Hydrogen Tunneling in the Flavoenzyme Monoamine Oxidase B†

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ABSTRACT: Competitive k_H/k_T and k_D/k_T kinetic isotope effects on *p*-methoxybenzylamine oxidation by the 8α-*S*-cysteinyl flavin adenine dinucleotide (FAD)-dependent enzyme monoamine oxidase B (MAO-B) have been measured as a function of temperature. At pH 7.5, exponents relating observed k_H/k_T and k_D/k_T isotope effects indicate the presence of a temperature-dependent change in rate-limiting step. At lower temperature (e.g., 2 °C), the presence of multiple rate-limiting steps (commitments) is clearly indicated from the size of the exponent and individual isotope effects. Noncompetitive k_H/k_D isotope effect measurements indicate a trend in observed isotope effects between pH 9.0 and 6.0, with isotope effects increasing at lower pH. Primary and secondary k_H/k_T and k_D/k_T isotope effects were therefore measured as a function of temperature at pH 6.1. Exponents relating primary and secondary k_H/k_T and k_D/k_T in the 10−43 °C range are 3.13 ± 0.04 and 2.36 ± 0.13, respectively, and do not systematically change with temperature. These data indicate that commitments, if present, remain constant across this temperature range. The temperature dependence of the observed primary isotope effects gives values for the ratios of Arrhenius prefactors of 0.13 ± 0.03 (A_H/A_T) and 0.52 ± 0.05 (A_D/A_T). Both values are well below the lower limits predicted in the absence of tunneling contributions to the reaction coordinate, indicating that both deuterium and protium tunneling take place in this reaction. The presence of a temperature independent commitment contribution cannot be rigorously ruled out; however, the effect of such a commitment on the observed A_H/A_T and A_D/A_T values is shown to be quite small.

During the last several years clear evidence has emerged for the phenomenon of hydrogen tunneling in the reactions catalyzed by yeast alcohol dehydrogenase (YADH)[1] (Cha et al., 1989; Rucker et al., 1992), horse liver alcohol dehydrogenase (HLADH) (Bahnson et al., 1993), and bovine serum amine oxidase (BSAO) (Grant & Klinman, 1989; Rucker et al., 1992). These reactions involve hydride transfer to a nicotinamide cofactor (alcohol dehydrogenases) and proton transfer to an active site base (bovine serum amine oxidase). This report extends previous studies to include bovine liver monoamine oxidase B (MAO-B), a flavin-dependent mitochondrial outer membrane enzyme. MAO-B catalyzes the oxidative deamination of a large number of biogenic amines (Singer, 1987, 1991; Trevor et al., 1987). The nature of the hydrogen abstraction step is not known, but the two mechanisms suggested for substrate oxidation involve either a proton abstraction (Silverman et al., 1980; or possibly hydrogen atom abstraction, Silverman & Zelechonok, 1992) from a nitrogen radical cation generated by a one-electron oxidation of substrate or a direct hydrogen atom transfer from substrate (Walker & Edmondson, 1994). The nature of the hydrogen transfer step in the MAO-B reaction therefore differs from other enzyme systems for which hydrogen tunneling has been established.

In previous studies (Bahnson et al., 1993; Cha et al., 1989; Grant & Klinman, 1989; Rucker et al., 1992), two experimental probes have been used for the detection of hydrogen tunneling in enzyme systems. The first involves a comparison of observed k_H/k_T and k_D/k_T isotope effects. As originally shown by Saunders (1985), the semiclassical relationship $(k_D/k_T)^{3.26} = k_H/k_T$ is expected to break down in the direction of observed exponents larger than 3.26 when tunneling is moderate and contributes to the hydrogen transfer step. Studies on the alcohol dehydrogenase reaction have, indeed, resulted in observed exponents significantly larger than 3.26, an indication of hydrogen tunneling for enzymes from both yeast and horse liver (Bahnson et al., 1993; Cha et al., 1989). Alternatively, the temperature dependence of isotope effects can be used to probe for hydrogen tunneling. In the absence of tunneling contributions, the Arrhenius prefactor ratio A_L/A_T, where L can be either H or D, is expected to be close to unity. Observed values smaller than the lower limits in the absence of tunneling [about 0.6 for A_H/A_T and 0.9 for A_D/A_T (Bell, 1980; Schneider & Stern, 1972)] provide evidence that quantum effects are contributing to the reaction under study. This method has been used extensively for nonenzymatic reactions in solution (Bell, 1980; Melander and Saunders, 1987) and more recently also for the bovine serum amine oxidase reaction (Grant and Klinman, 1989; Rucker et al., 1992). In the latter example, exponents relating k_H/k_T and k_D/k_T were found to be normal, suggesting differences in the degree of tunneling for the reactions catalyzed by alcohol dehydrogenases and serum amine oxidase (Grant and Klinman, 1989).

An unambiguous demonstration of tunneling requires that the hydrogen transfer step be fully rate limiting in the temperature range studied. In the case of enzyme-catalyzed reactions, this is often not the case. It is therefore important

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[1] Abbreviations: MAO-B, monoamine oxidase B; YADH, yeast alcohol dehydrogenase; HLADH, horse liver alcohol dehydrogenase; BSAO, bovine serum amine oxidase; HPLC, high-performance liquid chromatography; CMC, critical micelle concentration.

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to understand how the experimental probes described above are affected by partial rate limitation of steps other than the hydrogen transfer step. As originally shown by Cha et al. (1989), the exponential relationship $(k_D/k_T)^{3.26} = k_H/k_T$ breaks down in the direction of smaller exponents when kinetic complications are present. This is opposite to what is expected in the case of hydrogen tunneling and therefore eliminates the possibility of a false signature of tunneling from observed exponents. The temperature dependence of isotope effects is also affected by kinetic complications; however, the effect depends strongly on whether the hydrogen transfer step is equally rate limiting in the temperature range studied. If there is a change in the degree of rate-limitation across the temperature range, large deviations in observed values for A_L/A_T from unity may occur, even if there are no tunneling contributions to the reaction studied. On the other hand, in the absence of changes in the degree of rate limitation by the hydrogen transfer step, only minor deviations in observed A_L/A_T from its intrinsic value are expected (see Results). Exponents relating observed k_H/k_T and k_D/k_T isotope effects are very sensitive to changes in rate-limiting step and therefore can serve as a probe of the degree of rate limitation of the hydrogen transfer step in the temperature range under investigation.

