On the Mechanism of Action of Cytochrome P450: Evaluation of Hydrogen Abstraction in Oxygen-Dependent Alcohol Oxidation†

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ABSTRACT: The mechanism of oxidation of primary and secondary benzylic alcohols to the corresponding carbonyl compounds by purified rabbit liver cytochrome P450 forms 2B4 and 2E1 in a reconstituted enzyme system has been examined by linear free energy relationships, intramolecular and steady-state deuterium isotope effects, and the incorporation of an O_2-derived oxygen atom or solvent-derived deuterium. The k_{cat} and K_m values were found to be relatively insensitive to the presence of electronic perturbations at the para position. The Hammett reaction constants for the oxidation of benzyl alcohols by P450s 2B4 and 2E1 are −0.46 and −0.37, respectively, and with 1-phenylethyl alcohols the corresponding reaction constants are −1.41 and −1.19, respectively. With [1-$^2H_1$]benzyl alcohol, P450s 2B4 and 2E1 show similar intramolecular deuterium isotope effects of 2.6 and 2.8, respectively, whereas with [1-$^2H_2$]benzyl alcohol under steady-state conditions, the deuterium isotope effects on the catalytic constants are 2.8 and 1.3, respectively. No significant isotope effect on the catalytic constant was noted for either form of P450 with 1-phenylethyl alcohol. In D_2O, acetophenone formed by either form of P450 from 1-phenylethyl alcohol does not contain a deuterium atom at the methyl group, whereas under an atmosphere of $^{18}O_2$ approximately 30% of the labeled oxygen is incorporated into the carbonyl group with either form of the cytochrome. The results are consistent with a mechanism that involves stepwise oxidation of the alcohol to a carbon radical α to the alcohol function, followed by oxygen rebound to yield the *gem*-diol, dehydration of which gives the carbonyl product. However, the rate-determining step is dependent on the alcohol substrate and the form of cytochrome P450 that is examined. Carbon–hydrogen bond cleavage in benzyl alcohol is clearly rate-limiting with P450 2B4 and partially rate-limiting with P450 2E1, whereas in 1-phenylethyl alcohol this step is not rate-limiting with either cytochrome.

P450[1] heme proteins constitute a class of highly versatile biological catalysts that utilize molecular oxygen and NADPH to oxidize diverse organic compounds of endobiotic and xenobiotic origin (Coon et al., 1992). The various reactions result in the insertion of an atom of molecular oxygen into a hydroxylated product or in other types of oxidation at functional groups such as amines, ethers, esters, aldehydes, and alcohols (Guengerich, 1987). A general mechanistic scheme developed for such P450-catalyzed reactions accounts for various aspects of the catalytic cycle and for the insertion of a molecular oxygen-derived oxygen atom into the oxidized product (White & Coon, 1980). In the oxidation of alcohols to carbonyl products, however, some exceptions to the predicted incorporation of an atom of molecular oxygen into the carbonyl product have been observed by various laboratories. Partial or complete lack of incorporation of O_2-derived oxygen into the carbonyl product has been observed that is apparently not explainable by exchange with water (Akhtar et al., 1982; Cheng & Schenkman, 1983; Suhara et al., 1984; Wood et al., 1988). This has resulted in various mechanistic hypotheses for the oxidation of alcohols by P450 such as oxidative dehydrogenation (Cheng & Schenkman, 1983; Wood et al., 1988) or stereospecific dehydration of a transient *gem*-diol such that the inserted oxygen is specifically lost (Suhara et al., 1984). Ekström et al. (1987) have reported that cleavage of the C_1–H bond of ethanol appears to be a rate-determining step in catalysis by the ethanol-inducible form of P450.

