Structure–Activity Relationships in the Oxidation of Benzylamine Analogues by Bovine Liver Mitochondrial Monoamine Oxidase B[†,‡]

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ABSTRACT: The influence of *para* and *meta* substitution of benzylamine on its interaction with bovine liver mitochondrial monoamine oxidase B (MAO B) has been investigated by steady-state and reductive half-reaction anaerobic stopped-flow kinetic approaches. Steady-state kinetic properties of each benzylamine analogue suggest that *para* or *meta* substitution does not alter the mechanistic pathway of catalysis [Husain, M., *et al.* (1982) *Biochemistry 21*, 595–600]. All analogues tested exhibited $^{D}k_{cat}$ values ranging from 5.5 to 8.9 and $^{D}[k_{cat}/K_m(\text{amine})]$ values ranging from 3.3 to 8.1. $^{D}[k_{cat}/K_m(O_2)]$ values of ~1 are observed for all substrate analogues. Values for K_d were calculated from steady-state isotope effect data [Klinman, J. P., & Matthews, R. G. (1985) *J. Am. Chem. Soc. 107*, 1058–1060] and are in good agreement with K_s values determined from analysis of the rate of MAO B reduction as a function of benzylamine analogue concentration in reductive half-reaction experiments. A linear correlation of benzylamine analogue K_d values with the hydrophobicity parameter (π) is observed for the *para*-substituted analogues where the binding affinity increases with increasing hydrophobicity of the substituent. Statistical treatment of the correlation shows a small negative contribution to binding by the van der Waals volume (V_W) of the *para* substituent. *meta*-Substituted benzylamine analogues show a decreased binding affinity with the V_W of the substituent and no correlation with the hydrophobicity value of the substituents tested. No spectral evidence was found for any flavin radical intermediates during the time course of MAO B flavin reduction in anaerobic reductive half-reduction stopped-flow experiments with any of the α,α-diprotio- or α,α-dideuteriobenzylamine analogues tested. The limiting rates of enzyme reduction exhibit large ^{D}k values (6.5–14.1) for all of the analogues tested. *para*-Substituted benzylamine analogues reduce MAO B with limiting rates that correlate with the steric influence (E_s value) of the substituent. Statistical analysis shows the rate of MAO B reduction by *para*-substituted analogues to be retarded by increased values of E_s and, with a smaller contribution, by the hydrophobicity value of the substituent. The rate of MAO B reduction by *meta*-substituted benzylamine analogues is essentially independent of the nature of the substituent. No evidence was found for any electronic contribution to the rate of MAO B flavin reduction by any of the analogues tested. These data demonstrate the steric orientation of the substrate to be important in the rate of amine oxidation by MAO B and that ring *meta* substituents favor this orientation. The relevance of these results to the proposed radical mechanism for MAO catalysis [Silverman, R. B. (1991) *Biochem. Soc. Trans. 19*, 201–206] is discussed.

Monoamine oxidase (EC 1.4.3.4, MAO)[1] is an integral membrane-bound flavoenzyme situated on the mitochondrial outer membrane. The enzyme catalyzes the oxidative deamination of biogenic amines using O_2 as electron acceptor. The A and the B isozymic forms of MAO have been shown to be separate gene products (Bach *et al.*, 1988) and to be expressed in a tissue-specific manner (Weyler *et al.*, 1990). MAO B is readily purified from bovine liver mitochondria and has been the subject of numerous structural and mechanistic studies. A considerable body of kinetic evidence (Husain *et al.*, 1982; Pearce & Roth, 1985; Edmondson *et al.*, 1993) suggests that the mechanistic pathway for benzylamine oxidation by MAO B is as depicted in Scheme 1. As illustrated in this scheme, O_2 reacts with the reduced enzyme–imine complex, and the imine product dissociates from the enzyme before non-enzymatic hydrolysis. In the case of phenethylamine (Husain *et al.*, 1982) and its analogues (Pearce & Roth, 1985), the imine product dissociates from the reduced enzyme prior to O_2 reoxidation.

Our current knowledge regarding the mechanism of MAO-catalyzed amine oxidation has come mainly from studies on the interaction of MAO with mechanism-based inhibitors (Silverman, 1991). From these studies, Silverman and colleagues (1991) have proposed a radical mechanism for MAO involving an aminium cation substrate radical and a flavin radical as the initial reaction intermediates, followed by α-CH deprotonation, radical migration to the α-carbon of the substrate, and electron transfer either directly to the flavin radical or *via* an amino acid residue to result in formation of the imine product and flavin hydroquinone (Scheme 2). Experimental support for this mechanism is indirect, and no evidence is currently available for the formation and decay of enzyme–flavin or substrate radicals at times consistent with MAO catalytic turnover. Thus, further work is required to test critically the validity of the mechanism proposed in Scheme 2.

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[1] Abbreviations: MAO, monoamine oxidase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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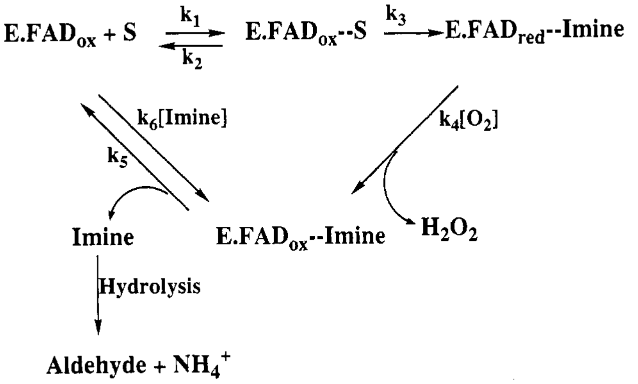
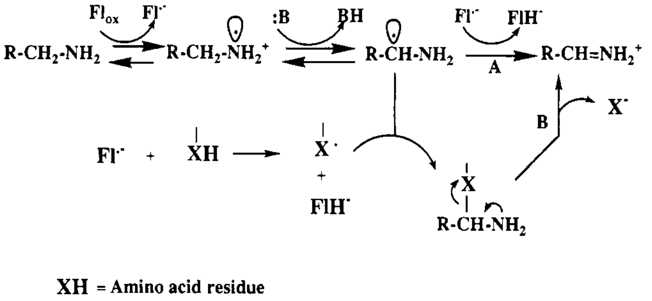
Scheme 1: Reaction Pathway for the MAO B-Catalyzed Oxidation of Benzylamine[a]



[a] Husain *et al.* (1982); Edmondson *et al.* (1993).

Scheme 2: Proposed Radical Mechanism for MAO B Catalysis[a]



[a] Adapted from Silverman (1991).

One of the various approaches that can be exploited to investigate oxidation–reduction enzyme mechanisms includes the examination of enzyme reactivity as a function of substrate structure. Effects of substituents on enzyme catalytic rates have been shown to be influenced by electronic effects (σ), hydrophobicity parameters (π) (Fujita *et al.*, 1964), and steric parameters [E_s or V_W, where E_s is the Taft steric parameter (Hansch & Leo, 1979), and V_W is the van der Waals volume of the substituent (Bondi, 1964)]. The effect of these parameters on intrinsic rates of C–H bond cleavage or on substrate analogue binding is defined by the following expression:

$$\log k \text{ (or } K_d) = \rho\sigma + A\pi + BE_s \text{ (or } V_W) + C \qquad (1)$$

where ρ, A, and B are parameter coefficients, and C is a constant. Such structure–activity relationships have been employed as part of an examination of the enzyme mechanisms of alcohol dehydrogenase (Klinman, 1976), dopamine-β-monooxygenase (Miller & Klinman, 1985), and bovine plasma amine oxidase (Hartmann & Klinman, 1991). A key element to the successful application of this approach to enzyme systems is the determination of the microscopic rate constant for C–H bond cleavage using kinetic isotope effects and the determination of rate constants using stopped-flow kinetic approaches.

The effects of substitution at the aromatic ring on the rate of MAO-catalyzed oxidation of benzylamine have been investigated previously (Hellerman & Erwin, 1968; Hellerman *et al.*, 1972; Zeller *et al.*, 1971). However, the results of these earlier studies are not readily interpretable since V_{max} values at only a single [O$_2$] were determined, and it is not clear whether the differences observed as a result of the nature of the substituent reflect alterations in the C–H bond breakage step during catalysis or in other contributing steps. In the present study, approaches similar to those discussed by Klinman and co-workers have been employed to investigate the influence of *para* and *meta* substituents on the oxidative deamination reaction catalyzed by bovine liver MAO B. Anaerobic reductive half-reaction stopped-flow experiments have been performed to determine directly the influence of *para* and *meta* substituents on the rate of 8-α-*S*-cysteinyl-FAD reduction in MAO B. Both α,α-diprotio and α,α-dideuteriobenzylamine analogues are compared in steady-state as well as stopped-flow kinetic experiments in order to determine the extent to which carbon–hydrogen bond breakage is rate limiting during catalysis. The results provided in this study show a differential interaction of *para*- and *meta*-substituted benzylamine analogues with MAO B.

