Membrane and Cytosolic Components Affecting Transport of the Precursor for Ornithine Carbamyltransferase into Mitochondria*

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A higher molecular weight precursor (M, = 39,000) to the liver mitochondrial matrix enzyme, ornithine carbamyltransferase (M, = 36,000), is imported and processed by heart mitochondria in vitro in a manner similar to liver mitochondria. In both systems, however, an additional 37-kDa ornithine carbamyltransferase polypeptide appears, but this arises from nonspecific events and, therefore, does not represent a bona fide intermediate in the overall processing sequence. Our experiments demonstrate that the outer mitochondrial membrane of mitochondria contains a protease-sensitive (5 μg of trypsin or chymotrypsin/ml, 15 min at 2 °C), salt-resistant (1.0 M KCl) protein which is required to maintain import functions. In addition, functional post-translational import requires a component of the reticulocyte lysate (i.e., cytosol) that is used for initially synthesizing precursor enzyme. The component is retained by Sephadex G-25. Import of Sephadex G-25-excluded precursor is restored by fresh reticulocyte lysate but not by a combination of other additives, including Mg²⁺, K⁺, ATP, ADP, P₃, succinate, and total translation mixture (minus lysate).

Mitochondria acquire the majority of their proteins by importing nuclear-coded products in the form of precursor polypeptides from the cytoplasm. The overall pathway involves synthesis by free 80 S ribosomes, rapid transport through the aqueous cytosolic compartment of the cell, and transmembrane uptake of the precursor across either one or both mitochondrial membranes by an exclusively post-translational mechanism (1–5); the same pathway is also followed for precursor polypeptides which are destined for insertion into the outer membrane of mitochondria (6, 7). How newly synthesized precursor proteins are selectively channeled to mitochondria, however, is not presently known, although it is generally agreed that receptors of some kind must be involved since cytoplasmic precursors to mitochondrial proteins enter this organelle and no other. Nor is it known whether or not selective channeling to mitochondria is mediated by a soluble SRP, analogous to the type which targets nascent secretory proteins (8–10) to their ER receptor (11). As far as SRPs are concerned, however, it should be emphasized that a major function for the secretory SRP complex is to ensure that secretory polypeptides are not prematurely deposited in the cytosol, and thus trapped there, before having a chance to react with ER (10); such a problem does not exist for precursors to mitochondrial proteins since the cytosolic compartment constitutes part of the normal post-translational pathway for transport of these proteins to their final destination (1–7, 12–14).

In the present communication, we show that import functions of isolated mitochondria are inhibited by certain perturbations to the mitochondrial surface (e.g., mild proteolysis) but not by others (e.g., salt washes). The evidence indicates that it is intrinsic-like protein(s) of the outer membrane which mediates recognition and/or uptake of the cytoplasmic precursor to the mitochondrial matrix enzyme of rat liver, ornithine carbamoyltransferase (EC 2.1.3.3). Additionally, a cytosolic factor(s) appears to play some role in the overall process.

MATERIALS AND METHODS

General—For most of the routine procedures used in this study, the methods followed have been outlined elsewhere (13, 16). These include protein measurements, extraction of mRNA by the chloroform/phenol method, protein synthesis in a messenger-dependent (17) cell-free protein-synthesizing system derived from rabbit reticulocytes (15), and SDS-polyacrylamide gel electrophoresis and fluorography of dried gels. Where individual protocols were employed only for a specific experiment, they are described in detail in the appropriate figure legend. Male Sprague-Dawley rats (120–200 g, body weight) were used throughout this investigation.