As shown herein, the temperature dependence of competitive primary k_H/k_T and k_D/k_T isotope effects on the oxidation of *p*-methoxybenzylamine catalyzed by MAO-B leads to values for A_H/A_T and A_D/A_T which fall far below the lower limits expected in the absence of a tunneling contribution. Due to observed exponents relating k_H/k_T and k_D/k_T isotope effects slightly smaller than 3.26, we have not been able to eliminate the possibility that the hydrogen transfer step is somewhat less than fully rate limiting. However, observed exponents do not change systematically in the experimental temperature range, indicating a similar temperature dependence for all kinetically significant steps. Under these conditions, observed Arrhenius prefactor ratios are expected to be only marginally altered from their intrinsic values, allowing us to conclude that both protium and deuterium undergo tunneling in the monoamine oxidase B reaction.

EXPERIMENTAL PROCEDURES

Materials

Monoamine oxidase was isolated from bovine liver mitochondria as described by Salach (1979) and further modified by Weyler and Salach (1981). Horse liver alcohol dehydrogenase, catalase, lactate dehydrogenase, and NADH were from Boehringer Mannheim. NAD^+ and pyruvate were from Sigma, $LiAlH_4$, $NaCNBH_3$ and $NaBH_4$ from Aldrich, semicarbazide hydrochloride and $LiAl^2H_4$ from Fluka (>99% D), reduced Triton X-100 from Calbiochem, and hydrochloride gas from Matheson. All solvents were of HPLC grade from Baker and were used without further purification except diethyl ether (Fisher), which was dried over molecular sieves (5 Å) prior to use, and absolute methanol, which was purchased from Aldrich.

Synthesis of Radiolabeled p-Methoxybenzylamines. Synthesis of randomly tritiated [1,1-$^1H_2$-1-^3H]-*p*-methoxybenzylamine and [1,1-$^2H_2$-1-^3H]-*p*-methoxybenzylamine as well as [1,1-$^1H_2$-UL-^{14}C]-*p*-methoxybenzylamine and [1,1-$^2H_2$-UL-^{14}C]-*p*-methoxybenzylamine has been previously described (Rucker et al., 1992). It became necessary to synthesize additional [1,1-$^1H_2$-UL-^{14}C]-*p*-methoxybenzylamine, and that was done according to the following procedure. Previously synthesized [1,1-$^1H_2$-UL-^{14}C]-*p*-methoxybenzyl alcohol (Rucker et al., 1992) (20 μCi, 40 μmol) was enzymatically converted to [1-^{1}H-UL-^{14}C]-*p*-methoxybenzaldehyde in a coupled enzyme system at room temperature containing, in addition to the alcohol, 270 mg of pyruvate (25 mmol), 85 mg of NAD^+ (150 μmol), 12.7 mg of liver alcohol dehydrogenase, and 1 mg of lactate dehydrogenase (500 units), in 1 mL of 0.10 M potassium phosphate buffer, pH 7.2. After 9 h the reaction was >92% complete, as judged by HPLC (C18 column, eluted with 13% MeOH, 13%CH_3CN, 74% H_2O). It was then quenched by adding 5 mL of ether, and the ether layer was removed and the aqueous layer washed three times with 5 mL of ether. Combined etheral layers were dried over Na_2SO_4, and the volume was reduced to approximately 0.5 mL by gently blowing $N_{2(g)}$ over the solution. The resulting [1-^{1}H-UL-^{14}C]-*p*-methoxybenzaldehyde (16 μCi, 32 μmol) was then converted to [1,1-$^1H_2$-UL-^{14}C]-*p*-methoxybenzylamine by a modified method of Borch et al. (1971). Ammonium acetate (0.77 g, 10 mmol) and $NaCNBH_3$ (35 mg, 565 μmol), dissolved in 3 mL absolute methanol, were added to the aldehyde solution, and the reaction mixture was stirred at room temperature for 13 h, or until complete by HPLC. The resulting [1,1-$^1H_2$-UL-^{14}C]-*p*-methoxybenzylamine was purified by HPLC [C18, eluted with 97% (0.2% TFA in H_2O), 3% THF]. The final overall yield after purification was about 40%, and the specific activity was 0.5 μCi/μmol, based on the starting alcohol.

Synthesis of [1,1-^{1} H_2]-p-Methoxybenzylamine and [1,1-$^2H_2$]-p-Methoxybenzylamine. All glassware was dried in an oven for 1 h prior to use. The synthesis was performed according to a modification of the procedure of Nystrom (1955). A suspension of 0.91 g of $LiAlH_4$ (24 mmol) or 1.00 g $LiAl^2H_4$ (24 mmol) in 3 mL of anhydrous ether was added dropwise to a solution of *p*-methoxybenzonitrile in 4 mL of anhydrous ether over a period of 45 min. After further stirring at room temperature for 1hr, the reaction was quenched by adding ca. 1 mL of H_2O. One mL of 10 M KOH was added and the ether layer removed. The aqueous layer was washed with ether, and the combined ether layers were washed with 0.1M KOH and dried over anhydrous Na_2SO_4. Hydrochloride salts were formed by bubbling hydrochloride gas through the solution. The *p*-methoxybenzylamine hydrochlorides were filtered, washed with ether, and recrystallized from methanol. Final yields: [1,1-$^1H_2$]-*p*-methoxybenzylamine, 61%; [1,1-$^2H_2$]-*p*-methoxybenzylamine, 53%. The deuterated *p*-methoxybenzylamine was estimated to contain >99% D in the benzyl position, as the amount of protium material was too small to be determined by NMR.