We have chosen to examine the oxidation of benzylic alcohols by P450 2E1 and 2B4 as a mechanistic model since reactions at benzylic positions are sensitive to electronic perturbations by substituents on the aromatic ring, and the magnitude of this effect on the rate constant is a useful indicator of the intermediate generated at the benzylic position (Jaffe, 1953). Two further advantages are the large extinction coefficients of benzaldehydes and acetophenones that permit sensitive quantitative analysis by reversed-phase HPLC and the enolization rate, hydration equilibrium, and electron impact mass fragmentation pattern of acetophenone that permit a sensitive measurement of the incorporation of a solvent proton at the methyl group or of an $^{18}O_2$-derived oxygen atom into the carbonyl group. In this study, we have determined (a) the linear free energy relationship for the oxidation of a series of para-substituted benzyl and 1-phenylethyl alcohols to the aldehydes and ketones, respectively; (b) the intramolecular deuterium isotope effect for the oxidation of benzyl alcohol to benzaldehyde; (c) the steady-state deuterium isotope effect on the catalytic constants for the oxidation of benzyl and 1-phenylethyl alcohols to benzaldehyde and acetophenone, respectively; and (d) the incorporation into acetophenone of a solvent-derived proton at the methyl group or an $^{18}O_2$-derived oxygen atom at the carbonyl group.

Our results establish that the oxidation of benzyl alcohols by P450 2B4 proceeds by the rate-determining formation of

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[1] Abbreviations: P450, cytochrome P450; reductase, NADPH-cytochrome P450 reductase; DLPC, dilauroylglyceryl-3-phosphorylcholine; HPLC, high-pressure liquid chromatography. P450 2B4 and P450 2E1 are the currently recommended names (Nelson et al., 1993) for the rabbit liver microsomal isoforms originally designated LM_2 and LM_{3a}, respectively.

a benzylic radical as an intermediate and that oxidative desaturation of 1-phenylethyl alcohol to an enol is not an intermediate with either P450 2B4 or 2E1. Our findings also indicate that both forms of P450 oxidize primary or secondary benzylic alcohols to the corresponding carbonyl compounds by the same sequence of reactions involving an intermediate benzyl radical and oxygen rebound to form the *gem*-diol, dehydration of which yields the carbonyl compounds. However, the rate-limiting step in the overall reaction is dependent on the alcohol substrate as well as the isozyme of P450.

MATERIALS AND METHODS

Substrates and Reagents. NADPH and DLPC were obtained from Sigma and Calbiochem, respectively. Sodium borodeuteride, lithium aluminum deuteride, and primary and secondary benzyl alcohols were obtained from Aldrich. The commercially obtained alcohols were examined for contamination by the corresponding carbonyl compounds and were redistilled or recrystallized from 40% hot aqueous ethanol when necessary. Other alcohols were synthesized as described below from aldehydes or ketones obtained from Aldrich. $^{18}O_2$ of 98% isotopic purity was obtained from Cambridge Isotope Laboratories.

Synthesis of para-Substituted and Deuterium-Labeled Benzyl and 1-Phenylethyl Alcohols. To a solution of the aldehyde or ketone (50 mmol in 100 mL of 20% aqueous ethanol), sodium borohydride (100 mmol) was added, and the reaction mixture was stirred overnight at room temperature. The solution was then concentrated at room temperature under reduced pressure, diluted with 50 mL of water, and extracted twice with 25-mL portions of CH_2Cl_2. After the combined extract had been dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the residue was vacuum distilled (at 18 mmHg) to yield the desired alcohol in an overall yield that ranged from 60 to 80%. In all cases, the alcohol contained less than 0.01% of the starting aldehyde or ketone as determined by HPLC. For the synthesis of [1-$^2H_1$]-1-phenylethanol and [1-$^2H_1$]benzyl alcohol, the method was essentially as above, except that sodium borodeuteride (98% isotopic purity, from Aldrich) was used in a 2.5-fold equivalent excess and the acetophenone or benzaldehyde was 5.0 M in 20% ethanol. For the synthesis of [1-$^2H_2$]-benzyl alcohol, benzoic acid (24.6 mmol) was dissolved in 25 mL of dry ether that had been freshly distilled from lithium aluminum hydride, and the solution was injected into 250 mL of dry ether containing lithium aluminum deuteride (23.8 mmol, 98% isotopic purity, from Aldrich). The mixture was maintained under an atmosphere of dry nitrogen and stirred overnight at room temperature, after which 100 mL of ice-cold 2 N HCl was added to decompose the excess reductant. The ether layer was separated, washed once with saturated aqueous sodium bicarbonate, and dried over anhydrous sodium sulfate. The ether was then removed under reduced pressure at room temperature, and the residue was vacuum distilled (at 18 mmHg) to yield the labeled alcohol in 60% yield, based on the starting benzoic acid.