The binding of *para*-substituted benzylamine analogues to MAO B increases in affinity with increasing hydrophobicity of the substituent, while the binding affinity of *meta*-substituted analogues decreases with increasing molecular volume of the substituent. The limiting rate for flavin reduction is invariant with the nature of the *meta* substituent, whereas the rate of MAO B reduction is decreased with increasing values of the E_s parameter of the *para* substituent. Electron-accepting or -withdrawing nature of the substituent (*meta* or *para*) appears to have little influence on the rate of C–H bond cleavage. The relationship of these results with the current views on the mechanism of C–H bond cleavage in MAO B catalysis (Silverman, 1991) is discussed.

EXPERIMENTAL PROCEDURES

Enzyme. MAO B was purified from mitochondrial preparations of fresh bovine livers obtained at a local slaughterhouse by the method outlined by Salach (1979) as further modified by Weyler and Salach (1981). The specific activity of preparations used in this study was 4 units/mg when assayed as described by Salach (1979). All enzyme preparations showed >95% functionality as demonstrated by the level of flavin reduction following the addition of excess substrate under anaerobic conditions relative to the degree of flavin reduction following the addition of sodium dithionite. Storage and manipulation of purified enzyme samples followed general procedures described by Ramsay *et al.* (1987).

Substrate Analogues. The ring-substituted [^{1}H]benzylamine analogues used in this study were purchased from Aldrich or Pfaltz and Bauer, Inc., with the following exceptions. *p*-Carboxybenzylamine was synthesized using *p*-cyanobenzoic acid (Aldrich) as starting material according to Wakamatsu *et al.* (1980). *p*-Hydroxybenzylamine was synthesized as described by Hartmann and Klinman (1991). α,α-Dideuteriobenzylamines were synthesized by reduction of the corresponding nitriles (obtained from Aldrich) in dry diethyl ether with LiAlD$_4$ (98% D, obtained from Sigma) (Ammundsen & Nelson, 1951). All benzylamine analogues were recrystallized at least three times as their hydrochloride salts from ethanol. Purity was monitored by thin-layer chromatography on silica gel using *n*-butanol/acetic acid/H$_2$O, 4/1/5 (v/v/v, upper layer), as developing solvent. Visualization of compounds on TLC plates used either staining with I$_2$ vapors or fluorescence after derivatization with fluorescamine. All α,α-diprotio- and α,α-dideuteriobenzylamine analogues used in this study exhibited spectra consistent with their respective structures and the extent of isotopic incorporation when examined by ^{1}H NMR (using a Nicolet 360-MHz instrument) and by ^{13}C NMR at 50.3 MHz (using an IBM/Bruker WP200 SY instrument). All samples were dissolved in D$_2$O as their hydrochoride salts for NMR analysis. The dideuteriobenzylamine analogues used in this study showed deuterium incorporation in excess of 95%.

Steady-State Enzyme Assays. All kinetic experiments were performed at 25 °C in 50 mM HEPES, pH 7.5, containing 5 mg/mL RTX-100 (a catalytically hydrogenated form of the non-ionic detergent Triton X-100) (Tiller *et al.*, 1984), which was purchased from Sigma. This detergent permits spectrophotometric monitoring of ring-substituted benzaldehyde production in the near-UV wavelength range which is otherwise inaccessible in the presence of Triton X-100 due to its absorption spectral properties. Oxygen concentrations were measured polarographically using a Yellow Springs instrument. Instrument calibrations were performed as described by Robinson and Cooper (1970) using the phenazine ethosulfate-mediated O_2 oxidation of NADH. Spectrophotometric assays of substituted benzaldehyde formation were performed using a Gilford update of a Beckmann DU spectrophotometer. Operational δ molar absorption extinction coefficients for calculation of the amount of substituted benzaldehyde formed were determined by comparison of O_2-uptake rates with δ absorbance rates under identical conditions. These values are the following: benzylamine, 250 nm, 12 800 M^{-1} cm^{-1} (Neumann *et al.*, 1975); *p*-methoxybenzaldehyde, 281 nm, 12 600 M^{-1} cm^{-1}; *p*-methylbenzaldehyde, 259 nm, 12 800 M^{-1} cm^{-1}; *p*-fluorobenzaldehyde, 250 nm, 14 200 M^{-1} cm^{-1}; *p*-chlorobenzaldehyde, 257 nm, 21 800 M^{-1} cm^{-1}; *p*-bromobenzaldehyde, 264 nm, 21 700 M^{-1} cm^{-1}; *p*-(trifluoromethyl)benzaldehyde, 243 nm, 11 800 M^{-1} cm^{-1}; *m*-methoxybenzaldehyde, 253 nm, 11 900 M^{-1} cm^{-1}; *m*-methylbenzaldehyde, 252 nm, 15 700 M^{-1} cm^{-1}; *m*-chlorobenzaldehyde, 247 nm, 16 300 M^{-1} cm^{-1}; and *m*-(trifluoromethyl)benzaldehyde, 242 nm, 15 600 M^{-1} cm^{-1}.

Stopped-Flow Kinetic Experiments. Anaerobic stopped-flow experiments were performed using an apparatus purchased from Kinetics Instrument, Ann Arbor, MI. The dead time for the instrument was determined to be 1 ms using the myoglobin–azide reaction. Kinetic absorbance traces were digitized and recorded using a Nicolet 4094 digital oscilloscope. Data were transferred to an IBM XT computer *via* an IEEE-488 interface for subsequent data analysis. Solutions of enzyme and of substrate were degassed in tonometers and stored under an argon atmosphere. The argon used was purified of any residual oxygen by passage through a column of heated BASF catalyst. All solutions contained 50 mM glucose, 22 nM glucose oxidase, and 100 units/mL catalase to consume any remnants of O_2. In some cases, the glucose/glucose oxidase system was substituted with 240 μM hypoxanthine and 10 nM xanthine oxidase. Drive syringes in the stopped-flow apparatus were made anaerobic by incubation for 2 h with the above O_2-scavenging system in degassed buffer before introduction of the enzyme and substrate.

Data Analysis. Steady-state kinetic data were fit to a nonlinear regression program for data conforming to the Michaelis–Menton equation as outlined by Duggleby (1981) to obtain V_{max} and K_m values. Stopped-flow kinetic data for anaerobic half-reaction experiments were fit to a monoexponential decay function using software provided by EMF Software, Baltimore, MD (MEDAS 87). Initial estimates of substrate binding (K_s) and the rate constant for enzyme reduction (k_{red}) according to the mechanism

$$E + S \overset{K_s}{\rightleftharpoons} ES \xrightarrow{k_{red}} E_r{*}P \qquad (2)$$

were determined as described by Strickland *et al.* (1975). Further analysis of k_{obs} vs [S] plots was performed using a fitting program written in Basic and supplied by Dr. Gordon Tollin, University of Arizona (Simondsen *et al.*, 1982). This program solves for the theoretical best fit to the observed dependence of observed rate with [S] using the equations derived for the above mechanism (Frost & Pearson, 1961).

Substituent parameters (σ, π, and E_s) were used as listed in Hansch and Leo (1979). V_W values were obtained from Bondi (1964) or calculated as described in his paper. Statistical fits (one or two parameter) of rate or binding data correlations with substituent parameters were performed using the regression subroutine in the statistics subroutine of Asystant data analysis software (Macmillan Software Co.) and run on an IBM XT computer.

RESULTS

Substituent Effects on Steady-State Kinetic Properties. Although previous studies (Zeller *et al.*, 1971; Hellerman *et al.*, 1972; Hellerman & Erwin, 1968) have examined the effect of *para* and *meta* substitution on the V_{max} of MAO-catalyzed oxidation of benzylamines, a ready interpretation of their results is precluded in that kinetic data were obtained only at a single [O_2] (air saturation). The K_m for O_2 may vary with the nature of the substituent, and the observed maximal velocities may differ depending on the relative $K_m(O_2)$ for the substituted benzylamine tested. In addition, since a number of kinetic steps may contribute to k_{cat}, any alteration in rate constants at steps other than the α-C–H bond breaking step as a result of substrate modification would further complicate data interpretation to obtain mechanistic insights. Previous work, however, has demonstrated a large deuterium kinetic isotope effect on k_{cat} in MAO-catalyzed benzylamine oxidations (Husain *et al.*, 1982). These data suggest that the step involving α-C–H bond cleavage should contribute substantively to k_{cat}, and therefore, if all of the substituted benzylamine analogues were to exhibit comparably large isotope effects on k_{cat}, meaningful information might be obtained from steady-state kinetic data to assess the role of substituent effects.

