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Mutagenic Probes of the Role of Serine 209 on the Cavity Shaping Loop of Human Monoamine Oxidase A

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Summary

Literature reports implicating human monoamine oxidase A (MAO A) in apoptotic processes report levels of MAO A protein that do not correlate with activity suggesting unknown mechanisms may be involved in regulation of catalytic function. Bioinformatic analysis suggests Ser209 as a possible phosphorylation site that may be relevant to catalytic function since it is adjacent to a six-residue loop termed the “cavity shaping loop” from structural data. To probe the functional role of this site, MAO A Ser209Ala and Ser209Glu mutants were created and investigated. In its membrane-bound form, the MAO A Ser209Glu phosphorylation mimic exhibits similar catalytic and inhibitor binding properties as wild-type MAO A. Solubilization in detergent solution and purification of the Ser209Glu mutant results in considerable decreases in these functional parameters. In contrast, the MAO A Ser209Ala mutant exhibits similar catalytic properties to those of wild-type enzyme when purified. Compared with purified wild-type and Ser209Ala MAO A proteins, the Ser209Glu MAO A mutant shows significant differences in covalent flavin fluorescence yield, circular dichroism spectra and thermal stability. These structural differences in the purified MAO A Ser209Glu mutant are not exhibited in QSAR patterns using a series of *para*-substituted benzylamine analogues which are similar to wild-type enzyme. These data suggest that serine 209 in MAO A does not appear to be the putative phosphorylation site for regulation of MAO A activity and demonstrate the membrane environment plays a significant role in stabilizing the structure of MAO A and its mutant forms.

Keywords

monoamine oxidase A; phosphomimic; cavity-shaping loop; mutagenesis; membrane

Monoamine oxidase A (MAO; EC 1.4.3.4) serves an important role in the degradation of serotonin and has been the object of intense experimental interest as this enzyme has been implicated in human conditions ranging from aggressive trait disorders [1-3] to cardiovascular disease [4-6]. Although a considerable amount of structural and functional information is known [7,8] regarding this membrane-bound mitochondrial flavoenzyme, very little is known about any possible processes that could regulate its function. The involvement of MAO A in pro-apoptotic signaling pathways is suggested by a variety of studies that have shown staurosporine (a kinase inhibitor) induces MAO A-sensitive apoptosis [9]. Ou *et al.* [10] have shown that MAO A and a protein (R1) that inhibits the MAO A promoter are downstream of p38 MAP kinase functions and support their

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involvement(s) in an apoptotic signaling pathway. MAO A catalysis appears to be an important factor in induction of apoptosis since treatment of cells with clorgyline (a specific MAO A inhibitor) appears to have a protective role. Data from several studies [9,11,12] reveal that the level of MAO A expression does not correlate well with MAO A catalytic activity levels. These observations suggest investigation of any regulatory post-translational modification of MAO A that might influence its catalytic activity to be a worthwhile endeavor.

Protein phosphorylation is a well-known mechanism for the regulation of the functional activity [13,14] of enzymes and the following observations provide the rationale for the experiments presented here. The sequence of MAO A was subjected to NetPhos [15] (a bioinformatic neural network method) to predict potential phosphorylation sites. The results shown in Fig. S1 suggest 8 serine sites predicted to be available for phosphorylation, of which serine 81 and serine 209 exhibit the highest prediction ranking scores (0.994 and 0.990, respectively). Of these two sites, serine 209 is of interest since the crystal structures of human MAO A [16,17] have shown differing conformations of a six-residue loop that is termed the “cavity shaping loop”. One conformer is more extended and the other is in a more coiled structure similar to that of MAO B (Fig. 1). Serine 209 is situated adjacent to the “cavity shaping loop” and its proximity from the carboxyl of glutamate 216 would result in electrostatic repulsion if serine 209 were to be phosphorylated. This “cavity shaping loop” may serve to alter the shape of the catalytic site of MAO A, which would result in alterations in MAO A catalytic function and serve a regulatory function. Therefore, serine 209 could be a target for phosphorylation. We chose to investigate the functional consequences of serine 209 phosphorylation in human MAO A.

To date, there are no published data demonstrating the *in vivo* phosphorylation of MAO A. In order to investigate potential influences of serine 209 phosphorylation on MAO A catalytic function, we report here studies on two mutant proteins in which serine 209 is substituted with either a glutamate residue thereby generating a “phosphorylation mimic” [18-20] or an alanine residue which precludes any phosphorylation on this residue. The structural and functional consequences of these mutations are determined and compared with wild-type enzyme. The results of this study demonstrate a remarkable stabilizing influence in the mitochondrial outer membrane environment on the Ser209Glu MAO A and suggest that phosphorylation of serine 209 likely does not occur as a primary mode of enzyme regulation *in vivo*.

