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Cloning of a Novel Monoamine Oxidase cDNA from Trout Liver

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SUMMARY

A trout liver monoamine oxidase (MAO) cDNA was cloned by screening a cDNA library with a human MAO-A cDNA probe. The trout MAO cDNA encodes 499 amino acids, with a molecular mass of 56.6 kDa. The deduced amino acid sequence of trout MAO shows 70% and 71% identity with those of human MAO-A and MAO-B, respectively. Trout MAO contains the pentapeptide sequence Ser-Gly-Gly-Cys-Tyr, to which the cofactor FAD is covalently bound. Transient expression of the cDNA in COS-7 cells shows that trout MAO oxidizes both serotonin [5-hydroxytryptamine (5-HT)] and β-phenylethylamine (PEA), unlike human MAO-A and MAO-B, which oxidize only 5-HT and PEA, respectively. The K_m for 5-HT is similiar for trout MAO (130 ± 17 mM) and human MAO-A (68 ± 4 mM). The K_m for PEA is similiar for trout MAO (12.5 ± 2.0 mM) and human MAO-B (1.5 ± 0.2 mM). When 5-HT is used as a substrate, trout MAO is more sensitive to clorgyline (IC_{50}, 2.8 ± 0.2 × 10^{-8} M) than deprenyl (IC_{50}, 1.0 ± 0.1 × 10^{-6} M), a result similiar to the inhibtion selectivity of human MAO-A. However, trout MAO is less sensitive to clorgyline than is human MAO-A (IC_{50}, 5.8 ± 0.1 × 10^{-10} M). Trout MAO is less sensitive to deprenyl (IC_{50}, 4.6 ± 0.3 × 10^{-7} M) than is human MAO-B (IC_{50}, 1.4 ± 0.1 × 10^{-9} M) when PEA is used as the substrate. These results indicate that trout MAO displays substrate and inhibitor selectivities that are not identical to those of either MAO-A and -B, and it therefore represents a novel type of MAO. The structure of trout MAO will provide insights into the substrate and inhibitor selectivities of the MAOs.

MAO (EC 1.4.2.4) is a flavoenzyme, located in the outer mitochondrial membrane (1), that catalyzes the oxidative deamination of many neurotransmitters and dietary amines. Two types of MAO (A and B) have been described, based on substrate preferences and inhibitor selectivity (2–5). MAO-A preferentially oxidizes biogenic amines such as 5-HT, norepinephrine, and epinephrine and is irreversibly inactivated by lower concentrations of clorgyline than is MAO-B. MAO-B preferentially oxidizes PEA and benzylamine and is inactivated by lower concentrations of deprenyl than is MAO-A. Dopamine, tyramine, and kynuramine are common substrates for MAO-A and -B.

Cloning of the H-MAO-A (6, 7) and H-MAO-B cDNAs (6) and their expression in mammalian cells (8) provided unequivocal evidence that the substrate and inhibitor selectivities of MAO-A and B reside in their primary amino acid sequences. Comparisons of the deduced amino acid sequences from the H-MAO-A, R-MAO-A, and B-MAO-A and the H-MAO-B and R-MAO-B cDNAs show greater identity for the same MAO subtype from different species, compared with the identity between MAO-A and -B from the same species (9). Both forms of MAO have been detected in birds, reptiles, and amphibia (10). Sea urchins (11) and starfish (12) contain a single form of MAO that oxidizes both 5-HT and PEA but shows a higher affinity for PEA. Furthermore, this MAO activity is more sensitive to deprenyl than clorgyline regardless of the substrate used, which suggests that it is like the B subtype. Similarly, MAO activity found in octopus oxidizes both 5-HT and PEA, but the latter has a higher affinity (13). However, octopus MAO is equally sensitive to clorgyline and deprenyl regardless of the substrate, suggesting that it does not correspond to the A or B subtype. Teleosts, including goldfish (14), perch (15), carp (16, 17), trout (17), and pike (18), contain a single form of MAO, unlike tetrapods, which contain both MAO-A and -B subtypes. Evidence for a single form of MAO in teleosts is based on the observation that MAO inhibitors display single-phase sigmoidal inhibition curves. In contrast, tetrapods display a double-phase sigmoidal inhibition curve using either clorgyline or deprenyl, which indicates the presence of two MAO subtypes. It is believed that MAO-B confers an advantage for a terrestrial mode of life (10).

The sensitivity of teleost MAO to inhibition by MAO-A-selective inhibitors such as harmaline and clorgyline suggests a type A-like enzyme (10, 14, 15). However, other studies using MAO-A- and -B-selective substrates and inhibitors show that teleost MAO is equally sensitive to clorgyline and deprenyl and is capable of oxidizing both 5-HT and PEA (16–18). This paper

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ABBREVIATIONS: MAO, monoamine oxidase; 5-HT, 5-hydroxytryptamine; PEA, β-phenylethylamine; T-MAO, trout monoamine oxidase; R-MAO, rat monoamine oxidase; B-MAO, bovine monoamine oxidase; H-MAO, human monoamine oxidase.

was designed to further evaluate the enzymatic properties of teleost MAO by cloning and expressing a T-MAO cDNA. It is of great interest to determine the primary structure of T-MAO for comparisons at the amino acid level, to help identify domains important for substrate and inhibitor selectivity.

Materials and Methods

Chemicals. [α-^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham. [^{3}H]5-HT (26.7 Ci/mmol) and [^{14}C]PEA (58 Ci/mmol) were purchased from DuPont.

Screening of a cDNA library. A λZapII cDNA library constructed from trout liver poly(A)$^+$ mRNA was kindly provided by Dr. T. T. Chen. Approximately 1 × 10^6 plaques were screened by hybridization to a ^{32}P-labeled *Sac*I restriction fragment that represents nucleotides 553–1201 of the H-MAO-A cDNA (6).

Three positive clones were initially selected and partially sequenced to identify sequence homologies with MAO. A clone containing the largest positive cDNA insert (2.8 kilobases) was sequenced on both strands by the dideoxy method, using Sequenase version 2.0 (United States Biochemicals) (19). Universal and reverse primers, in addition to primers based on obtained sequences, were used for DNA sequencing.

