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The Requirement of Heat Shock Cognate 70 Protein for Mitochondrial Import Varies among Precursor Proteins and Depends on Precursor Length

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The cytosolic heat shock cognate 70-kDa protein (hsc70) is required for efficient import of ornithine transcarbamylase precursor (pOTC) into rat liver mitochondria (K. Terada, K. Ohtsuka, N. Imamoto, Y. Yoneda, and M. Mori, Mol. Cell. Biol. 15:3708–3713, 1995). The requirement of hsc70 for mitochondrial import of various precursor proteins and truncated pOTCs was studied by using an in vitro translation import system in which hsc70 was completely depleted. hsc70-dependent import of pOTC was about 60% of the total import, while import of the aspartate aminotransferase precursor, the serine:pyruvate aminotransferase precursor, and 3-oxoacyl coenzyme A thiolase was about 50, 30, and 0%, respectively. The subunit sizes of these four precursor proteins were 40 to 47 kDa. When pOTC was serially truncated from the COOH terminal, the hsc70 requirement decreased gradually and was not evident for the shortest truncated pOTCs of 90 and 72 residues. These truncated pOTCs were imported and proteolytically processed rapidly in 0.5 to 2 min at 25°C, and the processed mature portions and the presequence portion were rapidly degraded. Sucrose gradient centrifugation analysis followed by import assay showed that pOTC synthesized in rabbit reticulocyte lysate forms an import-competent complex of about 11S in an hsc70-dependent manner. S values of import-competent forms of aspartate aminotransferase precursor, serine:pyruvate aminotransferase precursor, and 3-oxoacyl coenzyme A thiolase were 9S, 9S, and 4S, respectively. Thus, the S value decreased as the hsc70 dependency decreased. Precursor proteins were coimmunoprecipitated from the reticulocyte lysate containing the newly synthesized precursor proteins with an hsc70 antibody. The amount of coimmunoprecipitated proteins was much larger in the absence of ATP than in its presence. Among the four precursor proteins, the amount of coimmunoprecipitated protein decreased as the hsc70 dependency decreased.

Most mitochondrial proteins are synthesized on cytosolic free ribosomes as larger precursors with NH_2-terminal presequences, released into the cytosolic pool, and then imported into mitochondria. The presequence portions of precursor proteins are proteolytically cleaved in the mitochondrial matrix, and the mature portions are folded and assembled into final conformations. Prior to import, the precursor proteins must be maintained in an unfolded conformation in the cytosol (34).

Members of the hsp70 family and other factors in the cytosol are required for mitochondrial protein import (18, 35). Presequence-specific cytosolic factors have been identified as candidates for the latter factors (10, 20). However, the role of the hsp70 family in mitochondrial protein import is not well understood. Generally, cytosolic hsp70s (hsp70 and hsc70 in higher animals) transiently interact with newly synthesized polypeptides emerging from ribosomes (3) and may prevent them from aggregation or misfolding. Deshaies et al. (5) noted that the precursor of ATPase β subunit accumulated in yeast cells depleted of cytosolic hsp70s. Since urea denaturation of some hybrid precursor proteins markedly stimulates mitochondrial import, cytosolic hsp70s were conceptually regarded as an "unfoldase" that works posttranslationally. However, this does not seem to be the case. Studies in our laboratory (33) and elsewhere (14) revealed that the cotranslational action of hsc70 is more likely to be an event in the formation of import-competent forms of precursor proteins.

We developed an in vitro translation-mitochondrial import system in which cytosolic hsc70 was completely depleted from the rabbit reticulocyte lysate (33). In this system, import of ornithine transcarbamylase precursor (pOTC) was markedly reduced by hsc70 depletion, and this reduction was almost completely overcome when purified hsc70 was added back during translation but not during import. Thus, hsc70 seems to associate with the nascent pOTC polypeptide chain during translation and to maintain it in an import-competent form.

We report here that the hsc70 requirement varies among the four precursor proteins tested and is not evident for one of them. The hsc70 requirement decreases gradually as pOTC is serially truncated from the COOH terminal. A relationship between hsc70 dependency and the size of import-competent forms of the precursor proteins seems apparent. Coimmunoprecipitation of the precursor proteins with hsc70 is also reported.

MATERIALS AND METHODS

Materials. Nuclease-treated rabbit reticulocyte lysate was purchased from Promega. [^{35}S]Pro-mix (>37 TBq of [^{35}S]methionine per mmol) was purchased from Amersham. Murine hsc70 was purified from Ehrlich ascites fluid by ATP-agarose column chromatography and Superdex gel filtration column chromatography. mRNAs for rat pOTC, pig aspartate aminotransferase precursor (pAAT), rat serine:pyruvate aminotransferase precursor (pSPT), and rat 3-oxoacyl coenzyme A (CoA) thiolase were synthesized by in vitro transcription of the recom-

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binant plasmids pSPT18/pOTC (19), pSP65/pmAAT (23), pAS321 (24), and pSPT19/T1 (1), respectively. For preparation of truncated pOTC mRNAs, pSPT18/pOTC was digested with appropriate restriction enzymes at truncating positions in the coding region of pOTC. The resultant templates were transcribed with a MEGAscript kit (Ambion Inc.). Antibodies to rat 3-oxoacyl-CoA thiolase (16), bovine OTC (1), and rat SPT (25) were those described previously. Antibody-conjugated Sepharose was prepared by coupling an hsc70-specific monoclonal antibody (1B5) or purified rat immunoglobulin G (Chemicon International Inc., Temecula, Calif.) to cyanogen bromide-activated Sepharose 4FF (Pharmacia Biotechnology) at 4.0 mg/ml as specified by the manufacturer.

Depletion of hsc70 from rabbit reticulocyte lysate and in vitro translation. hsc70-depleted rabbit reticulocyte lysate was prepared by treatment with 1B5 antibody-conjugated Sepharose resin, as described previously (33). Mitochondrial precursor proteins and truncated pOTCs were translated in vitro in the presence of [^{35}S]methionine for 60 min at 25°C. After translation, truncated pOTCs were released from ribosomes by adding 1 mM puromycin. The protein-synthetic activity of the reticulocyte lysate was decreased by 20 to 70% by the procedure of hsc70 depletion. This reduction can probably be attributed to irreversible binding of ribosomes to the Sepharose matrix (12). For the hsc70 readdition experiment, purified mouse hsc70 was added to the hsc70-depleted lysate at 150 μg/ml prior to translation.