Isolation of Heart Mitochondria—A single heart (0.8–1.1 g) was quickly removed from a rat, placed in 10 ml of ice-cold medium A (10 mM Hepes, pH 7.5, 220 mM mannitol, 70 mM sucrose, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraaceteate, 2 mg/ml of bovine serum albumin), and homogenized for 3 s with a Brinkmann Polytron at a setting of 6.5 (modified from Refs. 18 and 19). All further manipulations were performed at 2–4 °C. The homogenate was diluted to 40 ml with medium B (medium A minus bovine serum albumin) and centrifuged in a swing-out IEC 279 rotor at 1800 rpm for 10 min. The upper three-quarters of the supernatant was removed and further centrifuged in a Sorvall SS-34 rotor at 7000 rpm for 10 min. The resulting pellet was uniformly suspended in 10 ml of medium B and centrifuged at 1800 rpm for 10 min. The supernatant was centrifuged at 7000 rpm to collect purified mitochondria. They were suspended in a medium containing 10 mM Hepes, pH 7.4, 0.25 mM sucrose, 1 mM dithiothreitol, 10 mM Na succinate, 0.16 mM ADP, and 2.5 mM K₂HPO₄, pH 7.4.

In Vitro Import and Processing by Heart Mitochondria—Translation of rat liver mRNA was performed in a reticulocyte cell-free system (17) for 60 min at 30 °C, after which the reaction mixture was adjusted to 0.25 M sucrose and 10 μg/ml of cycloheximide. Freshly suspended heart mitochondria (106 μl) were then combined with 100 μl of translation products to give a final mitochondrial protein concentration of 2.5–3.0 mg/ml. The mixture was incubated at 30 °C for various periods of time up to 90 min.

Immunoprecipitation—Reaction mixtures were diluted 1:4 with Trion medium (1.20% Trion X-100, 25 mM methionine, 12.5 mM

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ethylendiaminetetraacetic acid, 0.025% NaN₃, and phosphate-buffered saline). NaCl was added to give a final concentration of 1.4 M and insoluble material was removed by centrifugation in a Beckmann Ti-75 rotor at 45,000 rpm for 45 min. An excess of nonspecific antibody to ornithine carbamyltransferase (20) was added and the mixtures were incubated overnight at 4 °C. Following addition of Protein A-Sepharose (Pharmacia), the samples were incubated, with washing, for 1 h at room temperature. They were then centrifuged at 12,000 × g for 2 min. The pellets were washed three times with Triton medium and two times with 0.9% NaCl. The final pellets were resuspended in 30 μl of SDS sample buffer (15), heated at 100 °C for 3 min, and recentrifuged. The supernatants were applied to a 30-cm SDS-polyacrylamide slab gel (15) and, following electrophoresis at 30 mA, the gel was stained, impregnated with ENHANCE (New England Nuclear), dried, and fluorographed for 1-5 days.

RESULTS AND DISCUSSION

The precursor to ornithine carbamyltransferase is one of a limited number of mammalian mitochondrial proteins which have proven amenable to studies of import and processing by isolated mitochondria in vitro (4, 5, 13, 21-24, 26). As such, it represents one of the best understood, with the mechanisms involved conforming closely to what is known concerning import of precursor proteins by mitochondria in lower eukaryotes (25).

In the present communication, we describe import of precursor ornithine carbamyltransferase, a product of hepatocyte mRNA, by isolated heart mitochondria in vitro. Heart mitochondria do not contain ornithine carbamyltransferase (see Ref. 26) and, therefore, provide a useful heterologous system where the background of processed ornithine carbamyltransferase is zero. The system is described in Fig. 1, where kinetics of incorporation and processing of precursor ornithine carbamyltransferase are presented. In an earlier report, we showed that processing by heart and liver mitochondria demonstrate a very similar pattern with respect to precursor ornithine carbamyltransferase, both qualitatively and quantitatively (26). Processing occurs in the presence of cycloheximide (Fig. 1) and involves post-translational uptake of the primary translation product of ornithine carbamyltransferase mRNA (Mr ~ 39,000), which is then converted to the mature form of the enzyme (Mr ~ 36,000) upon entry into mitochondria (Figs. 1 and 2; see Refs. 4, 5, 13, 21-24, and 26).

