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The “gating” residues Ile199 and Tyr326 in human monoamine oxidase B function in substrate and inhibitor recognition

Erika M. Milczek^{1,3}, Claudia Binda², Stefano Rovida², Andrea Mattevi², and Dale E. Edmondson¹

¹Departments of Chemistry and Biochemistry, Emory University, Atlanta, Georgia 30322 USA

²Department of Genetics and Microbiology, University of Pavia, via Ferrata 1, Pavia 27100 Italy

Summary

The major structural difference between human monoamine oxidases A (MAO A) and B (MAO B) is that MAO A has a monopartite substrate cavity of ~550 Å³ volume and MAO B contains a dipartite cavity structure with volumes of ~290 Å³ (entrance cavity) and ~400 Å³ (substrate cavity). Ile199 and Tyr326 side chains separate these two cavities in MAO B. To probe the function of these gating residues, Ile199Ala and Ile199Ala Tyr326Ala mutant forms of MAO B were investigated. Structural data on the Ile199Ala MAO B mutant show no alterations in active site geometries compared to WT enzyme while the Ile199Ala-Tyr326Ala MAO B mutant exhibits alterations in residues 100–103 which are part of the loop gating the entrance to the active site. Both mutant enzymes exhibit catalytic properties with increased amine K_M but unaltered k_{cat} values. The altered K_M values on mutation are attributed to the influence of the cavity structure in the binding and subsequent deprotonation of the amine substrate. Both mutant enzymes exhibit weaker binding affinities relative to WT enzyme for small reversible inhibitors. Ile199Ala MAO B exhibits an increase in binding affinity for reversible MAO B specific inhibitors which bridge both cavities. The Ile199Ala-Tyr326Ala double mutant exhibits inhibitor binding properties more similar to those of MAO A than to MAO B. These results demonstrate the bipartite cavity structure in MAO B plays an important role in substrate and inhibitor recognition to distinguish its specificities from those of MAO A and provides insights into specific reversible inhibitor design for these membrane-bound enzymes.

Keywords

monoamine oxidase B; structure of methylene blue complex; inhibitor specificity; mutations of gating residues; dipartite to monopartite cavity conversion

Introduction

Monoamine oxidases [EC 1.4.3.4] (MAOs) are mitochondrial outer membrane-bound flavoenzymes that catalyze the oxidative deamination of biogenic amines and amine neurotransmitters (serotonin, dopamine, and epinephrine) [1]. They exist in two isoforms in mammals (MAO A and MAO B) as separately encoded X-linked gene products with amino acid sequences that are ~70% identical [2]. Age-related increases in MAO B levels in

Correspondence: Dr. Dale E. Edmondson, Department of Biochemistry, Emory University, 1510 Clifton Road, Atlanta, Georgia USA 30322, Tel.001-404-727-5972, Fax. 001-404-727-2738, deedmon@emory.edu.

³Present Address: Department of Chemistry, Princeton University, Princeton, NJ 08544 USA

Database

The atomic coordinates and structural factors have been deposited in the Protein Data Bank under the accession number **3zyx**.

neuronal tissues and resulting catalytic production of H_2O_2 (leading to reactive oxygen species) is thought to contribute to cellular apoptosis and subsequent neurodegenerative diseases [3]. Therefore, specific inhibitors of MAO B function as neuro-protectants.

Previous work has shown that long, planar compounds with extended π -conjugation, like 8-(3-chlorostyryl)caffeine [4] and *trans,trans*-farnesol [5], exhibit high specificities for reversible inhibition of human MAO B and do not bind to human MAO A. Investigations of the structures of human MAO B and MAO A show the main difference is the dipartite active site cavity in MAO B and monopartite active site cavity in MAO A to explain this differential binding behavior [6]. Ile199 of human MAO B functions as a conformational gate between the two cavities and is substituted by a Phe in bovine MAO B [5]. Of interest, bovine MAO B does not bind the above-mentioned compounds.

High-resolution crystal structures of human MAO B show Ile199 adopts distinct conformations depending on the nature of the inhibitor bound [7]. When small inhibitors are bound within the substrate cavity, the side chain of Ile199 rotates into a closed conformation which creates the bipartite active site. With larger inhibitors such as *trans,trans*-farnesol bound in the active site, the side chain of Ile199 occupies an open conformation resulting in the fusion of the two cavities to one of $\sim 700 \text{ \AA}^3$. These observations provide the groundwork for the suggestion that Ile199 serves as a structural determinant for substrate and inhibitor recognition [5]. Such a “gating” function is not observed with MAO A since it contains a monopartite active site cavity.