Transient expression of MAO cDNAs. T-MAO, H-MAO-A, and H-MAO-B cDNAs were subcloned into the *Eco*RI site of the mammalian expression vector pECE (yielding T-MAO/pECE, H-MAO-A/pECE, and H-MAO-B/pECE, respecitvely) (8). The correct orientation of the cDNAs with respect to the simian virus 40 promoter was determined by endonuclease digestion. The resulting plasmids were purified by two CsCl gradients and were transiently transfected into COS-7 cells by a high efficiency, calcium phosphate transfection method (20). Fifty milligrams each of H-MAO-A/pECE, H-MAO-B/pECE, and T-MAO/pECE were transfected under the same conditions.

Determination of MAO-A and -B catalytic activity. Catalytic activities of T-MAO and H-MAO-A and -B were determined by using whole-cell homogenates 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. The final concentrations in the 1-ml assay mixture were 50 mM sodium phosphate buffer, pH 7.4, 100 μM [^{3}H]5-HT or 10 μM [^{14}C]PEA, and 40 μl of 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) (for 5-HT) or toluene (for PEA). Each tube was vortexed for 30 sec and centrifuged at 2000 × *g* for 10 min. Four milliliters of the organic layer were withdrawn and mixed with 5 ml of scintillation fluid. The radioactivity of the reaction product was determined by liquid scintillation counting.

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 point. K_m and V_{max} values were determined by the direct linear estimate method, as implemented in the Enzyme Kinetics program (D. G. Gilbert, dogStar Software, Biology Department, University of Indiana, Bloomington, IN).

Inhibition of the expressed wild-type and mutant MAO-A and -B by deprenyl and clorgyline was performed by preincubating various concentrations of the inhibitors with whole-cell homogenates (~8 × 10^5 cells) at 37° for 30 min before addition of the substrate. The concentration of deprenyl or clorgyline required for 50% inhibition of MAO activity (IC_{50}) was calculated by Hill analysis. The IC_{50} values were the mean of two determinations. The protein concentration of the cell homogenate was determined by the method of Lowry *et al.* (22).

Results

Isolation of the T-MAO cDNA. A trout liver cDNA library was screened by hybridization to a ^{32}P-labeled H-MAO-A cDNA representing nucleotides 553–1201 (6). Three clones were identified; they differ only in the lengths of their 3′ ends. The nucleotide sequence and deduced amino acid sequence of the longest clone, of 2.8 kilobases (T-MAO-2.8), are shown in Fig. 1. The open reading frame of T-MAO-2.8 contains an ATG initiation codon at nucleotide 100 and a TAG stop codon at

