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THE PRECURSOR TO ORNITHINE CARBAMYL TRANSFERASE IS TRANSPORTED TO MITOCHONDRIA AS A 5S COMPLEX CONTAINING AN IMPORT FACTOR

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The precursor to ornithine carbamyl transferase (Mr = 40,000) was synthesized in a rabbit reticulocyte lysate system and purified by immunoaffinity chromatography. Import of purified precursor by isolated mitochondria depended upon the presence of import factor(s) in fresh reticulocyte lysate. Velocity sedimentation analyses indicated that import factor binds to precursor to form a 5S complex (~ 90 kDa); in this form, precursor was efficiently imported by isolated mitochondria. The ability of the 5S complex to deliver precursor into mitochondria was not affected by pretreatment with high concentrations of RNase. Import factor did not bind to mitochondria in the absence of precursor; upon binding of precursor to mitochondria in the presence of import factor, subsequent transmembrane uptake of precursor did not require the continued presence of additional lysate components.

Whether or not a soluble recognition element, analogous to the signal recognition particle (SRP) of the ER[1] system (1), functions during targeting of precursor proteins to mitochondria remains an open question. Three reports (2-4) have demonstrated that a soluble cytosolic component(s), with properties typical of a protein, is required for precursor import and processing in vitro, and more recently it has been suggested that, in addition to protein factor, RNA is required for import (5). Whether the import factor functions as a mitochondrial recognition element or perhaps as a helper protein required for translocating precursor proteins across mitochondrial membranes, is not presently known. In the present study, we extend our earlier findings (2) on the requirement for cytosolic components from reticulocyte lysate during in vitro import of the

[1]Abbreviations: ER, endoplasmic reticulum; OCT, ornithine carbamyl transferase; pOCT, precursor to OCT; 37K, a 37-kDa fragment of pOCT; EDTA, ethylenediamine tetraacetate; PMSF, phenylmethylsulfonylfluoride; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone; CCCP, carbonylcyanide 3-chlorophenylhydrazone; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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precursor (6-9) to the rat liver mitochondrial matrix enzyme, ornithine carbamyl transferase (OCT, Mr = 36,000, EC 2.1.3.3). Here, we provide evidence that import factor present in reticulocyte lysate associates with the precursor to the enzyme (pOCT, Mr = 40,000) to form a 5S complex and show that the functional complex does not contain an RNA moiety requisite for precursor import. Evidence is also provided that import factor does not bind to mitochondria in the absence of precursor; upon binding of pOCT to mitochondria in the presence of reticulocyte lysate, however, subsequent import of pOCT no longer requires the continued presence of lysate.

MATERIALS AND METHODS

General. For most of the routine procedures used in this study, the methods followed have been outlined elsewhere (2,9 and references cited therein). These include protein measurements, extraction of mRNA, protein synthesis in a messenger-dependent cell-free system derived from rabbit reticulocytes and containing [^{35}S] methionine (~ 1000 Ci/mmol, NEN Corp.), isolation of rat heart mitochondria, immunoprecipitation, and SDS-PAGE and fluorography of dried gels.

Purification of In Vitro Synthesized pOCT. Rat liver mRNA was translated in the reticulocyte system at 30° for 60 min; the mixture was then centrifuged at 175,000xg for 45 min to remove ribosomes. Aliquots (500 µl) of the translation mixture were diluted with an equal volume of phosphate-buffered saline, 40 mM methionine, 20 mM EDTA, and 0.04% (w/v) NaN_3; NaCl was then added to give a final concentration of 1.4 M and PMSF, TPCK and TLCK were added to give a final concentration each of 0.25 mM. This was followed by the addition of 350 µg anti-OCT IgG covalently coupled to Protein A Sepharose (7 mg IgG/ml swollen Sepharose). The mixture was rotated at 22° for 2 h and then centrifuged at 12,000xg for 5 seconds. The pellet was washed ten times with 1 ml of medium containing 1.0 M NaCl, 20 mM methionine, 10 mM EDTA, 0.02% NaN_3, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 100 units/ml aprotinin, 0.1 mM each of TPCK, PMSF and TLCK, 0.02 mM each of leupeptin and chymostatin, and phosphate-buffered saline. This was followed by five 1 ml washes with the same medium minus NaCl. The final pellet was incubated for 2 minutes with 500 µl of 5 mM Tris, pH 7.5, 80 mM lithium 3,5-diiodosalicylate (LIS), 2 mg/ml bovine serum albumin, and centrifuged at 12,000xg for 5 seconds. The supernatant, containing pOCT, was removed and dialyzed for 2 h against 500 ml of 5 mM Tris, pH 7.5, 40 mM LiCl. This was repeated twice with fresh buffer. Dialysis was then continued for 18 h against two changes of 500 ml of 20 mM Hepes, pH 7.5, 80 mM KCl, and 1 mM Mg acetate.

