A Key Amino Acid Responsible for Substrate Selectivity of Monoamine Oxidase A and B

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Monoamine oxidase (MAO) oxidizes biologically important amines including neurotransmitters and plays a central role in the regulation of intracellular levels of these amines. Two distinct forms of MAO (MAO A and MAO B) were defined based on differences in substrate and inhibitor specificities. We earlier reported that the region between about residues 120 and 220 of rat MAO is responsible for determination of the substrate selectivity of MAO A and B (Tsugeno, Y, Hirashiki, I., Ogata, F., and Ito, A. (1995) J. Biochem. (Tokyo) 118, 974–980). To determine the essential amino acids in this region that participate in substrate recognition, a series of mutant enzymes in which amino acid residues that are conserved among various species but are different between the two forms of the enzyme were replaced with the corresponding amino acids of the counterpart and were engineered from the cDNAs of rat liver MAO A and B, and affinities for several substrates were examined. A single mutation in which Phe-208 in MAO A was substituted by the corresponding residue of Ile in MAO B was sufficient to convert the A-type substrate selectivity, and the reverse was exactly the case. Phe at this position was replaceable with Tyr for the A-type specificity and Ile was replaceable with Val and Ala for the B-type. Thus, aromatic and aliphatic residues seem to contribute to render substrate selectivity of MAO A and MAO B, respectively.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were purchased from Biologica Co. (Nagoya, Japan). Restriction endonucleases and DNA-modifying enzymes were purchased from Nippon Gene (Toyama, Japan), Toyobo (Osaka, Japan), Takara Shuzo (Kyoto, Japan), and New England Bio-labs Inc. (Beverly, MA). Zymolyase 100T was from Seikagaku Kogyo (Tokyo, Japan). Clorglyline and (-)-deprenyl were obtained from Sigma and Research Biochemicals Inc. (Natick, MA), respectively. The radiochemicals were purchased from American Radiolabeled Chemicals Inc. (St Louis, MO), Amersham International (Amersham, England), and ICN Pharmaceuticals Inc. (Irvine, CA). Affinity chromatography resins were purchased from Calbiochem-Behring (La Jolla, CA). The radiochemicals used here were of the highest grade commercially available.

Site-directed Mutation of MAOs—The cDNAs for rat MAO A (21) and MAO B (22) were subcloned into the pBluescript vector. Site-directed mutagenesis was performed with synthetic oligonucleotides, according to the procedure used by Kunkel et al. (25). Oligonucleotides CACTTGTTAACGGCTCAGCATGCGACG, CTCTTCGATGCGGTGCCTTCC, GCTGATCAGCAGCCCTG, and GTTGTTGAGAAGAT-GCTATGGC were purchased from New England Nu-clear (Boston, MA). All other chemicals used here were of the highest grade commercially available.

Expression of Mutant MAOs in Yeast Cells and Preparation of Mito-
chondria from the Cells—Transformation of yeast cells, Saccharomyces cerevisiae strain TD-1 (his4–38, ura3–52, trp1–288, can1), MAK(k) (cir+) with plasmid pYcDE2, was done according to Ito et al. (27), and Trp" transformants were selected on SD synthetic medium containing 2% glucose, 0.67% yeast nitrogen base without amino acids, 20 mg/ml each of adenine sulfate and uracil, and 0.5% casamino acid. The transformed cells were cultured to the mid-logarithmic phase in YPD medium with vigorous shaking, and then the cells were harvested by centrifugation at 2,200 × g for 10 min. Mitochondrial fraction was prepared as described (21). Briefly, after treatment of the cells with Zymolyase 100 T (2 mg/g wet cells) for 60 min at 30 °C, the resultant was suspended in 10 mM HEPES-KOH buffer, pH 7.4, containing 0.65 mM sorbitol, 1 mM EDTA, and a protease inhibitor mixture (0.1 mM chymostatin, 2 mg/ml aprotinin, 1 mM pepstatin, 1.1 mg/ml phosphoramidon, 7.2 mg/ml E-64, 0.5 mg/ml leupeptin, 2.5 mg/ml antipain, 0.1 mM benzamidine, 1 mM phenylmethysulfon fluoride) with a Dounce homogenizer. The lysate was centrifuged at 1,000 × g for 10 min, and the supernatant was further centrifuged at 10,000 × g for 10 min to obtain the mitochondrial fraction. The mitochondrial pellet was finally suspended in 10 mM HEPES-KOH buffer, pH 7.4, containing 15% glycerol, 0.65 mM sorbitol, and 1 mM EDTA and stored at −80 °C until use.