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1     GGAATTCGCGGCCGCCGAGGCAACGGCAGGCAGGCTTGGTAGAGAGCGAGAGATAGACAG   60
61    TGACAGTGAGAAAGAGAGAGCGAGAGCCATAACAACACCATGACAGCACAGAACACATTC  120
                                                M  T  A  Q  N  T  F
121   GACGTTATAGTTATTGGAGGAGGAATATCAGGGCTGAGTGCAGCTAAGCTGTTGAAGGAG  180
      D  V  I  V  I  G  G  G  I  S  G  L  S  A  A  K  L  L  K  E
181   AAAGGGCTGAGTCCTGTGGTTCTGGAAGCTAGAGACCGGGTTGGTGGACGGACCTTCACC  240
      K  G  L  S  P  V  V  L  E  A  R  D  R  V  G  G  R  T  F  T
241   GTACAGAATGAGCAGACTAAGTATGTTGACCTGGGCGGGGCCTACGTCGGGCCTACTCAG  300
      V  Q  N  E  Q  T  K  Y  V  D  L  G  G  A  Y  V  G  P  T  Q
301   AATCGTATCCTCCGATTGGCTAAAGAGTGTGGAGTCAAGACCATCAAAGTCAACGAGGAG  360
      N  R  I  L  R  L  A  K  E  C  G  V  K  T  I  K  V  N  E  E
361   GAGAGGCTGGTGCATTATGTAAAGGGAAGTCCTACCCATTCAAAGGCTCCTTCCCCCCC  420
      E  R  L  V  H  Y  V  K  G  K  S  Y  P  F  K  G  S  F  P  P
421   ATGTGGAACCCATTCGCCCTGATGGACTACAACAATCTGTGGAGGAAGATGGATGAGATG  480
      M  W  N  P  F  A  L  M  D  Y  N  N  L  W  R  K  M  D  E  M
481   GGCAGCGAGATCCCCAGAGAGGCTCCATGGAAGGCTCCACATGCTGAGGAGTGGGACAAG  540
      G  S  E  I  P  R  E  A  P  W  K  A  P  H  A  E  E  W  D  K
541   ATGACCATGAAGCAGCTCTTTGATAAGATCTGCTGGACCAGTTCTGCGAGGCGGTTCGCA  600
      M  T  M  K  Q  L  F  D  K  I  C  W  T  S  S  A  R  R  F  A
601   ACTCTGTTTGTGAACGTGAATGTGACCTCTGAACCCCACGAGGTGTCTGCCCTGTGGTTC  660
      T  L  F  V  N  V  N  V  T  S  E  P  H  E  V  S  A  L  W  F
661   CTCTGGTACGTCAAGCAGTGTGGAGGGACCATGAGGATCTTCTCCACCACCAACGGAGGA  720
      L  W  Y  V  K  Q  C  G  G  T  M  R  I  F  S  T  T  N  G  G
721   CAGGAGAGAAAGTTTTTGGGAGGTTCCAGCCAGATCAGTGAGTGTATGGCCAAAGAGCTG  780
      Q  E  R  K  F  L  G  G  S  S  Q  I  S  E  C  M  A  K  E  L
781   GGTGAGCGGGTCAAGATGGAGTCTCCTGTCTACAAGATCGACCAGACAGGGGACATGGTC  840
      G  E  R  V  K  M  E  S  P  V  Y  K  I  D  Q  T  G  D  M  V
841   GAGGTCGAGACGCTCAACAAGGAAACATACAAGGCCAAGTATGTGATTGTGGCGACCCCT  900
      E  V  E  T  L  N  K  E  T  Y  K  A  K  Y  V  I  V  A  T  P
901   CCTGGTCTGAACTTGAAGATGCACTTCAACCCCGAGCTACCCCCTCTCAGAAACCAGCTG  960
      P  G  L  N  L  K  M  H  F  N  P  E  L  P  P  L  R  N  Q  L
961   ATCCACAGGGTCCCCATGGGCTCTGTCATCAAATGTATTGTCTACTACAGAGAGAACTTC 1020
      I  H  R  V  P  M  G  S  V  I  K  C  I  V  Y  Y  R  E  N  F
1021  TGGAGGAAGAAAGGGTACTGTGGCACTATGGTGATCGAGGAGGAAGAGGCCCCCATTGGC 1080
      W  R  K  K  G  Y  C  G  T  M  V  I  E  E  E  E  A  P  I  G
1081  TTGACTCTGGATGACACAAAGCCCGATGGGACTGTGCCCGCCATTATGGGCTTCATCCTG 1140
      L  T  L  D  D  T  K  P  D  G  T  V  P  A  I  M  G  F  I  L
1141  GCCCGCAAGTGCAGGAAGCTATGTGGACTGACGAAAGAGGAGAGGAAGAAGAGGATCTGT 1200
      A  R  K  C  R  K  L  C  G  L  T  K  E  E  R  K  K  R  I  C
1201  GAGATCTACTCCAGAGTGCTAGGCTCAGAGGAGGCTCTGCATCCTGTGCACTATGAGGAG 1260
      E  I  Y  S  R  V  L  G  S  E  E  A  L  H  P  V  H  Y  E  E
1261  AAGAACTGGTGTGAGGAGGAGTATTCTGGAGGCTGCTACACTGCTTACTTCCCCCCTGGT 1320
      K  N  W  C  E  E  E  Y  S  G  G  C  Y  T  A  Y  F  P  P  G
1321  ATACTCACCCAGTACGGCAAGGTTCTCAGGGAGCCAGTAGGCAGGCTGTACTTTGCAGGA 1380
      I  L  T  Q  Y  G  K  V  L  R  E  P  V  G  R  L  Y  F  A  G
1381  ACAGAGACAGCTACTGAGTGGAGTGGCTATATGGAGGGGCTGTTCAGGCAGGGGAGAGG 1440
      T  E  T  A  T  E  W  S  G  Y  M  E  G  A  V  Q  A  G  E  R
1441  GCCGCCAGAGAGGTCATGTATGAAATGGGCAGGATTCCACAGAGTCAGATCTGGCAAACA 1500
      A  A  R  E  V  M  Y  E  M  G  R  I  P  Q  S  Q  I  W  Q  T
1501  GAGCCTGAGTCAGTGGAAGTCCCAGCACTCCCGTTGTCACCACCTTCTGGGAGCGAAAC 1560
      E  P  E  S  V  E  V  P  A  L  P  F  V  T  T  F  W  E  R  N
1561  CTTCCATCTGTGGGCGGGTTCATCAACTTCCTGGCGTAGCCTCCGTCCTGTCTGTTGCAA 1620
      L  P  S  V  G  G  F  I  N  F  L  A
1621  CTGCAGCAGGCATGTTGGCCTATCAGAAGGGGCTTCTCACCCGGAGCTAAACCTAAACCC 1680
1681  CTCCCACCACCACCTCTCTCCTGCATCTCAGAGGGCAATTCTCTGACGGCACACTATTCC 1740
1741  CTTTACAATGCACTTCTTTTGACCACAGCTAGAGTAGTGCTCCACTTTTGACCACATAGA 1800
1801  GTTCTGGTATAAAGTAATGCACTTTATAGGAACAGGTTGCATGTGAGACGTTGCCACTCT 1860
1861  GTGTATAGGCTGGTAAGACTGTTCAGCTCTGTCATTCACTACTGTACGTCATTGTTCTAA 1920
1921  ACTCATCTCACAGTGTCAACAAGGACTATAAGCAGCTCACAATATATGATGTTGCTAAGT 1980
1981  GCTTGATACTAGAGTGGAAGTGAGAGAGAGTCTAGCAAATAGTAGTTAAGTTACATACAT 2040
2041  AGAGAGCACT 2050
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Fig. 1. T-MAO cDNA sequence and deduced amino acid residues (one-letter code).

nucleotide position 1548. It codes for a polypeptide of 499 amino acids, with a molecular mass of ~56.6 kDa.

Catalytic activity and K_m values for T-MAO expressed in transfected COS-7 cells. T-MAO expressed in COS-7 cells was capable of oxidizing both 5-HT and PEA (Table 1). In contrast, cells transfected with the H-MAO-A or -B cDNA showed only 5-HT or PEA oxidation, respectively. No endogenous MAO enzymatic activity towards 5-HT or PEA was detected in COS-7 cells.

The kinetic parameters K_m and V_{max} were determined from homogenates prepared from COS-7 cells transfected with the T-MAO, H-MAO-A, or H-MAO-B cDNA (Table 1). The K_m values for 5-HT were similar for T-MAO (130 ± 17 μM) and H-MAO-A (68 ± 4 mM). Likewise, the K_m values for PEA were similar for T-MAO (12.5 ± 2.0 μM) and H-MAO-B (1.5 ± 0.2 mM). The V_{max} values obtained by using 5-HT as a substrate were similar for T-MAO (22 ± 2 nmol/20 min/mg of protein) and H-MAO-A (21 ± 1 nmol/20 min/mg of protein), as were the V_{max} values for T-MAO (8.4 ± 0.9 nmol/20 min/mg of protein) and H-MAO-B (4.8 ± 0.2 nmol/20 min/mg of protein) obtained by using PEA as the substrate. Of interest was the difference in V_{max} values of T-MAO for 5-HT (22 ± 2 nmol/20 min/mg of protein) and for PEA (8.4 ± 0.9 nmol/20 min/mg of protein). This difference was not due to transfection efficiencies, because the same transfections were used to measure both 5-HT and PEA oxidation. Despite a higher K_m for 5-HT, compared with PEA, T-MAO oxidizes 5-HT at a higher rate than it does PEA.