The data in Table 1 show the influence of *para* and *meta* substitution on k_{cat}, K_m(amine), and $K_m(O_2)$ for the MAO B-catalyzed oxidation of benzylamine. All substrate analogues exhibited parallel-line Lineweaver–Burk plots on variation of [O_2] as is normally observed when benzylamine is the substrate [cf. Husain *et al.* (1982)]. The *para*-substituted substrate analogues exhibit a 50-fold range in k_{cat}, with the *p*-CF_3 analogue showing the slowest rate and benzylamine showing the fastest rate. Of interest is the finding that the *p*-carboxybenzylamine is neither a substrate nor a competitive inhibitor. This observation suggests that placing a negative charge at the *para* position of the substrate prevents binding to the active site. Experiments with *p*-nitrobenzylamine demonstrate that it is a very slow substrate, too slow to be readily examined by polarographic or spectrophotometric techniques. Kinetic data on the oxidation of this analogue by MAO were obtained from anaerobic half-reaction studies, which will be described below. However, *p*-nitrobenzylamine is an excellent competitive inhibitor of benzylamine oxidation with a K_i value of 7 μM. In contrast to the lower k_{cat} values exhibited by the *para*-substituted analogues, *meta*-substituted benzylamines all exhibited values considerably higher than that observed with benzylamine (Table 1).

The $K_m(O_2)$ values determined for each benzylamine analogue did vary with the nature of the substituent (Table 1). While the *meta*-substituted analogues exhibited quite similar $K_m(O_2)$ values, the *para*-substituted analogues exhibited $K_m(O_2)$ values that varied ~30-fold. These data

Table 1: Steady-State Kinetic Constants for the MAO B-Catalyzed Oxidation of *para*- and *meta*-Substituted Benzylamine Analogues

substituent	k_{cat} (min^{-1})	K_m(amine) (μM)	K_m(O_2) (μM)	$\Phi_{O_2}^{-1}$ (M^{-1} min^{-1})
H	640 ± 56	500 ± 60	280 ± 40	2.5×10^6
p-MeO	240 ± 8	330 ± 40	120 ± 8	1.6×10^6
p-Me	62 ± 1	120 ± 10	24 ± 2	2.1×10^6
p-F	390 ± 12	240 ± 20	110 ± 9	3.3×10^6
p-Cl	170 ± 11	130 ± 9	80 ± 11	1.8×10^6
p-Br	80 ± 1	80 ± 7	50 ± 2	1.6×10^6
p-CF_3	13 ± 0.4	140 ± 12	9 ± 0.3	$\sim 1.3 \times 10^6$
p-COO^-		neither a substrate nor a competitive inhibitor		
p-NO_2		competitive inhibitor with benzylamine (K_I = 7 μM)		
m-MeO	720 ± 30	630 ± 70	510 ± 35	1.4×10^6
m-Me	790 ± 80	340 ± 40	650 ± 90	1.3×10^6
m-Cl	660 ± 20	210 ± 20	490 ± 29	1.4×10^6
m-CF_3	900 ± 110	1010 ± 130	540 ± 88	1.6×10^6

Table 2: Steady-State Kinetic Constants and Deuterium Isotope Effects for the MAO B-Catalyzed Oxidation of *para*- and *meta*-Substituted α,α-D_2-Benzylamine Analogues

α,α-D_2-benzylamine substituent	k_{cat} (min^{-1})	${}^{D}k_{cat}$	${}^{D}[k_{cat}/K_m(\text{amine})]$	K_m(O_2) (μM)	${}^{D}[k_{cat}/K_m(O_2)]$
H	78 ± 2	8.2 ± 0.9	5.2 ± 0.4	30 ± 4	1.0 ± 0.1
p-MeO	27 ± 1	8.9 ± 0.6	8.1 ± 1.0	13 ± 3	1.0 ± 0.2
p-Me	7 ± 0.1	8.8 ± 0.1	6.8 ± 0.7	<10	
p-F	51 ± 0.7	7.6 ± 0.4	5.2 ± 0.5	11 ± 0.1	0.8 ± 0.1
p-Cl	26 ± 1	6.5 ± 0.6	5.5 ± 1.3	18 ± 2	1.4 ± 0.2
p-Br	11 ± 0.3	7.3 ± 0.1	7.4 ± 1.0	11 ± 0.4	1.5 ± 0.1
m-MeO	120 ± 5	6.0 ± 0.5	2.9 ± 0.4	80 ± 9	0.9 ± 0.1
m-Me	130 ± 5	6.1 ± 0.8	3.6 ± 0.4	105 ± 10	1.0 ± 0.2
m-Cl	120 ± 4	5.5 ± 0.3	3.3 ± 0.5	86 ± 8	0.9 ± 0.1
m-CF_3	130 ± 3	6.9 ± 0.9	7.5 ± 0.6	78 ± 3	1.0 ± 0.1

further underscore the importance of determining steady-state kinetic data for substrate analogues at saturating concentrations of both MAO B substrates. Previous studies (Husain *et al.*, 1982) have shown that the Dalziel coefficient for O_2 $\Phi_{O_2}^{-1}$ is a reasonable measure of the rate of O_2 reaction with the reduced enzyme [cf. Bright and Porter (1975)]. In the case of substrates (such as phenethylamine) which follow a binary complex mechanism, the $\Phi_{O_2}^{-1}$ value of 4.3×10^5 M^{-1} min^{-1} is quite similar to the second-order rate constant (3.6×10^5 M^{-1} min^{-1}) for O_2 oxidation of the free, reduced enzyme (Husain *et al.*, 1982). The $\Phi_{O_2}^{-1}$ values determined for the benzylamine analogues tested in this study (Table 1) are all greater than that found for phenethylamine and are similar to the value exhibited by benzylamine. These data support the view that O_2 reacts with the reduced enzyme–imine complex rather than the free reduced enzyme for all of the analogues listed in Table 1. The kinetic pathways for catalytic turnover of the benzylamine analogues tested appear to follow that depicted in Scheme 1.

Effect of α,α-^2H Substitution on Steady-State Kinetic Properties. Previous studies (Husain *et al.*, 1982) demonstrated that MAO B exhibited reasonably large steady-state deuterium kinetic isotope effects with benzylamine as a substrate but not with phenethylamine. In the case of phenethylamine, the rate of O_2 reoxidation of the reduced enzyme is the slowest step in turnover, which accounts for the low ${}^{D}k_{cat}$ value observed. A series of α,α-dideuterated *meta*- and *para*-substituted benzylamine analogues were synthesized, and the steady-state parameters k_{cat}, K_m(amine), and K_m(O_2) were determined (Table 2). Significant ${}^{D}k_{cat}$ values (a composite of both 1° and 2° kinetic isotope effects) and ${}^{D}(k_{cat}/K_m)$ values are observed for all of the substituted benzylamine analogues tested. The ${}^{D}k_{cat}$ values range from 7 to 9 for the *para*-substituted analogues and are slightly lower (5.6–7.3) for the *meta*-substituted analogues (Table 2). In most of the analogues tested, ${}^{D}k_{cat}$ values are greater than ${}^{D}[k_{cat}/K_m(\text{amine})]$ values (*p*-bromo- and *m*-(trifluoromethyl)-benzylamine analogues being exceptions).

