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Site-Directed Mutagenesis of Monoamine Oxidase A and B: Role of Cysteines

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SUMMARY

Nine cysteines are found in the deduced amino acid sequences of both human liver monoamine oxidase (MAO)-A and MAO-B. The role of these cysteine residues in MAO-A and -B catalytic activity was studied by site-directed mutagenesis, whereby each cysteine residue was converted to serine. The wild-type and mutant cDNAs were then transiently transfected into COS cells and assayed for MAO-A and -B catalytic activity using 5-[^{3}H]-hydroxytryptamine and [^{14}C]phenylethylamine, respectively, as substrates. Catalytic activities were retained in seven MAO-A cysteine to serine mutants (mutations at residues 165, 210, 266, 306, 321, 323, and 398) and in six MAO-B cysteine to serine mutants (mutations at residues 5, 172, 192, 297, 312, and 389). Kinetic parameters (K_m) of these mutants were also similar to those of the wild-type enzymes, indicating that these cysteines are not necessary for enzymatic activity. Substitution of MAO-A Cys-374 and -406 and MAO-B Cys-156, -365, and -397 with serine resulted in complete loss of MAO-A and -B catalytic activity. The loss of catalytic activity was not due to unsuccessful transfection of the mutants, as indicated by either Northern blot or Western blot analysis. The loss of catalytic activity in the MAO-A Ser-406 and MAO-B Ser-397 mutants may be due to the prevention of covalent binding of the enzyme to the cofactor FAD, which is necessary for catalytic activity. The loss of catalytic activity of MAO-A Ser-374 and MAO-B Ser-156 and -365 suggests that these cysteines are important for catalytic activity, but whether they are involved in forming the active site or are important for the appropriate conformation of MAO-A and -B remains to be studied.

MAO (EC 1.4.2.4), located in the outer mitochondrial membrane, catalyzes the oxidative deamination of many neurotransmitters and dietary amines (1, 2). Two types of MAO (A and B) have been described based on substrate preferences and inhibitor specificities (3–5). MAO-A preferentially oxidizes biogenic amines such as serotonin, norepinephrine, and epinephrine and is irreversibly inactivated by the acetylenic inhibitor clorgyline. MAO-B preferentially oxidizes PEA and benzylamine and is inactivated by the irreversible inhibitors pargyline and deprenyl. Human liver MAO-A and MAO-B cDNAs (6) and genes (7) have been isolated. Comparison of the deduced amino acid sequences shows that the A and B forms have subunit molecular weights of 59,700 and 58,800, respectively, and share 70% sequence identity (6). Both sequences contain the pentapeptide Ser-Gly-Gly-Cys-Tyr where the obligatory cofactor FAD covalently binds to the cysteine (8). Furthermore, a catalytically active enzyme can be expressed from MAO-A or -B cDNA in COS cells (9), suggesting that the substrate and inhibitor sensitivity of MAO-A or -B activity is contained within a single polypeptide that exists as either a monomer or a homodimer but not a heterodimer. Interestingly, the genes for MAO-A and -B have strikingly similar structures; both consist of 15 exons and exhibit identical exon-intron organizations, suggesting that MAO-A and MAO-B are derived from duplication of a common ancestral gene (7).

The active site of MAO has been suggested to be composed of two domains, one bearing the FAD prosthetic group and the other comprising the substrate binding site (10). The use of affinity reagents, e.g., 4-fluoro-3-nitrophenylazide (11, 12) and 2-chloro-2-phenylethylamine (13), for MAO has not yet resulted in the identification of the active site residues. The sulfhydryl-modifying reagent dipyridyldisulfide has been shown to inhibit purified placenta MAO-A catalytic activity (14), and 5,5′-dithiobis(2-nitrobenzoic acid), *N*-ethylmalemide, and iodoacetamide (15–17) have been shown to decrease purified bovine MAO-B catalytic activity. These results indicate that the cysteine residues are important for the catalytic activity of MAO-A and MAO-B. Furthermore, one cysteine residue was suggested to be directly involved in the MAO-B catalytic reaction (18). The deduced amino acid sequences show that both MAO-A and -B contain nine cysteine residues (6). In this paper we have studied the role of each cysteine residue by mutating