Import of in vitro-synthesized precursor proteins into isolated mitochondria. The import mixture (50 μl) containing 3.0 to 5.0 μl of the reticulocyte lysate containing ^{35}S-labeled proteins was incubated with isolated rat liver mitochondria (100 μg of protein) at 25°C for the indicated time. The reaction was stopped by adding 1.0 ml of ice-cold mitochondrial isolation buffer (33) containing 0.1 mM dinitrophenol, and the mitochondria were reisolated by centrifugation. The pelleted mitochondria were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (13) or Tricine-SDS-polyacrylamide gel electrophoresis (30). The radioactive polypeptides were visualized and quantitated by imaging plate analysis with FUJIX BAS2000 (Fuji Photo Film Co.).

Sucrose gradient centrifugation of in vitro-synthesized precursor proteins and import of fractionated precursor proteins into isolated mitochondria. ^{35}S-labeled pOTC, pAAT, pSPT, and 3-oxoacyl-CoA thiolase, synthesized in a rabbit reticulocyte lysate system (0.2 ml), were layered on linear 5 to 20% sucrose gradients (4.8 ml) containing 50 mM *N*-2-hydroxymethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.4), 2 mM ATP, 5 mM magnesium acetate, 50 μM antipain, and 50 μM leupeptin. Centrifugation was performed for 3.8 h at 4°C at 50,000 rpm in a Hitachi 55P ultracentrifuge with an RPS55T rotor. Marker proteins and mitochondrial matrix fraction (1.0 mg of protein) were centrifuged in the same experiments. Fractions were collected through the bottom of the tubes, and 100-μl portions were incubated with 100 μl of 2× import mixture (240 mM potassium acetate, 4.4 mM dithiothreitol, 10 mM NADH, 50 μg of creatine kinase [Sigma], 10 μg of bovine serum albumin, rat liver mitochondria [300 μg of protein]) at 25°C for 12 min. The import reaction was terminated by adding ice-cold mitochondrial isolation buffer containing dinitrophenol, and the mitochondria were reisolated by centrifugation.

Other methods. The mitochondrial matrix fraction was prepared from isolated rat liver mitochondria by sonication followed by centrifugation at 100,000 × *g* for 30 min. AAT activity was measured with a GOT-UV assay kit (Wako Pure Chemical Industries, Osaka, Japan). OTC, SPT, and 3-oxoacyl-CoA thiolase were detected by immunoblot analysis with enhanced chemiluminescence kits (Amersham).

RESULTS

Requirement of hsc70 for import of various precursor proteins into mitochondria. In previous studies, we found that the import of pOTC into isolated rat liver mitochondria was reduced by 67 to 75% when hsc70 was depleted from the reticulocyte lysate prior to translation (33). We then asked if hsc70 is also required for mitochondrial import of other precursor proteins. Rat pOTC, pig pAAT, rat pSPT, and rat 3-oxoacyl-CoA thiolase, which are destined for the mitochondrial matrix, were synthesized in untreated or hsc70-depleted reticulocyte lysates and subjected to import assay. Essentially complete depletion of hsc70 was confirmed by immunoblot analysis, as described previously (33).

When pOTC was synthesized in the hsc70-depleted lysate, pOTC import into the mitochondria was reduced by about 66% (Fig. 1A). This reduction was almost completely recovered when purified hsc70 was added back to the depleted lysate prior to translation. These results are in accord with our previous data. When pAAT import was assayed in the same set of experiments, it was reduced by about 50% by hsc70 depletion and the reduction was almost completely recovered by hsc70

