Monoamine oxidase (MAO) is responsible for the oxidation of biogenic and dietary amines. It exists as two isoforms, A and B, which have a 70% amino acid identity and different substrate and inhibitor specificities. This study reports the identification of residues responsible for conferring this specificity in human MAO A and B. Using site-directed mutagenesis we reciprocally interchanged three pairs of corresponding nonconserved amino acids within the central portion of human MAO. Mutant MAO A-I335Y became like MAO B, which exhibits a higher preference for β-phenylethylamine than for the MAO A preferred substrate serotonin (5-hydroxytryptamine), and became more sensitive to deprenyl (MAO B-specific inhibitor) than to clorgyline (MAO A-specific inhibitor). The reciprocal mutant MAO B-Y326I exhibited an increased preference for 5-hydroxytryptamine, a decreased preference for β-phenylethylamine, and, similar to MAO A, was more sensitive to clorgyline than to deprenyl. These mutants also showed a distinct shift in sensitivity for the MAO A- and B-selective inhibitors Ro 41-1049 and Ro 16–6491. Mutant pair MAO A-T245I and MAO B-I236T and mutant pair MAO A-D328G and MAO B-G319D reduced catalytic activity but did not alter specificity. Our results indicate that Ile-335 in MAO A and Tyr-326 in MAO B play a critical role in determining substrate and inhibitor specificities in human MAO A and B.

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Substrate and Inhibitor Specificities for Human Monoamine Oxidase A and B Are Influenced by a Single Amino Acid*

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Monoamine oxidase (MAO); amine:oxygen oxidoreductase (deaminating) (flavin-containing); EC 1.4.3.4) catalyzes the oxidative deamination of biogenic and xenobiotic amines and plays an important role in regulating their levels. MAO is a flavin-adenine dinucleotide-containing enzyme located on the mitochondrial outer membrane (1–3). It exists in two forms, A and B. MAO A preferentially oxidizes serotonin (5-hydroxytryptamine, 5-HT) and is inhibited by low concentrations of clorgyline (4) and Ro 41–1049 (5), whereas MAO B preferentially oxidizes β-phenylethylamine (PEA) and is inhibited by low concentrations of deprenyl (6) and Ro 16–6491 (7). Dopamine is a common substrate (8). MAO A and B are composed of 527 and 520 amino acids, respectively, and have a 70% amino acid identity (9). They are closely linked on the X-chromosome (10) and have an identical intron-exon organization, indicating that they are derived from a common ancestral gene (11). Higher 5-HT and norepinephrine levels and a phenotype characterized by increased aggressive behavior is observed when the MAO A gene is deficient in humans (12) and in mice (13). Disruption of the MAO B gene in mice results in increased PEA but not 5-HT, norepinephrine, or dopmanie and confers a resistance to the Parkinsonism-inducing toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (14). Because MAO is an integral membrane protein, it is difficult to crystallize, and its three-dimensional structure has not been reported.

To determine the region(s) responsible for the substrate and inhibitor specificities of the two isoenzymes, we and other groups have made point mutations and chimeric MAO A/B enzymes (15–21). It has been shown that reciprocally interchanging amino acids Phe-208 in MAO A and its corresponding residue in MAO B, Ile-199, was sufficient to partially reverse the substrate and inhibitor specificities of rat MAOs (22) but not human MAOs (21). This indicated that different amino acid residues may determine specificity in human and rat MAOs. We also found that the residues that may be important for specificity in human MAOs are within a 161-amino acid segment (amino acids 215–375 in MAO A and 206–266 in MAO B) (21). This segment contains 32 amino acids that are nonconserved between human MAO A and B, of which 15 are conserved among the different species of MAO A or B. Trout MAO shares a 70 and 71% amino acid identity with MAO A and B, respectively. However because the substrate and inhibition profile of trout MAO is much closer to that of MAO A than to that of MAO B (23), classifying it as such allows us to reduce the number of amino acids potentially responsible for specificity from 15 to 5 (Fig. 1). Of these five, two were mutated as part of an earlier experiment to the corresponding residue in the other MAO, expressed in Saccharomyces cerevisiae cells, and did not exhibit any kinetic differences from the wild-type enzyme. This indicated that they do not play an important role in specificity. For the other three amino acids we have made six reciprocal mutants. We found that when two corresponding amino acids, Ile-335 and Tyr-326 in human MAO A and B, respectively, were reciprocally interchanged, the substrate and inhibitor specificities were also switched. This result suggests that Ile-335 and Tyr-326 in human MAO A and B, respectively, play a key role in conferring substrate and inhibitor specificities in human MAOs.