Results

Kinetics Properties of Human Wild-type MAO A and MAO A Ser209Glu Mutant in Membrane-bound Form

Preliminary studies showed the Ser209Glu mutant, but not the Ser209Ala mutant, of MAO A to be unstable to purification unless measurements were performed on freshly purified enzyme and the preparation was kept on ice. Therefore, initial comparative studies of this mutant with wild-type enzyme were performed in membrane preparations. Previous studies of Tyr444 mutants of MAO A showed their membrane-bound forms to be stable while the purified forms readily inactivate [21]. To determine active site concentrations so that k_{cat} values could be calculated, we conducted titration of membrane particles of wild-type MAO A and of the MAO A Ser209Glu mutant with clorgyline. As shown in Fig. 2A and 2B, MAO concentrations in membrane particles of wild-type and the Ser209Glu mutant are 11.5 μ M and 6.5 μ M, respectively. It should be noted that the differences of MAO concentrations (wild-type and the mutant enzymes) in membrane particles result from differences in total protein concentrations in these experiments. Both wild-type MAO A and the MAO A Ser209Glu mutant in membrane preparations exhibit similar specific activities. Another

interesting phenomenon we found is that membrane particles of the MAO A Ser209Glu mutant show a 10-fold lower activity in potassium phosphate buffer containing 0.5% reduced Triton X-100 than in potassium phosphate buffer in which the detergent was omitted, while wild-type MAO A in membrane-bound form exhibits similar activities in the presence and absence of 0.5% reduced Triton X-100.

Using four different substrates, comparison of the MAO A Ser209Glu mutant in membrane-bound form (Table 1) with wild-type MAO A shows similar turnover numbers (k_{cat}) and catalytic efficiencies (k_{cat}/K_m). Similar binding affinities of MAO A specific reversible inhibitors are observed for both the MAO A Ser209Glu mutant as well as wild-type MAO A. These catalytic and binding data demonstrate that, in their membrane bound forms, substitution of serine 209 with a negatively charged glutamate residue does not alter the catalytic and structural properties of the active site of the protein. However, as will be demonstrated below, solubilization and purification of the mutant enzyme in detergent solution results in considerable changes in these parameters.

UV-Vis Spectral Properties of Human MAO A Serine 209 Mutants

The purified human MAO A Ser209Ala and Ser209Glu mutants show absorption spectral properties expected for covalent flavin cofactors (the solid lines in Fig. S2A and S2B). Addition of the acetylenic inhibitor clorgyline results in the conversion of the oxidized flavin cofactors to their respective N(5) flavocyanine adducts [22] which exhibit characteristic absorption maximum at 415 nm with an $\epsilon = 23,400 \text{ M}^{-1}\text{cm}^{-1}$ (the dashed lines in Fig. S2A and S2B). These data demonstrate that the freshly purified mutant enzymes exhibit >90% functionality and that they react stoichiometrically with irreversible inhibitors in a manner similar to that observed with wild-type MAO A.

Thermal Stability of Human MAO A Serine 209 Mutants

Since the purified Ser209Glu mutant exhibits lowered stability relative to the wild-type and the Ser209Ala enzymes, their respective thermal stabilities were compared to establish conditions that would facilitate further comparisons. At five different temperatures: 0°C, 10°C, 15°C, 25°C and 30°C, the purified MAO A Ser209Ala mutant exhibits stability comparable to wild-type MAO A. (Fig. 3A). At 25°C, the purified MAO A Ser209Ala mutant lost ~40% activity in 120 minutes while at 30°C, ~50% of MAO A Ser209Ala mutant activity is lost. In contrast, the purified MAO A Ser209Glu mutant is only thermally stable at 0°C (Fig. 3B). After incubation for 120 min at 10 and 15°C, this mutant retains 70% and 55% activity, respectively. Increasing the incubation temperature to 25 and 30°C results in greater losses in activity (~40% of and 25% of activity remaining, respectively). These data demonstrate that substituting Ser209 with Glu markedly reduces the stability of human MAO A.

Comparison of Kinetic Properties of Detergent-Solubilized Forms of Human Wild-Type MAO A and of the MAO A Serine 209 Mutants

Although no major functional effect of placing a negative charge at position 209 in MAO A is observed in membrane-bound forms of the enzyme, large differences are observed on comparison of the purified forms in detergent solution. Comparison of the steady state kinetic parameters for the oxidation of benzylamine, kynuramine, phenylethylamine and serotonin for the human wild-type MAO A, the Ser209Ala MAO A mutant and the Ser209Glu MAO A mutant are shown in Table 2. For the MAO A Ser209Ala mutant, only modest changes in catalytic efficiencies are observed (~1.5–3.7-fold lower than wild-type MAO A). In contrast, the k_{cat} values of the MAO A Ser209Glu mutant for these substrates are >10-fold lower and the respective K_m values are >10-fold higher than those exhibited by the wild-type enzyme. Therefore, the relative catalytic efficiencies (k_{cat}/K_m values) for these

substrates tested with the Ser209Glu mutant are in the range of 0.5%-1% of those determined for the wild-type MAO A.

A similar pattern is observed with several MAO competitive inhibitors. The MAO A Ser209Ala mutant exhibits similar K_i values (a 1-2 fold difference) to those of wild-type MAO A (Table 3). Large changes in inhibition affinities were observed on comparison of the wild-type MAO A and MAO A Ser209Glu mutant (Table 3). d-Amphetamine and isatin, which are nonselective reversible MAO inhibitors, inhibit the human MAO A Ser209Glu mutant with much lower affinities (160-fold and 20-fold, respectively) than observed for the wild-type enzyme (Table 3), phentermine binds to the Ser209Glu mutant with a K_i of 6,682 μ M, which is 13-fold lower than that found for wild-type MAO A. The MAO A specific reversible inhibitors, harmaline, pirlindole and tetrindole are also bound to the Ser209Glu mutant much more weakly than the values observed with either wild-type or the Ser209Ala MAO A mutant. These results demonstrate that, in purified preparations of MAO A, placing a negative charge at position 209 has a major influence on the properties of the substrate binding site of MAO A which suggest structural alterations occurring in the conformation of the cavity shaping loop (Fig. 1).