Mitochondrial Import Incubations. Rat heart mitochondria were purified and employed for in vitro import of pOCT as previously described (2,9). Standard import incubations contained either 25 µl of liver mRNA-directed reticulocyte lysate translation products (in 75 µl total) or 25 µl of purified pOCT (in 150 µl total), plus the following relevant constituents: 0.5 mg/ml mitochondrial protein, 0.25 M sucrose, 40 mM KCl, 1.0 mM Mg acetate, 0.5 mM ATP, 10 µg/ml cycloheximide, 5 mM Hepes, pH 7.5, 0.25 mM dithiothreitol, 2.5 mM Na succinate, 0.04 mM ADP, and 1.0 mM K_2HPO_4, pH 7.5. Import was performed at 30° for 60 minutes.

Sucrose Density Gradient Centrifugation. Samples (0.5 ml) were layered on 5-30% linear sucrose gradients (13 ml) containing 10 mM Hepes, pH 7.5, 40 mM KCl, and 1 mM Mg acetate and centrifuged in a Beckman SW40 rotor at 180,000xg for 23 h at 4°. Fractions (0.8 ml) were collected using an ISCO (model 640) gradient fractionator and the sucrose concentration in each fraction was determined using

a Bausch and Lomb refractometer. When gradient fractions were utilized for in vitro import assays, the final concentration of relevant constituents was the same as for standard import incubations (see above) except that mitochondria were added at a final concentration of 0.25 mg protein/ml.

RESULTS

As demonstrated previously (2,3), reticulocyte lysates employed to synthesize pOCT in vitro contain a factor(s) which is required for subsequent post-translational import of pOCT by isolated mitochondria. The cytosolic import factor(s) shows properties typical of a protein, e.g., import does not occur if reticulocyte lysate is treated with protease or is heated at 90° for 4 min. (ref. 3 and unpublished results).

In Vitro Import of Purified pOCT. Following synthesis in a rabbit reticulocyte cell-free system, pOCT was purified by immunoaffinity chromatography, in which exhaustive washing was carried out to free precursor from endogenous reticulocyte lysate components; essentially all lysate protein and greater than 85% of non-pOCT translation products were removed by this procedure. This highly enriched preparation of pOCT was not imported and processed by isolated mitochondria in vitro (Fig. 1, Panel B); the conditions employed were identical to those employed for standard import assays (Fig. 1, Panel A), except that lysate was absent from the import incubation. During standard import and conversion of pOCT (Mr = 40,000) to mature enzyme (Mr = 36,000), a minor 37-kDa product was also formed (see Fig. 1, Panel A and refs. 6-9), but earlier experiments (2) suggested that this latter product arises non-specifically as a result of limited damage to mitochondria in the presence of reticulocyte lysate.

When purified pOCT was incubated with mitochondria, but in the presence of reticulocyte lysate, it was imported and processed (Fig. 2) in a manner similar to pOCT which had not undergone prior purification (cf. Fig. 1). Import was abolished by treatment with CCCP (Fig. 2, lane f) and mature OCT co-sedimented with mitochondria and was resistant to external protease treatment (not shown).

Association between Import Factor and pOCT. To determine if reticulocyte lysate import factor associates physically with pOCT prior to interaction of the precursor with the surface of mitochondria, velocity sedimentation studies were

