ = +168.5; for S-(−)-1-(4-hydroxyphenyl)ethanol, [α]$_D$ = −47.5, [α]$_{578}$ = −49.5, [α]$_{546}$ = −56.9, [α]$_{436}$ = −101.0, [α]$_{365}$ = −168.4, the R,S-($\pm$)-1-(4-hydroxyphenyl)(1-$^2H_1$)ethanol was 99.2 atom % ^2H; for R-(+)-1-(4-hydroxyphenyl)(1-$^2H_1$)ethanol (99.0 atom % ^2H), [α]$_D$ = +49.1, [α]$_{578}$ = +51.4, [α]$_{546}$ = +58.5, [α]$_{436}$ = +103.7, [α]$_{365}$ = +173.4; for S-(−)-1-(4-hydroxyphenyl)(1-$^2H_1$)ethanol (99.0 atom % ^2H), [α]$_D$ = −48.8, [α]$_{578}$ = −50.9, [α]$_{546}$ = −58.3, [α]$_{436}$ = −103.6, [α]$_{365}$ = −173.4, the R,S-($\pm$) -4-(1′-$^2H_1$)ethylphenol was 97.1 atom % ^2H; for R-(+)-4-(1′-$^2H_1$)ethylphenol (96.7 atom % ^2H), ([α] values were measured at 20 °C; C = 25.00, in ethanol) [α]$_D$ = 0.16, [α]$_{578}$ = 0.17, [α]$_{546}$ = 0.20, [α]$_{436}$ = 0.37, [α]$_{365}$ = 0.690); for S-(−)-4-(1′-$^2H_1$)-ethylphenol (98.3 atom % ^2H for each of two preparations), [α]$_D$ = −0.247 and −0.240, [α]$_{578}$ = −0.242 and −0.260, [α]$_{546}$ = −0.295 and −0.290, [α]$_{436}$ = −0.563 and −0.505, [α]$_{365}$ = −1.04 and −0.927. Unfortunately, we could not devise a method, nor was one found in the literature, for determining the exact enantiomeric purity of the R- or S-isomer. For the purposes of this report, we assumed that the S-4-ethylphenol preparation with the highest optical rotation was 100% S-isomer, and this preparation was used for our kinetic studies. With this assumption, we estimated the purity of the R-isomer to be 66% ee (enantiomeric excess). All alcohols and all 4-ethylphenols were pure as judged by HPLC using a 50- × 4.50-mm Hypersil 3-μm ODS column: alcohols [including the R,S-($\pm$)-alcohol], t_R = 1.7 min, 0.8 mL/min flow rate, 7:3 $H_2O/MeOH$; 4-ethylphenols, t_R = 1.9 min, 1 mL/min flow rate, 45:55 $H_2O/MeOH$. Optical rotations were recorded on a Perkin-Elmer model 141 polarimeter with a standard 1-dm, temperature-thermostated cell.

Steady-State and Stopped-Flow Kinetic Assays with 1-(4-Hy-droxyphenyl)ethanols. All steady-state kinetic assays were done in 50 mM Tris-HCl, pH 7.6, I = 0.05 (KCl), at 25 °C, as described earlier.[14] The electron-accepting substrate for PCMH was PES, and the reactions were monitored at 600 nm, which measured the reduction of DCIP (initial concentration, 95 μM) by reduced PES. Assays were initiated by addition of enzyme to cuvettes containing buffer, phenolic substrate, and dyes.

(45) Dale, W. J.; Hennis, H. E. *J. Am. Chem. Soc.* **1958**, *80*, 3645–3649.

(46) Everhart, E. T.; Craig, J. C. *J. Chem. Soc., Perkins Trans. 1* **1991**, 1701–1707.

(47) Everhart, E. T.; McIntire, W. S.; Craig, J. C., manuscript in preparation.

Stopped-flow experiments were performed as described previously.[15,48] The experiments were done in 50 mM Tris-HCl buffer containing 10 mM D-glucose, pH 7.6, $I = 0.05$ (KCl), at 25 °C. The solutions became anaerobic due to ther presence of catalase and glucose oxidase in the buffer.[48]