Flavin Fluorescence and Circular Dichroism Spectral Properties of Human Wild-Type MAO A and of the MAO A Serine 209 Mutant Proteins

To investigate whether any differential structural alterations occur in the catalytic site of MAO A as a consequence of these mutations, the spectral properties of the active site covalent flavin coenzyme was compared for wild-type MAO A and the two Ser209 mutant enzymes. As shown in Fig. 4A, both human wild-type MAO A (the solid line) and MAO A Ser209Ala mutant (the dashed line) exhibit similar fluorescence intensities and emission maxima. However, for the MAO A Ser209Glu mutant (the dotted line), a marked decrease in the fluorescence intensity and a blue-shift (maximum emission at 510 nm) are observed. The fluorescence intensity of the covalent flavin is known to be influenced by solvent dielectric [23] and by other environmental influences [24-26]. If the observed fluorescence spectral properties reflect their differential environments, denaturation of the proteins should result in samples exhibiting identical spectral properties. Unfolding of the proteins by incubation with guanidine chloride resulted in all three enzyme samples exhibiting essentially identical fluorescence emission intensities and maxima (Fig. 4B). Thus, the covalent flavin cofactors in all denatured proteins are present in identical levels and are now in identical environments. The fluorescence intensities of all denatured proteins are higher than that shown in Fig. 4A, demonstrating that the quantum yields of fluorescence are higher in their respective denatured forms than in their native forms. Therefore, the fluorescence spectral differences observed in the native forms of the proteins reflect structural alterations to the active site on incorporating the mutations.

To further investigate the environment of flavin cofactor in the active site of MAO A, circular dichroism (CD) spectroscopy was used to monitor the alterations in the ellipticity of the bound flavin chromophore in visible region (300-550 nm). Since the flavin ring is optically inactive, any alterations in CD spectral properties reflect alterations of the asymmetric protein environment about the flavin binding site. The CD spectra in Fig. 5 show the oxidized form of the flavin in either human wild-type MAO A (the solid line) or in the MAO A Ser209Ala mutant (the dashed line) to exhibit quite similar dichroic spectra: two positive bands at 380 and 460 nm, respectively. The CD spectrum of the MAO A Ser209Glu mutant shows the band at 460 nm to exhibit a negative signal (the dotted line). Since in the UV-Vis absorption spectrum of the MAO A Ser209Glu mutant (Fig. S2B, the solid line), the purified enzyme showed characteristic absorption of oxidized flavin at 456 nm which does not differ from wild-type enzyme, the negative absorption at 460 nm in CD spectrum does not result from the introduction of other chromophoric forms of the flavin

(i.e. semiquinone or hydroquinone redox forms) or other components exhibiting absorption in this spectral region. These CD results agree with the observed different fluorescence spectrum of the MAO A Ser209Glu mutant, indicating a structural change in the active site which affects the interaction of the isoalloxazine ring of the FAD cofactor with its surrounding environment.

Structure/Activity Studies of Human MAO A Serine 209 Mutants as a Probe of Active Site Structure

The above spectroscopic and catalytic studies of the MAO A Ser209Glu mutant enzyme suggest considerable alterations of the catalytic site affected by this mutation in the solubilized form of the enzyme. A probe to provide further information on the nature of these alterations is to probe the behavior of the mutant enzyme with *para*-substituted benzylamine substrate analogues. Previous studies from this laboratory have shown wild-type MAO A catalyzes the oxidation of these analogues with the following properties. Large deuterium kinetic isotope effects are observed demonstrating C-H bond cleavage is rate limiting in catalysis. A Hammett plot of $\log k_{cat}$ versus the electronic parameter of the *para*-substituent exhibits a ρ value of $+1.89 \pm (0.43)$ demonstrating a H^+ abstraction mechanism for C-H bond cleavage, and a correlation of $\log K_d$ for substrate analogue binding with the van der Waals volume of the *para*-substituent (where a higher affinity is observed with an increase in substituent volume) [27]. These Quantitative Structure-Activity Relationship (QSAR) approaches were applied to the Ser209 mutant forms of MAO A as a sensitive probe of active site structures. The steady-state kinetic parameters for catalyzed oxidation of seven *para*-substituted benzylamine analogues by the MAO A Ser209Ala and Ser209Glu mutants were determined and their respective values of k_{cat} and K_m are shown in Table 4. The turnover numbers ($k_{cat}(H)$) of the MAO A Ser209Ala and Ser209Glu mutants determined for each substrate show a marked dependence on the nature of the *para*-substituent. The k_{cat} and K_m values determined for the MAO A Ser209Ala mutant for these analogues are quite similar to those previously published for wild-type MAO [27]. In contrast, significant decreases in k_{cat} values and increases in K_m values for the MAO A Ser209Glu mutant enzyme are observed (Table 4). These data demonstrate that substitution of serine 209 with glutamate dramatically reduces the catalytic efficiency of human MAO A as shown above for catalytic activity data of the solubilized mutant enzyme with other amine substrates (Table 2).