During post-translational assays with both heart and liver mitochondria, however, a second 37-kDa ornithine carbamyltransferase polypeptide appears, in addition to fully processed enzyme (Fig. 1; see also Refs. 4, 5, 22, and 24), but this polypeptide product remains sensitive to exogenous proteases under conditions where the fully processed enzyme is protected (not shown), it does not co-sediment with mitochondria following in vitro import assays (Fig. 2), and does not "chase" into mitochondria when recovered from in vitro postimport assays and added to fresh mitochondria (Fig. 3). Thus, within the detection limits of these types of experiments, there is no compelling evidence to assume that the 37-kDa ornithine carbamyltransferase polypeptide represents a bona fide intermediate in the overall processing of precursor ornithine carbamyltransferase (but see Ref. 24). We suggest that the 37K product arises in vitro due to leakage from mitochondria of the soluble matrix endoprotease described by Mori et al. (4); this protease was shown to convert precursor ornithine carbamyltransferase to 37K product, not to fully processed enzyme (4).

Effects of Mitochondrial Surface Perturbations on Import of Precursor Ornithine Carbamyltransferase by Heart Mitochondria—In elucidating the nature of receptors in ER which are responsible for mediating transmembrane uptake of nascent precursors of secretory proteins, two initial approaches were taken: removing salt-labile components from the cytoplasmic surface of ER microsomes (8-10) and subjecting the surface of ER to exogenous proteolysis (11). We have applied similar perturbations to intact mitochondria. In contrast to the situation for ER microsomes, treatment of heart mitochondria with high concentrations of salt, up to 1 M KCl, had no substantial effect on the ability of the mitochondria to maintain import and processing functions (Fig. 4). Mild proteolysis with trypsin and chymotrypsin, however, completely

![FIG. 1](left) In vitro processing of pOCT by isolated rat heart mitochondria. Reticulocyte lysates containing products directed by rat liver mRNA were incubated with rat heart mitochondria in the presence of cycloheximide for 0, 10, 20, 30, and 60 min (lanes c-g, respectively). Products were then subjected to immunoprecipitation with anti-ornithine carbamyltransferase and precipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane a, total translational products were immunoprecipitated with pre-immune IgG; lane b, as in lane a but immunoprecipitation was with anti-ornithine carbamyltransferase IgG. OCT, ornithine carbamyltransferase.

![FIG. 2](left) Mature ornithine carbamyltransferase, but not 37K ornithine carbamyltransferase polypeptide, is recovered with mitochondria in vitro. Liver mRNA-directed translation products were incubated with heart mitochondria for 0 (lane a) and 60 min (lane b). Mitochondria were then recovered by centrifugation at 8000 × g for 10 min. Supernatant (S) and pellet (P) were subjected to immunoprecipitation with anti-ornithine carbamyltransferase and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. OCT, ornithine carbamyltransferase.

![FIG. 3](right) 37K ornithine carbamyltransferase polypeptide is not further processed to mature ornithine carbamyltransferase by heart mitochondria in vitro. A reticulocyte translation mixture containing products synthesized under the direction of liver mRNA were incubated for 90 min with heart mitochondria. The mitochondria, containing fully processed ornithine carbamyltransferase, were then removed by centrifugation, leaving a supernatant that contained 37K ornithine carbamyltransferase polypeptide, but no precursor ornithine carbamyltransferase or fully processed ornithine carbamyltransferase. The supernatant was then further incubated in the absence of mitochondria for 0 min (lane a) or with fresh mitochondria for 60 min (lane b), and product was recovered by precipitation with anti-ornithine carbamyltransferase and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. Lane c, markers for ornithine carbamyltransferase for pOCT, 37K ornithine carbamyltransferase, and fully processed ornithine carbamyltransferase. The radioautography would detect less than 10% conversion of 37K ornithine carbamyltransferase to fully processed ornithine carbamyltransferase. OCT, ornithine carbamyltransferase.
abolished such functions (Fig. 5). When tested separately, both proteases were effective (not shown). And for both enzymes, it could be shown that their effect on import functions could not be accounted for by contaminating phospholipases. The two enzyme preparations were shown to be free of phospholipase activity as assayed by their ability to hydrolyze phosphatidylcholine in unilamellar vesicles; no breakdown was detected following thin layer chromatography of the phospholipid (not shown). Finally, the protease-sensitive component of intact mitochondria, which is required for import, appears to be restricted to the outer membrane. Firstly, under the very mild conditions used for proteolysis, trypsin or chymotrypsin would not be expected to penetrate this membrane and, secondly, stripping the outer membrane from mitochondria by the digitonin procedure (27) renders the resulting mitoplasts incapable of importing and processing precursor ornithine carbamyltransferase (not shown, see also Ref. 4).