Inhibitor sensitivity of T-MAO expressed in COS-7 cells. The inhibitory effects of clorgyline and deprenyl on T-MAO, H-MAO-A, and H-MAO-B expressed in transfected COS-7 cells are shown in Fig. 2. The difference in sensitivity between these two inhibitors was more pronounced for H-MAO-A or H-MAO-B than T-MAO when either 5-HT or PEA was used as a substrate (Table 2). T-MAO was approximately 36-fold more sensitive to inhibition by clorgyline (IC_{50}, 2.8 ± 0.2 × 10^{-8} M) than deprenyl (IC_{50}, 1.0 ± 0.1 × 10^{-6} M) when 5-HT was used as a substrate. However, H-MAO-A was approximately 2000-fold more sensitive to inhibition by clorgyline (IC_{50}, 5.8 ± 0.1 × 10^{-10} M) than deprenyl (IC_{50}, 1.2 ± 0.1 × 10^{-6} M). The difference in the relative potencies of these two inhibitors for inhibition of T-MAO and H-MAO-A was due to the fact that T-MAO was approximately 48-fold less sensitive to inhibition by clorgyline than was H-MAO-A. The two were equally sensitive to inhibition by deprenyl when 5-HT was used as the substrate. Although T-MAO and H-MAO-A display similar K_m and deprenyl IC_{50} values for the oxidation of 5-HT, they possess different sensitivities towards clorgyline.

TABLE 1

Kinetic parameters of T-MAO, H-MAO-A, and H-MAO-B expressed in COS-7 cells

The cDNAs for these proteins were transfected into mammalian COS cells as described in the text. K_m and V_{max} values of the expressed enzymes were determined as described in Materials and Methods, using [^{14}C]PEA (1–10 μM) and [^{3}H]5-HT (10–100 μM) as the substrates. The protein concentration of the cell homogenate was determined by the method of Lowry et al. (22). The K_m and V_{max} values are the means of two determinations.

	[^{3}H]5-HT		[^{14}C]PEA	
	K_m	V_{max}	K_m	V_{max}
	μM	nmol/20 min/mg of protein	μM	nmol/20 min/mg of protein
T-MAO	130 ± 17	22 ± 2	13 ± 2	8.4 ± 0.9
H-MAO-A	68 ± 4	21 ± 1	ND[a]	ND
H-MAO-B	ND	ND	1.5 ± 0.2	4.8 ± 0.2

[a] ND, activity not detected.

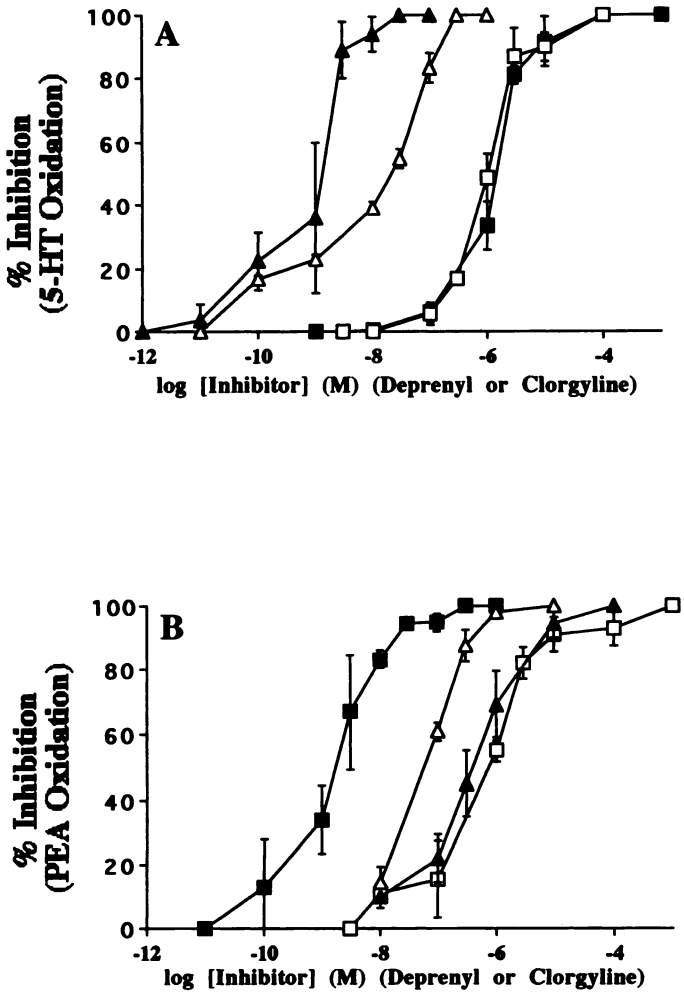

Fig. 2. Clorgyline and deprenyl inhibition of T-MAO (△, □) and H-MAO-A (▲, ■) activity expressed in COS cells, using 5-HT (100 mM) as the substrate (A), and of T-MAO (△, □) and H-MAO-B (▲, ■) activity expressed in COS cells, using PEA (10 mM) as the substrate (B). See Materials and Methods for a detailed description of the assay. △, ▲, clorgyline; □, ■, deprenyl.

When PEA was used as a substrate, T-MAO was approximately 8-fold more sensitive to inhibition by clorgyline (IC_{50}, 5.5 ± 0.1 × 10^{-8} M), compared with deprenyl (IC_{50}, 4.6 ± 0.3 × 10^{-7} M). However, H-MAO-B was about 290-fold more sensitive to deprenyl (IC_{50}, 1.4 ± 0.1 × 10^{-9} M) than clorgyline (IC_{50}, 4.0 ± 0.2 × 10^{-7} M). Taken together, these data indicate that T-MAO is capable of oxidizing both 5-HT and PEA. Furthermore, T-MAO is more sensitive to inhibition by clorgyline, regardless of whether 5-HT or PEA is used as the substrate, suggesting that T-MAO is different from either H-MAO-A and H-MAO-B but is more like H-MAO-A.

Comparison of the amino acid sequence of T-MAO with those of H-MAO-A, B-MAO-A, R-MAO-B, H-MAO-B, and R-MAO-B. To understand the structural requirements underlying MAO-A and -B substrate and inhibitor selectivity, the amino acid sequence of T-MAO was compared with those of R-MAO-A (23), H-MAO-A (6), B-MAO-A (24), H-MAO-B (6), and R-MAO-B (25). The percentage identity between the

TABLE 2

IC_{50} values for T-MAO, H-MAO-A, and H-MAO-B expressed in COS cells

IC_{50} values were determined in the presence of various concentrations of deprenyl and clorgiyline. Inhibitors were preincubated for 30 min before addition of the substrates. The IC_{50} values are the means of two determinations.