In addition to conformational alterations of the Ile199 “gating” residue, the side chain of Tyr326 exhibits modest conformational changes on inhibitor binding (such as rasagiline) to human MAO B [8]. Tyr326 and Ile199 thus serve to separate these two cavities in human MAO B. To probe the respective roles of Ile199 and Tyr326 in the maintenance of the active site geometry as well as function in substrate and inhibitor recognition, the Ile199Ala and Ile199Ala-Tyr326Ala mutant forms of human MAO B were constructed, expressed, and purified. An Ile199Ala mutation would permanently “open” the gate with unknown functional consequences. A double mutant involving Ile199 and Tyr326 is proposed to create a large monopartite active site in MAO B which should dramatically alter its substrate and inhibitor binding specificities. As shown in this paper, these alterations of MAO B convert the enzyme to one with functional properties more similar to those of MAO A and provide new insights into the gating function of the protein loop guarding the opening to the entrance cavity.

Results

Structural Determination of the Ile199Ala-Tyr326Ala Mutant Form of MAO B

Previous structural work from this laboratory has defined the $1.65 \text{ \AA}$ structure of WT recombinant human MAO B [7] and the $2.0 \text{ \AA}$ structure of the Ile199Ala mutant enzyme [9]. These data show that replacing the isopropyl side chain of Ile199 with a methyl group has no deleterious influence on the structural integrity of the enzyme's active site. In designing crystallization trials with the Ile199Ala-Tyr326Ala double MAO B, preliminary experiments showed that methylene blue, a strong MAO A inhibitor [10], binds to the double mutant with an affinity higher than that observed with WT MAO B. As will be discussed below, the increase in size of the active site cavity and its conversion from a dipartite to monopartite structure are responsible for this increase in affinity. The methylene blue-inhibited Ile199Ala-Tyr326-Ala MAO B mutant readily formed crystals which diffract to $2.2 \text{ \AA}$ resolution. The structure was solved by molecular replacement. Crystallographic data statistics are shown in Table 1 and the structure is shown in Figure 1A–C. These data show that replacement of the isopropyl and phenolic side chains of Ile199 and Tyr326 with the

methyl groups of alanyl residues have no major effect on the structure of the enzyme or on the conformations of other residues about the active site. The major effect of these mutations is to alter the dipartite cavity structure of MAO B to one that is monopartite with a calculated volume of 732 Å³. Notably, the loop and especially Phe103 shift by about one Angstrom toward the side chain at position 199. In this regard, the structure of the double mutant exhibits dual structural consequences as a single cavity is observed (as in the complexes with large inhibitors such as safinamide [11]) and Phe103 adopts a conformation similar to that found in the MAO B structures in complexes with small inhibitors where the Ile199 side chain gate is in its “closed” position [7].

Catalytic properties of the mutant forms of MAO B

To examine the effects of both single and double mutations on the catalytic properties, the kinetic properties for the oxidation of the substrates kynuramine, benzylamine, and of several phenylalkylamines were determined (Table 2). Kynuramine is oxidized by either MAO A or MAO B and the various phenylalkylamines allow comparisons of side chain length on catalytic turnover. Similar turnover numbers (k_{cat}) are observed for wild type and the two mutant enzymes; however, the K_M values observed for the mutant enzymes are found to be considerably higher than those for WT enzyme. Phenethylamine, a traditional MAO B substrate, exhibits a 4-fold increase in K_M with the single mutant while the double mutant exhibits a K_M value 75-fold higher than that found with WT enzyme (Table 2). The alteration in K_M values with the substrates tested result in catalytic efficiencies (k_{cat}/K_M) values that are between 1–27 % of those observed with WT enzyme. Benzylamine is a poor substrate for the double mutant with a K_M value too high to measure at pH 7.5. The rates of oxidation catalyzed by the double mutant are linearly proportional to the concentration of benzylamine in the assay (Figure 2). Increasing the pH of the assay medium to a value of 9.3 results in a decrease in the K_M value ($K_M = 866 \mu\text{M}$) suggesting that the reason for non-saturation with this substrate at lower pH is due to the inability of the double mutant enzyme either to bind the protonated form of the substrate or to facilitate deprotonation of the amine moiety in the ES complex for catalysis to efficiently proceed. These results suggested a role for these gating residues in presenting the deprotonated amine substrate for the reductive half-reaction with the flavin cofactor.

