Stereochemical Dynamics of Aliphatic Hydroxylation by Cytochrome P-450

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Abstract: Previous studies on the stereochemistry of hydroxylation by cytochrome P-450 enzymes have been contradictory and confusing. Therefore, the hydroxylation of four isotopically substituted phenylethane substrates has been examined with a single isozyme of rabbit liver microsomal cytochrome P-450. In each case the corresponding 1-phenylethanol was essentially the only product. With ordinary phenylethane, the product was 48% (*R*)-1-phenylethanol and 52% the *S* isomer. With (*R*)-phenylethane-*1-d*, the product was 42% *R* alcohol, while with (*S*)-phenylethane-*1-d* the product was 70% *R* alcohol. When the substrate was phenylethane-*1,1-d*$_2$, 50% *R* alcohol was produced. The alcohols from the single-deuterium-substituted substrates were highly enriched in deuterium, indicating the operation of a large deuterium isotope effect on hydrogen removal. Most importantly, 23–40% of the hydroxylation events resulted in alcohol with configuration opposite to that of the original hydrocarbon substrate. These "crossover" events require the intermediacy of a discrete tricoordinate carbon intermediate. These data unambiguously demonstrate that hydroxylation stereospecificity must be enforced by the surrounding protein tertiary structure and is not an inherent feature of the cytochrome P-450 reaction mechanism.

The cytochrome P-450 family of enzymes continues to provide an interesting challenge to chemists seeking to understand the mechanism by which molecular oxygen is reductively split under mild aqueous conditions in biological systems. As a result of this process, one atom of the dioxygen molecule is reduced to a molecule of water, while the second is introduced into an organic molecule, frequently with high regio- and stereoselectivity. Several particular P-450 enzymes exhibit an *absolute* stereospecificity such as the adrenal steroidogenic enzymes[1,2] and the camphor-hydroxylating cytochrome from *Pseudomonas putida*.[3] Early work on the stereochemistry of phenylethane hydroxylation by McMahon[4] indicated that the reaction proceeded with *net conservation* of the stereochemistry of the hydrogen that had been replaced by hydroxyl. Groves,[5] however, provided evidence that the hydroxylation of norbornane involved significant *loss* of the original stereochemistry of the replaced hydrogen atom. This result was followed by the work of Caspi,[6] showing that terminal hydroxylation of [1-^{3}H,^{2}H,^{1}H]octane by liver microsomal suspensions proceeded with complete retention of the original stereochemistry. Most recently, Sligar[3] found that in the 5-hydroxylation of *d*-camphor by cytochrome P-450$_{cam}$ hydrogen removal could occur from either the 5-exo or 5-endo face of the bicyclic molecule, but oxygen delivery occurred exclusively to the exo face. As a result of such disparate results in the literature, a widespread belief exists that the cytochrome P-450 enzymes perform their hydroxylation reactions with complete retention of the original configuration at the oxidized carbon atom.[7]

The question of the net stereochemistry of the hydroxylation event is of some importance, since it has profound mechanistic consequences. A concerted event must proceed with either complete retention of configuration (as in a direct singlet carbene insertion) or complete inversion (as in an S_N2 displacement). Of course, net retention of configuration can be the result of two successive concerted inversions, as Knowles has demonstrated for some phosphoryl transferases.[8] A nonconcerted reaction (i.e., one involving a discrete intermediate with a finite lifetime), on the other hand, can lead to retention, inversion, or racemization. In the particular case of enzymatic hydroxylations of aliphatic carbon atoms, a tricoordinate intermediate (carbenium ion, carbanion, or carbon radical) may, in principle, combine with a hydroxyl group on either of the two sides, although the local chirality of the enzyme active site may favor oxygen delivery to only one side. Thus, the discovery of net retention of configuration following the hydroxylation is mechanistically *uninformative*, since the enzyme may provide rigid steric constraints to enforce production of only one enantiomer. As an example, Englard et al.[9] recently demonstrated that γ-butyrobetaine hydroxylase operates with complete stereospecificity while utilizing a carbon radical intermediate. On the other hand, the discovery of inversion provides *some* information, and the discovery of either partial or complete racemization *requires* the existence of a tricoordinate carbon intermediate. The previous literature, then, leaves us uncertain whether P-450-catalyzed hydroxylations proceed through an intermediate and whether the exact mechanism may be dependent on the particular substrate molecule and P-450 isozyme combination.

We have undertaken a study of the stereochemistry of hydroxylation by a single isolated enzyme, P-450$_{LM2}$. The results are unambiguous and in complete accord with the previous conclusion by Groves et al.[5] that significant loss of the original stereochemistry occurs during the hydroxylation event. Furthermore, we are able to provide an unprecedentedly detailed quantitative description of the stereochemical course of the reaction, including the inherent preference of the enzyme for *pro-R* and *pro-S* hydrogen removal, probabilities of stereochemical crossover, and the deuterium kinetic isotope effects associated with *pro-R* and *pro-S* hydrogen removal.

Experimental Section

General Analytical Procedures. Infrared spectra were recorded on a Perkin-Elmer 735 instrument as thin films or as potassium bromide pellets, with use of the 1601-cm^{-1} band of polystyrene for frequency calibration. Proton NMR spectra were determined on a Varian EM-360 instrument at 60 MHz or an IBM WP-200SY at 200 MHz, referenced to tetramethylsilane as internal standard. Gas chromatography (GC) was performed on a Varian 3700 chromatograph equipped with a flame ionization detector. The following 2 m × 2 mm columns were used: column A, 3% SP2330 on Supelcoport (80/100); column B, 3% Carbowax 20 M on Supelcoport (80/100). The carrier gas for GC was N_2 (30 mL/min) and for GC–MS was He (13 mL/min). Integration of gas chromatographic peaks was handled by a Hewlett-Packard 3990A integrator; the internal standard technique was employed for absolute

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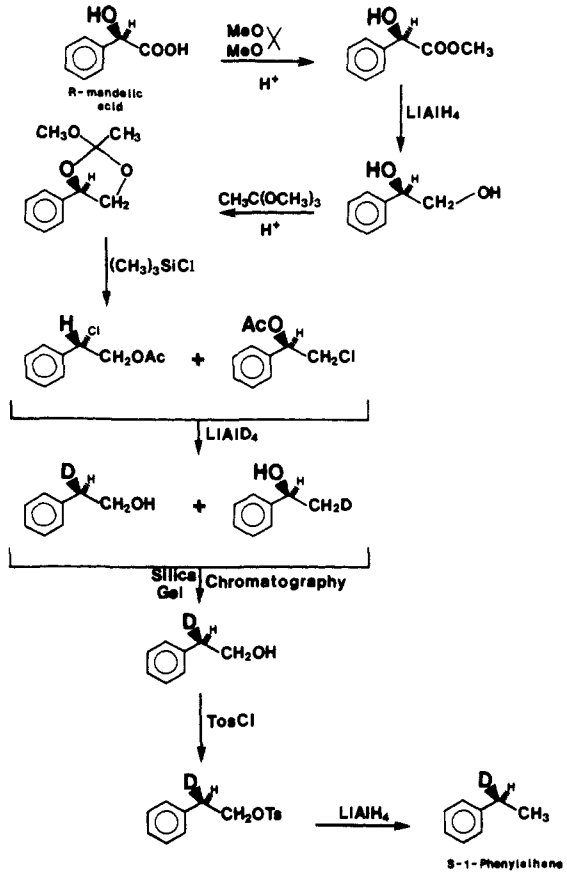
Scheme I



quantitation. Gas chromatography–mass spectrometry (GC–MS) was performed on a Hewlett-Packard 5992 instrument with the use of electron-impact ionization at an energy of 70 eV. Melting points were measured on a Thomas-Hoover melting point apparatus. High-pressure liquid chromatography (HPLC) was accomplished on a Perkin-Elmer Series 4 pump equipped with a Micromeritics 788 dual-variable UV detector. Optical rotations were measured in 1-dm cells on a Rudolph Model 80 high-precision polarimeter.

