In Vitro Import into Mitochondria of the Precursor of Mitochondrial Aspartate Aminotransferase*

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The import of the precursor of mitochondrial aspartate aminotransferase was reconstituted in vitro with isolated mitochondria thus corroborating the earlier conclusion of a post-translational uptake. The higher M₄ precursor was synthesized in a reticulocyte lysate programmed with free polysomes from chicken liver. After incubation with intact mitochondria from chicken heart about 50% of the precursor was converted to the mature form in a time-dependent process, its rate being a function of the amount of mitochondria added. The same amount of precursor was processed to the mature form on addition of a mitochondrial extract. No conversion to the mature enzyme took place when the precursor was incubated with intact mitochondria in the presence of the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone or of the chelator o-phenanthroline which does not diffuse into the mitochondrial matrix. In contrast, the chelator bathophenanthroline disulfonate which does not differentiate to the mature form on addition of a mitochondrial precursor was synthesized in a reticulocyte lysate and the precursor accumulated in the cytosol of chicken embryo fibroblasts (6); 3) in the presence of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) pre-mAspAT accumulated in the cytosol of these cells and on dilution of CCCP was quantitatively taken up into the mitochondria (6). In the present paper we extend previous studies on the uptake of in vitro synthesized pre-mAspAT into isolated mitochondria (7, 8) and show that the in vitro translocation of chicken pre-mAspAT follows the mechanism delineated for precursors of other matrix and inner membrane proteins. Extensive studies particularly with yeast and Neurospora (for reviews, see Refs. 9 and 10) have shown that the import of these proteins occurs post-translationally (11, 12), and requires an energized inner mitochondrial membrane for transport (13-16) as well as a metal-dependent matrix protease for processing (17, 18). In addition, the aggregation state of in vivo and in vitro synthesized pre-mAspAT was investigated.

EXPERIMENTAL PROCEDURES

Preparation of Polysomes—Estradiol treatment of roosters for the preparation of polysomes from liver and disruption of liver tissue was as described (5) except for the composition of the media. Medium A contained 20 mM Tris chloride, 75 mM potassium chloride, 100 mM potassium acetate, 5 mM magnesium acetate (pH 7.6). Medium B contained in addition to medium A, 4 mg ml⁻¹ of yeast RNA, 4 mg ml⁻¹ of heparin, 200 mM sucrose, and 5 mM dithiothreitol. The tissue suspension was homogenized with a Dounce homogenizer by six strokes with the loosely fitting pestle A and two strokes with the tightly fitting pestle B. Subsequently, the homogenate was centrifuged in a Sorvall SS-34 fixed angle rotor at 10,000 rpm (12,000 × gav) for 5 min at 0 °C. The supernatant was again centrifuged in the same rotor at 12,000 rpm (17,400 × gav) for 10 min at 0 °C. For isolation of free polysomes, the supernatant was overlaid onto a discontinuous gradient comprising 6 ml each of 1.38 and 2 M sucrose containing 40 mM Tris chloride, 25 mM potassium chloride, 5 mM magnesium acetate, 4 mg ml⁻¹ of yeast RNA, 5 mM dithiothreitol (pH 7.6). The 1.38 M sucrose solution contained in addition 4 mg ml⁻¹ of heparin. After centrifugation in a Sorvall T'85 rotor at 52,000 rpm (200,000 × gav) for 20 h at 2 °C the polysomal pellets were resuspended in polysome buffer (50 mM Tris chloride, 25 mM potassium chloride, 5 mM magnesium acetate, 2 mM dithiothreitol, pH 7.6) and stored at −70 °C. To assess polysome size and integrity, their sedimentation profiles were analyzed on 15-32.5% sucrose isokinetic gradients as described (5), except that the sucrose solutions contained the same salt concentrations as the polysome buffer, and centrifugation was in a Kontron TST 41 rotor at 40,000 rpm (200,000 × gav) for 40 min at 4 °C.

