FURTHER STUDIES ON THE SYNTHESIS OF A-FORM MONOAMINE OXIDASE

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Abstract—The relation between precursors and restoration of A-form MAO activity in rat liver after administration of clorgyline to rats was investigated by measuring the rates of recovery of A-form MAO activity after treatment with the inhibitor. The half-lives of mitochondrial and microsomal A-form MAO were estimated as 3.5 and 2.0 days, respectively. MAO activity and the amount of MAO molecules were completely restored within 14 days. However, the values attained did not exceed the control values in a period of 14 days. Clorgyline plus cycloheximide or chloramphenicol did not prevent the recovery of MAO activity in the microsomes, but did not delay the appearance of enzyme activity in the mitochondria. A and B-form-like MAO were also observed in the microsomal and supernatant fractions, with clorgyline as inhibitor. These results suggest that the microsomal enzyme is a precursor of the mitochondrial enzyme, that the levels of A-form and B-form MAO are regulated genetically, and that the two forms of MAO may be synthesized separately.

It is of interest to investigate the development of various membrane-bound enzymes in submitochondrial fractions. Recently, labelled amino acids have often been used in studies on the turnover rates of mitochondrial membrane proteins (1). Studies have also been done using irreversible inhibitors of enzymes associated with mitochondrial membranes. There are many reports on the effects of various monoamine oxidase (MAO) inhibitors (2-4), since the first report of Barondes (5) on the rate of regeneration of MAO activity in rat brain mitochondria after administration of JB-516. Pargyline, a more specific and irreversible MAO inhibitor has also been used, because it binds covalently to the enzyme (6). Clorgyline, a specific A-form MAO inhibitor, and deprenyl, a specific B-form MAO inhibitor, also inhibit the enzyme by forming a covalently linked inhibitor flavin-adduct in a ratio of 1:1 (7), like that formed with pargyline.

In preliminary studies we found (8) that the rates of recovery of MAO in rat liver mitochondria and microsomes after administrations of clorgyline and deprenyl are similar to those after administration of pargyline (6) and that MAO shows distinctly different turnover rates in mitochondrial and microsomal fractions. These results suggest that the microsomal enzymes may be precursors of mitochondrial enzymes or that the enzymes in the two fractions are distinctly different.

The finding that treatment with cycloheximide inhibits restoration of MAO activity in pargyline treated animals (4, 6) prompted us to study the details of the origin of mitochondrial MAO in relation to restoration of MAO activity in microsomes.
and its restoration in mitochondria after inhibition by inhibitors using irreversible MAO inhibitors and inhibitors of protein synthesis.

MATERIALS AND METHODS

Preparation of enzyme: Male Wistar rats weighing 100–150 g were used. A dose of 20 mg/kg of clorgyline (a specific inhibitor of the A-form MAO) in 0.5 ml saline was given (i.p.). At the indicated times after the injection the rats were anesthetized with 10 mg/kg of sodium pentobarbital and the livers were quickly removed. Homogenates (10%) were prepared in 0.25 M sucrose adjusted to pH 7.2. Crude mitochondrial and microsomal fractions were prepared by the centrifugation methods described by Schneider and Hogeboom (9). The mitochondrial and microsomal pellets were then resuspended in the homogenization medium, washed twice and used as enzyme preparations. The supernatant fractions obtained by centrifugation at 100,000xg for 60 min were also used as enzyme preparations. These operations were carried out at 4°C.

To study the effects of inhibitors of protein synthesis, rats were given cycloheximide at 1 mg/kg twice daily i.p. and then at 0.5 mg/kg twice daily, or were given chloramphenicol i.p. at 50 mg/kg every 24 hr for 5 days.

MAO activity: MAO activity was measured using labelled substrates, as described earlier (10). The incubation medium contained a suitable amount of the enzyme to give a linear reaction for at least 40 min in a total volume of 275 μl of potassium phosphate buffer, pH 7.2. The reaction was started by adding 25 μl of labelled substrate and incubation was carried out for 20 min at 37°C. Then the reaction was stopped by adding 2N HCl. With serotonin as substrate, the reaction product was extracted with ether, while with β-phenylethylamine (β-PEA) as substrate the product was extracted with toluene. Samples of the extract were mixed with Aquasol as scintillation liquid and their radioactivities were measured with a Packard-TriCarb Liquid Scintillation Spectrometer. Enzyme activity was expressed in disintegrations per min (dpm) extracted after 20 min of incubation. Values for dpm were linearly proportional to the incubation time.