Materials. The homogeneous enzymes cytochrome P-450$_{LM2}$ and NADPH-cytochrome P-450 reductase were isolated from rabbit liver as described previously.[10,11] The phospholipid, dilauroylglycero-3-phosphorylcholine, was purchased from Sigma Chemical Co. and used as a sonicated suspension in water. Sodium borodeuteride (minimum 98 atom % D), lithium aluminum deuteride (minimum 98 atom % D), and Super-Deuteride were obtained from Aldrich Chemical Co. D-(−)- and L-(+)-mandelic acids were purchased as the crystalline grades from Sigma Chemical Co. (*R*)-(+)- and (*S*)-(−)-*sec*-phenethyl alcohols (99%) were purchased from Aldrich. Ethylbenzene was freed of traces of *sec*-phenethyl alcohol from autoxidation by passage over a short silicic acid column. Pyridine was dried over molecular sieves. Other chemicals were commercial products of high quality.

(*R*)-(−)-Phenylethane-*1-d* and (*S*)-(+)-Phenylethane-*1-d*. The synthetic scheme developed by Elsenbaumer and Mosher[12] was followed, starting from chirally pure mandelic acid (Scheme I). D-(−)-Mandelic acid (having the *R* configuration) yields (*S*)-phenylethane-*1-d*, while L-(+)-mandelic acid (having the *S* configuration) yields (*R*)-phenylethane-*1-d* in this scheme. Parallel syntheses of both (*R*)- and (*S*)-phenylethane-*1-d* were carried out. The following summary applies to both series of synthetic steps, whether starting from D- or L-mandelic acid. Therefore, the configuration at the stereogenic carbon atom is not indicated. Methyl mandelate [mp 55–56 °C (lit. mp 55–56 °C)] was obtained in 90% yield from mandelic acid. Phenylethylene glycol [mp 63–64 °C (lit. mp 59–63 °C] was obtained in 74% yield from methyl mandelate. 2-Methoxy-2-methyl-4-phenyl-1,3-dioxolane [bp 90–100 °C (0.2–0.3 Torr) [lit. bp 87 °C (0.02 Torr)]] was obtained in 87% yield from phenylethylene glycol. 2-Chloro-2-phenethyl acetate [bp 70 °C (0.25 Torr) [lit. bp 72–74 °C (0.07 Torr)]] was obtained in 98% yield from the dioxolane. The literature procedure was followed except that the reaction mixture was cooled to −78 °C prior to addition of chlorotrimethylsilane and then allowed to warm to 0 °C. 2-Phenylethanol-*2-d* was obtained in 81% yield from the chloroacetate. The contaminating 1-phenylethanol-*2-d* was efficiently removed by silica gel chromatography, and differential crystallization of the 2,4-dinitrobenzoates was not needed. 2-Phenylethyl-*2-d* tosylate [mp 38 °C (lit. mp 37.5–38.5 °C)] was obtained in 57% yield from the alcohol. 1-Phenylethane-*1-d* was obtained in 84% yield from the tosylated. This material was distilled from bulb to bulb under vacuum, and the final sample was obtained by collection from the gas chromatograph with column B. (*S*)-(+)-Phenylethane-*1-d*: 200-MHz NMR spectrum ($CDCl_3$) 1.23 (3 H, d t, J_H = 7.5 Hz, J_D = 0.85 Hz, CH_3), 2.62 (1 H, quartet of t, J_H = 7.5 Hz, J_D = 2.0 Hz, CHD), 7.20 ppm (5 H, m, C_6H_5); mass spectrum, *m/z* (% abundance) 108 (2.7), 107 (34), 106 (15), 93 (9), 92 (100), 79 (5), 78 (8), 77 (6), 66 (9), 52 (5), 51 (11). The NMR and mass spectra of (*R*)-(−)-phenylethane-*1-d* were virtually identical with those of the *S* isomer, and both spectra indicated essentially 100% deuterium content in the desired position.

Phenylethane-*1,1-d*$_2$. Thionyl chloride (13.0 g, 110 mmol) was added dropwise to a stirring solution of (*R,S*)-phenylethanol-*1-d* (12 g, 100 mmol) in ether (50 mL) at 0 °C. After 10 min, GC analysis indicated complete conversion of the alcohol to a mixture of 95% *sec*-phenethyl chloride and 5% styrene. The solution was washed with sodium bicarbonate followed by water and then was dried, first over sodium sulfate and then over magnesium sulfate. The dried alkyl chloride solution was deaerated with a dry nitrogen stream and Super-Deuteride (lithium triethylborodeuteride, 100 mmol as a 1 M solution in THF) was added via a needle, septum, and air-tight syringe. The reaction mixture was stirred over nitrogen for 2 h until GC indicated complete reduction of the alkyl chloride. The reaction mixture was quenched by dropwise addition of water (5 mL) and washed successively with saturated sodium bicarbonate, water, and saturated sodium chloride. The ether/THF solution was dried over sodium sulfate followed by magnesium sulfate and submitted to fractional distillation through a Vigreux column. The fraction most enriched in ethylbenzene, as determined by GC, was taken for the final purification by collection from the gas chromatograph with column B: 60-MHz NMR spectrum in ($CDCl_3$) 1.23 (br s, 3 H, CH_3), 7.40 ppm (s, 5 H, C_6H_5); mass spectrum, *m/z* (% abundance) 109 (2.6), 108 (31), 107 (12), 93 (100), 92 (42), 79 (10), 78 (11), 86 (14), 52 (11), 51 (23), 50 (13). Both the NMR and mass spectra indicated essentially 100% deuterium content in the 1-position.

(*R,S*)-1-Phenylethanol-*1-d*. Acetophenone (12 g, 100 mmol) was reduced with sodium borodeuteride (1.75 g, 46 mmol) in 0.2 M NaOH (50 mL) at room temperature for 3 h, at which point HPLC indicated complete conversion of the ketone. The reaction mixture was extracted with ether, and the ether solution was dried over magnesium sulfate. Removal of ether by distillation afforded 12.1 g of the alcohol, which was pure by GLPC analysis: 60-MHz NMR spectrum ($CDCl_3$) 1.47 (br s, 3 H, CH_3), 2.43 (br s, 1 H, OH), 7.48 (s, 5 H, C_6H_5); IR spectrum (thin liquid film) 3390 (s, OH), 3075, 3040, 2985, 2940, 2140 (w, C-D), 1488, 1445, 1365, 1232, 1132, 1072, 935, 745, 693 cm^{-1}; mass spectrum, *m/z* (% abundance) 124 (2.3), 123 (21), 122 (3.6), 108 (66), 107 (5.7), 105 (9), 80 (100).

(*S*)-*O*-Propionylmandelic Acid. The method of Thayer[13] was modified. L-(+)-Mandelic acid (5 g, 33 mmol) was dissolved in dry pyridine (40 mL) and the resultant solution cooled to 0 °C in a flask fitted with a calcium chloride drying tube. Propionyl chloride (3.1 g, 33 mmol) was added slowly with stirring, and the solution was stirred overnight at room temperature. The solution was taken up in ether and washed with ice-cold 1 N HCl and with water. Removal of the ether on the rotary evaporator left an oily residue that was washed with a small quantity of ice-cold 1 N HCl, causing a white solid to separate. The white solid was washed with water, dissolved in ether, and dried with anhydrous magnesium sulfate. Rotary evaporation afforded a waxy, white solid, (mp 68–71 °C), identified as (*S*)-*O*-propionylmandelic acid: IR spectrum 3700–2400 (br, s, COOH), 1745 (s, ester C=O), 1715 (s, carboxylic acid (C=O), 1180 and 1115 cm^{-1}; 60-MHz NMR spectrum ($CDCl_3$) 1.22 (t, 3 H, CH_3), 2.58 (q, 2 H, CH_2), 6.15 (s, 1 H, CHO), 7.65 (s, 5 H, C_6H_5), 8.87 ppm (s, 1 H, COOH, exchangeable with D_2O).