	IC_{50}			
	[^{3}H]5-HT		[^{14}C]PEA	
	Clorgyline	Deprenyl	Clorgyline	Deprenyl
	M			
T-MAO	$2.8 \pm 2 \times 10^{-8}$	$1.0 \pm 0.1 \times 10^{-6}$	$5.5 \pm 0.1 \times 10^{-8}$	$4.6 \pm 0.3 \times 10^{-7}$
H-MAO-A	$5.8 \pm 0.8 \times 10^{-10}$	$1.2 \pm 0.1 \times 10^{-6}$	ND[a]	ND
H-MAO-B	ND	ND	$4.0 \pm 0.2 \times 10^{-7}$	$1.4 \pm 0.1 \times 10^{-9}$

[a] ND, not determined.

amino acid sequence of T-MAO and those of R-MAO-A, H-MAO-A, and B-MAO-A ranges from 69 to 70%, which is similar to the identity between T-MAO and H-MAO-B or R-MAO-B (Table 3). The highest levels of amino acid identity were found for the same form of MAO from different species, i.e., MAO-A (85–88%) and MAO-B (88%) (Table 3).

Alignment of T-MAO, R-MAO-A, H-MAO-A, B-MAO-A, H-MAO-B, and R-MAO-B is shown in Fig. 3. There are four highly conserved regions among T-MAO and MAO-A and -B from various species (Fig. 3). The first is located near the amino terminus of all MAO molecules, corresponding to T-MAO amino acids 8–45. This region is predicted to fold as an ADP-binding $\beta\alpha\beta$ unit, based on a set of rules describing the type of amino acid required at 11 specific positions of a peptide fragment (26). Position 11 of the ADP "fingerprint" region hydrogen bonds with the 2'-OH of the FAD cofactor and must be either aspartic acid or glutamic acid. All MAO molecules have an aspartic acid residue in this position (amino acid residue 39 for T-MAO) except for B-MAO-A, which contains glutamic acid. The second region is located at T-MAO residues 180–223 and currently has no known function. The third region is located at T-MAO residues 382–453 and represents the most conserved region among all MAO molecules regardless of the subtype. This region contains the pentapeptide Ser-Gly-Gly-Cys-Tyr, in which the obligatory cofactor FAD is covalently bound to cysteine. The fourth region is structurally conserved for all species except for trout. It is located at the carboxyl terminus (R-MAO-A amino acids 500–518) and consists of a transmembrane segment that was predicted, by the method of Rao and Argos (27), using the PCGENE software program RAOARGOS (Intelligenetics, Mountain View, CA), to form a membrane-associated α-helix. Furthermore, this motif is followed by eight or nine amino acids, three of which are basic (Fig. 3). The mitochondrial targeting signal for R-MAO-B is located in the carboxyl-terminal 29 amino acids (28). Strikingly, T-MAO lacks a transmembrane segment and the basic residues found in the mammalian forms of MAO.

TABLE 3

Comparison of the overall percentage identity among the amino acid sequences of T-MAO, R-MAO-A, H-MAO-A, B-MAO-A, H-MAO-B, and R-MAO-B

The Genetics Computer Group molecular biology software program GAP was used to determine the percentage identity.

	Identity				
	T-MAO	R-MAO-A	H-MAO-A	B-MAO-A	H-MAO-B
	%				
T-MAO	100				
R-MAO-A	70	100			
H-MAO-A	70	87	100		
B-MAO-A	69	85	88	100	
H-MAO-B	71	72	73	72	100
R-MAO-B	71	71	72	70	88

Amino acid residues that are conserved between species but different between MAO-A and MAO-B, especially the nonconserved substitutions, may represent residues that are important for conferring substrate and inhibitor selectivity. Among these nonconserved amino acid substitutions between MAO-A and -B, the amino acids conserved between T-MAO and MAO-A might be important for conferring MAO-A characteristics (H-MAO-A amino acids Leu-26, Glu-58, Ala-68, Tyr-100, Ala-120, His-148, Lys-154, Phe-173, Asn-181, Ser-184, Pro-186, Phe-208, Glu-286, Met-300, Pro-347, Lys-370, and Asn-494) and those amino acids conserved between T-MAO and MAO-B may confer MAO-B characteristics (H-MAO-B amino acids Ala-72, Glu-84, Thr-201, Glu-232, Ala-257, Ser-293, Thr-314, Arg-350, Thr-356, Glu-359, Leu-407, Pro-458, Gln-464, Val-470, and Pro-476) (Fig. 3).

Comparison of the secondary structures of T-MAO and H-MAO-A and -B. The secondary structures of H-MAO-A and -B and T-MAO were predicated by the method of Garnier *et al.* (29), using the PCGENE software program GARNIER (Intelligenetics). As shown in Fig. 4, the four highly conserved amino acid regions, consisting of the ADP-binding motif, a putative substrate binding region, the site of FAD covalent attachment, and a membrane-spanning segment, also show high levels of similarity in predicated secondary structure. Other regions of similar secondary structure for all MAO molecules correspond to T-MAO amino acids 53–72 and 138–155 (Fig. 4). The differences between the secondary structure of T-MAO and those of the other MAO forms seem to be scattered throughout the polypeptide. However, there are clusters of secondary structures that are conserved only between T-MAO and H-MAO-A or between T-MAO and MAO-B. For example, regions corresponding to T-MAO residues 174–177, 339–343, and 473–476 form structures that are conserved with MAO-A but not MAO-B. Although the amino acid identity between T-MAO and both MAO subtypes (69–71%) is similar to the identity between MAO-A and MAO-B subtypes (70–73%), it appears that the secondary structure is more conserved between the MAO-A and MAO-B subtypes. Whether these differences in secondary structure impart inhibitor or substrate selectivity remains to be studied.