To determine whether these mutations altered the relative rates of C-H bond cleavage, the oxidation of the α,α [2H]-benzylamine analogues was determined (Table 4). $^Dk_{cat}$ values in the range of 5-13 (Table 4) are observed for each mutant enzyme demonstrating the C-H bond cleavage step (in the reductive half-reaction) remains rate-limiting in catalysis [27]. Kinetic isotope effects on k_{cat}/K_m ($^D(k_{cat}/K_m)$) values range from 6-12 for both mutants. Analysis of these kinetic data provides the basis for comparison of QSAR substituent effects both on the mechanism of C-H bond cleavage and substrate analogue binding parameters to the mutant enzymes.

Linear regression analysis of the rate of steady state turnover of the MAO A Ser209Ala and Ser209Glu mutants with the electronic substituent parameter (σ) was performed using the data set obtained for seven benzylamine substrate analogues (Table 4). The correlations of $\log k_{cat}$ with σ are shown in Fig. 6. For both mutant enzymes, a linear correlation of rate with electron withdrawing ability of the *para*-substituent is observed. The following correlations for the two mutant enzymes are:

$$\begin{aligned} \text{MAO A Ser209Ala} \quad \log k_{cat} ([^1H]) &= 2.30(\pm 0.41)\sigma + 0.61(\pm 0.11) \\ \log k_{cat} ([^2H]) &= 2.31(\pm 0.46)\sigma - 0.40(\pm 0.12) \end{aligned}$$

$$\begin{aligned}\text{MAO A Ser209Glu} \quad \log k_{\text{cat}} ([^1\text{H}]) &= 1.58(\pm 0.29)\sigma - 0.36(\pm 0.08) \\ \log k_{\text{cat}} ([^2\text{H}]) &= 1.39(\pm 0.34)\sigma - 1.19(\pm 0.09)\end{aligned}$$

A lower p value is observed with the Ser209Glu mutant enzyme than with either wild-type MAO A or the Ser209Ala mutant, but, given the error in the estimation of this value, one can conclude that no major effects on the mechanism of C-H bond cleavage result from these mutations. The higher p value observed for the Ser209Ala mutant enzyme is also within experimental uncertainty of wild-type enzyme. No significant correlations of $\log k_{\text{cat}}$ with other QSAR parameters (hydrophobicity or steric effects) are observed with either mutant enzyme and the correlations with electronic parameter are not improved in two component correlations.

With the knowledge of deuterium kinetic isotope effect data for both mutant enzymes, the apparent substrate dissociation constants which represent all pre-isotopically sensitive steps could be calculated by the method of Klinman and Matthews [28]. Since MAO A binds only the deprotonated form of the amine substrates [29], the dissociation constant K_d values are corrected according to McEwen [30]. Correlations of these calculated binding data with QSAR parameters and comparison with available data on wild-type MAO A provide insights into any environmental changes in the active sites of the mutant enzymes. QSAR analysis of *para*-substituted benzylamine analogue binding to the two mutant enzymes was performed using the data in Table 4. Linear correlations of *para*-substituted benzylamine analogue binding affinities to the MAO A Ser209Ala and Ser209Glu mutants are observed only with the van der Waals volume (V_w) of each substituent (Fig. 7). The values of V_w are scaled by a factor of 0.1 to make their magnitudes similar with other substituent parameters. The QSAR binding correlations for the MAO A serine 209 mutants are described by the following relationships:

$$\begin{aligned}\text{MAO A Ser209Ala} \quad \log K_d &= -0.58(\pm 0.27)(0.1 \cdot V_w) - 4.58(\pm 0.33) \\ \text{MAO A Ser209Glu} \quad \log K_d &= -0.62(\pm 0.24)(0.1 \cdot V_w) - 3.46(\pm 0.29)\end{aligned}$$

By comparison, wild-type MAO A exhibits the following relationship [27]:

$$\log K_d = -0.45(\pm 0.05)(0.1 \cdot V_w) - 4.8(\pm 0.1)$$

Therefore, within experimental uncertainty, essentially parallel correlations of $\log K_d$ with the van der Waals volume (V_w) of the *para* substituent are observed for wild-type and the Ser209 mutant forms of MAO A. These data suggest similar structures of the substrate binding sites for both mutant and wild-type enzymes. Substitution of Ser209 with Ala has only minor effects on benzylamine binding affinity while the glutamate substitution decreases the apparent affinity approximately 10-fold. Therefore, the observed conformational alteration in the active site in the Glu mutant enzyme decreases the binding affinities of both substrates and reversible inhibitors. Paradoxically, the QSAR properties of wild-type enzyme appear to be maintained. The molecular basis for these observations remains for future investigation.

