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^{2}H Kinetic Isotope Effects and pH Dependence of Catalysis as Mechanistic Probes of Rat Monoamine Oxidase A: Comparisons with the Human Enzyme

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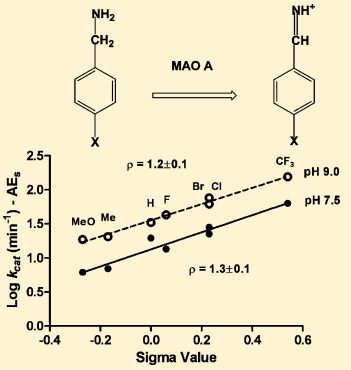
ABSTRACT: Monoamine oxidase A (MAO A) is a mitochondrial outer membrane-bound flavoenzyme important in the regulation of serotonin and dopamine levels. Because the rat is extensively used as an animal model in drug studies, it is important to understand how rat MAO A behaves in comparison with the more extensively studied human enzyme. For many reversible inhibitors, rat MAO A exhibits K_i values similar to those of human MAO A. The pH profile of k_{cat} for rat MAO A shows a pK_a of 8.2 ± 0.1 for the benzylamine ES complex and pK_a values of 7.5 ± 0.1 and 7.6 ± 0.1 for the ES complexes with *p*-CF_3-^{1}H- and *p*-CF_3-^{2}H-benzylamine, respectively. In contrast to the human enzyme, the rat enzyme exhibits a single pK_a value (8.3 ± 0.1) with k_{cat}/K_m for benzylamine versus pH and pK_a values of 7.8 ± 0.1 and 8.1 ± 0.2 for the ascending limbs, respectively, of k_{cat}/K_m versus pH profiles for *p*-CF_3-^{1}H- and *p*-CF_3-^{2}H-benzylamine and 9.3 ± 0.1 and 9.1 ± 0.2 for the descending limbs, respectively. The oxidation of para-substituted benzylamine substrate analogues by rat MAO A has large deuterium kinetic isotope effects on k_{cat} and on k_{cat}/K_m. These effects are pH-independent and range from 7 to 14, demonstrating a rate-limiting α-C−H bond cleavage step in catalysis. Quantitative structure−activity correlations of log k_{cat} with the electronic substituent parameter (σ) at pH 7.5 and 9.0 show a dominant contribution with positive ρ values (1.2−1.3) and a pH-independent negative contribution from the steric term. Quantitative structure−activity relationship analysis of the binding affinities of the para-substituted benzylamine analogues for rat MAO A shows an increased van der Waals volume (V_w) increases the affinity of the deprotonated amine for the enzyme. These results demonstrate that rat MAO A exhibits functional properties similar but not identical with those of the human enzyme and provide additional support for C−H bond cleavage via a polar nucleophilic mechanism.



Monoamine oxidase A (MAO A) is an outer mitochondrial membrane-bound flavoenzyme that regulates the concentrations of important neurotransmitters in cells, such as dopamine and serotonin.[1] Alteration of the physiological levels of MAO A is implicated in neuropsychiatric disorders such as aggressive trait disorder[2,3] and depression[4,5] and may contribute to cardiomyopathies.[6] Therefore, MAO A is a clinically proven drug target for treatment of neurological disorders and may be a future drug target for cardioprotective drugs.[7]

Rat MAO A is found to be ∼90% identical in sequence with the human enzyme. Structural data show that human MAO A crystallizes as a monomer[8] whereas rat MAO A is dimeric.[9] Recent pulsed EPR data show that both human and rat MAO A's exist as dimers in their membrane-bound forms and that either dimer partially dissociates into monomers (∼50%) on detergent solubilization and purification.[10] The explanation for their crystallographic differences is that the monomeric form of human MAO A more readily crystallizes, whereas it is the dimeric form of rat MAO A that does so. The high levels of sequence identity of human and rat MAO A serve as the premise for using the rat as an animal model for the human enzyme in drug development studies. Recent comparative studies of para-substituted amphetamine analogue binding to rat and human MAO A show differences in their structure−activity correlations.[11] Despite extensive studies on human and rat MAO A's, a detailed functional comparison of these two enzymes has not been performed. Mechanistic studies[12] of human MAO A with a series of para-substituted benzylamine analogues led to these findings. (1) Large ^{2}H kinetic isotope effects suggest that α-C−H bond cleavage is rate-limiting in catalytic turnover. (2) Steady-state and stopped-flow kinetic studies show that the rate of reduction of the flavin increases with improved electron withdrawing ability of the substituent, and this result has led to a mechanistic description of the reductive half-reaction as one following a polar nucleophilic mechanism. (3) A linear correlation of increased binding affinity with the van der Waals volume (V_w) of the para substituent is comparable with the monopartite nature of MAO A's active site cavity. Recent pH-dependent studies of human MAO A catalysis show catalytically sensitive pK_a values for both ES and E,[13] which may or may not be the same for the rat enzyme.

