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# The in vitro insertion of monoamine oxidase B into mitochondrial outer membranes

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Bovine monoamine oxidase (MAO) B has been synthesized in vitro using a reticulocyte lysate translation system directed by bovine liver poly(A)$^+$ RNA. The newly synthesized enzyme apparently lacks a cleavable N-terminal extension, but MAO B is readily incorporated into mitochondria or isolated mitochondrial outer membranes prepared from rat liver. ATP is not required for the binding of the newly synthesized enzyme to the outer membranes, but is necessary for the insertion of MAO B into these membrane vesicles. The ATP is not required to generate a mitochondrial membrane potential as assembly occurs under conditions that preclude either the formation or the maintenance of the potential. MAO B will bind to but not become incorporated into outer membrane vesicles which have been treated with trypsin, suggesting that the insertion of MAO B also depends on protein factors present on the outer membranes.

Mitonchondrial outer membrane; Monoamine oxidase; Mitochondrial biogenesis

## 1. INTRODUCTION

The overwhelming majority of the proteins in mitochondria and all of the proteins of the outer membrane of the mitochondria are encoded on nuclear genes and synthesized on cytoplasmic ribosomes [1]. The import of a number of proteins into the interior mitochondrial compartments, matrix, inner membrane and space between the inner and outer membranes, has been well studied. Although there are some exceptions, most precursor proteins contain N-terminal signal peptides which are cleaved in the matrix after import, and the import process requires an electrochemical potential across the inner membrane [1], extramitochondrial ATP [2,3] and trypsin-sensitive factors on the mitochondrial outer membrane [4,5]. Although the insertion of proteins into the outer membrane has been less well studied, the process appears to differ in some important respects. For example, porin, the pore-forming protein of the outer membrane, has been shown, in studies with *Neurospora* and yeast, not to contain an N-terminal signal peptide [6,7], nor is a membrane potential required for its insertion [7]. However, very recently, a requirement for extramitochondrial ATP has been demonstrated for porin assembly [8]. It is less certain whether trypsin-sensitive factors are necessary for the membrane insertion of porin. In yeast, trypsin treatment prevents import of the $\beta$-subunit of the $F_1$ ATPase (inner membrane) and cytochrome $b_2$ (intermembrane space) under conditions that do not diminish the insertion of porin [7]. On the other hand, in *Neurospora*, porin assembly requires trypsin-sensitive outer membrane components [8].

Although the insertion of several outer membrane proteins has been studied in both yeast and *Neurospora*, less has been done with mammalian systems, and, at least in the case of porin, the mammalian process has been reported not to be strictly in parallel with the process in fungi [9]. We have studied the insertion of monoamine oxidase B (MAO B) into mammalian mitochondria and outer membranes. MAO B is one of two immunologically and catalytically distinct MAO isoenzymes [10].

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These proteins have different peptide maps, and both are located in the outer membranes of mammalian mitochondria [11].

## 2. EXPERIMENTAL

### 2.1. *Preparation of poly(A)$^+$ RNA from bovine liver*

A portion of liver was removed, cut into approx. 2 $cm^3$ blocks and frozen on dry ice. This step took less than 4 min from the time the animal was slaughtered. The importance of obtaining and freezing liver samples within a few minutes cannot be overemphasized. The blocks of frozen liver could then be stored at −70°C for at least 6 months.

RNA was extracted from 1 or 2 of the frozen blocks according to a published procedure using guanidine thiocyanate-phenol-chloroform [12]. Poly(A)$^+$ RNA was isolated from the total RNA by existing methods [13] using a column of oligo(dT) linked to dextran beads. The purified poly(A)$^+$ RNA was stored in water at −70°C. RNA concentrations were estimated spectrophotometrically assuming an absorbance of 20 at 260 nM for a 1 mg/ml RNA solution.

