Enzymic and Molecular Characteristics of a New Form of Monoamine Oxidase, Distinct from Form-A and Form-B*

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Abstract—The present study was undertaken to clarify the enzymic and molecular properties of monoamine oxidase (MAO) in carp brain. In particular, its sensitivities to selective MAO inhibitors, kinetic properties and molecular weight were compared with those of the enzyme in carp liver. The selective and potent MAO-A and MAO-B inhibitors FLA 788(+), FLA 336(+), MD 780236 and benzylcyanide caused dose-dependent inhibitions of MAO activity in both carp brain and liver; the inhibition curves were all single-sigmoidal, and the degrees of inhibition of the activities towards 5-hydroxytryptamine (5-HT, selective MAO-A substrate), tyramine (substrate for both forms of MAO) and \( \beta \)-phenylethylamine (PEA, selective MAO-B substrate) were similar. This was also the case for inhibition of activity in carp brain by the irreversible and selective MAO-A and MAO-B inhibitors clorgyline and l-deprenyl, indicating the presence in both preparations of a single MAO which differs from either form of MAO. Studies on the substrate specificities and \( K_m \) values for these three substrates and the inhibitory effects of some compounds suggested that the enzymic characters of MAO in carp preparations were similar and that these enzymes might be FAD-containing enzymes, like MAO in various mammals. By labelling the preparations with radioactive pargyline and then subjecting them to sodium dodecyl sulfate electrophoresis, the apparent molecular weights of carp brain and liver MAO were estimated as 60,000 daltons. The same value was also obtained for rat brain and liver mitochondrial MAO-B. These results indicate that by the present definitions of MAO-A and MAO-B, MAO in carp brain and liver is similar to, but distinct from, both these forms of MAO.

Mitochondrial monoamine oxidase [MAO, monoamine: O\(_2\) oxidoreductase (deaminating) (flavine-containing) EC 1.4.3.4] exists in many tissues of various species, where it is thought to play an important role in physiological functions of the central and peripheral nervous systems by catalyzing oxidative deamination of biogenic amines such as norepinephrine, dopamine and 5-hydroxytryptamine (1). MAO is classified into two forms, MAO-A and MAO-B, on the basis of different sensitivities to inhibition by the selective inhibitors clorgyline and l-deprenyl: MAO-A is highly sensitive to clorgyline (2) and less sensitive to l-deprenyl, whereas MAO-B is highly sensitive to l-deprenyl, but less sensitive to clorgyline (3). However, MAO in carp (Cyprinus carpio) liver mitochondria shows similar sensitivities to clorgyline and l-deprenyl as reported previously (4, 5). Therefore, this carp liver MAO may be a new type that is distinct from either MAO-A or MAO-B and may be useful in studies on the genesis of the two forms of mammalian MAO (4–6).

In the present study, we investigated whether carp brain MAO is similar to the MAO
in carp liver, particularly in terms of its sensitivities to some selective MAO inhibitors other than clorgyline and I-deprenyl. We also examined the molecular weights, kinetic properties and sensitivities to several compounds of MAO in carp brain and liver.

Materials and Methods

Enzyme preparations: Carp, weighing 900–1,200 g, were used in this study. They were killed by decapitation, and their brains and livers were rapidly removed, cut into small pieces and homogenized in 5 vol. of 0.32 M sucrose-10 mM phosphate buffer, pH 7.8 (brain) or 0.25 M sucrose-10 mM phosphate buffer (liver) first in a Waring blender and then in a glass homogenizer on ice. The brain homogenate was then centrifuged at 600 × g for 10 min to remove nuclei and cell debris, and the resulting supernatant was used as the enzyme preparation. The 600 × g supernatant obtained from the liver homogenate was centrifuged at 8,500 × g for 30 min, and the resulting pellet was washed by suspension in sucrose buffer and recentrifugation as before. The final pellet was then suspended in the same sucrose buffer and used immediately or stored frozen in aliquots until use as the liver mitochondrial preparation.

Rat brain and liver mitochondria were also prepared by different centrifugations as described above.