With the availability of purified recombinant rat MAO A,[14] it was of interest to determine whether the mechanistic insights determined for the human enzyme would also be true for the rat enzyme. As shown in this study, the functional properties of rat MAO A are similar but not identical with those of the human enzyme. Both enzyme sources exhibit pH-dependent behaviors consistent with the proposed polar nucleophilic

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mechanism. These comparative data should be important in future drug development studies using the rat as an animal model for the human.

EXPERIMENTAL PROCEDURES

Materials. Benzylamine, *d*-amphetamine, isatin, kynuramine, methylene blue, phenylethylamine, serotonin, glycerol, Bis-Tris propane, and potassium phosphate were purchased from Sigma-Aldrich. β-Octyl glucopyranoside was from Anatrace Inc., and reduced Triton X-100 was from Fluka. Harmane, pirlindole mesylate, and tetrindole mesylate were purchased from TOCRIS Bioscience. All benzylamine analogues used in this study were either purchased from Sigma-Aldrich or synthesized in this laboratory as described previously.[15] Recombinant rat liver MAO A was expressed in and purified from cells of *Pichia pastoris* strain KM71 as described previously.[14] Purified rat MAO A was stored in 50 mM potassium phosphate containing 20% glycerol and 0.8% (w/v) β-octyl glucopyranoside (pH 7.2). *d*-Amphetamine, a reversible MAO A inhibitor used for stabilizing the enzyme during purification, was removed prior to all kinetic measurements.

Determinations of k_{cat}, K_m, and K_i Values. Steady-state kinetic measurements of rat MAO A were conducted at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 0.5% (w/v) reduced Triton X-100 using kynuramine as a substrate. The rate of kynuramine oxidation by MAO A is followed spectrophotometrically by the formation of the product 4-hydroxyquinoline at 316 mm ($\Delta\varepsilon$ = 12600 M^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 μmol of kynuramine to 4-hydroxyquinoline in 1 min. All kinetic measurements were performed at air saturation (~240 μM O_2), which is assumed to be saturating conditions.

Values of k_{cat} and K_m were obtained by global fits of steady-state kinetic data to the Michaelis−Menten equation using GraphPad Prism version 5.0. Competitive K_i values were determined by measuring initial rates of substrate oxidation in the presence of varying concentrations of inhibitor and the data analyzed also using GraphPad Prism version 5.0. The K_i value for methylene blue was obtained using the equation developed by Morrison[16] for tight-binding inhibitors.

pH-dependent kinetic experiments were performed in 20 mM Bis-Tris propane buffer containing 0.5% (w/v) reduced Triton X-100, 50 mM NaCl, and 20% glycerol. The pH of the buffer was adjusted in the range of 6.5−9.5 by the addition of HCl or NaOH.

Steady-State Kinetic Measurements of Para-Substituted Benzylamine Analogue Oxidation. All steady-state kinetic measurements of para-substituted benzylamine analogue oxidation with rat MAO A were also performed either in 50 mM potassium phosphate buffer (pH 7.5) or in 20 mM Bis-Tris propane buffer containing 0.5% (w/v) reduced Triton X-100 at 25 °C. The steady-state rate of benzylamine analogue oxidation to the corresponding benzaldehyde was measured spectrophotometrically. Monitoring wavelength and molar absorption extinction coefficients for each aldehyde are given in ref 15. The low oxidation rates of *p*-F-BA, *p*-Me-BA, *p*-MeO-BA, and α,α-[^{2}H]benzylamine analogues precluded accurate determinations of their rates of product formation by following aldehyde accumulation. Therefore, the Amplex Red−peroxidase coupled assay ($\Delta\varepsilon_{560}$ = 54000 M^{-1} cm^{-1}) was used because it has a 5-fold higher level of sensitivity.

Data Analysis. Values of the substituent parameters σ, π, and E_s were obtained from ref 17 and V_w values from ref 18. K_d values for the benzylamine analogues were determined from steady-state ^{2}H kinetic isotope effect data as described by Klinman and Matthews.[19] Multivariate linear regression analysis of rate and binding data as a function of substituent parameters was performed using the StatView software package (Abacus Concepts). Fits of pH-dependent kinetic data were analyzed as described by Dunn et al.[13] by fitting the data to the appropriate equations using GraphPad Prism version 5.0.

