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SOLUBLE AND MEMBRANE-BOUND PIG LIVER MITOCHONDRIAL MONOAMINE OXIDASE: THERMOSTABILITY, TRYPTIC DIGESTABILITY AND KINETIC PROPERTIES

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Abstract—It has previously been shown that pig liver monoamine oxidase (monoamine: O_2 oxidoreductase, EC 1.4.3.4) can be rendered soluble in buffer by extraction with methylethyl ketone. The soluble enzyme can be rebound to delipidated membranes in the presence of highly acidic phospholipids.

A comparison of the thermostabilities of the enzyme bound to the mitochondria and the soluble form showed that the soluble enzyme was much more labile than the bound enzyme. Rebinding of the soluble enzyme to relipidated membranes partially restored the thermostability. The soluble enzyme was more sensitive to tryptic digestion than the enzyme bound to mitochondria. Rebinding of the soluble enzyme to replipidated membranes did not decrease its sensitivity to trypsin. The role of membrane components in stabilizing the enzyme molecule is discussed.

The change in the rate of tyramine oxidation with temperature was the same for both forms of the enzyme.

Harmaline was found to reversibly inhibit both bound and soluble enzyme to the same degree. This was true both with tyramine and serotonin as substrate. However, serotonin oxidation by either form of the enzyme was inhibited more than tyramine oxidation. Pargyline irreversibly inhibited tyramine and serotonin oxidation to the same degree, both with the bound and the soluble enzyme. The molecular activity of the enzyme did not change after liberation from the membrane. The possibility that there are multiple forms of monoamine oxidase in the pig liver is discussed in the light of these findings.

MITOCHONDRIAL monoamine oxidase is localized in the outer membrane of the mitochondria.[1] The enzyme is firmly bound to the membrane and can not be extracted into aqueous media,[2] unless use is made of detergents,[3–5] or sonication, freezing and thawing.[6] The finding that the enzyme can be rendered soluble after extraction with organic solvent[7] indicates that interaction with phospholipids is of importance for the binding of the enzyme. This interpretation is supported by the finding of Oreland and Olivecrona[8] that the liberation of monoamine oxidase and the extraction of acidic phospholipids were well correlated. Furthermore, Olivecrona and Oreland[9] have shown that the soluble enzyme could be rebound to lipid-depleted membranes in the presence of acidic phospholipids.

There have been many studies on the properties of monoamine oxidase purified after solubilization (see Ref. 10). An implicit assumption in some of these studies has been that the properties of the enzyme are not changed by the solubilization or liberation procedure. However, as regards monoamine oxidase liberated from the mitochondrial structure by extraction with organic solvents, the loss of some membrane

constituent of possible importance for its function might affect the properties of the enzyme. When detergents are used they seem to remain in the enzyme preparation throughout the purification procedure.[3] Thus, in those cases there is also the possibility that the detergent may alter the properties of the enzyme.

In the present communication we have compared some properties of the enzyme in its membrane-bound form and after liberation from the membrane by extraction with aqueous methylethyl ketone.[7] Experiments have also been carried out with enzyme rebound to membrane fragments by addition of phospholipid.

MATERIALS AND METHODS

Chemicals. Trypsin (bovine pancreas, type III) and catalase (bovine liver) were obtained from Sigma Chemical Company, St. Louis, U.S.A. Pargyline HCl was a kind gift of Abbott Laboratories, North Chicago, U.S.A. Cardiolipin was purchased from Pierce Chemical Co., Rockford, Ill. U.S.A.