Discussion

Serine 209 as a Site for the Putative Regulation of MAO A Activity by Phosphorylation

Other than studies of regulation of MAO A activity by gene promoter activation/deactivation, there are no reports of any regulatory mechanism. Yet there are numerous studies documenting levels of MAO A expression that do not correlate with levels of catalytic activity observed. One example relating to a human condition is the study of placental tissues from pre-eclampsic patients where low levels of MAO A activity are observed (relative to placental tissues from normal patients) while MAO A levels as

detected immunochemically or by mRNA analysis appear normal [31]. Other studies outlined in the introduction to this paper document low correlations of MAO A catalytic activity with levels of enzyme expression. To date, no definitive evidence exists for phosphorylated forms of MAO A in a biological system and its putative influence on catalytic activity. This study attempts to address this question through the generation of a “phospho-mimic” form of MAO A by a glutamate substitution of a Ser residue identified through a bioinformatics analysis and structural analysis as a reasonable candidate for phosphorylation. The evidence presented in this study demonstrates the predicted effects on structure and catalytic properties for the purified solubilized form of the enzyme. This, however, is not reflected in the membrane-bound form.

The structure and activity of MAO A has been known for some time to be much more stable in its membrane environment as compared to a detergent-containing aqueous solution. The replacement of Ser 209 with Ala has little effect on either MAO A structure or activity whereas its replacement with Glu has considerable effect on its non-membrane bound form. Interestingly, both mutants of MAO A appear to fold properly on expression and to incorporate covalently bound FAD cofactors. Previous data from this laboratory has demonstrated that the apo-(deflavinated) (Cys406Ala MAO A) mutant form is capable of proper folding and incorporation in the mitochondrial outer membrane in *S. cerevisiae* cells and activity can be reconstituted by addition of FAD [32]. Therefore, we predict that the apoform of WT and the Ser209 mutant forms of MAO A are also incorporated into the mitochondrial outer membrane prior covalent flavin incorporation (although not determined in this study). Structural studies of MAO A [17] demonstrate it is held to the mitochondrial outer membrane via a single trans-membrane C-terminal α -helix. Other protein-membrane interactions are also likely to occur which are currently not well-defined. The results in this study demonstrate that such membrane-protein interactions are important for the stable conformation of the six-residue “cavity shaping loop”. This loop does not appear to be in direct contact with the membrane (see Fig. 1) and therefore long-range interactions are probably involved as suggested in a recent theoretical study on rat MAO A [33]. Indeed, placing a negative charge at a residue predicted to be electrostatically repulsed by a nearby glutamate residue does not seem to influence the structure in the membrane-bound form, but certainly does in the detergent solubilized form. Presumably, the membrane could be acting as a ‘pseudo-scaffold’ for MAO that restricts its conformation and charge effects in the membrane or neutralize this unstable electrostatic interaction whereas placement of the mutant enzyme in a micelle of a neutral detergent does not.

The major conclusion from this study is that a putative phosphorylation of Ser209 in MAO A does not appear to be a viable post-translational mechanism for regulation of enzyme activity, at least not in its membrane-bound form. It is difficult at this point to state with any certainty whether such a modification would serve a purpose, such as in the case of the non-mitochondrial MAO A observed in pre-eclamptic tissue [31] as no phosphorylated form of MAO has been found *in vivo*. No dramatic effects are observed on the membrane-bound form of the enzyme either via catalytic turnover or sensitivity to active site directed inhibitors. If one were to only investigate the detergent soluble, purified form of the enzyme, a quite different conclusion would be reached. This conclusion also assumes that mammalian tissue mitochondrial outer membranes have properties similar to those exhibited by the *Pichia* outer mitochondrial membranes. This is probably an incorrect assumption. In addition, our knowledge of differences and similar properties of outer mitochondrial membranes from different tissues in the same mammalian organism is inadequate for any definitive conclusions. Therefore, it remains for future investigations to determine if MAO A is phosphorylated *in vivo*, and if it is, then what site is targeted for phosphorylation and what influence does it exert on catalytic activity. The results presented here emphasize the usefulness of studies of both membrane-bound as well as purified, detergent solutions of

mutant forms of MAO A (or of MAO B) and this caveat should be extended to other membrane-associated enzymes/receptors as well.

Experimental procedures

Reagents

The QuikChange XL Site-Directed Mutagenesis Kit was obtained from Stratagene. The plasmid (pPIC3.5K), strain (KM71) and Amplex Red reagent were obtained from Invitrogen Corp. β -Octylglucopyranoside was from Anatrace Inc. Reduced Triton X-100 was from Fluka. Potassium phosphate, glycerol, phenylmethylsulfonyl fluoride, triethylamine, isatin, benzylamine, kynuramine, β -phenylethylamine, serotonin, d-amphetamine, phentermine, horse radish peroxidase (HRP), and guanidine chloride were purchased from Sigma-Aldrich. Dithiothreitol was from US Biological (Swampscott, MA). Harmane, pirlindole mesylate and tetrindole mesylate were purchased from Tocris Bioscience (Ellisville, MO). DEAE Sepharose™ Fast Flow resin was obtained from Amersham Biosciences (Uppsala, Sweden). All benzylamine analogues were synthesized as described previously [34].