RESULTS

Competitive Inhibition by MAO A Reversible Inhibitors. The K_i values exhibited by rat MAO A for the reversible MAO A-specific inhibitors, harmane, pirlindole, tetrindole, and methylene blue are essentially identical with those exhibited by human MAO A under identical conditions of pH, buffer, and temperature (Table 1). MAO B-specific reversible and irreversible inhibitors bind weakly (K_i > 10 μM) to rat MAO A as they do to the human enzyme with only minor differences in observed K_i values (data not shown). These data demonstrate that rat MAO A exhibits affinities for reversible MAO A-specific inhibitors similar to those of the human enzyme, and the rat enzyme shows no unexpected affinities for any of the MAO B-specific inhibitors

Table 1. Comparison of Competitive Inhibition Constants for Human and Rat MAO A for a Series of Reversible MAO A Inhibitors

	K_i (μM)	
inhibitor	human MAO A	rat MAO A
harmane	0.58 ± 0.02	0.75 ± 0.04
pirlindole mesylate	0.92 ± 0.04	0.77 ± 0.02
tetrindole mesylate	5.3 ± 0.24	3.6 ± 0.34
methylene blue	0.027 ± 0.0029[a]	0.026 ± 0.0015

[a]Data taken from ref 34.

Effects of pH on Steady-State Kinetic Parameters. Previous pH-dependent steady-state kinetic studies of human MAO A by Dunn et al.[13] demonstrated that a group in the ES complex with benzylamine is deprotonated for optimal activity and that two deprotonation steps (for either free enzyme or free substrate) influence catalytic turnover with both an enhancement and subsequently a decrease in the rate of the enzyme in enzyme-catalyzed reaction with an increasing assay medium pH. To determine whether rat MAO A exhibits pH-dependent behavior and pK_a values similar to that of the human enzyme, the effects of pH on steady-state kinetic parameters for the oxidation of benzylamine and of *p*-CF_3-benzylamine (α,α-^{1}H and α,α-^{2}H) were determined. The reason for comparison of these two benzylamine analogues is that they exhibit differing pK_a values (ΔpK_a = 0.6) for their unbound forms, with benzylamine exhibiting a pK_a of 9.3 and the *p*-CF_3 analogue a pK_a of 8.8, which provides observable differences in the pH−activity profiles. Because the O_2 concentration is saturating with MAO A at pH 7.5, it was assumed that saturating conditions are maintained at all pH values. As shown in Figure 1A−C (solid line), the k_{cat} values for benzylamine and α,α-[^{1}H]- and α,α-[^{2}H]-*p*-CF_3-benzylamines increase and exhibit sigmoidal dependences with increasing pH, indicating the presence of a single macroscopic ionization with pK_a values of 8.2 ± 0.1, 7.5 ± 0.1,

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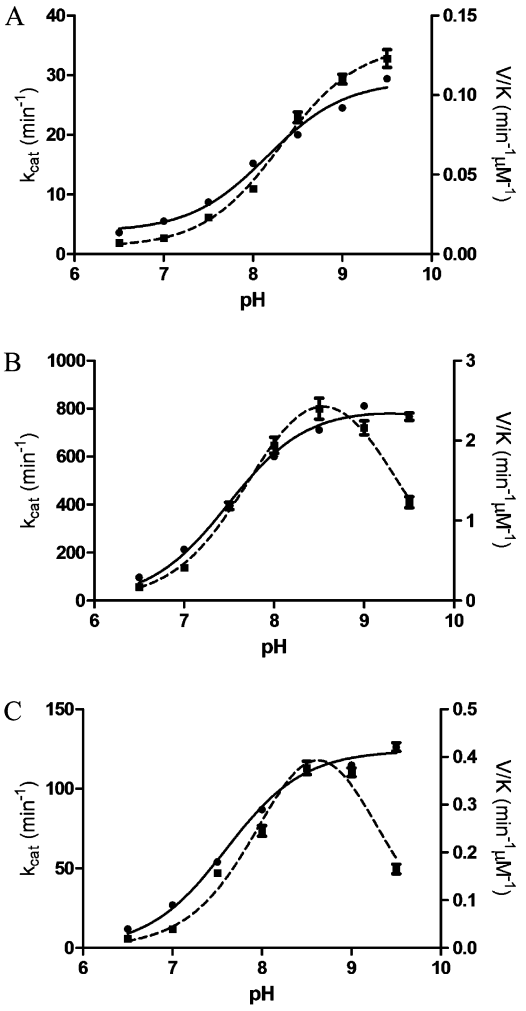

Figure 1. pH dependence of the steady-state kinetic parameters of rat MAO A-catalyzed oxidation of benzylamine (A), α,α-[^{1}H]-*p*-CF_3-benzylamine (B), and α,α-[^{2}H]-*p*-CF_3-benzylamine (C). Values of k_{cat} (●) and V/K (■) are shown.