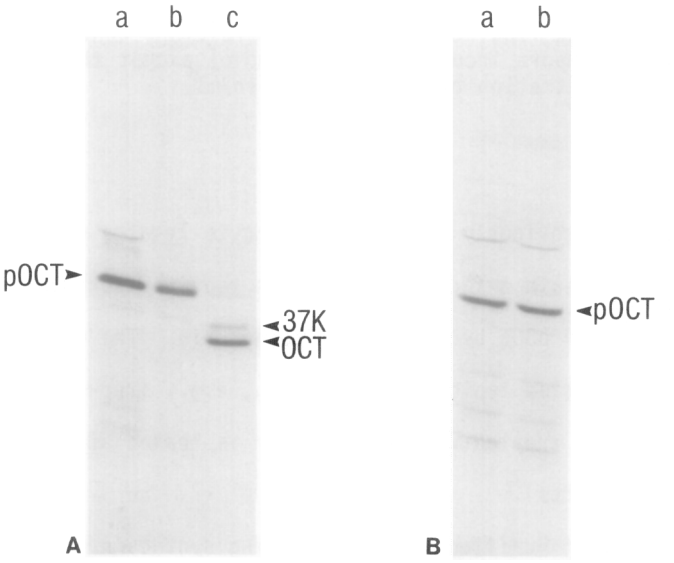

Figure 1. Purified pOCT is not imported and processed by rat heart mitochondria. Panel A. Rat liver mRNA was translated in a rabbit reticulocyte lysate system containing [^{35}S]methionine; lysate products (25 µl) were added to a standard mitochondrial import assay mixture (total volume = 75 µl) containing rat heart mitochondria (see Materials and Methods) and incubated for 0 (lane b) or 60 (lane c) minutes at 30°. The import mixture was then subjected to immunoprecipitation with mono-specific anti-OCT antibody and radioactive products were visualized following SDS-PAGE and fluorography. Lane a, input pOCT (not incubated with mitochondria). Panel B. pOCT was purified from total liver mRNA-directed reticulocyte lysate translation products; aliquots containing 20,000 cpm (25 µl) were either not incubated with mitochondria (lane a) or were incubated with mitochondria for 60 min at 30° (total volume = 150 µl). Radioactive products were precipitated in ice-cold 10% TCA and analyzed by SDS-PAGE and fluorography. The positions of pOCT, 37K product, and mature OCT are indicated.

performed (Fig. 3). Centrifugation was performed under identical ionic conditions to those employed for *in vitro* import assays. The sedimentation profile of pOCT in gradient 1 showed that about 75% of the precursor sediments between 4S and 6S, peaking at 5S (Fig. 3, Panel A). When the 5S fraction containing pOCT from gradients 1 and 2 was added directly to mitochondria, import and processing of pOCT was observed (Fig. 3, Panel B, lanes a, c). Moreover, addition of fresh lysate to these incubations only slightly improved import (Fig. 3, Panel B, lanes b, d). In contrast, when the corresponding fraction from gradient 3 (lysate minus pOCT) was assayed for its ability to support import of purified pOCT, import did not occur (Fig. 3, Panel B, lanes e, f). This indicates that import factor must be associated with pOCT in gradients 1 and 2, rather than simply co-sedimenting with the precursor.

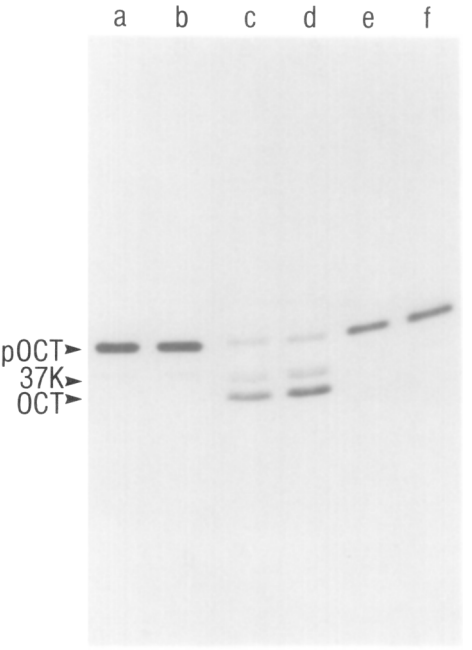

Figure 2. Reticulocyte lysate is required for in vitro import of purified pOCT by heart mitochondria. Purified [^{35}S]pOCT (approx. 20,000 cpm) was incubated with mitochondria at 30° for 0 (lane b) or 60 (lanes c-f) min, in a total volume of 150 µl. Import incubations contained the following additions: 35 µl reticulocyte lysate (lane c), 70 µl reticulocyte lysate (lane d), standard concentrations of complete in vitro translation cocktail components minus lysate (lane e), or 70 µl reticulocyte lysate plus 1 µM CCCP (lane f). Following import incubations, the total mixtures were subjected to immunoprecipitation with anti-OCT antibody and analyzed by SDS-PAGE and fluorography. Lane a, input purified pOCT (not incubated with mitochondria). The positions of pOCT, 37K product, and mature OCT are indicated.