### 2.2. *In vitro translation of poly(A)$^+$ RNA and assays for membrane assembly of MAO B*

Poly(A)$^+$ RNA was used to charge a commercially obtained (NEN Research Products, Boston, MA) reticulocyte lysate translation kit. The kit essentially reproduces the conditions described by Pelham and Jackson [14]. Our assays were performed in a volume of 30–50 $\mu$l and contained from 60 to 90 $\mu$g poly(A)$^+$ RNA/ml. In most of our experiments, the incubation was allowed to proceed for 60 min, and then mitochondria or mitochondrial outer membranes were added at a concentration of 100 $\mu$g protein/ml. The preincubations were necessary because the membrane preparations contained ribonucleases that interfered with translation. The incubations were terminated by immersing the tubes in an ice bath. Usually, the mixtures were divided into two equal portions. One was incubated at 37°C for 30 min with 10.0 $\mu$g/ml of proteinase K and then the protease was inhibited with 2 mM PMSF. Otherwise, both aliquots were handled identically.

When neither mitochondria nor outer membranes were added, IgG prepared from monospecific polyclonal antiserum against MAO B was added directly. When membranes were included, 40 $\mu$g mitochondria were added as a carrier, and the mixture was centrifuged at 10000 × $g$ for 10 min to remove the membranes. The pellets were then solubilized by sonication in 400 $\mu$l of 50 mM Tris-HCl/154 mM NaCl (pH 7.4) containing 1.0% Triton X-100, and then a 1/300 dilution of the anti-MAO B IgG was added.

The antibody-containing mixtures were incubated for 60 min at 37°C and then enough staphylococcal protein A (which had been bound to agarose beads) was added to ensure the complete removal of all of the IgG. This mixture was incubated at 4°C for 60 min with stirring. The IgG-protein A-agarose complex was removed by centrifugation and washed by resuspension and centrifugation twice with about 1 ml of 10 mM Tris-HCl (pH 7.4)/1.0% Triton X-100, twice more with 1.0 M sucrose dissolved in the same buffer and finally two to four times more with the original Tris/Triton X-100 buffer (i.e. until the radioactivity in the supernatant approached background).

The immune complexes were then dissolved by boiling in SDS-mercaptoethanol for 10 min, and subjected to electrophoresis (SDS-PAGE) as described by Laemmli [15]. The gels were soaked in a commercially available mixture of fluors (NEN Research Products, Boston, MA), dried and used to expose X-ray film. Often, purified MAO B which had been labelled with 5 × $10^{-5}$ M [$^3$H]pargyline (NEN) was included in the electrophoretograms as a reference [16]. Pargyline is a mechanism-based inhibitor that forms a covalent adduct with MAO B.

### 2.3. *Preparation and trypsin treatment of mitochondrial outer membranes*

Mitochondria and mitochondrial outer membranes were prepared as we have previously described [17]. In some instances, the outer membranes were treated with trypsin according to an existing method [18]. Briefly, 5 mg/ml outer membrane protein was incubated on ice for 30 min with 100 $\mu$g/ml bovine pancreatic trypsin in 0.25 M sucrose/4 mM EGTA/50 mM Tris-HCl (pH 7.5). Soya trypsin inhibitor was added to a final concentration of 100 $\mu$g/ml. This treatment will cause extensive proteolysis of outer membranes. On the other hand, the membrane barrier is intact, since similar treatment of mitochondria has a negligible effect on the recovery of adenylate kinase, a marker for the intermembrane space.

### 2.4. *Other methods*

MAO B was purified from bovine mitochondria according to Salach [19]. Antisera to MAO B were raised in New Zealand white rabbits as reported [10] except that no injections were made in the feet. A crude IgG fraction was prepared by precipitation with 50% ammonium sulfate. The precipitate was washed with 50% ammonium sulfate and, finally, dialyzed against 10 mM $K_2HPO_4$ (pH 7.5).

### 2.5. *Materials*

Except when otherwise indicated, materials were purchased from Sigma (St. Louis, MO) and of the highest purity available.

## 3. RESULTS

### 3.1. *Newly synthesized MAO B is incorporated into mitochondria and mitochondrial outer membranes*

As can be seen from fig.1, a radiolabelled immunoprecipitable MAO B was synthesized by a reticulocyte lysate translation system that contained [$^{35}$S]methionine and had been charged with bovine liver poly(A)$^+$ RNA. The newly synthesized MAO B had approximately the same electrophoretic mobility as did authentic MAO B which had been purified from bovine liver mitochondria and then reacted with [$^3$H]pargyline so the enzyme could be identified by fluorography.