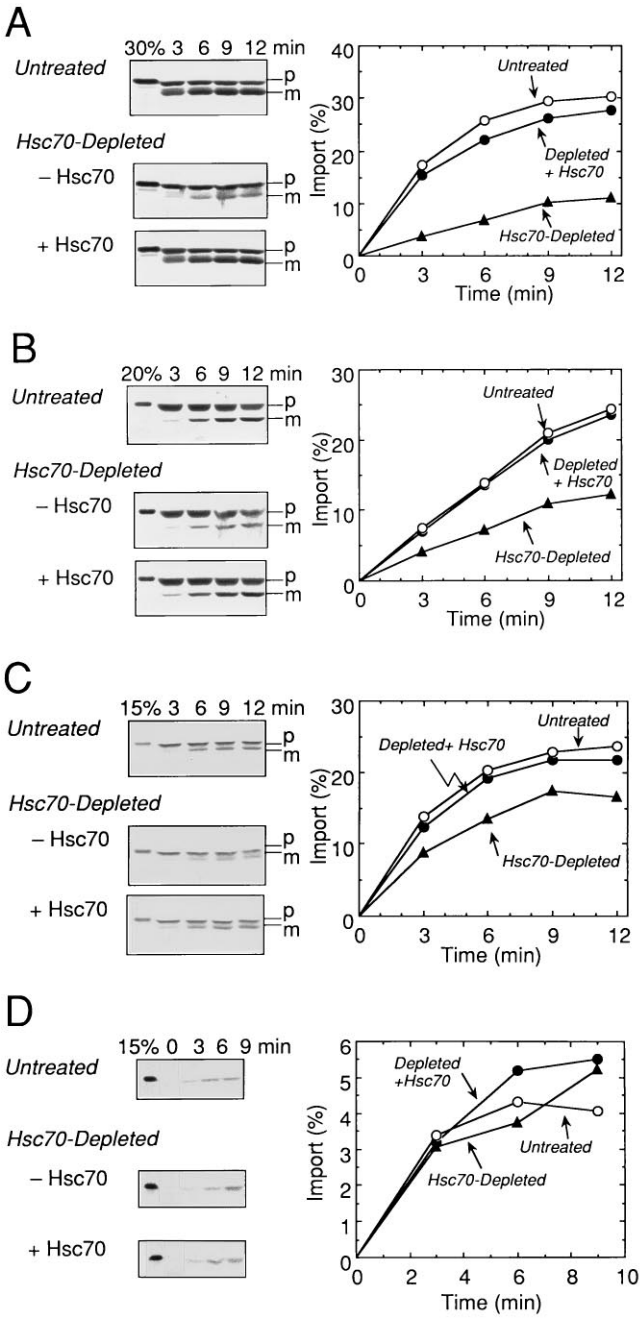

FIG. 1. Effect of hsc70 depletion and readdition on the import of various mitochondrial proteins. Rat pOTC (A), pig pAAT (B), rat pSPT (C), and rat 3-oxoacyl-CoA thiolase (D) were synthesized in the untreated or the hsc70-depleted rabbit reticulocyte lysate without (−Hsc70) or with (+Hsc70) readdition of 10 μg of purified mouse hsc70. The import mixtures (50 μl) containing 5 μl of the lysate containing ^{35}S-labeled proteins were incubated with isolated rat liver mitochondria (100 μg of protein) at 25°C for the indicated time. The radioactivities of ^{35}S-labeled proteins used for each import mixture were 7.8×10^5 to 10.0×10^5 dpm (A), 4.7×10^5 to 12.0×10^5 dpm (B), 1.8×10^5 to 5.0×10^5 dpm (C), and 0.5×10^5 to 1.9×10^5 dpm (D). The import was halted by diluting the import mixture into 1.0 ml of ice-cold mitochondrion isolation buffer containing 0.1 mM dinitrophenol. For 3-oxoacyl-CoA thiolase (D), the diluted import mixture was further treated with 40 μg of proteinase K per ml for 20 min on ice, and digestion was terminated with 1 mM phenylmethylsulfonyl fluoride. The mitochondria were reisolated and subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide). The radioactive bands were visualized and quantitated by imaging-plate analysis with FUJIX BAS2000. p, precursor form; m, mature form; 30%, 20%, and 15% in lanes 1, 30, 20, and 15% of the input ^{35}S-labeled protein, respectively.

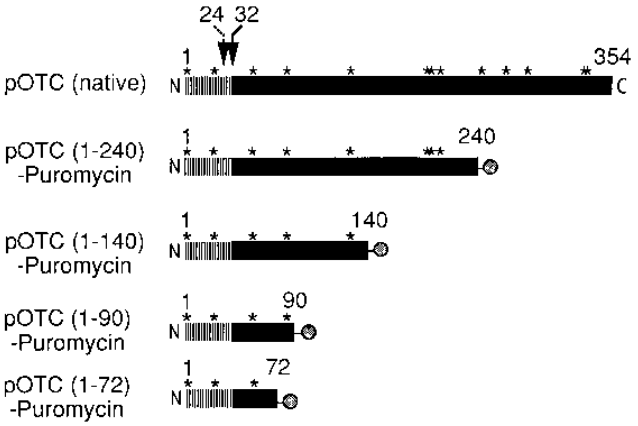

FIG. 2. Structures of truncated pOTCs. A rat pOTC plasmid for in vitro transcription, pSPT18/pOTC, was digested with appropriate restriction enzymes which cut at the truncating positions in the coding region of pOTC. Resultant templates were transcribed in vitro with SP6 RNA polymerase. About 5 μg of in vitro-synthesized mRNA was translated in the reticulocyte lysate to generate the corresponding truncated pOTC. After translation, truncated pOTCs were released from ribosomes with 1 mM puromycin. Puromycin is incorporated into the COOH terminal of the truncated polypeptides and is indicated as a shaded circle. The presequence portion (Met-1 to Gln-32) is indicated by a shaded bar, and the mature portion is indicated by a solid bar. Asterisks indicate positions of methionine residues. Residue 24 is the putative processing site to generate the intermediate form (31).

readdition (Fig. 1B). Import of pSPT was reduced by about 30% by hsc70 depletion under practically identical conditions (Fig. 1C). On the other hand, import of 3-oxoacyl-CoA thiolase, which has no cleavable presequence (1), was apparently not reduced by the hsc70 depletion (Fig. 1D). Thus, the hsc70 requirement seems to vary from one precursor to another, even though the tested precursor proteins have similar sizes of subunits (40 to 47 kDa). This is in accord with the proposal that hsc70 prevents unproductive folding (such as aggregation or misfolding) of nascent polypeptides and stabilizes unfolded proteins (9). The precursor proteins are likely to have different tendencies to aggregate or misfold.

Requirement of hsc70 for import of truncated pOTCs. We next asked whether the hsc70 requirement varies depending on the length of the precursors. Since pOTC showed the highest dependence on hsc70, we examined the import of a series of truncated pOTCs (Fig. 2). The truncated pOTCs were generated from the corresponding truncated mRNAs, which had been prepared from template DNA digested at appropriate truncating positions in the coding region of pOTC. Synthesis was carried out for 60 min in the untreated reticulocyte lysate or in the hsc70-depleted lysate, and then the truncated pOTCs were released from ribosomes with puromycin.

Import of full-length pOTC into the mitochondria was reduced by 67% by depletion of hsc70 from the reticulocyte lysate but recovered after the hsc70 readdition (Fig. 3A). The addition of 1 mM puromycin after translation had no apparent effect on import of full-length pOTC. When pOTC(1–240)-puromycin was imported into mitochondria, the 27-kDa precursor form was processed to the 24-kDa mature form (Fig. 3B). The sizes of these forms were close to the expected ones. Import of pOTC(1–240)-puromycin was reduced by about 33% by hsc70 depletion but recovered almost completely when hsc70 was readded prior to translation.

Import of pOTC(1–140)-puromycin was analyzed by Tricine-SDS-polyacrylamide gel electrophoresis (Fig. 3C). When this truncated precursor of 16 kDa synthesized in the untreated lysate was incubated with isolated mitochondria, the mature form of 12 kDa appeared rapidly and reached a plateau in 2 min (Fig. 3C). An intermediate-sized product of 13 kDa, which is apparently the processing intermediate (17, 31), appeared within 0.5 to 1 min and disappeared within 4 min. Concomitant with formation of the intermediate form, a fragment of about 2.7 kDa, which is presumably the presequence portion (residues 1 to 24), appeared and then disappeared. Import of pOTC(1–140)-puromycin was reduced by about 40% by the hsc70 depletion but recovered almost completely after the hsc70 readdition.

