Structure−Activity Relations in the Oxidation of Phenethylamine Analogues by Recombinant Human Liver Monoamine Oxidase A[†]

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ABSTRACT: The interaction of recombinant human liver monoamine oxidase A (MAO A) with a series of phenethylamine substrate analogues has been investigated by steady-state and stopped-flow kinetic techniques. Substrate analogues with para substituents exhibit large deuterium kinetic isotope effect on k_{cat}, on k_{cat}/K_m, and on the limiting rate of enzyme reduction in reductive half-reaction experiments. These kinetic isotope effect values range from 5 to 10 with the exception of tyramine, which exhibited smaller steady-state isotope effects (2.3−3.5) than that observed on the rate of flavin reduction (6.9). The stopped-flow data show that imine release from the reduced enzyme is slower than the rate of catalytic turnover. Phenethylamine oxidation by MAO A can be described as the C−H bond cleavage step being rate limiting in catalysis and with oxygen reacting with the reduced enzyme−imine complex. In the case of tyramine, the product release from the oxidized enzyme−imine complex contributes to the rate limitation in catalysis. The binding affinities of a series of para-substituted phenethylamine analogues to MAO A show an increase in affinity of the deprotonated amine with increasing van der Waals volume of the substituent. The limiting rate of enzyme reduction decreases with increasing van der Waals volume of the substituent in a linear manner with no observable electronic contribution as observed previously with benzylamine reduction of MAO A [Miller, J. R., and Edmondson, D. E. (1999) *Biochemistry 38,* 13670−13683]. Examination of side chain analogues of phenethylamine show 3-phenylpropylamine to be oxidized 2.5-fold more slowly and bound 75-fold more tightly than phenethylamine. 4-Phenylbutylamine is not a substrate for MAO A but is a good competitive inhibitor with a K_i value of 31 ± 5 μM. Analysis of the effect of alkyl side chain alterations on binding affinities of a series of arylalkylamine analogues taken from this study and from the literature show a linear correlation with the Taft steric value (E_s) of the side chain. These results suggest that the binding site for the aryl ring is identical for phenethylamine and for benzylamine analogues and that steric interactions of the alkyl side chain with the enzyme strongly contribute to the binding affinities of a series of reversible inhibitors of MAO A.

Recent studies from this laboratory have probed the structures of the substrate binding sites and the mechanisms of catalysis of monoamine oxidases A and B (MAO A and B, EC 1.4.3.4) using quantitative structure−activity relationship (QSAR)[1] approaches (*1*, *2*). These structure−activity relationships are based on measurements of the binding affinities and the rates of covalent flavin reduction of these enzymes using a series of para-substituted benzylamine analogues. The results from these studies have demonstrated that an increase in the size of the para substituent increases the analogue binding affinity in MAO A while the hydrophobicity of the substituent facilitates binding in MAO B. The rate of flavin reduction of MAO A shows a clear correlation with the electron-withdrawing ability of the para substituent, therefore suggesting that C−H bond cleavage occurs by a H^+ abstraction mechanism (*2*). No detectable electronic effects are observable in reductive half-reaction studies of MAO B (*1*), which have been interpreted to be a result of the orientation of the bound substrate in a manner that suppresses the transmission of electronic effects from the para position (*2*). These studies have identified defined distinct differences between these two enzymes although they share ∼70% sequence identities (*3*) that may be valuable in the development of specific inhibitors possessing clinical relevance.

To further investigate the catalytic mechanism of MAO A that is known to oxidize neurotransmitters such as serotonin and dopamine, we initiated a study to investigate the effect of para substitution of phenethylamine analogues on MAO A catalysis and compare directly with previous data on benzylamine analogues. The rationale for this approach is that the natural substrates for MAO A contain ethylamine side chains attached to an aromatic ring; thus, the results from phenethylamine may provide additional insights into our understanding of the specificity of binding and the mechanism of oxidation of the above neurotransmitters. In addition, it is of interest to determine if the observations and predictions of the benzylamine data with MAO A could be

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[1] Abbreviations: QSAR, quantitative structure−activity relationships; MAO A, monoamine oxidase A; MAO B, monoamine oxidase B.

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extended to the phenethylamine class of substrate analogues. Previous studies with MAO B (*4*) have shown that phenethylamine reduces the enzyme-bound flavin 50-fold faster than the rate observed with benzylamine and that the imine-reduced enzyme complex readily dissociates so that the free reduced enzyme is the species that reacts with O_2 in a step that is rate limiting in catalysis. In addition, it was shown that ^Dk for MAO B reduction for phenethylamine is considerably lower than that of benzylamine, which suggests some alterations in transition-state structure may occur simply by increasing the side chain by one methylene group.

With these observed differences between the kinetic properties of benzylamine oxidation and phenethylamine oxidation with MAO B (*4*), it is of interest to determine if similar differences are observed with MAO A. The results presented in this paper on the steady-state and reductive half-reaction kinetic studies of MAO A oxidation of a series of para-substituted phenethylamine analogues provide additional insights into the specificity of substrate binding and the mechanism of the enzyme. In addition to para-substituted phenethylamine analogues, we have also used 3-phenylpropylamine and 4-phenylbutylamine as substrate/inhibitor analogues to asses the role of alkyl side chain in analogue binding and in the oxidation of these analogues by MAO A. The relevance of these results in the prediction of compounds that could be developed as high-affinity reversible inhibitors of MAO A is discussed.