It should be pointed out that the K_m(O_2) values measured for α,α-dideuteriobenzylamine analogues are significantly *lower* (Table 2) than those observed with the protio analogues (Table 1). The consequence of large ${}^{D}k_{cat}$ values with similarly large influences of deuteration on K_m(O_2) is that the values for ${}^{D}[k_{cat}/K_m(O_2)]$ for the benzylamine analogues tested are all very close to unity. A practical consequence of this observation is that K_m(O_2) decreases commensurate with k_{cat} as a consequence of α,α-dideuteration. Since K_m(O_2) varies considerably from values greater than that at air saturation to values in the micromolar region with the various benzylamine analogues, it would be prudent for other investigators in MAO B catalysis to be cognizant of changes in K_m(O_2) when investigating the interaction of the enzyme with differing substrate analogues. Using the mechanistic pathway outlined in eqs 3 and 4,

$$E_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{ox}S' \underset{*k_6}{\overset{*k_5}{\rightleftharpoons}} E_{red}P' \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_{red}P \qquad (3)$$

*denotes isotope-sensitive step

$$E_{red}P + O_2 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_{red}PO_2 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_{ox}P\text{–}H_2O_2 \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}}$$

$$E_{ox}P + H_2O_2 \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} E_{ox} + P \qquad (4)$$

the following expression can be derived for ${}^{D}[k_{cat}/K_m(O_2)]$ (eq 5):

$$^{D}\left(\frac{k_{cat}}{K_m(O_2)}\right) = \frac{^{D}K_{eq} + \left(\frac{k_5}{k_6}\right)\left(\frac{1+k_7/k_8}{1+k_4/k_3}\right)}{1 + \left(\frac{k_5}{k_6}\right)\left(\frac{1+k_7/k_8}{1+k_4/k_3}\right)} \qquad (5)$$

By inspection of eq 5, $^{D}[k_{cat}/K_m(O_2)]$ will equal unity when $^{D}K_{eq} \sim 1$ or when k_6 and/or k_8 approaches zero (i.e., the C–H bond cleavage step or the step immediately after C–H bond cleavage is irreversible). As will be shown below, anaerobic reductive half-reaction experiments for all of the analogues tested show behavior consistent with the suggestion that the reductive step is irreversible, and therefore the value for k_6 and/or k_8 is very small.

Stopped-Flow Kinetic Studies. Anaerobic reductive half-reaction kinetic experiments were carried out to determine the effect of benzylamine substitution on the rate of reduction of the covalent FAD coenzyme of MAO B. For all benzylamine analogues tested, the rate of enzyme reduction was determined under pseudo-first-order conditions and the effect of substrate concentration on k_{obs} was determined. In all kinetic experiments, the rate of enzyme-bound flavin reduction was monitored at 450 nm. Kinetic traces at other wavelengths showed no detectable spectral intermediates due to flavin neutral or anionic semiquinone for any of the analogues tested. An example of this is shown in Figure 1 for the reduction of MAO B by *p*-fluorobenzylamine where kinetic traces were followed at 450, 580 (where neutral flavin semiquinone would be expected to absorb), and 375 nm (where anionic flavin semiquinone has an increased absorbance relative to the oxidized flavin). Previous studies (Edmondson *et al.*, 1981) have demonstrated that 8α-substituted neutral and anionic flavin semiquinones have absorption spectral properties similar to those of normal flavins. In all cases, the spectral changes observed are as expected for the reduction of the oxidized flavin to its hydroquinone form with no observable anionic or neutral flavin semiquinone intermediates (Figure 1). Thus, if any flavin radical intermediates are operative in the MAO-catalyzed oxidation of the benzylamine analogues tested in this study, they must occur at concentrations too low to permit spectral observation.

The observed rate constants for flavin reduction displayed a hyperbolic dependence on substrate concentration. As discussed by Strickland *et al.* (1975), such rapid reaction kinetic data can readily be interpreted using the following general mechanism where enzyme reduction is essentially irreversible:

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \xrightarrow{k_{red}} E_{red}P \qquad (6)$$

The limiting rate constant for flavin reduction is represented by k_{red}, and the apparent binding constant, by K_s, defined as $(k_{off} + k_{red})/k_{on}$ using the steady-state condition or would be equal to k_{off}/k_{on} using the equilibrium condition (Strickland *et al.*, 1975). In the latter situation, K_s is directly equal to K_d. The kinetic constants K_s and k_{red} can be obtained from the slope and *y*-intercept in plots of $1/(k_{obs})$ versus $1/[S]$ (Strickland *et al.*, 1975).

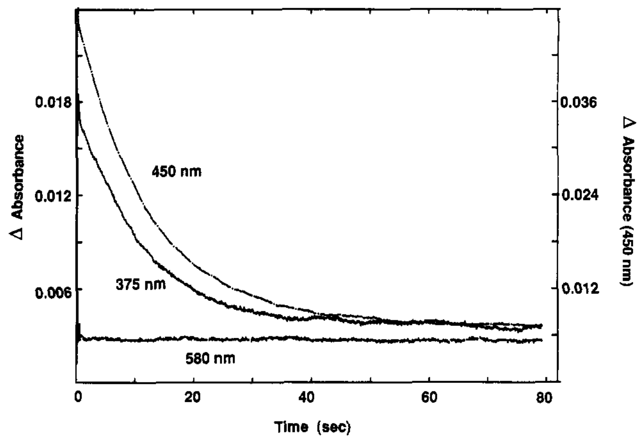


FIGURE 1: Absorbance changes on mixing 2.8 μM MAO B with *p*-F-benzylamine in the stopped-flow apparatus under anaerobic conditions. The absorbance changes at 450, 580, and 375 nm were followed in the presence of 0.1 mM amine. The absorbance increase at the *left* axis denotes absorbance changes during flow as monitored during the 200-ms time period before triggering.

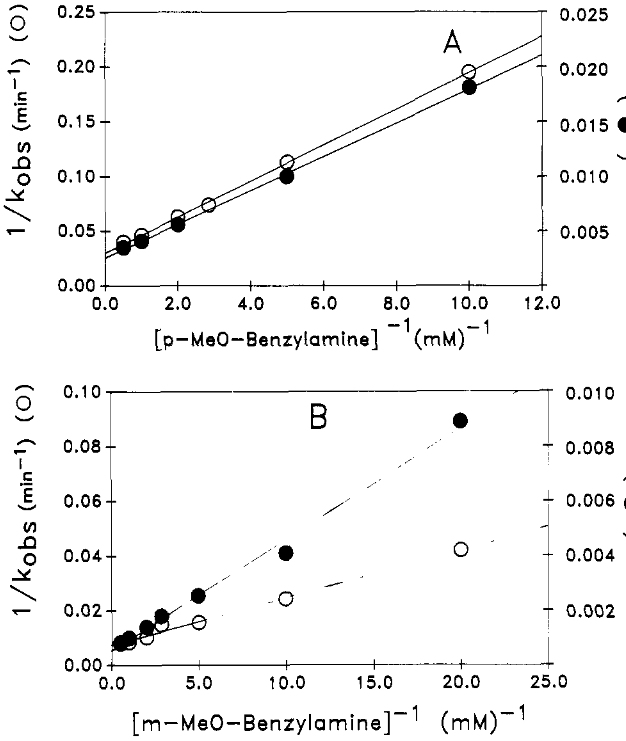


FIGURE 2: Reciprocal plots of the influence of benzylamine analogue concentration on the observed rate of MAO B reduction under anaerobic conditions. (A) Observed rates with *p*-MeO-benzylamine: α,α-diprotio (●), right ordinate; α,α-dideuterio (○), left ordinate. (B) Observed rates with *m*-MeO-benzylamine: α,α-diprotio (●), right ordinate; α,α-dideuterio (○), left ordinate.

When the reciprocal of the observed rate constant is plotted as a function of reciprocal benzylamine analogue concentration, linear plots are obtained for all analogues tested (Figure 2). Such linear behavior is in agreement with the results of Husain *et al.* (1982) but is in contrast with the stopped-flow data of Ramsay *et al.* (1987). In the latter case the curvature seen in the double-reciprocal plots of MAO B reduction by benzylamine (Ramsay *et al.*, 1987) does not conform to predicted behavior (Strickland *et al.*, 1975) in that the departure from linearity curves upward at low substrate concentrations. A ready explanation for such deviation from linearity is that the observed upward curvature in these experiments is due to the presence of residual O_2 in the stopped-flow apparatus which would slow the observed rate of flavin reduction at low concentrations of amine but have less of an effect on observed rates at higher amine concentrations.