Import of pOTC(1–90)-puromycin was accompanied by a similar two-step processing pattern (Fig. 3D). The precursor form of 11 kDa was converted to the intermediate form of 8.2 kDa, and the latter was further processed to the mature form of 7.2 kDa. The amount of the mature form reached a maximum in 1 min and then decreased. The mature form was degraded in the mitochondrial matrix, probably because the mature form (58 residues plus puromycin) was too short to form a stable higher-order structure. Import of pOTC(1–90)-puromycin was reduced only slightly by hsc70 depletion. Import of pOTC(1–72)-puromycin and degradation of the processed mature portion were more rapid (Fig. 3E). The amount of the intermediate form and/or the mature form reached its maximum level within 0.5 min and then decreased rapidly. In contrast, the fragment corresponding to the presequence showed a similar fate for the shortest three truncated precursors. Import of pOTC(1–72)-puromycin was not affected by hsc70 depletion or readdition. These results indicate that the hsc70 dependency decreases as the size of pOTC decreases and disappears for very short truncated pOTCs.

Sucrose gradient centrifugation analysis of in vitro-translated precursor proteins and import-competent forms. We found earlier that pOTC synthesized in the reticulocyte lysate sediments as a ca. 11S complex and that the formation of this 11S complex depends on the presence of hsc70 (33). In the present work, we analyzed sedimentation profiles of other precursor proteins and import-competent forms. To minimize the loss of import competence of the precursor proteins, ultracentrifugation was done at 4°C. Mature OTC is composed of three identical subunits of 36 kDa and sediments with a coefficient of 6S (Fig. 4A) (15). pOTC synthesized in vitro sedimented with a broad peak of about 11S in a sucrose gradient, in agreement with previously obtained data (15, 33). When the fractionated samples were subjected to import assay, the 11S peak fraction showed the highest import activity. Assuming that this complex is spherical, 11S corresponds to a molecular mass of about 200 kDa. Therefore, pOTC appears to be maintained in an import-competent structure in this large complex, and this complex is likely to be composed of multiple components.

Matrix-localized mature AAT, a homodimer of 45-kDa subunits (29), sedimented at 5.7S (Fig. 4B). In vitro-synthesized pAAT sedimented with a peak of 7S, whereas the import activity was found in fractions with a peak of around 9S. Mature SPT, a homodimer of 43-kDa subunits (26), sedimented with a peak of 6S (Fig. 4C). In vitro-synthesized pSPT sedimented with a peak of 9S, and import activity was found in fractions centered at 9S. 3-Oxoacyl-CoA thiolase, a homotetramer of 42-kDa subunits (2), sedimented with a peak of 8S (Fig. 4D). In vitro-synthesized 3-oxoacyl-CoA thiolase sedimented with a peak of 5S. Import activity was observed in fractions centered at 4S, a value which corresponds roughly to the molecular mass of the 42-kDa subunit. Thus, the import-competent form is likely to be a monomer.

These results indicate that the sedimentation coefficient of the import-competent form varies among the four precursor