EXPERIMENTAL PROCEDURES

Expression and Purification of Monoamine Oxidase A. Recombinant human liver MAO A was expressed in *Saccharomyces cerevisiae* (RH218) and purified as described previously (*2*). Typical enzyme yields from a 24-L yeast culture was about 100 mg of pure MAO A with a specific activity of 1.5 units/mg. *d*-Amphetamine, a competitive inhibitor that stabilizes the enzyme during purification, was removed prior to all kinetic measurements.

This procedure involved dilution of the stock enzyme sample 10-fold with cold 50 mM potassium phosphate buffer (pH 7.5) containing 0.8% (w/v) octyl glucoside, followed by 10-fold concentration using an Amicon ultrafiltration cell (PM30 membrane). The dilution and concentration cycle was repeated twice more, followed by addition of buffer to give appropriate enzyme concentration.

Steady-State Assay Procedures. Standard enzyme assays were performed at 30 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 5 mg/mL reduced Triton X-100 using kynuramine as a substrate. Enzyme-catalyzed kynuramine oxidation is followed spectrophotometrically by the increase in absorption at 316 nm (absorption maximum of the product 4-hydroxyquinoline, extinction coefficient 12 600 M^{-1} cm^{-1}) (*5*). One unit of the enzyme activity is defined as the amount of enzyme needed to oxidize 1 μmol of kynuramine to 4-hydroxyquinoline in 1 min under standard assay conditions.

Phenethylamine Analogues. The following para-substituted phenethylamine analogues were purchased from Aldrich: *p*-H, *p*-NO_2-, *p*-OMe-, *p*-Me-, *p*-Cl-, *p*-F-, and *p*-OH (tyramine). The analogues 3-phenylpropylamine and 4-phenylbutylamine were also purchased from Aldrich and converted to their HCl salts by bubbling HCl though a cold diethyl ether solution of the amine. *p*-Br- and *p*-CF_3-phenethylamines were synthesized by reduction of the corresponding benzylnitriles (purchased from Aldrich) with $LiAlH_4$ in dry diethyl ether (*6*). *p-tert*-Butylphenethylamine was synthesized by converting the commercially available benzylbromide to the nitrile (*7*) followed by reduction with $LiAlH_4$. The α,α-dideuterophenethylamine analogues used in this study were synthesized by reduction of the corresponding benzylnitriles in dry diethyl ether with $LiAlD_4$ (obtained from Sigma). α,α-Dideutero-*p*-NO_2-phenethylamine was synthesized by reduction of the nitrile with $NaBD_4/BF_3$ (*8*). α,α-Dideutero-*p*-OH-phenethylamine was synthesized by reduction of the *p*-methoxybenzylnitrile with $LiAlD_4$ and subsequent cleavage of the *p*-methoxy ether in concentrated HCl (*9*). All the phenethylamine analogues were recrystallized as their hydrochloride salts from ethanol. The purity and structures of the analogues were confirmed by spectral analysis using MS and ^1H NMR. No detectable α-hydrogens are observed in the deuterophenethylamine analogues demonstrating >95% ^2H substitution.

Steady-State Kinetic Studies of p-Substituted Phenethylamine Analogues. All steady-state kinetic experiments were performed at 11 °C in 50 mM potassium phosphate buffer containing 0.8% (w/v) octyl glucoside. The rates of phenethylamine oxidation by MAO A were measured by monitoring the amount of hydrogen peroxide generated in the reaction by a peroxidase-coupled assay system as described by Holt et al. (*10*). All the steady-state kinetic experiments were performed using a Gilford update of a Beckmann DU spectrophotometer. Oxygen uptake studies were performed polarographically using a Yellow Springs Oxygraph interfaced to a Nicolet 4094 digital oscilloscope for acquiring digital output of the rate data, which were then transferred to a PC for data analysis using the program Enzfitter.

Single-Wavelength Stopped-Flow Kinetic Experiments. Anaerobic stopped-flow kinetic experiments were performed on a single-wavelength stopped-flow apparatus (Kinetic Instruments, Ann Arbor, MI) with a measured deadtime of 1 ms. The stopped-flow apparatus is equipped with a 2-cm path length cell, and the time-dependent absorption changes were recorded on a Nicolet 4094 digital oscilloscope. The instrument was made anaerobic by incubating the entire flow line of the apparatus with argon-purged anaerobic buffer containing 50 mM glucose, 22 nM glucose oxidase, and 100 U/mL catalase for 2−3 h prior to the experiments.

Anaerobic solutions of MAO A and substrate analogues were prepared in tonometers by repeated degassing and purging with oxygen-free argon that had been purified by passage through a heated column of copper chromite catalyst. All the solutions contained 22 nM glucose oxidase, 50 mM glucose, and 100 U/mL catalase to remove any residual traces of oxygen. The rate of MAO A reduction was monitored by absorption changes at 450 nm under pseudo first-order conditions ([MAO A] ≪ [substrate]). The digital data files were transferred to a Dell 386 computer for subsequent analysis. Reaction traces were analyzed according single- or double-exponential rate equations with offset using the GRAFIT graphing program.