Assay of MAO Activity—Because each mutant enzyme had a different stability after solubilization of the mitochondrial membranes, we used fresh mitochondrial fractions for MAO activity. The activity was determined by the radiometric procedure described by Wurtmann and Axelrod (28), using as substrates serotonin, tryptamine, tyramine, and PEA. The assay mixture contained the mitochondrial fraction (50 μg of protein) in a total volume of 60 μl of 50 mM phosphate buffer, pH 7.4. The reaction, initiated by adding the substrate, was run for 20 min at 37 °C and was stopped by the addition of 40 μl of 2 M HCl. When tryptamine and PEA were used as substrate, the reaction time was set within the period (2 min) for which the reaction progressed linearly. The product was extracted with water-saturated ethyl acetate-toluene (1:1 v/v), and that in the organic solvent phase was estimated in a Packard Tri-Carb liquid scintillation spectrometer. In inhibition experiments, mitochondria were preincubated with various concentrations of clorgyline or deprenyl in 50 mM phosphate buffer, pH 7.4, at 37 °C for 30 min, and the remaining activity was determined as described above.

RESULTS

Comparison of the primary structures of the two forms of rat MAO with those from other species revealed a greater degree of similarity between the same form from different species than between different forms from the same species. The amino acid residues that are identical in different species in each form can be inferred to have been conserved during evolution because of their functional importance. The residues that are conserved among various species but different between the two forms might be those responsible for substrate selectivity of MAO. In a region between residues 120 and 220 that has been determined to be important in conferring substrate selectivity of MAO A and MAO B (21), only nine amino acid residues are identical in each form of MAO but are different between the two forms (amino acid residues boxed in Fig. 1). Five nonconservative amino acid residues in MAO B (shaded in Fig. 1) were replaced with the corresponding amino acids in MAO A, by site-directed mutation using oligonucleotides. The cDNAs encoding the wild-type and mutated MAOs were introduced into yeast cells, and the mitochondrial fractions were isolated from the cells expressing the enzymes, as described under “Experimental Procedures.” Monoamine oxidase activities in the mitochondrial fractions were measured using as substrates serotonin, tryptamine, tyramine, and PEA.

As described in the previous report (21), the specific activities of the mutant proteins based on mitochondrial protein varied, and the mutants derived from MAO B, even the parent MAO B, usually had very low activity in yeast cells with every substrate used. Thus, a comparison of the Vmax values among the mutant enzymes would be invalid. In the present study, affinity for four substrates (Km) served as a criterion for ascertaining which type of the substrate selectivity was rendered to the mutant enzymes (Table I). The MAO B variants carrying a His residue instead of Leu at position 139, MAO B(L139H), has substantially the same affinity profile for the substrates as that of the wild-type MAO B, that is, high affinity to PEA and no detectable activity toward serotonin. Affinity for the substrates of the mutant with triple substitutions at positions 172, 175, and 177, MAO B(C172N/A175S/T177P), was also unaffected. However, the mutant B(1199F), in which Ile-199 was converted to Phe, exhibited a dramatic increase in affinity for serotonin and tyramine, and their Km values were close to those of MAO A, although practically no change was observed in the affinity for tryptamine but with some decrease in that for PEA. The result indicates that the amino acid residue at position 199 in MAO B is important for selective binding of some substrates such as serotonin and tyramine. Because the above data pointed to Ile-199 as the single most important determinant of substrate selectivity of MAO B, we asked whether the corresponding amino acid in MAO A plays the same role in the substrate recognition. Phe at position 208 in MAO A was mutated to Ile to make the counter mutant of B(I199F), A(F208I). As shown in Table I, the mutation caused a marked reduction in affinity for serotonin and tyramine, to the same level of that in MAO B with practically no change in the affinity for tryptamine and PEA. This confirmed that an amino acid residue at the position 208 in MAO A and the residue at the corresponding position in MAO B plays a vital role in determination of substrate selectivity of MAO A and MAO B.