Assay of monoamine oxidase (MAO) activity: MAO activity was assayed radiochemically as reported previously (7, 8) with $[14\text{C}]$-labelled amines, diluted with the respective unlabelled amines, as substrates. The enzyme reaction was performed at 37 °C and pH 8.0 for 10 min. Metabolites formed in this enzyme reaction were extracted with an ethyl acetate/toluene mixture (1:1 v/v) saturated with water, and radioactive activity in the extract was measured in a Tricarb liquid scintillation spectrometer. In this assay, enzyme activity was found to be linearly proportional to the amount of protein and incubation time. Specific MAO activities were corrected for the efficiencies of extraction of metabolites into the extraction mixture (8).

In experiments in which the concentration of oxygen was changed, the reaction mixture (100 μl final volume in a 10 ml glass tube) was aerated for 10 sec with mixtures of different proportions of oxygen and nitrogen.

In inhibition studies, enzyme preparations were preincubated with various concentrations of the test compound at 37 °C, unless otherwise specified, for 30 min and remaining MAO activity was determined radiochemically as described above. In this study, the final substrate concentrations used were approximately equivalent to their $K_m$ values, determined previously (5).

Protein concentration was determined by a modified biuret method (9) with bovine serum albumin as a standard.

Sodium dodecyl sulfate-gel electrophoresis: The molecular weights of MAO in carp brain and liver preparations labelled with $[^3\text{H}]$-pargyline were determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel (5%) electrophoresis with RNA polymerase B ($z=39,000$, $\alpha=42,000$, $\kappa=100,000$, $\beta=140,000$ and $\beta'=180,000$, SDS Page Marker I, Seikagaku Kogyo, Co., Ltd., Tokyo) as marker proteins. In all cases, 0.1% SDS was included in both the electrode buffer and the gel buffer. Briefly, MAO in carp brain and liver (23.2 mg protein of brain and 20.4 mg of liver) was labelled with $[^3\text{H}]$-pargyline by preincubating the enzyme preparations with 0.33 nmol labelled inhibitor at 37 °C for 2 hr. Then unbound radioactive pargyline was removed by three washings with sucrose-buffer and then recentrifugation at 28,000 × g for 10 min. After three washings, the pellet preparations were resuspended in a loading solution containing 4% SDS, 2% 2-mercaptoethanol, 40% glycerol in 10 mM phosphate buffer, pH 7.2, and samples were heated at 100 °C for 4 min before application to gels. The samples, each containing 100–300 μg of protein, were layered on top of the gels and subjected to electrophoresis towards the anode at a current of 1 mA per tube for about 10 min and then at 3 mA for about 10 hr. The gels used for examination of the distribution of radioactivity were cut with a razor blade into approximately 3 mm thick slices. The radioactivity in each slice was determined after solubilization of the gel in NCS-tissue solubilizer.

Labelling of rat brain and liver mitochondrial MAO-B with $[^3\text{H}]$-pargyline and
then electrophoresis were performed in the same way, but using about 10 mg protein of brain and of liver mitochondria for denaturation with SDS. This amount of labelled pargyline almost completely inhibited rat MAO-B activity, but did not significantly affect that of MAO-A. MAO-A in the two rat preparations was not labelled in this study. Non-specific binding of $^3$H-pargyline was determined by preincubating these carp and rat preparations with $10^{-4}$ M unlabelled pargyline before adding $^3$H-pargyline, and the observed radioactivities were corrected for non-specific binding.

**Chemicals:** $^{[14]}$C-Tyramine hydrochloride (50 mCi/mmol), $^{[14]}$C-$\beta$-phenylethylamine hydrochloride (PEA, 48.3 mCi/mmol), $^{[14]}$C-5-hydroxytryptamine binoxalate (5-HT, 51.5 mCi/mmol) and $^{[14]}$C-tryptamine bisuccinate (51.5 mCi/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. $^{[14]}$C-Dopamine hydrochloride was from The Radiochemical Centre, Amersham, U.K. Clorgyline hydrochloride was kindly provided by May & Baker Ltd., Dagenham, U.K.; and 1-deprenyl hydrochloride was a gift from Dr. J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary. MD 780236 (±) was a gift from Le Centre de Recherche Delalande, France, through Drs. P. Dostert and M. Strolin-Benedetti. FLA 336 (+) (amiflamine) and FLA 788 (+) were from Astra Läkemedel, Sweden, through Dr. H. Selander. Iproniazid hydrochloride, o-phenanthroline hydrochloride, cuprizone (biscyclohexanone oxalyldihydrazone) and isoniazid were purchased from Tokyo Chemical Industries, Co., Ltd., Japan. Semicarbazide hydrochloride was from Koso Chemical Co., Ltd., Japan; harmine hydrochloride from Sigma Chemical Co., Ltd., St. Louis, MO, U.S.A.; and pheniprazine hydrochloride from Chugai Pharmaceutical Co., Ltd., Japan.