Methods

Noncompetitive Kinetic Isotope Effects. Buffers used were 0.10 M borate (pH 9.0) 0.10 M HEPES (pH 7.5) or 0.10 M sodium phosphate (pH 6.0) all containing 5 mg/mL reduced Triton X-100. Initial rates for *p*-methoxybenzaldehyde production were determined by monitoring the absorbance at 281 nm on a Cary spectrophotometer [$\Delta\epsilon$ = 12.6 mM^{-1} cm^{-1} (Walker, 1987)]. The O_2 concentration was ~260 μM

Table 1: Observed Isotope Effects and Exponential Relationships for *p*-Methoxybenzylamine Oxidation by Monoamine Oxidase B, pH 7.5[a]

temp (°C)	1° k_H/k_T	1° k_D/k_T	2° k_H/k_T	2° k_D/k_T	1° exp[b]	2° exp[b]
2.0	13.03 ± 0.51	2.707 ± 0.040	1.115 ± 0.012	1.095 ± 0.007	2.57 ± 0.05	1.20 ± 0.15
25.0	21.43 ± 0.81[c]	2.703 ± 0.102[c]	1.272 ± 0.030	1.119 ± 0.034	3.08 ± 0.12	2.14 ± 0.36
43.2	16.87 ± 0.35	2.464 ± 0.069	1.233 ± 0.010	1.078 ± 0.015	3.13 ± 0.10	2.79 ± 0.53

[a] Reported numbers are the average of one experiment (5−7 timepoints), with standard deviation, unless otherwise stated. [b]

$$\text{exponent} = \frac{\ln(k_H/k_T)}{\ln(k_D/k_T)}$$

$$\text{error} = \sqrt{\left[\frac{\Delta(H/T)}{(H/T)}\frac{1}{\ln(D/T)}\right]^2 + \left[\frac{\Delta(D/T)}{(D/T)}\frac{\ln(H/T)}{(\ln(D/T))^2}\right]^2}$$

where H, D and T represent the corresponding rate constants. [c] Average of all time points of five individual experiments.

(air saturation), and the temperature was kept at 25.0 ± 0.1 °C with a Neslab waterbath.

Competitive Kinetic Isotope Effects. As in noncompetitive experiments, these experiments were performed in 0.10 M phosphate (pH 6.1) or 0.10 M HEPES (pH 7.5), using air-saturated buffers. Approximately 0.1−0.5 units of MAO-B and 6500 units of catalase were used in each experiment. The final concentration of *p*-methoxybenzylamine was ca. 0.5−1.0 mM, with a tritium to C-14 ratio of approximately 10. In most experiments, 5 mM semicarbazide hydrochloride was also included, to convert product *p*-methoxybenzaldehyde to the corresponding semicarbazone. In each experiment, three samples at $t = 0$ and 6−8 samples at various reaction times were removed and quenched with 0.7 mM $HgCl_2$ and 200−400 mM semicarbazide (final concentrations), the latter to drive semicarbazone formation to completion. In other experiments, product aldehyde was converted to the corresponding alcohol with alcohol dehydrogenase and NADH, followed by the addition of NaOH and $NaBH_4$ to complete the reduction of aldehyde to alcohol. Samples were then frozen on dry ice and stored at −70 °C until analyzed by HPLC (C18, 1mL/min. Solvent A: 98% (0.2% TFA in H_2O), 2% THF; solvent B: 58% (0.2% TFA in H_2O), 2% THF, 40% MeOH; linear gradient from 100% A to 100 %B over 20 min). Tritiated water eluted at 3 min, *p*-methoxybenzylamine at 9 min, and *p*-methoxybenzaldehyde semicarbazone at 30 min. No *p*-methoxybenzoic acid (elutes at 33 min) was detected. Isotope effects were calculated according to

$$\frac{k_L}{k_T} = \frac{\ln(1 - f_L)}{\ln\left(1 - f_L\frac{R_P}{R_\infty}\right)} \quad (1)$$

where L represents H or D, f_L is the fractional conversion of substrates to products (as determined by ^{14}C), and R_P and R_∞ are $^3H/^{14}C$ ratios in products at fractional conversions and 100% conversion, respectively. Since $R_0/2$ routinely equaled R_∞, the former was used to calculate isotope effects. The temperature was kept to within ±0.1 °C in a Neslab or Thermomix waterbath. The pH of the phosphate buffer used at pH 6.1 changed only by 0.04 pH units in the 10−43 °C range, which is a negligible change. The detergent concentration used is approximately 25 times the critical micelle concentration (CMC) of Triton X-100 at 25 °C (Crook et al., 1964; Ray & Némethy, 1971), and since the CMC changes only by approximately 0.1 mg/mL in our experimental temperature range (Ray & Némethy, 1971), it is safe to assume that there are no significant micellar changes in this range. Correction of isotope effects due to protium contamination in deuterated substrates was done as described by Grant and Klinman (1989), assuming a protium contamination in [1,1-$^2H_2$-UL-^{14}C]-*p*-methoxybenzylamine of 3%, as previously determined (Rucker et al., 1992). All isotope effects are on the second order rate constant V_{max}/K_M, since tritium is a trace label in these experiments (Klinman, 1978).

Table 2: Steady-State Deuterium Isotope Effects on *p*-Methoxybenzylamine Oxidation by MAO-B as a Function of pH at 25.0 °C[a]

	pH 6.0	pH 7.5	pH 9.0
$^D(V_{app})$	7.5 ± 1.3	5.9 ± 0.3	6.0 ± 0.3
$^D(V_{max}/K_M)$	11.1 ± 3.9	8.9 ± 1.6	4.8 ± 1.0

[a] Data analyzed using a hyperbolic fit of initial rate vs [S]. Reported numbers are for O_2 concentrations of ~260 μM (air saturation). The reported value for K_M (O_2) at 25 °C and pH 7.5 is 120 μM (Walker, 1987). The values for $^D(V_{app})$ may therefore be slightly lower than for $^D(V_{max})$ (O_2 is saturating). Observed isotope effects on V_{max}/K_M should not be affected by lack of saturation by O_2.

Determination of Activation Parameters. Initial rates for [1,1-$^2H_2$]-*p*-methoxybenzylamine oxidation by MAO-B were measured in 0.10 M sodium phosphate, pH 6.1, at 10, 25, and 40 °C, as described for noncompetitive kinetic isotope effects.