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ABBREVIATIONS: MAO, monoamine oxidase; 5-HT, 5-hydroxytryptamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; PEA, phenylethylamine; 1-PCPA, 1-phenylcyclopropylamine; MOPS, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid.

each to serine. Our results show that, in addition to the FAD binding site (Cys-406 in MAO-A and Cys-397 in MAO-B), Cys-374 plays an important role in MAO-A catalytic activity, whereas Cys-156 and Cys-356 are important for MAO-B catalytic activity. When the rest of the cysteine residues were mutated to serine, catalytic activity was retained and the K_m values of the mutant enzymes for their substrates were similar to those of the wild-type enzyme, suggesting that those cysteines may not be important for enzymatic activity.

Experimental Procedures

Materials. [α-^{32}P]ATP (3000 Ci/mmol) and the oligonucleotide-directed mutagenesis kit were from Amersham, [^{3}H]5-HT (26.7 Ci/mmol) and [^{14}C]PEA (58 Ci/mmol) were obtained from DuPont, the DNA sequencing kit and restriction endonucleases were from United States Biochemicals., the poly(A)$^+$ mRNA isolation kit was from Invitrogen, and the Immunopure ultrasensitive ABC staining kit was from Pierce.

Oligonucleotide-directed site-specific mutagenesis. The 2.1-kb human liver MAO-A cDNA and 2.5-kb human liver MAO-B cDNA in pUC18 were excised with *Eco*RI and subcloned into the *Eco*RI restriction site of the replicative form DNA of M13 mp18. Single-stranded DNA of the recombinant phage was used as the starting material for the mutagenesis of the MAO-A and -B genes. The mutagenic primers used in this study (Table 1) were synthesized with an Applied Biosystems 380A DNA synthesizer. Each mutant was prepared by using the reagents and protocol of the Amersham oligonucleotide-directed *in vitro* mutagenesis kit. The mutated cDNAs were used to transform *Escherichia coli* TG1 cells.

Screening of mutant cDNA. After transformation of competent TG1 cells with the mutated cDNAs, five plaques from each mutant were picked and single-stranded DNAs were prepared. Dideoxy DNA sequencing (19) was then performed to identify positive plaques containing the desired mutation.

Construction of mutant cDNA in expression vector and transfection. The replicative forms of the positive plaques containing the desired mutation were purified. The mutant MAO-A and -B cDNAs were excised with *Eco*RI and then subcloned into the *Eco*RI site in the polylinker region of the pECE expression vector (9). This plasmid contains the SV40 early promoter, a selectable ampicillin resistance gene, and an origin of replication for propagation in bacteria. The correct orientations of the mutant MAO-A cDNA and MAO-B cDNA in pECE were determined by restriction enzyme analysis using *Hind*III alone and *Hind*III plus *Pst*I, respectively. Each mutant was sequenced again to confirm that the correct mutant was cloned into pECE. The resulting plasmids were transiently transfected into COS cells by the high efficiency calcium-phosphate transfection method described by Chen and Okayma (20). At each transfection an equal amount (50 μg) of wild-type MAO-A or -B cDNA in pECE was transfected into COS cells as a positive control for comparison with the mutant enzyme. Thus, the catalytic activities of a mutant enzyme and a wild-type enzyme expressed in COS cells transfected under the same conditions were assayed and compared.