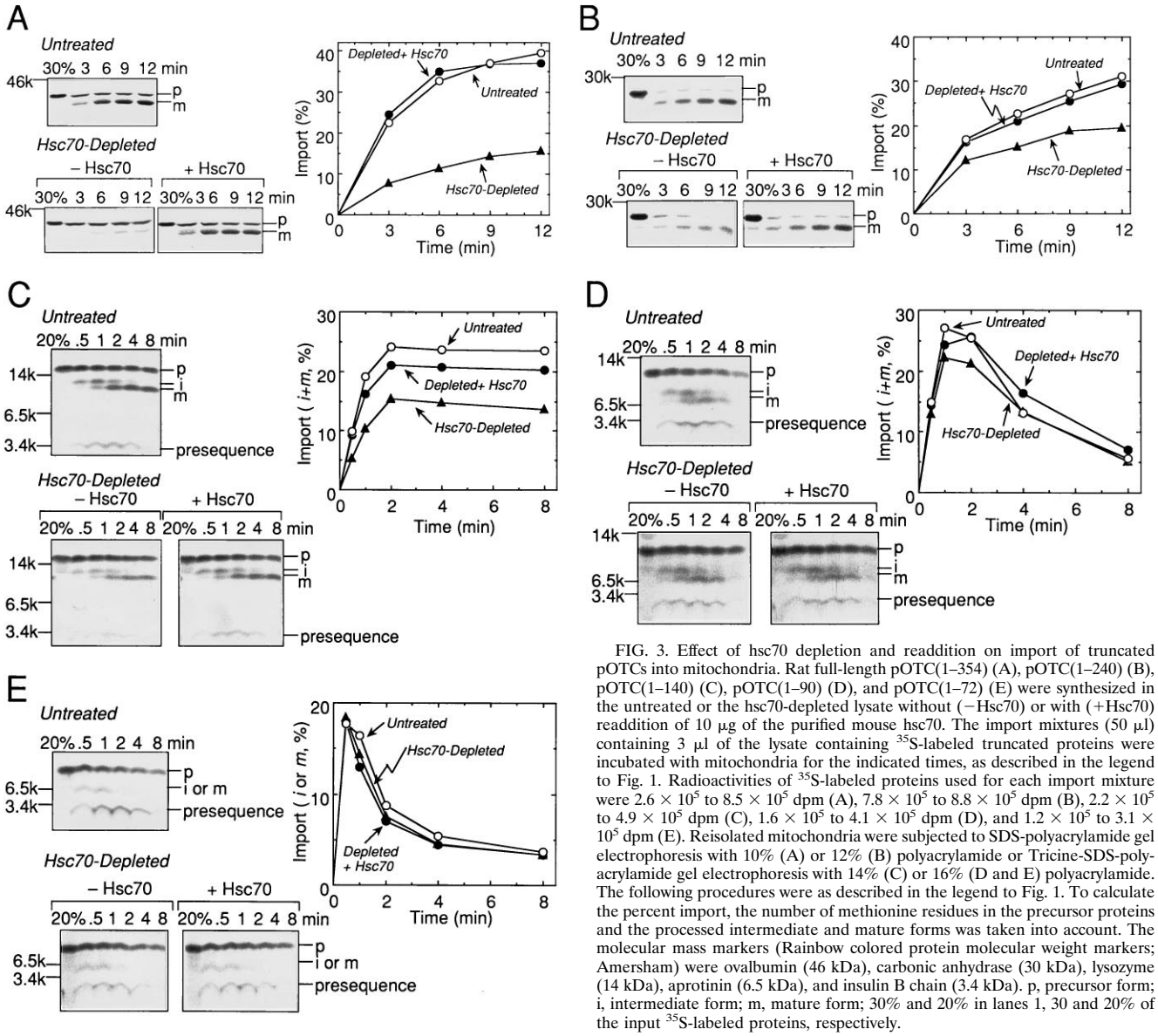

FIG. 3. Effect of hsc70 depletion and readdition on import of truncated pOTCs into mitochondria. Rat full-length pOTC(1–354) (A), pOTC(1–240) (B), pOTC(1–140) (C), pOTC(1–90) (D), and pOTC(1–72) (E) were synthesized in the untreated or the hsc70-depleted lysate without (−Hsc70) or with (+Hsc70) readdition of 10 μg of the purified mouse hsc70. The import mixtures (50 μl) containing 3 μl of the lysate containing ^{35}S-labeled truncated proteins were incubated with mitochondria for the indicated times, as described in the legend to Fig. 1. Radioactivities of ^{35}S-labeled proteins used for each import mixture were 2.6×10^5 to 8.5×10^5 dpm (A), 7.8×10^5 to 8.8×10^5 dpm (B), 2.2×10^5 to 4.9×10^5 dpm (C), 1.6×10^5 to 4.1×10^5 dpm (D), and 1.2×10^5 to 3.1×10^5 dpm (E). Reisolated mitochondria were subjected to SDS-polyacrylamide gel electrophoresis with 10% (A) or 12% (B) polyacrylamide or Tricine-SDS-polyacrylamide gel electrophoresis with 14% (C) or 16% (D and E) polyacrylamide. The following procedures were as described in the legend to Fig. 1. To calculate the percent import, the number of methionine residues in the precursor proteins and the processed intermediate and mature forms was taken into account. The molecular mass markers (Rainbow colored protein molecular weight markers; Amersham) were ovalbumin (46 kDa), carbonic anhydrase (30 kDa), lysozyme (14 kDa), aprotinin (6.5 kDa), and insulin B chain (3.4 kDa). p, precursor form; i, intermediate form; m, mature form; 30% and 20% in lanes 1, 30 and 20% of the input ^{35}S-labeled proteins, respectively.

proteins from 11S to 4S, even though they have similar subunit molecular masses of 40 to 47 kDa.

hsc70 directly interacts with in vitro-translated precursor proteins. We finally examined whether hsc70 directly interacts with in vitro-translated precursor proteins. Beckmann et al. (3) showed that cytosolic hsp70 family members interact transiently and cotranslationally with numerous proteins. Since various [^{35}S]methionine-labeled cellular proteins could be coimmunoprecipitated with hsp70s in an ATP-dependent manner (3), we tested four precursor proteins synthesized in vitro for coimmunoprecipitation with hsc70 in the absence and presence of ATP. When [^{35}S]pOTC synthesized in rabbit reticulocyte lysate was subjected to coimmunoprecipitation with hsc70-specific antibody (1B5)-coupled Sepharose, 4.1% of the pOTC was coprecipitated in the absence of ATP whereas 1.2% of the precursor was coprecipitated in the presence of ATP (Fig. 5). Higher coimmunoprecipitation in the absence of ATP than in its presence was also observed for pAAT, pSPT, and T1. These results are in accord with the previous observation (3). However, the amounts of coimmunoprecipitated precursors in the absence of ATP varied from one precursor protein to another; those of pAAT, pSPT, and T1 were 2.9, 1.4, and 1.0%, respectively. Thus, among the four precursor proteins, the degree of coimmunoprecipitation correlated well with that of hsc70 dependency in mitochondrial import.

DISCUSSION

The requirement of cytosolic hsc70 for mitochondrial import of pOTC and three other precursor proteins was examined in an in vitro translation-mitochondrial import system in which hsc70 had been depleted. These precursors showed different extents of hsc70 dependency, with the highest being for pOTC (about 70%) and the lowest being for 3-oxoacyl-CoA thiolase (about 0%). Because hsc70 depletion was complete and because reductions in the mitochondrial import were almost fully recovered by the readdition of hsc70, we could correctly evaluate the extent of hsc70 dependency. Differences in hsc70