Rapid-Scanning Stopped-Flow Kinetic Measurements. Time-dependent changes in absorption spectra upon mixing anaerobic solutions of MAO A and tyramine were obtained using an OLIS-RSM 1000 stopped-flow spectrophotometer

Table 1: Steady-State Kinetic Parameters for Oxidation of para-Substituted Phenethylamines by MAO A (WT) at 11 °C

	proteo		deutero		isotope effects		
substituent	k_{cat} (min^{-1})	K_m (μM)	k_{cat} (min^{-1})	K_m (μM)	$^Dk_{cat}$	$^D(k_{cat}/K_m)$	K_d [b] (μM)
H	64 ± 1.2	1250 ± 69	7.55 ± 0.26	1410 ± 170	8.5 ± 0.3	9.6 ± 1.3	1433 ± 216
OH	83.7 ± 1.9	423 ± 42	35.8 ± 0.6	633 ± 42	2.3 ± 0.1	3.5 ± 0.4	813 ± 128
CF_3	4.5 ± 0.1	755 ± 54	0.71 ± 0.03	1190 ± 120	6.3 ± 0.3	9.9 ± 1.3	1268 ± 199
F	111.5 ± 1.8	1060 ± 55	21.1 ± 0.3	2050 ± 75	5.3 ± 0.1	10.2 ± 0.7	2268 ± 2 00
Cl	26.6 ± 0.4	320 ± 20	3.45 ± 0.07	380 ± 28	7.7 ± 0.2	9.2 ± 0.9	392 ± 47
Br	17.1 ± 0.5	226 ± 22	2.06 ± 0.04	260 ± 20	8.3 ± 0.3	9.6 ± 1.2	266 ± 43
Me	18.6 ± 0.3	150 ± 8	2.05 ± 0.02	152 ± 7	9.1 ± 0.2	9.2 ± 0.7	152 ± 15
NO_2	200 ± 3.1	4820 ± 200	38.3 ± 1.8	8270 ± 910	5.2 ± 0.3	9.0 ± 1.1	9181 ± 1298
MeO[a]							52.3 ± 8.3
EtO[a]							80.6 ± 6.9
tert-butyl[a]							195 ± 13.3

[a] Values indicated are K_i values determined using *p*-CF_3-benzylamine as a competitive substrate analogue. [b] Calculated from steady-state kinetic isotope effect data (*15*).

(Olis, Inc., Bogart, GA) in the laboratory of W. David Wilson (Department of Chemistry, Georgia State University, Atlanta, GA). Kinetic and spectral data were analyzed using software provided by OLIS.

Data Analysis. Steady-state kinetic parameters were obtained by analyzing the steady-state kinetic data according to Michaelis−Menten equation using Enzfitter. Determination of limiting rates for the reductive half-reaction of MAO A and dissociation constants for each amine substrate were performed as described by Strickland (*11*). All kinetic data were analyzed using approaches described previously (*1, 2*) for both steady-state and pre-steady-state data. Values for the substituent parameters (σ, π, and E_s) were obtained from Hansch et al. (*12*). Values for the van der Waals volumes (V_w) of each substituent were calculated as described by Bondi (*13*). Multivariate linear regression analysis of rate and binding data with substituent parameter were performed using the Statview software package (Abacus Concepts).

RESULTS

Steady-State Kinetic Data for MAO A Oxidation of Phenethylamine Analogues. The steady-state kinetic parameters for the MAO A catalyzed oxidation of eight para-substituted phenethylamine analogues were determined, and the respective values for k_{cat} and K_m are shown in Table 1. Those analogues tested which function as substrates exhibit Michaelis−Menten behavior with the $[O_2]$ at air saturation. No increase in rate is observed at higher concentrations of O_2. When the reaction was followed polarographically by monitoring O_2 consumption, the rates were linear for greater than 80% of the trace at saturating phenethylamine concentrations, which demonstrates that the K_m $[O_2]$ is much lower than the O_2 concentration at air saturation (240 μM). The k_{cat} values for the analogues tested range from a minimum of 4.5 min^{-1} for the *p*-CF_3 analogue to maximum of 200 min^{-1} for the *p*-NO_2 analogue. All analogues tested exhibit measurable $^Dk_{cat}$ and $^D(k_{cat}/K_m)$ values (Table 1). Isotope effects on k_{cat} range from a low of 2.3 for the *p*-OH analogue to a maximum of 9.1. With the exception of the of *p*-OH analogue, MAO A oxidation of the phenethylamine analogues is characterized by $^D(k_{cat}/K_m)$ values in the range of 9−10. High $^Dk_{cat}$ and $^D(k_{cat}/K_m)$ values are also observed for the MAO A-catalyzed oxidation of benzylamine analogues (*2*).

Three analogues in which the para substituent has a large van der Waals volume (MeO, EtO, and *tert*-butyl) exhibit no detectable activity as substrates for MAO A. They are, however, bound to the active site of the enzyme and function as competitive inhibitors of *p*-CF_3-benzylamine oxidation by MAO A with K_i values in the 50−200 μM range (Table 1). These data demonstrate that, for the phenethylamine class of substrate analogues, para substituents exhibit a major role in catalytic turnover and binding affinity.

Stopped-Flow Kinetic Studies of para-Substituted Phenethylamine Analogue Oxidation by MAO A. To provide a more detailed insight into the rate of C−H bond cleavage for this class of amine analogues, reductive half-reaction studies were carried out under anaerobic conditions in the stopped-flow apparatus as described previously (*2*), and the data were analyzed in accord with the methods published by Strickland et al. (*11*):

$$E + S \underset{K_S}{\rightleftharpoons} ES \xrightarrow{k_3} E_rP \qquad (1)$$

Enzyme reduction rates (k_{obs}) were determined under pseudo-first-order conditions ([amine] ≫ [MAO A]) by following the rate of enzyme-bound flavin reduction at 450 nm with time at varying concentrations of the amine analogue.