Taken together, therefore, the evidence indicates that one or more proteins located in the outer membrane of heart mitochondria facilitate import of precursor ornithine carbamyltransferase. Since the protein(s) is not liberated by 1 M KCl, it must be tightly bound to the outer membrane, perhaps embedded in the bilayer, but presumably with a critical portion or domain which is exposed at the surface of the organelle and, therefore, accessible to added proteases.

Despite considerable effort, however, we have not been able to achieve reconstitution of import functions by adding proteolysates back to proteolysed mitochondria. Nor have we found that wide spectrum antisera raised in rabbits against alkali-soluble and alkali-insoluble proteins of outer membrane of liver mitochondria are able to neutralize in any specific manner the import of precursor ornithine carbamyltransferase. Despite the fact that extensive cross-reactivity was shown to exist for heart and liver outer membrane proteins, as judged by the nitrocellulose blotting technique (not shown). Either the receptor protein(s) is non-antigenic in rabbits or those cross-reacting antigenic determinants which might influence receptor activity are buried in the bilayer and, therefore, remain unavailable to the antibodies.

**Involvement of Cytosolic Factor(s) in the Recognition and/or Uptake of Precursor Ornithine Carbamyltransferase by Heart Mitochondria in Vitro—** Because incorporation of cytoplasmic precursor polypeptides into mitochondria occurs by an exclusively post-translational route, questions arise as to the minimum requirements for import of a precursor polypeptide by mitochondria in vitro. For example, does import simply require the presence of a precursor polypeptide on the one hand and functionally intact mitochondria on the other, or are there additional components which are required and which are normally provided by the rather complex translational systems which are invariably present in all import assays reported to date? In the present case, our translational mixture derives from a reticulocyte lysate, the composition of which is considered to represent a standard "cytosolic" fraction. Isolated mitochondria are maintained in a functional state simply by suspending them in a medium containing a pH buffer (pH 7.4), an iso-osmoticum (sucrose), and substrates for oxidative phosphorylation (ADP, P) and the citric acid cycle (succinate) (see "Materials and Methods"). Our standard reconstitution assay involves mixing these mitochondria with a reticulocyte translational mixture containing freshly made precursor ornithine carbamyltransferase.

Fig. 6 shows that a factor, or factors, present in reticulocyte lysate is required for import of precursor ornithine carbamyltransferase by heart mitochondria in vitro. The experiment involved passing a reticulocyte translational mixture containing newly synthesized precursor ornithine carbamyltransferase through a Sephadex G-25 column and testing whether the excluded fraction, which contained the precursor, was still capable of supporting transport of the precursor into freshly prepared mitochondria. It was not (Fig. 6). The G-25 column

![Fig. 4 (left). Pretreatment of heart mitochondria with high concentrations of KCl does not inhibit subsequent import and processing of pOCT.](http://www.jbc.org/)

![Fig. 5 (right). Pretreatment of heart mitochondria with trypsin and chymotrypsin prevents subsequent import and processing of pOCT.](http://www.jbc.org/)
Uptake of Precursor Enzyme by Mitochondria