Cell-free Protein Synthesis—Read-out translation of free polysomes in a reticulocyte lysate in the presence of [³⁵S]methionine was performed as described (5) except that incubation was at 29 °C for 40 min. Protein synthesis was stopped by chilling or, when specified, by the addition of 0.2 mM cycloheximide. Postribosomal supernatants

Two isoenzymes of aspartate aminotransferase occur in animal cells, one being located exclusively in the cytosol (cAspAT'), the other in the mitochondrial matrix (mAspAT). The isoenzymes are homologous proteins occurring as α₂-dimers with M₄ = 2 × 45,000. Both the precursor synthesized in a rabbit reticulocyte lysate and the precursor accumulated in the cytosol of chicken embryo fibroblasts were found to exist as homodimer or hetero-oligomer and high M₄ complexes (M₄ > 300,000).

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The abbreviations used are: cAspAT, cytosolic aspartate aminotransferase; mAspAT, mitochondrial aspartate aminotransferase; pre-mAspAT, precursor of mAspAT; SDS, sodium dodecyl sulfate; CCCP, carbonyl cyanide m-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid.
were obtained by centrifuging the chilled translation mixture in a Kontrotn TST 60 rotor at 45,000 rpm (190,000 × g) for 1 h at 2 °C.

**In Vitro Uptake into Mitochondria and Processing of Pre-mAspAT**—The incubation mixture for in vitro import contained 60 μl of the labeled reticulocyte lysate (4 × 10^6 cpm of [35S]methionine incorporated into newly synthesized mAspAT) or 80 μl of postribosomal supernatant which had been adjusted to 210 mM sucrose, 110 mM potassium acetate, and 1.5 mM magnesium acetate. Mitochondria were isolated from chicken heart as described (19) except that EGTA was omitted from the isolation medium. The freshly prepared mitochondria (250 μg of mitochondrial protein in 25 μl of isolation medium) were added to the translation products and incubated at 29 °C for 4 h. The mitochondria were then reisolated by centrifugation in a SS-34 rotor at 12,500 rpm (15,000 × g) for 15 min at 4 °C. To both the mitochondrial pellet (suspended in 50 μl of water) and the supernatant were added 80 μl of homogenization buffer containing 0.1% SDS and 1 mg ml^-1 of methionine (the Staphylococcus aureus (20) and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the incubation continued for 1 h at room temperature. After washing with Triton buffer (1% Triton X-100, 0.1% SDS) and elution with 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride, pH 7.5, to 0.1% SDS. The particulate material was removed by centrifugation at 45,000 rpm (140,000 × g) for 1 h at 15 °C, and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C. After incubation, 100–200 μl of a 10% (v/v) suspension of Staphylococcus aureus (20) were added and the supernatant was mixed with 50–100 μl of anti-mAspAT antiserum for 14–16 h at 4 °C.

For processing assays, the labeled translation products were incubated with the supernatant (15,000 × g, 10 min, 4 °C) of the homogenization buffer was adjusted to pH 7.4 and contained no protease inhibitors chymostatin, antipain, leupeptin, pepstatin, 175 μg ml^-1 of apronin, 5 mM o-phenanthroline, 2 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride (pH 7.1). Homogenization was performed by 20 strokes with a Teflon pestle in a mitochondrial extract (17) as described above for the import assay. After incubation, the total mixture was dissociated in SDS and subjected to immunoprecipitation.

**Preparation of Samples for Sucrose Gradient Centrifugation**—For analysis of pre-mAspAT synthesized in vivo five Petri dishes of cultured chicken embryo fibroblasts were pulsed with 0.3 mCi ml^-1 of [35S]methionine for 15 min at 37 °C in the presence of 20 μM CCCP (6). The cells were harvested in the cold, centrifuged for 7 min at 2,400 rpm, and taken up in 300 μl of homogenization buffer containing 80 μg ml^-1 each of chymostatin, antipain, leupeptin, pepstatin, 175 μg ml^-1 of apronin, 5 mM o-phenanthroline, 2 mM EGTA, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride (pH 7.1). Homogenization was performed by 20 strokes with a Teflon pestle in a Brij 58 homogenizer at 1750 rpm for 1 h at 4 °C. A postribosomal supernatant was obtained by centrifugation in an Eppendorf centrifuge for 10 min at 12,000 rpm (10,000 × g) and 4 °C.

The sample for chromatography of pre-mAspAT synthesized in a cell-free system was obtained by diluting the postribosomal supernatant of a [35S]methionine-labeled reticulocyte lysate containing 120 μg ml^-1 each of chymostatin, antipain, leupeptin, pepstatin, and 260 μg ml^-1 of apronin, 1 mM dithiothreitol with 1 volume of 150 mM potassium chloride, 1 mM dithiothreitol, 10 mM Tris chloride (pH 7.1).