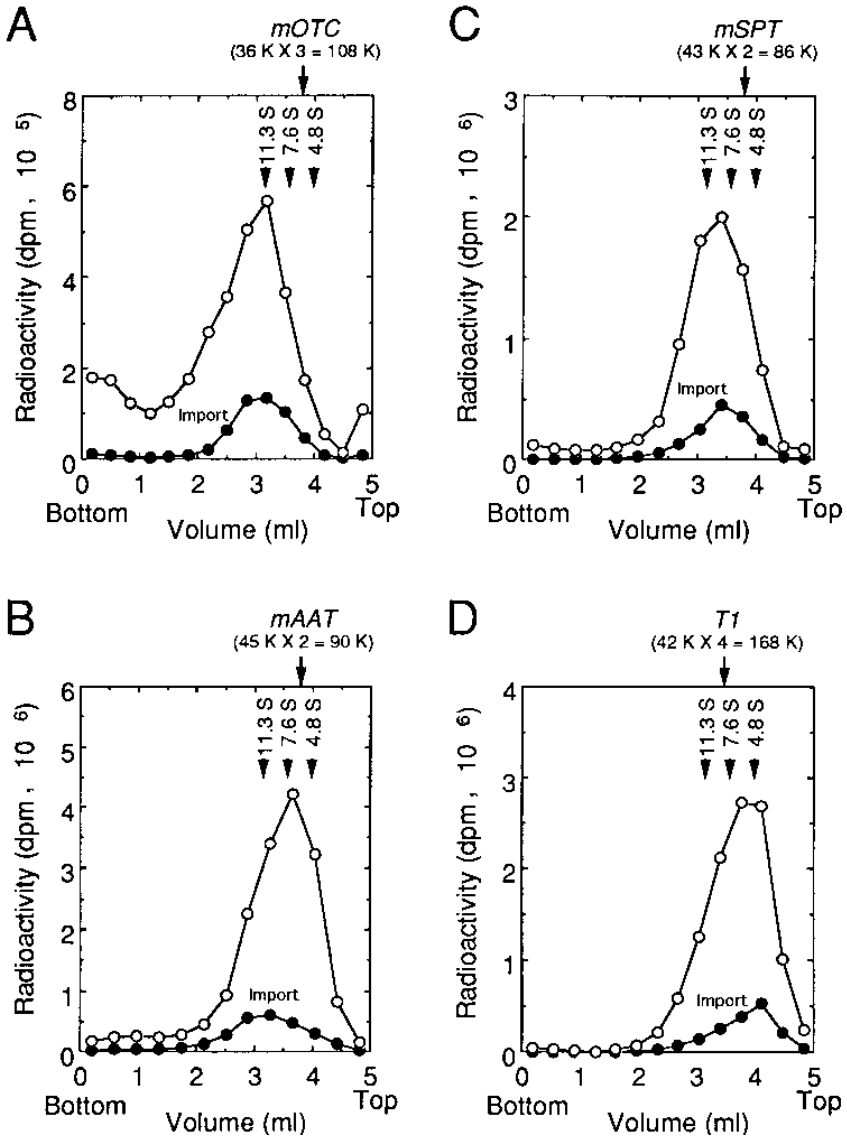

FIG. 4. Sucrose gradient centrifugation of precursor proteins synthesized in rabbit reticulocyte lysate and import of fractionated precursors into mitochondria. Samples (0.20 ml) of ^{35}S-labeled pOTC (6.5×10^6 dpm) (A) pAAT (3.7×10^7 dpm) (B), pSPT (1.8×10^7 dpm) (C), and 3-oxoacyl-CoA thiolase (T1) (2.9×10^7 dpm) (D) synthesized in untreated rabbit reticulocyte lysate were layered on linear 5 to 20% sucrose gradients (4.8 ml). Centrifugation was performed for 3.8 h at 4°C at 50,000 rpm in a Hitachi 55P ultracentrifuge with an RPS55T rotor. Fractions (0.33 ml) were collected through the bottom of the tube, and 100-μl portions were immediately subjected to an import assay. Open circles represent radioactivities of precursor proteins in the fractions; solid circles represent radioactivities of imported proteins. The recoveries of ^{35}S-labeled precursor proteins were about 60% in all cases. The positions of sedimentation of the assembled mature proteins in the mitochondrial matrix are shown at the top of each panel by an arrow. Arrowheads show the positions of bovine catalase (11.3S), yeast alcohol dehydrogenase (7.6S), and bovine serum albumin (4.8S).

dependency may be because of different tendencies of the precursor proteins to fold, misfold, or aggregate. A high hsc70 dependency of pOTC may be related to our findings (22) that purified recombinant pOTC readily aggregates. On the other hand, the import competence of 3-oxoacyl-CoA thiolase seems to be maintained without the participation of hsc70.

hsc70 interacts with unfolded segments of polypeptides, perhaps in the form of nascent polypeptides emerging from ribosomes (3). hsc70 may bind different polypeptide segments with a wide spectrum of affinities, and the cycles of hsc70 binding, release, and rebinding may prevent polypeptides from misfolding and promote polypeptides to carry out productive folding, perhaps an intrinsic nature of cytosolic proteins (9). In the case of mitochondrial proteins, however, productive folding may be partly perturbed by NH_2-terminal presequence portions of the precursor proteins, but this would not be sufficient (14, 22). In our experiments with a series of truncated pOTCs, the hsc70 dependency was lost halfway between pOTC(1–354) (full length) and pOTC(1–240)-puromycin and almost completely between pOTC(1–140)-puromycin and pOTC(1–90)-puromycin. Thus, hsc70-acting sites appear to reside in residues 90 to 140 and residues 240 to 354, not in the presequence portion. These results agree with data (28) that chemically synthesized presequences of pOTC and aldehyde dehydrogenase precursor were imported rapidly into the mitochondria in the absence of reticulocyte lysate but not with the report (27) that import of the chemically synthesized presequence of ornithine aminotransferase precursor was markedly stimulated by the reticu-