(*S*)-*O*-Propionylmandelyl Chloride. Thionyl chloride (0.25 g, 2.2 mmol) was added slowly to (*S*)-*O*-propionylmandelic acid (0.13 gm, 0.6 mmol) in a sealed vial. The vial was kept at room temperature overnight; then, HCl and excess thionyl chloride were removed by flushing a slow flow of dry nitrogen gas through the vial via a septum and two hypodermic needles. The residual red liquid was used directly in subsequent derivatization reactions: IR spectrum 3075 (w), 3040 (w), 2990 (w),

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2945 (w), 2890 (w), 1802 (s, C=OCl), 1754 (s, C=OOC), 1456 (m), 1360 (m), 1160 (s, COC), 1085 (m), 960 (m), 915 (m), 810 (s), 750 (s, C_6H_5), 695 cm^{-1} (s, C_6H_5); 60-MHz NMR spectrum ($CDCl_3$) 1.18 (t, 3 H, CH_3), 2.55 (q, 2 H, CH_2), 6.32 (s, 1 H, CHO), 7.63 ppm (br, 5 H, C_6H_5). The mild conditions used for the acylation and chlorination of mandelic acid avoided racemization of the stereogenic carbon, a problem with conventional methods.

(*S*)-1-Phenylethyl *O*-Propionylmandelate. 1-Phenylethanol (*R* or *S* or *R,S*; 220 mg, 2 mmol) was mixed with dry pyridine (0.6 mL) in a small septum-sealed vial that was then cooled in ice. (*S*)-*O*-Propionylmandelyl chloride (450 mg, 2 mmol) was added via a syringe. The mixture was shaken, allowed to stand for 1 h, and then was dissolved in ether (25 mL). The ether solution was washed with hydrochloric acid (1 M, 2 × 10 mL), with sodium bicarbonate (5%, 10 mL), and finally with water (10 mL). The ethereal solution was dried over anhydrous magnesium sulfate, and the ether was removed by rotary evaporation at 35 °C. The residual yellow oil was used without further purification for chromatography: IR spectrum 3075 (w), 3045 (w), 2990 (w), 2950 (w), 2900 (w), 1743 (s, C=O), 1499 (w), 1455 (m), 1370 (m), 1262 (m), 1209 and 1172 (s, CO), 1609 (m), 752 and 699 cm^{-1} (s, $C_6H_5^-$); 60-MHz NMR spectrum ($CDCl_3$) 1.0–1.6 (m, superimposed d and t, 6 H, 2 $-CH_3$), 2.50 (q, 2 H, $-CH_2-$), 3.53 (q, 1 H, MeCHO), 6.17 [s, 1 H, OCH(Ph)COO], 7.45 ppm (broad s, 10 H, 2 C_6H_5); mass spectrum, *m/z* (% abundance) 163 (12), 105 (51), 77 (14), 57 (100). The molecular ion was not detectable.

The individual *R* and *S* enantiomers of 1-phenylethanol allowed us to assign identities to the diasteromeric chromatographic peaks and also allowed us to develop a derivatization procedure that avoided racemization at the stereogenic carbon atoms of mandelic acid and of 1-phenylethanol. An irreducible amount (ca. 11%) of the optical antipode was always found by our method in the commercial samples of (*R*)- or (*S*)-1-phenylethanol we used, despite the fact that these samples displayed a specific rotation (40°) near the literature value of 43.6°.[14] This contamination was also observed when we used the method of Pirkle,[15] which is based on NMR shift differences of chirotopic hydrogens in esters of α-[1-(9-anthryl)-2,2,2-trifluoroethoxy]acetic acid. We concluded that previous samples of this alcohol have similarly been optically impure, a reasonable circumstance in view of the preparative resolution of the enantiomers by means of differential crystallization of brucine salts.

Enzymic Hydroxylation of Phenylethane. Reaction mixtures contained cytochrome P-450 (0.3 nmol), NADPH-cytochrome P-450 oxidoreductase (0.6 nmol), dilauroyl glycero-3-phosphorylcholine (30 μg, added as a sonicated suspension in water), potassium phosphate (100 μmol, pH 7.4), phenylethane or a deuterated analogue (65 μmol, added as the pure liquid), catalase (6000 units), and a NADPH-generating system in a total volume of 1 mL. The NADPH-generating system consisted of isocitric dehydrogenase (0.8 unit), $NADP^+$ (400 nmol), isocitric acid (5 μmol), and magnesium chloride (10 μmol). Under these conditions cytochrome P-450 was the rate-limiting component. Reactions were initiated by the addition of isocitric dehydrogenase and were allowed to proceed at 25 °C in sealed 15-mL tubes for 12 h with occasional gentle shaking. In one experiment, the reaction period was varied from 2 to 96 h to judge the maximum yield of alcohol attainable. Organic materials were extracted with 1 mL of chloroform. If the absolute yield of 1-phenylethanol was to be measured, then *p*-methylbenzyl alcohol or *p*-cresol was added as an internal standard prior to extraction. The chloroform solution was dried over anhydrous sodium sulfate and then over molecular sieves. The dried solution was then concentrated to ca. 20 μL and used for direct analysis of alcohols or for diastereomeric derivatization. The total yield of 1-phenylethanol was determined by GC on column A at 140 °C, while the total deuterium content of the alcohol was determined by GC–MS on column A at 160 °C.

Diasteromeric Derivatization of Enzymically Formed Alcohols. The dried and concentrated solution of 1-phenylethanol from the enzyme reaction was cooled in ice in a septum-sealed vial. Dry pyridine (4 μL) was added, followed by (*S*)-*O*-propionylmandelyl chloride (20–50 μL of a 120 mM solution in chloroform). An excess of the acid chloride was always used, as well as sufficient reaction time, to ensure quantitative conversion of alcohol to ester. The vial was shaken and allowed to stand at room temperature for 1 h. The resulting solution was directly analyzed for enantiomeric composition by GC on column A at 180 °C and for the deuterium content of the resolved enantiomers by GC–MS on column A at 210 °C.

Determination of Deuterium Contents. Determination of the deuterium content of the enzymatic alcohol product was accomplished by GC–MS of the phenylethyl mandelate derivative and of the alcohol directly. The mandelate did not exhibit a detectable molecular ion (*m/z* 312) with electron-impact ionization (70 eV), so that we were forced to use a fragment in determining the deuterium content. A moderate peak at *m/z* 163 (12%) was assigned the probable structure of $[C_6H_5CHOC(O)CH_2CH_3]^+$ by the obervations of a shift of this ion to *m/z* 149 when using the *O*-acetylmandelate ester and of no shift in position when synthetic 1-phenylethanol-*1-d* was used as the alcohol. Thus, the *m/z* 163 ion is useless for deuterium assessment since it does not contain the phenylethyl portion of the ester. A major peak at *m/z* 105 (51%), on the other hand, was found by the same criteria to represent the $[C_6H_5CHCH_3]^+$ ion. The ratio of intensities at *m/z* 105 and 106, then, is proportional to the ratio of abundances of hydrogen-containing and deuterium-containing alcohols in the mixture. A calibration curve (Figure 1) was compiled by measuring the 105/106 ratio for a series of known standards, prepared by esterification of mixtures of weighed amounts of the pure isotopic alcohols. This curve showed good concordance with the calculated theoretical curve. Deuterium contents could also be obtained from direct analysis of the alcohols, without esterification. The mass spectrum of 1-phenylethanol shows an abundant molecular ion (*m/z* 122, 21%) and a strong fragment ion (*m/z* 107, 66%, M – CH_3). Either ion is suitable for deuterium analysis, and, in fact, both the 123/122 and 108/107 ratios from the alcohol were used to confirm the deuterium contents indicated by the 105/106 ratio from the ester.

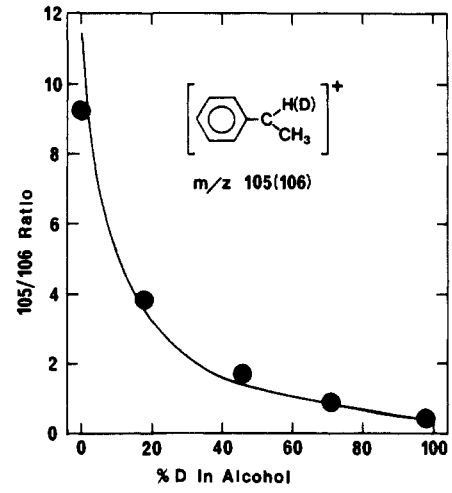

Figure 1. Calibration curve for mass spectral estimation of deuterium content of 1-phenylethyl *O*-propionylmandelate. The ratio of peak intensities of the *m/z* 105 to *m/z* 106 ions was determined for several samples of known deuterium content. The curve is the theoretical dependence of the ratio *m/z* 105/106 on percent D, assuming the normal P + 1 contribution ($^{13}C[C_6H_5CHCH_3]^+$) to 106 and P – 1 contribution ($[C_6H_5CD{=}CH_2]^+$) to *m/z* 105 measured from the mass spectrum of ordinary 1-phenylethyl *O*-propionyl mandelate.