To provide more in depth insights into the pH-dependent behavior of MAO B and its mutant forms, the rates of oxidation of the substrates benzylamine ($\text{pK}_a = 9.33$) and *p*-CF₃-benzylamine ($\text{pK}_a = 8.75$) were determined in the pH range of 7.0 – 9.5. These two substrates have amine pK_a values differing by 0.58 pK_a units which differ enough to be observable on comparison of pH-dependent kinetic parameters for WT and mutant enzymes. Plots of V/K vs pH provide estimates of pK_a values of groups important in catalysis for the free enzyme and the ionization form amenable for catalysis for the free substrate. Jones et al. [12] found pK_a values of 7.1 for MAO B indicating an enzyme group deprotonation to enhance catalytic activity and a value of 9.97 for the pK_a of a group where deprotonation diminishes catalytic activity in plots of the pH-dependence of V/K for the MAO B-catalyzed oxidation of phenethylamine. These results were interpreted [12] to suggest that deprotonation of a group with a pK_a of 7.1 on the free enzyme is required for activity and that deprotonation of the amine ($\text{pK}_a = 9.97$) of the substrate diminishes catalytic function suggesting that the protonated form of the substrate is bound in the active site. It should be pointed out that assignment of higher pK_a to the free form of the substrate was based on the similarity of the observed pK_a with the known pK_a of phenethylamine.

Using two substrates with differing pK_a values for their respective amine moieties (see above), we find that WT MAO B and the two gate mutants exhibit ascending catalytic activities with pH using either benzylamine or with *p*-CF₃-benzylamine as substrates (Figure 2B and C). Analysis of the V/K data using the method outlined by Fersht [13] show identical

values of 8.7 ± 0.1 using either benzylamine or *p*-CF₃-benzylamine for WT MAO B (Figure S-2 and Table 3). The identities observed demonstrate that the assignment of these pK_a values to be due to a catalytically essential group on the free enzyme species (E). If these pK_a values were due to the ionization of the free substrate, then a differential pK_a (~0.6) would be observed on comparison of benzylamine and *p*-CF₃-benzylamine data. Examination of the Ile199Ala mutant shows pK_a values of 8.8 ± 0.2 and 8.6 ± 0.3 for the free enzyme and the same substrates (Figure 2b, Figure S-2, and Table 3). Thus, little or no perturbations of pK_a value of this catalytically essential group in the free form of MAO B is observed on mutation of the Ile199 gate. The pH-dependence of V/K with the double mutant show a value of 8.7 ± 0.2 with benzylamine and a value of 8.2 ± 0.3 with *p*-CF₃-benzylamine. The pK_a values for the single and double mutant form have more uncertainty due to the lower levels of V/K values exhibited for each mutant. Extrapolation of V/K for the fully deprotonated free enzyme forms show, with benzylamine, the single mutant is 42 % and the double mutant is ~5 % of WT activity. Using *p*-CF₃-benzylamine as substrate, the single mutant exhibits 33 % and the double mutant 24 % WT activity. These data demonstrate that the presence of a *p*-CF₃ substituent on the substrate enhances catalytic turnover relative to the unsubstituted ring of benzylamine in the double mutant. Whether this is due to electronic effects of the electron withdrawing substituent or to the H-bonding capability of the substituent is not known.

The observed pK_a values of E with WT and mutant enzymes (~8.6) are quite different from that observed with human granulocyte MAO B (pK_a = 7.1) [12] using phenethylamine as substrate. The main difference in experimental protocols is that Jones et al. [12] used membrane preparations of MAO B while this work used purified preparations of recombinant human MAO B. Thus, we propose that the differences in observed values may reflect the influence of the membrane environment on the pK_a of this residue whose deprotonation is required for optimal activity. There is no obvious residue in the substrate cavity other than Cys172 that could ionize in this pH range, however, previous mutagenesis studies [14] have shown this residue is not required for catalytic activity. Therefore, the location of the residue whose ionization is responsible is likely to be near the surface of the protein in or near the loop (residues 99–112) that shields the entrance cavity. Possible candidates include Lys95 or Lys93. There are His residues (residues 90, 91, and 115) flanking this shielding loop that are possible but less likely candidates.

