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Do MAO A and MAO B utilize the same mechanism for the C-H bond cleavage step in catalysis? Evidence suggesting differing mechanisms

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Summary

The detailed molecular mechanism proposed for the MAO-catalyzed oxidation of amines has been controversial with the basic assumption that both MAO A and MAO B follow the same pathway for the C-H bond cleavage step. Using the mechanistic approach of investigation of electronic effects of various benzylamine ring substituents in experiments at pH=9.0, human MAO A exhibits a kinetic behavior characteristic of a H^+ abstraction while human MAO B exhibits kinetic properties characteristic of a H^- abstraction. These results lead to the conclusion that the assumption that MAO A and MAO B follow identical mechanisms is incorrect.

Keywords

monoamine oxidase A; monoamine oxidase B; human; mechanistic properties

Introduction

The detailed molecular mechanism for the substrate C-H bond cleavage step in flavin-dependent amine oxidases has been a controversial area of research. Three different proposed mechanisms include a hydride ion abstraction (Fitzpatrick, 2010), a proton abstraction involving the transient covalent addition of the substrate to the flavin (Miller and Edmondson, 1999), and a proton abstraction following 1-electron transfer from the amine to the flavin (Lu *et al.*, 2002).

The mammalian monoamine oxidases (MAO A and MAO B) have been the most intensely studied members of this enzyme class by virtue of their pharmacological importance. The crystallographic structures of human MAO A and MAO B show them to exhibit similar α chain folds as might be expected from their ~70% sequence identity (Edmondson *et al.* 2009). Since the structures about their respective FAD sites are essentially identical, the general assumption in the literature is that they catalyze amine oxidation by identical mechanisms. Structure-activity studies on human and rat MAO A at pH values of 7.5 and 9.0 using a series of *para*-substituted benzylamine analogues show the C-H bond cleavage step to exhibit a positive p values which is consistent with C-H bond cleavage to occur by a H^+ abstraction from the carbon α to the amine (Miller and Edmondson, 1999; Wang and Edmondson, 2011). A similar behavior is also observed with zebrafish MAO (Aldeco *et al.*, 2010). In contrast, previous studies of bovine MAO B showed no electronic influence of the *para* substituent on the rate of flavin reduction (Walker and Edmondson, 1994). Studies with other flavin-dependent amine oxidizing enzymes have led to the proposal that C-H bond

cleavage occurs by a hydride ion mechanism (Fitzpatrick, 2010). Model system studies of benzylamine oxidation by hydride ion acceptors (Dubey *et al.* 2002; Shukla *et al.* 2003) show negative ρ values (-1.7), in contrast to the behaviors exhibited by human and rat MAO A. Recent ^{15}N kinetic isotope effect studies on human MAO B (McMillar *et al.* 2011) show that C-H bond cleavage is a catalytic step separate from C-N bond order changes which demonstrates the two steps are not concerted as might be expected for a hydride ion transfer mechanism. With the availability of purified human MAO A and MAO B in our laboratory as well as a series of *para*-substituted ^1H and ^2H benzylamine analogues, we investigated the kinetic behavior of human MAO B to determine whether it follows the behavior of MAO A or that of bovine MAO B. The data obtained provide evidence that human MAO B functions by a mechanism different from that of MAO A. Therefore, the general assumption in the literature that both enzymes function by identical mechanisms of C-H bond cleavage may be incorrect.

Materials and methods

Materials

Recombinant human MAO A and B were expressed in *Pichia pastoris* strain KM71 and purified using methods described previously (Newton-Vinson *et al.* 2001; Li *et al.* 2002). β -octylglucopyranoside was from Anatrace Inc., potassium phosphate and Bis-Tris from Sigma-Aldrich, and reduced Triton X-100 from Fluka. Benzylamine analogues were synthesized as described previously (Walker and Edmondson, 1994).

Determination of kinetic parameters

k_{cat} and K_{m} values were obtained by fitting steady state kinetic data to the Michaelis-Menten equation using GraphPad Prism 5.0. In cases where apparent substrate inhibition was observed the data were fit to the equation for substrate inhibition in GraphPad which is from equation 5.44 in Copeland, 2000. The resulting fits provided values of k_{cat} , K_{m} , and K_{is} where K_{is} is the inhibition constant for substrate inhibition. All assays were performed in air-saturated solutions. All steady state kinetic measurements of *para*-substituted benzylamine analog oxidation with human MAO B were performed in 50 mM Bis-Tris buffer (pH=9.0) containing 0.5% (w/V) reduced Triton X-100 at 25°C. The steady state rates of benzylamine analogue oxidation to the corresponding benzaldehyde were measured spectrophotometrically. Wavelengths and molar absorption extinction coefficients for each aldehyde are given by Walker and Edmondson (1994). Stopped flow data were acquired using an OLIS RM-1000 rapid scan instrument with all kinetic and spectral data processed using OLIS software.