Results

The enzyme used, $P\text{-}450_{LM2}$, is a homogeneous protein of known primary structure.[16,17] Combination of this enzyme with its physiological partner, NADPH-cytochrome P-450 reductase, in an artificial phospholipid milieu provides a reconstituted hydroxylase system useful for study of the enzymatic hydroxylation reaction.[18,19] The substrate chosen for stereochemical studies was phenylethane. This compound is hydroxylated by $P\text{-}450_{LM2}$ more than 99% at the benzylic position, affording 1-phenylethanol. Only traces of 2-phenylethanol or of ethylphenols are found. In addition, phenylethane is a reasonably good substrate for $P\text{-}450_{LM2}$, with a turnover number of about 10 min^{-1}. Finally, a good synthetic scheme is already available for the two derivatives of phenylethane, which are chiral by virtue of deuterium substitution at the 1-position. Thus, the deuterium serves as a marker for attack by $P\text{-}450_{LM2}$ at the *pro-R* or *pro-S* positions. Some previous work with $P\text{-}450_{LM2}$ has indicated relatively little stereochemical preference[18] and significant racemization[5] of the prostereogenic[20]

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Table I. Observed Net Stereochemical and Isotopic Composition of Enzymatically Formed 1-Phenylethanol[a]

substrate	mole percent product				% *pro-R* abst	alc total % D
	R_H	R_D	S_H	S_D		
$PhCH_2CH_3$	47.6 ± 2.5 (n = 5)		52.4 ± 2.5 (n = 5)			*b*
(*R*)-$PhCHDCH_3$	5.3 ± 0.5 (n = 4)	37.0 ± 1.0 (n = 4)	1.2 ± 1.2 (n = 4)	56.5 ± 1.8 (n = 4)	6.5 ± 1.7	93.5 (n = 1)
(*S*)-$PhCHDCH_3$	6.0 ± 0.9 (n = 5)	64.5 ± 3.2 (n = 5)	10.0 ± 1.2 (n = 5)	19.5 ± 2.1 (n = 5)	84.0 ± 5.3	92.8 (n = 1)
$PhCD_2CH_3$		49.5 ± 0.7 (n = 4)		50.5 ± 0.7 (n = 4)		100[c]

[a] The hydroxylation product from each indicated substrate was analyzed for deuterium content by GC–MS using the ratios m/z 107/108 and 122/123. The result is listed in the table as alc total % D. Then, the alcohol was esterified with (*S*)-*O*-propionylmandelyl chloride. The enantiomeric composition was obtained from the GC–MS-resolved mandelate diastereomers, and the deuterium content of the resolved enantiomers was obtained from the ratio m/z 105/106. The data allowed us to calculate the population of enantiomeric and isotopic forms. Values are given as the mean ± standard deviation for n replicate experiments. Within each experiment the values were measured three times. [b] Normal background abundance of deuterium. [c] No hydrogen-containing alcohol was measurable in the mass spectrum.

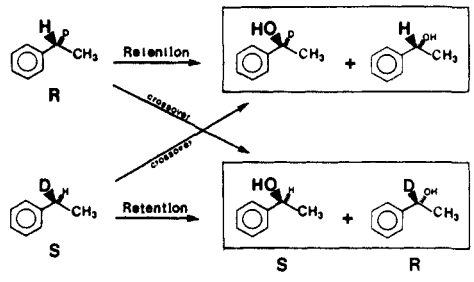


Figure 2. Possible stereochemical courses of phenylethane-*1-d* hydroxylation. Any combination of the four alcohols shown is possible, starting from either phenylethane-*1-d* stereoisomer. Horizontal arrows show the products froma stereochemical retention mechanism, while angled arrows show the results of crossover or a stereochemical inversion mechanism.

position during hydroxylation of bicyclic compounds. Thus, hydroxylation of phenylethane could yield (*R*)-1-phenylethanol, (*S*)-1-phenylethanol, or a mixture of the two. If the phenylethane is chirally deuterated, then potentially a mixture of deuterated and nondeuterated *R* and *S* alcohols could result (Figure 2). Determination of the four components for both (*R*)- and (*S*)-phenylethane-*1-d* will allow a complete description of the stereochemical course of the hydroxylation event. The problem, then, consisted of three parts: synthesizing enantiomerically pure (*R*)- and (*S*)-phenylethanes-*1-d*; resolving the *R* and *S* enantiomeric 1-phenylethanols; determining the deuterium contents of the resolved enantiomers.

An elegant synthesis of enantiomerically pure phenylethane-*1-d* has been described by Mosher,[12] as explained in the Experimental Section. Enantiomeric analysis of the *R* and *S* stereoisomers of 1-phenylethanol was accomplished by gas chromatography of the diastereomeric esters formed by the reaction of the alcohols with either (*R*)- or (*S*)-*O*-propionylmandelyl chloride. The latter derivatizing agent was prepared from (*R*)- or (*S*)-mandelic acid (100% enantiomeric excess) via two mild reactions not involving the stereogenic carbon, as shown in Scheme II. (*S*)-*O*-Acetylmandelyl chloride was equally useful. Deuterium contents of the resolved alcohols were determined by mass spectrometry as described in the Experimental Section.

A series of preliminary experiments established that phenylethane is efficiently hydroxylated by this reconstituted system, with no nonenzymatic hydroxylation (eq 1). The optimal amount

$$PhCH_2CH_3 + O_2 + NADPH + H^+ \rightarrow PhCH(OH)CH_3 + H_2O + NADP^+ \quad (1)$$

of phenylethane in the reactions was found to be 8 μL (65 μmol) in a 1-mL volume. The time evolution of the accumulation of 1-phenylethanol is shown in Figure 3. The initial zero-order kinetic period was about 2 h (turnover number about 10 min^{-1}), followed by a declining rate up to 12 h, at which point no further product accumulated. The rate undoubtedly falls off because one or more components become exhausted, but we did not pursue this point.

(20) Mislow, K.; Siegel, J. *J. Am. Chem. Soc.* **1984**, *106*, 3319–3328.

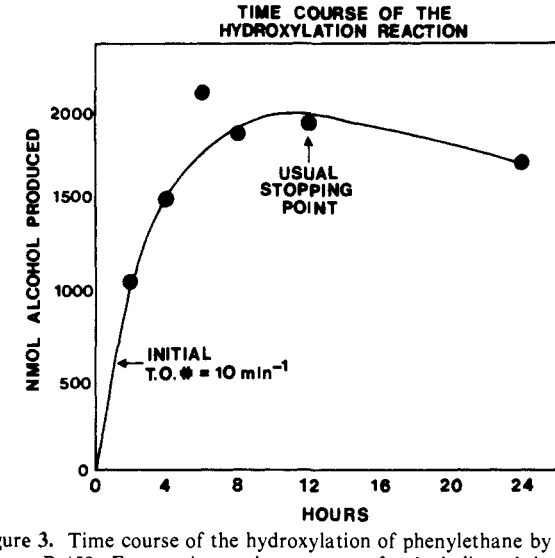
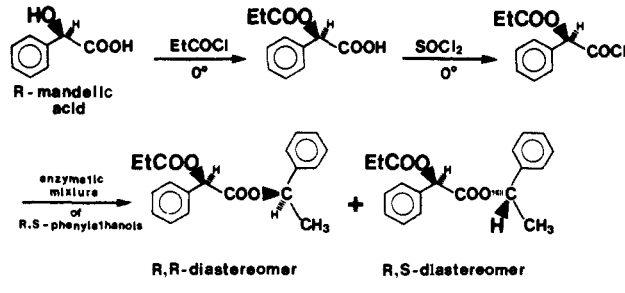


Figure 3. Time course of the hydroxylation of phenylethane by cyctochrome P-450. Enzymatic reactions were run for the indicated times and then stopped and analyzed for 1-phenylethanol. Reaction conditions were as described in the Experimental Section.