Enzymes. P450 forms 2B4 and 2E1 and the reductase were purified from rabbit liver by methods previously described by this laboratory (Coon et al., 1978; French & Coon, 1979; Koop et al., 1982). The individual preparations were homogeneous as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the specific contents were 15.7, 18.8, and 12.2–13.1 nmol/mg of protein, respectively. Stock solutions of these enzymes were 70.5, 24.0, and 53.0 μM, respectively.

were mixed in a 1:1 molar ratio, distributed into sufficient tubes for each assay, and maintained at −20 °C until used. A typical steady-state kinetic assay was as follows. In a final volume of 0.5 mL, the reaction mixture contained 25 μmol of potassium phosphate buffer, pH 7.4, 30 μg of freshly dispersed DLPC, 0.1 nmol of P450 2E1 or 0.2 nmol of P450 2B4 with an equimolar amount of the reductase, substrate at the appropriate concentration, and 0.5 μmol of NADPH as the final addition. After incubation for 20 min at 30 °C, 0.25 mL of 6% perchloric acid was added, and the mixture was kept on ice for 30 min prior to centrifugation at 5000 rpm for 10 min. A 50-μL aliquot of the supernatant solution was then analyzed by HPLC. Each substrate concentration was assayed in duplicate along with a blank from which the NADPH was omitted. Under these assay conditions, product formation was found to be linear for 45 min. The rate of the reaction was also found to be linear with respect to the level of P450 in the range of 0.05–0.7 nmol for a period of 20 min (data not shown).

HPLC Analysis. Quantitative analysis of the enzymatically formed aldehyde or ketone was done with a Waters μBondapack C-18 reversed-phase analytical column with use of an automated HPLC system consisting of a Waters WISP Model 710 autosampler, a Model 600 solvent delivery system, and a Model 480 UV/visible detector set at the appropriate wavelength maximum of the carbonyl product, and a Hewlett-Packard Model 3600 integrator. An isocratic solvent system consisting of acetonitrile and water containing 0.1% trifluoroacetic acid was used for all determinations, with the concentration of acetonitrile adjusted so that the retention time of the aldehyde or ketone was between 7 and 9 min. Solvent mixtures that led to elution of the product earlier than 6 min caused it to appear as a shoulder on the front of the NADP/NADPH peak, frequently resulting in incorrect recognition of the peak for automated integration. Elution times longer than 10 min were also undesirable, since the product was eluted as a broad peak, resulting in decreased sensitivity. Standards of the carbonyl product were run with each assay in the range from 20 to 600 pmol; in this range, the integrated area of the peak was found to be directly proportional to the amount of the standard. Typically, the lower limit of accurate product quantitation was 30 pmol. Each sample was injected in duplicate, and the mean integration value was used to quantitate the carbonyl product.

Intramolecular Deuterium Isotope Effect with [1-$^2H_1$]-Benzyl Alcohol Determined by GC/MS. A typical reaction mixture contained 50 μmol of potassium phosphate buffer, pH 7.4, 60 μg of DLPC, 0.5 nmol of P450 form 2E1 or 1.0 nmol of form 2B4 reconstituted with reductase in a 1:1 molar ratio as described, 1.5 or 6.0 μmol of [1-$^2H_1$]benzyl alcohol for reactions with 2E1 or 2B4, respectively, and 5 μmol of NADPH in a final volume of 1.0 mL. The mixture was incubated at 30 °C for 2 h, after which time it was extracted by vigorous mixing with 2 mL of CH_2Cl_2. The CH_2Cl_2 layer was dried over anhydrous sodium sulfate, reduced to a volume of approximately 0.5 mL under a stream of nitrogen, and applied to an analytical silica gel HPLC column (25 × 0.4 cm) previously equilibrated with CH_2Cl_2. The column was treated with CH_2Cl_2 at a flow rate of 1 mL/min, and the fraction eluted between 4.0 and 6.0 min was collected and concentrated to approximately 20 μL under a stream of dry nitrogen. (Benzaldehyde and benzyl alcohol standards were eluted at 4.5 and 12.2 min, respectively, under these conditions.) A 4-μL aliquot was injected onto a 30-m DB-5 fused