and 7.6 ± 0.1, respectively. These values represent pK_a values of their respective ES complexes. The difference in pK_a values for the catalytic parameters on comparison of benzylamine with the *p*-CF_3 analogue (0.6) is within experimental uncertainty of the difference in pK_a values for the free forms of these substrates. As shown with the human MAO A-catalyzed oxidation of benzylamine, the k_{cat}/K_m data for rat MAO A oxidation of proteo and deutero *p*-CF_3-benzylamines are best fit by bell-shaped profiles, as described by Dunn et al.[13] (Figure 1B,C), with estimated pK_a values of 7.8 ± 0.1 and 9.3 ± 0.1, and 8.1 ± 0.2 and 9.1 ± 0.2, respectively (Table 2). A small alkaline shift of the pK_{a1} value (0.3−0.4) is observed for ^{2}H-*p*-CF_3-benzylamine relative to the ^{1}H form. In contrast, with benzylamine, the k_{cat}/K_m versus pH profile exhibits a single ionization (8.3 ± 0.1) (Figure 1A, dashed line) in the pH range covered rather than two pK_a values observed with both the human enzyme and the *p*-CF_3-benzylamine analogue. Therefore, if a pH-dependent deprotonation occurs that is detrimental to catalysis with the rat enzyme, it must occur in a region more alkaline than pH 9.5. Considering the differences in pK_a values found upon comparison of other ionizations of benzylamine with the *p*-CF_3 analogue, this pK_a is expected to occur at ≥10.

Table 2. pK_a Values Estimated from Fits of the pH Dependence of Kinetic Parameters of Rat MAO A

	ES complex	free E or S	
substrate	pK_a	pK_{a1}	pK_{a2}
benzylamine	8.2 ± 0.1	8.3 ± 0.1	–
α,α-[^{1}H]-*p*-CF_3-benzylamine	7.5 ± 0.1	7.8 ± 0.1	9.3 ± 0.1
α,α-[^{2}H]-*p*-CF_3-benzylamine	7.6 ± 0.1	8.1 ± 0.2	9.1 ± 0.2

Steady-State Kinetic Parameters for Rat MAO A-Catalyzed Oxidation of Para-Substituted Benzylamine Analogues and the ^{2}H Kinetic Isotope Effects. The steady-state kinetic parameters for rat MAO A-catalyzed oxidation of seven para-substituted benzylamine analogues and their corresponding α,α-[^{2}H] analogues were determined at pH 7.5, and their k_{cat} and K_m values are listed in Table 3. The pH 7.5 conditions were chosen to allow direct comparison with published values for human MAO A.[12] Turnover numbers [k_{cat}(H)] of rat MAO A determined for each substrate analogue show a marked dependence on the nature of the substituent. The k_{cat} values range from 9.2 min^{-1} for the *p*-MeO analogue to 333.5 min^{-1} for the *p*-CF_3 analogue. Catalytic rates determined for the α, α-[^{2}H]benzylamine analogues exhibit large isotope effects on both $^D k_{cat}$ and $^D(k_{cat}/K_m)$ (Table 3). Human MAO A also exhibits large isotope effects on k_{cat} in the range of 6.7−14.3, demonstrating that α-C−H bond cleavage (the reductive half-reaction) is rate-limiting in catalytic turnover.[12] The large kinetic isotope effects observed for para-substituted benzylamine oxidation by rat MAO A show this conclusion is also valid for rat MAO A.

The pH dependence of k_{cat} and k_{cat}/K_m shows maximal values at pH 9.0 (Figure 1), and therefore, the substituent effect for benzylamine oxidation was also assessed at this pH value. Previous mechanistic conclusions from substituent effect data have been criticized[20] via the suggestion that the positive ρ value observed with the human enzyme at pH 7.5 originates from electronic effects on amine pK_a values in the ES complex. Performing these measurements at pH 9.0 would minimize this contribution because the ES complex is in its deprotonated form. Values for the kinetic parameters at pH 9.0 for both human and rat MAO A are listed in Table 4. In agreement with data collected at pH 7.5, the k_{cat} values are higher for those analogues with electron-withdrawing substituents. Correlations of k_{cat} values for both the rat and human enzymes at pH 7.5 and 9.0 with electronic, hydrophobic, and steric parameters are presented and discussed below.

Effects of Substituents on the Rates of Catalytic Turnover. Previous data on human MAO A[12] show that k_{cat} values and the rates of flavin reduction are mainly dependent on the electronic parameter (σ) of the para substituent at pH 7.5. The steady-state kinetic isotope values observed demonstrate that k_{cat} values are good approximations of the intrinsic rate of the C−H bond cleavage step. These data constitute the most powerful argument that supports the C−H bond cleavage step in MAO A catalysis as occurring via a H^+ abstraction that is part of a polar nucleophilic mechanism.[12] Correlation analysis using k_{cat} values to assess the influence of substituent parameters on the rates of benzylamine analogue oxidation was performed using the data in Table 4, and the results are presented in Table 5. The statistical *F* values for these correlations allow for identification of the most appropriate