The uptake of precursor enzymes by mitochondria is illustrated in Fig. 6. A factor(s) present in reticulocyte lysate is required for import of precursor ornithine carbamyltransferase by heart mitochondria. Rat liver mRNA was translated in the reticulocyte system and, after 60 min, the mixture was passed through a Sephadex G-25 column equilibrated with 10 mM Hepes, pH 7.5 (ratio of lysate to bed volume = 1:10). Aliquots of the excluded (void) volume were adjusted to 0.25 M sucrose, and KCl, Mg acetate, and ATP were added either singly or in combination to give the same final concentrations as those present in standard import assays (see "Materials and Methods"). The aliquots were then mixed with suspended mitochondria, incubated for 60 min, and analyzed in the standard way (see "Materials and Methods"). A, lane d, 40 mM KCl, minus Mg, minus ATP; lane e, 40 mM KCl, 1.0 mM Mg, minus ATP; lane f, 40 mM KCl, 1.0 mM Mg, 0.5 mM ATP; for lanes g-i, conditions were the same as for lane f except the reaction volume was 300 µl instead of 200 µl and contained either 50 µl of fresh reticulocyte lysate (lane g), 50 µl of additional translation mixture minus lysate (lane h), or fresh lysate plus translation mixture (lane i); lane c, no additives to void volume, no postincubation with mitochondria. For comparisons, a standard processing assay was performed in this experiment using unfraccionated lysate products: incubation in the absence of mitochondria for 0 min (lane a) and in the presence of mitochondria for 60 min (lane b), B, following translation, reaction mixtures were either untreated (lanes a and b) or passed through a G-25 column (lanes c and d). Cycloheximide was added to both and further incubation was carried out at 30°C for 0 min (lanes a and c) or 60 min (lanes b and d). OCT, ornithine carbamyltransferase.

was equilibrated in 10 mM Hepes, pH 7.4, and would be expected to retain components with a molecular mass <5000 Da, including inorganic salts, nucleotides, and a range of other components (e.g. small proteins, small RNAs, free fatty acids, etc.). At least with respect to Mg<sup>2+</sup>, K<sup>+</sup>, and ATP, however, these standard ingredients did not represent the missing component, i.e. when Mg<sup>2+</sup>, K<sup>+</sup>, and ATP, either singly or in combination, were added to the column flow-through, import of precursor ornithine carbamyltransferase by heart mitochondria was not restored (Fig. 6A, lanes d-f). Nor could the failure to import be accounted for by instability of the precursor (Fig. 6F) or by alterations in pH or osmoticum or by deficiencies in mitochondrial respiration and oxidative phosphorylation, since these requirements are all provided by the mitochondrial suspension medium (see "Materials and Methods"). The factor, however, derives from the reticulocyte lysate itself rather than from other translation mixture additives (e.g. mixture, GTP, etc.) since addition of the former (Fig. 6A, lanes g and i) but not the latter (Fig. 6, lane h) completely restored the ability of precursor ornithine carbamyltransferase in column eluates to enter mitochondria.

CONCLUSIONS

It seems clear that mechanisms must exist in vivo to selectively channel precursors of mitochondrial proteins to their final destination because, once released from their site of synthesis on free 80 S ribosomes, the primary translation product enters mitochondria, and no other organelle. The details of these mechanisms, however, are not presently known. In the present communication, therefore, we report findings on the properties of a putative mitochondrial surface receptor(s) and a cytosolic factor(s), both of which appear to be required for some aspect of recognition and/or uptake of precursor ornithine carbamyltransferase by mitochondria in vitro. The evidence that a tightly bound (intrinsic like) protein, or proteins, exposed at the surface of mitochondria mediates transport of precursor into the organelle, is based on the fact that controlled and mild proteolysis of intact mitochondria completely abolished their import function (Fig. 5); the protein(s) is not liberated from the mitochondrial outer membrane by high concentrations (1 M) of KCl (Fig. 4). In addition, however, a soluble cytosolic factor appears to play some role as well, in so far as fractionation of a standard reticulocyte lysate translational mixture (i.e. a "cytosolic" fraction) by Sephadex G-25 chromatography rendered precursor ornithine carbamyltransferase incapable of entering mitochondria. The factor(s) is presumably retained by the column either because it is relatively small (<5000 Da) or because of nonspecific (e.g. hydrophobic, ionic) interactions with Sephadex beads. Whether the factor(s) functions by acting on mitochondria, on the precursor, or on both remains to be determined.

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