Time Course Reactions. A typical reaction mixture contained 3−4 mM of the phenolic substrate, 30 μM horse heart cytochrome c, 1 mg of total protein/mL of the crude cytochrome c oxidase, 40−70 μg/mL of PCMH in 20−30 mL of 10 mM Tris-HCl buffer, pH 7.6, $I = 0.01$. At various times over the course of a reaction, 1-mL aliquots were removed and quickly mixed with 0.15 mL of saturated $(NH_4)_2SO_4$ to quench the reaction. (It was found that cytochrome c is an excellent electron acceptor at $I \leq 0.01$, but at $I \geq 0.1$, the reaction is halted.) The quenched samples were centrifuged at 16000g for 3 min. The proteins were separated from small molecules using Centricon-10 centrifuge filters; 30 min at 5000g at 4 °C. The filtered liquid was analyzed directly by HPLC; for example, 5 μL was injected onto a Burdick-Jackson C-18 column (0.4 × 25 cm; 5-μm particle size), and the column was eluted with 2:3 (v/v) MeOH/H$_2$O at a 1 mL/min flow rate, with 254- and 280-nm detection. The chromatograms were recorded and peaks were integrated using an Altex model C-R1A integrator (Beckman Instruments, Inc., Fullerton, CA). For preparative runs, 1 mL was injected onto the same column, and the appropriate fractions were collected. The MeOH was removed from these samples using a stream of dry, filtered N$_2$. The remaining aqueous phase was extracted four times with 0.25−0.5 mL of diethyl ether. The ether was dried with anhydrous Na$_2$SO$_4$ and the solvent removed in a stream of N$_2$. Dried samples were taken up in 50 μL of dry MeOH and stored at −70 °C. For analysis of the enantiomeric composition of the aryl carbinols, a small volume of the sample collected directly from the C-18 column (before MeOH evaporation and ether extraction) was injected onto a Chiralcel OB column (cellulose tribenzoate-derivatized macroporous silica gel, 0.4 × 25 cm, 5-μm particles; Daicel Chemical Industries, Ltd., Japan) and eluted with n-hexane/2-propanol (various mixtures from 7:1 to 19:1, v/v), with 254- and 280-nm detection.[24,25] This column gave nearly baseline separation of the R- and S- isomers of the arylcarbinols. In early experiments, the enantiomeric composition was determined using a Pirkle Type 1-A column (0.4 × 25 cm, 5-μm particles; Regis Chemical Co., Morton Grove, IL).[22,23] However, for analyses on this column, the phenolic aryl carbinols had to be diacetylated in order to separate the R- and S-isomers. Diacetylation of the extracted and dried 1-mL aliquot samples was carried out with 0.1 mL of acetic anhydride and 0.1 mL of triethylamine at room temperature for 2 h. The reagents were removed in a stream of dry N$_2$. Samples were dissolved in 0.1 mL of n-hexane, and 20 μL was injected onto the Pirkle column and eluted with n-hexane/2-propanol (various ratios from 9:1 to 35:1, v/v), with 254- and 280-nm detection.

PCMH was anaerobically incubated with 4-vinylphenol or 1-(4-hydroxyphenyl)ethanol (20 °C under Ar). At time zero, 30-mL reaction mixtures contained 3.4 mM alcohol or 2.4 mM 4-vinylphenol, 0.11 μM glucose oxidase, 20 μg/mL catalase, 0.1 M D-glucose, 8.06 μg/mL PCMH in 10 mM Tris-HCl, pH 7.6. For the 4-vinylphenol reaction, at $t = 0$, PCMH was reduced with a stoichiometric amount of 1-(4-hydroxyphenyl)ethanol. At various times over a 28−50-h period, aliquots were removed, processed, and analyzed by HPLC as described in the previous paragraph.

Oxygen Electrode Assays. Assays for the enzymic oxidation of 4-ethylphenol were done at 25 °C in air-saturated 0.01 M Tris-HCl buffer, pH 7.6. The reactions were monitored with a Clark electrode using a Gilson Oxygraph equipped with a 1.6-mL reaction cell.[26] In addition to 4-ethylphenol and PCMH, the reaction mixture contained horse cytochrome c and beef heart cytochrome c oxidase (vide supra).

Derivation of Equations Used To Analyze Data: **Derivation of k_{cat}/K Expressions.** The example presented here is for the most complicated case, that is, for (+)-R-4-(1-^{2}H$_1$)ethylphenol, which is 83% R-isomer and 97.6 atom % ^{2}H. The reactions and equations given below were derived using Scheme 4 in the text.
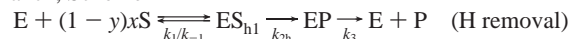
(48) Ramsay, R. R.; Koerber, S. C.; Singer, T. P. *Biochemistry* **1987**, *26*, 3045−3050.