Expression and Purification of Human MAO A Ser209Ala and Ser209Glu Mutants

Recombinant human liver MAO A Ser209Ala and Ser209Glu mutants were generated using the Stratagene QuikChange® XL Site-Directed Mutagenesis Kit. The desired sequence alterations were confirmed by DNA sequence analysis. The mutant enzymes were expressed in *Pichia pastoris* (strain KM71) using methods described previously [35]. The process for purification of the MAO A Ser209Ala mutant is identical to that for the wild-type enzyme [35]. However, purification of the MAO A Ser209Glu mutant required some modifications. Briefly, the DEAE Sepharose™ Fast Flow anion exchange column was pre-equilibrated with 10 mM potassium phosphate containing 20% (v/v) glycerol and 0.5% (w/v) Triton X-100 (pH 7.2). During the Triton extraction step, homogenized pellets were suspended in 10 mM potassium phosphate (pH 7.2). d-Amphetamine, a reversible MAO inhibitor, was added in the elution step to stabilize enzyme activity. Purified enzyme was stored in 50 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol, 0.8% (w/v) β -octylglucopyranoside, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 30 μ M dithiothreitol (DTT). The purified mutant enzymes exhibit homogeneous bands on SDS-polyacrylamide gels and migrated with an apparent molecular weight of 60 kD. Both mutants contain covalently bound flavin cofactors as detected by Western blot analysis using antisera specific for the covalent flavins [36].

Preparation of Membrane Particles of human Wild-type MAO A and MAO A Ser209Glu Mutant

Yeast cells from a 0.5 liter of culture was suspended in 0.5 liter of breakage buffer with an equal volume of silica-zirconia beads (0.5 mm in diameter) and then disrupted in Biospec Beadbeater (Bartlesville, OK) with 6 cycles of 2-min beating and 5-min chilling on ice. After removal of glass beads by filtration through a layer of Miracloth (Calbiochem), the cell lysate (separated from unbroken cells and large cell debris by centrifugation at $1500 \times g$ for 10 min at 4°C) was centrifuged at $100,000 \times g$ for 30 min at 4°C to isolate the membrane fraction. The pellets were suspended in 0.1 M triethylamine (TEA) (pH 7.2). Protein concentration was determined using the Biuret Method [37].

To determine the stoichiometry of catalytic sites of MAO A in membrane-bound preparations, suspensions of membrane preparations of the recombinant enzymes were incubated overnight at 4°C with various molar ratios of clorgyline and levels of catalytic activity remaining were determined. Linear extrapolation of the activity versus moles

clorgyline results in plots that allowed the determination of active site concentrations of MAO A and mutant forms.

Spectroscopic Experiments

All UV-Vis absorption spectral studies of human MAO A serine 209 mutants (~10 μ M) in 50 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside were carried out on a Cary 50 UV-Vis spectrophotometer.

Steady-state fluorescence measurements of both the wild-type MAO A and MAO A Ser209 mutants were conducted on an AMINCO-Bowman Series 2 luminescence spectrometer equipped with a 150 W Xenon lamp. The flavin fluorescence signal was excited at 450 nm and emission recorded from 480 to 600 nm. All protein samples were in 50 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside. Denaturation of the wild-type MAO A and MAO A serine mutants was achieved by dilution of the stock protein solution with guanidine chloride in protein buffer leading to final denaturant concentrations of 4 M.

Circular dichroism spectral measurements were performed at 0°C using an Aviv model 62DS spectrophotometer. A quartz cell with pathlength of 1 cm was used in the 500 to 300 nm region at a scan rate of 5 nm/sec at a bandwidth of 1.5 nm with a 1 s dwell-time. All samples were in 50 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside and were analyzed with concentrations ranging from 20 to 35 μ M. A total of five repetitive scans were averaged, and the spectra smoothed using an adjacent-point averaging function.

Thermal Stability of Human MAO A Serine 209 Mutants

Human MAO A Ser209Ala mutant and MAO A Ser209Glu mutant in 50 mM potassium phosphate (pH 7.5) containing 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside were incubated at five different temperatures: 0°C, 10°C, 15°C, 25°C and 30°C. The loss of enzyme activity was determined over a 2-hour period. For the MAO A Ser209Ala mutant, 5 μ L aliquots were removed every 10 minutes for the determination of catalytic activity using kynuramine as substrate. The rate of 1 mM kynuramine oxidation in 50 mM potassium phosphate with 0.5% reduced Triton X-100 (pH 7.5) was monitored at 316 nm (product 4-hydroxyquinone absorbance, $\epsilon = 12,000 \text{ M}^{-1}\text{cm}^{-1}$) [38] over time using a Perkin Elmer Lambda 2 spectrophotometer. One unit activity of MAO A was defined as the amount of enzyme that is able to catalyze the formation of 1 mole 4-hydroxyquinone per minute. Since the enzymatic activity of the MAO A Ser209Glu mutant was much lower than wild-type MAO A, the oxidation rate of kynuramine by the purified Ser209Glu mutant was too low to accurately monitor product formation. Amplex Red-peroxidase coupled assays, which increase the detection sensitivity approximately 5-fold, were used to monitor the loss of enzyme activity of the MAO A Ser209Glu mutant. Briefly, 20 μ L aliquots of the MAO A Ser209Ala mutant were removed from the incubation buffer every 10 minutes and applied to Amplex Red-peroxidase coupled assay.

Steady-State Enzymatic Activity Assays

All steady-state enzymatic activity assays of the purified human MAO A Ser209 mutants were performed in 50 mM potassium phosphate assay buffer (pH 7.5) with 0.5% reduced Triton X-100 and the conversion of substrates was spectrophotometrically determined using a Perkin-Elmer Lambda 2 UV-Vis spectrophotometer at 25°C. The concentrations of the purified MAO A mutants were determined by the absorbance of flavin cofactor ($\epsilon_{456\text{nm}} = 11,800 \text{ M}^{-1}\text{cm}^{-1}$). d-Amphetamine was removed by gel filtration (Sephadex G-25) before

performing steady-state kinetic studies. All assays were performed in air-saturated solutions (oxygen concentration is $\sim 240 \mu\text{M}$).