Scheme II. Analysis of Enantiomers



Enantiomeric analysis of the enzymatic alcohol from phenylethane is shown in Figure 4 and Table I. Approximately equal quantities of the *R* and *S* isomers were present. The enantiomeric composition was invariant with time, indicating the alcohols did not undergo further stereochemical changes during exposure to the enzymes subsequent to their formation. When (*R*)-phenylethane-*1-d* was the substrate, the *S* alcohol predominated (Figure 5), while when (*S*)-phenylethane-*1-d* was the substrate, the *R* alcohol predominated (Figure 6). The phenylethane-*1,1-*d_2 produced about equal quantities of *R* and *S* alcohols again (Figure 7). Deuterium contents were obtained for the alcohols from the single-deuterium-substituted phenylethanes and are shown in Table I as well. One immediately notices that with both (*R*)- and (*S*)-phenylethane-*1-d* almost all hydroxylation occurred by way of hydrogen removal. The alcohols from both substrates were enriched in deuterium. Clearly, a significant deuterium isotope effect must be operative.

These observed enantiomeric compositions and deuterium contents have little meaning as they stand. However, we can

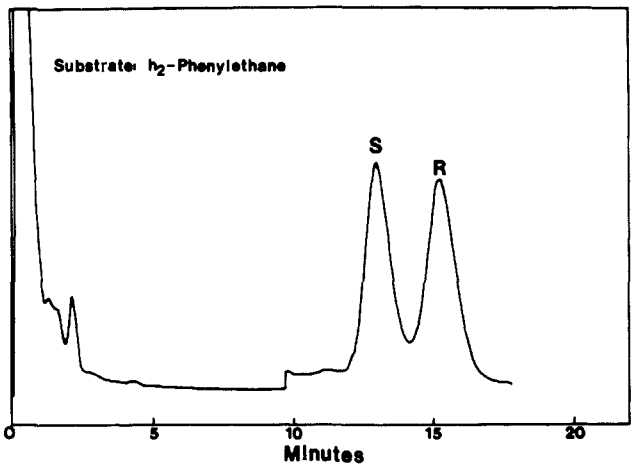


Figure 4. Gas chromatogram of diastereomeric (*S*)-*O*-propionylmandelyl esters of alcohol mixture from hydroxylation of phenylethane by cytochrome P-450. After the enzymatic hydroxylation had proceeded for 12 h, the alcohol product was extracted with chloroform, derivatized with (*S*)-*O*-propionylmandelyl chloride, and subjected to gas chromatography on column A at 180 °C. (*S*)-1-phenylethyl *O*-propionylmandelate has a retention of about 13 min, while (*R*)-1-phenylethyl (*S*)-*O*-propionylmandelate elutes at about 16 min.

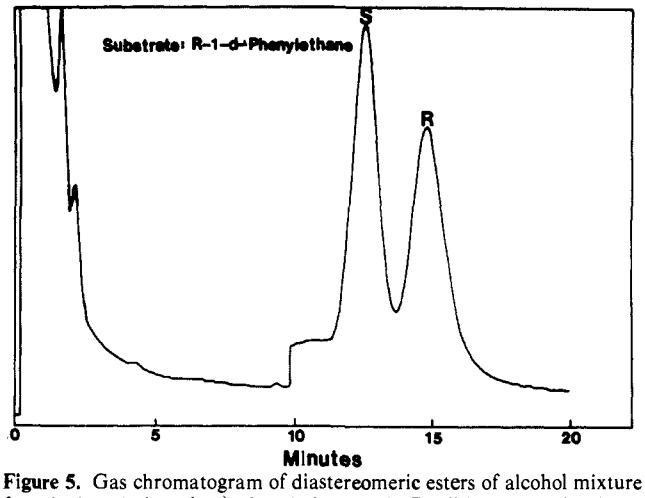


Figure 5. Gas chromatogram of diastereomeric esters of alcohol mixture from hydroxylation of (*R*)-phenylethane-*1-d*. Conditions are as in Figure 4.

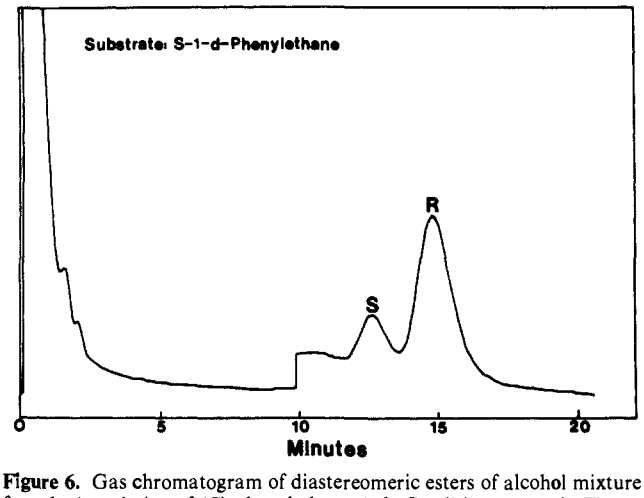


Figure 6. Gas chromatogram of diastereomeric esters of alcohol mixture from hydroxylation of (*S*)-phenylethane-*1-d*. Conditions are as in Figure 4.

dissect them into more fundamental descriptors of the stereochemical course through the following analysis. We define the following constants and parameters following the formalism of Gelb et al.:[3] k_R^H, rate constant for abstraction of *pro-R* hydrogen;

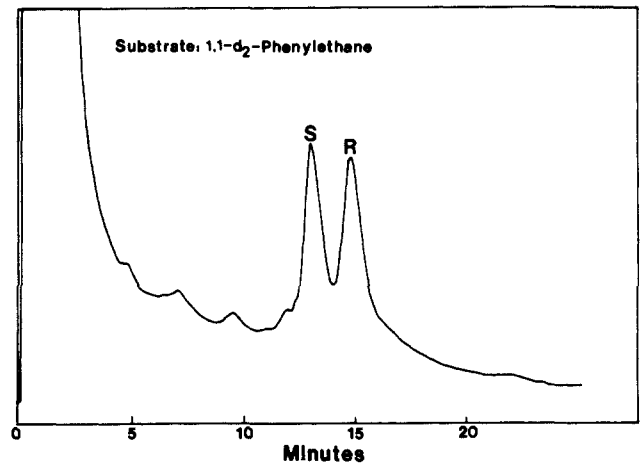


Figure 7. Gas chromatogram of diastereomeric esters of alcohol mixture from hydroxylation of phenylethane-*1,1-d*$_2$. Conditions are as in Figure 4.

k_S^D, rate constant for abstraction of *pro-S* deuterium; other rate constants, similarly labeled; G_1 (eq 2–4), intrinsic ratio of *pro-R*

$$G_1 = k_R^H/k_S^H \quad \text{range 0–infinity} \tag{2}$$

$$G(R\text{-}d_1) = k_R^D/k_S^H \tag{3}$$

$$G(S\text{-}d_1) = k_R^H/k_S^D \tag{4}$$

to *pro-S* hydrogen abstraction with phenylethane; $P_{re\text{-}si}$, probability that a tricoordinate carbon intermediate initially having the *re* face toward the oxygen delivery trajectory (i.e., *pro-R* abstraction) will cross over to the *si* face (range 0–1); $P_{si\text{-}re}$, probability that a tricoordinate carbon intermediate initially having the *si* face toward the oxygen delivery trajectory (i.e., *pro-S* abstraction) will cross over to the *re* face (range 0–1); *R*-KIE (eq 5), deuterium

$$R\text{-KIE} = k_R^H/k_R^D \tag{5}$$

kinetic isotope effect for *pro-R* hydrogen abstraction; *S*-KIE (eq 6), deuterium kinetic isotope effect for *pro-S* hydrogen abstraction.