Table 1: Components Required and Effect of Catalase and Superoxide Dismutase on Oxidation of Benzyl Alcohol by P450 2E1

system	activity [nmol min^{-1} (nmol of P450)$^{-1}$]
complete[a]	3.12 ± 0.02
cytochrome P450 omitted	0.0
reductase omitted	0.0
NADPH omitted	0.0
complete + catalase (10 units)	3.20 ± 0.04
complete + catalase (100 units)	3.05 ± 0.04
complete + superoxide dismutase (180 units)	3.20 ± 0.05]
complete + superoxide dismutase (720 units)	3.10 ± 0.04

[a] The complete system was as described in the text, with 1.5 μmol of benzyl alcohol. In the other experiments, the components indicated were selectively omitted or added to the complete system.

splitless injector was maintained at 200 °C, and helium was used as the carrier gas at a head pressure of 10.0 psi. The column temperature was maintained at 50 °C for 2 min and then raised to 275 °C at 10 °C/min. Benzaldehyde was eluted at 7.4 min under these conditions. The gas chromatograph was attached to a Finnigan 4021 mass spectrometer operating at 70 eV. Data aquisition and processing were done with the Finnigan INCOS data system.

Solvent Deuterium Incorporation into Acetophenone. A reaction mixture similar to that described above for determination of the intramolecular isotope effect with benzyl alcohol was used, except that the medium was 92% deuterium oxide and the substrate was 1-phenylethanol at a concentration of 3.0 or 6.0 mM with P450 2E1 or 2B4, respectively. A comparable reaction mixture lacking NADPH and the substrate but containing 1.5 μmol of acetophenone served as a control for the exchange of deuterium with the methyl hydrogens. Acetophenone was isolated by HPLC on silica gel and analyzed by GC/MS as described earlier for benzaldehyde. The retention times of acetophenone and 1-phenylethanol on the silica gel column were 6.5 and 14.0 min, respectively; the retention time of acetophenone on the DB-5 fused silica capillary column was 8.5 min.

Incorporation of Oxygen from $^{18}O_2$ into Acetophenone. A typical 10-mL reaction mixture contained 0.5 mmol of potassium phosphate buffer, pH 7.4, 0.6 mg of DLPC, 60 μmol of 1-phenylethanol, and 5.0 nmol of P450 2B4 or 2.5 nmol of P450 2E1 reconstituted with reductase as described. The mixtures were made anaerobic by repeated purging with oxygen-free nitrogen, $^{18}O_2$ was then introduced, and NADPH (0.1 mmol) was injected as an aqueous anaerobic solution to initiate the reaction. The incubation was at 30 °C for 2.5 h, after which time the reaction mixtures were extracted with two 5-mL aliquots of CH_2Cl_2. The extract was dried over anhydrous sodium sulfate and then evaporated under reduced pressure to 0.5 mL, and the acetophenone was purified by silica gel chromatography and analyzed by GC/MS as described above. To determine the extent of $^{16}O_2$ isotope dilution that might have occurred under the experimental conditions, the hydroxylation of toluene to benzyl alcohol by P450 2B4 was determined. GC/MS analysis of the resulting benzyl alcohol showed 96% ^{18}O incorporation (data not given), indicating that insignificant isotopic dilution took place under the experimental conditions used.

RESULTS

Components Required. The requirements for the oxidation of benzyl alcohol to benzaldehyde by P450 2E1 in the

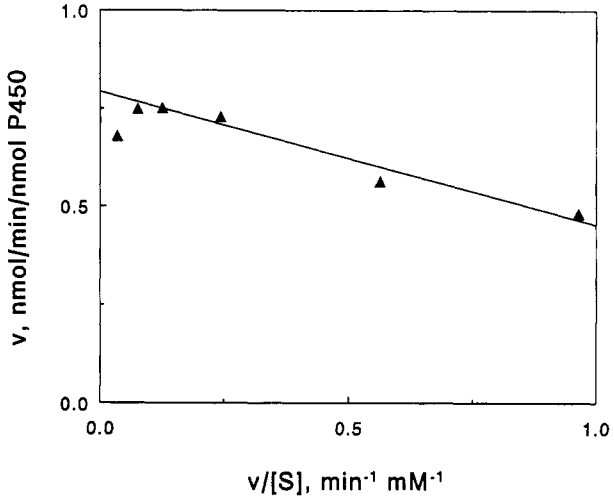

FIGURE 1: Typical Woolf–Augustinsson–Hofstee plot as shown for *p*-fluorophenylethyl alcohol with P450 2B4, from which kinetic constants were obtained. The plot indicates the inhibition observed at the highest substrate concentrations. K_m and k_{cat} values were obtained from those concentrations at which substrate inhibition was not observed.