All other chemicals used were of the highest grade available commercially.

**Results**

In preliminary experiments, using carp brain homogenate pretreated with $10^{-3}$ M semicarbazide to inhibit another amine oxidase, benzylamine oxidase (BZ0), which is present in carp liver and oxidizes PEA (4, 5), the inhibitory effects of clorgyline and 1-deprenyl on MAO were investigated after preincubation at 37°C for 30 min. The results obtained indicated that the sensitivities to these selective and irreversible MAO-A and MAO-B inhibitors were similar, irrespective of which selective substrate of MAO-A or MAO-B, such as 5-HT, tyramine or PEA, was
used, as observed in earlier studies on carp liver mitochondrial MAO (data not shown).

Next, the inhibitory effects of some other selective MAO inhibitors on the MAO activities in both carp brain and liver pretreated with semicarbazide were determined with 5-HT, tyramine and PEA as substrates after preincubation with the inhibitors at 37°C for 30 min. The results in Fig. 1 show that MD 780236, a selective and potent MAO-B inhibitor (10), dose-dependently inhibited the MAO activities in both carp preparations. This compound at a concentration of 10^{-3} M completely inhibited all activity and gave single-sigmoidal inhibition curves with all three substrates. The degrees of inhibition of either the brain or liver preparation were similar irrespective of whether 5-HT, tyramine or PEA was used as the substrate. Similar inhibitory effects of different concentrations of FLA 788 (+), a selective and potent MAO-A inhibitor (11–13), on MAO activity in both preparations were also observed with these three substrates. The results in Fig. 2 indicate that the inhibitions of both carp preparations by FLA 788 (+) all gave the same single-sigmoidal curve, as observed for inhibition by MD 780236, regardless of which of these three substrates was used. The inhibitory effects of two other selective MAO inhibitors, FLA 336 (+) (MAO-A inhibitor) (11–13) and benzylcyanide (MAO-B inhibitor) (14, 15),

![Graphs showing inhibition of carp brain and liver MAO activity towards 5-HT, tyramine and PEA by various concentrations of FLA 788(+) and FLA 336(+).](image)

Fig. 2. Inhibition of carp brain and liver MAO activity towards 5-HT, tyramine and PEA by various concentrations of FLA 788(+). The procedure was as for Fig. 1, but with FLA 788(+), instead of MD 780236, as the inhibitor. The symbols are as for Fig. 1. Each point is the mean for two duplicate determinations.

![Graphs showing inhibition of carp brain and liver MAO activity towards 5-HT, tyramine and PEA by various concentrations of FLA 336(+).](image)

Fig. 3. Inhibition of carp brain and liver MAO activity towards 5-HT, tyramine and PEA by various concentrations of FLA 336(+). The procedure was as for Fig. 1, but with FLA 336(+) as the inhibitor. The symbols are as for Fig. 1. Each point is the mean for two duplicate determinations.
on the enzyme activities in carp brain and liver were studied, and the results with FLA 336 (+) are shown in Fig. 3. The curves for inhibition by FLA 336 (+) were also single-sigmoidal, and the degrees of inhibition with these substrates were similar. Similar results were obtained on inhibition by benzylcyanide (data not shown).

The $K_m$ values calculated from Lineweaver-Burk plots of carp brain and liver MAO activities towards 5-HT, tyramine and PEA, after pretreatment with semicarbazide ($10^{-3}$ M), were determined and are shown in Table 1. The values for these respective amine substrates of carp brain and liver MAO are similar (Table 1).