Plots of k_{cat} vs pH are expected to follow ionizations of the ES complexes that would have catalytic functional importance in MAO B and its mutant forms. As pointed out by Jones et al. [12] the fact that [O₂] at air saturation in the catalytic assays is not saturating with MAO B brings an additional level of complexity in the estimation of pK_a values from such data. The influence of these mutations on O₂ reactivity and $K_{\text{M}}\text{O}_2$ behavior is not known and will be investigated in future studies since the monopartite MAO A exhibits a low $K_{\text{M}}\text{O}_2$ and its reaction with O₂ is dependent on the presence of substrate/products in the active site of the reduced enzyme [15]

It is of interest that phenylbutylamine (Table 2) exhibits saturation behavior at pH 7.5 with the double mutant in contrast to what was observed with benzylamine. This behavior is not a reflection of their respective differences in amine pK_a values which are quite similar. The longer alkyl side chain may permit interactions of the phenyl ring with the residues in the nascent entrance cavity site of the double mutant leading to an environment facilitating amine deprotonation of the free enzyme for optimal catalysis.

Inhibitor binding properties of the mutant forms of MAO B

To determine the influence of the gating residues on inhibitor binding, several classes of MAO inhibitors (MAOIs) were tested (for structures, see Figure S1). Isatin is a non-specific

reversible inhibitor that binds to WT MAO B in the substrate cavity [7]. It competitively binds to the Ile199Ala single mutant ($K_i = 12 \mu\text{M}$) with an affinity similar to that of MAO A ($K_i = 15 \mu\text{M}$) [16], however, it inhibits the Ile199Ala-Tyr326Ala double mutant ($K_i = \sim 360 \mu\text{M}$) with a ~ 100 -fold weaker affinity than to WT MAO B ($K_i = 3 \mu\text{M}$) (Table 4). Tranylcypromine binding is not dramatically influenced by mutations to the MAO B cavity structure and this observation is consistent with its known non-specificity for MAO A or MAO B [17]. Weak binding of aminoindane or methylaminoindane (R or S isomers) to the MAO B double mutant is also observed which were shown previously to also bind solely to the substrate cavity of WT MAO B [18] (data not shown). Therefore, removal of one “wall” (i.e. the Ile199 and Tyr 326 side chains; Fig. 2) of the substrate cavity of MAO B results in an enzyme with a considerable loss of affinity for compounds that bind within the MAO B substrate cavity.

Previous work has shown that specific MAO B inhibitors bind by traversing both the entrance and the substrate cavities, which forces Ile199 into an open conformation allowing fusion of the two cavities [5,6]. These inhibitors exhibit no observable binding to WT MAO A thus demonstrating the role of the bipartite cavity structure of MAO B in specificity. Removal of the gating residue in MAO B (the Ile199Ala mutant form) results in either increased (7-to 24-fold higher) or no difference in affinities for the MAO B-specific inhibitors safinamide ($K_i = 21 \text{ nM}$), farnesol ($K_i = 2.7 \mu\text{M}$), 1,4-diphenyl-2-butene ($K_i = \sim 9 \mu\text{M}$) and 1,4-diphenyl-1,3-butadiene ($K_i = 1.0 \mu\text{M}$) (Table 5). The double mutant exhibits decreased affinities for these dual cavity spanning specific inhibitors binding safinamide ($K_i = 4 \mu\text{M}$) with a ~ 10 -fold weaker affinity than WT MAO B. A weaker binding of *trans,trans*-farnesol and no detectable binding of 1,4-diphenyl-2-butene or of 1,4-diphenyl-1,3-butadiene is found with the double mutant. In agreement with other functional data described above, loss of the dipartite cavity results in an enzyme that functionally resembles human MAO A in inhibitor binding properties.