EXPERIMENTAL PROCEDURES

Materials—The QuikChange® site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The oligonucleotides were from Operon Technologies Inc. (Alameda, CA). 5-HT, PEA, clorgyline, and B-Y326I exhibited an increased preference for 5-hydroxytryptamine; PEA, β-phenylethylamine.
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and (−)-deprenyl were from Sigma. 5-12-14C]Hydroxytryptamine binoxalate (57.80 mCi/mmol) and β-ethyl-1-14C]Phenylethylamine hydrochloride (52.00 mCi/mmol) were from PerkinElmer Life Sciences. Ro 41–1049 and Ro 16–6491 were from Research Biochemicals International, (Natick, MA). The pVL1392 transfer vector and the linearized BaculoGold® DNA were from PharMingen (San Diego, CA). Gentamycin, antibiotic-antimycotic solution, and fetal bovine serum were from Life Technologies, Inc. The 3,3′-diaminobenzidine was from Pierce. The EagleSight software was from Stratagene.

**Construction of the Baculovirus Transfer Vector**—Full-length human MAO A and B cDNAs were subcloned into the EcoRI site of pVL1392 transfer vector and named pVL1392-hMAOA and pVL1392-hMAOB, respectively. The clone was verified for proper insertion by restriction digest analysis. Recombinant MAO A and B-encoding virus was produced by homologous recombination and cotransfection of Sf21 insect cells with the transfer vector and the linearized baculovirus DNA via the calcium phosphate method (24). Recombinant baculovirus was isolated by plaque purification and amplified by infection of insect cells. Recombinant virus was added at a multiplicity of infection of 2. The cells were incubated at 27 °C for 72–80 h and harvested by centrifugation for 10 min at 5000 × g. Cell pellets were homogenized in 5 ml of 20 mM sodium phosphate buffer, pH 7.4 containing 0.5 mM sodium azide and 150 mM sodium chloride. The homogenates were divided into 0.5-ml aliquots, frozen by dipping into liquid nitrogen, and stored at −80 °C.

**Expression of the Mutant Clones**—Western analysis using rabbit anti-MAO A and anti-MAO B antibodies coupled to hydrogen peroxidase. Signals were developed using a peroxidase-conjugated goat IgG as secondary antibody followed by exposure to 3,3′-diaminobenzidine and H2O2. A highly purified bovine serum albumin standard was electrophoresed in parallel with the homogenates at nine concentration points ranging from 0.1 to 8 μg. The densities of the MAOs and the bovine serum albumin bands were quantitated using the EagleSight software, and the MAO concentrations were calculated according to the linear bovine serum albumin standard.

**Determination of the Kinetic Constants**—The kinetic constants for the oxidation of 5-HT and PEA and the inhibition by clorgyline, deprenyl, Ro 41–1040, and Ro 16–6491 were determined by the radiochemical method as previously described (26) using O2-saturated 50 mM sodium phosphate buffer. For the determination of the Michaelis-Menten equation, the following concentrations were used: [S] = 0.1 to 50 μM, v = 0 to 50 nmol/min. Values of v were plotted as a function of [S], and the kinetic parameters were determined by fitting all the data to the Michaelis-Menten equation. The values of K_m and V_max were calculated according to the linear bovine serum albumin standard.

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versus activity curve to the Michaelis-Menten equation and the calculated concentration of the enzyme from the quantitation assay. The IC_{50} values for the irreversible inhibitors clorgyline and deprenyl were determined by preincubating the inhibitor with the homogenate for 30 min at 37 °C and assaying for the remaining activity as described (26). The reversible inhibitors were incubated with PEA without the 30-min preincubation. Inhibitor concentrations ranged from 10^{-4} to 10^{-11} M, and the IC_{50} values were determined by Hill analysis.

**RESULTS**

We reciprocally interchanged amino acids Thr-245, Asp-328, and Ile-335 individually in human MAO A with their corresponding amino acids in human MAO B to produce the mutants A-T245I, A-D328G, and A-I335Y. Their equivalent mutants in MAO B (B-I236T, B-G319D, and B-Y326I) were also made.