Data analysis

Values of the substituent parameters for σ and π were obtained from Hansch *et al.* (1995) and for V_{w} (van der Waals volumes) from Bondi (1964). Multivariate linear regression analysis of rate data as a function of substituent parameters was performed using the StatView software package (Abacus Concepts). The pKa values for the ES complex were determined from rates of flavin reduction as a function of pH. Values for the pKa of E and S were obtained from plots of $k_{\text{cat}}/K_{\text{m}}$ vs pH. All pKa values were obtained from fits to equation 1 in Dunn *et al.* (2008) which was entered into GraphPad Prism 5.0 software

Results and discussion

pKa of ES complex of benzylamine with human MAO B

The effect of pH on MAO B activity has been shown to increase with pH and interpreted to reflect the ionization of the amine substrate. Jones *et al.* (2007) have shown the pH

dependence of k_{cat}/K_m for phenethylamine oxidation by membrane-bound MAO B exhibits pKa values of 7.1 and 9.3 which were interpreted to reflect the pKa values of the enzyme and substrate respectively. The pKa of the ES complex requires the determination of k_{cat} values at various pH values and would require determinations at saturating O_2 concentrations which is technically more challenging due to the high K_{mO_2} for MAO B. Our approach was to measure the pH dependence for the rate of MAO B flavin reduction under anaerobic conditions thus eliminating complications from the oxygen reaction. The data in Fig. 1a show the rate of flavin reduction of MAO B by benzylamine increases ~3-fold with pH. Analysis of the rate vs. pH data (Fig. 1b) shows an apparent pKa of 7.3 ± 0.3 which is assigned to the ionization of the ES complex. This value differs from that determined for MAO A which is found to be 8.0–8.2 for the human and rat enzymes (Wang and Edmondson, 2011; Dunn *et al.*, 2008). These results provide the experimental conditions to determine the steady state kinetic parameters on a series of *para*-substituted benzylamine analogues, independent of the differing pKa values of the respective ES complexes. Therefore, the influence of electron withdrawing or donating substituent on the benzylamine ring on k_{cat} was determined at pH=9.0.

Benzylamine substituent effects on steady state kinetic parameters

An unforeseen complication is observed when rate data for benzylamine analogue oxidation are collected at pH 9.0. The data in Fig. 2a show plots of rate vs. *p*-Br-benzylamine concentration at pH 7.5 and at pH 9.0. It is apparent from inspection of these plots that substrate inhibition is exhibited at pH 9.0 but not at 7.5. This behavior is not observed with MAO A or with the MAO B Ile199Ala-Tyr326Ala mutant where the bipartite cavity has been altered to a monopartite one (Milczek *et al.* 2011). Subject to further investigation, our interpretation of these results is the following. At pH 9.0, ~50% of the substrate is in its deprotonated form in solution. If the deprotonated substrate can enter the entrance cavity unhindered by the protein loops guarding the entrance, then one could imagine that two substrate molecules could sequentially bind to MAO B with one in the substrate cavity undergoing oxidation to the imine and the other bound in the entrance cavity. The binding of this second substrate in the entrance cavity would hinder release of the imine product, thus resulting in substrate inhibition as observed at pH 9.0. The apparent K_{IS} values estimated for substrate inhibition of MAO B at pH 9.0 are in the range of 1–2 mM for the different benzylamine analogues. The data in Table 1 show steady state kinetic parameters for the oxidation of a series of *para*-substituted benzylamine analogues by human MAO B at pH=9.0. All substrate analogues exhibit measurable deuterium kinetic isotope effects on k_{cat} and on k_{cat}/K_m therefore demonstrating that the C-H bond cleavage step contributes substantially to these kinetic constants as observed previously with MAO A (Miller and Edmondson, 1999; Wang and Edmondson, 2011).