Table 3. Steady-State Kinetic Constants for Rat MAO A-Catalyzed Oxidation of Para-Substituted Benzylamine Analogues at pH 7.5

para substituent	k_{cat}(H) (min^{-1})	K_m(H) (μM)	$^{D}k_{cat}$	k_{cat}(D) (min^{-1})	K_m(D) (μM)	$^{D}(V/K)$
H	20 ± 0.2	211.1 ± 18.7	6.7 ± 0.2	2.9 ± 0.1	423 ± 51.9	14 ± 2.1
CF_3	334 ± 5.4	194 ± 9.9	8.4 ± 0.2	40 ± 0.7	273 ± 13.7	12 ± 0.9
Br	62 ± 1.0	34 ± 3.2	13 ± 0.2	4.7 ± 0.1	30 ± 1.6	12 ± 1.3
Cl	44 ± 1.0	54 ± 6.2	14 ± 0.4	3.1 ± 0.1	32 ± 3.2	8.2 ± 1.3
F	14 ± 0.4	174 ± 13.6	11 ± 0.4	1.2 ± 0.1	109 ± 8.7	7.1 ± 0.8
Me	16 ± 0.1	55 ± 2.2	11 ± 0.2	1.5 ± 0.1	42 ± 3.9	8.1 ± 0.8
MeO	9.2 ± 0.2	80 ± 9.5	11 ± 0.5	0.8 ± 0.1	60 ± 10.2	8.4 ± 1.8

Table 4. Steady-State Kinetic Constants for Rat and Human MAO A-Catalyzed Oxidation of α,α-[^{1}H]-Para-Substituted Benzylamine Analogues at pH 9.0

	rat MAO A		human MAO A	
para substituent	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
H	33 ± 0.16	137 ± 2.5	9.6 ± 0.091	13 ± 0.29
CF_3	811 ± 11	2161 ± 87	283 ± 5.7	381 ± 18
Br	171 ± 1.4	3527 ± 142	61 ± 0.79	326 ± 17
Cl	120 ± 1.5	2089 ± 123	39 ± 0.49	223 ± 11
F	59 ± 0.84	386 ± 23	18 ± 0.33	38 ± 2.2
Me	48 ± 0.30	1074 ± 39	25 ± 0.24	347 ± 15
MeO	28 ± 0.22	296 ± 12	11 ± 0.081	100 ± 3.2

substituent parameters (electronic, hydrophobicity, or steric). Statistical analysis of the rat and human MAO A k_{cat} data at pH 7.5 and 9.0 (Table 5) shows that the best single-parameter correlation is found with the electronic parameter σ, although the rat MAO A data at pH 9.0 also show a good correlation with the Taft steric parameter (E_s). Two-parameter correlations of log k_{cat} values of human and rat MAO A show marked improvements in the statistical F values for a combination of electronic and steric parameters (Table 5).

Analysis of k_{cat} values with the various substituent parameters for data taken at pH 9.0, which provides conditions for maximal catalytic rate, is also presented in Table 5. The best single-parameter correlation for rat MAO A is with the electronic substituent parameter with a ρ value of 1.8 ± 0.3 and is also true for human MAO A with a ρ value of 1.6 ± 0.4. Substantial increases in F values are observed when two-parameter correlations are performed with the inclusion of steric terms (either E_s or V_w). The following two-parameter equations are presented to describe rat and human MAO A k_{cat} values at pH 9.0:

$$\text{rat: } \log k_{cat} = 1.2(\pm 0.1)\sigma - 0.3(\pm 0.1)E_s + 1.55(\pm 0.03)F_{2,5} = 335, \quad P < 0.0001$$

$$\text{human: } \log k_{cat} = 0.8(\pm 0.1)\sigma - 0.4(\pm 0.1)E_s + 1.01(\pm 0.03)F_{2,5} = 337, \quad P < 0.0001$$

Both enzyme systems show a correlation with positive ρ values and a negative steric effect for the oxidation of the deprotonated substrates. There is a small decrease in ρ observed for the human enzyme with an increase in pH, but no change is observed for the rat enzyme. In both cases, the coefficient for the steric term remains constant at both pH values. Plots of log k_{cat} corrected for steric contributions versus σ are shown in Figure 2. Because the reaction is facilitated by the para electron-withdrawing groups, these data provide further support for the polar nucleophilic mechanism[12] in which the *pro-R* benzyl proton is abstracted as a H^+. If C−H bond cleavage were to occur by a hydride transfer mechanism, one would expect to observe a negative ρ value as shown for the oxidation of benzylamine analogues (ρ = −1.72 to −2.03)[21,22] in model system studies using hexamethylenetetramine-bromine or cetylmethylammonium permanganate as the hydride acceptor. Recent ^{15}N kinetic isotope effect measurements of human MAO B oxidation of benzylamine show that C−H bond cleavage and N-rehybridization are not concerted in the reaction, which also argues against a hydride mechanism for MAO B.[23]

Quantitative Structure−Activity Relationships of the Binding of Para-Substituted Benzylamine Analogues to Rat MAO A. The K_d values for rat and human MAO A binding to the deprotonated forms of the para-substituted benzylamine analogues were compared. The kinetic isotope effects observed for these benzylamine analogues (Tables 3) permit the calculation of substrate dissociation constants (which are a function of all pre-isotopically sensitive steps) and have been shown to correlate well with K_s values obtained from reductive half-reaction stopped-flow data.[12,15] Dissociation constants for all of the benzylamine analogues were calculated from the data in Table 6, as described by Klinman and Matthews:[19]

$$\frac{^{D}k_{cat} - 1}{^{D}(k_{cat}/K_m) - 1} = K_m/K_d \quad (1)$$

where K_d is the amine substrate dissociation constant from all complexes occurring prior to the isotope-sensitive C−H bond cleavage step.