In contrast to previous data on the reduction of MAO A by benzylamine analogues that exhibited only monophasic kinetic behavior (*2*), the decrease in flavin absorbance occurs in a biphasic manner for most of the phenethylamine analogues tested. Figure 1 shows the time-dependent spectral changes in the visible region on mixing MAO A with tyramine in a rapid-scanning stopped-flow spectrophotometer under anaerobic conditions. The inset shows the time-dependent change in absorption at 450 nm, which exhibits biphasic kinetic behavior. The faster phase accounts for the majority of the magnitude of the spectral change, and the rate of this phase is hyperbolically dependent on the substrate concentration. In contrast, the slower phase, which represents about 25% of the total spectral change, is independent of substrate concentration and is in the range of 4−9 min^{-1} for all of the analogues tested. This slow phase is not observed at low concentrations of substrate where the observed rate of flavin reduction is slower and therefore is similar in rate, which precludes its observation. The spectral changes in the slow kinetic phase are monotonic. These data show that the slow phase is not due to intermolecular reduction of any nonfunctional enzyme by functional MAO A since the spectral changes associated with the slow phase do not

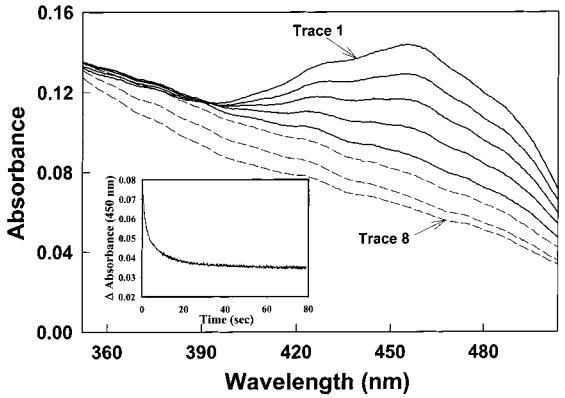


FIGURE 1: Rapid-scanning stopped-flow spectra for the reaction of MAO A with tyramine under anaerobic conditions. The spectra were obtained upon mixing 5 μM MAO A with 5 mM tyramine (after mixing concentrations). The solid lines (traces 1−5) represent the fast phase, and the broken lines (traces 6−8) represent the slow phase. The time courses for traces 1−8 are as follows: 1, 66, 130, 259, 549, 1195, 3775, and 9679 ms, respectively. Inset: The time-dependent absorption changes at 450 nm for the reaction of 2.5 μM MAO A with 5 mM tyramine in a single-wavelength stopped-flow spectrophotometer. The solid smooth line is the best fit of the experimental data according to the two-exponential rate equation.

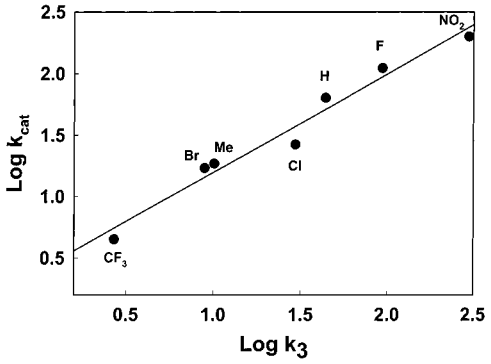


FIGURE 2: Linear correlation of turnover rate (k_{cat}) and rate of MAO A reduction (k_3) for the MAO A-catalyzed oxidation of phenethylamine analogues.

exhibit those of an oxidized to reduced flavin conversion. The spectral changes also do not exhibit any characteristics of the intermediate formation of any anionic or neutral flavin semiquinone during the reductive half-reaction. No kinetic isotope effect is observable on this slow phase since the slow rate of flavin reduction with α,α-dideuterophenethylamines preclude its direct observation. These data can be explained in the following manner. The fast kinetic phase is attributed to the reduction of the enzyme-bound flavin by the substrate as depicted in eq 1. The origin of the observed slow kinetic phase is suggested to be due to the dissociation of the imine product from the reduced form of the enzyme. The rate of this phase is slower than catalytic turnover (Table 1), which demonstrates that it not to be catalytically significant. Similar types of spectral changes in the 450-nm region due to perturbation of the absorption spectrum of the flavin hydroquinone by the imine product have been observed previously in reductive half-reaction experiments with bovine liver MAO B using *p*-*N*,*N*-dimethylamino-*N*-methylbenzylamine as substrate (*14*), which was also interpreted to be due to the dissociation of the imine product from the reduced enzyme. These data also suggest that the mechanistic pathway of catalytic turnover of MAO A using phenethylamine analogues as substrates follows a pathway where oxygen reacts with the reduced enzyme−imine complex rather than the free, reduced form of the enzyme in contrast with previous conclusions from studies on the oxidation of phenethylamine by bovine liver MAO B (*4*). For the balance of this paper, only the rate data for the rapid phase of the reaction are used for QSAR analysis.

The kinetic data for substrate reduction of MAO A are shown in Table 2 and include the limiting rate of flavin reduction (k_3) and the binding constant of the analogue to the enzyme by its K_s value (*11*). K_s values for both proteo and deutero substrate analogues provide a measure of substrate analogue binding affinities and compare well with the K_d values calculated from the steady-state kinetic isotope effect data (*15*) (Table 1).