Effect of *para* substituents on the rates of steady state turnover

To investigate if human MAO B exhibits the same effect of *para* substituent on the rate of turnover of benzylamine oxidation as does human MAO A, a linear regression analysis of $\log k_{\text{cat}}$ as a function of the electronic parameter σ was performed and compared with published values of human MAO A (Miller and Edmondson, 1999; Wang and Edmondson 2011). As shown in Fig. 3, the results from oxidation rates of 7 *para*-substituted benzylamine analogues were used for a linear regression analysis which exhibits a negative dependence on the electronic parameter and is best described by the relation:

$$\text{Log } k_{\text{cat}} = -0.9(\pm 0.1)\sigma + 2.3(\pm 0.1)$$

The addition of substituent parameters describing hydrophobicity or steric terms did not improve the statistical parameters for the correlation. The slope of this plot is $\rho = -0.9 \pm 0.10$ (correlation coefficient of 0.94), showing the rate of turnover *decreases* with electron-withdrawing substituents, which is opposite to the behavior exhibited by human MAO A at pH=9.0 ($\rho = +0.8 \pm 0.1$) (Wang and Edmondson, 2011). Therefore, human MAO B exhibits quite different catalytic QSAR parameters from that of MAO A which suggests the modes of C-H bond cleavage to occur via different mechanisms.

This conclusion is also apparent in $\log k_{\text{cat}}$ MAO A vs $\log k_{\text{cat}}$ MAO B for the same series of benzylamine analogues under identical experimental conditions (Fig. 4). If the enzymes utilized the same mode of C-H bond cleavage, one would expect similar responses to electron withdrawing or donating groups in the para position of the substrate. The plot in Fig. 4 shows a linear correlation ($r^2=0.9$) for the various analogues tested. Those substituents that show the highest turnover with MAO A are the poorest substrates for MAO B.

Taken together, these kinetic data provide reasonable evidence that MAO A and MAO B catalyze amine oxidations by differing mechanisms of C-H bond breakage. The observed electronic effects of the *para* substituents on catalytic rates support MAO B to function via a non-concerted H^- abstraction while MAO A functions *via* a H^+ abstraction which is likely via a polar nucleophilic mechanism. These proposed mechanistic alternatives are presented in the Scheme in Fig. 5. Additional evidence is required to provide added support for this proposal. If further experiments support this proposal, then the outstanding question that arises is what factors are employed to favor a given path.

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Fig. 1.

A. Stopped flow absorbance trace of the rate of MAO B flavin reduction at pH=6.8 (trace A) and at 8.8 (trace B). B. Influence of pH on the rate of MAO B reduction rate. The solid line is a fit of the data to the equation 1 in Dunn *et al.* (2008). All kinetic data were obtained at 25°C in 20 mM Bis-Tris propane buffer containing 0.5% (w/V) reduced Triton X-100 and 10% (w/V) glycerol

Fig. 2.

Plots of initial rates vs. substrate concentration for the MAO B-catalyzed oxidation of *p*-Br-benzylamine at pH = 7.5 and at pH = 9.0. The rates were determined at 25°C in 20 mM Bis-Tris propane buffer containing 0.5% (w/V) reduced Triton X-100 by following the formation of *p*-Br-benzaldehyde at 264 nm, $\Delta \epsilon_M = 21,700 \text{ M}^{-1}\text{-cm}^{-1}$

Fig. 3. Correlations of the steady state (k_{cat}) rates of MAO B oxidation of para-substituted benzylamines (k_{cat}) at pH= 9.0 with the substituent electronic value (σ). The statistical parameters for the correlation are: $F_{1,6} = 67.4$, $r^2=0.93$, and $p=0.0004$

Fig. 4.

Comparison of log k_{cat} values for human MAO B and human MAO A oxidation of seven *para*-substituted benzylamine analogues at pH =9.0. The linear plot shows a slope of -0.46 ± 0.07 with the following statistical parameters: $F_{1,6}=42$, $p=0.0013$, and $r^2=0.9$

Fig. 5.

Proposed schemes for modes of C-H bond cleavage steps in MAO A and in MAO B

Table 1

Steady State Kinetic Constants for MAO B Catalyzed Oxidation of p-Substituted Benzylamine Analogues at pH=9.0

Substituent	k_{cat} (min^{-1})	K_m (μM)	Dk_{cat}	$D(V/K)$
p-H	240 $\pm$ 4.4	77 $\pm$ 5.5	2.25	1.99
p-MeO	347 $\pm$ 3.7	49.3 $\pm$ 1.9	1.86	2.59
p-Me	243.8 $\pm$ 3.9	32.73 $\pm$ 2.3	2.67	4.73
p-F	182.5 $\pm$ 4.5	26.2 $\pm$ 3.6	1.44	1.75
p-Cl	167.3 $\pm$ 4.5	22 $\pm$ 1.7	2.10	1.90
p-Br	119.3 $\pm$ 6	19.7 $\pm$ 2.9	2.12	2
p-CF ₃	62.2 $\pm$ 3	15.4 $\pm$ 2.9	2.40	3.9