$$S\text{-KIE} = k_S^H/k_S^D \tag{6}$$

We also define the "selectivity" function, *S*, which has the value 1 when only *pro-R* hydrogen is abstracted by the enzyme, the value −1 when only *pro-S* hydrogen is taken and 0 when *pro-R* and *pro-S* are equally likely to be taken (eq 7). Nonintegral values of *S*

$$S_1 = (k_R^H - k_S^H)/(k_R^H + k_S^H) \tag{7}$$

indicate a preference for *pro-R* or *pro-S* hydrogen but not an absolute specificity. The *intrinsic* selectivity refers to the value of *S* for the nondeuterated substrate. The basic equation governing the net observed enantiomeric composition of the 1-phenylethanol product is eq 8.

$$\%\ R \text{ alcohol} = (\%\ \textit{pro-R} \text{ abst})(1 - P_{re\text{-}si}) + (\%\ \textit{pro-S} \text{ abst})(P_{si\text{-}re}) \tag{8}$$

Intuitively, eq 8 states that the number of *R* alcohol molecules produced is equal to the number of *pro-R* hydrogen abstractions that lead to alcohol *without* crossover plus the number of *pro-S* abstractions that lead to alcohol *with* crossover. Equation 8 may be rewritten as shown in eq 9.

$$P_{si\text{-}re} = \frac{(\%\ R \text{ alc}) - (\%\ \textit{pro-R} \text{ abst})(1 - P_{re\text{-}si})}{100 - \%\textit{pro-R} \text{ abst}} \tag{9}$$

Equation 9 may be used to calculate the two crossover probabilities because we can experimentally measure % *R* alc and % *pro-R* abst for the two singly deuterated phenylethanes, thus generating two equations in two unknowns. % *pro-R* abst is obtained from the total deuterium content of the alcohol (*R* + *S*) when either (*R*)-phenylethane-*1-d* or (*S*)-phenylethane-*1-d* is hydroxylated. When *S*-d_1 is used, % *pro-R* abst = % D in the

Table II. Fundamental Stereochemical Descriptors for Phenylethane Hydroxylation by Cytochrome P-450$_{LM2}$[a]

substrate	% *pro*-R abst	*G*	*S*
	Fundamental Descriptors		
$PhCH_2CH_3$	21.5 ± 14.1	0.274 ± 0.159	−0.570 ± 0.282
(*R*)-$PhCHDCH_3$	6.5 ± 1.7	0.0695 ± 0.0191	−0.870 ± 0.034
(*S*)-$PhCHDCH_3$	84.0 ± 5.3	5.25 ± 1.56	0.680 ± 0.106
$PhCD_2CH_3$	26.6 ± 10.6	0.362 ± 0.172	−0.468 ± 0.212
	Fundamental Constants		
$P_{re\text{-}si}$ = 0.232 ± 0.037	*R* KIE = 4.0 ± 1.3	G_1 = 0.274 ± 0.159	
$P_{si\text{-}re}$ = 0.396 ± 0.023	*S* KIE = 19 ± 10	S_1 = −0.570 ± 0.282	

[a] The fundamental descriptors and constants describing the stereochemical course of the hydroxylation event were calculated as indicated in the text from the observed data in Table I.

alcohol, while when *R*-d_1 is used, % *pro-R* abst = 100 − % D in the alcohol. The use of eq 9 with two different substrates provides one means of obtaining numerical values for $P_{re\text{-}si}$ and $P_{si\text{-}re}$ by measuring only the net enantiomeric composition of the alcohol and the total deuterium content in the alcohol. Alternatively, one can obtain the crossover probabilities from either *R*-d_1 or *S*-d_1 alone if one can also measure the respective deuterium contents of the resolved *R* and *S* alcohols. For instance, the fraction of *R* to *S* crossover events is the number of *pro-R* abstractions that yields *S* alcohol divided by the total number of *pro-R* abstractions. In the case of *R*-d_1, then, we use eq 10 and 11, and in the case of *S*-d_1, we use eq 12 and 13.

$$P_{re\text{-}si} = \frac{\%\ S\text{-}h\ \text{alc}}{\%\ R\text{-}h\ \text{alc} + \%\ S\text{-}h\ \text{alc}} \tag{10}$$

$$P_{si\text{-}re} = \frac{\%\ R\text{-}d\ \text{alc}}{\%\ R\text{-}d\ \text{alc} + \%\ S\text{-}d\ \text{alc}} \tag{11}$$

$$P_{re\text{-}si} = \frac{\%\ S\text{-}d\ \text{alc}}{\%\ R\text{-}d\ \text{alc} + \%\ S\text{-}d\ \text{alc}} \tag{12}$$

$$P_{si\text{-}re} = \frac{\%\ R\text{-}h\ \text{alc}}{\%\ R\text{-}h\ \text{alc} + \%\ S\text{-}h\ \text{alc}} \tag{13}$$

Equation 10–13 allow us to estimate $P_{re\text{-}si}$ and $P_{si\text{-}re}$ with both *R*-d_1 and *S*-d_1 and compare the estimates. If crossover probabilities are independent of the presence of deuterium, then we should measure the same values for $P_{re\text{-}si}$ and for $P_{si\text{-}re}$ with either substrate. With (*R*)-phenylethane-*1*-*d* we measured $P_{re\text{-}si}$ = 0.185 and $P_{si\text{-}re}$ = 0.396, while with (*S*)-phenylethane-*1*-*d* we measured $P_{re\text{-}si}$ = 0.232 and $P_{si\text{-}re}$ = 0.375. This correspondence of *P* values proves that deuterium atoms do not perturb the probability of crossover. Once we know the crossover probabilities, we can apply eq 8 to the nondeuterated substrate. This time we solve for % *pro-R* abst.

$$\%\ \textit{pro-R}\ \text{abst} = \frac{\%\ R\ \text{alc} - 100P_{si\text{-}re}}{1 - P_{re\text{-}si} - P_{si\text{-}re}} \tag{14}$$

Given % *pro-R* abst for the nondeuterated substrate, then

$$G = \%\ \textit{pro-R}\ \text{abst}/(100 - \%\ \textit{pro-R}\ \text{abst}) \tag{15}$$

Values of *G* are calculated for each of the four phenylethanes by means of eq 15 using the appropriate % *pro-R* abst measured or calculated in each case. Deuterium kinetic isotope effects are obtained as simple ratios of *G* values (eq 16 and 17).

$$R\ \text{KIE} = k_R^H/k_R^D = \frac{k_R^H/k_S^D}{k_R^D/k_S^H} = G_1/G(R\text{-}d_1) \tag{16}$$

$$S\ \text{KIE} = k_S^H/k_S^D = \frac{k_R^H/k_S^D}{k_R^H/k_S^H} = G(S\text{-}d_1)/G_1 \tag{17}$$

Table II compiles the fundamental stereochemical descriptors calculated with eq 7–17. The G_1 value for phenylethane hydroxylation is 0.27, meaning that the enzyme active site favors *pro-S* hydrogen abstraction over *pro-R* by about 4 to 1. When deuterium occupies the *pro-R* position, the intrinsic selectivity and the isotope effect operate in the same direction, such that $G(R\text{-}d_1)$ drops close to 0. With this substrate the enzyme is almost stereospecific with respect to hydrogen removal. On the other hand, when deuterium occupies the *pro-S* position, the intrinsic selectivity and the isotope effect are in opposition. If *S*-KIE were about 4, then the selectivity and the isotope effect would cancel, and we would expect about 50% *pro-R* abstraction (expected *S* value = 0). The experimentally measured value of *pro-R* abstraction for *S*-d_1 is 84%. Thus, *S* KIE must be substantially greater than 4 in order for $S(S\text{-}d_1)$ to be as high as 0.7. The calculated value of *S* KIE is, in fact, 19 while that for *R* KIE is 4.0.

Values of the crossover probabilities are also shown in Table II. The probability of a *pro-R* abstraction leading to *S* alcohol is nearly 25% while the figure for *pro-S* abstraction leading to *R* alcohols is 40%. These values represent considerable loss of stereochemistry during the hydroxylation event and can only mean that *a discrete tricoordinate carbon intermediate is allowed enough freedom of movement in the active site to turn completely over and present the opposite face for recombination 25–40% of the time.* This result is unambiguous since the magnitude of the crossover is greater than any possible experimental error in the system. For instance, deuterium contents of the alcohols were measured by two independent methods with good agreement. The error in estimation of enantiomeric compositions was shown in control experiments to not exceed 11% and was probably near 0%. The deuterium contents of the d_1 substrates were confirmed to be approximately 100% by mass spectrometry and NMR. Finally, stereochemical impurity of the phenylethane-*d* substrates would introduce an indeterminate error. Limits to such an error can be assessed as follows below.