Early studies suggested the deprotonated form of the amine[24,25] binds to the active site of MAO A, and because the pH of enzyme assay buffer used in this study is 7.5, we

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Table 5. Comparative Correlations of k_{cat} Values for the Rat and Human MAO A with Para-Substituted Benzylamine Analogues at pH 9.0 and 7.5 with Steric, Electronic, and Hydrophobic Substituent Parameters

	rat MAO A log k_{cat}, pH 9.0				human MAO A log k_{cat}, pH 9.0				rat MAO A log k_{cat}, pH 7.5				human MAO A log k_{cat}, pH 7.5[a]			
parameter	slope	R^2	F^b	significance	slope	R^2	F	significance	slope	R^2	F	significance	slope	R^2	F	significance
σ	1.8 ± 0.3	0.94	38	0.002	1.6 ± 0.4	0.88	17	0.0096	1.8 ± 0.3	0.94	38	0.002	1.9 ± 0.4	0.90	23	0.005
E_s	−0.6 ± 0.1	0.88	18	0.008	−0.6 ± 0.1	0.94	41	0.0014	−0.6 ± 0.2	0.84	12	0.019	−0.7 ± 0.2	0.88	18	0.009
V_w	0.5 ± 0.3	0.61	3	0.145	0.6 ± 0.3	0.69	4.6	0.084	0.5 ± 0.3	0.56	2.3	0.192	0.5 ± 0.3	0.53	2	0.218
π	1.1 ± 0.3	0.84	12	0.019	1.2 ± 0.3	0.88	18	0.0085	1.1 ± 0.4	0.80	9.2	0.029	1.3 ± 0.3	0.88	17	0.009
$\sigma + E_s$	1.2 ± 0.1, −0.3 ± 0.1	0.99	335	<0.0001	0.8 ± 0.1, −0.4 ± 0.1	0.99	336	<0.0001	1.3 ± 0.3, −0.3 ± 0.1	0.98	45	0.002	1.2 ± 0.3, −0.4 ± 0.1	0.98	38	0.002
$\sigma + \pi$	1.3 ± 0.4, 0.4 ± 0.2	0.97	28	0.004	0.9 ± 0.4, 0.7 ± 0.3	0.95	18	0.01	1.5 ± 0.4, 0.4 ± 0.3	0.96	22	0.007	1.2 ± 0.4, 0.7 ± 0.3	0.96	25	0.006
$\sigma + V_w$	1.6 ± 0.1, 0.3 ± 0.1	0.99	129	0.0002	1.4 ± 0.2, 0.4 ± 0.1	0.98	40	0.0023	1.7 ± 0.2, 0.2 ± 0.1	0.98	41	0.002	1.7 ± 0.4, 0.2 ± 0.2	0.94	14	0.015

[a]Taken from ref 12. [b]The F value is a statistical term relating the residuals 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 value of F indicates a better fit. The significance is calculated from the F value and represents the fractional chance that the correlation is meaningless.

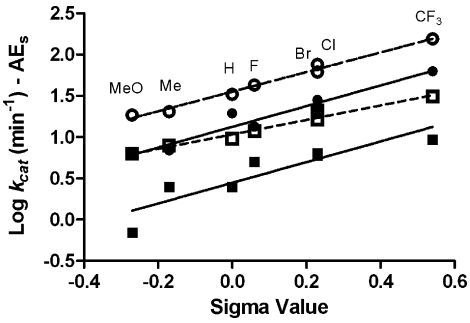

Figure 2. Correlation of log k_{cat} with AE_s of MAO A turnover of α,α-[^{1}H]-para-substituted benzylamine analogues with the substituent electronic parameter (σ): rat MAO A at pH 7.5 (●), rat MAO A at pH 9.0 (○), human MAO A at pH 7.5 (■), and human MAO A at pH 9.0 (□). A is the coefficient determined for the E_s contribution and is −0.3 ± 0.1 for the rat enzyme and −0.4 ± 0.1 for the human enzyme.