The substrate specificities of carp brain and liver MAO, after pretreatment with semicarbazide ($10^{-3}$ M), were studied using tyramine, 5-HT, dopamine and tryptamine as substrates. As shown in Table 2, MAO in both preparations oxidized tyramine most rapidly. The activities with 5-HT and tryptamine as substrates were about 40–50% of that with tyramine in carp liver and 70–80% of that with tyramine in carp brain, and the activities of both preparations with dopamine and PEA were about 20–25% of those with tyramine.

The activities of both carp preparations towards tyramine were assayed at various oxygen concentrations. The results (Figs. 4A and B) gave parallel lines in Lineweaver-Burk plots with $K_m$ values of about 150 $\mu$M (146 $\mu$M±22.6 $\mu$M for brain and 150±21.9 $\mu$M for liver) for oxygen ($K_{O2}^{m}$), calculated from secondary plots. Similar parallel lines were obtained when the oxygen tension was varied at a series of fixed tyramine concentrations with a $K_{m}^{t}$ of about 130 $\mu$M (133 $\mu$M for brain and 125 $\mu$M for liver). These results indicate that MAO in both preparations reacts according to the double-displacement or ping-pong reaction mechanism shown for mammalian MAO-A (16) and MAO-B (7, 17–19).

The effects of various compounds on MAO in carp brain and liver were determined, and the results are shown in Table 4. Pheniprazine, harmine, o-phenanthroline, and iproniazid strongly inhibited the activity in both preparations, but two carbonyl reagents, semicarbazide and isoniazide, and a copper chelator, cuprizone, were only weakly in-

| Table 1. $K_m$ values of carp brain and liver MAO for 5-HT, tyramine and PEA |
|-------------------|-------------------|-------------------|
|                   | $K_m$ value ($\mu$M) Tyramine | $K_m$ value ($\mu$M) 5-HT | $K_m$ value ($\mu$M) PEA |
| Carp brain        | 59.9±4.2           | 149.9±20.5         | 89.25±8.4            |
| Carp liver        | 44.2±5.8           | 163.8±13.9         | 83.7±5.0             |

Both carp preparations used had been pretreated with semicarbazide ($10^{-3}$ M) at 37°C for 30 min. Values were determined from Lineweaver-Burk plots. Values are means±S.E.M.

| Table 2. Substrate specificities of carp brain and liver MAO |
|-------------------|-------------------|-------------------|-------------------|-------------------|
|                   | MAO activity (nmol product/min/mg protein) |
|                   | Tyramine | 5-HT | PEA | Dopamine | Tryptamine |
| Carp Liver        | 4.00(100) | 1.67(41.7) | 0.83(21.8) | 0.99(24.8) | 1.97(49.3) |
| Carp Brain        | 0.071(100) | 0.049(89.0) | 0.014(19.7) | 0.015(21.1) | 0.055(77.5) |

Both carp preparations had been pretreated with semicarbazide, as described for Table 1. Values are expressed as nmol product formed/min/mg protein. Values in parentheses indicate activities as percentages of that with tyramine. Substrates were added at concentrations of 1 mM. Values are means for two duplicate determinations.
Fig. 4. Lineweaver-Burk plots of MAO activity in carp brain (A) and liver (B) assayed at different concentrations of oxygen (left) and tyramine (right). Ordinate, 1/activity, expressed as dpm·10⁻²; abscissa, 1/mM tyramine and oxygen, respectively. For this study, the preparations were not pretreated with semicarbazide since as reported previously (4, 5), no appreciable BZO activity was found with tyramine as substrate. Oxygen concentrations used: ▲—▲ (0.055 mM), ⋄—⋄ (0.110 mM), △—△ (0.217 mM) and ○—○ (1.085 mM), respectively. Tyramine concentrations: ▲—▲ (0.02 mM), ⋄—⋄ (0.05 mM), △—△ (0.1 mM) and ○—○ (0.2 mM). Points are means for two duplicate determinations in two groups of preparations, each derived from ten carp.
Table 3. Effects of several compounds on carp brain and liver MAO