of benzaldehyde is dependent on P450, the reductase, and NADPH; omission of any of these components results in no observable oxidation of the alcohol. Catalase and superoxide dismutase have no effect on product formation, indicating that hydrogen peroxide and superoxide, which are produced in the reconstituted enzyme system (White & Coon, 1980), are not involved in a nonenzymatic, Fenton-type reaction with the alcohol. Similar results were obtained with all of the other alcohols examined in this study, including the experiments with P450 2B4, thus showing that alcohol oxidation occurs within the catalytic site of the cytochrome.

Steady-State Kinetics and Linear Free Energy Correlation Analysis. The activities of benzyl alcohol and seven para-substituted derivatives and of 1-phenylethanol and eight para-substituted derivatives were examined in each case at six concentrations, the range of which depended on the form of P450 being studied. With P450 2B4, the substrate concentrations varied from 0.3 to 10.0 mM, and with 2E1 they were from 0.1 to 3.0 mM. The kinetic constants were obtained from linear regression analysis of the initial rates fitted to Lineweaver–Burk and Woolf–Augustinson–Hofstee plots (Segel, 1975); the latter is shown in Figure 1 for *p*-fluorophenylethyl alcohol. Some substrates showed inhibition at high concentrations with both cytochromes. Accordingly, such results were not included in the determination of kinetic constants, but at least four data points were used for each value calculated. Both plots gave correlation coefficients greater than 0.99, and the kinetic constants obtained by the two analytical methods were in good agreement. Tables 2 and 3 summarize the steady-state kinetic constants determined for the oxidation of the series of benzyl alcohols and the series of 1-phenylethyl alcohols, respectively, by both P450 2B4 and P450 2E1. The K_m values vary from 0.11 to 7.3 mM for the benzyl alcohols and from 0.05 to 7.5 mM for the phenylethyl alcohols, with no obvious correlation with the partition coefficient, the P450 used, or the rate of oxidation. The k_{cat} values were used for linear free energy correlation analysis as shown in Figure 2A for the oxidation of para-substituted benzyl alcohols and in Figure 2B for the oxidation of para-substituted 1-phenylethyl alcohols by the two cytochromes. The reaction constants for the oxidation of benzyl alcohols obtained from these plots are –0.46 (correlation coefficient = –0.59) and

Table 2: Steady-State Kinetic Constants for Oxidation of para-Substituted Benzyl Alcohols by P450s 2E1 and 2B4[a]

para-substituent	Hammett constant (σ^o_p)	P450 2E1 k_{cat} (min^{-1})	P450 2E1 K_m (mM)	P450 2B4 k_{cat} (min^{-1})	P450 2B4 K_m (mM)
OCH_3	–0.27	4.20	1.00	1.20	4.31
CH_3	–0.17	3.37	0.31	0.79	0.33
H	0.00	3.59	0.45	3.38	7.28
F	0.06	2.89	0.32	0.61	1.28
Br	0.23	2.08	0.13	0.75	0.13
Cl	0.23	2.37	0.11	0.52	0.13
CN	0.66	2.31	0.69	0.49	1.48
NO_2	0.78	2.90	0.43	0.73	1.90

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations. The Hammett substituent constants are taken from Hansch (1973).

Table 3: Steady-State Kinetic Constants for Oxidation of para-Substituted 1-Phenylethyl Alcohols by P450s 2E1 and 2B4[a]

para-substituent	Hammett constant (σ^o_p)	P450 2E1 k_{cat} (min^{-1})	P450 2E1 K_m (mM)	P450 2B4 k_{cat} (min^{-1})	P450 2B4 K_m (mM)
OCH_3	–0.27	3.50	0.64	3.46	3.12
CH_3	–0.17	3.38	0.44	4.37	0.72
H	0.00	4.04	0.49	6.03	1.88
F	0.06	2.35	0.26	2.28	0.65
Br	0.23	0.92	0.06	1.59	0.06
Cl	0.23	1.76	0.05	1.93	0.27
COOH	0.45	0.62	7.54	0.31	6.09
CN	0.66	1.19	0.42	0.97	1.19
NO_2	0.78	1.06	0.13	1.00	2.31

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations. The Hammett substituent constants are taken from Hansch (1973).