Wild-type MAO A and B, as well as the six mutants, were successfully overexpressed in insect cells using recombinant baculovirus. The turnover number ($k_{cat}$) and the affinity ($K_m$) toward the MAO A-specific substrate 5-HT and the MAO B-specific substrate PEA were determined. We used the specificity constant, $k_{cat}/K_m$, to depict the specificity of an enzyme toward 5-HT or PEA.

As shown in Table I, MAO A wild type had a 6-fold higher $k_{cat}$ for 5-HT than for PEA but a similar $K_m$ for both substrates, resulting in a $k_{cat}/K_m$ value for 5-HT that is about seven times that of PEA (Fig. 2). MAO B wild type, on the other hand, had a 19-fold higher $k_{cat}$ for PEA than for 5-HT and a much lower $K_m$ for PEA, resulting in a $k_{cat}/K_m$ for PEA that is about 40,000 times that for 5-HT. Similarly, MAO A and B had a higher $k_{cat}/K_m$ for 5-HT and PEA respectively. These results are consistent with literature findings that classify 5-HT as MAO A-specific and PEA as MAO B-specific (16, 17, 26).

In contrast to MAO A, mutant MAO A-I335Y showed a higher $k_{cat}$ for PEA than for 5-HT. It also exhibited a 35-fold increase in its $K_m$ for 5-HT to 2801 μM (which is similar to the 5-HT $K_m$ of MAO B of 3891 μM); thus the $K_m$ for 5-HT was lower than for PEA (Table I). This produced a larger $k_{cat}/K_m$ for PEA than for 5-HT (Fig. 2). In effect, A-I335Y acquired an MAO B-like substrate specificity.

Mutant B-Y326I had similar $k_{cat}$ values for 5-HT and PEA and, compared with MAO B, exhibited a 7-fold decrease in $K_m$ for 5-HT and a 5-fold increase in $K_m$ for PEA (Table I). The resulting $k_{cat}/K_m$ values (Fig. 2) indicate that this mutant retained MAO B-like substrate specificities. However, the $k_{cat}/K_m$ of the mutant was only about 75-fold higher for PEA than for 5-HT, compared with an ~40,000-fold difference in the MAO B wild type. Therefore even though B-Y326I retained a higher specificity for PEA than for 5-HT, it exhibited a significant shift in specificity toward MAO A. In summary, Ile-335 in MAO A and Tyr-326 in MAO B play an important role in the substrate specificity of human MAO A and B.

A switch in sensitivities for MAO A and B irreversible inhibitors clorgyline and (−)-deprenyl was also observed (Fig. 3). MAO A has an IC_{50} value of 1.2 × 10^{-9} M for clorgyline and 1.3 × 10^{-6} M for deprenyl, whereas MAO B has an IC_{50} value of 6.3 × 10^{-7} M for clorgyline and 4.3 × 10^{-9} M for deprenyl. Compared with MAO A, A-I335Y exhibited about a 6000-fold decrease in sensitivity toward clorgyline (IC_{50} = 7.1 × 10^{-6} M) and a 10-fold increase in sensitivity toward deprenyl (IC_{50} = 1.2 × 10^{-7} M) (Fig. 3). Thus the inhibitor sensitivity of this MAO A mutant became MAO-B like. Similarly, MAO B-Y326I was MAO A-like and was more sensitive to clorgyline (IC_{50} = 2.8 × 10^{-8} M) than to deprenyl (IC_{50} = 1.8 × 10^{-7} M) (Fig. 3). Therefore Ile-335 and Tyr-326 determine clorgyline and deprenyl sensitivities.

We also studied enzyme specificity toward the reversible inhibitors Ro 41–1049 (MAO A-specific) and Ro 16–6491 (MAO B-specific). As shown in Fig. 4A, A-I335Y had about a 4000-fold decreased sensitivity toward Ro 41–1049 compared with MAO A (IC_{50} = 5.6 × 10^{-8} M for MAO A and 2.5 × 10^{-4} M for A-I335Y), becoming MAO B-like. Similarly, B-Y326I exhibited a 1000-fold higher sensitivity for the MAO A-specific inhibitor Ro 41–1049 compared with MAO B (IC_{50} = 2.5 × 10^{-4} M for MAO B and 2.5 × 10^{-6} M for B-Y326I) and became more like MAO A. However, for the MAO B-specific inhibitor Ro 16–6491, both mutants exhibited a decrease in sensitivity when compared with their parent enzyme, and no reversal in specificity was observed.