Known MAO A-specific inhibitors are characterized by bulky fused ring systems which exhibit higher affinities for its larger monopartite active site. The reversible inhibitors pirlindole, harmaline, and methylene blue inhibit MAO A with high affinities [10,19] and demonstrate weak inhibitory behavior with WT MAO B (Table 6). The Ile199Ala mutant also exhibits weak (micromolar) or no affinities for these inhibitors indicating this gating residue between the bipartite cavities does not play an important role in this specificity difference. Removal of the dipartite cavity structure results in a MAO B form that exhibits higher affinities for these MAO A specific reversible inhibitors than either WT or the single mutant (Table 6). In the case of pirlindole, neither MAO B nor the Ile199Ala single mutant are inhibited, while the Ile199Ala-Tyr326Ala double mutant is competitively inhibited in the low μM range ($K_i = 4.1 \mu\text{M}$). Methylene blue is a tight binding inhibitor of MAO A ($K_i = 25 \text{ nM}$) [10], the single mutant ($K_i = 250 \text{ nM}$), and of the Ile199Ala-Tyr326Ala mutant ($K_i = 143 \text{ nM}$). In contrast, it is bound with lower affinity to MAO B ($K_i = 1 \mu\text{M}$). Examination of the crystal structure of the methylene blue complex with the double mutant shows that the orientation of the bound ligand (Fig. 1 A–C) would clash with the side chain of Tyr326 in either WT or the Ile199Ala mutant forms. Therefore, we predict the conformation of bound methylene blue in these enzymes would be altered to accommodate the observed binding. The inhibitory properties of the double mutant with MAO A-specific inhibitors show a “gain of function” which results from formation of a large, monopartite cavity. This structural change also results in a “loss of function” as the MAO B specific inhibitor zonisamide ($K_i = 3.1 \mu\text{M}$) [20] exhibits no detectable inhibition of the double mutant.

Discussion

The structural and functional results of this study present insights into the role of the dipartite cavities in human MAO B and on the function of the gating residue (Ile 199) whose conformation is relevant for inhibitor and substrate function. Binding small inhibitors like isatin to WT MAO B results in the Ile199 gate rotating to a closed conformation [5]. Permanently opening the 'gate' through mutation of Ile199 to an Ala results in 3–4-fold reduced binding affinity of smaller inhibitors that bind only in the substrate cavity. MAO B specific inhibitors that traverse both the entrance cavity and the substrate cavity force the Ile199 'gate' to rotate into its open conformation resulting in the fusion of the two cavities. The Ile199Ala mutation has a positive effect on binding of this class of inhibitors with an increase of up to 24-fold higher affinity than to WT. The results of inhibitor binding to the MAO B double mutant is consistent with the structural data in Figure 1 showing that the cavity structure of this form is monopartite rather than bipartite. MAO A has a monopartite active site cavity and therefore, analysis of MAO A specific inhibitors provides insights into the properties of the Ile199Ala-Tyr326Ala active site. MAO A-specific inhibitors bind to the double mutant with increased affinities relative to WT MAO B but with lower affinities than observed with MAO A. Smaller inhibitors like isatin bind to the double mutant considerably more weakly than to WT enzyme. MAO B-specific inhibitors which favor a bipartite active site bind to the double mutant with ~10-fold weaker affinities or not at all.

Structural data on the double mutant enzyme show interesting structural effects that deserve some comment. Phe103 is situated on the protein loop that guards access to the entrance cavity. When Ile199 is in an "open" position, the side chain of Phe103 is forced into a conformation that results in the protein loop closing off access to the entrance cavity. When Ile199 is in its "closed" conformation, the Phe103 side chain does not experience this steric clash and is now in a conformation resulting in an "open" conformation of this "guarding" protein loop. Presumably, in the ligand free-state, these conformations are rapidly inter-converting thus providing access to the active site. Conversion of Ile199 to an Ala residue results in this Phe103 side chain to be in a conformation that favors opening the "guarding" loop in front of the entrance cavity. Visualization of this process can be observed in Figures 1B and C. Comparison of the cavity structure in the double mutant with those determined previously with bound inhibitors where both "open" and "closed" conformations were present show that these mutations have now resulted in a larger volume (~65 Å³) of a monopartite cavity as well as increased its volume relative to that found when the Ile199 gate is open.

The functional consequences of this structure in the double mutant are evident with altered ligand binding properties and in catalytic properties as shown in Figure 2. The alteration in K_M value for benzylamine in the double mutant (Figure 2A) is best explained by the failure of this mutant enzyme to facilitate deprotonation of the ES complex. Since neither mutated residue is expected to exhibit pKa values in the range observed for WT enzyme and the pKa values of the residue required for optimal catalysis in the free enzyme is not changed, the difference probably originates from alterations in catalytic site hydrophobicity and/or water content.