Preparation and assay of monoamine oxidase. Soluble monoamine oxidase was prepared from water-washed pig-liver mitochondria as described earlier.[7] Briefly, the extraction procedure involves two steps, each consisting of a ketone extraction and a subsequent buffer extraction. The first step extracts about 80 per cent of the phospholipids but does not liberate any enzyme. In the second step, in which ammonium sulphate is present during the extraction with ketone, about 25 per cent of the original enzyme activity can be extracted by the buffer. In all experiments described in this communication the soluble enzyme preparation had been chromatographed on Sephadex G-200 before use. After this, the degree of purification was about 200-fold as compared with the homogenate.[11]

Monoamine oxidase activity was assayed either by the spectrophotometric method of Tabor, Tabor and Rosenthal[12] with benzylamine as substrate or by using an oxygen electrode with tyramine or serotonin as substrate. In the former case the activity is expressed in nmoles of benzaldehyde formed per minute in a cuvette containing 10 μmoles of benzylamine in 3 ml of 0·1 M potassium phosphate buffer, pH 7·5, at 24°. One unit of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 nmole of benzaldehyde/min. When the oxygen electrode was used the activity is expressed as nmoles of O_2 consumed per min in a reaction vessel containing 5 μmoles of substrate (tyramine or serotonin), 100 units of catalase, 20 μmoles of semicarbazide, 2 μmoles of KCN in 2·3 ml of 0·1 M potassium phosphate buffer, pH 7·5, at 37°. The oxygen electrode used was that of Rank Brothers (Cambridge) with the electronic equipment from a Gilson Model K oxygraph.

Preparation of soluble enzyme bound to relipidated membranes. Mitochondrial membranes were rendered virtually devoid of phospholipids by repeating the methylethyl ketone extraction procedure four times.[7] To about 100 mg of delipidated membranes were then added 10 mg dispersed cardiolipin[9] and about 2500 units of soluble monoamine oxidase, all in 2 ml 0·1 M phosphate buffer, pH 7·5. After incubation for 30 min at 37° the relipidated membranes were spun down by centrifugation at 40,000 *g* for 10 min. Usually 30–50 per cent of the enzyme activity was found in the pellet and could not be removed from the membranes by washing with buffer.[9] Less than 20 per cent of the enzyme activity found in the pellet was due to enzyme remaining in the delipidated membranes.

Protein estimation. Protein was estimated by the method of Lowry *et al.*,[13] with human serum albumin as standard.

RESULTS

Thermostability

In these experiments mitochondria, soluble monoamine oxidase, the residue after the second extraction step or monoamine oxidase rebound by cardiolipin, to delipidated mitochondrial residues (see Methods), was diluted in buffer preheated to 50° and then kept at this temperature in a water bath. At intervals an aliquot of the sample was withdrawn and placed into an ice-bath. The enzyme activity was then measured within 20 min. As shown in Fig. 1, monoamine oxidase activity in the mitochondria did not decrease after heating for 1 hr. This is in contrast to the activity of the soluble enzyme, which had almost disappeared after about 1 hr at 50°.

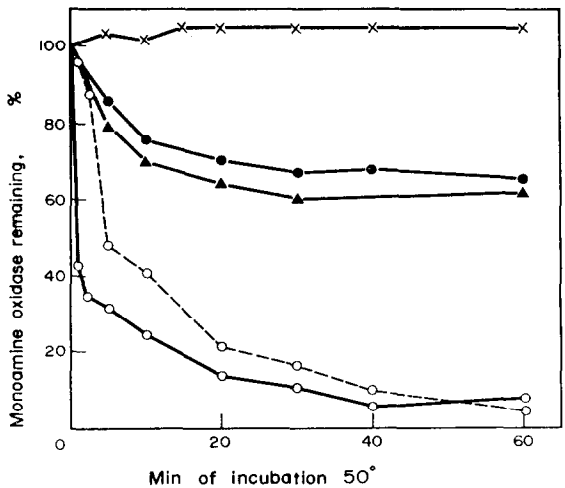

FIG. 1. Thermostability of bound and soluble forms of monoamine oxidase. About 500 units of monoamine oxidase bound to mitochondria: ×—×—×; in the soluble form: ○—○—○; bound to the residue after the ketone extraction procedure: ▲—▲—▲ and rebound to relipidated membranes: ●—●—● was diluted in 2 ml of 0·1 M potassium phosphate buffer, pH 7·5. The buffer had been preheated to 50° and the sample was then kept at this temperature in a water-bath. At the times indicated aliquots were withdrawn and chilled in an ice-bath. Monoamine oxidase activity was determined within 20 min with tyramine as substrate (see Methods). The activity of the soluble enzyme was also determined after 24 hr: ○- - -○- - -○.