Table 6. Correction of Para-Substituted Benzylamine Rat MAO A Binding Constants for the Selective Binding of the Deprotonated Forms

para substituent	amine pK_a[a]	calcd K_d (μM)	pK_a-corrected K_d (μM)[b]	log K_d (M)
H	9.3	460	6.7	−5.2
CF_3	8.8	284	15	−4.8
Br	9.1	30	0.76	−6.1
Cl	9.1	29	0.75	−6.1
F	9.3	103	1.7	−5.8
Me	9.5	41	0.39	−6.4
MeO	9.6	58	0.43	−6.4

[a]Benzylamine analogue pK_a values were taken from ref 12. [b]pK_a-corrected binding constants were calculated using eq 2.

found it necessary to correct the calculated K_d values to reflect the concentration of the deprotonated amine to observe reasonable correlations. Equation 2 is used to correct dissociation constants of each benzylamine analogue with MAO A's:

$$K_{d(corrected)} = \frac{K_{d(calculated)}}{1 + \text{antilog}(pK_a - pH)} \quad (2)$$

where pH is that of the assay buffer for each analogue and the corrected K_d value for each analogue is listed in Table 6.

Linear regression analysis of these K_d values with substituent parameters shows the best correlation to involve the steric parameter (V_w) (Table 7). No additional improvement is observed with two-parameter analysis. Essentially identical correlations of increased binding affinity with increased size of the para substituent are observed with the log K_d versus V_w plots previously observed for human MAO A[12] and shown with rat MAO A (Figure 3). The following equations do show that the rat enzyme binds these benzylamine analogues with an ~10-fold higher affinity than does the human enzyme but that both are largely dependent

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Table 7. Correlations of Binding Affinities (K_d) of Deprotonated Para-Substituted Benzylamine Analogues to Rat MAO A with Steric, Electronic, and Hydrophobic Substituent Parameters

parameter	correlation (slope)	*y*-intercept	correlation coefficient	*F*	significance
σ	0.60 ± 1.10	−6.00 ± 0.20	0.26	0.3	0.613
E_s	0.78 ± 0.29	−5.42 ± 0.24	0.81	7.4	0.053
V_w	−0.77 ± 0.15	−5.15 ± 0.18	0.93	25	0.007
π	−0.54 ± 0.54	−5.79 ± 0.28	0.45	1.0	0.373
$\pi + V_w$	−0.04 ± 0.29, −0.76 ± 0.20	−5.15 ± 0.21	0.93	9.6	0.050
$\pi + E_s$	1.21 ± 0.44, 1.62 ± 0.36	−5.26 ± 0.18	0.95	13	0.032
$\sigma + V_w$	0.18 ± 0.48, −0.76 ± 0.18	−5.16 ± 0.21	0.93	10	0.047
$\sigma + \pi$	2.06 ± 0.86, −1.29 ± 0.47	−5.53 ± 0.22	0.86	4.1	0.139

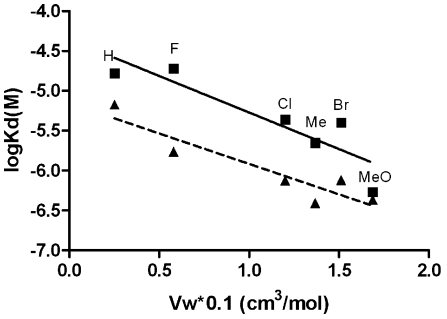

Figure 3. Comparison of correlations of the binding of para-substituted benzylamine analogues to human MAO A (■)[12] and rat MAO A (▲) with the van der Waals volume (V_w) of the para substituent. All binding constants are corrected for selective binding of the deprotonated amine.

on the steric parameter V_w:

$$\text{rat: } \log K_d = -0.8(\pm 0.2)(0.1V_w) - 5.2(\pm 0.2)F_{1,6} = 25,\ P = 0.007$$

$$\text{human: } \log K_d = -0.9(\pm 0.2)(0.1V_w) - 4.4(\pm 0.3)F_{1,6} = 16,\ P = 0.016$$

DISCUSSION

Although rat MAO A is ∼90% identical in sequence with the human enzyme, a number of differences in properties warranted this comparative functional study. The rat enzyme crystallizes as a dimer,[26] while the human enzyme crystallizes as a monomer,[8,27] even though both enzymes are found to be dimeric in their membrane-bound forms.[10] Other differences include a more solvent accessible active site for the human enzyme[28] and different dependencies of para substituent size in the binding of *p*-thioalkyl amphetamines.[11] Finally, the altered surface charge of the rat enzyme relative to the human enzyme is evident in its weaker binding to anion exchange columns on purification[14] versus that observed for the human enzyme.[29] In spite of these observed differences in properties, the results in Table 1 show that the rat MAO A specificities and affinities for the "MAO A" selective reversible inhibitors are similar to those of human MAO A. This behavior is consistent with available structural data showing the monopartite active site cavities of the two enzymes are quite similar.[8,26]