**RESULTS**

**In Vitro Uptake of the Precursor of Mitochondrial Aspartate Aminotransferase by Chicken Heart Mitochondria**—A rabbit reticulocyte lysate was programmed with free polysomes from chicken liver in the presence of [35S]methionine. After translation, a sample of the lysate was incubated with isolated mitochondria. The incubation mixture was centrifuged, and the supernatant and the mitochondrial pellet were analyzed for labeled pre-mAspAT and mature mAspAT (Fig. 1A). The supernatant contained only precursor, whereas the mitochondrial pellet contained both precursor and mature mAspAT. Apparently, part of the precursor had been taken up by the organelles and processed to the mature protein while part of it had been merely bound to the mitochondria. Exogenous mature mAspAT did not compete with the precursor for uptake into mitochondria. The possibility to inhibit the import in chicken embryo fibroblasts by CCCP (see Introduction) offered a second source of precursor for the in vitro study of the translocation process. However, the precursor in the cell homogenate was neither imported into isolated mitochondria nor was it processed by a mitochondrial extract (not shown).

**Rates of Import and Processing of Pre-mAspAT**—On incubation of a labeled translation mixture with exogenous mAspAT the precursor in the supernatant decreased concomitantly with an increase of labeled mature mAspAT in the
mitochondria during the first 90 min of incubation (Fig. 2A). No mature labeled or unlabeled mAspAT was detected in the supernatant indicating that most mitochondria remained intact during the incubation. A quantitative evaluation of the results is given in Fig. 2B. After an incubation of 120 min about 50% of the total precursor had been converted to the mature form. The percentage of pre-mAspAT co-sedimenting with mitochondria increased from -10% after 15 min to -20% after 120 min. The co-sedimented precursor, like the precursor remaining in the supernatant, was completely digested by trypsin in a very low concentration (10 μg ml⁻¹, 20 min, 4 °C; not shown); apparently it had not entered the mitochondria but was merely bound to the outer mitochondrial membrane. The proteolytic susceptibility of pre-mAspAT (see also Ref. 6) is in marked contrast to the resistibility of the mature protein either obtained by in vitro processing or as isolated from chicken heart (see Ref. 26).

Incubation of a labeled reticulocyte lysate with increasing amounts of mitochondria or with corresponding increasing amounts of hypotonic mitochondrial extract, results in an accelerated generation of mature mAspAT (Fig. 3, A and B, respectively). However, at 200–400 μg of mitochondrial protein added a maximum value of imported or processed precursor is reached. This maximum value corresponds to 50% of the total precursor and equals the maximum fraction of pre-mAspAT found to be imported on prolonged incubation (Fig. 2). Addition of unlabeled reticulocyte lysate (nuclease-treated or untreated; up to 6 times the amount of labeled reticulocyte lysate) as well as addition of chicken heart cytosol and chicken fibroblast cytosol to the import assay failed to increase the yield of import. Such additions were reported to stimulate the in vitro import of the precursors of ornithine transcarbamylase (27) and of cytochrome b₂ (28). Addition of Zn²⁺ or Co²⁺ to the mitochondrial extract (18) also did not increase the fraction of processed precursor. Apparently, under the present experimental conditions the extent of import or processing of pre-mAspAT is limited neither by time nor by the amount of mitochondria or mitochondrial extract added nor by auxiliary factors. Only part of the precursor synthesized in the reticulocyte lysate is present in a form that can be imported or processed. The same fraction of importable precursor was obtained with different preparations of polysomes. However, the fraction was even smaller when other batches of reticulocyte lysate were used.

Inhibitors of Import and Processing—In order to show that
The processing assays B were made described (5). After 90 min at 29 °C the mitochondrial proteins did not increase the amount of imported or processed mAspAT; 0, pre-mAspAT in the supernatant of import assay A or a supernatant of hypotonically disrupted mitochondria. In the case of the in vivo synthesized product, faint bands of pre-mAspAT were detected which corresponded to a Mₐ of ~40,000 (Fig. 5B) suggesting a small pool of monomer.