The limiting rates of flavin reduction are (with the exception of the *p*-OH analogue) 1.7-fold lower than the corresponding turnover values (Tables 1 and 2) as observed previously using benzylamine analogues as substrates (*2*). These rate differences are due to the higher enzyme concentrations required for stopped-flow experiments and the decrease in rate with increased enzyme concentrations as observed previously (*2*). For this reason, all stopped-flow experiments were performed at a constant MAO A concentration (5 μM) to avoid any artifactual differences in rate that would arise from comparing rates determined with different enzyme concentrations. The reductive half-reaction rates follow a similar behavior as the k_{cat} values for the different analogues. This is demonstrated in Figure 2 where a linear correlation is observed between k_{cat} and k_{red} for all of the phenethylamine analogues tested with the exception of tyramine in which the rate-limiting step is proposed to be product release (see below). Deuterium kinetic isotope effect data on k_{red} show values ranging from 5.2 to 8.8, which compare well with those determined from steady-state measurements and provide direct evidence for the reduction of the covalent flavin by the substrate analogues as being

Table 2: Kinetic Parameters for the Anaerobic Reduction of MAO A (WT) by para-Substituted Phenethylamines at 11 °C

	proteo		deutero		isotope effects	
substituent	k_{red} (min^{-1})	K_s (μM)	k_{red} (min^{-1})	K_s (μM)	$^{D}k_{red}$	$^{D}(k_{red}/K_s)$
H	43.8 ± 3.7	1660 ± 430	6.00 ± 0.3	1370 ± 194	7.3 ± 0.7	6.0 ± 1.8
OH	168 ± 10	1930 ± 250	24.3± 0.3	760 ± 24	6.9 ± 0.4	2.72 ± 0.4
CF_3	2.7 ± 0.2	460 ± 97	0.31 ± 0.08	nd[a]	8.65 ± 2.3	nd
F	93.6 ± 4.7	1450 ± 230	10.7 ± 0.5	1520 ± 200	8.75 ± 0.6	9.2 ± 1.2
Cl	29.4 ± 1.1	297 ± 43	4.2 ± 0.1	274 ± 28	7.0 ± 0.3	6.5 ± 1.2
Br	8.9 ± 0.5	162 ± 40	1.7 ± 0.1	169 ± 35	5.2 ± 0.4	5.5 ± 1.8
Me	10.1 ± 0.5	104 ± 25	1.69 ± 0.03	105 ± 7	6.0 ± 0.3	6.0 ± 1.5
NO_2	296.4 ± 44.4	11 300 ± 2 700	nd	nd	nd	nd

[a] nd, not determined.

Table 3: Kinetic Parameters of Arylalkylamine Analogues as MAO A Substrates

analogue	K_s (mM)	K_d (mM)	k_{red} (min^{-1})	k_{cat} (min^{-1})
benzylamine[a]	1.24 ± 0.16	1.15	1.42 ± 0.20	2.54 ± 0.08
phenethylamine	1.66 ± 0.43	1.43	43.8 ± 3.7	64.0 ± 1.2
3-phenylpropylamine	0.022 ± 0.001	nd[c]	20.0 ± 0.2	25.1 ± 0.6
4-phenylbutylamine[b]	competitive inhibitor		$K_i = 0.031 \pm 0.005$ mM	

[a] From ref *2*. [b] The value indicated is the K_i value using *p*-CF_3-benzylamine as a substrate analogue. [c] nd, not determined.

rate limiting and also provides a rationale for using these rate data to reflect the rate of C−H bond cleavage in phenethylamine oxidation by MAO A.

The major outlier in comparison of steady state with stopped-flow kinetic isotope effects (Tables 1 and 2) is the larger ^Dk value observed in stopped flow (6.9) than on k_{cat} (2.3) or k_{cat}/K_m (3.5) with the *p*-OH-phenethylamine analogue (tyramine). Suppression of expressed isotope effects is often encountered in enzyme systems and has been discussed in detail (*16*). The reduction of $^Dk_{cat}$ is suggested to be due to a contribution of product release (from the oxidized enzyme) as a slower step than reduction of the flavin (the ratio of catalysis) while commitment factors (the commitment to catalysis) are present to suppress $^D(k_{cat}/K_m)$. Assuming that the value of $^Dk_3$ is close to the value of the intrinsic isotope effect for the *p*-OH analogue, the rate of product release is estimated to be ~3-fold slower than the rate of flavin reduction, and the commitment to catalysis (k_3/k_2) is estimated to be slightly over one (1.4). Consistent with this view, the *p*-OH-phenethylamine analogue is the only one where the value of k_{cat} is significantly lower than the value of k_3.

Influence of Chain Length on Kinetic Parameters of MAO A Oxidation. Comparison of the steady state and stopped-flow data with para-substituted phenethylamine analogues (Tables 1 and 2) with those determined previously with benzylamine analogues (*2*) suggested that an increase in chain length by one methylene group results in a substantial increase in the rate of MAO A-catalyzed oxidation. To provide more insight into this trend, the interaction of MAO A with 3-phenylpropylamine was investigated by steady-state and stopped-flow kinetic studies. As depicted in Table 3, this analogue is a reasonable substrate for MAO A with a k_{cat} value of 25 ± 0.6 min^{-1} and a limiting rate of flavin reduction (k_3) of 20 ± 0.2 min^{-1}. Thus, increasing the chain length by one methylene group over that of phenethylamine decreases the rate of substrate oxidation by a factor of ~2.5. The K_s value for binding of this analogue is found to be 22 μM, which is 75-fold tighter than the value determined for phenethylamine (Table 2). These data suggest that the increase in binding affinity results from interactions of the side chain with amino acid residues in the catalytic site.

To probe whether the above conclusion has any validity, the substrate analogue 4-phenylbutylamine was tested as a substrate for MAO A. No detectable oxidation of this analogue by MAO A was found; however, it was found to be an effective competitive inhibitor of MAO A with a K_i value of 31 ± 5 μM (Table 3). This affinity is of a similar order of magnitude as found for 3-phenylpropylamine with the difference being that the analogue with the 3-carbon side chain is a substrate while that with a 4-carbon side chain is not. These data suggest that increasing the binding interactions of the side chain of the substrate analogue with the enzyme leads to a reduction in the formation of catalytically productive complexes and an increase in the binding affinity. Analysis of this behavior with that of known reversible inhibitors of MAO A will be discussed below.