Determination of MAO-A and -B activity. MAO activity of cysteine mutants and wild-type enzyme were determined by using the whole-cell homogenate of the transfected cells, as described previously (21). Approximately 5×10^6 transfected COS cells were homogenized in 250 μl of 50 mM sodium phosphate buffer (pH 7.4). A 40-μl aliquot of the homogenate (corresponding to ~8×10^5 cells) was used to measure MAO activity. The final concentration of the 1-ml assay mixture contained 50 mM sodium phosphate buffer (pH 7.4), 100 μM [^{3}H]5-HT (for MAO-A) or 10 μM [^{14}C]PEA (for MAO-B), and the transfected cell homogenate. After a 20-min incubation at 37°, the reaction was terminated by the addition of 0.1 ml of 6 N HCl. The reaction product was extracted with 6 ml of ethyl acetate/benzene (1:1) (MAO-A) or toluene (MAO-B). Each tube was capped and shaken for 30 sec. The tubes were centrifuged at 2000 rpm for 10 min to separate the two phases. Four milliliters of the organic layer were withdrawn and mixed with 5 ml of scintillation fluid (National Diagnostics). The radioactivity of the reaction product was determined by liquid scintillation spectrometry.

For determination of kinetic parameters, [^{3}H]5-HT (10–100 μM) and [^{14}C]PEA (1–10 μM) were varied over a 10-fold concentration range and approximately the same number of cells (~8×10^5) were used for each assay. Apparent K_m values were calculated from the linear intercept and slope of double-reciprocal plots ($1/V$ versus $1/S$). The K_m values are the mean of at least two determinations.

Northern blot analysis. Poly(A)$^+$ mRNA was obtained from approximately 10^7 transfected COS cells by using the Micro-Fast Tract mRNA isolation kit (Invitrogen). Two micrograms of mRNA thus isolated were denatured by incubation in 3 volumes (v/v) of denaturing solution [10× MOP, pH 7.2 (200 mM MOPS, 10 mM EDTA, pH 8.0, 100 mM sodium acetate)/formaldehyde/formamide, 133:238:677] at 65° for 15 min and were then electrophoresed at 50 V for 3 hr in a 1% agarose gel containing 2.2 M formaldehyde. After fractionation of RNA through electrophoresis, the gel was transferred to a nylon membrane by blotting for ~24 hr in 20× standard saline citrate (3 M sodium chloride, 0.5 M sodium citrate, pH 7.0). When the transfer was completed, the RNA membrane was baked for 1 hr at 80° in a vacuum oven, prehybridized for 4 hr at 37° in 50% formamide prehybridization buffer, and then hybridized with the random-priming ^{32}P-labeled *Sac*I-*Sac*I fragment of the MAO-A cDNA probe.

Western blot analysis. One hundred micrograms of transfected mutant and wild-type whole-cell homogenates were denatured by boiling for 5 min in SDS sample buffer and were then subjected to SDS-PAGE according to the method of Laemmli (22). After electrotransfer of the proteins from the SDS-PAGE gel to a nitrocellulose membrane, Western blot analysis was carried out using the Pierce Immunopure Ultrasensitive ABC staining kit. In brief, the membrane was blocked for 4 hr at room temperature with normal goat serum in TBS (pH 7.5). The blocked membrane was rinsed twice with 3% (w/v) nonfat milk in TBS (TBSC), incubated overnight at room temperature in the presence of polyclonal rabbit anti-MAO-B antiserum at a 500-fold dilution, and then washed twice for 7 min with TBSC. The membrane was incubated

TABLE 1

Oligonucleotides used for site-directed mutagenesis of human MAO-A (A) and human MAO-B (B) genes

Oligonucleotides are complementary to the cDNA sequence except for nucleotide substitution positions, which are underlined.