RESULTS

Table 1 summarizes competitive isotope effects measured for *p*-methoxybenzylamine oxidation at pH 7.5. At 25 °C, the primary k_H/k_T isotope effect is 21.4 ± 0.8, which is comparable to a k_H/k_D isotope effect of 8.4 from the Schwain−Schaad relationship (Swain et al., 1958), $(k_H/k_D)^{1.44} = k_H/k_T$. This value is in good agreement with a previously determined value for k_H/k_D using stopped flow kinetics (Walker, 1987) and with the $^D(V_{max}/K_M)$ value determined from steady-state kinetic measurements (Table 2). Analogous to the original Schwain−Schaad expression, multiple isotope effects can be related by the expression $(k_D/k_T)^{3.26} = k_H/k_T$. As first pointed out by Saunders (1985), the exponent relating k_H/k_T to k_D/k_T is expected to be especially sensitive to tunneling, with exponential values greater than 3.26 characteristic of this phenomenon. Subsequently, Cha et al. (1989) demonstrated that the exponent will fall below 3.26 when the hydrogen abstraction step is only partially rate limiting. In the monoamine oxidase B reaction, exponents relating observed primary and secondary isotope effects at pH 7.5 and 25 °C are reduced from the semiclassical value

Table 3: Observed Isotope Effects and Exponential Relationships for *p*-Methoxybenzylamine Oxidation by Monoamine Oxidase B in the 10−43 °C Range at pH 6.1[a]

temp (°C)	1° k_H/k_T	1° k_D/k_T	2° k_H/k_T	2° k_D/k_T	1° exp[b]	2° exp[b]
10.0	28.89 ± 1.10	2.896 ± 0.132	1.388 ± 0.019	1.149 ± 0.019	3.16 ± 0.14	2.32 ± 0.31
15.0	27.59 ± 0.70	2.892 ± 0.054[c]	1.359 ± 0.016	1.132 ± 0.018[c]	3.12 ± 0.05	2.47 ± 0.31
25.0	21.98 ± 1.16[c]	2.690 ± 0.086[d]	1.339 ± 0.037[c]	1.131 ± 0.012[d]	3.20 ± 0.12	2.37 ± 0.30
30.0	21.49 ± 0.95	2.631 ± 0.044	1.341 ± 0.011	1.127 ± 0.010	3.17 ± 0.07	2.45 ± 0.19
35.0	18.52 ± 0.99	2.569 ± 0.070	1.340 ± 0.013	1.129 ± 0.032	3.09 ± 0.11	2.41 ± 0.57
40.0	17.42 ± 0.40	2.509 ± 0.067	1.286 ± 0.028	1.109 ± 0.042	3.11 ± 0.09	2.43 ± 0.91
43.0	17.02 ± 0.52	2.507 ± 0.133	1.263 ± 0.029	1.118 ± 0.015	3.08 ± 0.18	2.09 ± 0.33
average:					3.13 ± 0.04	2.36 ± 0.13

[a] Reported numbers are the average of one experiment (5−7 timepoints), with standard deviation, unless otherwise stated. [b] For calculation and error, see Table 1. [c] Average and standard deviation of all time points of two individual experiments. [d] Average and standard deviation of all time points of three individual experiments.

of 3.26 to 3.08 and 2.14, respectively (Table 1), indicating the probable presence of more than one rate-limiting step. When the temperature was lowered to 2 °C, observed exponents were lowered even further (to 2.57 and 1.20 for primary and secondary isotope effects, respectively), and the observed k_H/k_T isotope effect decreased to a value below that at 25 °C. Since isotope effects are expected to increase with decreasing temperature, this is a clear indication that step(s) other than the hydrogen transfer step are becoming increasingly rate limiting at low temperatures. Consider the kinetic scheme in eq 2:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES' \xrightarrow{k_5} E + P \qquad (2)$$

where the first two equilibria represent substrate dissociation and an equilibrium between two enzyme bound reactant species, respectively, and k_5 is the hydrogen transfer rate constant. As first described by Northrop (1977), multiple rate-limiting steps reduce the observed V_{max}/K_M isotope effect from its intrinsic value according to

$$^T(V_{max}/K_M)_{obs} = (^Tk + C)/(1 + C) \qquad (3)$$

where C, the commitment, represents the degree of rate limitation by steps other than the hydrogen transfer step and ^Tk represents the intrinsic isotope effect.

To search for conditions that might give observed isotope effects closer to intrinsic values, $^D(V_{app})$ and $^D(V_{max}/K_M)$ were measured at pH 6.0, 7.5, and 9.0 (Table 2). It can be seen that while the experimental scatter is large, there is a general trend with observed isotope effects increasing as the pH is lowered. This suggests that observed isotope effects are closer to their intrinsic values at pH 6.0 than at pH 7.5. Additionally, V_{max}/K_M decreases approximately 20-fold as the pH is lowered from 7.5 to 6.0 (data not shown), conditions expected to reduce the contribution of an external commitment to the overall rate.[2]

Encouraged by these results, we pursued measurement of k_H/k_T and k_D/k_T isotope effects at pH 6.1. Table 3 summarizes observed isotope effects and exponents in the 10−43 °C range. Experiments were not pursued below 10 °C, due to a very slow reaction with deuterated substrate. At 25 °C, both isotope effects and exponents are found to be similar to those measured at pH 7.5, indicating that if commitments are present at this temperature, they are not significantly altered by the large change in rate that accompanies the reduction in pH. *Of particular note is the observation that observed exponents do not change systematically in the 10−43 °C range*, the average values being 3.13 ± 0.04 and 2.36 ± 0.13 for primary and secondary exponents, respectively. This shows that in contrast to pH 7.5 there are no temperature-dependent changes in the level of expression of intrinsic isotope effects. The fact that the primary exponent is virtually unchanged, despite a combined 260-fold change in rate (20-fold due to a change in pH from 7.5 to 6.1 and approximately 13-fold due to change in temperature when going from 10 to 43 °C), supports the view that *external* commitments, which are expected to be sensitive to experimental conditions, do not contribute to the measured isotope effect.