DISCUSSION

QSAR Approaches to MAO A Catalysis. The analysis of binding and rate data for the phenethylamine class of substrates to MAO A uses approaches that have been used previously with MAO A (*2*), MAO B (*1*), and the quinoprotein plasma amine oxidase (*17*) using the general equation

$$\log k \text{ (or } K) = \rho\sigma + A\pi + BV_w + C \qquad (2)$$

where σ is the electronic (electron-withdrawing or -donating) contribution of the substituent, π is the hydrophobicity parameter (*18*), and V_w is the van der Waals volume of the substituent (*13*) or the steric term, which is also modeled by other parameters such as E_s (*19*).

Our approach is to attempt single correlations initially, find the best fit, and then attempt to improve the correlations by inclusion of a second parameter. Three parameter fits are not attempted since the number of substituents used in these experiments are insufficient for this extent of statistical analysis. The results from analysis of phenethylamine binding and rate data are compared with the previous analysis of benzylamine analogue interaction with MAO A (*2*).

Binding Parameters of para-Substituted Phenethylamines to MAO A. Using the approach outlined above, the apparent binding constants for the phenethylamine analogues were correlated with either one or two parameters of eq 2. The results are tabulated in Table 4. Of the single parameter correlations, the best fit is that of steric effect (either V_w or E_s). Poor correlations are observed with hydrophobicity (π) or even worse correlations with the electronic parameter (σ). The similarity of V_w and E_s are expected since these two steric parameters exhibit collinearity with one another. The addition of a second parameter to the steric term in a two-parameter fit does not improve the *F* value over that observed with a single-parameter fit; therefore, the binding of para-substituted phenethylamine analogues can be adequately predicted as being favored with increasing size of the para substituent. No electronic influence on binding is observed as found with the benzylamine analogues (*2*), which is explained by the lack of an electronic effect on the pK_a of the primary amine in the phenethylamine class. The binding of the phenethylamine analogues is expected to follow other studies that have documented the deprotonated form of the amine as the species bound to the active site of MAO A. Therefore, our final analysis correlated the K_d calculated for the level of deprotonated amine at pH 7.5 using (*20*):

$$K_d \text{ (corrected)} = \frac{K_d \text{ (observed)}}{1 + \text{antilog } (pK_a - pH)} \qquad (3)$$

Table 4: Correlations of Binding Affinities (K_s or K_d) of Deprotonated para-Substituted Phenethylamines for MAO A with Steric, Electronic, and Hydrophobic Substituent Parameters[a]

parameter	correlation slope	correlation *y*-intercept	correlation coeff	F[b]	significance[c]
π	-0.57 ± 0.20	-5.49 ± 0.11	0.33	8.3	0.0104
σ	-0.12 ± 0.56	-5.63 ± 0.12	0.003	0.1	0.83
E_s	0.96 ± 0.11	-4.88 ± 0.11	0.82	72	<0.0001
V_w	-0.99 ± 0.11	-4.65 ± 0.12	0.84	87	<0.0001
$V_w + \pi$	-0.95 ± 0.13 -0.06 ± 0.12	-4.68 ± 0.13	0.84	41	<0.0001
$V_w + \sigma$	-1.00 ± 0.11 0.16 ± 0.23	-4.64 ± 0.12	0.84	42	<0.0001

[a] K_s values used in linear regression analysis were obtained from proteo and dideutero phenethylamine analogues. K_d values were calculated from steady-state isotope effects as described by Klinman and Mathews (*15*). All values are corrected to reflect the selective binding of the deprotonated amines to the enzyme according to eq 3. Correlations do not include *p*-NO_2-, *p*-CF_3-, *p*-EtO-, and *p-tert*-butylphenethylamine. [b] The F value is a statistical term relating the residual of each point to the fitted line to the residuals of each point to the mean value. F is weighted for the number of variables in the correlation and the number of data points. A higher F value indicates a better fit. [c] Significance is calculated from the F value and represents the fractional chance that the derived correlation is meaningless.

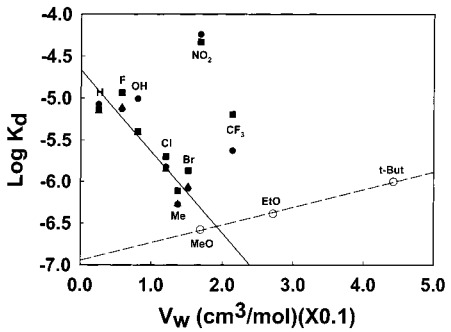


FIGURE 3: Correlation of phenethylamine analogue binding to MAO A with van der Waals volume of the para substituent. Note that *p*-CF_3 and *p*-NO_2 analogues are not included in the analysis. The binding constants were obtained as K_s for α,α-diproteo- (●) or α,α-dideuterophenethylamines (▲), as K_d (■), or as K_i (○). The solid smooth line is the linear best fit of the experimental data excluding *p*-NO_2-, *p*-CF_3-, *p*-EtO-, and *p-tert*-butylphenethylamine analogues. The broken line is the linear best fit of the experimental data for the *p*-MeO-, *p*-EtO-, and *p-tert*-butylphenethylamine analogues.

The results of the single-parameter correlation with V_w are shown in Figure 3. The equation describing this correlation is

$$\log K_d = -0.99 \pm (0.11)(0.1V_w) - 4.65 \pm (0.12) \quad (4)$$

$$R^2 = 0.84, \quad F_{1,18} = 87, \quad p = <0.0001$$

Note that the analogues *p*-NO_2, *p*-CF_3, *p*-ethoxy, and *p-tert*-butyl are omitted from the correlation. The anomalous behavior of *p*-NO_2- and *p*-CF_3-phenethylamine analogues are suggested to be due to additional interactions with the active site of the enzyme such as H-bonding. As shown in Figure 3, the size of the *p*-ethoxy and *tert*-butyl substituents actually decrease the affinity of phenethylamine binding, which suggests that there are size limitations about the para position of the bound phenethylamine analogue. When the size of the substituent increases above ~15 cm^3/mol, the orientation of the bound substrate is such that it is unable to form a productive complex for catalysis (although it can bind tightly to the enzyme's active site).