If the activity of the soluble enzyme was determined 24 hr after the heat treatment, reactivation was found to have occurred in the aliquots exposed to 50° for less than 60 min (Fig. 1). This reactivation was greater the shorter the time of heat treatment. Thus, in the sample treated for 1 min the activity increased from 43 to 96 per cent of the original activity, while the 10 min sample was only reactivated from 25 to 41 per cent.

To exclude the possibility that the decreased thermostability of the soluble enzyme was due to the low concentration of protein,[14] the residue after the second buffer extraction, inactivated at 55° for 30 min, was added to give the same protein concentration as in the mitochondrial preparation. We know from earlier experiments that

the soluble enzyme does not bind to such membrane fragments in the absence of acidic phospholipids.[9] This addition of protein, however, did not increase the thermostability of the soluble enzyme.

Previous studies have shown that the soluble enzyme can form a soluble complex with highly acidic phospholipids.[9] However, when 5 mg of dispersed cardiolipin was added to 1 ml of the undiluted preparation of soluble enzyme, no change in its thermostability was observed.

When the thermostability of the enzyme remaining in the lipid-depleted membrane fragments after the second extraction was tested, it was found to be less than that of the mitochondrial preparation but considerably greater than that of the soluble enzyme. That is, about 35 per cent of the enzyme activity was lost after 60 min exposure to 50° (Fig. 1).

When monoamide oxidase bound to relipidated mitochondrial membranes (see Methods) was treated in the same way, its thermostability was found to be about the same as that of the enzyme in the residue after the second extraction step (Fig. 1).

Effect of temperature on reaction rates. Our previous studies have shown that the soluble and the bound enzyme seem to have the same substrate specificity but that, at least for benzylamine, there is a difference in the Michaelis constant.[11] Figure 2 shows that the response of the reaction rate to temperature was the same for both forms of the enzyme when tyramine was used as substrate. The slight separation of the

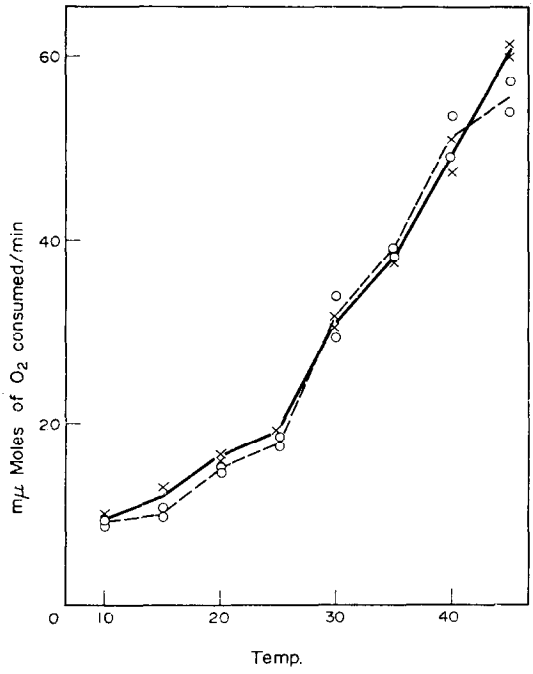

FIG. 2. Effect of temperature on the rate of oxidation of tyramine by bound and soluble forms of monoamine oxidase. Monoamine oxidase activity in mitochondria: ×—×—×, and in the preparation of soluble enzyme: ○- - -○- - -○ was measured at the temperatures indicated. In all cases the buffer in the reaction vessel was saturated with air at the temperature indicated, and the reaction was started by the addition of tyramine.

curves above 40° is probably due to the difference in thermostability of the two enzyme preparations (Fig. 1).