In Figure 1 we show the temperature dependence of observed primary isotope effects at pH 6.1. From the Arrhenius behavior of isotope effects as a function of temperature,

$$\ln (k_L/k_T) = \ln (A_L/A_T) - \Delta E_a/RT \qquad (4)$$

the Arrhenius prefactor ratio A_L/A_T can be obtained by a linear extrapolation to infinite temperature.[3] The values for the primary A_H/A_T and A_D/A_T obtained are 0.13 ± 0.03 and 0.52 ± 0.05, respectively. These values are both well below the lower limits of 0.6 and 0.9 expected in the absence of tunneling contributions (Schneider & Stern, 1972; Bell, 1980).

Although the data in Figure 1 provide support for tunneling in the MAO-B reaction, the small size of the exponent relating isotope effects (Table 3) raises the possibility of a temperature-independent commitment. Therefore the data were reanalyzed to see how observed isotope effects change as a function of temperature when commitments are present. In eq 5 we show the partial derivatives of eq 3, assuming that C is independent of temperature:

[2] Commitments can be either due to substrate (or product) dissociation steps (first equilibrium of eq 2), in which case they are said to be *external*, or they can be directly related to the hydrogen transfer step (second equilibrium of eq 2). In this latter case they are said to be *internal*. External commitments are expected to change with pH, as the pH dependence of substrate release is frequently different from that of steps representing catalysis. Additionally, external commitments are likely to have different enthalpies of activation as compared to hydrogen transfer, and thus they are expected to change with temperature.

[3] For graphical purposes, we present our data in the linear form according to eq 4, while reported Arrhenius parameters are obtained by a nonlinear fit to the Arrhenius equation, $k_L/k_T = A_L/A_T \exp(-\Delta E_a/RT)$. We note that linear and nonlinear fitting of our data gives the same results.

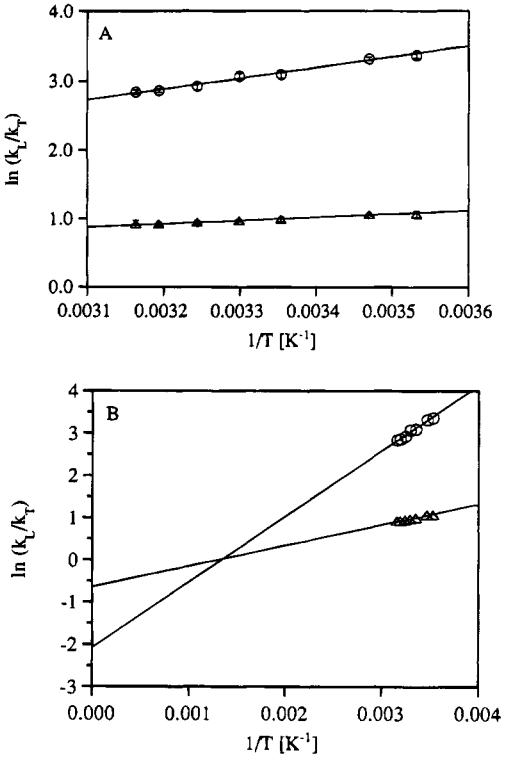

FIGURE 1: (A) Arrhenius plot of primary k_H/k_T (○) and k_D/k_T (△) isotope effects. The data were fit to the equation $\ln(k_L/k_T) = \ln(A_L/A_T) - \Delta E_a/RT$. Error bars represent errors reported in Table 3. (B) Expanded plot, showing the y intercepts.

$$\frac{\partial}{\partial(1/T)}\left[\ln\left(\frac{{}^{T}k + C}{1 + C}\right)\right] = \frac{\partial}{\partial(1/T)}[\ln({}^{T}k + C) - \ln(1 + C)] = \frac{1}{{}^{T}k + C}\frac{\partial}{\partial(1/T)}({}^{T}k) \quad (5)$$

Similarly, the partial derivative of $\ln {}^{T}k$ as a function of $1/T$ is given in

$$\frac{\partial}{\partial(1/T)}[\ln {}^{T}k] = \frac{1}{{}^{T}k}\frac{\partial}{\partial(1/T)}({}^{T}k) \quad (6)$$

The ratio of eqs 6 and 7 is $({}^{T}k + C)/{}^{T}k$, which in our experiments is expected to be very close to unity since the value of C is expected to be small relative to ${}^{T}k$. A temperature-independent commitment therefore does not change the slope of ln(isotope effect) vs $1/T$ appreciably. The intercept $\ln(A_L/A_T)$ can be written as

$$\ln(A_L/A_T) = \ln(k_L/k_T)_{T^*} + \text{slope}(1/T^*) \quad (7)$$

where the slope is given by eqs 5 or 6 and T^* is an arbitrary temperature. Since the slope is not appreciably affected by a small, temperature-independent commitment, the difference between the observed and intrinsic values for $\ln(A_L/A_T)$ can be written according to

$$\ln(A_L/A_T)_{obs} - \ln(A_L/A_T)_{int} \approx \ln\left(\frac{{}^{T}k + C}{1 + C}\right) - \ln({}^{T}k) = \ln\left(\frac{({}^{T}k + C)/(1 + C)}{{}^{T}k}\right) \quad (8)$$

Once again, for values of C which are small relative to ${}^{T}k$,

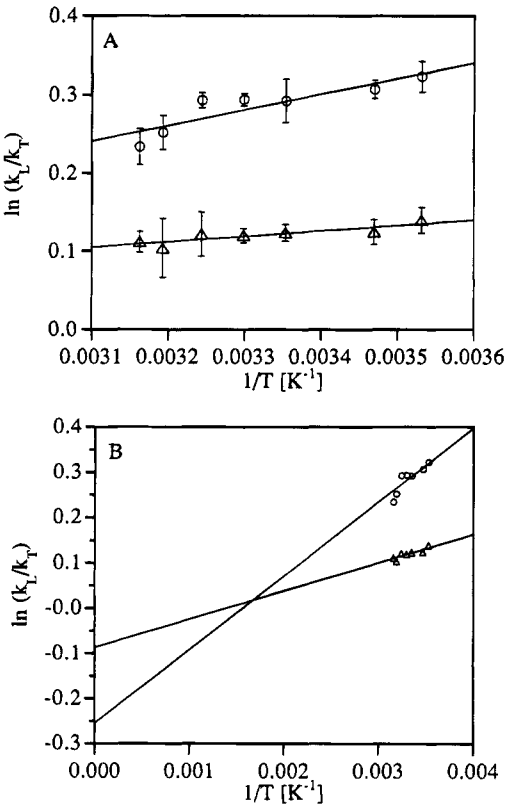

FIGURE 2: (A) Arrhenius plot of secondary k_H/k_T (○) and k_D/k_T (△) isotope effects. The data were fit to the equation $\ln(k_L/k_T) = \ln(A_L/A_T) - \Delta E_a/RT$. Error bars represent errors reported in Table 3. (B) Expanded plot, showing the y intercepts.