Pretreatment of 5S Precursor Complex with RNase Does Not Prevent Import of pOCT. In view of a recent report that import of pOCT was prevented by pretreatment of reticulocyte lysate with high concentrations of RNase A at 37° (5), similar tests were carried out with the 5S precursor complex recovered from a sucrose density gradient. RNase treatment of the 5S complex did not inhibit subsequent import (Fig. 4, Panel A, lanes b, c), suggesting that a RNase-sensitive component is not a constituent part of the 5S complex nor is RNA required for delivery of pOCT to mitochondria per se. Pretreatment of reticulocyte lysate with RNase at 37°, however, diminished the ability of the lysate to support import of exogenously-added, purified pOCT (Fig. 4, Panel B, lanes c,d).

Import Factor does not Bind to Mitochondria in the Absence of pOCT. Fig. 5 shows results of an experiment in which lysate, either alone or containing

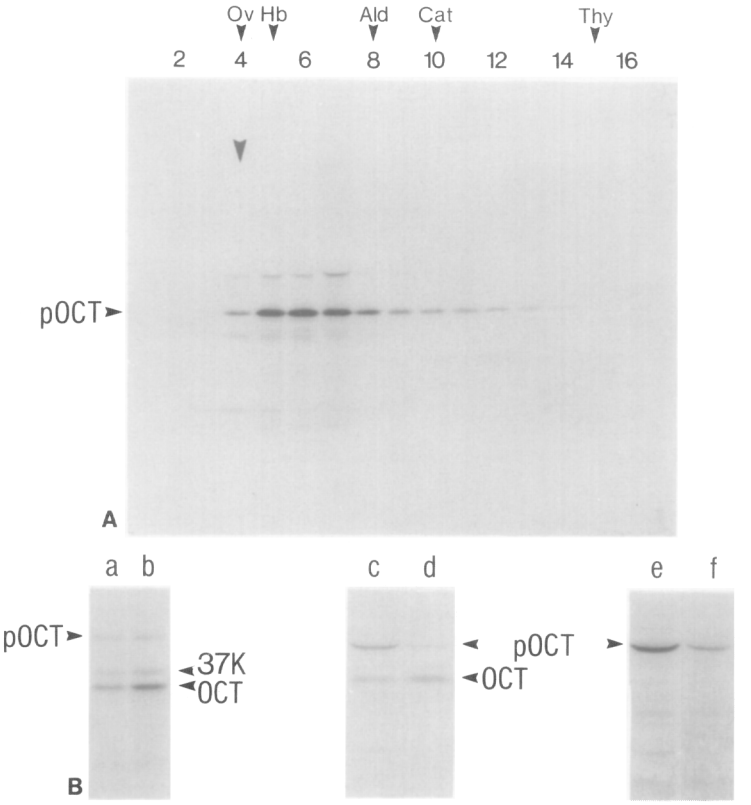

Figure 3. Cytosolic import factor from reticulocyte lysate interacts with pOCT. The following samples were prepared for velocity sedimentation analysis in sucrose density gradients: gradient 1, 500 μl of liver mRNA-directed total translation products, pre-centrifuged to remove ribosomes and containing 250 μl reticulocyte lysate; gradient 2, 500 μl containing purified [^{35}S]pOCT (approx. 200,000 cpm), 250 μl post-ribosomal reticulocyte lysate, 10 mM Hepes, pH 7.5, 40 mM KCl, and 1.0 mM Mg acetate; the mixture was incubated at 30° for 30 minutes prior to loading on the gradient; gradient 3, as in gradient 2 except that pOCT was omitted (lysate alone). The samples were sedimented in 5-30% sucrose density gradients as described in Materials and Methods. Additional gradients contained the following markers: thyroglobulin (Thy, 19.3S), catalase (Cat, 11.3S), aldolase (Ald, 7.4S), hemoglobin (Hb, 4.2S), and ovalbumin (Ov, 3.6S). The position at which monomeric pOCT sedimented in these gradients is indicated by the downward pointing arrow at fraction #4 in Panel A. PANEL A. Equal aliquots (200 μl) of each fraction from gradient 1 were immunoprecipitated with anti-OCT antibody; precipitates were analyzed by SDS-PAGE and fluorography. PANEL B. Fraction #6 from all three gradients was analyzed either for import of constituent pOCT in this fraction (gradients 1 and 2) or for the ability of fraction #6 to support import of exogenous purified pOCT (gradient 3). Import incubations (600 μl) each contained 200 μl from fraction 6 of the individual gradients and was carried out for 60 minutes at 30° at which time immunoprecipitation with anti-OCT antibody was performed (lanes a and b) or mitochondria were isolated by centrifugation and analyzed directly by SDS-PAGE (lanes c-f). Lane a, import employing fraction #6 from gradient 1 (containing liver mRNA-directed reticulocyte lysate translation products); lane b, as in lane a except the import incubation was supplemented with 240 μl fresh reticulocyte lysate. Lanes c and d, as in lanes a and b, respectively, except that fraction #6 from gradient 2 (containing purified [^{35}S]pOCT plus lysate) was employed for import. Lanes e and f, 25 μl of purified [^{35}S]pOCT was incubated with mitochondria in the absence (lane e) or presence (lane f) of 200 μl from fraction #6 of gradient 3 (containing lysate alone); the ratio of input purified pOCT to lysate protein in fraction #6 of gradient 3 was the same as the pOCT: lysate protein ratio in fraction #6 of gradient 2.