Results similar to those of gel permeation chromatography were also obtained by zone velocity centrifugation in sucrose gradients. About half of pre-mAspAT from the reticulocyte lysate sediments in a Mₐ range around 85,000 and the other half in the high Mₐ range of the gradient (Mₐ > 240,000), suggesting that under the conditions used pre-mAspAT exists partly as a homodimer or hetero-oligomer and partly as a higher Mₐ aggregate (Fig. 6, A and D). The distribution of pre-mAspAT between the two forms was not constant; in another experiment the ratio between the two peaks was 4:1 in favor of the putative dimeric form. On centrifugation of the postmitochondrial supernatant from CCCP-treated chicken embryo fibroblasts, the precursor was distributed over a Mₐ range from ~40,000 to >240,000 corresponding to 1 to 6 times its Mₐ in SDS containing gels (Fig. 6, B and D). For determining the Mₐ of newly processed mAspAT, a homogenate of pulsed chicken embryo fibroblasts was sonicated in order to release the labeled enzyme from the mitochondria. After centrifugation, mAspAT was detected as a distinct peak in the 85,000 region of the gradient (Fig. 6, C and D) indicating that the newly processed mAspAT exists exclusively as a homodimer. Mature endogenous unlabeled mAspAT was immunoprecipitated from the same fractions as newly processed mAspAT (not shown).

**FIG. 3.** Rates of import and processing of pre-mAspAT as functions of the amount of mitochondria or mitochondrial extract, respectively. Four samples of [³⁵S]methionine-labeled translation products were incubated with (A) varying amounts of isolated mitochondria or (B) corresponding amounts of a supernatant of hypotonically disrupted mitochondria (final volume 85 μl; for details, see "Experimental Procedures"). After 90 min at 29 °C the supernatant and mitochondrial fractions of the import assays A and the processing assays B were made 4% (w/v) in SDS and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. The bands corresponding to pre-mAspAT and mature endogenous unlabeled mAspAT were cutout from the gels and the radioactivity counted as described (5). 0, mAspAT in the mitochondria or processed mAspAT, respectively; O, pre-mAspAT in the supernatant of import assay A or pre-mAspAT in processing assay B; △, pre-mAspAT associated with the mitochondria; ■, sum of total pre-mAspAT plus mAspAT. Prolonged incubation (2 h) of the samples containing 400 μg of mitochondrial proteins did not increase the amount of imported or processed mAspAT.

**FIG. 4.** Effect of inhibitors on import and processing of pre-mAspAT. Mitochondria (A) or a supernatant (B) of hypotonically disrupted mitochondria were preincubated with the indicated inhibitors for 5 min on ice: —, no addition; C, 10 μM CCCP; BP, 1 mM bathophenanthroline disulfonate; OP, 2 mM o-phenanthroline. The mitochondria or the mitochondrial extract were then added to the [³⁵S]methionine-labeled reticulocyte lysate previously adjusted to the same inhibitor concentration. Incubation was for 60 min at 29 °C. Supernatants (S) and mitochondrial pellets (P) of the import assay suspensions and the processing assays were made 4% (w/v) in SDS and subjected to immunoprecipitation. Marker proteins (M) are the same as in Fig. 2A.

As expected, CCCP did not prevent the generation of mature enzyme if pre-mAspAT was incubated with a mitochondrial extract (Fig. 4B). Of the two chelating agents tested only the more hydrophobic o-phenanthroline which penetrates the mitochondrial membranes, but not the charged bathophenanthroline disulfonate which cannot enter the mitochondria, inhibited the appearance of mature enzyme in intact mitochondria (Fig. 4A). When tested with the mitochondrial extract both agents inhibited the processing of pre-mAspAT (Fig. 4B).

**Aggregation State of in Vitro and in Vico Synthesized Pre-mAspAT—** A postribosomal supernatant of a labeled reticulocyte lysate or a postmitochondrial supernatant of chicken embryo fibroblasts pulsed in the presence of CCCP (6) were applied to a high performance gel permeation chromatography column. In both cases, the bulk of pre-mAspAT was eluted with the void volume (Fig. 5, A and B) corresponding to a Mₐ > 300,000. Apparently, after synthesis pre-mAspAT becomes part of a high molecular weight complex. A minor fraction of pre-mAspAT from the reticulocyte lysate was eluted in a broad peak corresponding to an apparent Mₐ of 75,000 (Fig. 5A). This elution volume corresponds with that of mAspAT (Fig. 5C) which for unknown reasons does not elute corresponding to its Mₐ of 90,000. In the case of the in vivo synthesized product, faint bands of pre-mAspAT were detected which corresponded to a Mₐ of ~40,000 (Fig. 5B) suggesting a small pool of monomer.