pH-Dependent Activity Profiles. Dunn et al.[13] have deduced from the pH−k_{cat}/K_m profiles for human MAO A that there are two distinct ionizations influencing catalytic activity with benzylamine analogues: an enhancing deprotonation occurring at a pK_a of 8.5 ± 0.1 and an inhibitory ionization with a pK_a of 9.2 ± 0.1. A similar pH-dependent k_{cat}/K_m profile with a pK_a of 7.5 ± 0.1 has been reported by Jones et al.[30] for the oxidation of kynuramine by human MAO A, which is somewhat lower (but probably within the error involved in fitting the data) than the value of 8.0 ± 0.2 reported by Dunn et al.[13] The conclusion reached by Jones et al.[30] that the protonated form of the substrate binds to the enzyme and undergoes a deprotonation before reaching the active site for oxidation is an interesting suggestion and requires additional investigation for verification. The hydrophobicities of the active sites of rat and human MAO A would favor entrance of a neutral form of the substrate rather than its protonated form. As shown in this study, effects of substituents on the pK_a of the ES complex and the perturbation of the macroscopic pK_a (by ∼2 pK units) provide evidence of a deprotonation step of the ES complex that must involve an initial binding of the protonated amine substrate prior to the chemical steps in catalysis. As stated above, the detailed molecular basis for this deprotonation step requires further investigation.

Rat MAO A exhibits a single activity-enhancing pK_a of 8.3 ± 0.1 with benzylamine, while the second (unobserved) inhibitory ionization presumably occurs with a pK_a of >10. Thus, on the free enzyme there are small differences in macroscopic pK_a values for groups that influence catalytic activity. Similar differences are observed with the single ionizations (due to the ES complexes with benzylamine) observed in k_{cat} versus pH profiles, which show a pK_a of 7.9 ± 0.1 for the human enzyme and a pK_a of 8.2 ± 0.1 for the rat enzyme. These observations are consistent with the conclusion reached by Dunn et al.[13] that binding of the amine substrate to the active site of either MAO A perturbs the pK_a of the amino group by 1−2 pK_a units. Rat MAO A oxidation of *p*-CF_3-benzylamine exhibits a pK_a for the ES complex (k_{cat}) that is ∼0.6 unit lower than that with benzylamine. This result is consistent with the influence of the substituent on the pK_a of the free amine. This behavior shows the ability of electronic substituent effects to be transmitted through the benzyl ring and to influence the reactivity of the benzyl protons as well as the amine N in the bound (ES) form of the substrate.

Structure−activity correlations of the rates of benzylamine analogue oxidation by rat MAO A show that the electronic parameter of the para substituent is a major determinant of alterations in reactivity with an observed ρ value of ∼1.0, which is consistent with the C−H bond cleavage rate being limited by a H^+

abstraction. If the cleavage mode were to follow a H^- transfer mechanism as suggested by a number of papers,[20,30] then a negative ρ value would be observed, which has been observed in model reactions of benzylamine analogue oxidation by hydride ion acceptors.[21,22] In a recent review[30] and in a previous paper on mouse polyamine oxidase,[20] Fitzpatrick and co-authors argued that the large positive ρ values observed with human MAO A can be attributed to electronic effects of the para substituents on the pK_a values of the amine group, because the structure−activity data were determined at pH 7.5 rather than at higher pH values where deprotonated amine levels would be independent of the nature of the para substituent. The basis for this criticism is that they observe a 0.5 decrease in ρ (from −0.09 to −0.59) for polyamine oxidase when the pH increases from 6.6 to 8.6.[31] In response to this criticism, our data with rat and human MAO A show the differences in ρ values to be somewhat smaller, because of increased contributions from steric parameters. However, they exhibit positive values (~1 or greater) as would be predicted for a mechanism involving a H^+ abstraction. The magnitudes of measured $^D(k_{cat})$ and $^D(k_{cat}/K_m)$ values are found to be independent of pH and of the nature of the para substituent, suggesting that there are no observable changes in mechanism over the pH range used and that the observed kinetic isotope effects are close to being intrinsic.

In conclusion, the results of this study further support the view that the functional properties of rat MAO A are similar to but not identical to those of the human enzyme. These differences include a 10-fold increased substrate binding affinity, 2−3-fold higher k_{cat} values for most substrates, and small differences in pK_a values for E and ES. QSAR behavior with para-substituted benzylamine analogues is nearly identical for the rat and human enzymes. In addition, this study provides additional support for the view that the reductive half-reaction in MAO A catalysis occurs with C−H bond cleavage involving a H^+ abstraction via a polar nucleophilic mechanism as suggested by the work on the human enzyme[12] and by arguments presented in recent reviews.[32,33]

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ABBREVIATIONS

MAO A, monoamine oxidase A; OMM, outer mitochondrial membrane; QSAR, quantitative structure−activity relationships; σ, substituent Hammett electronic constant; V_w, substituent van der Waals volume; π, substituent hydrophobicity constant; E_s, substituent Taft steric constant.

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