A. Desired mutation	Primer sequence
Cys-165 → Ser	3′-CTGTTTTAGTCGACCTGTTTC-5′
Cys-201 → Ser	3′-CACTTCGTCTCGCCCCCGTGG-5′
Cys-266 → Ser	3′-GTAATACTCTCGTTTATGCAT-5′
Cys-306 → Ser	3′-CAGTAATTCTCGTACTACATA-5′
Cys-321 → Ser	3′-CTAATGAGACCGACGTACTAG-5′
Cys-323 → Ser	3′-ACACCGTCGTACTAGTAACTT-5′
Cys-374 → Ser	3′-TTCTTTTAGAGACTCCAGATA-5′
Cys-398 → Ser	3′-TTCTTGAACAGACTCCTCGTC-5′
Cys-406 → Ser	3′-AGACCCCCGTCGATGTGCCGG-5′

B. Desired mutation	Primer sequence
Cys-5 → Ser	3′-TCGTTGTTTTCGCTGCACCAG-5′
Cys-156 → Ser	3′-CTGTTCGAGTCGACCTGACTT-5′
Cys-172 → Ser	3′-CACTTGGACAGACAGTGACGT-5′
Cys-192 → Ser	3′-CACTTCGTCAGACCTCGGTGT-5′
Cys-297 → Ser	3′-CAGTAGTTCAGATATCAAATA-5′
Cys-312 → Ser	3′-TTCCTAATGAGACCTTGGTAC-5′
Cys-365 → Ser	3′-TTCTTTGAAAGACTTGAGATA-5′
Cys-389 → Ser	3′-CTTTTCTTGACCAGACTCCTC-5′
Cys-397 → Ser	3′-AGACCCCCGAGGATGTGTAGA-5′

for 4 hr at room temperature with a second biotinylated anti-rabbit antibody in 0.05% Tween 20 in TBS (TBST) and was then washed twice for 7 min with TBST. The membrane was then incubated for 4 hr at room temperature with an avidin-biotinylated horseradish peroxidase macromolecular complex in TBST. The membrane was rinsed twice for 7 min in TBS and then overlaid with a high-sensitivity enzygraphic web (International Inc.) for color reaction and band visualization.

Results

Expression and catalytic activity of MAO-A and -B cysteine mutants. In these experiments, the *Eco*RI fragment of human liver MAO cDNA (2.1 kb for MAO-A and 2.5 kb for MAO-B) was ligated into M13 mp18. Single-stranded DNA of this recombinant phage vector was then used as a template for creating the mutations. Each of the nine cysteine residues in the MAO-A and -B cDNAs was converted to serine by using the synthetic oligonucleotides listed in Table 1. The constructed mutant MAO-A and -B cDNAs in M13 mp18 were further analyzed by the dideoxy DNA-sequencing method to confirm that the desired mutations had taken place and that there was no contamination with wild-type MAO-A or -B cDNA.

The sequence-verified mutant MAO-A and -B cDNAs were excised from M13 mp18, subcloned into the pECE expression vector, and then transiently transfected into COS cells. The catalytic activities and kinetic parameters of these MAO-A and -B cysteine mutants are shown in Table 2.

As shown in Table 2A, there was no detectable catalytic activity in MAO-A Ser-374 and Ser-406 mutants. These results were repeated in three transfection experiments. Cys-406 is known to be involved in the FAD binding site as part of the pentapeptide Ser-Gly-Gly-Cys-Tyr. Cys-374 may play an important role in the catalytic activity of MAO-A. Other mutants that exhibited catalytic activity, ranging from 48 to 112% of that of the wild-type enzyme, were Ser-165 (58%), Ser-201 (69%), Ser-266 (48%), Ser-306 (61%), Ser-321 (62%), Ser-323 (112%), and Ser-398 (49%). The K_m values (Table 2A) estimated from Lineweaver-Burk plots for these mutants (Ser-165, 97 μM; Ser-201, 194 μM; Ser-266, 106 μM; Ser-306, 133 μM; Ser-321, 132 μM; Ser-323, 161 μM; Ser-398, 102 μM) were similar to that of the wild-type enzyme (127 μM), suggesting that these cysteine residues may not be important for MAO-A catalytic activity.