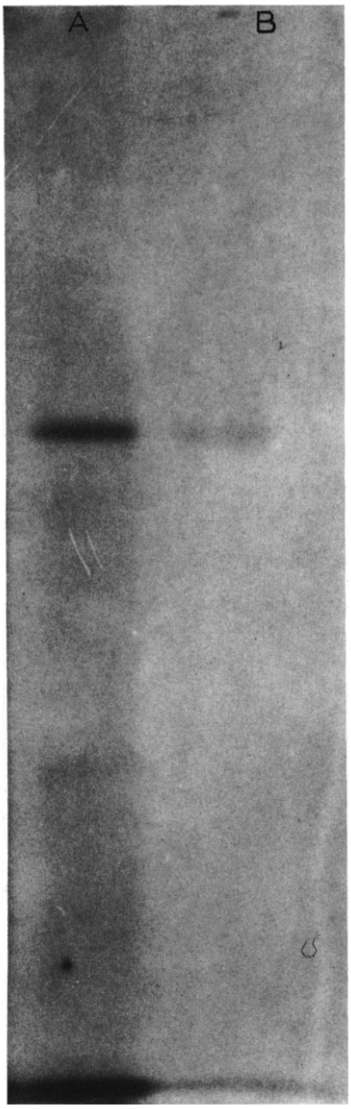


Fig.1. Newly synthesized MAO B has the same apparent size as the mature enzyme. A reticulocyte lysate fortified with an ATP-generating system and containing [$^{35}$S]methionine was charged with bovine poly(A)$^+$ RNA and allowed to incubate at ambient temperature for 60 min. This mixture was reacted with IgG prepared from antiserum to bovine MAO B, and the immune complex was isolated and analyzed by electrophoresis and fluorography as described in section 2. Newly synthesized MAO B (A) and MAO B that had been purified from bovine liver mitochondria and then reacted with [$^3$H]pargyline (B) are compared.

It is not surprising that newly synthesized MAO B and the mature enzyme apparently have the same molecular mass. Others have observed that rat MAO B [20] is not synthesized with an N-terminal extension; however, it was of interest to us to determine whether the newly synthesized MAO B could also be incorporated into mitochondrial outer membranes. To test this, the complete