R-^{2}H

upper branch, Scheme 4
$$E + yx\text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ES}_\text{d} \xrightarrow{k_{2d}} \text{EP} \xrightarrow{k_3} E + P \quad \text{(D removal)}$$

lower branch, Scheme 4
$$E + yx\text{S} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \text{ES}_\text{h} \xrightarrow{k_{ii}} \text{EP} \xrightarrow{k_{iii}} E + P \quad \text{(H removal)}$$

S-^{2}H

upper branch, Scheme 4
$$E + (1-y)x\text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ES}_{\text{h1}} \xrightarrow{k_{2h}} \text{EP} \xrightarrow{k_3} E + P \quad \text{(H removal)}$$

lower branch, Scheme 4
$$E + (1-y)x\text{S} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \text{ES}_{\text{d1}} \xrightarrow{k_{iid}} \text{EP} \xrightarrow{k_{iii}} E + P \quad \text{(D removal)}$$

HH

upper branch, Scheme 4
$$E + (1-x)\text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ES}_{\text{h2}} \xrightarrow{k_{2h2}} \text{EP} \xrightarrow{k_3} E + P \quad \text{(H removal)}$$

lower branch, Scheme 4
$$E + (1-x)\text{S} \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} \text{ES}_{\text{h3}} \xrightarrow{k_{iih}} \text{EP} \xrightarrow{k_{iii}} E + P \quad \text{(H removal)}$$
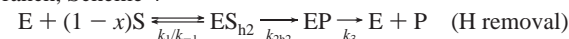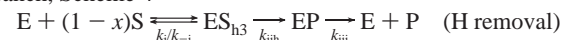
In these reactions, it is assumed that the rate constants for binding and catalysis are different for the upper and lower branches but the intrinsic primary and secondary isotope effects are the same for both branches. Therefore, $k_{2d} = pk_2$, $k_{ii} = s\epsilon k_2$, $k_{2h} = sk_2$, $k_{iid} = p\epsilon k_2$, $k_{2h2} = k_2$, $k_{iih} = \epsilon k_2$, $k_i = \delta k_1$, $k_{-i} = \gamma k_{-1}$, x = fraction of ^{2}H in substrate (97.6 atom %), and y = fraction R-^{2}H isomer (83%).

$$d[E]/dt = -k_1[\text{S}][E] - \delta k_1[\text{S}][E] + k_{-1}[\text{ES}_\text{d}] + \gamma k_{-1}[\text{ES}_\text{h}] + \\ k_{-1}[\text{ES}_{\text{h1}}] + \gamma k_{-1}[\text{ES}_{\text{d1}}] + k_{-1}[\text{ES}_{\text{h2}}] + \gamma k_{-1}[\text{ES}_{\text{h3}}] + \\ k_3[\text{EP}] = 0 \quad (6)$$

$$d[\text{ES}_\text{h}]/dt = yxk_1[\text{S}][E] - (k_{-1} + pk_2)[\text{ES}_\text{h}] = 0 \quad (7)$$

$$d[\text{ES}_\text{d}]/dt = yx\delta k_1[\text{S}][E] - (\gamma k_{-1} + s\epsilon k_2)[\text{ES}_\text{d}] = 0 \quad (8)$$

$$d[\text{ES}_{\text{h1}}]/dt = (1-y)xk_1[\text{S}][E] - (k_{-1} + sk_2)[\text{ES}_{\text{h1}}] = 0 \quad (9)$$

$$d[\text{ES}_{\text{d1}}]/dt = (1-y)x\delta k_1[\text{S}][E] - (\gamma k_{-1} + p\epsilon k_2)[\text{ES}_{\text{d1}}] = 0 \quad (10)$$

$$d[\text{ES}_{\text{h2}}]/dt = (1-x)k_1[\text{S}][E] - (k_{-1} + k_2)[\text{ES}_{\text{h2}}] = 0 \quad (11)$$

$$d[\text{ES}_{\text{h3}}]/dt = (1-x)\delta k_1[\text{S}][E] - (\gamma k_{-1} + \epsilon k_2)[\text{ES}_{\text{h3}}] = 0 \quad (12)$$

$$d[\text{EP}]/dt = pk_2[\text{ES}_\text{d}] + \epsilon sk_2[\text{ES}_\text{h}] + sk_2[\text{ES}_{\text{h1}}] + \epsilon pk_2[\text{ES}_{\text{d1}}] + \\ k_2[\text{ES}_{\text{h2}}] + \epsilon k_2[\text{ES}_{\text{h3}}] - k_3[\text{EP}] = 0 \quad (13)$$

$$[E_t] = [E] + [\text{ES}_\text{h}] + [\text{ES}_\text{d}] + [\text{ES}_{\text{h1}}] + [\text{ES}_{\text{d1}}] + [\text{ES}_{\text{h2}}] + \\ [\text{ES}_{\text{h3}}] + [\text{EP}] \quad (14)$$