As shown in Table 2B, there was no detectable catalytic activity, compared with the wild-type enzyme, with MAO-B mutants Ser-156, Ser-365, and Ser-397. These results were consistent in three different transfection experiments. Cys-397 is known to be involved in the FAD binding site as part of the pentapeptide Ser-Gly-Gly-Cys-Tyr. Cys-156 and Cys-365 may play important roles in the catalytic activity of MAO-B. Other mutants that exhibited catalytic activity, ranging from 26 to 71% of that of the wild-type enzyme, were Ser-5 (68%), Ser-172 (59%), Ser-192 (56%), Ser-297 (26%), Ser-312 (71%), and Ser-389 (67%). The K_m values (Table 2B) for these mutants (Ser-5, 1.64 μM; Ser-172, 3.38 μM; Ser-192, 2.29 μM; Cys-297, 1.79 μM; Cys-312, 1.78 μM; Cys-389, 1.27 μM) were similar to that of the wild-type enzyme (1.92 μM), suggesting that these cysteine residues may not be important for MAO-B catalytic activity.

Northern blot analysis of wild-type and mutant MAO-A transfected in COS cells. To verify that Ser-374 and Ser-406 mutants were being transfected and transcribed, Northern blot analysis using mRNA isolated from COS cells and COS cells transfected with wild-type MAO-A cDNA or one of these two mutant cDNAs was performed. As illustrated in Fig. 1, full length MAO-A-specific mRNA was detected in COS cells transfected with wild-type cDNA (Fig. 1, *lane 1*) or mutant cDNAs (Fig. 1, *lanes 2* and *3*). The mRNA transcripts of the mutants were the same size as that of the wild-type enzyme (2.1 kb) and were expressed at levels comparable to that of the wild-type

TABLE 2

Catalytic activities and kinetic parameters of MAO-A cysteine mutants (A) and MAO-B cysteine mutants (B) expressed in COS cells

The enzymatic activities of the wild-type and mutant enzymes were determined as described in Experimental Procedures, using [^{3}H]5-HT as the substrate of MAO-A and [^{14}C]PEA as the substrate for MAO-B. The protein concentration of the cell homogenate was determined by the method of Lowry *et al.* (28). The activities are the mean of three experiments. The K_m values of the wild-type and mutant enzymes were determined as described in Experimental Procedures, using [^{3}H]5-HT ranging from 10 to 100 μM and [^{14}C]PEA ranging from 1 to 10 μM as the substrates. The K_m values are the mean of at least two determinations.

A. Amino acid substitution	MAO-A activity		K_m
	nmol/20 min/mg of protein	*% of wild-type activity*	*μM*
Wild-type	14.6 ± 3.0	100	127
Cys-165 → Ser	8.0 ± 1.6	58 ± 18	97
Cys-201 → Ser	9.7 ± 0.2	69 ± 15	194
Cys-266 → Ser	6.8 ± 0.6	48 ± 9	106
Cys-306 → Ser	8.3 ± 3.0	61 ± 31	133
Cys-321 → Ser	8.7 ± 1.2	62 ± 21	132
Cys-323 → Ser	16.1 ± 2.0	112 ± 10	161
Cys-374 → Ser	0	0	
Cys-398 → Ser	7.2 ± 2.7	49 ± 12	102
Cys-406 → Ser	0	0	
B. Amino acid substitution	**MAO-B activity**		**K_m**
	nmol/20 min/mg of protein	*% of wild-type activity*	*μM*
Wild-type	3.7 ± 3.0	100	1.92 ± 0.68
Cys-5 → Ser	2.7 ± 2.6	68 ± 17	1.64 ± 0.56
Cys-156 → Ser	0	0	
Cys-172 → Ser	2.1 ± 1.4	59 ± 8	3.38 ± 0.45
Cys-192 → Ser	2.2 ± 1.9	56 ± 4	2.29 ± 0.66
Cys-297 → Ser	1.0 ± 0.7	26 ± 6	1.79 ± 0.68
Cys-312 → Ser	2.1 ± 0.6	71 ± 29	1.78
Cys-365 → Ser	0	0	
Cys-389 → Ser	2.4 ± 1.6	67 ± 9	1.27
Cys-397 → Ser	0	0	