Sensitivity to trypsin digestion. In these experiments the possibility that the binding of the enzyme to the mitochondrial membrane was of importance for its sensitivity to trypsin digestion was investigated. When about 150 mg of the mitochondrial preparation was incubated with 7·5 mg of trypsin in 3 ml of phosphate buffer, pH 7·5, at 37°, the monoamine oxidase activity slowly decreased compared to that of mitochondria incubated without trypsin. About 35 per cent of the activity had disappeared after 24 hr (Fig. 3). When the same experiment was performed with soluble monoamine oxidase the enzyme activity was more rapidly destroyed; only about 20 per cent of the activity remained after 10 hr. This increased sensitivity was not due to a lower protein concentration since residue after the second buffer extraction, inactivated at 55° for 30 min, had been added to give 150 mg total protein. When soluble monoamine oxidase was rebound to mitochondrial membranes relipidated with cardiolipin, its sensitivity to trypsin digestion did not decrease (Fig. 3). In neither of these experiments did the activity of the enzyme preparation incubated without trypsin decrease more than 20 per cent in 24 hr.

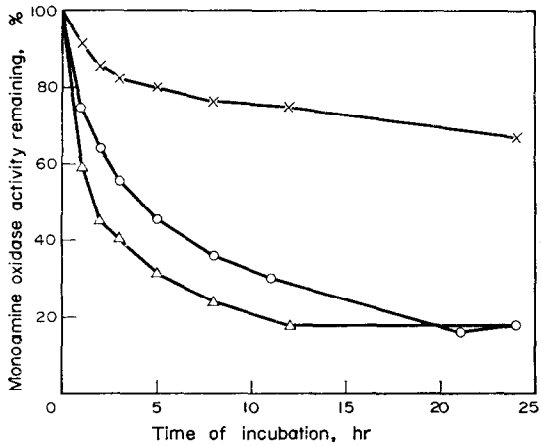

FIG. 3. Sensitivity to trypsin digestion of bound and soluble forms of monoamine oxidase. About 750 units of monoamine oxidase bound to mitochondria: ×—×—×; in the soluble form: ○—○—○ and rebound to relipidated membranes: △—△—△ was incubated with 5 mg of trypsin in 3 ml 0·1 M phosphate buffer, pH 7·5, at 37°. At the times indicated the activity was measured with tyramine as substrate (see Methods). The values are expressed as per cent activity of a control incubated under the same conditions but without trypsin.

Inhibition by pargyline. In order to see whether the bound and the soluble enzyme differed in their sensitivities towards monoamine oxidase inhibitors, experiments were performed with an irreversible inhibitor, pargyline[15] and a reversible inhibitor, harmaline.[16] The irreversibility of the pargyline inhibition was tested by plotting reaction velocity versus amount of enzyme as suggested by Ackermann and Potter.[17] In this plot inhibitors which merely "titrate" the enzyme should produce a line that intercepts the x-axis at a point to the right of the origin, corresponding to the amount of enzyme bound by the inhibitor. Furthermore, the slope of the line should be the same as that obtained with the uninhibited enzyme. Reversible inhibition should produce a

line passing through the origin and having a decreased slope as compared to the uninhibited reaction. Figure 4 shows that the curve obtained after preincubation of enzyme plus 10^{-7} M pargyline for 30–40 min at 24° passed through the origin, indicating reversible inhibition. After preincubation for 3–4 hr at 37° with 6×10^{-7} M pargyline, however, as in the experiments illustrated in Fig. 5, the curve was parallel to that of the uninhibited enzyme, indicating irreversible inhibition.