Table 5: Correlations of Rate Data of para-Substituted Phenethylamines for MAO A with Steric, Electronic, and Hydrophobic Substituent Parameters[a]

parameter	correlation slope	correlation *y*-intercept	correlation coeff	F	significance
π	-0.82 ± 0.16	1.72 ± 0.10	0.68	26	0.0003
σ	-1.34 ± 0.39	1.53 ± 0.11	0.50	12	0.0048
E_s	0.64 ± 0.10	2.05 ± 0.13	0.76	38	<0.0001
V_w	-0.78 ± 0.12	2.31 ± 0.16	0.77	40	<0.0001
$V_w + \pi$	-0.53 ± 0.15 -0.40 ± 0.17	2.16 ± 0.15	0.85	30	<0.0001
$V_w + \sigma$	-0.64 ± 0.35 -0.50 ± 0.32	2.18 ± 0.17	0.81	24	0.0001

[a] Correlations include both k_{cat} and k_3 taken from Tables 1 and 2. Correlations do not include *p*-NO_2-phenethylamine.

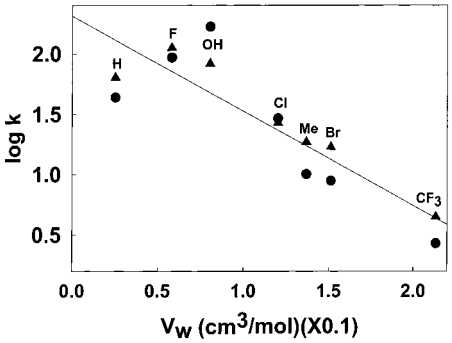


FIGURE 4: Correlation of the rate of phenethylamine oxidation by MAO A with the van der Waals volume of the para-substituent. Rates of phenethylamine oxidation were obtained from either steady-state kinetic data (▲) or from the reductive half-reaction experiments (●).

Comparison of the binding parameters of para-substituted phenethylamine analogues to MAO A with those determined previously for the binding of para-substituted benzylamine analogues to MAO A (*2*) show comparable behavior. Therefore, the binding site for the aromatic rings of these two classes of substrates appears to be the same rather than a situation of alternate binding modes that have been demonstrated in the interaction of different classes of benzylamine analogues to MAO B (*21*).

Factors Influencing the Rate of Flavin Reduction. To assess the influence of substituent parameters on the rate of flavin reduction in MAO A catalysis, we performed correlations using both values for k_{cat} and k_{red} since the linear correlation in Figure 2 and the kinetic isotope effects demonstrate that both measurements reflect the rate of flavin reduction in phenethylamine catalysis (with the exception of tyramine). The one- and two-parameter correlations are shown in Table 5 where the best correlation is that the rate of flavin reduction decreases with increasing size of the para substituent as shown in Figure 4 and is best described by

$$\log k_{red} \text{ (or } k_{cat}) = -0.78 \pm (0.12)(0.1V_w) + 2.31 \pm (0.16) \quad (5)$$

$$R^2 = 0.77, \quad F_{1,13} = 40, \quad p = <0.0001$$

In contrast with the results with benzylamine analogues ($\rho = +2$) (*2*), no electronic influence of the para substituent

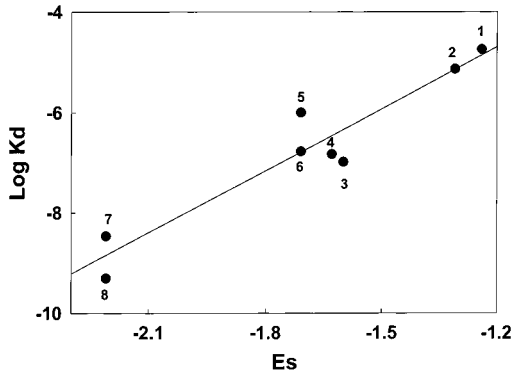


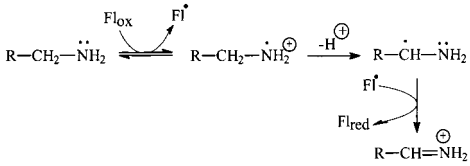
FIGURE 5: Correlation of the arylalkylamine binding affinity to MAO A with Taft steric parameter (E_s) of the alkyl side chain of the analogues. The arylalkylamine analogues included in the analysis are as follows: 1, benzylamine (from ref *2*); 2, phenethylamine; 3, phenylpropylamine; 4, phenylbutylamine; 5, (*R*)-amphetamine (from ref *22*); 6, (*S*)-amphetamine (from ref *22*); 7, (−)-*trans*-phenylcyclopropylamine (from ref *23*); and 8, (+)-*trans*-phenylcyclopropylamine (from ref *23*).

on the rate of MAO A flavin reduction by the phenethylamine analogues is detected. The graphical representation of the relationship of eq 5 is shown in Figure 4. The reaction rate of flavin reduction is dominated by the van der Waals volume of the para substituent with slower rates occurring with larger substituent size. Classically the insertion of a methylene group between the aromatic ring and the reaction site of a molecule leads to a reduction of the substituent electronic effect but not its total elimination [$\rho = +1.0$ for the ionization of benzoic acid and $\rho = +0.59$ for the ionization of phenylacetic acid (*12*)]. The results presented here with MAO A suggest that the steric effects dominate the reduction rate and any electronic effect that may be present is too small to detect. The observed similarity in binding behavior of phenethylamine and benzylamine analogues diminish (but do not eliminate) the possibility that steric effects may alter the ring conformation in the active site resulting in a further suppression of the transmission of electronic effects to the reaction site.