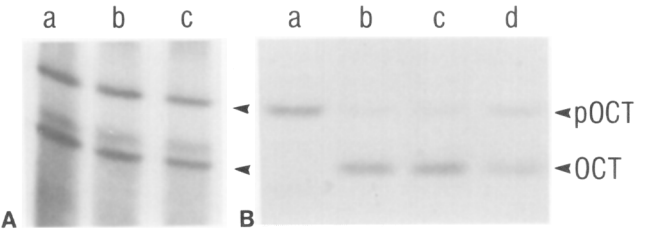

Figure 4. RNase treatment of 5S pOCT complex isolated from a sucrose gradient does not inhibit subsequent import and processing of pOCT. PANEL A. Lane a, 100 µl of fraction #6 from gradient 1 (Fig. 4) was incubated at 37° for 10 min, then incubated with mitochondria (total volume = 300 µl) at 30° for 60 min; lane b, as in lane a except that fraction #6 was preincubated with 80,000 units RNase A/ml at 37° for 10 min prior to import incubation. Total import assay mixtures were subjected to immunoprecipitation with anti-OCT prior to analysis by SDS-PAGE and fluorography. PANEL B. Lane a, purified [^{35}S]pOCT (20,000 cpm) (not incubated with mitochondria); lane b, purified [^{35}S]pOCT incubated at 30° for 60 min with mitochondria and reticulocyte lysate (50 µl) in a total volume of 150 µl; lane c, as in lane b except that lysate was preincubated at 37° for 10 min prior to import incubation; lane d, as in lane b except that lysate was preincubated with 80,000 units RNase A/ml at 37° for 10 min prior to import incubation. Mitochondrial pellets were obtained from import incubations (lanes b-d), dissolved in SDS sample buffer, and analyzed directly by SDS-PAGE fluorography.

exogenous purified pOCT, was incubated with mitochondria at 4° for 30 min, at which time the mitochondria were recovered by centrifugation. At 4°, about 50% of newly-synthesized pOCT binds to mitochondria and is recovered with the organelle following centrifugation; none, however, is imported (results not shown). Following a temperature shift to 30°, mitochondria which had been preincubated at 4° with reticulocyte lysate alone were incapable of subsequently importing purified pOCT at 30° (Fig. 5, Panel A, lane b). In contrast, pOCT which had been pre-bound to mitochondria at 4° in the presence of lysate was subsequently imported and processed to mature enzyme in the absence of lysate (Fig. 5, Panel B, lane a), and import was not further stimulated by the addition of fresh lysate to the 30° import incubation (Fig. 5, Panel B, lane b).