eq 8 reduces to

$$\ln(A_L/A_T)_{obs} - \ln(A_L/A_T)_{int} \approx \ln\left(\frac{1}{1 + C}\right) \quad (9)$$

A common feature of enzyme reactions is the appearance of multiple steps characterized by comparable free energy barriers. This feature often leads to overall commitment factors in the range of 0.5 to 5.0 (cf. Cleland, 1975; Albery and Knowles, 1976; Burbaum et al., 1989), with a concomitant reduction in C when the reaction is studied under nonoptimal conditions. Since the data summarized in Table 3 for MAO-B were collected under nonoptimal conditions of pH, we expect an internal commitment, if present, to be relatively small. A value for C of 0.6 brings observed secondary exponents in the MAO-B reaction (Table 3) close to the semiclassical value of ~3.3 (See Discussion). Using this value for C changed the data of Figure 1 very little, yielding the results shown in and Figure 3A. Extrapolating these data to infinite temperature gives A_H/A_T of 0.19, which can be compared to the experimentally observed value of 0.13. It should be noted that the presence of $C = 0.6$ has a far greater effect on the individual isotope effect values, elevating intrinsic isotope effects above measured values in Table 3, e.g., to a primary k_H/k_T of 35.6 and a secondary k_H/k_T of 1.54 at 25 °C. The magnitude of the calculated secondary k_H/k_T alone would implicate tunneling, since it is too large to rationalize easily from semiclassical effects.

As first pointed out by Grant and Klinman (1989), the temperature dependence of k_D/k_T can provide an excellent control in situations where small commitments may arise in measured k_H/k_T parameters. Since the commitment contributing to k_D/k_T is reduced by the size of the primary k_H/k_D isotope effect (approximately 10-fold with monoamine

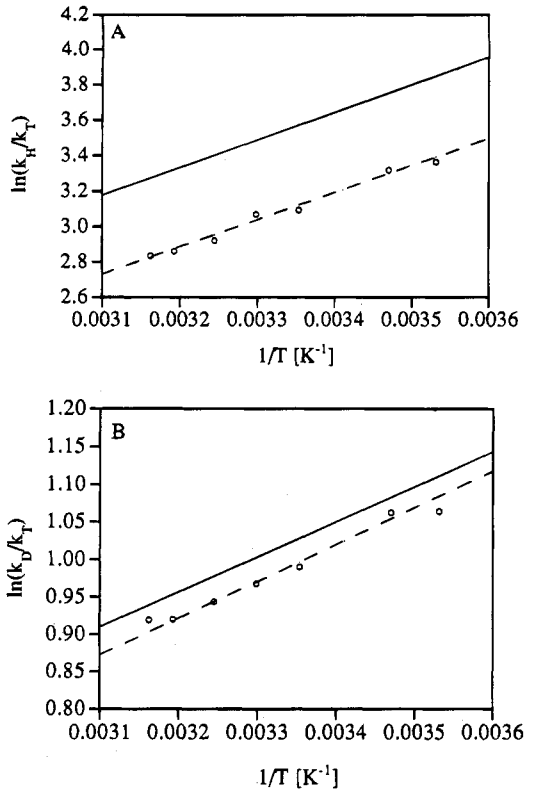

FIGURE 3: (A) Simulation of intrinsic primary k_H/k_T isotope effects in the MAO-B reaction, assuming a temperature independent commitment. The solid line represents calculated intrinsic isotope effects, from $(k_H/k_T)_{intrinsic} = (k_H/k_T)_{observed}(1 + C) - C$, where the commitment C is assumed to have a fixed value of 0.6. The open circles represent the experimental data of Table 3 and the dashed line represents the least-squares fit through the data, as described in the legend to Figure 1. The calculated intrinsic k_H/k_T isotope effects can be described by $\ln(k_H/k_T) = 1561.3/T - 1.665$, while a least-squares fit of observed isotope effects (dashed line) gives $\ln(k_H/k_T) = 1536.7 - 2.036$. The corresponding values for A_H/A_T are 0.19 (calculated) and 0.13 (observed), respectively. (B) Simulation of intrinsic primary k_D/k_T isotope effects. For this calculation, C was calculated according to $C = 0.6/[(k_H/k_T)_{intrinsic}]^{1/1.44}$ [from the Swain–Schaad relationship, $(k_H/k_D)1.44 = (k_H/k_T)$ (Swain et al., 1958)]. The open circles represent the data of Table 3 and the dashed line the least-squares fit through the data. The calculated intrinsic k_D/k_T isotope effect can be described by $\ln(k_D/k_T) = 466.02/T - 0.535$, while a least squares fit of observed isotope effects (dashed line) gives $\ln(k_D/k_T) = 489.85/T - 0.647$. The corresponding values for A_D/A_T are 0.59 (calculated) and 0.53 (observed), respectively.

oxidase B), there are little ambiguities with respect to the A_D/A_T value. However, it should be noted that if the commitment for protium transfer is temperature-independent, the corresponding commitment for deuterium transfer has to undergo a slight change with temperature. This originates in the fact that a temperature independent commitment implies that the energy of activation for hydrogen transfer and other rate-limiting steps are identical, which cannot be simultaneously true for the reaction of two different isotopes, due to their different zero point energies. The net result of a small change with temperature of the commitment for deuterium transfer is, however, quite small. As illustrated in Figure 3B, a graph of $(k_D/k_T)_{int}$ vs $1/T$ leads to a value for $(A_D/A_T)_{int}$ of 0.59, which can be compared to the experimentally observed value of 0.53. This shows that A_D/A_T is well below the limit of 0.9 expected in the absence of tunneling, indicating clearly a role for hydrogen tunneling in the monoamine oxidase B reaction.