We can check the internal consistency of our results by calculating the expected enantiomeric composition of the alcohol from hydroxylation of phenylethane-*1*,*1*-d_2. The expected % *R* abst may be calculated from eq 18 and 19.

$$G(d_2) = k_R^D/k_S^D = G_1(S\ \text{KIE}/R\ \text{KIE}) \tag{18}$$

$$\%\ R\ \text{abst} = 100G(d_2)/[1 + G(d_2)] \tag{19}$$

Given values for $P_{re\text{-}si}$, $P_{si\text{-}re}$, and % *R* abst, we can use eq 8 to calculate the expected value for % *R* alc with the d_2 substrate. This calculated value is 60% *R* alcohol, which is to be compared with the experimental value of 50% listed in Table I. The relative error is 20%, representing the composite of errors in $P_{re\text{-}si}$, $P_{si\text{-}re}$, G_1, *R* KIE, and *S* KIE. Another effect, ignored until this point, contributes to the error as well. In addition to the primary deuterium isotope effect on deuterium abstraction, a secondary isotope effect operates on hydrogen abstraction when deuterium is the geminal atom at the benzylic methylene. If the secondary isotope effect is greater than unity,[21] the net effect is actually to suppress the magnitude of the observed isotope effect as measured here. Thus, $R\ \text{KIE}_{obsd} = R\ 1°\text{-KIE}/S\ 2°\text{-KIE}$. However, when deuterium occupies *both* positions at methylene, then an additional secondary isotope effect enters to further reduce the rate constant for deuterium removal, so that the equation in that case becomes $R\ \text{KIE}_{obsd} = (R\ 1°\text{-KIE})(R\ 2°\text{-KIE})/(S\ 2°\text{-KIE})$. The result is that our *calculated* value of $G(d_2)$ will be wrong by the factor $(S\ 2°\text{-KIE})/(R\ 2°\text{-KIE})$ and our value of % *R* abst calculated by eq 19 will correspondingly be in error. We have no independent means to assess this ratio of secondary isotope effects, but we can estimate limits for its magnitude. While secondary isotope effects may in theory be as high as 1.7,[22] they rarely exceed 1.2[23] and may be unity. Given the significant difference measured between *R* KIE and *S* KIE, we expect the secondary isotope effects to be

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(22) Thornton, E. K.; Thornton, E. R. In *Isotope Effects in Chemical Reactions*; Collins, C. J., Bowman, N. S., Eds.; Van Nostrand Reinhold: New York, 1970; pp 213–285.
(23) Sunko, D. E.; Borcic, S. In *Isotope Effects in Chemical Reactions*; Collins, C. J., Bowman, N. S., Eds.; Van Nostrand Reinhold: New York, 1970; pp 160–212.

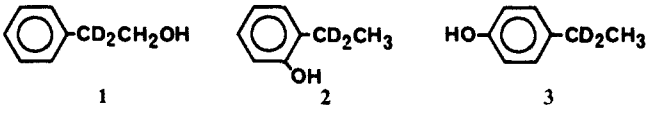
Table III. Regioselectivity of Phenylethane Hydroxylation by Cytochrome P-450$_{LM2}$[a]

substrate	product	abund, %	rate, min^{-1}
phenylethane	1-phenylethanol	99.8	9.0 ± 0.1
	2-phenylethanol	0.08	0.0072 ± 0.0048
	o-ethylphenol	0	0
	p-ethylphenol	0.13	0.012 ± 0.008
phenylethane-*1,1*-d_2	1-phenylethanol	96.4	7.1 ± 0.2
	2-phenylethanol	1.1	0.078 ± 0.003
	o-ethylphenol	0.6	0.048 ± 0.009
	p-ethylphenol	1.9	0.077 ± 0.008

[a] Enzymatic hydroxylation reactions were conducted as described in the Experimental Section, but the alcohols were not derivatized to the mandelate esters. Instead, they were directly analyzed by gas chromatography on column A or by reversed-phase high-pressure liquid chromatography. Abundances represent the percent contribution of the particular product to the total product from that substrate. Absolute rates of formation of the various products are expressed as turnover numbers and are given as the average ± deviation for three replicate experiments.

different as well so that their ratio will not be 1. Therefore, 0.8 < (*S* 2°-KIE)/(*R* 2°-KIE) < 1.2. However, we cannot further specify the ratio. In any event, it is clear that with the magnitude of potential error in our calculation of % *R* alcohol from the d_2 substrate, 20% relative error between theory and experiment is reasonable agreement. Therefore, we believe that our values of the fundamental stereochemical constants in Table II are essentially correct.

Another nuance of the stereochemical dynamics in the P-450$_{LM2}$–phenylethane hydroxylation system also came to light during experiments with the d_2 substrate: the relative yields of three minor products were increased. These were identified from GC and HPLC retention times as the 2-phenylethanol (**1**), the



1 **2** **3**

o-ethylphenol (**2**), and the *p*-ethylphenol (**3**). As shown in Table III, the total relative yield of these three products increased from 0.2% to 3.6% when both benzylic hydrogens were replaced with deuterium. The *absolute* rates of formation of all three products were also increased, so the four products apparently result from competition for the reactive oxygen during the same substrate binding event. This sort of "metabolic switching" has been reported before with P-450 enzymes, most recently by Harada et al.[24] in the oxidation of 7-ethoxycoumarin-*1,1*-d_2 and by Eble and Dawson[25] in the hydroxylation of 5,5-difluorocamphor by P-450$_{cam}$. This phenomenon is usually interpreted as the selective reduction of one or more hydrogen abstraction rate constants, with other rate constants being unaltered. In our case, then, the presence of deuterium at the benzylic methylene allows the slower hydrogen abstraction at the methyl and addition to the aromatic ring to be expressed with a greater frequency in the total product due to the kinetic isotope effect. We were not surprised that **1** and **2** were increased, given their proximity to the favored hydroxylation site; but for **3** to be increased indicates an additional degree of freedom of movement of the phenylethane in the active site such that the other end of the phenyl ring is occasionally exposed to the reactive oxygen before site selection occurs. A deuterium isotope effect of 18 ± 8 for methylene hydrogen abstraction may be estimated from the data in Table III, with the considerable error due to the large relative error for the low-yield products. The deuterium isotope effect expressed in k_{cat} for benzylic hydroxylation is only $^{D}V = 1.28$, indicating that at least one enzymic step with a large "commitment to catalysis"[24] precedes hydrogen abstraction.

The presence of products **1**–**3** raises the possibility that some benzyl alcohol arose via hydrogen abstraction at the terminal

Figure 8. Approach of phenylethane to the iron–oxo unit. The central hatched atom is iron, with an oxygen atom above and sulfur below. Carbon atoms are solid black. The lines represent the *x* and *y* axes through iron, and the four atoms in the *xy* plane are the pyrrole nitrogens: (A) best approach for benzylic hydrogen abstraction; (B) possible approach for para hydroxylation, which involves phenyl epoxidation rather than hydrogen abstraction.

methyl or ring carbons, followed by hydrogen migration to give benzyl radicals. If such migration occurred with the d_2 substrate, then the major product, 1-phenylethanol, should retain *both* deuterium atoms of the substrate. If the 1-phenylethanol can only result from initial benzylic hydrogen abstraction, then it should contain only a *single* deuterium. Mass spectrometry of the enzymically formed 1-phenylethanol from phenylethane-*1,1*-d_2 showed the normal-abundance ratio of *m/z* 124/123 (10.8%) expected for the molecular ion with one deuterium atom. The 107/108 ratio of 8.6% for the ($M^+ - CH_3$) ion was the same as in the spectrum of authentic 1-phenylethanol-*1*-*d*. Thus, the enzymic alcohol was formed exclusively by initial benzylic hydrogen abstraction.