Discussion

The present study clearly demonstrates that transient expression of a T-MAO cDNA can generate deprenyl and

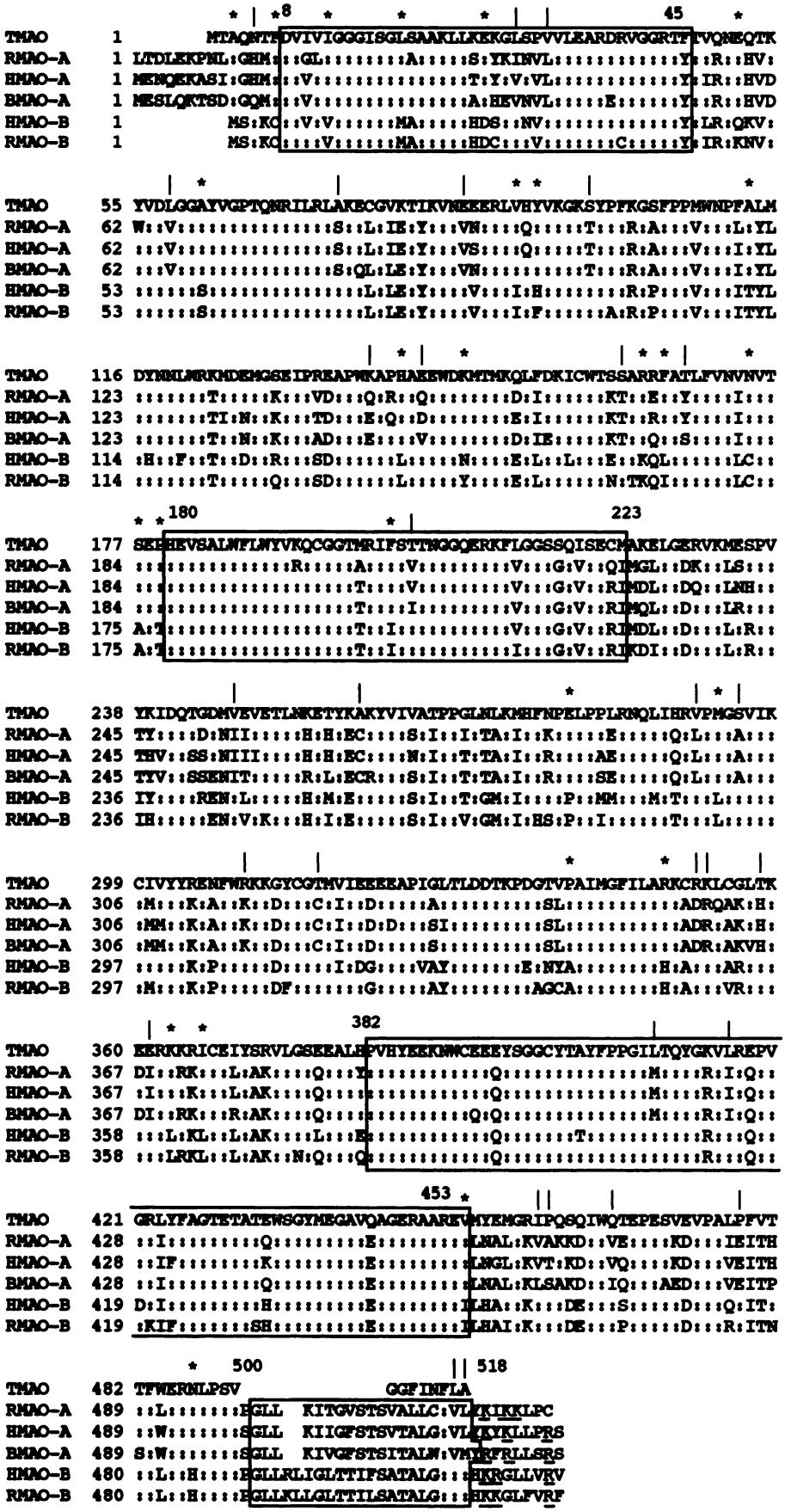

Fig. 3. Alignment of amino acid sequences for T-MAO, R-MAO-A, H-MAO-A, B-MAO-A, H-MAO-B, and R-MAO-B. Sequences were aligned using the Genalign program (Intelligenetics). *, Nonconserved amino acids that are identical specifically between T-MAO and MAO-A. *Vertical lines*, nonconserved amino acids that are identical specifically between T-MAO and MAO-B. *Boxed regions*, highly conserved regions; *underlined amino acids*, basic amino acids.