The substrate and inhibitor specificities exhibited by A-I335Y and B-Y326I suggest that specificity may be determined by the presence of either an aliphatic or an aromatic side chain at this position. To confirm this, we made mutants A-I335F and B-Y326F. A-I335F had $k_{cat}/K_m$ values of 3.2 × 10^{-4} M for 5-HT and 0.03 for PEA, making it similar to A-I335Y. In addition, B-Y326F had $k_{cat}/K_m$ values of 0.042 for 5-HT and 2.71 for PEA, which are similar to values for B-Y326I. For the inhibitors, A-I335F had IC_{50} values of 6.8 ± 0.7 × 10^{-6} for clorgyline and 3.8 ± 0.4 × 10^{-7} for deprenyl, which are similar to values for A-I335Y, and equivalently B-Y326F had IC_{50} values of 2.4 ± 0.8 × 10^{-8} for clorgyline and 2.2 ± 0.5 × 10^{-7} for deprenyl, which are similar to values for B-Y326I. These results show that it is the presence of either an aromatic or aliphatic side chain that is responsible for the inverse specificity effect.

The other two pairs of mutants, A-T245I and B-I326T and A-D328G and B-G319D, showed a decrease in $k_{cat}$ values for both 5-HT and PEA compared with their parent enzymes. A-T245I and A-D328G showed a $k_{cat}$ of 12.6 ± 1.6 and 1.6 ± 0.2 min^{-1}, respectively, for 5-HT (comparing with 67.4 ± 5.4 min^{-1} for MAO A wild type) and 4.2 ± 0.5 and 0.3 ± 0.1 min^{-1}, respectively, for PEA (comparing with 11.2 ± 0.4 min^{-1} for MAO A wild type). B-I326T and B-G319D showed $k_{cat}$ values of 1.8 ± 0.1 and 2.2 ± 0.1 min^{-1}, respectively, for 5-HT (comparing with 5.1 ± 0.1 for MAO B wild type) and 19.4 ± 0.8 and 14.3 ± 2.0 min^{-1}, respectively, for PEA (comparing with 98.4 ± 5.2 for MAO B wild type). However, they retained the $K_m$ values for both substrates, thus resulting in unaltered specificities.

Their IC_{50} values for clorgyline, deprenyl, Ro 49–1049, and Ro 16–6491 were also unchanged compared with their parent enzyme. These results suggest that the mutations decrease the

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (min^{-1})</th>
<th>PEA (μM)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$</th>
<th>$K_m$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAO A wild type</td>
<td>67.4 ± 3.4</td>
<td>11.2 ± 0.4</td>
<td>80 ± 4</td>
<td>91 ± 4</td>
<td></td>
</tr>
<tr>
<td>MAO A-I335Y</td>
<td>0.7 ± 0.1</td>
<td>1.8 ± 0.3</td>
<td>2801 ± 6</td>
<td>87 ± 15</td>
<td></td>
</tr>
<tr>
<td>MAO B wild type</td>
<td>5.1 ± 0.1</td>
<td>98.4 ± 5.2</td>
<td>3891 ± 17</td>
<td>1.9 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>MAO B-Y326I</td>
<td>21.3 ± 3.3</td>
<td>27.3 ± 3.5</td>
<td>569 ± 32</td>
<td>9.5 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE I**

$k_{cat}$ and $K_m$ values of wild-type and mutant MAOs for the substrates 5-HT and PEA. The $k_{cat}$ and $K_m$ values were determined as described under “Experimental Procedures” for the MAO A-prefering substrate 5-HT and the MAO B-prefering substrate PEA. The values given are the means of at least three experiments ± S.E.
catalytic activity by either directly or indirectly affecting the active site. However, they do not affect substrate or inhibitor specificity.

It should be noted that even though the substrate and inhibitor specificities of A-I335Y and B-Y326I were switched, their kinetic and inhibitory constants were not identical to those of the other MAO; the $K_m$ values for PEA were not greatly affected. The IC$_{50}$ values for the inhibitors shift toward, but do not become identical to, the opposite MAO, and no specificity change was observed for Ro 16–6491. This suggests that other amino acids also play a role in determining substrate and inhibitor specificities.