Alterations in structure of the guarding "entrance loop" by mutagenesis or by membrane interactions are thought to have important influences on the properties of the active site. In this respect, it would be of interest to measure the pKa for deprotonation of recombinant MAO B in its membrane associated form to compare with purified preparations to address the question of membrane influence of pKa values. A more detailed experimental and theoretical description of the dynamics of this process will be the subject of further work.

Materials and Methods

Reagents

The reagents used in this study were obtained from commercially available sources unless otherwise stated. All benzylamine analogues used in this study were purchased from commercial sources or were synthesized using procedures previously described [21]. MAO A and MAO B inhibitors that are commercially available were purchased from Tocris Bioscience, Ellisville, Missouri, USA or from Sigma-Aldrich, St. Louis, Missouri, USA. Safinamide was a gift from Newron Pharma (Milan, Italy). *trans,trans*-Farnesol and 8-(3-cholorostyryl)caffeine were generously supplied by Dr. Neal Castagnoli, Virginia Tech. University. The structures of the various MAO inhibitors used in this study are shown in Supplementary Information.

Enzymes

Recombinant WT human liver MAO B [22] and MAO A [23] were expressed in *Pichia pastoris* and purified by published protocols. The Ile199Ala MAO B and Ile199Ala-Tyr326Ala mutant enzymes were prepared by gene mutations using the Stratagene Quik-Change XL Site-Directed Mutagenesis kit and confirmed by gene sequence analysis. The mutant enzymes were expressed and purified using the protocol for WT enzyme.

Catalytic Assays

Standard MAO A and MAO B activity assays were performed spectrophotometrically using *p*-CF₃-benzylamine ($\Delta\epsilon = 11,800 \text{ M}^{-1}\text{-cm}^{-1}$, $\lambda = 243 \text{ nm}$) and benzylamine ($\Delta\epsilon = 12,800 \text{ M}^{-1}\text{-cm}^{-1}$, $\lambda = 250 \text{ nm}$), respectively, at 25 °C in 50 mM phosphate buffer (pH 7.5) containing 0.5% (w/v) reduced Triton X-100. Assays with other benzylamine analogues were performed as described previously [21,24]. MAO B Ile199Ala assays were performed in the same buffer using *p*-CF₃-benzylamine as substrate and MAO B Ile199Ala-Tyr326Ala assays using *p*-Br-benzylamine ($\Delta\epsilon = 21,700 \text{ M}^{-1}\text{-cm}^{-1}$, $\lambda = 264 \text{ nm}$) at 25 °C in 50 mM CHES (pH 9.3) and 0.5% (w/v) reduced Triton X-100. Phenethylamine and other phenylalkylamine oxidations were monitored spectrally using the horse radish peroxidase coupled Amplex red assay ($\Delta\epsilon = 54,000 \text{ M}^{-1}\text{-cm}^{-1}$, $\lambda = 560 \text{ nm}$). Kynuramine oxidation was monitored spectrally ($\Delta\epsilon = 12,000 \text{ M}^{-1}\text{-cm}^{-1}$, $\lambda = 316 \text{ nm}$).

The interaction of various inhibitors with the WT and mutant enzymes were determined by measuring the initial rates of substrate oxidation (six different concentrations) in the presence of varying concentrations of inhibitor (a minimum of four different concentrations). K_i values were determined using global fit analysis of the hyperbolic fits of enzyme activity with inhibitor concentrations using Graphpad Prism 5.0 software.

pH Studies were performed spectrophotometrically using *p*-CF₃-benzylamine or benzylamine as substrates at 25 °C in a buffer containing 50 mM potassium phosphate, 50 mM sodium pyrophosphate phosphate, and 50 mM CHES with 0.5% (w/v) reduced Triton X-100. The pH was adjusted to 7.5, 8.0, 8.5, 9.0, or 9.5 depending on the assay. pKa Estimations from steady state kinetic data used approaches and equations described by Dunn et al. [25] and by Fersht [13]. Fits of experimental data to these equations were performed using GraphPad Prism 5.0 software.