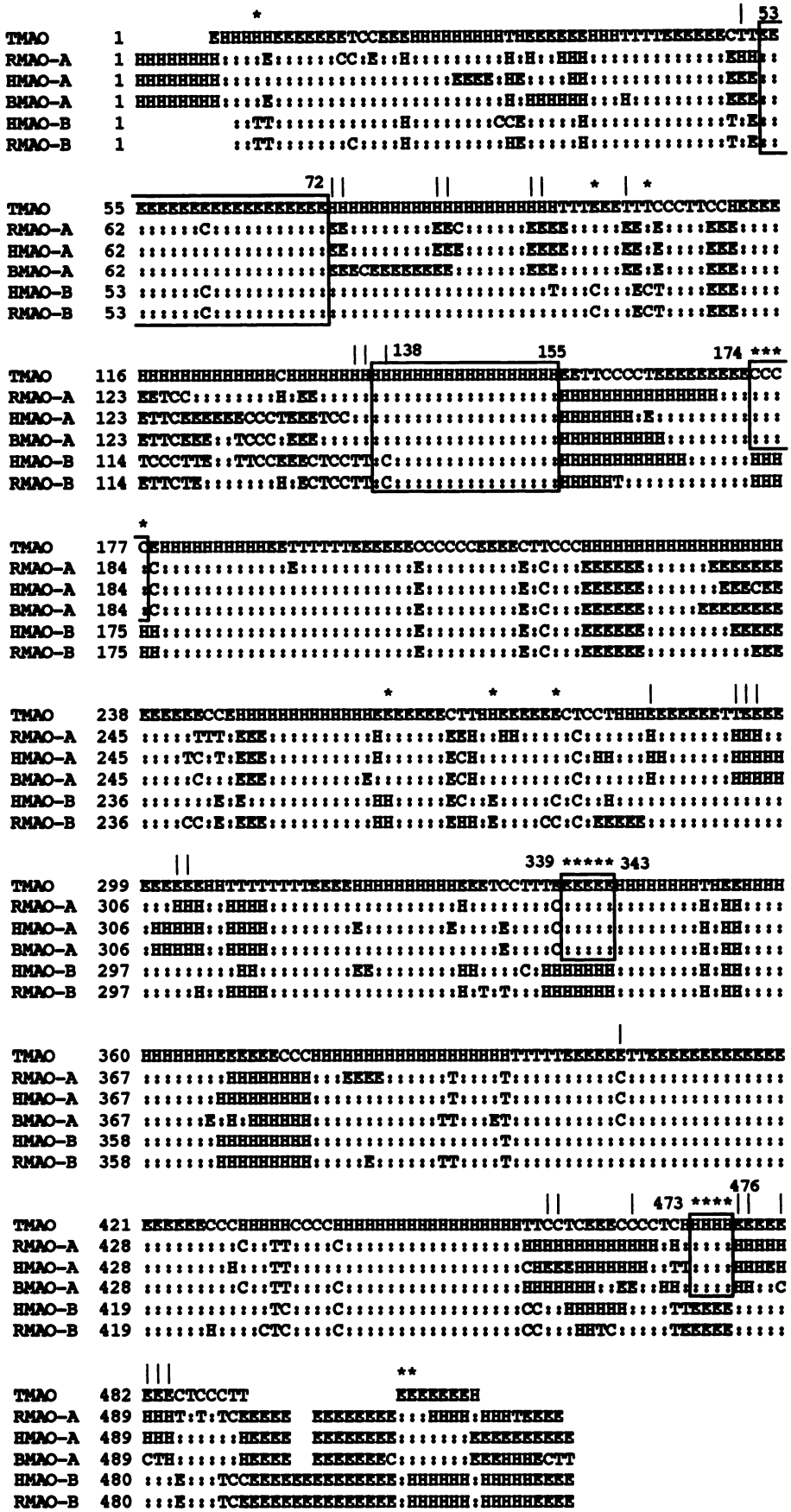

Fig. 4. Alignment of protein secondary structures predicted by the Garnier method (PCGENE) for T-MAO, R-MAO-A, H-MAO-A, B-MAO-A, H-MAO-B, and R-MAO-B. *, Secondary structures that are identical specifically between T-MAO and MAO-A. *Vertical lines*, secondary structures that are identical specifically between T-MAO and MAO-B. *C*, coil; *E*, extended; *H*, helix; *T*, turn. *Boxed regions*, highly conserved regions.

clorgyline inhibition curves similar to those found in trout eggs (17), liver, brain, kidney, and intestine (30). The single-phase sigmoidal inhibition curve provides evidence for a single enzyme in homogenized tissue and mitochondrial isolates (17). Our results show that T-MAO has properties more like those of the MAO-A form, for the following reasons: 1) T-MAO is capable of oxidizing both 5-HT and PEA in transient expression assays, unlike the MAO-A or -B subtypes, with which only 5-HT or PEA, respectively, is deaminated, and 2) T-MAO is more sensitive to inhibition by clorgyline than deprenyl, regardless of whether 5-HT or PEA is used as substrate (Fig. 2). These features of T-MAO are very similar to those of placenta H-MAO-A (31), which can oxidize both 5-HT and PEA and is more sensitive to clorgyline than deprenyl. However, T-MAO shares 68% and 69% amino acid homology with MAO-A and -B, respectively, which does not reflect any more resemblance to MAO-A (Fig. 3; Table 3).

Although the mechanism determining the selectivity of the two forms of MAO remains uncertain, the increasing number of primary structures from different species provide insight for relating structure to enzymatic selectivity. Which residues confer MAO-A or -B enzymatic activity is a central question that remains to be answered. Comparison of the sequences of the different forms (MAO-A and -B) with those from other species (human, cow, and rat) (Fig. 3) showed that there is a greater degree of similarity between the same form from different species than between different forms from the same species. For example, R-MAO-B and H-MAO-B have approximately 90% identical amino acid residues, whereas H-MAO-A and -B share only approximately 70% of their amino acid residues. Those amino acid residues that are identical in different species and subtypes can be inferred to have been conserved during evolution because they are functionally important. More importantly, those residues that are conserved between species but are different in the MAO subtypes, especially nonconserved substitutions, could be residues that are important in conferring the selectivity of the two types of MAO. Among these nonconserved substitutions, T-MAO shares homology with both H-MAO-A and H-MAO-B (Fig. 3).

There are only 15 nonconservative amino acid residues that are identical in T-MAO and MAO-B but not in MAO-A (H-MAO-B amino acid residues Ala-72, Glu-84, Thr-201, Glu-232, Ala-257, Ser-293, Thr-314, Arg-350, Thr-356, Glu-359, Leu-407, Pro-458, Gln-464, Val-470, and Pro-476). It is tempting to speculate that these amino acids may partly contribute to the MAO selectivity. Our intron-exon mutagenesis studies on H-MAO-A show that Ala-257 does not affect MAO-A or MAO-B enzymatic selectivity. Mutagenesis of the remaining 14 amino acids of T-MAO to those of MAO-A may impart more MAO-A-specific properties.

T-MAO has 499 amino acids, which is the shortest among all the cloned MAOs (Fig. 3). The carboxyl-terminal 29 amino acids have been shown to be the mitochondrial targeting signal in liver R-MAO-B (28). It is intriguing that the carboxyl terminus of T-MAO is not predicted to form a membrane-associated α-helix but T-MAO is localized to the mitochondria. However, the last 10 amino acids in T-MAO are all hydrophobic and may favor membrane anchorage. The absence of basic residues in the carboxyl terminus of T-MAO may decrease the rate of import to the mitochondria. The yeast outer mitochondrial membrane protein 70 molecule contains at its amino terminus a "signal-anchor" motif made up of a short stretch of basic residues followed by a membrane-spanning region (32). The mitochondrial import rate of this molecule is dependent on the amino-terminal basic region.

In summary, we have cloned the T-MAO cDNA and expressed it in mammalian cells to characterize its substrate and inhibitor selectivities. Our results demonstrate that T-MAO 1) oxidizes both 5-HT and PEA (but the former is the preferred substrate), 2) is inhibited by lower concentrations of clorgyline than deprenyl, 3) has similar levels of amino acid identity to both MAO subtypes, and 4) lacks carboxyl-terminal structural homology to mammal MAOs. It is for these reasons that T-MAO represents a novel type of MAO.