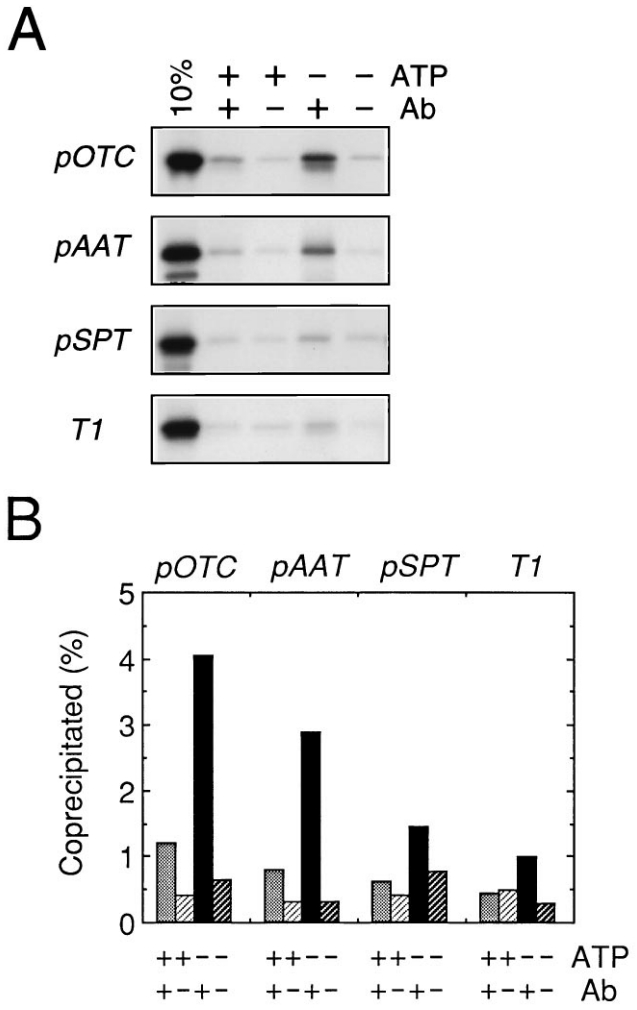

FIG. 5. Coimmunoprecipitation of precursor proteins synthesized in rabbit reticulocyte lysate with hsc70. ^{35}S-labeled pOTC, pAAT, pSPT, and 3-oxoacyl-CoA thiolase (T1), synthesized in rabbit reticulocyte lysate system, were divided in half. One portion was treated with an ATP hydrolytic enzyme apyrase (Sigma type IV; 10 U/ml) for 30 min at 4°C to deplete ATP, while the other was placed at 4°C. Then 5 mM 5′-adenylylimidodiphosphate (AMP-PNP) was added to one portion (lanes ATP−) while 5 mM magnesium ATP was added to the other (lanes ATP+). Samples (10 μl) of each portion were added to 1.0 ml of a 1% suspension of antibody-coupled Sepharose beads in immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 3 μg aprotinin per ml, and 1.5 μg of leupeptin per ml in phosphate-buffered saline). Immunoprecipitation was performed with either control rat IgG- (lanes Ab−) or rat 1B5 antibody (Ab+)-coupled Separose. The radioactivities of ^{35}S-labeled proteins used for each immunoprecipitation were 1.6×10^6 dpm for pOTC, 7.3×10^5 dpm for pAAT, 5.0×10^5 dpm for pSPT, and 3.9×10^5 dpm for T1. After a 1-h incubation with gentle mixing at 4°C, Sepharose beads were pelleted by centrifugation at 2,000 × *g* for 1 min in a refrigerated microcentrifuge. The beads were washed five times with 1.0 ml of immunoprecipitation buffer. Then the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) followed by fluorography. Portions of the fluorograms are shown in panel A. Lane 10%, 10% of the ^{35}S-labeled proteins was used for immunoprecipitation. The results were quantitated by imaging plate analysis with a FUJIX BAS2000 analyzer and are shown in panel B.

locyte lysate. These discrepancies may due to different presequences. Because mitochondrial presequences can induce the aggregation of unfolded proteins (6), the possibility that hsc70 binds to the presequence portion of pOTC and prevents it from aggregation cannot be ruled out. Recognition sequences of hsc70 are reported to be heptameric peptide sequences with some common features (7, 32). It will be interesting to test which part of the pOTC sequence is recognized by hsc70.

Analysis of mitochondrial import of truncated pOTCs revealed an unequivocal two-step processing. Furthermore, the apparent time required for maximum accumulation of the intermediate form and/or the mature form was very short (within 2 min at 25°C) for the shortest three truncated precursors. We speculate that the mature portion of the precursor protein delays translocation of the presequence portion across mitochondrial membranes.

pOTC synthesized in rabbit reticulocyte lysate forms an 11S complex in sucrose gradient centrifugation in an hsc70-dependent manner. This 11S complex was shown to be the import-competent form of pOTC. The sedimentation coefficient of the import-competent form of pOTC and other precursors differed from one precursor to another, even though the subunit sizes are similar. It is notable that the sedimentation coefficient and therefore probably the size of the import-competent form of the precursors increases as the hsc70 dependency of mitochondrial import increases. The composition of the import-competent complexes remains to be elucidated.

We could demonstrate not only a functional requirement of hsc70 for mitochondrial import of precursor proteins by using our hsc70-depletion system but also direct interaction of hsc70 with precursor proteins by coimmunoprecipitation experiments. The four precursor proteins we tested showed different degrees of hsc70 dependency in mitochondrial import and of hsc70 interaction. Coprecipitation with hsc70 showed an ATP dependency, as shown for the bulk of newly synthesized proteins, which is in accord with the previous observation (3). Furthermore, a good correlation was found between functional hsc70 dependency in mitochondrial protein import and direct hsc70 interaction with precursor proteins.

In the presence of ATP, interaction between hsc70 and precursor proteins seems to be transient. hsc70 interacts with newly synthesized proteins at an early stage of the folding process (3, 8). Productive folding of cytosolic proteins is postulated to be promoted by the cycles of hsc70 binding, release, and rebinding (9). The same process may be involved in the case of precursor proteins targeted to mitochondria, although hsc70 interacts with the precursor proteins to maintain a loosely folded state which is appropriate for translocation. Several members of the DnaJ family have been found in mammals, but the functions of most of them are unknown (4). A mammalian DnaJ homolog, hsp40, is proposed to mediate the initial folding process in the cytosol with hsc70 (8). Cytosolic proteins are folded properly by a set of these chaperones (hsc70-hsp40-TriC), whereas mitochondrion-targeted proteins must bypass the subsequent folding processes in the cytosol. We speculate that the import competence of mitochondrion-targeted proteins is obtained by cooperation of hsc70, a DnaJ homolog(s), and a putative organelle-targeting factor(s) such as presequence-binding factor (20, 21) and mitochondrial import stimulation factor (10, 11).