Linearity in plots of $1/k_{obs}$ versus $1/[\text{amine}]$ has important consequences in the interpretation of these kinetic results. Such behavior demonstrates that flavin reduction is irrevers-

Table 3: Effect of *para* or *meta* Substitution on the Rates of Reduction of MAO B by Protio- and Deuteriobenzylamine Analogues and on Their Binding Affinities to the Catalytic Site[a]

substituent	k_{red}(H) (min^{-1})	k_{red}(D) (min^{-1})	$^{D}k_{red}$	K_s(H) (μM)	K_s(D) (μM)	K_d[b] (μM)
H	760 ± 2	75 ± 0.4	10.1 ± 0.1	430 ± 4	360 ± 6	290
p-OH	117 ± 3			2370 ± 160		
p-MeO	365 ± 4	32 ± 0.5	11.4 ± 0.3	530 ± 17	490 ± 22	300
p-Me	62 ± 1	7.3 ± 0.3	8.5 ± 0.5	100 ± 4	100 ± 1	120
p-F	600 ± 8	59 ± 0.4	10.2 ± 0.2	240 ± 10	90 ± 3	150
p-Cl	308 ± 5	38 ± 0.6	8.1 ± 0.3	90 ± 6	56 ± 3	110
p-Br	123 ± 1	19 ± 2	6.5 ± 0.7	46 ± 2	20 ± 2	80
p-CF_3	11.5 ± 0.4			130 ± 1		
p-NO_2	3.8 ± 0.2			25 ± 4		
p-$N(Me)_2$	148 ± 5	12 ± 0.6	12.3 ± 1.0	640 ± 55	450 ± 70	
m-MeO	1580 ± 29	145 ± 2	10.9 ± 0.4	610 ± 27	245 ± 11	240
m-Me	1900 ± 28	158 ± 1	12.0 ± 0.3	450 ± 16	106 ± 4	170
m-Cl	2260 ± 50	160 ± 2	14.1 ± 0.5	290 ± 21	60 ± 3	105
m-CF_3	2020 ± 12	150 ± 2	13.5 ± 0.2	1490 ± 26	680 ± 28	1200

[a] Error analyses of k_{red} and K_s values were determined by nonlinear least squares analysis of the equation describing the reductive half-reaction (Strickland *et al.*, 1975): $k_{obs} = k_{red}[\text{amine}]/([\text{amine}] + K)$. Each data set includes the observed rate constants for 5–10 different amine concentrations. [b] Calculated from steady-state kinetic isotope effect data (Tables 1 and 2) as described by Klinman and Matthews (1985).

ible. For the reaction scheme denoted in eqs 3 and 4, this requires that k_6 and/or k_8 are negligibly small relative to k_7. The finding of linear plots for all of the substituted benzylamine analogues in reductive half-reaction experiments shows the reduction step to be essentially irreversible for all of the substituents, consistent with a similar conclusion based upon $^{D}[k_{cat}/K_m(O_2)]$ values of near unity from steady-state kinetic data (Table 2).

The rates for enzyme reduction (k_{red}) and the K_s values for each substituted benzylamine analogue (both α,α-diprotio and α,α-dideuterio) are listed in Table 3. There is considerable variation in the rates of enzyme reduction (~200-fold) among the various *para*-substituted benzylamine analogues with all exhibiting slower rates than that of benzylamine. In contrast, *meta*-substituted benzylamines all reduce MAO B much faster than benzylamine with little variation in their individual rates. The apparent binding constants obtained from stopped-flow data can be compared directly to experimentally determined K_d values. Steady-state isotope effect data presented in Table 2 permit calculation of K_d values for the various analogues using the following expression, as described by Klinman and Matthews (1985):

$$\frac{^{D}k_{cat} - 1}{\left(\dfrac{^{D}k_{cat}}{K_m(\text{amine})}\right) - 1} = \frac{K_m}{K_d} \tag{7}$$

where K_d represents the substrate dissociation constant from all complexes prior to the isotope-sensitive step. The calculated K_d values show trends similar to those of the K_s values for the various analogues. In most cases, the deviation in values is within a range of 2–3-fold.

The deuterium kinetic isotope effects on enzyme reduction (a product of both primary and secondary isotope effects) are large for all analogues tested. In the case of *para*-substituted analogues the isotope effect on the rate of reduction either equals or is slightly greater than $^{D}k_{cat}$ (Table 2). The *meta*-substituted analogues exhibit $^{D}k_5$ values approximately twice that observed on $^{D}k_{cat}$, which suggests the presence of commitment factors that suppress full expression of the isotope effect on catalytic turnover for this class of substrate analogues.

Structure–Activity Relationships in Benzylamine Analogue Binding to MAO B. Substrate analogue dissociation constants with MAO B have been determined by two different approaches: they have been calculated from deuterium isotope effects on steady-state kinetic data (Table 3) and estimated by K_s values from reductive half-reaction stopped-flow kinetic studies (Figure 2 and Table 3). As described in the introduction, log K_d can be correlated with the following substituent parameters: σ, π, and E_s (or V_W) (eq 1). An assessment of the dependence of log K on these parameters was initially attempted with single-parameter correlations. After a number of attempts, it was found that the best correlations are found when the data from the *para*-substituted analogues are separated from those of the *meta*-substituted analogues. As shown in Figure 3A, the binding constants for the *para*-substituted benzylamine analogues show a good correlation with the hydrophobicity (π) parameter of each substituent. Using both steady-state and stopped-flow derived binding constants, a linear correlation with the π value is found with a slope of –1.02 ± 0.12 (correlation constant of 0.79). Only *p*-nitrobenzylamine exhibits an anomalous fit and was not included in the analysis. This correlation also provides an explanation for the inert behavior of *p*-carboxybenzylamine toward MAO B (Table 1). The π value for an ionized carboxyl group is –4.36 (Hansch & Leo, 1979). From the plot in Figure 3A, the K_d for *p*-carboxybenzylamine binding to MAO B is estimated to be ~3 M. This correlation demonstrates that *para*-substituents on the benzylamine ring will be bound with increasing affinity when the hydrophobicity parameter is increased.

In contrast to the *para*-substituted analogues, *meta*-substituted benzylamines show a decreased affinity for MAO B with increases in the van der Waals volume of the substituent (Figure 3B). The correlation in Figure 3B shows a slope of +0.98 ± 0.22 (correlation coefficient of 0.67) when both steady-state and stopped-flow kinetically derived binding constants are correlated with V_W. Poorer correlations are observed with σ, π, or E_s. Note that *m*-H (benzylamine) is not included in the analysis. *meta*-Substituted benzylamine analogues can clearly be differentiated from *para*-substituted analogues on the basis of factors important in binding affinity. This difference is also observed in substituent effects on the rates of enzyme reduction (see below).

Substituent Effects on the Rate of MAO B Reduction. The values for k_{red} of the various *para*- and *meta*-substituted benzylamine analogues also show differing patterns. All *meta*-substituted benzylamines reduce the flavin in MAO B at rates faster than those of the *para*-substituted analogues (Table 3). In addition, there is little effect of the nature of the *meta* substituent on the rate of enzyme reduction, whereas the *para*-substituent analogues exhibit markedly different values for

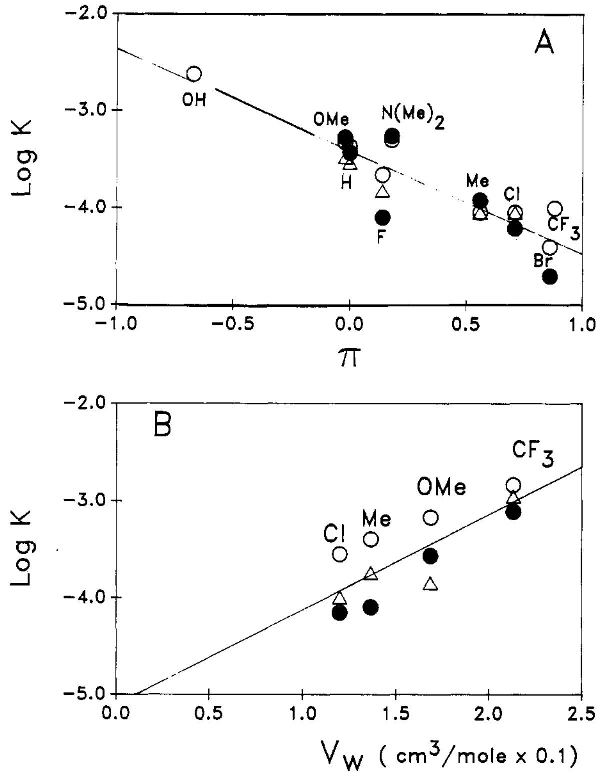


FIGURE 3: Correlation of K_s and K_d values of benzylamine binding to MAO B with substituent parameters. (A) *para*-Substituted benzylamine binding to MAO B correlated with the hydrophobicity value for the substituent: (○) K_s value from stopped-flow experiments with α,α-diprotiobenzylamine analogues; (●) K_s value from stopped-flow experiments with α,α-dideuteriobenzylamine analogues; (▲) K_d values calculated from steady-state kinetic isotope effect data as described by Klinman and Matthews (1985). (B) *meta*-Substituted benzylamine analogue binding to MAO B correlated with the van der Waals volume of the substituent. The symbols depict the K_s and K_d values as described in panel A.

k_{red}. Since the values of k_{red} for the *meta*-substituted analogues are essentially invariant (within a factor of 1.4), the rates of flavin reduction by these analogues are independent of electronic, steric, and/or hydrophobic effects of the *meta* substituent.