The temperature dependence of observed secondary isotope effects (Figure 1B) does not give A_H/A_T or A_D/A_T values significantly smaller than 1, the values being 0.77 ± 0.15 and 0.92 ± 0.04, respectively. While the small deviations from unity could be due to some coupled motion between the hydrogen being transferred and the secondary hydrogen (Huskey and Schowen, 1983), this is unlikely to play a significant role in the MAO-B reaction.

Analysis of kinetic data obtained with dideuterated *p*-methoxybenzylamine at various temperatures gives a value for the enthalpy of activation of 63 kJ/mol (graph not shown). An enthalpy of activation for protonated substrate can the be estimated from $E_a(D) - E_a(H) = [E_a(D) - E_a(T)] - [E_a(H) - E_aT)] = 9$ kJ/mol (using the slopes in Figure 1), giving a value for the enthalpy of activation for protonated substrate of 54 kJ/mol.

DISCUSSION

Generality of Tunneling. The data presented herein demonstrate that tunneling takes place in the hydrogen abstraction step of *p*-methoxybenzylamine oxidation by MAO-B, bringing the number of enzymes for which hydrogen tunneling has been demonstrated by measurements of competitive k_H/k_T and k_D/k_T isotope effects to four. This suggests that tunneling is a general feature of hydrogen abstraction by enzymes, since the enzymes investigated so far catalyze very different reactions. Two are NADH-dependent dehydrogenases (YADH and LADH) (Bahnson et al., 1993; Cha et al., 1989; Rucker et al., 1992), one is a quinoprotein (BSAO) (Grant and Klinman, 1989; Rucker et al., 1992) and one is flavin-dependent (MAO-B, this study). It is becoming increasingly clear that a full treatment of enzyme-catalyzed hydrogen transfer reactions must take into account quantum effects. Since tunneling is, by itself, temperature independent, an important question to address is the role of thermal activation in these enzyme reactions. The energy of activation (E_a) for the MAO-B hydrogen transfer step is 54 kJ/mol, which is very similar to the activation energy for the hydrogen transfer steps in the YADH and BSAO reactions (Grant & Klinman, 1989; Rucker et al., 1992). A model which assumes that hydrogen transfer occurs solely by quantum mechanical tunneling from a vibrationally activated enzyme–substrate complex has been proposed by Bruno and Bialek (1992). In this view, thermal fluctuations of the protein lead to continuous sampling of configurations for bound reactants. These fluctuations increase the tunneling rate by shortening the transfer distance for the hydrogen atom. This model was able to reproduce successfully the temperature dependence of primary isotope effects in the BSAO reaction (Bruno and Bialek, 1992). It is, however, likely to be incomplete in that it does not allow for partitioning of thermal energy into the reactive C–H bond and also does not take into account classical transfer of hydrogen over the potential barrier. A more complete approach to describe hydrogen transfer reactions has been proposed by Truhlar and co-workers (Garrett and Truhlar, 1980; Truhlar and Gordon, 1990). In their view, hydrogen transfer occurs by a combination of classical and quantum mechanical behavior by corner cutting near the top of the barrier. In such a description, the enthalpy of activation reflects a partitioning of energy into a wide range of modes which leads to changes in protein geometry, van der Waals interactions, hydrogen bonding, bond angles of the reacting

substrate, etc. It is to be expected that tunneling in reactions with these properties will be relatively insensitive to the nature of $K_{eq(int)}$, the equilibrium constant for the hydrogen transfer step. This latter view of hydrogen transfer is consistent with the effect of changes in $K_{eq(int)}$ on the degree of tunneling observed in the YADH and BSAO reactions (Rucker et al., 1992). In both enzymes, tunneling was shown to be probable with substrates of different structure and different values for $K_{eq(int)}$. Of some interest, tunneling in the YADH reaction was shown to correlate better with $\Delta H°$ than $\Delta G°$, increasing in probability as $\Delta H°$ approached zero (Rucker et al., 1992).

Magnitude of Exponential Relationships. We were surprised to find that the exponents relating secondary isotope effects in this study all fall well below 3.26. Although we do not yet have a satisfactory explanation for this observation, there are three possibilities under consideration. The first is that a small temperature-independent commitment is present under all conditions of measurement. Since secondary isotope effects, by nature of their smaller size, are more sensitive to commitments than primary effects, this could explain the greater reduction in the exponent relating secondary isotope effects. As the commitment does not appear to change appreciably with pH at 25 °C nor with temperature at pH 6.1, it would probably have to be an internal commitment related to the hydrogen transfer process (as compared to external commitments, which include substrate and product binding steps).[2] While secondary isotope effects are more sensitive to commitments, we cannot bring the secondary exponent to a value close to 3.26 by invoking a commitment without bringing the primary exponent to a value larger than 3.26, i.e., in the direction expected in the presence of tunneling (Cha et al., 1989; Saunders, 1985). For example, assumption of a C value for protium transfer of 0.6 and an intrinsic secondary exponent at 25 °C of 3.31 gives an intrinsic primary exponent of 3.44 and observed primary and secondary exponents of 3.10 and 2.34, respectively. It is therefore possible to explain the unusually small secondary exponents with commitment factors, but only if breakdown of the primary exponent in the direction of tunneling is allowed to take place. Additionally, intrinsic k_H/k_T isotope effects would have to be quite large under these conditions; the intrinsic primary effect is estimated as 36, while the secondary effect as 1.54. The secondary effect is very large, even larger than the equilibrium effect for sp^3 to sp^2 rehybridizations (Cleland, 1980); this latter feature would also implicate tunneling (Cha et al., 1989). Recalling that a small temperature independent-commitment does not change observed isotopic Arrhenius prefactor values appreciably (eq 9), it becomes clear that the presence of a small commitment under our experimental conditions would not alter the interpretation of the data.