Competitive inhibition experiments with the purified MAO A Ser209Ala mutant were performed using kynuramine as substrate while the purified MAO A Ser209Glu mutant was performed using *p*-CF₃-benzylamine as substrate (Amplex Red-peroxidase coupled assay).

The kinetics studies of (kinetic property and competitive inhibition) membrane particles of wild-type MAO A and MAO A Ser209Glu mutant were performed in 50 mM potassium phosphate assay buffer (pH 7.5). The concentration of MAO A protein in the membrane particles was determined by titration with the irreversible inhibitor, clorgyline, as described above.

All steady state kinetic measurements of *para*-substituted benzylamine analogue oxidation with the purified MAO A serine 209 mutants were performed in 50 mM potassium phosphate (pH 7.5) 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 were given by Walker and Edmondson [34]. It is noted that since the oxidation rates of *p*-F-BA, *p*-Me-BA, *p*-MeO-BA as well as α,α -[²H]benzylamine analogues are too low to accurately monitor the formation of the corresponding aldehyde, Amplex Red-peroxidase coupled assays were again used to achieve higher sensitivity. In addition, as mentioned above, the MAO A Ser209Glu mutant exhibits very low enzymatic activity, Amplex Red-peroxidase coupled assays were performed to obtain all steady-state kinetic data of the MAO A Ser209Glu mutant.

Data analysis

All steady-state kinetic data were fit either by Michaelis-Menten equation (hyperbolic equation) or by a Lineweaver-Burk plot (linear fit) using the program Origin 7.0 Pro (Microcal Inc.) to calculate turnover number (k_{cat}) and Michaelis constant (K_m). Inhibition constant values (K_i) were calculated by analyzing the apparent K_m of substrates at various concentrations of inhibitor. Values of substituent parameters σ and V_w were obtained from Hansch *et al.* [39] and from Bondi [40], respectively. Binding data for the benzylamine analogues were determined from steady-state deuterium kinetic isotope effect data as described by Klinman and Matthews [28]. Multivariate linear regression analysis of rate and binding data as a function of substituent parameters was performed using the StatView software package (Abacus Concepts)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MAO A	Monoamine Oxidase A
CD	circular dichroism
QSAR	Quantitative Structure-Activity Relationship

Fig. 1.

The different conformations of the cavity-shaping loop in two human MAO A crystal structures. The two crystal structures by De Colibus (in green) and by Son (in cyan) were superimposed. For quality of viewing specific residues, the superimposed structures are displayed in 60% translucent mode. Flavin cofactor is in yellow. The cavity shaping loops in De Colibus' and Son's structure are shown in red and black, respectively. Serine 209 and glutamate 216 are indicated in stick mode. The figure was drawn using PyMOL (W.L. Delano (2002) PyMOL, Delano Scientific, San Carlo, CA. <http://www.pymol.org>).

Fig. 2. Determination of MAO A active site concentrations in membrane particles by titration with clorgyline. (A) Wild-type MAO A; (B) MAO A Ser209Glu Mutant.

Fig. 3.

Comparison of thermal stabilities of the purified human MAO A Ser209Ala mutant (**A**) and Ser209Glu mutant (**B**). Loss of catalytic activities versus incubation time at 0, 10, 15, 25 and 30°C are shown (enzyme buffer: 50 mM potassium phosphate, 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside, pH 7.5).

Fig. 4. Fluorescence spectra of human wild-type MAO A (—), MAO A Ser209Ala mutant (---) and MAO A Ser209Glu mutant (···) before (**A**) and after (**B**) guanidine chloride denaturation. All spectral data were acquired in 50 mM potassium phosphate containing 20% glycerol and 0.8% (w/v) β -octylglucopyranoside, pH 7.5. The concentrations of all samples were normalized to 20 μ M.

Fig. 5. Visible circular dichroism spectra of the oxidized human wild-type MAO A (—), MAO A Ser209Ala mutant (---) and MAO A Ser209Glu mutant (···). All spectral data were acquired in 50 mM potassium phosphate containing 20% (v/v) glycerol and 0.8% (w/v) β -octylglucopyranoside, pH 7.5.

Fig. 6. Hammett plots of k_{cat} values of human MAO A Ser209Ala mutant (—, ■) and MAO A Ser209Glu mutant (---, ○) for the oxidation of *para*-substituted benzylamine analogues (σ). $F_{1,6}$ values for the human MAO A Ser209Ala and Ser209Glu mutants are 35 and 28, respectively. Purified enzyme preparations were used and the k_{cat} values measured at air saturation.

Fig. 7. Correlations of calculated K_d values for the binding of *para*-substituted benzylamine analogues to human MAO A Ser209Ala mutant (—, ■), MAO A Ser209Glu mutant (---, ○) with the van der Waals volume (V_w) of the *para* substituent. $F_{1,5}$ values for the human MAO A Ser209Ala and Ser209Glu mutants are 4.6 and 6.5, respectively. All binding constants are corrected for the concentration of deprotonated amine in the assays.