To determine whether structural differences between Ile and Phe, a hydrophobic branched chain and an aromatic ring, respectively, contribute to different affinities for the substrates, Phe-208 in MAO A was replaced with Tyr, Val, and Ala. Mutation of Phe to Tyr had no effect on affinity for all the substrates used. However, mutants in which Phe was converted to the residues with aliphatic side chains, A(F208V) and A(F208A), exhibited exactly the same affinity profile as that of the mutant A(F208I) discussed above. Thus, substrate selectivity of MAO B can be explained by the different aromatic and aliphatic side chains of amino acids at this position.

Effects of the type-specific inhibitors on the mutation at position 208 in MAO A were examined using clorgyline and deprenyl as inhibitors for MAO A and MAO B, respectively. PEA was used as the substrate because all the enzymes oxidized this substrate. IC50 values of clorgyline and deprenyl for MAO A were reported to be 0.025 μM and 5.0 μM, respectively, and those for MAO B were reported to be 79 μM.
and 0.13 μM, respectively (21). 0.5 and 1 μM of clorgyline and deprenyl, respectively, were then used to examine the different sensitivity of the mutants to the two inhibitors (Fig. 2). At these concentrations of inhibitors, MAO A activity was abolished by clorgyline, whereas the full activity remained even in the presence of deprenyl. The reverse was true for MAO B. As expected from data on the substrate selectivity of B-type, B(L139H) and B(C172N/A175S/T177P) were inhibited only with deprenyl. However, B(I199F) had substantially the same inhibition pattern as those mutants and MAO B, although the enzyme did exhibit an A-type nature at least with regard to the affinity for serotonin and tyramine. On the contrary, patterns of sensitivity to inhibitors of A(F208I), A(F208V), and A(F208A) converted to that of the B-type enzymes with the single amino acid mutation, as in the case for substrate selectivity. A(F208Y) showed the same inhibitor sensitivity as the parent enzyme. Thus, except for B(I199F), all mutant enzymes had inhibitor sensitivity expected from their substrate selectivity.

**DISCUSSION**

We obtained evidence that an amino acid residue at position 208 in MAO A and the residue at the corresponding position 199 in MAO B plays an important role in determination of substrate selectivity of MAO A and MAO B. An enzyme with aromatic amino acids, such as Phe and Tyr, at this position has a similar affinity for most substrates with aromatic amino acids, such as Phe and Tyr, at this position has a similar affinity for most substrates with aromatic amino acids. A good correlation between aromatic amino acid at this position and properties in substrate specificity and inhibitor sensitivity more like those of mammalian MAO A-type substrate specificity was seen in trout MAO (29), which has Phe at this position and properties in substrate specificity and inhibitor sensitivity more like those of mammalian MAO A, although it does share a similar extent of homology (about 70%) with both mammalian MAO A and MAO B.