The best correlation for k_{red} with substituent parameter for the *para*-substituted benzylamine analogues is with E_s (the Taft steric parameter) (Figure 4A), which reflects the van der Waals radius of the first atom of the substituent. A reasonable correlation of k_{red} for the *para*-substituted analogues is also observed with V_W (the van der Waals radius of the substituent), although -NO_2 and CH_3O-substituents are outliers with the linear correlation. Note also that similar correlations are observed with α,α-dideuteriobenzylamine analogues as with the α,α-diprotio forms (Figure 4A). The deuterio *meta*-substituted analogues show little or no rate dependence on the nature of the substituent, as is also observed for the protio analogues. The *para*-substituted deuterated benzylamines also exhibit a rate dependence on the steric parameter similar to that observed with the protio analogues (Figure 4B). This similarity in correlation on deuteration of the substrate analogues suggests that the kinetic isotope effect observed in reductive half-reaction experiments approximates the intrinsic isotope effect on C–H bond cleavage.

DISCUSSION

Statistical Analysis of Structure–Activity Correlations. The single-parameter correlations show that binding of *para*-substituted benzylamine analogues to MAO B is highly correlated with the hydrophobicity parameter of the substituent in which the affinity is increased with increasing hydrophobicity (Figure 3A). The regression data in Table 4 further document this conclusion. On comparison of single-parameter fits, the correlation of π with log K_d shows the best correlation coefficient, the highest F value, and the lowest probability that the correlation is zero (significance). The F value of 76 is much greater than the 99% confidence level. The only

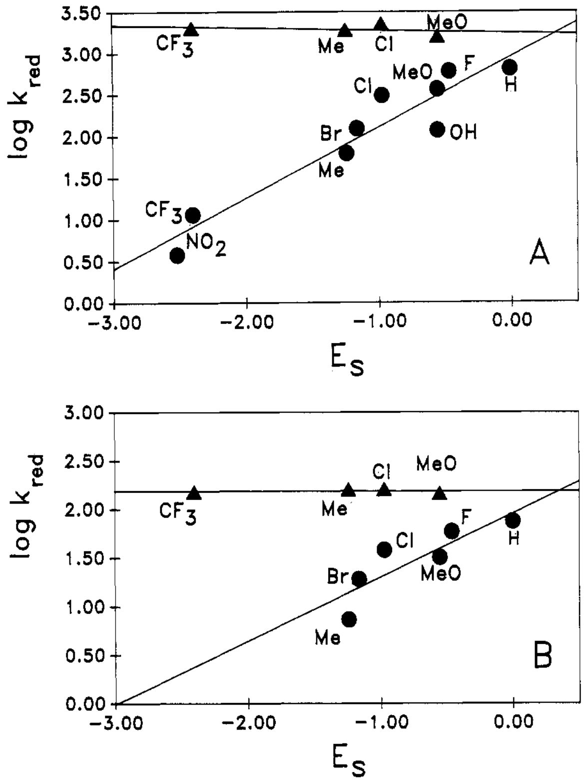


FIGURE 4: Correlation of the limiting rate of MAO B reduction with the steric parameter (E_s) of the substituent. (A) α,α-Diprotiobenzylamine analogues: (●) *para* substituents and (▲) *meta* substituents. (B) α,α-Dideuteriobenzylamine analogues: (●) *para* substituents and (▲) *meta* substituents.

Table 4: Correlation Analysis of log K_s or log K_d with Hydrophobic, Steric, and Electronic Parameters in the Interaction of *para*-Substituted Benzylamine Analogues with MAO B[a]

parameter	correlation	*y*-intercept	correlation coeff	*F* value[b]	significance[c]
π	−1.02 ± 0.12	−3.42 ± 0.06	0.79	76	2.9 × 10^{-8}
V_W	0.03 ± 0.13	−3.79 ± 0.20	0.002	0.04	0.84
σ	−0.94 ± 0.23	−3.81 ± 0.08	0.45	16.3	7 × 10^{-4}
π	−1.10 ± 0.10			124	9.3 × 10^{-10}
+		−3.61 ± 0.08	0.87		
V_W	0.17 ± 0.05			10.7	4.1 × 10^{-3}
π	−0.86 ± 0.13			42.3	3.1 × 10^{-6}
+		−3.49 ± 0.07	0.83		
σ	−0.33 ± 0.16			4.1	5.7 × 10^{-2}
V_W	−0.23 ± 0.10			5.0	3.8 × 10^{-2}
+		−3.52 ± 0.15	0.56		
σ	−1.22 ± 0.25			24.4	9.1 × 10^{-5}

[a] The analysis used K_s values determined for both protio- and deuteriobenzylamine analogues as well as K_d values calculated from steady-state kinetic data (Table 3). A total of 22 values were used. [b] The F value is the ratio of the regression sum of squares (explained by model) to the residual sum of squares (not explained by model) weighted by the number of data points and the degrees of freedom. Therefore, the higher the F value, the better the fit. [c] The significance is the probability that the model parameters are zero. Therefore, the smaller the number, the higher the confidence level in the fit.

Table 5: Correlation Analysis of log k_{red} with Hydrophobic, Steric, and Electronic Parameters in the Reduction of MAO B with *para*-Substituted Benzylamine Analogues[a]

parameter	correlation	*y*-intercept	correlation coeff	*F* value	significance
E_s	0.86 ± 0.11	2.97 ± 0.14	0.91	67	7.9×10^{-5}
V_W	−0.92 ± 0.35	3.18 ± 0.48	0.50	7.0	0.034
σ	−1.43 ± 0.57	2.20 ± 0.21	0.47	6.2	0.041
π	−0.06 ± 0.54	2.05 ± 0.31	0.002	0.01	0.91
E_s	1.02 ± 0.17				
+		3.10 ± 0.18	0.92	35	0.001
σ	0.47 ± 0.40			1.4	0.28
E_s	0.93 ± 0.07			166	1.3×10^{-5}
+		2.96 ± 0.09	0.97		
π	0.37 ± 0.11			10.3	0.018
σ	−1.57 ± 0.63			6.2	0.047
+		2.14 ± 0.24	0.51		
π	0.30 ± 0.44			0.5	0.523

[a] Correlations were performed using k_{red}(H) values from Table 3. A total of 9 values were used. The value for the *p*-$(CH_3)_2$-*N*-substituted benzylamine was not included due to lack of a reliable E_s value for that substituent. Other values are defined in Table 6.

two-parameter fit that provides a better statistical correlation is that of π and V_W (Table 4). (The number of substituents tested is too few to allow for a three-parameter fit). Note that the correlation coefficient is improved over the single-parameter correlation with π and that the *F* values and significance values also show a better fit. Therefore, the best description of *para*-substituted benzylamine binding to MAO B is one in which a small contribution from the van der Waals volume is included with π:

$$\log K_d = (-1.10 \pm 0.01)\pi + (0.17 \pm 0.05)V_W - (3.61 \pm 0.08) \quad (F_{2,19} = 61.9) \quad (8)$$

Thus, substrate binding to MAO B is largely favored by an increase in hydrophobicity of the *para* substituent; however, an increase in substituent volume will have a negative effect on binding. Similar studies on *para*-substituted benzylamine binding to bovine plasma amine oxidase (Hartmann & Klinman, 1991) also demonstrated that the correlation was highly dependent on hydrophobicity of the *para* substituent with little or no increase in statistical fit on adding the electronic parameter σ. Thus, the substrate binding site for the flavoenzyme MAO B and the quinoprotein plasma amine oxidase appear to be similar as regards *para*-substituted benzylamine analogue binding. No multiparameter statistical analysis was performed on the binding of *meta*-substituted benzylamine analogues to MAO B since data for only four substituents were obtained. The plot in Figure 3B demonstrates that a reasonable correlation of binding affinity with V_W is observed with *meta* substituents. The larger the *meta* substituent, the weaker the binding affinity.