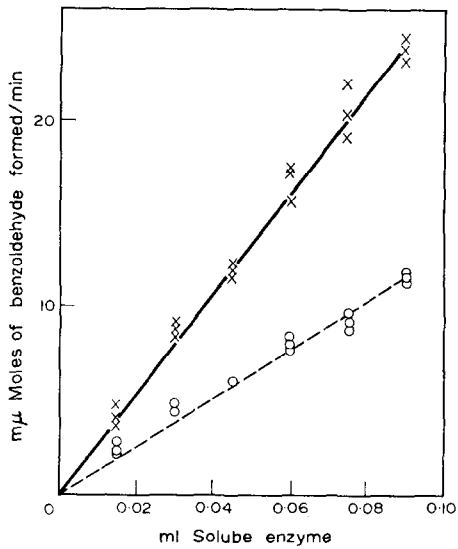

FIG. 4. Reversible inhibition of monoamine oxidase by pargyline. Various amounts of the preparation of soluble enzyme were diluted to 3 ml in 0·1 M phosphate buffer, pH 7·5, and the activities measured after addition of 10 μmoles of benzylamine, ×—×—×. The same amounts of enzyme were then diluted to 3 ml in the buffer containing 10^{-7} M pargyline. After incubation for 30–40 min at 24° the activities were measured with benzylamine as substrate, O- - -O- - -O.

Using monoamine oxidase preparations from other species, it has previously been shown that pargyline inhibits the oxidation of tyramine and serotonin to different degrees (see Discussion). Such a difference, however, was not found in the experiments shown in Fig. 5, in which the reaction between enzyme and pargyline was allowed to go to completion. Figure 5A shows that the same amount of mitochondria was "titrated" by 0·3 nmoles of pargyline both with tyramine and serotonin as substrate and Fig. 5B shows that this was also the case when the soluble enzyme preparation was used.

Molecular activity of bound and soluble enzyme. It has previously been shown that pargyline reacts with the active site of monoamine oxidase.[15] It has also been shown that pargyline binds selectively to monoamine oxidase even in such a crude enzyme preparation as mitochondria.[18] Thus there is reason to believe that the "titration" described above can be used to measure the amount of monoamine oxidase active site in a certain amount of mitochondrial or partially purified enzyme preparation. Furthermore, since one mole of flavin prosthetic group was found per mole of enzyme,[11] the amount of enzyme active site probably equals the amount of enzyme. If the values given in Fig. 5A and B are used in this way, the molecular activity, defined as the number of moles of substrate transformed/min/mole of enzyme,[19] was