Crystallographic methods

Crystallographic studies were performed as previously described [7]. Briefly, mutant enzyme solutions in the presence of methylene blue in 25 mM potassium phosphate pH 7.5 and 8.5 mM Zwittergent 3–12 (Anatrace, Affymetix, Maumee, Ohio USA) were crystallized by mixing equal volumes of protein sample and reservoir solution (12% (w/v) polyethylene

glycol 4000, 100 mM N-(2-acetamido)-2-iminodiacetic acid buffer pH 6.5, and 70 mM lithium sulphate. Diffraction data were collected at 100 K at the European Synchrotron Radiation Facility in Grenoble, France. Data processing and scaling were carried out using MOSFLM [26] and programs of the CCP4 package [27]. Crystallographic refinements were performed with the programs REFMAC5 [28] and COOT [29]. Structural illustrations were produced using Pymol (www.pymol.org).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MAO A	monoamine oxidase A
MAO B	monoamine oxidase B
CHES	2-(cyclohexylamino)-ethane sulfonic acid
PDB	Protein Data Bank
CSC	8-(3-Chloro)styrylcaffeine
DPB	1,4-Diphenyl-2-butene
DPBD	1,4-Diphenyl-1,3-butadiene

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Figure 1.

A. Ribbon overall structure of the human MAO B monomer (Ile199Ala/Tyr326Ala double mutant) in complex with methylene blue. The FAD cofactor is represented in yellow, whereas the methylene blue inhibitor bound to the active site cavity (semitransparent gray surface) is in blue. B. Active site residues of human MAO B Ile199Ala/Tyr326Ala double mutant in complex with methylene blue. Nitrogen atoms are blue, oxygens are red, sulfurs are yellow; and carbon atoms are gray. FAD is in yellow and methylene blue is in blue for residues. Water molecules are shown as red spheres. Unbiased electron density map (contoured at 1.2 σ) is shown for the inhibitor, the FAD cofactor and the double mutation sites. C. Zoomed view of the double mutant MAO B structure (in blue, same orientation as in Fig. 1a) superposed to the wild type protein bound to safinamide (in cyan; PDB code 2v5z). The sites of mutation (Ile199Ala and Tyr326Ala) are shown. Phe103 is also drawn to highlight the different conformation of the side chain and of the cavity gating loop (residues 99–104). Previous human MAO B structures have demonstrated that the conformation of this loop depends on the dipartite nature of the active site cavity which in turn is related to the Ile199 conformation and the inhibitor bound.

Figure 2.

Comparisons of catalytic behaviors of WT MAO B (■), Ile199Ala MAO B (▲) and Ile199Ala/Tyr326Ala MAO B (●). A. WT MAO B and Ile199Ala/Tyr326Ala velocities in response to benzylamine concentrations in their respective assays. B. Effect of pH on k_{cat}/K_M of WT MAO B, Ile199Ala MAO B, and Ile199Ala/Tyr326Ala MAO B using benzylamine as substrate. C. Effect of pH on k_{cat}/K_M of WT MAO B, Ile199Ala MAO B, and Ile199Ala/Tyr326Ala MAO B using *p*-CF₃-benzylamine as substrate.

Table 1

Data collection and refinement statistics for the structure of human MAO B I199A/Y326A double mutant complex with methylene blue

Space group	C222
Unit cell axes (Å)	$a = 131.9, b = 225.0, c = 86.6$
Resolution (Å)	2.2
$R_{\text{sym}}^{a,b}$ (%)	11.0 (33.0)
Completeness ^b (%)	98.2 (89.8)
Unique reflections	64,424
Redundancy	4.4 (2.5)
I/σ^b	10.8 (2.8)
N° of atoms for protein/FAD/ligand/water	7894/2×53/2×20/534
Average B value for ligand atoms (Å ²)	59.2
R_{cryst}^b (%)	19.0 (24.1)
R_{free}^b (%)	24.4 (32.2)
Rms bond length (Å)	0.013
Rms bond angles (°)	1.38

^a $R_{\text{sym}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of i^{th} observation and $\langle I \rangle$ is the mean intensity of the reflection.

^b Values in parentheses are for reflections in the highest resolution shell.

^c $R_{\text{cryst}} = \sum |F_{\text{obs}} - F_{\text{calc}}| / \sum |F_{\text{obs}}|$ where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively. R_{cryst} and R_{free} were calculated using the working and test sets, respectively.