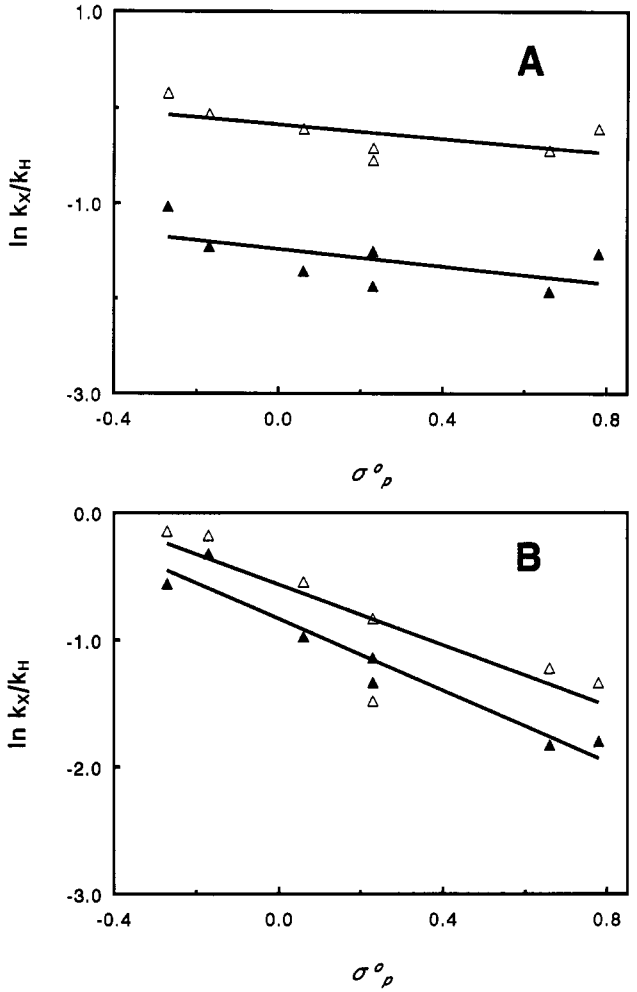

FIGURE 2: Linear free energy correlation diagrams for the P450 2E1-catalyzed (△) and P450 2B4-catalyzed (▲) oxidation of benzyl alcohols (panel A) and of 1-phenylethyl alcohols (panel B). Ln k_X/k_H was calculated from the rate constants shown in Tables 2 and 3, and values for σ^o_p were obtained from Hansch (1973).

responding reaction constants are –1.41 (correlation coefficient = –0.96) and –1.19 (correlation coefficient = –0.85), respectively. The results with *p*-carboxy-1-phenylethyl alcohol deviated significantly from linearity and were excluded from linear regression analysis of the data. The significance of the reaction constants in connection with the isotope effects to be presented is discussed below.

Deuterium Isotope Effect with Benzyl and Phenylethyl Alcohols. Table 4 shows the isotope effect on the steady-state parameters for the oxidation of benzyl alcohol as compared to its 1-dideuterio derivative and of 1-phenylethyl alcohol as compared to its 1-monodeuterio derivative by P450 2B4 and P450 2E1. With phenylethyl alcohol and either cytochrome no isotope effect on the catalytic constant was observed, suggesting that, with these enzymes, cleavage of the benzylic carbon–hydrogen bond is not rate-determining in the overall reaction. With the dideuterio benzyl alcohol, however, a significant isotope effect was obtained with both of the cytochromes. The effect on the catalytic constant in the case of P450 2B4 is 2.8, indicating that carbon–hydrogen bond breakage contributes to the enzymatic rate-limiting process. In contrast, the isotope effect on this parameter with P450 2E1 is 1.3, suggesting that hydrogen abstraction is only partially rate-limiting in the overall reaction. With respect to the K_m values, a significant isotope effect was seen only with P450 2B4 and benzyl alcohol; the cause of the 1.6-fold increase has not been studied in detail.