| Concentration (mM) | 5-HT brain | 5-HT liver | Tyramine brain | Tyramine liver | PEA brain | PEA liver |
|--------------------|------------|------------|----------------|----------------|-----------|-----------|
| Pheniprazine       | 0.1        | 96±2.8     | 100±0.2        | 100±0.0        | 99±1.4    | 100±0.0   |
|                    | 0.01       | 95±2.1     | 99±0.7         | 97±0.0         | 99±0.7    | 70±4.2    |
| Harmine            | 0.1        | 94±2.1     | 100±0.2        | 97±0.0         | 99±0.7    | 76±4.2    |
|                    | 0.01       | 93±2.1     | 99±0.7         | 94±1.4         | 98±0.0    | 55±7.1    |
| o-Phenanthidine    | 0.1        | 70±3.5     | 85±0.1         | 79±1.4         | 87±0.7    | 67±2.1    |
|                    | 0.01       | 13±10.6    | 36±1.7         | 29±2.1         | 46±3.5    | 25±1.4    |
| Iproniazid         | 1.0        | 92±2.1     | 99±0.2         | 93±2.1         | 98±0.4    | 98±1.4    |
|                    | 0.1        | 72±2.1     | 96±0.7         | 80±0.7         | 95±1.6    | 88±2.8    |
| Isoniazid          | 1.0        | 25±14.8    | 16±0.7         | 19±2.8         | 12±0.4    | 23.9      |
|                    | 0.1        | 0          | 5.7±8.0        | 6.0±5.7        | 3.0±2.8   | 22.0      |
| Semicarbazide      | 1.0        | 1.3±2.3    | 13±0.7         | 15±2.1         | 7.5±3.5   | 36.8      |
|                    | 0.1        | 1.0±1.7    | 5.9±1.7        | 1.5±0.7        | 5.1±3.9   | 32.7      |
| Cuprizone          | 1.0        | 19±2.1     | 20±2.1         | 18±2.5         | 17±0.4    | 34.6      |
|                    | 0.1        | 9.0±5.7    | 9.0±2.8        | 12±4.9         | 8.5±0.2   | 26.2      |

*Both carp preparations had been pretreated with semicarbazide (10⁻³ M) at 37°C for 30 min. The inhibitory effects of these compounds were determined and compared with those obtained without semicarbazide pretreatment. Values are means±S.E.M., where appropriate.

Discussion

On the basis of differences in sensitivities to inhibition by clorgyline and L-deprenyl, MAO in various tissues from many species has been classified into two forms, MAO-A and MAO-B. Thus, with the substrate tyramine, a common substrate of both forms of MAO, the inhibition curves obtained with these two acetylenic inhibitors are double-
sigmoidal when the tissue contains both forms of MAO. However, our previous studies showed that the inhibition curves of activity in carp liver mitochondria towards tyramine were single-sigmoidal and that the sensitivities to these two inhibitors were the same (4, 5). Similar results were obtained with 5-HT or PEA as substrate (4, 5). These results indicate that carp liver contains a single MAO, which is presumably neither MAO-A nor MAO-B, but that because it can oxidize the substrates for MAO-A and MAO-B, the carp enzyme may have the properties of both forms of MAO (4, 5). Thus, in our earlier study, using carp liver MAO as a model enzyme preparation for MAO-A and MAO-B, we studied the mechanisms of the inhibitory effects of some detergents (6).

The present study was undertaken to clarify the enzymic properties of MAO in carp brain and especially to compare this brain enzyme with MAO in carp liver. The MAO inhibitors FLA 788 (+) and FLA 336 (+) (amiflamine) used in this study have been shown to be specific and potent MAO-A inhibitors, and thus they are thought to be useful therapeutically for the treatment of depressive disorders (12, 13). These two FLA compounds cause reversible inhibition of MAO-A (12, 13). We also used MD 780236 which is a selective and potent MAO-B inhibitor (10). In vitro, it causes suicide inhibition of MAO-B because it acts as a substrate for both forms, and the resulting product then forms a stable adduct with the active site of MAO-B, whereas with MAO-A, the role of MD 780236 as a substrate predominates (20–22). Benzylcyanide, which was also used in this study causes selective inhibition of MAO-B (14, 15).