When inhibitors were used, the enzyme was preincubated for 20 min at 25°C with the inhibitor at the concentration indicated in legends to figures before addition of radioactive substances.

Titration with inhibitor (11): Various amounts of enzyme preincubated with inhibitor at 37°C for 2 hr in a total volume of 275 μl of potassium phosphate buffer, pH 7.2. As a control, the enzyme was preincubated in the same way, but in the absence of an inhibitor. After preincubation, 25 μl of labelled substrate was added to estimate MAO activity. The inhibitor concentrations used were 1×10^-8 M deprenyl with β-PEA as substrate and 1×10^-9 M clorgyline with serotonin.

Assays of other enzyme activities: For estimation of the damage of other enzymes in mitochondria and microsomes by clorgyline, glucose-6-phosphatase a marker of microsomal membranes was measured by the method of Swanson (12) and succinate dehydrogenase, a marker of mitochondrial membranes by the method of Singer and Kearney (13) in the respective fractions.

Estimation of protein: Protein contents of preparations were measured by the method of Lowry et al. (14) with bovine albumin as a standard. The protein concentration of enzyme preparations was adjusted to 10 mg/ml.

RESULTS

The MAO activities in rat liver mitochondria and microsomes were measured with serotonin and β-PEA as substrates after
administration of 20 mg/kg of clorgyline. As shown in Fig. 1, with serotonin as substrate, the activity in mitochondria was 10% of the control activity 12 hr after administration of clorgyline and then increased gradually, being 50% of the control value after 2 weeks. With β-PEA as substrate, the MAO activity was about 75% of the control value 12 hr after administration of clorgyline and then increased rapidly, being restored completely within 7 days. The curves for recovery of activity in microsomes with serotonin and β-PEA as substrates were similar to those of mitochondria, but the recoveries of both activities were faster.

No inhibition of succinate dehydrogenase, a mitochondrial membrane marker enzyme, or of glucose-6-phosphatase, a microsomal membrane marker enzyme, was observed after administration of clorgyline (Table 1).

The turnover rates of these MAOs were calculated from a plot of the percent inhibition of MAO activity vs the time after administration of clorgyline, on a semilogarithmic scale. The data for mitochondria and microsomes both fitted a straight line (Fig. 2), and the half-lives of mitochondrial and microsomal A-form MAO were estimated as 3.5 and 2.0 days, respectively.

The relationship between the recovery of

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**Table 1. Enzyme activities in rat liver mitochondria and microsomes after administration of clorgyline**

<table>
<thead>
<tr>
<th>Time after administration</th>
<th>Monoamine oxidase*</th>
<th>Succinic dehydrogenase</th>
<th>Monoamine oxidase**</th>
<th>Glucose-6-**phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>β-PEA</td>
<td>5-HT</td>
<td>β-PEA</td>
</tr>
<tr>
<td>Control</td>
<td>6.82±0.04</td>
<td>4.71±0.03</td>
<td>1.12±0.09</td>
<td>0.72±0.08</td>
</tr>
<tr>
<td>1 day</td>
<td>1.22±0.02</td>
<td>3.97±0.03</td>
<td>0.285±0.04</td>
<td>0.56±0.05</td>
</tr>
<tr>
<td>2 days</td>
<td>2.04±0.06</td>
<td>4.23±0.01</td>
<td>0.280±0.04</td>
<td>0.36±0.05</td>
</tr>
<tr>
<td>3 days</td>
<td>3.18±0.06</td>
<td>4.41±0.05</td>
<td>0.285±0.07</td>
<td>0.72±0.03</td>
</tr>
<tr>
<td>5 days</td>
<td>4.08±0.03</td>
<td>4.60±0.02</td>
<td>0.280±0.02</td>
<td>0.91±0.05</td>
</tr>
<tr>
<td>7 days</td>
<td>5.03±0.07</td>
<td>4.79±0.06</td>
<td>0.285±0.05</td>
<td>1.01±0.03</td>
</tr>
<tr>
<td>10 days</td>
<td>5.78±0.02</td>
<td>4.65±0.02</td>
<td>0.290±0.02</td>
<td>1.06±0.07</td>
</tr>
<tr>
<td>14 days</td>
<td>6.39±0.07</td>
<td>4.60±0.08</td>
<td>0.295±0.08</td>
<td>1.11±0.06</td>
</tr>
</tbody>
</table>