Discussion

The best evidence presently available suggests that the reactive oxygen intermediate of the cytochrome P-450 enzymes is an iron–oxo species that is two oxidation equivalents above the ferric state, formally equivalent to peroxidase compound I.[26] We will us this model in our discussion of the dynamics of aliphatic hydroxylation. This iron–oxo species is expected to have unpaired electron density in Fe–O π orbitals, which are perpendicular to the heme plane.[27,28] Hydrogen abstraction from a substrate C–H bond would produce a substrate radical and an iron–hydroxo complex. Grove-type[5] oxygen rebound results in C–O bond formation for the observed product alcohol.

Five independent stereochemical constraints govern the selection of the exact configuration of the phenylethane–heme–oxo transition state: (1) hydrogen transfer occurs most easily with a linear C–H–O arrangement;[29] (2) since the oxygen p orbitals should be perpendicular to the Fe–O σ bond, then the C–H bond should be approximately parallel to the heme plane and within the *xz* or *yz* planes for optimal alignment; (3) removal of hydrogen from the benzylic position of phenylethane will be easiest when the C–H bond being broken is nearly perpendicular to the phenyl plane so that the developing p orbital can begin to overlap the phenyl π system; (4) the plane of the phenyl group will tend to remain parallel to the heme place to maximize π–π interactions; (5) protein groups in the vicinity will not allow all possible substrate–heme geometries. By examination of space-filling CPK molecular models we can generate the best compromises among effects (1)–(4). However, we can only refer to the data to assess effect (5). When ethylbenzene is attacked at the 1-hydrogens, the approach in Figure 8A allows a favorable π–π stacking interaction between the parallel π systems and nearly linear hydrogen transfer. We therefore believe that Figure 8A probably represents the best configuration for removal of the 1-hydrogens. The difference in bond energies between the methyl and benzylic C–H bonds may account for the virtual total lack of hydroxylation at the 2-position. Attack on the phenyl ring itself to generate *p*-

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(25) Eble, K. S.; Dawson, J. H. *J. Biol. Chem.* **1984**, *259*, 14389–14393.
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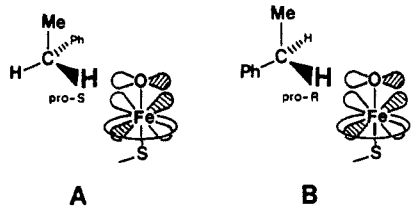

Figure 9. Two rotamers of phenylethane for which benzylic hydrogen abstraction is allowed. The approach of substrate to the oxo–heme is the same as in Figure 8A. In (A) the *pro-S* hydrogen is about to be removed, while in (B) the *pro-R* hydrogen will be removed.

ethylphenol will require an orientation similar to that of Figure 8B.

Concerning the accommodation of the observed selectivity for *pro-S* hydrogen abstraction, we must assume chirality of the enzyme active site near the iron atom. Obviously, two different orientations of the phenylethane molecules with respect to the fixed oxo–heme unit are required for *pro-R* and *pro-S* hydrogen abstraction, as seen schematically in Figure 9. The benzylic C–H bond is favorably positioned near the iron–oxygen pair with the phenyl group parallel to the heme and the methyl group vertical. The phenyl group must project to the right or left as viewed from the iron prior to *pro-S* or *pro-R* abstraction, respectively. Apparently, the local structure is such that phenyl projection to the right is the easier of the two orientations, since *pro-S* hydrogen abstraction is favored 4 to 1.

We can also consider the dynamics of the *crossover* process. Recombination will occur when the half-filled p orbital of the benzyl radical overlaps a half-filled π orbital of Fe–OH. Since the lobes of the benzyl p orbital are directed perpendicular to the plane of the phenyl ring, the crossover event can only occur by flipping of the entire substrate molecule, as shown in Figure 10. Notice that this process requires the phenyl ring to momentarily be perpendicular to the porphyrin plane. Since the P-450$_{LM2}$ active site is very open to solvent,[30] shows high conformational flexibility,[31] and can easily accommodate rather bulky tricyclic structures,[18] there is probably no appreciable barrier to limited movement vertical to the heme plane. On the other hand, crossover in which the ethyl group of the benzyl radical rotates with respect to a fixed phenyl group would still require some vertical movement and, in addition, would sacrifice conjugation energy between the radical center and the phenyl π system. The latter mode of crossover is, therefore, much less likely. Once the benzylic p orbital contacts the Fe–OH π orbital, the two radical species collapse, generating the new carbon–oxygen σ bond. The entire ensemble is now in the form of an iron-coordinated alcohol, which soon dissociates.

Our observation that the rate of *p*-ethylphenol formation was increased when the benzylic hydrogens were replaced with deuterium indicates that para attack directly competes with benzylic attack for the same reactive oxygen. If these were not in direct competition, we would expect the *absolute* rate of para hydroxylation to remain constant, although the *relative* yield of *p*-ethylphenol would still increase. The transition state for *para* attack should resemble that in Figure 8B. The interconversion of the benzylic and para transition states then corresponds to the interconversion of Figure 8A,B. Simple inspection shows that raising the ethyl end of the substrate molecule in Figure 8A will bring the para end near the reactive oxygen. Therefore, the interconversion of the two configurations does not require a large substrate movement such as leaving the active site and returning backwards.

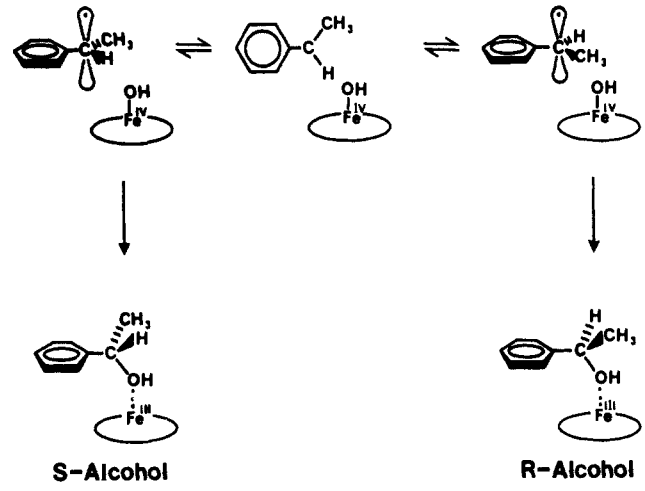

Figure 10. Movements of the phenethyl radical following hydrogen abstraction. The initial radical resulted from *pro-S* hydrogen removal. The initially formed benzylic radical can directly collapse to the *S* alcohol (retention) or crossover to present the *re* face before collapse to the *R* alcohol (inversion). Given the steric requirements of the recombination reaction, the only feasible crossover process involves rotation around the long axis of the benzyl radical. The final structures shown are the transient iron-coordinated alcohols prior to their diffusion from the active site.

The large deuterium isotope effect for *pro-S* hydrogen removal ($k^H/k^D = 19 \pm 10$) is the same as that seen by Groves et al.[5] previously with P-450, within experimental error. The large standard deviation on this number resulted from the high enrichment of deuterium in the alcohol, which made our estimate of the small amount of hydrogen-containing alcohol contain a large relative error. The crude measurement based on the increase of alternate hydroxylation products with $PhCD_2CH_3$ indicated a value of 18, approximately equal to the weighted average of *R* KIE and *S* KIE. With respect to the much smaller deuterium isotope effect of 4.0 observed for *pro-R* hydrogen removal, the same hypothetical steric effects that make *pro-R* hydrogen removal fourfold less favorable than *pro-S* could also substantially suppress a primary isotope effect by forcing a more angular C–H–O transition state.[32]

Conclusion

We have determined that phenylethane is hydroxylated by P-450$_{LM2}$ with very high regioselectivity at the benzylic position. A moderate preference (4/1) is exhibited for *pro-S* hydrogen removal. An appreciable fraction (25–40%) of the hydroxylation events involved crossover of stereochemistry between the hydrogen-abstraction and oxygen-delivery steps. This observation requires that a tricoordinate benzylic carbon atom had to have been a discrete intermediate. Assuming that all cytochrome P-450 enzymes generate essentially identical reactive oxygen intermediates, conservation of stereochemistry at the reacting carbon center is *not* a general and intrinsic characteristic of the P-450 hydroxylation mechanism. Therefore, those P-450 enzymes that do, in fact, produce stereospecific products must rely on constraints of the substrate binding site provided by the protein environment to enforce the desired stereochemistry.

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