Intramolecular Deuterium Isotope Effects with Benzyl Alcohol. The procedure used for examining the intramolecular pattern of benzaldehyde at 70 eV shows a molecular ion peak (m/z = 106) and an $(M - 1)^+$ ion peak (m/z = 105) of approximately the same intensity (Figure 3, panel A). With deuterium at the aldehydic position, produced in the enzymatic oxidation of the dideuterio benzyl alcohol, the fragmentation pattern has a molecular ion peak at m/z = 107 and an $(M - 2)^+$ peak at m/z = 105 of about the same intensity (panel B). The loss of one mass unit from benzaldehyde and two units from deuteriobenzaldehyde indicates that in this mass region the fragmentation pattern arises from the loss of the aldehydic hydrogen. The small m/z = 106 peak obtained with [^{2}H]benzaldehyde with an intensity approximately 8% of that of the m/z = 107 peak is due to the inherent contribution by ^{13}C (5.6%) and by the residual hydrogen present from the lithium aluminum deuteride (98% isotopic purity) used in the synthesis of [1-$^2H_2$]benzyl alcohol. Thus, in a mixture of benzaldehyde and deuteriobenzaldehyde, the ratio of the corrected signal intensities at m/z = 106 and 107 gives the relative abundance of the two species in the mixture. As shown by the scheme in Figure 4, benzaldehyde formed by enzymatic oxidation of (±) [1-$^2H_1$]benzyl alcohol by P450 would contain hydrogen and deuterium at the aldehydic position in amounts proportional to the rates of C–D and C–H bond cleavage, respectively. Therefore, the ratio of the corrected peak intensities at m/z = 107 and 106 corresponds to the

Table 4: Steady-State Kinetic Deuterium Isotope Effects on Oxidation of Benzyl Alcohol and 1-Phenylethyl Alcohol by P450 Cytochromes 2E1 and 2B4[a]

	P450 2E1				P450 2B4			
substrate	k_{cat} (min^{-1})	k_{cat} (H)/k_{cat} (D)	K_m (mM)	K_m (H)/K_m (D)	k_{cat} (min^{-1})	k_{cat} (H)/k_{cat} (D)	K_m (mM)	K_m (H)/K_m (D)
benzyl alcohol	4.26		0.54		3.22		7.82	
[1-$^2H_2$]benzyl alcohol	3.33	1.3	0.49	1.1	1.15	2.8	4.93	1.6
1-phenylethyl alcohol	3.95		0.50		5.86		1.87	
[1-$^2H_1$]-1-phenylethyl alcohol	4.28	0.9	0.53	0.9	5.38	1.1	1.68	1.1

[a] The k_{cat} and K_m values were obtained from Lineweaver–Burk and Woolf–Augustinsson–Hofstee plots and are the mean of two or three separate determinations.