Mitochondria* and microsomes** were used as enzyme preparations. Monoamine oxidase activity was expressed as nmoles/min/mg protein and succinic dehydrogenase and glucose-6-phosphatase activities were expressed as units/mg protein. Values are means±S.E. for three separate preparations.
MAO activity and the molecular amount of MAO was examined by titration experiments with clorgyline (for A-form MAO) or deprenyl (for B-form MAO). Clorgyline and deprenyl inhibit MAO irreversibly at a ratio of 1:1, i.e., the amount of enzyme inhibited was equal to the amount of inhibitor added on a molar basis. As shown in Fig. 3, the inhibition curves were parallel to that of control activity without inhibitor and intersected the abscissa on the right of the origin, corresponding to the amount of enzyme bound by the inhibitor. Figure 3 shows that with a mitochondrial preparation obtained 24 hr after administration of clorgyline, 33 μl of enzyme preparation was titrated with 0.3 pmoles of clorgyline with serotonin as substrate and that at later times after clorgyline administration the volume of the mitochondrial preparation titrated decreased gradually and was almost restored to the control value after 14 days. With β-PEA as

Fig. 2. Recovery of A-form MAO activities in rat liver mitochondria and microsomes after treatment with clorgyline. Mitochondria and microsomes were used as enzyme preparations. A-form MAO: mitochondria (▲—▲), microsomes (△—△) Abscissa: time after administration of clorgyline Ordinate: log of percentage inhibition of MAO activity.

Fig. 3. Titration of MAO in rat liver mitochondria with clorgyline and deprenyl using serotonin and β-PEA as substrates. Experiments were carried out as described in the text. Left: titration of B-form MAO (deprenyl 1 x 10^-6 M, β-PEA) Right: titration of A-form MAO (clorgyline 1 x 10^-6 M, serotonin). Mitochondrial fractions were prepared at various times after drug administration (□—□ 24 hr, ■—■ 2 days, ▲—▲ 5 days, △—△ 14 days) with control mitochondria (○—○ without inhibitor, ■—■ in the presence of inhibitor). The protein concentration of enzyme preparations was adjusted to 10 mg/ml.
The effects of inhibitors of protein synthesis

decreased rapidly, being restored completely within 5 days.

substrate, 21 μl of the same enzyme preparation was titrated with 3 pmol of deprenyl after 24 hr and then the volume decreased rapidly, being restored completely within 5 days.

Fig. 4. Effect of cycloheximide on recovery of MAO activity in rat liver mitochondria and microsomes after clorgyline administration. Cycloheximide was administered as described in the text. Clorgyline 20 mg/kg was administered at zero time. At the times indicated rats were killed and liver mitochondria and microsomal fractions were isolated as described in the text. Left: mitochondria. Right: microsomes. Cycloheximide alone (●—●, ○—○), cycloheximide plus clorgyline (■—■, □—□) and clorgyline alone (▲—▲, △—△). Values are means for triplicate experiments. Serotonin was used as substrate.

Fig. 5. Effect of chloramphenicol on recovery of MAO activity in rat liver mitochondria and microsomes after clorgyline administration. Experiments were performed as described in Fig. 4, except that chloramphenicol 50 mg/kg was administered. Chloramphenicol alone (●—●, ○—○), chloramphenicol plus clorgyline (■—■, □—□) and clorgyline alone (▲—▲, △—△). Left: mitochondria. Right: microsomes. Serotonin was used as substrate.
on the rates of return of MAO activity were investigated. Figure 4 shows that cycloheximide decreased the rate of restoration of MAO activity to the control level in the mitochondrial fraction. However, it did not completely block recovery of activity: the enzyme activity returned to about 50% of the control value 5 days after clorgyline administration, compared with 75% in the absence of cycloheximide. As shown in Fig. 4 (right), cycloheximide also did not completely block restoration of MAO activity in the microsomal membrane fraction, in which it was maintained at about 30% of the control activity. Figure 5 shows that when chloramphenicol was administrated to rats before treatment with clorgyline, the curves for restoration of MAO activity in the mitochondrial fraction were similar to those after administration of cycloheximide. Chloramphenicol also did not completely block restoration of MAO activity in microsomal membrane fractions.