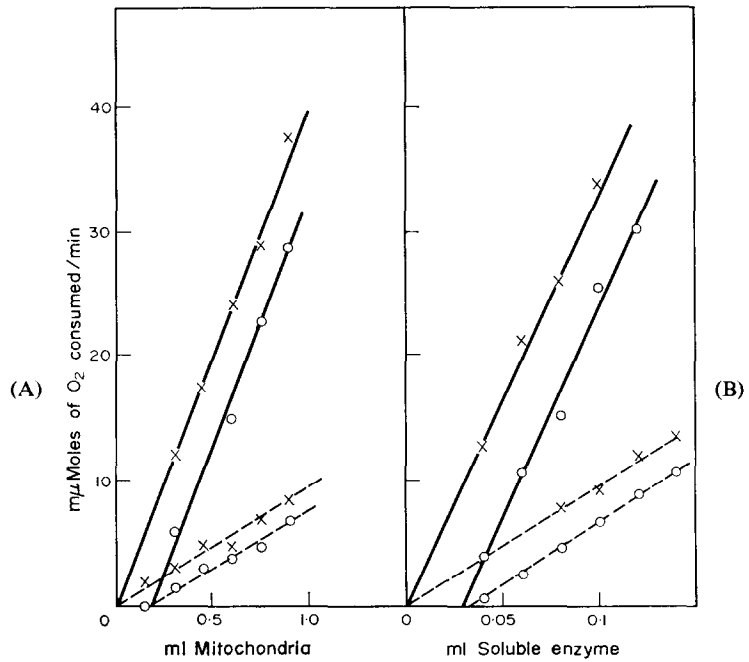

FIG. 5. Irreversible inhibition of bound and soluble monoamine oxidase by pargyline. In Fig. A various amounts of mitochondria were diluted to 1 ml in 0·1 M phosphate buffer, pH 7·5, and incubated for 3–4 hr at 37°. After incubation the activities were measured on 0·05 ml aliquots with tyramine, ×—×—×, and serotonin, ×– – –×– – –×, as substrates (see Methods). The same amounts of mitochondria were then incubated in the same way in the presence of $7{\cdot}5 \times 10^{-7}$ M pargyline and the activities measured on 0·05 ml aliquots with tyramine, ○—○—○, and serotonin, ○– – –○– – –○, as substrates. In Fig. B various amounts of the preparation of soluble enzyme were diluted to 0·5 ml in the buffer, incubated as described above, and the activities were measured on 0·1 ml aliquots using the above substrates. The same amounts of soluble enzyme were then incubated in the presence of 6×10^{-7} M pargyline as described above and the activities were again measured. The enzyme activities given in Fig. A and B are those measured in the aliquots.

400 for the bound enzyme and 350 for the soluble enzyme with tyramine as substrate. Thus there seems to be no singificant change in molecular activity after the ketone extraction procedure.

Inhibition by harmaline. When 0·2 mM harmaline was used as inhibitor in the Ackermann–Potter plot, both the soluble and the bound enzyme were reversibly inhibited to the same degree (Fig. 6A and B). Figure 6 also shows that the degree of inhibition by harmaline varied with the substrate used. Thus, with the mitochondrial preparation, the ratios of uninhibited enzyme activity to that in the presence of 0·2 mM harmaline were 1·6 and 4·0 respectively when tyramine and serotonin were used as substrates (Fig. 6A). When the soluble enzyme preparation was used the ratios were essentially the same, i.e. 1·6 and 3·5 for tyramine and serotonin respectively (Fig. 6B).

In order to exclude the possibility that the different degrees of inhibition of tyramine and serotonin oxidation by harmaline were due to different affinities of these substrates for the enzyme,[20] the experiments shown in Fig. 6 were also performed with tyramine and serotonin present in proportion to their K_m-values. In these experiments

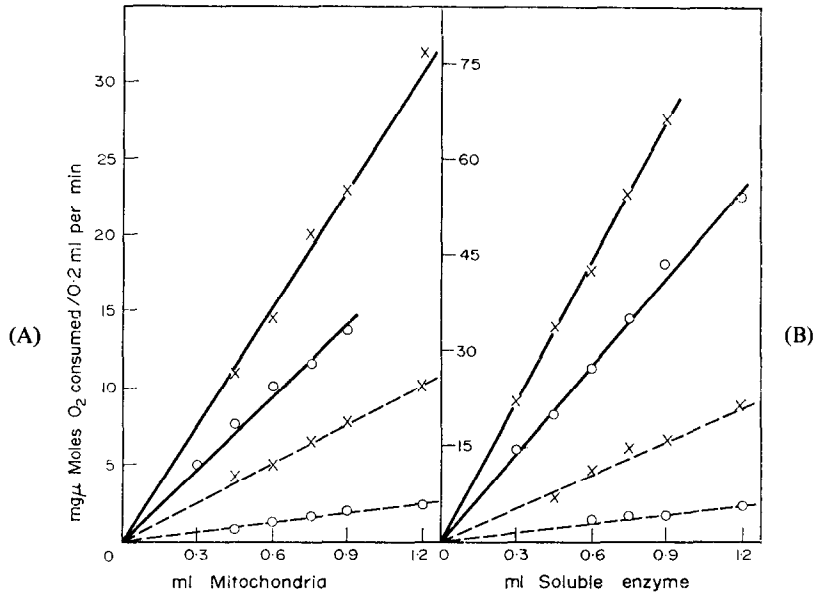

FIG. 6. Inhibition by harmaline of bound (A) and soluble (B) forms of monoamine oxidase. In Fig. A the various amounts of mitochondria indicated were incubated for 30 min at 37° in 3 ml of 0·1 M phosphate buffer, pH 7·5, and the enzyme activities were then measured on 0·2 ml aliquots with tyramine, ×—×—×, and serotonin, ×- - -× - - -×, as substrates (see Methods). The same incubations were also carried out with 0·2 mM harmaline present and the enzyme activities were measured on 0·2 ml aliquots with tyramine, ○—○—○, and serotonin, ○- - -○- - -○, as substrates, and, with 0·2 mM harmaline present in the reaction vessel. In Fig. B the same procedure was carried out using soluble enzyme preparation.