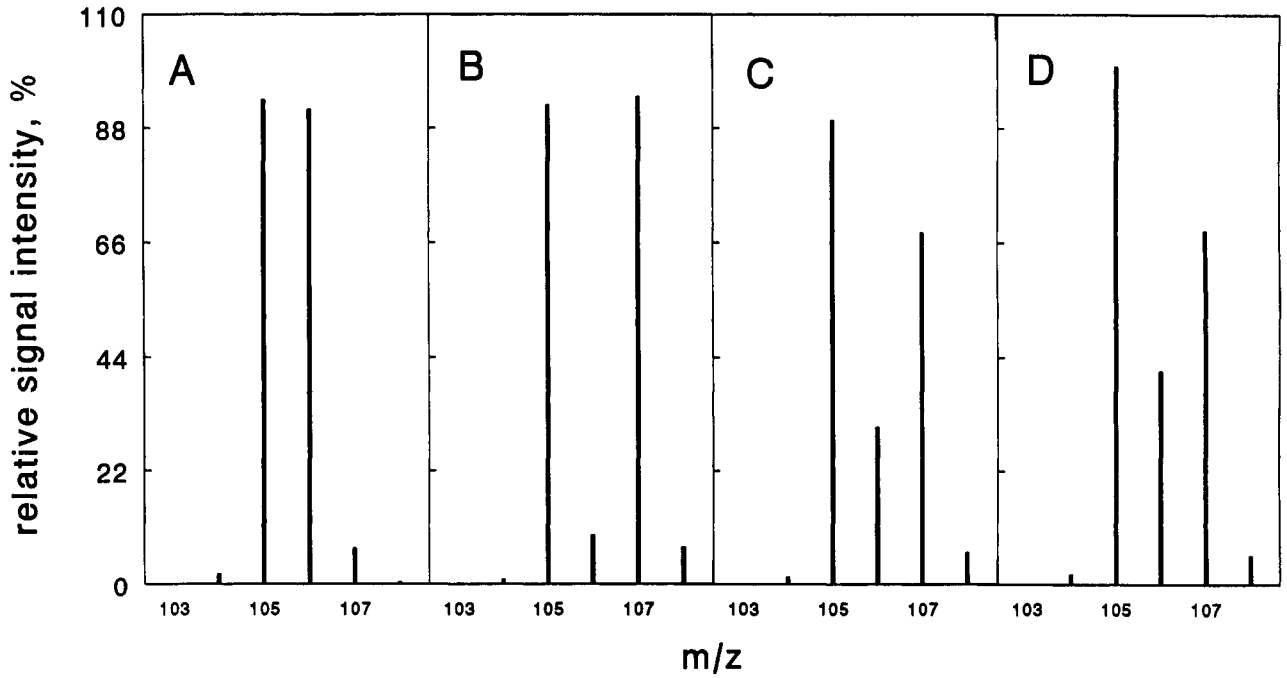

FIGURE 3: Mass spectral fragmentation pattern for benzaldehyde in the region from m/z = 102 to 109. Panel A, benzaldehyde; panel B, [1-$^2H_1$]benzaldehyde derived from P450 2B4-catalyzed oxidation of [1-$^2H_2$]benzyl alcohol; panel C, benzaldehyde obtained from P450 2E1-catalyzed oxidation of [1-$^2H_1$]benzyl alcohol; and panel D, benzaldehyde obtained from P450 2B4-catalyzed oxidation of [1-$^2H_1$]benzyl alcohol.

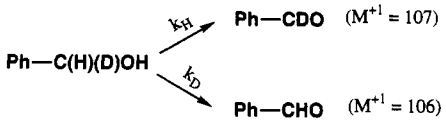

FIGURE 4: Molecular ion peak (m/z value) expected in mass fragmentation pattern of benzaldehyde formed by loss of hydrogen (k_H) or of deuterium (k_D) from [1-$^2H_1$]benzyl alcohol.

formed from (±) [1-$^2H_1$]benzyl alcohol by P450 2B4 and P450 2E1, respectively. The similar and relatively small magnitude of the intramolecular isotope effect for benzyl alcohol oxidation by 2E1 and 2B4, 2.8 and 2.6, respectively, suggests a comparable geometry for bond cleavage by the two forms of the cytochrome, but the significance with respect to transition states, as discussed by More O'Ferrall (1970) for chemical models, is not clear.

Isotope Incorporation into Acetophenone from Solvent or Molecular Oxygen in Enzymatic Oxidation of 1-Phenylethyl Alcohol. The ability of P450 to catalyze oxidative desaturation reactions (Nagata et al., 1986; Rettie et al., 1987, 1988) suggests the possibility of such a reaction with 1-phenylethyl alcohol to form the enol as a route to the carbonyl product. With this alcohol such a mechanism would result in the uptake of a solvent-derived proton into the methyl group of acetophenone. Accordingly, acetophenone formed enzymatically incorporation of an atom of deuterium into the enzymatically formed acetophenone would cause the m/z = 121 ion signal intensity to increase by approximately 92% and the m/z = 120 intensity to decrease by an equal amount. However, as shown in Figure 5, the intensity of m/z = 121 was not significantly increased relative to that of the molecular ion peak at m/z = 120. The results indicate that an oxidative desaturation mechanism does not operate with either form of P450 examined.

The possible incorporation of an atom of $^{18}O_2$ into acetophenone was determined to establish whether a *gem*-diol is formed as an intermediate in the oxidation of 1-phenylethyl alcohol by either form of P450. (The rapid exchange of oxygen at the aldehyde function precluded the estimation of ^{18}O incorporation into benzaldehyde formed from benzyl alcohol.) Figure 6 shows the relevant mass fragmentation region of acetophenone. The extent of oxygen incorporation was determined from the ion intensities at m/z 122 and 120 and at m/z 107 and 105. The results indicate that with P450 forms 2B4 and 2E1 the incorporation of oxygen from $^{18}O_2$ into the carbonyl group was 32 ± 2 and 29 ± 2%, respectively. A control experiment with acetophenone in $H_2{}^{18}O$ under similar conditions showed incorporation of 14 ± 2% ^{18}O due to solvent exchange; thus, it is obvious that the

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