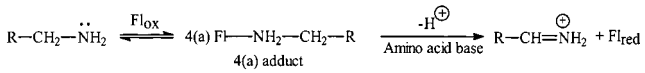
Analysis of Side Chain Steric Effects on Binding Affinity. The results in Table 3 demonstrate that substrate analogues with propyl or butyl side chains are bound with a much higher affinity to the active site of MAO A than benzylamine or phenethylamine. When probed for any correlations that could be identified with this behavior, steric parameters appeared to be the more likely determinants. The steric parameters (E_s) for methylene, ethylene, propylene, and butylene were initially used in correlations with the binding affinities calculated for each analogue with the reasonable assumption that the deprotonated form of the amine is selectively bound to MAO A. A reasonable correlation was indeed found for these four substrate analogues. Extension of these correlations to known inhibitors of MAO A include the R and S isomers of amphetamine (*22*) and the (−)- and (+)-trans isomers of phenylcyclopropylamine (*23*). The correlation is shown in Figure 5 where a linear correlation of log K_d with E_s is found for binding affinities spanning over a 10^4-fold range. The correlation for this linear relation is shown in

$$\log K_d = 4.11\ (\pm\ 0.56)E_s + 0.24\ (\pm\ 0.97) \qquad (6)$$

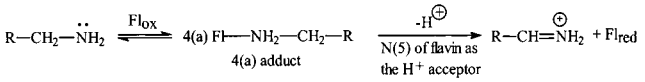
Scheme 1: Aminium Cation Radical Mechanism



Scheme 2: Polar Nucleophilic Mechanism



Scheme 3: Modified Polar Nucleophilic Mechanism



These data demonstrate that the side chain of the arylalkylamine exhibits a strong interaction with the enzyme, and this interaction is best correlated with steric factors. The E_s parameter has been correlated with the van der Waals radius of the substituent. In the case of the alkyl side chain analogues, the stronger this binding, the more likely this interaction results in the formation of a nonproductive complex, which is suggested to direct the amine functionality away from the active site flavin and may serve as the basis for the design of new reversible inhibitors of MAO A. Although we have not carried out as complete an analysis of MAO B, preliminary results show a shallower dependence of binding affinity with the E_s value of the side chain. Work is underway in this laboratory to further delineate this effect with MAO B.

Mechanistic Implications of These Results. The previous QSAR results with benzylamine analogues with MAO A provide direct evidence for H^+ abstraction as the mode of C−H bond cleavage in catalysis. This result is consistent with either model for the catalytic mechanism: (i) the aminium cation radical mechanism (*24*) (Scheme 1) or (ii) the flavin C(4a) addition mechanism (*25*) (Scheme 2). Using a different class of substrate analogues, no spectral evidence is found to suggest the intermediate formation of any flavin semiquinones as predicted by the aminium cation radical mechanism. In a previous publication (*2*), we favored a concerted version of the 4a-adduct mechanism to account for the failure to detect any spectral evidence for the substrate-flavin 4a adduct and to account for the observed isotope effects on k_{cat} and on k_{red} (Scheme 3).

One surprising aspect in comparing the rates of phenethylamine with benzylamine oxidation by MAO A is that the rate of flavin reduction (measure of the rate of C−H bond cleavage step as judged by the ^Dk values) is increased ∼30-fold with the phenethylamine substrate analogue. In contrast, the bond dissociation energy of the α-C−H of phenethylamine is estimated to be ∼10−15 kcal/mol greater than that of the benzyl C−H of benzylamine (*26*) and thus should be more difficult to cleave. This apparent contradiction can be explained by invoking contributions of steric flexibility of the phenethylamine side chain to promote C−H bond cleavage by the basic N(5) position formed in concert with the C(4a) adduct. Steric limitations of a similar benzylamine adduct would require specific orientations of the phenyl ring to place the benzyl C−H bond at a suitable location for H^+

abstraction. If the aminium cation radical mechanism were applicable, one might predict that the rates of flavin reduction be similar since the oxidation−reduction potential of the primary amine nitrogen in both classes of substrates is similar but would be influenced by para-substituent effects in the benzylamine class but not in the phenethylamine class of substrate analogues. In fact, the data show that phenethylamine oxidation by MAO A is faster than benzylamine oxidation with similar magnitudes of ^{D}k values. Thus, for this class of substrate analogues, no evidence can be found to support the aminium cation radical mechanism.

In conclusion, the results of this study provide added insights into the structure of the substrate binding site and into the catalytic mechanism of human liver MAO A. The results show that, for rapidly oxidized substrates, the reduced enzyme−imine product complex is the reactant with O_2, which contrasts with MAO B in which the free, reduced enzyme is the reductant of O_2. These conclusions support those published by Ramsay (*27*), who proposed that MAO A functions by a ternary complex mechanism and that enzyme reduction constitutes the rate-limiting step in catalytic turnover. The rate of MAO A reduction is governed by steric effects with the phenethylamine class of substrates and electronic effects with the benzylamine class. Binding affinities of substrate analogues by MAO A is favored by increased van der Waals volumes of the substituent and by increased E_s values of the alkyl side chain. Whether these steric effects are additive remains for future work and would be important in the design of specific reversible inhibitors of MAO A.

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