Since steady-state conditions are assumed, eqs 6−13 are set equal to zero. These equations are a series of linear homogeneous differential equations with regard to the enzyme species. [S] is assumed to be constant. Of eqs 6−13, eq 13 can be considered redundant. From eqs 6−12 and 14, matrix Z is constructed (ref 27, pp 73−92):

$$Z = \begin{bmatrix} -k_1(1+\delta)[S] & k_{-1} & \gamma k_{-1} & k_{-1} & \gamma k_{-1} & k_{-1} & \gamma k_{-1} & k_3 \\ xyk_1[S] & -k_{-1}-pk_2 & 0 & 0 & 0 & 0 & 0 & 0 \\ xy\delta k_1[S] & 0 & -\gamma k_{-1}-\epsilon s k_2 & 0 & 0 & 0 & 0 & 0 \\ x(1-y)k_1[S] & 0 & 0 & -k_{-1}-sk_2 & 0 & 0 & 0 & 0 \\ x(1-y)\delta k_1[S] & 0 & 0 & 0 & -\gamma k_{-1}-p\epsilon k_2 & 0 & 0 & 0 \\ (1-x)k_1[S] & 0 & 0 & 0 & 0 & -k_{-1}-k_2 & 0 & 0 \\ (1-x)\delta k_1[S] & 0 & 0 & 0 & 0 & 0 & -\gamma k_{-1}-\epsilon k_2 & 0 \\ 1 & 1 & 1 & 1 & 1 & 1 & 1 & 1 \end{bmatrix}$$

E ES_h ES_d ES_{h1} ES_{d1} ES_{h2} ES_{h3} EP

Next, a column vector, v, which is the solution to each of the differential equations, is constructed:

$$v = \begin{bmatrix} 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ 0 \\ E_t \end{bmatrix} \qquad (Z^{-1})v = \begin{bmatrix} N_E \\ N_{ES_h} \\ N_{S_d} \\ N_{S_{h1}} \\ N_{S_{d1}} \\ N_{S_h} \\ N_{S_{h3}} \\ N_{EP} \end{bmatrix}$$

The steady-state velocity is defined as $v = k_3[EP]_{ss}$, i.e., the rate of product formation. To determine the steady-state concentrations of each enzyme species, the following procedure is performed: $(Z^{-1})v$, where Z^{-1} is the inverse of Z. The resulting column vector is shown above. $[EP]_{ss}$ is defined as $N_{EP}/\sum N_i$, where N_i are all the components of the $(Z^{-1})v$ column vector. It turns out that the expression for $(k_{cat}/K)_R$ is $k_3(N_{EP})/(N_E[S])$. The expression for k_{cat}/K for unlabeled 4-ethylphenol is determined in the same fashion. For all the deuterated 4-ethylphenols, $k_{cat}/K = f(k_1,k_{-1},k_2,k_3,s,p,A,x,y)$, where $A = \delta\epsilon/\gamma$. If it is assumed that $C = k_2/k_{-1} = 0$, it follows that $k_{-1} \gg k_2$. This assumption simplifies the equations for k_{cat}/K for each deuterated and nondeuterated substrate. k_{cat}/K expressions for the deuterated 4-methylphenols were determined in the same manner. The matrix algebra was done by symbolic manipulation using MAPLE V, Release 4 software (Waterloo Maple Software, Waterloo, Ontario, Canada).

Steady-State Enzyme Reaction with Two Competing Substrates. With R and S as competing substrates, with P representing product as competitive inhibitor, and with unknown inhibitor, I, the following equations apply:

$$d[R]/dt = k_{catR}/\{1 + K_R/[R](1 + [S]/K_S)(1 + [P]/K_P)(1 + [I]/K_I)\}$$

$$d[S]/dt = k_{catS}/\{1 + K_S/[S](1 + [S]/K_R)(1 + [P]/K_P)(1 + [I]/K_I)\}$$

From these equations, $d[R]/d[S] = (k_{cat}/K)_R[R]/\{(k_{cat}/K)_S[S]\}$ (ref 27, pp 105−108), and $d[R] = (k_{cat}/K)_R[R]/\{(k_{cat}/K)_S[S]\}d[S]$. Integration of this equation from $[R]_0$ to $[R]$ and $[S]_0$ to $[S]$ yields $([R]/[R]_0)^n = [S]/[S]_0$, where $n = (k_{cat}/K)_S/(k_{cat}/K)_R$. Since the starting PCMH substrate is racemic R,S-1-(4-hydroxyphenyl)ethanol, $[R]_0 = [S]_0$. Thus, $([R]/[R]_0)^n = [S]/[R]_0 = (T − [R])/[R]_0$, where T is the total concentration of alcohol at any time. Rearranging this equation gives $T = [R]^n/[R]_0^{n-1} + [R]$.