The effect of increasing concentrations of clorgyline on the MAO activity in rat liver mitochondria is shown in Fig. 6, A. On incubation with the inhibitor at 25°C for 20 min, the MAO activity with serotonin as substrate (substrate for A-form MAO) was strongly inhibited, while the activity toward β- PEA (substrate for B-form MAO) was inhibited less. The activity with tyramine gave a double sigmoid curve, indicating that this substrate was oxidized by both the "A" and "B" forms of the enzyme. Similar inhibition curves were obtained using the microsomal and supernatant fractions obtained by centrifugation at 100,000 x g for 60 min as enzyme preparations (Fig. 6, B, C). However, with these preparations the inhibitions of tyramine oxidation and serotonin oxidation were less than with the mitochondrial preparation.

DISCUSSION

In this work, the MAO activities in rat liver mitochondria and microsomes were measured with serotonin and β- PEA as substrates after administration of 20 mg/kg clorgyline. As shown in Fig. 1, A-form MAO activity in mitochondria and was restored to almost the control value after 2 weeks. The curve for recovery of A-form MAO activity in microsomes with serotonin as substrate was similar, but the recovery was faster than that in mitochondria. B-form MAO in mitochondria and microsomes was also slightly inhibited by administration of 20 mg/kg clorgyline, but restoration of activity was rapid.

The turnover rates of these MAO's were
calculated by the method of Neff et al. (4) from a plot of the percent inhibition of MAO activity vs the time after administration of clorgyline on a semilogarithmic scale (Fig. 2). In this way the half-lives of the mitochondrial and microsomal A-form were estimated as 3.5 and 2.0 days, respectively. Pargyline is an irreversible MAO inhibitor, which has been shown to bind covalently to MAO in vivo. Thus it provides a useful tool for studying the rate of synthesis of MAO in various subcellular fractions. The turnover rates of MAO in the CNS are much slower than those in most other organs (brain, 11 days (4), liver, 3.5 days (6, 15), submaximal gland, 4 days (4)), although the half-life of MAO in heart is 12 days (16). Using clorgyline, an irreversible inhibitor of MAO that forms a covalent inhibitor flavine-adduct in a ratio of 1:1, we obtained a similar turnover rate for A-form MAO in mitochondria to that reported by Erwin and Deitrich (6). Results with this inhibitor showed that the recovery of A-form MAO activity was slower than that of B-form MAO activity.

The relationship between the recovery of MAO activity and the molecular amount of MAO was examined by titration experiments. The recovery progressed with time and was complete within 14 days, although the activity did not exceed the control value in this period. These results indicate that the levels of the A and B-forms or this molecular ratio are regulated genetically in rat liver mitochondria.

The rates of restoration of MAO activity after clorgyline administration differed in mitochondrial and microsomal fractions (Fig. 1), indicating that MAO has different turnover rates in the two fractions.

Cycloheximide did not completely prevent restoration of enzyme activity in the microsomal fraction and the activity in the mitochondrial fraction was restored after 2 days (Figs. 4 and 5). Similar results were obtained with chloramphenicol as inhibitor of protein synthesis. These results support the conclusion that the microsomal and mitochondrial enzymes are not different proteins and that the microsomal enzyme may be a precursor of the mitochondrial enzyme. Another possibility is that newly synthesized MAO on the microsomes is transferred directly to the mitochondrial and microsomal membranes and that it is these membranes that are restored after treatment with inhibitors. However, it is also possible that the microsomal and mitochondrial enzymes are different since microsomal MAO was inhibited completely by cycloheximide plus MAO inhibitor and had different Km values from mitochondrial MAO (6) and there is evidence that isozymes of MAO are present in mitochondria (17, 18).

Convincing evidence for the existence of at least 2 forms of MAO, named type A and B, was first obtained by Johnston (19). Type A MAO is strongly inhibited by clorgyline with serotonin as substrate while type B MAO is only inhibited by high concentrations of clorgyline with β-PEA. A double sigmoid curve was obtained with tyramine as substrate, indicating that this substrate was oxidized by both type A and B MAO. The above results were obtained with a mitochondrial preparation (see Fig. 6, A). Similar results were obtained with the microsomal and supernatant fractions obtained by centrifugation at 100,000 x g for 60 min. Thus both the A and B-forms may be present in microsomes and the supernatant and these forms may be synthesized separately in the microsomes. These enzymes in the supernatant fraction may have been liberated from the outer mitochondrial membranes or synthesized in this fraction.

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REFERENCES