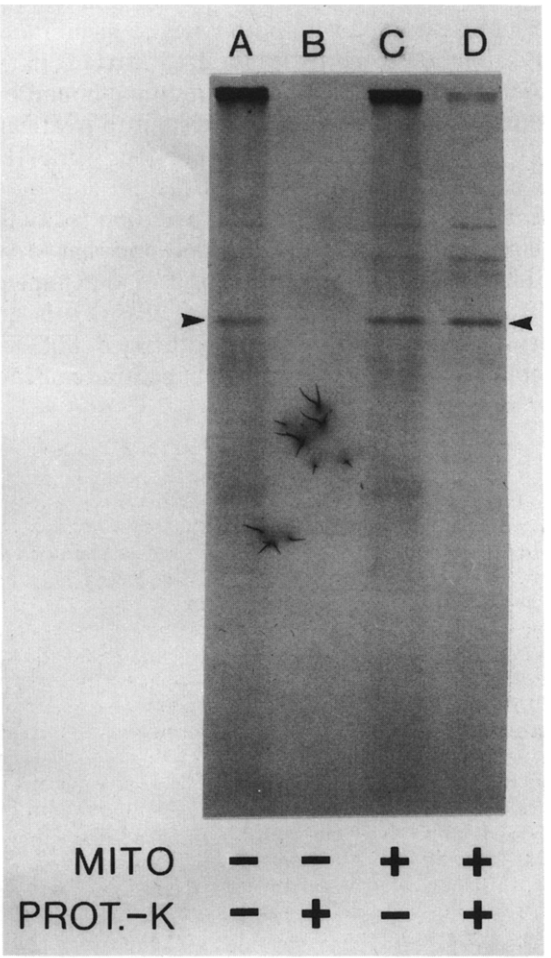


Fig.2. Newly synthesized MAO B will insert into mitochondrial membranes in a protease-resistant form. Reticulocyte lysates fortified with an ATP-generating system and containing [$^{35}$S]methionine were charged with bovine poly(A)$^+$ RNA and allowed to incubate at ambient temperature for 60 min. Two of the translation assays were put on ice after 60 min. Then 5 mg/ml mitochondrial protein was added to C and D, and these two samples were incubated for another hour. Samples B and D were treated with proteinase K (10.0 $\mu$g/ml for 30 min). Newly synthesized MAO B was immunoprecipitated either from the reaction mixture (A,B) or from mitochondria separated from the mix (C,D) and analyzed by electrophoresis and fluorography. The arrows indicate the electrophoretic mobility of purified [$^3$H]pargyline-labelled MAO B.

reticulocyte lysate system [i.e. containing [$^{35}$S]methionine and poly(A)$^+$ RNA] was pre-incubated for 60 min. Then, rat liver mitochondria or outer membranes (100 $\mu$g/ml) were added, and the incubation was continued. Virtually all of the newly synthesized MAO B was removed from solution (not shown) and, as can be seen from figs 2,3, became associated with both types of membranes. In distinction to newly synthesized MAO B in the absence of membranes, the membrane-bound enzyme was largely or entirely resistant to treatment with proteinase K (figs 2,3). In previous work [18], we have used proteases to show that isolated outer membranes have the same orientation as outer membranes in intact mitochondria and that MAO B in either isolated mitochondria or outer membranes is protected from proteases. So, it is not surprising that the in vitro synthesized MAO B would also become protected as it became embedded in the membrane. In several experiments (not shown), freeze-thawed mitochondria or rat liver microsomes replaced fresh mitochondria. The small amounts of MAO B that bound to microsomes were sensitive to protease treatment; however, even though the freeze-thawed mitochondria should be less able to maintain a membrane potential, newly synthesized MAO B was just as readily inserted into a protease-resistant form.

It may be noted that a few radioactive polypeptides aside from MAO B were occasionally detected in our immunoprecipitates. These appear irregularly and whether or not membranes are present. Since extensive washing greatly reduces or eliminates them, it is most likely that these unidentified proteins are strongly adsorbed to the immunoprecipitate.