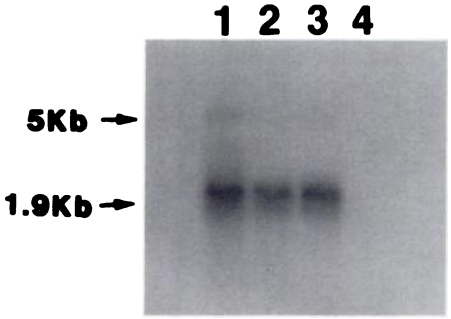

Fig. 1. Northern blot analysis of the wild-type and mutant MAO-A expressed in COS cells. Poly(A)$^+$ mRNA was extracted from transfected COS cells and analyzed for the expression of MAO-A transcripts as described in Experimental Procedures. Each lane contains 2 μg of poly(A)$^+$ mRNA from wild-type or mutant transfected cells. The values on the *left* indicate the size of transcript. *Lane 1*, wild-type MAO-A; *lane 2*, Ser-374 mutant; *lane 3*, Ser-406 mutant; *lane 4*, COS cells.

enzyme. COS cells themselves (Fig. 1, *lane 4*) do not contain the 2.1-kb MAO-A mRNA transcript. This result indicates that these two mutations did not interfere with the transcription of a full length MAO-A mRNA.

Western blot analysis of wild-type and mutant MAO-B enzymes. To test whether the lack of MAO-B activity in the Ser-156, Ser-365, and Ser-397 mutants was due to unsuccessful transfection, the expression products of the transfected cells were analyzed by Western blot using polyclonal anti-MAO-B antiserum. As shown in Fig. 2, wild-type MAO-B expressed in COS cells (Fig. 2, *lane 5*) migrated in SDS-PAGE at the same molecular weight as the MAO-B enzyme from human brain homogenate (~M_r 60,000) (Fig. 2, *lanes 2-4*). The intensity of each band was correlated with the enzyme concentration (compare Fig. 2, *lanes 3* and *4*); thus, this band represents MAO-B. Untransfected COS cells (Fig. 2, *lane 1*) contained no M_r 60,000 band; however, two faint bands with higher molecular weights were observed and may represent nonspecific binding of COS cell protein to the antiserum, because they were not seen in human brain homogenate (Fig. 2, *lane 2*). The M_r 60,000 band was observed in all mutants (Fig. 2, *lanes 6-14*) with similar intensity, suggesting that the transfection efficiencies for all mutants were similar. Thus, the lack of MAO-B activity in Ser-156 (Fig. 2, *lane 7*), Ser-365 (Fig. 2, *lane 12*), and Ser-397 (Fig. 2, *lane 14*) mutants is not due to lack of expression of the enzyme.

Discussion

The importance of cysteine residues for the catalytic activity of MAO-A and -B is suggested by the ability of sulfhydryl-modifying reagents to inhibit the activity of purified placenta MAO-A (14) and bovine MAO-B (15–17). The cloned human liver MAO-A and -B cDNAs (6) consist of 527 and 520 amino acids, respectively, within a single polypeptide, and both cDNAs code for nine cysteines. These nine cysteines are also conserved in the cloned bovine liver MAO-A (23) and rat liver MAO-B cDNA (24). To assess the role of cysteine residues in the catalytic activity of MAO-A and -B, each cysteine was substituted with serine by site-directed mutagenesis and the enzymatic properties of the mutant MAO-A and -B enzymes were characterized.

Role of cysteine in MAO-A. Our results show that the enzymatic activities of two MAO-A cysteine mutants (Ser-374 and Ser-406) were completely lost (Table 2A). Because Cys-406 is located in a pentapeptide of the enzyme (Ser-Gly-Gly-Cys-Tyr) that is involved in the binding of the cofactor FAD (8), mutation of Cys-406 to serine most likely prevents the covalent binding of FAD and thus results in a total loss of catalytic activity. The lack of enzymatic activity of the Ser-374 mutant suggests that Cys-374 may be involved in the active site or is important for the appropriate conformation of the enzyme. This interpretation is consistent with previous work in which the rate of inhibition of MAO-A by the sulfhydryl reagent dipyridyldisulfide (15) was shown to be retarded by the competitive inhibitor *d*-(+)-amphetamine.