MAO A has a similar affinity for most substrates with aromatic rings, yet over a 1000-fold difference in the affinity among substrates was observed for MAO B. The finding of participation of the aromatic side chain in substrate recognition of MAO A suggests that π-π interaction between aromatic rings of substrates and the enzyme plays a major part in their interaction and could explain why MAO A has a similar affinity for aromatic substrates. This was further confirmed by inhibition experiments using aromatic and nonaromatic compounds. Serotonin oxidizing activity of MAO A was competitively inhibited by PEA and 3-phenyl-1-propylamine with \( K_i \) values of about 150 μM, whereas the value of cyclohexane methylamine was over 10 times higher (about 2 mM). On the other hand, the presence of an amino acid with an aliphatic side chain at the position responsible for substrate selectivity of MAO B as well as large differences in its affinity to aromatic substrates indicate involvement of different interactions from the π-π interaction between substrates and the enzyme. PEA and tryptamine were good substrates for MAO B, whereas the enzyme showed practically no activity toward serotonin and tyramine, which have an extra phenolic hydroxyl group to the same carbon skeleton as tryptamine and PEA, respectively. Affinity of the enzymes for serotonin and tyramine was markedly reduced with a single mutation of residue 208 of MAO A from aromatic to aliphatic amino acids. It has been shown that C9-C10 linear aliphatic amines are preferentially oxidized by MAO B (30). Taken together, these results indicate that a hydrophobic van der Waals’ interaction, instead of an aromatic one, seems to be involved in recognition of substrates by MAO B and the phenolic hydroxyl group may strongly interfere such an interaction between substrates and the enzyme. When Ile at position 199 in MAO B was replaced with Phe, aromatic interaction may take place of hydrophobic one in substrate recognition of the mutant enzymes, and it acquired reasonable affinity to compounds with phenolic hydroxyl group. Although most mutant enzymes have the sensitivity to type-specific inhibitors expected from their substrate selectivity, one mutant,

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**Table I**

Affinity of wild-type and mutant MAOs for substrates

| Substrate   | MAO A (wild type) | MAO B (wild type) | MAO B (L139H) | MAO B (C172N/A175S/T177P) | MAO B (I199F) | MAO A (F208I) | MAO A (F208V) | MAO A (F208A) |
|-------------|------------------|------------------|----------------|---------------------------|---------------|---------------|---------------|---------------|
| Serotonin   | 0.31 ± 0.01      | NC               | NC             | NC                        | 0.19 ± 0.04   | NC            | NC            | NC            |
| Tyramine    | 0.40 ± 0.1       | 2.9 ± 0.6        | NC             | ND                        | 0.39 ± 0.01   | 5.8 ± 2       | 3.8 ± 0.5     | 3.5 ± 0.1     |
| Tryptamine  | 0.23 ± 0.1       | 0.067 ± 0.006    | ND             | ND                        | 0.069 ± 0.006 | 0.22 ± 0.01   | 0.34 ± 0.01   | 0.29 ± 0.03   |
| PEA         | 0.24 ± 0.05      | 0.010 ± 0.001    | 0.015 ± 0.001  | 0.010 ± 0.003             | 0.076 ± 0.02  | 0.12 ± 0.01   | 0.085 ± 0.007 | 0.12 ± 0.01   |

*NC, not calculated. \( K_i \) value was unable to be calculated because the enzyme had no detectable activity toward this substrate, even at 5 mM. ND, not determined.

**FIG. 2. Inhibition pattern of parent and mutant MAOs by type-specific inhibitors.** Yeast mitochondria (50 μg of protein) were preincubated at 37°C for 15 min in the presence of clorgyline (5.0 × 10⁻⁷ M) or deprenyl (1.0 × 10⁻⁶ M) in a total volume of 40 μl of 50 mM potassium phosphate buffer (pH 7.4). Labeled substrate (20 μl) was then added to estimate MAO activity. The remaining activity is expressed as a percentage of the control activity in the absence of inhibitors.
B(I199F), exhibited the B-type sensitivity, in contrast to its substrate preference of the A-type. Polarity of the two chloride groups in clorgyline may be too large and strong to be accepted in the substrate-binding pocket of MAO B, even after substitution of Ile-199 by an aromatic amino acid.

The present study provides the first experimental evidence for identification of a key amino acid participating the substrate selectivity of MAO. Our observations provide important information for molecular design of highly selective inhibitors.

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