Table 1
Steady-state kinetic properties of membrane-bound wild-type MAO A and the MAO A Ser209Glu mutant

Substrate	Wild-type MAO A				MAO A Ser209Glu			
	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)		k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	
Benzylamine	2.44±0.03	1.67±0.12	1.46±0.11		2.05±0.02	2.83±0.11	0.72±0.03	
Kynuramine	93.33±0.79	0.14±0.01	666.64±19.86		77.50±0.62	0.093±0.003	836.81±7.23	
Phenylethylamine	48.57±1.06	0.47±0.04	103.34±9.70		64.05±1.43	0.85±0.07	75.35±6.25	
Serotonin	145.77±1.80	0.094±0.004	1542.59±72.10		153.57±1.80	0.069±0.002	2221.75±79.58	
Competitive Inhibitor		K_i (μM)				K_i (μM)		
Harmane		0.14±0.03				0.18±0.02		
Pirlindole Mesylate		0.25±0.04				0.29±0.06		
Tetrindole Mesylate		2.43±0.15				2.76±0.11		

Comparison of steady state kinetic properties of the purified wild-type human MAO A and purified human MAO A Ser209Ala and Ser209Glu mutants.

Table 2

	Benzylamine	Kynuramine	Phenylethylamine	Serotonin
Human MAO A	k_{cat} (min^{-1})	2.5 ± 0.1^a	53.8 ± 1.0^c	175.1 ± 2.1^c
	K_m (mM)	1.04 ± 0.15^a	0.13 ± 0.01^b	0.30 ± 0.05^c
	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	2.4 ± 0.4^a	964.6 ± 98.9^b	583.7 ± 97.5^c
Human MAO A Ser209Ala	k_{cat} (min^{-1})	1.56 ± 0.03	18.84 ± 0.10	161.9 ± 4.0
	K_m (mM)	0.91 ± 0.09	0.15 ± 0.01	0.41 ± 0.04
	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	1.72 ± 0.17	262.8 ± 18.3	396.2 ± 35.4
Human MAO A Ser209Glu	k_{cat} (min^{-1})	0.226 ± 0.002	1.48 ± 0.04	25.38 ± 0.44
	K_m (mM)	9.73 ± 0.28	0.32 ± 0.02	19.11 ± 1.11
	k_{cat}/K_m ($\text{min}^{-1}\text{mM}^{-1}$)	0.023 ± 0.001	4.57 ± 0.37	7.17 ± 0.43

^aValues from Miller *et al.* [27].

^bValues from Nandigama *et al.* [41].

^cValues from Li *et al.* [35].

Table 3

Comparison of competitive inhibition constants for purified wild-type human MAO A and human MAO A Ser209Ala and Ser209Glu mutants.

	Human MAO A	Human MAO A Ser209Ala	Human MAO A Ser209Glu
	K_i (μM)		
d-Amphetamine	3.69±0.45	4.72±0.63	608.83±31.61
Isatin	15 ^a	24.5±5.6	314.67±2.13
Phentermine	498±60 ^b	944±25	6682±245
Harmaline	0.58±0.02	1.37±0.04	15.74±0.93
Pirlindole Mesylate	0.92±0.04	0.88±0.18	21.52±1.36
Tetrindole Mesylate	5.27±0.24	4.11±0.67	16.13±0.57

^aValue from Hubalek *et al.* [42].

^bValue from Nandigama *et al.* [43].

Comparison of steady-state kinetic constants for human MAO A Ser209Ala and Ser209Glu mutants catalyzed oxidation of *para*-substituted benzylamine analogues.

Table 4

<i>para</i> substituent	Human MAO A Ser209Ala				Human MAO A Ser209Glu			
	$k_{cat}(H)$ (min ⁻¹)	$K_m(H)$ (μM)	$D(k_{cat})$	$D(V/K)$	$k_{cat}(H)$ (min ⁻¹)	$K_m(H)$ (μM)	$D(k_{cat})$	$D(V/K)$
H	1.56 ± 0.03	905 ± 86	11.6 ± 0.2	12.2 ± 0.7	0.226±0.002	9734 ± 283	7.1 ± 0.2	6.2 ± 0.7
CF ₃	64.00 ± 0.43	948 ± 27	7.0±0.2	9.3 ± 0.9	3.36±0.10	6840 ± 607	7.7 ± 0.2	9.3 ± 0.9
Br	24.15 ± 0.56	278 ± 29	12.7 ± 0.3	10.8 ± 0.5	1.23±0.02	3529 ± 186	8.0 ± 0.1	6.1 ± 0.4
Cl	18.49 ± 0.61	341 ±51	13.7 ± 0.5	11.8 ± 2.0	0.703±0.008	1893 ± 112	9.0 ± 0.2	9.1 ± 0.6
F	3.38 ± 0.05	675 ± 35	10.7 ± 0.2	8.4 ± 0.8	1.05±0.37	14441 ± 1356	6.5 ± 2.3	10.5 ± 3.9
Me	3.22 ± 0.04	181±15	8.1 ± 0.1	8.8 ± 0.9	0.249±0.003	2586 ± 109	6.7 ± 0.2	8.9 ± 0.5
MeO	0.99 ± 0.02	249 ± 42	9.5 ± 0.2	7.9 ± 1.4	0.179±0.010	3273 ± 451	5.4 ± 0.3	5.7 ± 0.9