Single-parameter and two-parameter correlations of *para*-substituted benzylamine reduction of MAO B (log k_{red}) with electronic, steric, and hydrophobic substituent parameters are shown in Table 5. For a single-parameter fit, the best correlation is observed with E_s, as is also documented in Figure 4A. The statistical regression analysis of log k_{red} with E_s provides much better fit parameters than observed with V_W, σ, or π. Thus, the substituent effect on the rate of flavin reduction can be described for the *para*-substituted benzylamine analogues as

$$\log k_{red} = (0.86 \pm 0.11)E_s + (2.97 \pm 0.14) \quad (F_{1,7} = 67) \quad (9)$$

A slightly better two-parameter fit is observed with E_s and π where

$$\log k_{red} = (0.93 \pm 0.07)E_s + (0.37 \pm 0.11)\pi + (2.96 \pm 0.09) \quad (F_{2,6} = 83.2) \quad (10)$$

The probability for the π coefficient to be zero is only 1.8%, whereas in a two-parameter fit with E_s and σ, the probability of the coefficient for $\sigma(\rho)$ being zero is 28%. Thus, the analysis suggests that the electronic contribution of $\sigma(\rho)$ to log k_{red} is negligible for the MAO B oxidation of benzylamine analogues. These results are in contrast with those from the plasma amine oxidase reaction (Hartmann & Klinman, 1992) where the rate of C–H bond cleavage is dependent on σ ($\rho = 1.47 \pm 0.27$) and a negative contribution by π. A positive ρ value is expected for the plasma amine oxidase reaction since C–H bond cleavage occurs by a H^+ abstraction from an imine intermediate formed on reaction of the primary amine with the active site 6-hydroxydopa (topa) cofactor (Janes *et al.*, 1990). In the case of MAO B, the rate of substrate C–H bond cleavage of *para*-substituted benzylamine analogues is determined mainly by steric factors and a smaller positive contribution by hydrophobic parameters. When *meta* substituents are present, their contribution to log k_{red} in the MAO B reaction is independent of the substituent parameter (Table 3 and Figure 4). The small number of *meta*-substituted benzylamine analogues tested (4) and the observed independence of the rate of MAO B reduction on the nature of that substituent (Table 3) precluded any detailed statistical treatment.

Fuller *et al.* (1968) carried out an extensive study of the effect of *meta* and *para* substituents on the I_{50} for inactivation of rat liver mitochondrial MAO by a series of *N*-(phenoxyethyl)cyclopropylamine analogues. While direct comparison of their data to the results presented here is difficult since rat liver mitochondria contain equal amounts of MAO A and MAO B (Singer & Ramsay, 1993), Fuller *et al.* (1968) found that substituents in the *meta* position had a deleterious effect on inhibition presumably due to steric effects. Kutter and Hansch (1969) found that the data of Fuller *et al.* (1968) could be fit by an equation with significant contributions from E_s and σ and lesser contributions from π. Since it is not known what substituent effects will contribute to the interaction of MAO A with substrates and/or mechanism-based inhibitors, a detailed reinvestigation of the interaction of purified MAO A and of MAO B with this series of cyclopropylamine analogues would be of interest. Although structure–activity effects on the I_{50} for MAO inactivation by these *N*-(phenoxyethyl)cyclopropylamine analogues are not directly comparable to the substituent effects on benzylamine analogue binding to MAO B, the results of Fuller *et al.* (1968) do support one of the conclusions from this study in that *meta*-substituted analogues do interact with MAO B (and possibly MAO A) in a manner different from *para*-substituted analogues.

Applicability of the Structure–Activity Data on MAO B to the Proposed Aminium Cation Radical Mechanism for MAO B Catalysis. The proposed radical mechanism for MAO B-catalyzed amine oxidation was first put forth by Silverman and co-workers (Silverman *et al.*, 1980) and has been further supported by mechanism-based inhibitor studies in his laboratory during the intervening period until the present. This mechanism is shown in Scheme 2 and involves an initial electron abstraction from the amine lone pair, followed by a H^+ abstraction from the aminium cation radical intermediate, with subsequent α-carbon-based substrate radical formation and electron transfer to either the flavin coenzyme (path A) or an amino acid residue (with subsequent electron transfer to the flavin semiquinone) (path B) to form a flavin hydroquinone and an imine product. For the purposes of discussion of the proposed mechanism in Scheme 2 in the context of the data presented in this study, it is helpful to summarize the substituent effects on the rate of benzylamine oxidation by MAO B.

Both *para*- and *meta*-substituted benzylamines exhibit large deuterium kinetic isotope effects on k_{cat} or on k_{red}. The magnitude of ^{D}k is independent of the nature of the substituent, although $^{D}k_{red}$ values are larger for *meta* substituents than for *para* substituents. Model system studies of aminium cation radical deprotonation (Parker & Tilset, 1991; Xu *et al.*, 1991; Dinnocenzo & Banach, 1989) have also observed reasonably large kinetic isotope effects with the magnitude dependent on the nature of the abstracting base and on the nature of the *para* substituent. Thus, the observation of large ^{D}k values with MAO B does not disagree with the proposed mechanism in Scheme 2 but should also not be construed as support since other model amine oxidation reactions [e.g., the permanganate oxidation of benzylamine (Wei & Stewart, 1966)] also exhibit large deuterium kinetic isotope effects.

Stereoelectronic effects in model amine oxidations have been observed in H^+ abstraction from aminium cation radicals (Lewis, 1986) and in $H^{\bullet}$ abstraction reactions of amine α-C–H bonds by oxy radicals and the benzophenone triplet (Griller *et al.*, 1981). In both situations, α-C–H bond cleavage is promoted by overlap of the α-C–H bond with the amine nitrogen lone pair orbital. If C–H bond cleavage in MAO B catalysis were to occur by either mechanism, the rate would be influenced by the ability of the substrate to bind to the active site in a conformation facilitating maximal eclipsing of the *pro-R*-α-C–H (Yu *et al.*, 1986) with the amine lone pair nitrogen. The rate correlations shown in Figure 4 and Table 5 document that enzyme reduction rates are highly correlated with the E_s parameter for *para* substitutents, while the rate of enzyme reduction is independent of any parameter for the *meta* substituents. One interpretation of these results is that *meta* substitution results in the binding of the substrate in the active site in a conformation resulting in favorable overlap of the *pro-R*-α-C–H with the amine lone pair orbital. In the case of *para*-substituted substrate analogues, the nature of the *para* substituent appears to alter the binding of the substrate such that either nonproductive complexes are formed or the conformation of the substrate in the active site which could retard optimal orientation of the α-C–H with the nitrogen lone pair. Since no detailed structural information is currently available for MAO B, verification of this interpretation will ultimately require the X-ray structural determination of the enzyme with substrate analogues bound to the active site.

Our failure to observe the intermediate formation of flavin radical intermediates for any of the substrate analogues examined in reductive half-reaction kinetics experiments (Figure 1) provides, on the surface, contradictory evidence for the mechanism in Scheme 2. Simpson *et al.* (1982) have suggested that the initial electron-transfer step to form the flavin radical and the substrate aminium cation radical may be reversible and that the equilibrium constant may be unfavorable for observable radical formation in the enzyme-catalyzed reaction. In support of this notion are the unfavorable redox potentials for the electron-transfer step. The one-electron oxidation potentials of primary amines are in the range of ~1.5 V vs NHE (Barnes & Mann, 1967), while the one-electron reduction of the covalent flavin in MAO B has not been determined but, on the basis of analogy with other flavoenzymes (Stankovich, 1991), should be in the range of 0–0.2 V (versus NHE). The redox potential of the 6-*S*-cysteinyl-FMN of trimethylamine dehydrogenase has been shown to have a Fl_{ox}/Fl_{sq} redox potential of 0.04 V (Barber *et al.*, 1988) and is further increased to 0.23 V on binding tetramethylammonium chloride (Stankovich, 1991). A recent estimate of the Fl_{ox}/Fl_{sq} couple of MAO B has been reported to be 0.045 V (Ramsay *et al.*, 1993). The free energy difference (excluding any major changes in potential on enzyme binding) is estimated to be in the range of 21–34.5 kcal/mol, and therefore the equilibrium constant calculated for a reversible one-electron transfer between amine substrate and flavin would be in the range of 10^{15}–10^{25} in the direction of the reactants. By comparison, the redox potential for the iron–oxo species involved in one-electron oxidation of dimethylanilines in cytochrome P-450-catalyzed *N*-dealkylation reactions has been estimated to be 1.9–2.2 V (MacDonald *et al.*, 1989). Thus, redox potential considerations make one-electron oxidation of the amine by the MAO flavin very unlikely. Surdhar and Armstrong (1987) have directly measured the rate of the electron transfer from the neutral lumiflavin radical to piperazine aminium cation radical by pulse radiolysis and found the rate to be extremely fast ($k_{2nd\ order} = 2.7 \times 10^{9}$ $M^{-1}\ s^{-1}$) . Thus, the model flavin radical–aminium cation radical reaction shows the amine radical to be an effective *oxidizing* agent as also suggested by the above thermodynamic arguments.

If one of the precatalytic equilibria is energetically unfavorable as suggested above, the data presented here might provide some insight into the validity of that hypothesis. The kinetic isotope effect data determined for this series of benzylamine analogues can be used to provide information regarding equilibria preceding catalyses in enzyme systems (Northrop, 1981; Palcic & Klinman, 1983). For the enzyme mechanism involving multiple precatalytic equilibria,

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{{}^{*}k_7}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \xrightarrow{k_{11}} E_7 \xrightarrow{k_{13}} E_8 \tag{11}$$

where $^{*}k_7$ is the isotope-sensitive step and k_{11} and k_{13} are irreversible product release steps. The value for $k_8 \ll k_7$ from analysis of the reductive half-reaction stopped-flow data (see above).