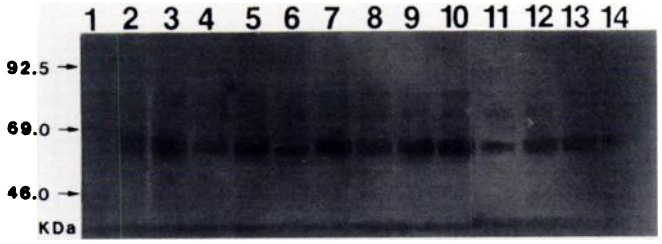

Fig. 2. Western blot analysis of the wild-type and mutant MAO-B expressed in COS cells. One hundred micrograms of homogenates of wild-type or mutant transfected cells were loaded in each lane. The values on the *left* indicate molecular mass. *Lane 1*, 100 μg of COS cells; *lane 2*, 85 μg of human brain homogenate; *lane 3*, 450 μg of human brain homogenate plus 100 μg of COS cells; *lane 4*, 85 μg of human brain homogenate plus 100 μg of COS cells; *lane 5*, wild-type MAO-B; *lane 6*, Ser-5 mutant; *lane 7*, Ser-156 mutant; *lane 8*, Ser-172 mutant; *lane 9*, Ser-192 mutant; *lane 10*, Ser-297 mutant; *lane 11*, Ser-312 mutant; *lane 12*, Ser-365 mutant; *lane 13*, Ser-389 mutant; *lane 14*, Ser-397 mutant.

Other mutants retained MAO-A catalytic activity. However, except for Ser-323 (112% of wild-type activity), the catalytic activities of the mutants (Ser-165, Ser-201, Ser-266, Ser-306, Ser-321, and Ser-398) were lower than that of the wild-type enzyme. Whether the lower activity seen with these mutants was due to lower transfection efficiency, altered protein folding, or lack of insertion into mitochondria is not clear at the present time. Nevertheless, similar K_m values were observed in these cysteine mutants (Ser-165, Ser-201, Ser-266, Ser-306, Ser-321, Ser-323, and Ser-398), suggesting that when these cysteines were changed to serine the binding of enzyme and substrate was not affected.

A Northern blot was used instead of a Western blot to assess the efficiency of the transfection of the MAO-A mutants because of the lack of a good antibody for MAO-A. In the absence of a Western blot, we cannot determine whether the mutant protein is being translated correctly at the same level as the wild-type enzyme. However, for MAO-A only two mutations resulted in losses of activity (Cys-374 and Cys-406). To demonstrate that these two mutants were transcribed, we performed a Northern blot analysis. Our results showed that similar amounts of wild-type and mutant mRNA were transcribed (Fig. 1). The MAO-B Western blot analysis showed that when the corresponding cysteines (Cys-365 and -397, respectively) in MAO-B were mutated to serine the same amount of MAO-B protein was expressed. Because MAO-A and -B are very similar, we feel that substitution of Cys-374 and -406 with serine in MAO-A may not affect the protein translation process. Thus, the Northern blot has demonstrated that the loss of activity is not due to nontransfection.

Role of cysteine in MAO-B. No activity was observed for three MAO-B cysteine mutants (Ser-156, Ser-365, and Ser-397) (Table 2B). The lack of MAO-B activity was not due to the lack of expression of the enzyme, because a similar amount of protein on the Western blot was observed for wild-type enzyme and mutants (Fig. 2). Cys-397 is located in a pentapeptide of the enzyme (Ser-Gly-Gly-Cys-Tyr) known to be involved in the binding of FAD (8). The substitution of Cys-397 by serine most likely prevents the covalent binding of FAD and thus results in a total loss of catalytic activity. Cys-156 and Cys-365 are also seen to be important for MAO-B catalytic activity.