Results on inhibition of carp brain and liver MAO that had been pretreated with semicarbazide to prevent the participation of BZO indicated that MAO in both carp preparations was highly sensitive to the selective MAO inhibitors tested. The inhibition curves obtained were all single-sigmoidal and almost identical, irrespective of which selective substrate of MAO-A or MAO-B, such as 5-HT (MAO-A), tyramine (MAO-A and MAO-B) or PEA (MAO-B), was used (23). Consistent with previous findings on identical sensitivities to clorgyline and l-deprenyl, the extents of inhibition of activity in the liver and brain by either of these selective inhibitors were almost identical with all three substrates. Similar results were obtained in preliminary experiments on inhibition of carp brain MAO by clorgyline and l-deprenyl. These results strongly suggest that by the present definition of MAO-A and MAO-B, the MAO in both carp preparations shows similar sensitivities to various selective inhibitors even with different selective substrates, and thus this enzyme is distinct from either MAO-A or MAO-B.

Carbonyl reagents such as semicarbazide and isoniazid and a copper chelator, cuprizone, strongly inhibit pyridoxal phosphate- and copper-containing amine oxidase(s), designated as BZO, clorgyline-resistant amine oxidase or, more recently, semicarbazide-sensitive amine oxidase (24–30). However, these reagents do not inhibit the mitochondrial FAD-containing amine oxidase, MAO. The present study showed that they did not significantly inhibit the activity of either carp preparation, even at the highest concentration used (10^-3 M) (Table 4). Moreover, after complete inhibition of BZO, no inhibition of MAO by the carbonyl reagent isoniazid or the copper chelator cuprizone was observed (Table 4), suggesting that carp brain, like carp liver (5) and many mammalian tissues (24–30), may contain both MAO and BZO.

Carp MAO may have FAD as its prosthetic group since it was also inhibited by the suicide MAO inhibitor pargyline, which during a sufficient preincubation time inhibits MAO by forming a stable adduct with FAD (31–33). The inhibition of carp brain and liver MAO by formation of a stable complex with pargyline was demonstrated by measuring remaining radioactivity of the labelled pargyline tightly bound to the protein band on SDS-gel electrophograms after repeated washings.

The substrate specificities and apparent K_m values of carp brain and liver MAO were in general similar, and their K_m values for tyramine (K_m^{tyramine}) and for oxygen (K_m^{O_2}).
determined at different oxygen and tyramine concentrations, were also similar. Thus the MAOs in these two organs are quite similar in enzymic and kinetic properties. When activities were assayed at various concentrations of oxygen and tyramine, Lineweaver-Burk plots of activity gave parallel lines, indicating that MAO in both carp preparations reacts according to the double-displacement or ping-pong reaction mechanism, as shown for various mammalian preparations of MAO-A and MAO-B (16–19).

The apparent molecular weights of carp brain and liver MAO, as determined from the position of tightly and specifically bound labelled pargyline, were both about 60,000 daltons (Fig. 5). A similar value was obtained for rat brain and liver MAO-B in the present study under the same conditions. These values for carp MAO are also similar to those for MAO-A and MAO-B (57,000–67,000 daltons) in various tissues of many species determined by SDS-gel electrophoresis (34–37).

Some investigators have suggested that the two forms of MAO may be due to a single enzyme with different allotropic properties conferred by the membrane lipid environment (38–40). MAO in carp brain and liver seems to have similar enzymic and molecular characters to those of MAO in various mammals, except that it is equally sensitive to selective inhibitors of MAO-A and -B such as clorgyline and I-deprenyl. Our previous studies showed that the lipid-environment in carp liver mitochondria probably does not play a role in the identical sensitivities of MAO to clorgyline and I-deprenyl (41). Thus carp brain and liver seem to contain a single MAO, which is apparently neither MAO-A nor MAO-B. Its active sites for bindings of these selective inhibitors may differ from those of MAO-A and MAO-B, suggesting from the evolutionary point of view that it may be a precursor of these two forms of mammalian MAO.

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