3 μmoles of tyramine and 9 μmoles of serotonin were used, since the K_m-value for both the bound and soluble enzyme was found to be 3 times higher with serotonin than with tyramine. These changes in the substrate concentrations, however, did not significantly change the ratio of the degrees of inhibition of tyramine and serotonin oxidation.

DISCUSSION

There was found to be a definite difference in thermostability between soluble and bound monoamine oxidase (Fig. 1). The activity of the enzyme *in situ* in mitochondria was unchanged after 1 hr at 50°, while the soluble enzyme had lost 85 per cent of its activity after 20 min. Such a difference in thermostability has also been found for the monoamine oxidase activity of whole lysed platelets and the purified platelet enzyme.[21] It is reasonable to assume that the conformation of the enzyme *in situ* in the membrane is protected from thermal denaturation by interaction with other membrane components. However, this interaction does not seem to affect the active site, since the molecular activity of the enzyme as well as its sensitivity to a reversible and an irreversible inhibitor was the same whether it was membrane-bound or in the soluble

state. When the enzyme was rendered soluble, the stabilizing interaction with other membrane components was lost and the enzyme became more labile. When the soluble enzyme was rebound to relipidated membranes its ability to resist heat denaturation was partially restored, which may be explained by a partial restoration of the supporting effect of other membrane components. As is shown in Fig. 1 the thermostability of the enzyme in the residue after the second extraction step was about the same as that of the rebound enzyme. This is in accordance with our suggestion, since the membranes after the second extraction step are poor in phospholipids.[7,8] The different thermostabilities of bound and soluble enzyme could, of course, result from preferential liberation of a thermolabile type among several monoamine oxidases by the ketone extraction procedure. This explanation, however, is very unlikely since the thermostability was partially restored by rebinding of the soluble enzyme. These results show that differences in the thermostability of different fractions of solubilized monoamine oxidase[22–25] do not necessarily mean that they contain different forms of the enzyme.

The soluble enzyme was more sensitive to trypsin digestion than the enzyme *in situ* in the mitochondria (Fig. 3). The interaction of the enzyme with other membrane components thus also protected it against digestion by trypsin. When the enzyme was rendered soluble by extraction of phospholipids an increased area of the enzyme protein was exposed to the proteolytic enzyme. The great sensitivity of the rebound enzyme suggests that a large area of enzyme protein was still available to trypsin after rebinding.

The irreversible inhibition by pargyline was preceded by a reversible phase (Fig. 4). When the reaction between enzyme and pargyline was allowed to go to completion the same amount of enzyme activity was titrated by pargyline irrespective of whether bound or soluble enzyme was used, and tyramine and serotonin oxidations were inhibited to the same degree (Fig. 5). Other groups have reported different degrees of inhibition of liver monoamine oxidase by pargyline, depending on whether serotonin or tyramine was used as substrate.[26–28] One explanation might be that in some species there are several forms of monoamine oxidases and in other species only one form; Hall *et al.*[29] have presented evidence that pig liver, in contrast to e.g. rat or human liver, contains a single form of monoamine oxidase. The present data support this conclusion.

Harmaline inhibited the membrane-bound and the soluble enzyme to the same extent. This was true both with tyramine and serotonin as substrate. However, both the bound and the soluble enzyme were more inhibited when serotonin was used as substrate (Fig. 6). Since the ratio between tyramine and serotonin oxidation was not changed by 100 times purification of the enzyme, it seems unlikely that the difference in inhibition could be due to the existence of several monoamine oxidases in the pig liver. That it could be due to differences in the affinities of the substrates for the enzyme was excluded by control experiments (see Results). It is interesting to note that differences in the degree of enzyme inhibition, apparently dependent on the substrates, but not correlated to their affinities for the enzyme, have been observed in another system.[30]

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