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References

1. Greenawalt, J. W., and C. Schnaitman. An appraisal of the use of monoamine oxidase as an enzyme marker for the outer mitochondrial membrane. *J. Cell. Biol.* **46**:173–179 (1970).
2. Johnson, J. P. Some observations upon a new inhibitor of monoamine oxidase in human brain. *Biochem. Pharmacol.* **17**:1285–1297 (1968).
3. Knoll, J., and K. Magyar. Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adv. Biochem. Psychopharmacol.* **5**:393–408 (1972).
4. Fowler, C. J., B. H. Callingham, T. J. Mantle, and K. F. Tipton. Monoamine oxidase A and B: useful concept? *Biochem. Pharmacol.* **27**:97–101 (1978).
5. Salach, J. I., K. Detmer, and M. B. H. Youdim. The reaction of bovine and rat liver monoamine oxidase with ^{14}C-clorgyline and ^{14}C-deprenyl. *Mol. Pharmacol.* **16**:234–241 (1979).
6. Bach, A. W. J., N. C. Lan, D. L. Johnson, C. W. Abell, M. E. Bembenck, S. W. Kwan, P. H. Seeburg, and J. C. Shih. cDNA cloning of human monoamine oxidase A and B: molecular basis of differences in enzymatic properties. *Proc. Natl. Acad. Sci. USA* **85**:4934–4938 (1988).
7. Hsu, Y.-P. P., W. Weyler, S. Chen, K. B. Sims, W. B. Rinehart, M. C. Utterback, J. F. Powell, and X. O. Breakefield. Structual features of human monoamine oxidase A elucidated from cDNA and peptide sequences. *J. Neurochem.* **51**:1321–1324 (1988).
8. Lan, N. C., C. Chen, and J. C. Shih. Expression of functional human monoamine oxidase A and B cDNAs in mammalian cells. *J. Neurochem.* **52**:1652–1654 (1989).
9. Grimsby, J., K. Chen, L.-J. Wang, N. C. Lan, and J. C. Shih. Human monoamine oxidase A and B genes exhibit identical exon-intron organization. *Proc. Natl. Acad. Sci. USA* **88**:3637–3641 (1991).
10. Hall, T. R., P. B. Yurgens, H. R. Figueroa, G. Uruena, J. M. Olcese, D. K. Newton, and S. R. Vorwald. Monoamine oxidase types A and B in the vertebrate brain. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **71**:107–110 (1982).
11. Nicotra, A., and M. Naccarato. Monoamine oxidase activity in *Paracentrotus lividus* sperm cells. *Int. J. Invert. Reprod.* **5**:101–105 (1982).
12. Nicotra, A., A. L. Serafino, and O. Senatori. Monoamine oxidase activity in tissues of the starfish *Maryhasterias glacialis*. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **84**:195–197 (1986).
13. Nicotra, A., O. Senatori, and G. Santangelo. Monoamine oxidase activity in hepatopancreas of *Octopus vulgaris*. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **106**:479–482 (1993).
14. Hall, T. R., and G. Uruena. Monoamine oxidase activity in several tissues of the goldfish, *Carassius auratus*. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **71**:145–147 (1982).
15. Hall, T. R., J. M. Olcese, H. R. Figueroa, and V. L. De Vlaming. Effects of inhibitors on monoamine oxidase activity in perch (*Perca flavescens*) brain *in vitro*. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **71**:141–144 (1982).
16. Kinemuchi, H., M. Sudo, M. Yoshino, T. Kawaguchi, Y. Sunami, and K. Kamijo. A new type of mitochondrial monoamine oxidase distinct from type-A and type-B. *Life Sci.* **32**:517–524 (1983).
17. Nicotra, A., and O. Senatori. Some characteristics of mitochondrial monoamine oxidase activity in eggs of carp and rainbow trout. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **92**:401–404 (1989).
18. Senatori, O., A. Nicotra, and R. Scopelliti. Monoamine oxidase activity in embryos of pike (*Esox lucius*). *Comp. Biochem. Physiol. C Comp. Pharmacol.* **96**:87–90 (1990).
19. Sanger, F., S. Nicklen, and A. R. Coulson. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467 (1977).
20. Chen, C., and H. Okayama. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**:2745–2752 (1987).
21. Chen, S., J. C. Shih, and Q.-P. Xu. Interaction of *N*-(2-nitro-4-azidophenyl)-serotonin with two types of monoamine oxidase in rat brain. *J. Neurochem.*

43:1680–1687 (1984).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Kuwahara, T., S. Takamoto, and K. Ito. Primary structure of rat monoamine oxidase A deduced from cDNA and its expression in rat tissues. *Argic. Biol. Chem.* **54**:253–259 (1990).
24. Powell, J. F., Y.-P. P. Hsu, W. Weyler, S. Chen, J. Salach, K. Andrikopoulos, J. Mallet, and X. O. Breakfield. The primary structure of bovine monoamine oxidase type A: comparison with peptide sequences of bovine monoamine oxidase type B and other flavoenzymes. *Biochem. J.* **259**:407–413 (1989).
25. Ito, A., T. Kuwahara, S. Inadome, and Y. Sagara. Molecular cloning of a cDNA for rat liver monoamine oxidase. *Biochem. Biophys. Res. Commun.* **157**:970–976 (1988).
26. Wierenga, R. K., P. Terpstra, and W. G. Hol. Prediction of the occurrence of the ADP-binding β-α-β-fold in proteins, using an amino acid sequence fingerprint. *J. Mol. Biol.* **187**:101–107 (1986).
27. Rao, M. J. K., and P. Argos. A conformation preference parameter to predict helices in integral membrane proteins. *Biochim. Biophys. Acta* **869**:197–214 (1986).
28. Mitoma, J.-Y., and A. Ito. Mitochondrial targeting signal of rat liver monoamine oxidase B is located at its carboxy terminus. *J. Biochem. (Tokyo)* **111**:20–24 (1992).
29. Garnier, J., D. J. Osguthorpe, and B. Robson. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**:97–120 (1978).
30. Edward, D., T. R. Hall, and J. A. Brown. The characteristics and distribution of monoamine oxidase (MAO) activity in different tissues of the rainbow trout, *Salmo gairdneri*. *Comp. Biochem. Physiol. C Comp. Pharmacol.* **84**:73–77 (1986).
31. Hsu, M.-C., and J. C. Shih. Photoaffinity labeling of human placenta monoamine oxidase-A by 4-fluoro-3-nitrophenylazide. *Mol. Pharmacol.* **33**:273–241 (1988).
32. McBride, H. M., D. G. Millar, J.-M. Li, and G. C. Shore. A signal-anchor sequence selective for the mitochondrial outer membrane. *J. Cell Biol.* **119**:1451–1457 (1992).

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