Table 2

Comparisons of substrate specificities of WT and mutant forms of MAO B at pH 7.5

Substrate	Enzyme	k_{cat} (min^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{min}^{-1}\mu\text{M}^{-1}$)
Kynuramine	WT MAO B	96 ± 1	27 ± 2	3.55 ± 0.27
	I199A	88 ± 2	94 ± 8	0.936 ± 0.082
	I199A-Y326A	64 ± 2	289 ± 23	0.22 ± 0.02
Benzylamine	WT MAO B	300 ± 8	150 ± 27	2.0 ± 0.4
	I199A	228 ± 13	2133 ± 409	0.107 ± 0.021
	I199A-Y326A ^a	2nd order rate =: $0.0106 \pm 0.0002 \mu\text{M}^{-1}\text{min}^{-1}$		
Phenylethylamine	WT MAO B	172 ± 3	9.4 ± 0.9	18 ± 2
	I199A	123 ± 2	40 ± 2	3.1 ± 0.2
	I199A-Y326A	110 ± 2	703 ± 58	0.16 ± 0.01
Phenylbutylamine	WT MAO B	110 ± 5	19 ± 3	5.8 ± 0.3
	I199A	135 ± 3	8.9 ± 0.7	15.2 ± 0.6
	I199A-Y326A	142 ± 1	55 ± 2	2.6 ± 0.1

^aThe kinetic values were determined to be $k_{\text{cat}} = 283 \pm 4 \text{ min}^{-1}$, $K_{\text{M}} = 866 \pm 34 \mu\text{M}$ in 50 mM CHES at pH 9.3 with 0.5% (w/v) reduced Triton X-100 buffer in the activity assay.

Table 3

Comparisons of pK_a values for deprotonation of a catalytically-essential group in WT and mutant forms of MAO B with benzylamine and *p*-CF₃-benzylamine as substrates

Enzyme	k_{cat}/K_M pK _a values with benzylamine	k_{cat}/K_M pK _a values with <i>p</i> -CF ₃ -benzylamine
WT MAO B	8.6 ± 0.1	8.7 ± 0.1
Ile199Ala MAO B	8.8 ± 0.1	8.6 ± 0.3
Ile199Ala/Tyr326Ala MAO B	8.7 ± 0.2	8.2 ± 0.3

Table 4

Comparisons of inhibition constants for non-specific MAO inhibitors with WT MAO B, WT MAO A, and mutant forms of MAO B

Enzyme	Isatin K_i (μ M)	Tranylepromine K_i (μ M)
WT MAO B	3 ^a	16 ^b
WT MAO A	15 ^a	19 ^b
MAO B Ile199Ala	12 $\pm$ 2	11 $\pm$ 3
MAO B Ile199Ala-Tyr326Ala ^c	360 $\pm$ 40	17 $\pm$ 3

^a Taken from work cited in reference [5].

^b Taken from work cited in reference [17].

^c Data collected at pH 7.5 for WT and single mutant. Double mutant data were collected at pH 9.3.

Table 5Inhibition constants for MAO B-specific reversible inhibitors with WT and mutant enzymes^a

Enzyme	K _i (μM)				
	Farnesol	DPB	Safinamide	CSC	DPBD
WT MAO B	2.3 ± 0.4 ^b	34.5 ± 1.4 ^b	0.50 ± 0.10 ^c	0.27 ± 0.08 ^b	7.0 ± 0.2 ^b
Ile199Ala MAO B mutant	2.7 ± 0.4	8.1 ± 1.6	0.021 ± 0.002	no inhibition	1.00 ± 0.03
Ile199Ala-Tyr326Ala MAO B mutant	18 ± 3	no inhibition	4.0 ± 0.6	1.7 ± 0.3	no inhibition

^aData collected at pH 7.5 for WT and single mutant. Double mutant data were collected at pH 9.3.^bTaken from reference [5].^cTaken from reference [11].

Table 6 K_i values for MAO A-specific reversible inhibitors^a

Enzyme	Pirlindole K_i (μ M)	Harmane K_i (μ M)	Methylene Blue K_i (μ M)
WT MAO A	0.92 ± 0.04^b	0.58 ± 0.02^b	0.025 ± 0.001^c
WT MAO B	No inhibition	140 ± 47	1.01 ± 0.05
MAO B Ile199Ala	No inhibition	550 ± 50	0.214 ± 0.032
MAO B Ile199Ala-Tyr326Ala	4.1 ± 0.2	165 ± 17	0.143 ± 0.014

^aData collected at pH 7.5 for WT and single mutant. Double mutant data were collected at pH 9.3.^baken from reference [19].^cTaken from reference [10]