One provocative question that arises is the chemical nature of the step that would generate a small commitment. At the present level of experimentation, it is not possible to describe this step, although, as discussed below (under Chemical Mechanism), we do not think this is due to a two 2-process for substrate oxidation (cf. eq 10). A step involving some protein reorganization could certainly precede $p-CH_3O-$benzylamine oxidation, with the caveat that $\Delta H^‡$ for this process would have to be similar to $\Delta H^‡$ for C−H bond cleavage. From the different steric effects observed for MAO-B catalyzed oxidation of *para*- vs *meta*-substituted benzylamines (Walker and Edmondson, 1994), it is also possible the p-CH_3O-benzylamine must reorient itself in the substrate binding pocket before it can undergo efficient C−H bond cleavage. This view is supported by the observation that *meta*-substituted benzylamines show consistently larger isotope effects than *para*-substituted substrates under conditions of the pre-steady-state (Walker and Edmondson, 1994).

A second, more speculative explanation for the anomalously small secondary exponents relates to the nature of the hydrogen transfer step itself. As described by Truhlar and co-workers (Truhlar and Gordon, 1990), tunneling leads to a phenomenon in which protium and deuterium cross the reaction barrier at different positions. Since secondary isotope effects reflect changes in force constant between the ground state and the point of barrier penetration, this could give rise to "different" secondary isotope effects for D transfer (k_D/k_T) than H transfer (k_H/k_T), particularly when tunneling is extensive. Under these conditions, the exponential relationship between k_D/k_T and k_H/k_T may be difficult to predict. As a third possibility, it is worth mentioning results of modeling of the alcohol dehydrogenase reaction by Huskey (1991). Using the program BEBOVIB and the Bigeleisen formulation of isotope effects, he calculates exponents relating k_D/k_T and k_H/k_T isotope effects for a model of the alcohol dehydrogenase reaction which assumes the same labeling scheme as in this work, i.e., dideutero substrates for k_D/k_T measurements and diprotio substrates for k_H/k_T measurements. Assuming no vibrational coupling between primary and secondary hydrogens, Huskey calculates an exponent of 2.8, which is attributed to breakdown of the rule of the geometric mean. This effect can be understood in terms of the sensitivity of secondary isotope effects to isotopic substitutions, since they contain many vibrational modes. Primary isotope effects, on the other hand, are usually dominated by a single vibrational mode, such that isotopic substitution in other positions have little effect on the observed isotope effect. Since secondary exponents are very sensitive to small changes in isotope effects (due to the fact that secondary isotope effects are small), relatively large deviations from 3.26 may occur in the secondary position while the primary exponents stay close to 3.26. In the case of MAO B, the most likely explanation for the small secondary exponents would involve a combination of a small commitment and one of the other possibilities mentioned.

Chemical mechanism of MAO-B. The large secondary isotope effects shown in Table 3 are close to equilibrium values seen for sp^3 to sp^2 rehybridizations (Cleland, 1980). Thus, it is possible that the oxidation of substrate occurs in two steps with an equilibrium secondary isotope effect on the first step and a large primary isotope effect on the second step. Silverman and co-workers have presented evidence for radical cation intermediates in the MAO-B reaction through the use of a variety of substrate analogs (Silverman, 1991, 1992; Silverman et al., 1980; Silverman and Zelechonok, 1992), cf., eq 10:

$$RCH_2NH_2 \rightleftharpoons RCH_2\dot{N}H_2^{+} \xrightarrow{-H^+} \dot{R}CHNH_2 \qquad (10)$$

According to the mechanism in eq 10, the magnitude of the secondary isotope effect would reflect a value for charge creation at the adjacent nitrogen, together with effects arising from the change in rehybridization at the α-carbon (sp^3 in

amine and sp^2 in the carbon radical). Although the formation of radical cation species may, in fact, be important in substrate analogs, there is no evidence for such intermediates in the course of benzylamine oxidation. Specifically, radical cation formation is expected to be accompanied by the formation of an equivalent of semiquinone at the flavin cofactor. However, as recently documented (Walker and Edmondson, 1994), no significant level of semiquinone (formed prior to the C−H abstraction step) could be detected by rapid mixing kinetic experiments using either α-protio- or α-deuterio-ring-substituted benzylamines. In the course of these studies, it was shown that substituents in either the *meta* or *para* position of the phenyl ring exerted little or no electronic effect on the hydrogen transfer step. This important observation, when taken with the failure to detect any reaction intermediates under stopped flow conditions, has led to the proposal of a direct hydrogen atom abstraction from substrate (Walker and Edmondson, 1994). Although the absence of a substituent effect on the hydrogen abstraction step could, in principle, reflect a very early transition state, the magnitude of the secondary isotope effects in this study (Table 3) argues against this being the case.[4]

Classically, secondary isotope effects are expected to vary between unity and the equilibrium isotope effect, the former limit being observed for very early transition states and the latter for late transition states (Klinman, 1978). In the case of coupled motion and tunneling, however, large secondary isotope effects can be observed even though the transition state is early, as observed in the YADH reaction (Cha et al., 1989). In that study, observed secondary exponents were significantly larger than the semiclassical value of 3.26, which is evidence for coupled motion and tunneling (Cha et al., 1989; Huskey and Schowen, 1983). The observation of secondary exponents considerably smaller than 3.26 argues against significant coupled motion in the MAO-B reaction. Additionally, from the temperature dependence of the secondary isotope effects (Figure 2), isotope effects on Arrhenius prefactors are close to unity; evidence of this nature has been used to rule out significant coupled motion in the BSAO reaction (Grant and Klinman, 1989), and a similar interpretation of the MAO-B reaction appears reasonable. Overall, the most straightforward mechanism which can be written for MAO-B involves a single-step hydrogen atom abstraction process characterized by a late transition state and accompanied by significant tunneling.

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[4] Within the context of a radical cation mechanism, eq 10, the absence of a substituent effect could arise for a very late transition state in which charge generation by one-electron oxidation is offset essentially completely by a subsequent proton abstraction. However, we cannot conceive of a proton abstraction from a poorly populated, energetically unfavorable radical cationic species as being characterized by a very late transition state. In fact, the expected energetics for such a proton abstraction (highly exoenergetic) would lead to an early transition state with charge properties reflecting the radical cation.

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