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REFERENCES

1. **Amaya, Y., H. Arakawa, M. Takiguchi, Y. Ebina, S. Yokota, and M. Mori.** 1988. A noncleavable signal for mitochondrial import of 3-oxoacyl-CoA thiolase. J. Biol. Chem. **263:**14463–14470.
2. **Arakawa, H., M. Takiguchi, Y. Amaya, S. Nagata, H. Hayashi, and M. Mori.** 1987. cDNA-derived amino acid sequence of rat mitochondrial 3-oxoacyl-CoA thiolase with no transient presequence: structural relationship with peroxisomal isozyme. EMBO J. **6:**1361–1366.
3. **Beckmann, R. P., L. E. Mizzen, and W. J. Welch.** 1990. Interaction of hsp 70 with newly synthesized proteins: implications for protein folding and assembly. Science **248:**850–854.
4. **Cyr, D. M., T. Langer, and M. G. Douglas.** 1994. DnaJ-like proteins—molecular chaperones and specific regulators of hsp70. Trends Biochem. Sci. **19:**176–181.
5. **Deshaies, R. J., B. D. Koch, M. Werner-Washburne, E. A. Craig, and R. Schekman.** 1988. A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptide. Nature (London) **322:** 800–805.
6. **Endo, T., S. Mitsui, and D. Roise.** 1995. Mitochondrial presequences can induce aggregation of unfolded proteins. FEBS Lett. **359:**93–96.
7. **Fourie, A. M., J. F. Sambrook, and M. J. Gething.** 1994. Common and divergent peptide binding specificities of hsp70 molecular chaperones. J. Biol. Chem. **269:**30470–30478.
8. **Frydman, J., E. Nimmesgern, K. Ohtsuka, and F. U. Hartl.** 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. Nature (London) **370:**111–117.
9. **Gething, M.-J., and J. Sambrook.** 1992. Protein folding in the cell. Nature (London) **355:**33–45.
10. **Hachiya, N., T. Komiya, R. Alam, J. Iwahashi, M. Sakaguchi, T. Omura, and K. Mihara.** 1994. MSF, a novel cytoplasmic chaperone which functions in precursor targeting to mitochondria. EMBO J. **13:**5146–5154.
11. **Hachiya, N., K. Mihara, K. Suda, M. Horst, G. Schatz, and T. Lithgow.** 1995. Reconstitution of the initial steps of mitochondrial protein import. Nature (London) **376:**705–709.
12. **Jackson, R. J., and T. Hunt.** 1983. Preparation and use of nuclease-treated rabbit reticulocyte lysate for the translation of eukaryotic messenger RNA. Methods Enzymol. **96:**50–74.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
14. **Lain, B., A. Iriarte, and M. Martinez-Carrion.** 1994. Dependence of the folding and import of the precursor to mitochondrial aspartate aminotransferase on the nature of the cell-free translation system. J. Biol. Chem. **269:**15588–15596.
15. **Miura, S., M. Mori, Y. Amaya, M. Tatibana, and P. P. Cohen.** 1981. Aggregation states of precursor proteins for mitochondrial carbamoyl-phosphate synthetase I and ornithine carbamoyltransferase. Biochem. Int. **2:**305–312.
16. **Mori, M., H. Matsue, S. Miura, M. Tatibana, and T. Hashimoto.** 1985. Transport of proteins into mitochondrial matrix. Evidence suggesting a common pathway for 3-ketoacyl-CoA thiolase and enzymes having presequences. Eur. J. Biochem. **149:**181–186.
17. **Mori, M., S. Miura, M. Tatibana, and P. P. Cohen.** 1980. Characterization of a protease apparently involved in processing of pre-ornithine transcarbamylase of rat liver. Proc. Natl. Acad. Sci. USA **77:**7044–7048.
18. **Murakami, H., D. Pain, and G. Blobel.** 1988. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. J. Cell Biol. **107:**2051–2057.
19. **Murakami, K., Y. Amaya, M. Takiguchi, Y. Ebina, and M. Mori.** 1988. Reconstitution of mitochondrial protein transport with purified ornithine carbamoyltransferase precursor expressed in Escherichia coli. J. Biol. Chem. **263:**18437–18442.
20. **Murakami, K., and M. Mori.** 1990. Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. EMBO J. **9:**3201–3208.
21. **Murakami, K., S. Tanase, Y. Morino, and M. Mori.** 1992. Presequence binding factor-dependent and -independent import of proteins into mitochondria. J. Biol. Chem. **297:**13119–13122.
22. **Murakami, K., F. Tokunaga, S. Iwanaga, and M. Mori.** 1990. Presequence does not prevent folding of a purified mitochondrial precursor protein and is essential for association with a reticulocyte cytosolic factor(s). J. Biochem. (Tokyo) **108:**207–214.
23. **Nishi, T., F. Nagashima, S. Tanase, Y. Fukumoto, T. Joh, K. Shimada, Y. Matsukado, Y. Ushio, and Y. Morino.** 1989. Import and processing of precursor to mitochondrial aspartate aminotransferase. Structure-function relationships of the presequence. J. Biol. Chem. **264:**6044–6051.
24. **Oda, T., T. Funai, and A. Ichiyama.** 1990. Generation from a single gene of two mRNAs that encode the mitochondrial and peroxisomal serine:pyruvate aminotransferase of rat liver. J. Biol. Chem. **265:**7513–7519.
25. **Oda, T., A. Ichiyama, S. Miura, and M. Mori.** 1984. Uptake and processing of serine:pyruvate aminotransferase precursor by rat liver mitochondria in vitro and in vivo. J. Biochem. (Tokyo) **95:**815–824.
26. **Oda, T., H. Miyajima, Y. Suzuki, and A. Ichiyama.** 1987. Nucleotide sequence of the cDNA encoding the precursor for mitochondrial serine:pyruvate aminotransferase of rat liver. Eur. J. Biochem. **168:**537–542.
27. **Ono, H., and S. Tuboi.** 1988. The cytosolic factor required for import of precursors of mitochondrial proteins into mitochondria. J. Biol. Chem. **263:** 3188–3193.
28. **Pak, Y. K., and H. Weiner.** 1990. Import of chemically synthesized signal peptides into rat liver mitochondria. J. Biol. Chem. **265:**14298–14307.
29. **Sakakibara, R., Y. Kamisaki, Y. Horio, and H. Wada.** 1983. Precursor of mitochondrial glutamic oxaloacetic transaminase isozyme exists as a dimer. Biochem. Int. **6:**231–238.
30. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368–379.
31. **Sztul, E. S., J. P. Hendrick, J. P. Kraus, D. Wall, F. Kalousek, and L. E. Rosenberg.** 1987. Import of rat ornithine transcarbamylase precursor into mitochondria: two-step processing of the leader peptide. J. Cell Biol. **105:** 2631–2639.
32. **Takenaka, I. M., S. M. Leung, S. J. McAndrew, J. P. Brown, and L. E. Hightower.** 1995. Hsc70-binding peptides selected from a phage display peptide library that resemble organellar targeting sequences. J. Biol. Chem. **270:**19839–19844.
33. **Terada, K., K. Ohtsuka, N. Imamoto, Y. Yoneda, and M. Mori.** 1995. Role of heat shock cognate 70 protein in import of ornithine transcarbamylase precursor into mammalian mitochondria. Mol. Cell. Biol. **15:**3708–3713.
34. **Wienhues, U., K. Becker, M. Schleyer, B. Guiard, M. Tropschug, A. L. Horwich, N. Pfanner, and W. Neupert.** 1991. Protein folding causes an arrest of preprotein translocation into mitochondria in vivo. J. Cell Biol. **115:**1601–1609.
35. **Zimmermann, R., M. Sagstetter, M. J. Lewis, and H. R. B. Pelham.** 1988. Seventy-kilodalton heat shock proteins and an additional component from reticulocyte lysate stimulate import of M13 procoat protein into microsomes. EMBO J. **7:**2875–2880.