Previous work showed that bovine MAO-B activity changed upon the addition of mercaptoethanol (25), suggesting that sulfhydryl groups are important for MAO-B activity. Gomes *et al.* (15) reported that there are four titratable sulfhydryl groups per monomer of MAO-B. In the presence of benzylamine, a substrate for MAO-B, the enzyme was protected from inacti-

vation by sulfhydryl-modifying reagents and the number of titratable sulfhydryl groups was reduced to three. Furthermore, a widely accepted catalytic mechanism for MAO-B has been proposed by Silverman and Zieske (25) that involves an electron transfer from the substrate amine to FAD to form a flavin radical anion intermediate and in which catalysis is dependent upon hydrogen atom donation from an active site amino acid of MAO-B to FAD. Experiments using the active site-directed MAO-B inhibitor 1-PCPA (18) suggest that cysteine is directly involved in the catalytic mechanism. In these studies, the titration of the cysteine residues of the denatured MAO-B enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) gave 6.2 cysteine residues in controls but only 5.2 residues in samples pretreated with 1-PCPA. Singer (26) confirmed these studies and found eight titratable cysteine residues in the absence of 1-PCPA and seven in its presence. Although these studies indicated different numbers of titratable cysteine residues, they all suggest that one cysteine may be involved in the active site of MAO-B. Based on these studies, either Cys-156 or Cys-365 may be involved in the active site or directly involved in the catalytic reaction, as suggested by Silverman and Zieske (18). It is also possible that they may be important for the appropriate conformation of the enzyme. The present studies cannot differentiate these two possibilities.

The other six MAO-B mutants, Ser-5, Ser-172, Ser-192, Ser-297, Ser-312, and Ser-389, showed catalytic activity. However, their catalytic activities were lower than that of the wild-type enzyme (especially that of the Ser-297 mutant). The Western blot showed that similar amounts of MAO-B were expressed in these mutants, compared with the wild-type enzyme (Fig. 2). However, Western blot indicates only the presence of the protein; the translocation, folding, and insertion of the protein may also affect the number of active enzymes and result in lower activity. It is tempting to speculate that these mutants may be able to insert into the mitochondria, because it has been shown (27) that the signal targeting MAO-B to the mitochondria is present within 29 carboxyl-terminal amino acid residues (positions 492–520). None of the cysteine residues of MAO-B are in this region. However, it is still possible that mutation of a cysteine residue may result in conformational changes at the carboxyl-terminal region and affect the insertion. The K_m values of these six MAO-B cysteine mutants (Ser-5, Ser-172, Ser-192, Ser-297, Ser-312, and Ser-389) were similar to that of the wild-type enzyme, suggesting that these cysteines did not affect the binding of the enzyme to the substrate PEA.

Both MAO-A and -B have nine cysteine residues in their primary sequences. Of these nine, seven are found at analogous sites. The structurally conserved cysteines between MAO-A and MAO-B are A165/B156, A201/B192, A306/B297, A321/B312, A374/B365, A398/B389, and A406/B397 (the FAD binding site). Our data indicate that the important cysteine residues for catalytic function in MAO-A and -B are among these conserved residues. In addition to the FAD binding site, MAO-A406/B397, the corresponding MAO-A374/B365 residues are important for MAO-A or -B catalytic activity. However, there is one additional cysteine residue important in MAO-B (Cys-156). The different characteristics of important cysteine residues between the two forms of the enzyme suggest that there is a structural or conformational difference in the active site of MAO-A and -B that may confer the different substrate and inhibitor specificities.

The present work has identified the essential cysteine residues for MAO-A and -B. With the availability of these cysteine mutants a large scale expression of the enzyme in *E. coli* or yeast can be performed for further purification. With purified mutants and the use of biochemical and molecular biological approaches, the question of whether these essential cysteine residues are directly involved in the active site of these two forms of the enzyme can be further studied.

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