The expression given by Northrop (1981) for ^{D}V for the above mechanism is

$$^{D}V = \frac{\frac{k_7\text{H}}{k_7\text{D}} + \frac{R_f}{E_f} + {}^{D}K_{eq}C_r}{1 + \frac{R_f}{E_f} + C_r} \tag{12}$$

where

$$R_f = \frac{k_7\text{H}}{k_5}\left(1 + \frac{k_4 + k_5}{k_3}\right) + \frac{k_7\text{H}}{k_{11}}\left(1 + \frac{k_{10} + k_{11}}{k_9}\right) + \frac{k_7\text{H}}{k_{13}} \tag{13}$$

$$E_f = 1 + \frac{k_6}{k_5}\left(1 + \frac{k_4}{k_3}\right) \tag{14}$$

If the precatalytic equilibrium were energetically unfavorable such that the $E_3 \rightleftharpoons E_4$ equilibrium were to lie far in the direction of E_3 as discussed above for the mechanism in Scheme 2, then

Table 6: Calculated Values for k_7/E_f and Their Ratios with k_{red} for Benzylamine Analogue Interaction with MAO B

substituent	k_7/E_f (min^{-1})[a]	$k_{red}/(k_7/E_f)$ [b]
H	809	0.9
p-MeO	316	1.2
p-Me	60	1.0
p-F	544	1.1
p-Cl	220	1.4
p-Br	70	1.8
m-MeO	1426	1.1
m-Me	1704	1.1
m-Cl	1921	1.2
m-CF_3	1907	1.1

[a] Calculated using the equation $k_7/E_f = k_{cat}(^{D}k - 1)/(^{D}k_{cat} - 1)$, where ^{D}k is assumed to be equal to $^{D}k_{red}$ (Table 3). $^{D}k_{cat}$ values are taken from Table 2; k_{cat} values, from Table 1. [b] k_{red} values are taken from Table 3.

k_6/k_5 might approximate 10^{15}–10^{25} and E_f would become a very large number. The term R_f/E_f would then be expected to be a small number and to have little influence on the value of ^{D}V. Palcic and Klinman (1983) have shown that kinetic isotope effect data can lead to the calculation of the microscopic rate constant for C–H bond cleavage according to the following equation using the mechanistic scheme of eq 12:

$$\frac{k_7}{E_f} = \frac{k_{cat}(^{D}k - 1)}{(^{D}k_{cat} - 1)} \qquad (15)$$

If E_f is a large number as discussed above, then $[k_{cat}(^{D}k - 1)]/(^{D}k_{cat} - 1)$ should also be a very small number.

Recent $^{H/T}k$ and $^{D/T}k$ values for *p*-MeO-benzylamine oxidation by MAO B (Jonsson *et al.*, 1993) show that the intrinsic isotope effect for C–H bond cleavage for this analogue is identical to the value determined by stopped flow (Table 3). From these data for the *p*-MeO-benzylamine analogue (Tables 1, 2, and 3) and eq 15, k_7/E_f is calculated to be 316 min^{-1} as compared to the limiting rate of k_{red} of 365 min^{-1} observed in stopped flow experiments. These considerations show that the value for the rate of flavin reduction is similar (within experimental uncertainty) to the value for the intrinsic rate of *pro-R-α*-C–H bond cleavage. Thus, the value of E_f is approximately unity. Therefore, the hypothesis that $k_5 \gg k_6$ is clearly not compatible with these considerations and cannot be the explanation for lack of observance of any flavin radical intermediates in stopped-flow experiments as being due to an energetically unfavorable electron-transfer equilibrium prior to C–H bond cleavage.

With the assumption that $^{D}k_{red}$ values (Table 3) approximate the intrinsic kinetic isotope effect values, calculations of k_7/E_f for all of the *para*- and *meta*-substituted benzylamine analogues used in this study show a close correspondence with k_{red} values determined for enzyme reduction (Table 6). This correspondence holds over a 40-fold range in rate of enzyme reduction with only *p*-Br- and *p*-Cl-benzylamine analogues exhibiting ratios of $k_{red}/(k_7/E_f) > 1$ (Table 6).

A requirement for the thermodynamic (but not necessarily kinetic) permissibility of an electron-transfer mechanism as shown in Scheme 2 is that $\Delta G^{\ddagger} > \Delta G_{et}$ (Dinnocenzo *et al.*, 1993). The $\Delta G^{\ddagger}$ value calculated for the limiting reduction rate of MAO B by the *meta*-substituted benzylamine analogues using the Eyering equation (Figure 4) is 12.3 kcal/mol, whereas the ΔG_{et} (see above) calculated from the difference in flavin–amine one-electron couples is in the range of 20–30 kcal/mol. Thus, the hypothesis of single-electron transfer from the amine to the flavin appears thermodynamically and kinetically improbable even with allowances for alterations in potential on binding to the enzyme. Substituents in the *para* and *meta* positions of *N,N*-dimethylbenzylamine analogues have been shown by Hull *et al.* (1969) to influence linearly the amine potential by 0.14 V/1σ unit. The substituent-induced shifts in one-electron potentials of primary benzylamines used in this study are expected to be altered by ~10%. The failure to observe any electronic contribution to the rate of enzyme reduction suggests this alteration in amine one-electron potential has little influence on the rates observed with the analogues tested. More notable shifts in amine one-electron potentials are found on alkylating the amine nitrogen to form 2° or 3° amines. Thus, *N,N*-dimethylbenzylamine has a potential ~0.5 V lower than that of benzylamine (Hull *et al.*, 1969) but is not a substrate for MAO B (although this amine analogue is a reasonable competitive inhibitor with benzylamine) (A. K. Bhattacharyya and D. E. Edmondson, unpublished data). This observation is also inconsistent with the mechanism in Scheme 2, which would predict MAO B to oxidize tertiary amines at a faster rate than primary amines. In this respect, it should be noted that MAO is capable of oxidizing cyclic tertiary amines such as MPTP (Ottoboni *et al.*, 1989) at quite reasonable rates of enzyme reduction ($k = 3.7$ s^{-1}) (Ramsay *et al.*, 1987). Thus, the apparent inability of MAO B to oxidize a structurally related *N,N*-dimethylbenzylamine suggests that steric effects may contribute. In accord with the results in this paper, *meta*-substituted *N,N*-dimethylbenzylamine analogues are substrates, albeit poor ones (k_{cat} = 6 min^{-1}), for MAO B (A. K. Bhattacharyya and D. E. Edmondson, manuscript in preparation), while *para* substitution results in analogues that are not substrates. Thus, the expected benefit of lowering the potential of the amine for one-electron transfer by methylation which would lead to an increased rate of oxidation is not experimentally observed under conditions where steric influences are minimized.

These considerations suggest that the proposed mechanism for MAO B catalysis as outlined in Scheme 2 is not supported by analysis of the kinetic data currently available. Thus, other possible mechanisms of C–H bond *cleavage* should be considered. The involvement of radicals in the catalytic mechanism of MAO B is strongly suggested from the work of Silverman and his colleagues (Silverman, 1991). Model system studies of primary amine oxidation by ClO_2 demonstrate that H$^{\bullet}$ abstraction occurs in addition to an electron-abstraction mechanism (Hull *et al.*, 1967). The observation of little or no correlation of the rate of MAO reduction with substituent electronic effects (Table 6) is supportive but not necessarily definitive evidence for a H$^{\bullet}$ mechanism in MAO B catalysis. For a H$^{\bullet}$ mechanism to be operative, an acceptor for H$^{\bullet}$ (which we will refer to as X) would be involved such that the bond dissociation energy of XH would have to be greater than the bond dissociation energy (75 kcal/mol) of the benzyl hydrogens of benzylamine (Cottrell, 1958). At present, no evidence for such a species (or such a reaction intermediate) capable of H$^{\bullet}$ abstraction in MAO B has been found. The oxidized flavin cofactor does not have the reactivity for H$^{\bullet}$ abstraction in its ground state. Amino acid radicals would be possible candidates and have been identified in other enzyme systems (e.g., ribonucleotide reductase; Stubbe, 1990) operating *via* a H$^{\bullet}$-abstraction mechanism. No definitive evidence is available for the presence of an amino acid radical in MAO B, although a recent spectroscopic study of the enzyme in its oxidized and anionic semiquinone forms (Yue *et al.*, 1993) is suggestive of the presence of a paramagnetic species in addition to the flavin radical. Further work on this aspect of MAO B structure–function is under investigation in this laboratory.

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