DISCUSSION

Velocity sedimentation analysis indicates that pOCT which has been newly synthesized in a rabbit reticulocyte lysate system migrates as a functional 5S complex (~ 90 kDa) whose constituent precursor is imported and processed when added to mitochondria in vitro. Assuming that the import complex contains a

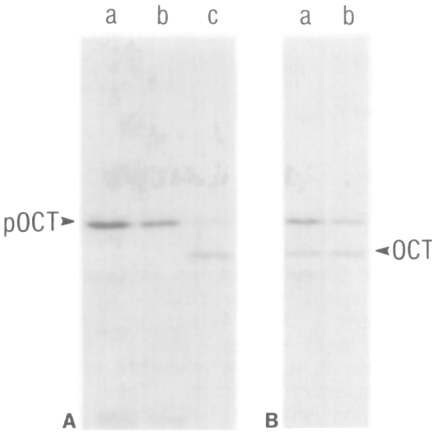

Figure 5. Import factor does not bind to mitochondria in the absence of pOCT. PANEL A. Lane b, mitochondria were preincubated with 70 μl of reticulocyte lysate (total volume = 150 μl) at 4° for 30 min in a reaction mixture which contained the same constituents as standard in vitro import assays (see Materials and Methods) except that [^{35}S]pOCT was not present. The mitochondria were recovered by centrifugation and incubated with [^{35}S]pOCT (20,000 cpm) at 30° for 60 min in a standard import reaction mixture minus reticulocyte lysate (total volume = 150 μl). Lanes a and c, mitochondria were preincubated at 4° for 30 min as in lane b except that reticulocyte lysate was not present. The mitochondria were recovered by centrifugation; the subsequent import incubation with [^{35}S]pOCT at 30° for 60 min contained either no reticulocyte lysate (lane a) or 70 μl of reticulocyte (lane c). PANEL B. lane a, purified [^{35}S]pOCT (20,000 cpm) was preincubated with mitochondria (total volume = 150 μl) in the presence of 70 μl of reticulocyte lysate at 4° for 30 min in a reaction mixture which contained the same constituents as standard in vitro import assays. Mitochondria (containing bound [^{35}S]pOCT) were recovered by centrifugation and incubated at 30° for 60 min in a standard import reaction mixture (total volume = 150 μl) minus reticulocyte lysate. Lane b, as in lane a except that import at 30° was performed in the presence of 70 μl of reticulocyte lysate. Total products were analyzed by SDS-PAGE and fluorography.

single molecule of pOCT with a monomeric molecular size of 40 kDa, cytosolic import factor presumably constitutes the remaining 50 kDa, an estimate which is very similar to that proposed for cytosolic import factor in yeast (4). Certainly, the size of the pOCT import complex precludes inclusion of any known cytoplasmic RNA as a constituent component; indeed, treatment of the 5S pOCT complex with high levels of RNase A does not inhibit subsequent import of pOCT by mitochondria in vitro (Fig. 4), indicating that RNA is not required for import of pOCT per se. Nevertheless, it is clear that RNase digestion of whole reticulocyte lysate reduces the ability of lysate to support import and processing of exogenously-added pOCT (Fig. 4, see also ref. 5). If a specific RNA species is playing some bona fide role, it must be functioning transiently at a very early

step in the import pathway, e.g., it may mediate the initial interaction between import factor and precursor prior to delivery of pOCT to mitochondria.

In contrast to results presented in this communication, Firgaira et al. (5) have recently estimated the size of a cytosolic pOCT complex in a reticulocyte lysate translation mixture to be quite large (~ 400 kDa). The complex was obtained and its size determined by molecular sieve chromatography. However, earlier reports (2,4) have demonstrated that a cytosolic component from reticulocyte lysate, requisite for import, is removed by Sephadex G-25 even in the presence of mitochondrial precursor polypeptides. The 400 kDa complex observed by Firgaira et al. (5) did not support import of its constituent pOCT in vitro whereas the 5S (~ 90 kDa) complex described here did; failure to import, together with the large aggregate size observed in ref. 5, presumably resulted from removal of essential small molecular weight components during chromatography.

Whether or not import factor is a requisite mitochondrial recognition element, functioning to mediate delivery of precursor to import receptor on the surface of the organelle, is not presently known; it may be, for example, that import factor bound to precursor functions only after the precursor makes contact with a mitochondrion, e.g., to help mediate insertion of the precursor polypeptide across the outer mitochondrial membrane. It appears, however, that import factor does not act by binding to mitochondria independent of precursor; moreover, once pOCT (presumably in association with import factor) binds to mitochondria, the continued presence of other components in reticulocyte lysate is not required to further support transmembrane uptake of the precursor (Fig. 5).

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Results of studies on the efficacy of Narcan® in reversing ethanol intoxication in humans and rodents are conflicting. Such data and their interpretation deserve reevaluation since possible antagonistic effects of naloxone on the consequences of ethanol oxidation may be obscured by the inhibition of ADH, which would potentiate intoxication.

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