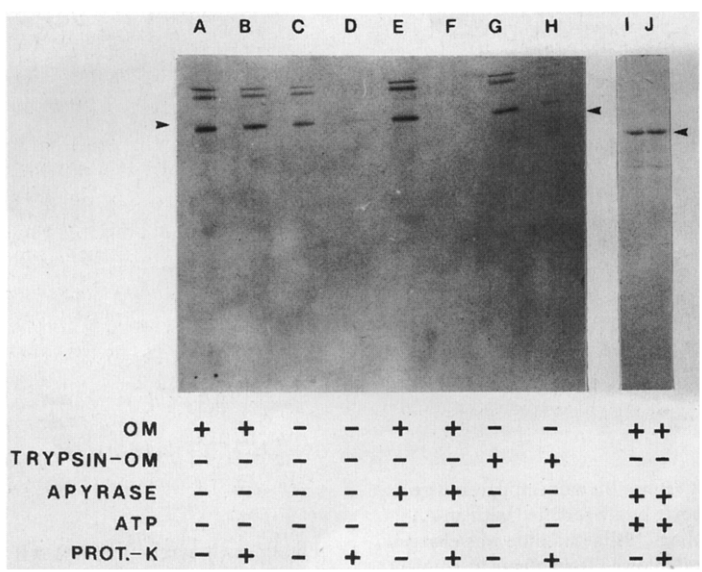


Fig.3. Both ATP and trypsin-sensitive membrane factors are required for MAO B insertion into mitochondrial outer membranes. Reticulocyte lysates fortified with an ATP-generating system and containing [$^{35}$S]methionine were charged with bovine poly(A)$^+$ RNA and allowed to incubate at ambient temperature for 60 min. Mitochondrial outer membranes (5 mg/ml protein) were added to A and B. No additions were made to C and D. Samples E and F were treated with 0.5 U/ml apyrase for 30 min before 5 mg/ml outer membrane protein was added. Samples I and J were also incubated with apyrase (0.5 U/ml for 30 min), but the concentration of ATP was readjusted to 1 mM immediately before and 20 and 40 min after the addition of 5 mg/ml outer membrane protein. Trypsin-treated outer membranes (5 mg/ml protein) were added to G and H. These samples were incubated for another hour after the membranes were added. Sample D and the membranes separated from samples B, F, H and J were treated with proteinase K (10.0 $\mu$g/ml for 30 min). Finally, C and D and the solubilized outer membranes from the other samples were reacted with antibodies to MAO B, and the immune complexes were analyzed by electrophoresis and fluorography. The arrows indicate the electrophoretic mobility of purified [$^3$H]pargyline labelled MAO B.

### 3.2. *Both trypsin-sensitive factors on the outer membranes and ATP are required for MAO B incorporation*

Experiments were performed to determine whether ATP was required for assembly of MAO B in the outer membranes. Again, a complete reticulocyte lysate was incubated for 60 min to permit synthesis of MAO B. Then, apyrase was added, and the mixture was incubated for 20 min to destroy the adenine nucleotides. Finally, outer membranes were added, and the incubation was continued for 40 min more. As can be seen from fig.3, MAO B was still able to bind to the outer membranes, but it remained accessible to proteinase K. In experiments in which ATP was restored after apyrase treatment (fig.3) or apyrase was boiled prior to its addition to the incubation (not shown), MAO B was inserted in a protease-resistant form. It is clear from these experiments that ATP is required for the insertion of MAO B in the outer membranes.

In another series of experiments, we treated isolated outer membranes with trypsin in amounts that we have shown remove proteins on the external aspect of the vesicle but which do not affect the membrane's integrity [18]. After the trypsin treatment, the protease was inhibited with soybean trypsin inhibitor, and these vesicles were substituted for intact outer membranes in the otherwise complete reticulocyte lysate system. These results are depicted in fig.3. Just as after ATP depletion, MAO B was able to bind to the trypsin-treated membranes but only a small portion of the enzyme was protected from proteinase K. That is, MAO B was not able to insert as well into the trypsin-treated vesicles as into intact outer membranes.

## 4. DISCUSSION

Our experiments show that newly synthesized MAO B can be assembled into mitochondrial outer membranes in spite of the fact that it apparently lacks a cleavable N-terminal extension. Furthermore, because it is readily assembled into isolated outer membranes which can neither generate nor maintain a proton gradient, the assembly of MAO B does not require a mitochondrial membrane potential.

On the other hand, insertion into the outer membrane does require ATP or energy derived from it. That is, although newly synthesized MAO B will bind to the membranes in the absence of ATP, it is not protease resistant like the mature membrane-bound form of the enzyme. In these respects, the insertion of MAO B into mammalian outer membranes resembles the similar process described for porin in fungi [6,7].

Our results also suggest that protein components on the exterior of the outer membranes may be involved in the insertion of MAO B. Outer membranes were treated with bovine trypsin under conditions that have been shown to remove exterior proteins, but not to breach the integrity of the vesicles [18]. When these trypsin-treated vesicles were used in the usual assay, MAO B would bind to the membranes, but was largely subject to digestion by proteinase K. A similar requirement for trypsin-sensitive factors has been reported for the insertion of porin into *Neurospora* mitochondrial outer membranes [8]. While it is certain that proteins in the outer membrane are necessary for insertion of MAO B, it is not clear that these proteins are directly involved in the insertion process. It is also possible that trypsin treatment may not specifically impair the insertion process, but rather, nonspecifically damage the outer aspect of the membrane sufficiently that it no longer accepts processed proteins.

While the assembly of MAO B into the outer membrane depends on ATP and perhaps on protein factors, neither of these seems to be essential for the initial protease-sensitive binding of the enzyme to the membranes. Instead, ATP and the protein factors seem to be involved in processes that occur after newly synthesized MAO B has been recognized by the outer membranes. Others have shown that porin probably undergoes a change in its conformation that permits insertion into the outer membranes [21]. Our data support the notion that ATP and possibly other factors are involved in a similar process that permits the incorporation of MAO B into the outer membranes.

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