In summary, the MAO A mutant A-I335Y acquired kinetic
parameters similar to those of MAO B. Similarly, the MAO B mutant B-Y326I acquired kinetic parameters more like those of MAO A. Therefore our results suggest that Ile-335 in MAO A and its corresponding residue in MAO B, Tyr-326, play an important role in conferring substrate and inhibitor preferences to human MAO A and B.

**DISCUSSION**

Using site-directed mutagenesis, we constructed six MAO mutants by reciprocally interchanging three corresponding amino acid pairs in human MAO A and B within a region thought to be important for conferring substrate and inhibitor specificities. The corresponding mutant pair A-I335Y and B-Y326I exhibited opposite specificities compared with their parent enzymes, MAO B, Tyr-326, play an important role in conferring substrate and inhibitor preferences to human MAO A and B.

The Ro 41–1049 (A) and 16–6491 (B) inhibition curves of wild-type MAOs and the MAO mutants A-I335Y and B-Y326I are plotted as percent inhibition versus log inhibitor concentrations. Error bars represent the S.E. of three experiments. The symbols ■, □, ●, and ○ represent MAO A, A-I335Y, MAO B, and B-Y326I, respectively.

FIG. 4. Ro 41–1049 and Ro 16–6491 inhibition of wild-type MAOs and the mutants A-I335Y and B-Y326I. The Ro 41–1049 (A) and 16–6491 (B) inhibition curves of wild-type MAOs and the MAO mutants A-I335Y and B-Y326I are plotted as percent inhibition versus log inhibitor concentrations. Error bars represent the S.E. of three experiments. The symbols ■, □, ●, and ○ represent MAO A, A-I335Y, MAO B, and B-Y326I, respectively.

However, this binding interaction may not be generalized to MAOs of other species. It was reported that switching Phe-208 and Ile-199 in rat MAO A and B, respectively, results in a partial inversion of specificities for some substrates and inhibitors (22). This result suggested that the structural feature responsible for determining specificity was the aromatic ring of Phe-208 in rat MAO A and the aliphatic side chain of Ile-199 in rat MAO B. This is the reverse of our present observation in human MAO, in which the aliphatic residue is in MAO A (Ile-335), and the aromatic residue is in MAO B (Tyr-326). Even though the data from the rat MAO mutants consisted only of $K_m$ values to ascertain substrate specificity and a single inhibitor concentration to determine inhibitor specificity, when compared with our results, these data may point to an MAO species difference in the way specificity is determined. In fact, we have previously found that the same amino acid substitutions made on human MAO, A-F208I and B-I199F, did not result in a change in specificities (21), as was observed in rats. Several reports indicate the existence of large differences in specificities among MAOs of the same subtype but from different mam-
malian species (27–30). Some oxadiazolone compounds have inhibitory potencies that vary four orders of magnitude between rat and bovine MAO B (28), whereas some antidepressant drugs show a B over A specificity in mouse and rat MAOs and the reverse specificity in rat and monkey MAOs (29).

These reports and our results suggest that the specificities of rat and human MAOs (and MAOs of other species) are determined by different amino acids. Indeed, a computer modeling study suggests spatial differences between the binding sites of MAO A and B that specificity may be determined by different specificities in human MAO A and B, respectively. Thr-245 and Asp-328 in MAO A and Ile-236 and Gly-319 in MAO A may interact with the active site but do not determine specificity.

The other four mutants (A-T245I, B-I236T, A-D328G, and B-G319D) did not result in any marked differences in their kcat values for both 5-HT and PEA were lower than those of the parent enzymes. Among them, a large decrease in kcat was observed in the two MAO A mutants, A-T245I and A-D328G. This indicates that these amino acid residues interact either directly or indirectly with the active site.

In summary, the present study identifies the corresponding amino acid pair Ile-335 and Tyr-326 as critical for the substrate and inhibitor specificities in human MAO A and B, respectively. Thr-245 and Asp-328 in MAO A and Ile-236 and Gly-319 in MAO A may interact with the active site but do not determine specificity.

3 Johan Wouters, Belgium, personal communication.

REFERENCES