In this study the import of pre-mAspAT into mitochondria was reconstituted with precursor that had been synthesized in a rabbit reticulocyte lysate programmed with free poly-somes from chicken liver and with mitochondria that had been isolated from chicken heart. The following lines of experimental evidence indicated that the observed processing of pre-mAspAT was executed by a chelator-sensitive protease in the mitochondrial matrix. 1) The uncoupling agent CCCP abolished the appearance of labeled mAspAT when pre-mAspAT was incubated with intact mitochondria. 2) Both chelators, bathophenanthroline disulfonate and o-phenanthroline (13, 29), inhibited the processing by a mitochondrial extract; however, only o-phenanthroline which in contrast to the charged bathophenanthroline disulfonate can diffuse...
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Fig. 5. Gel permeation chromatography of pre-mAspAT. (A) postribosomal supernatant of a reticulocyte lysate containing [35S]methionine-labeled pre-mAspAT or (B) a postmitochondrial supernatant of chicken embryo fibroblasts pulsed with [35S]methionine in the presence of 20 μM CCCP were applied to a high performance gel permeation chromatography column. To both samples, 4 μg of mAspAT were added as marker. The eluted fractions were analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. The Mr values indicated are from the calibration run of C. The denotation m indicates the elution volume of marker mAspAT as visualized by protein staining. It corresponds to a Mr of 75,000. The same elution volume of mAspAT was found in the calibration run. C, calibration of the column. The following Mr markers were used: catalase (240,000); lactate dehydrogenase (140,000); cytosolic aspartate aminotransferase (92,000); mitochondrial aspartate aminotransferase (90,000; however, its elution volume corresponds to a Mr of 75,000); carboanhydrase (30,000); myoglobin (17,000); cytochrome c (13,000). In some experiments including that of A, pre-mAspAT appeared as a double band. The additional band with a slightly lower Mr was analyzed by chemical cleavage of the protein in the gel slice and re-electrophoresis of the fragments (41). The peptide pattern was very similar to those of cleaved pre-mAspAT and mAspAT. The appearance of the additional band was sporadic and independent of the presence of mitochondria.

through the inner membrane inhibited the processing by intact mitochondria.

The reconstitution of the import of pre-mAspAT corroborates the previous conclusion, that the precursor is post-translationally translocated into the mitochondria (see Introduction). The notion of a post-translational uptake is confirmed by the finding that import occurred also with a postribosomal supernatant of a reticulocyte lysate where protein synthesis had been stopped by cycloheximide.

The estimated half-life of pre-mAspAT with respect to import in the in vitro system was ~30 min (Fig. 2), whereas in chicken embryo fibroblasts the half-life of the pre-mAspAT was found to be only 0.5 min (6). Similar slow in vitro translocation and processing has been observed in the case of several other precursors, e.g. pre-ornithine transcarbamylase (30, 31) pre-carbamoyl-phosphate synthetase (31, 32), and pre-methylmalonyl-CoA mutase (33).

The in vitro system is also less efficient than intact cells with respect to the yield of import. In CCCP-treated chicken fibroblasts pre-mAspAT which has been accumulated in the cytosol is quantitatively chased into mitochondria on release of the import block (6). In contrast, only about 50% of the total in vitro synthesized precursor was recovered as mature enzyme on incubation with intact mitochondria. The bulk of the nonimported precursor was about equally distributed between the mitochondrial fraction and the supernatant. Processing of in vitro synthesized pre-mAspAT to the mature form by a mitochondrial extract was also found to be limited to ~50% of total precursor (Fig. 3). The coincidence of the yields of import and processing suggests that the precursor itself rather than auxiliary factors is limiting. In agreement with this conclusion, addition of more mitochondria, more reticulocyte lysate, or a cytosol from chicken heart or fibroblasts all failed to increase the yield of import. Conceivably, the nascent polypeptide chains in the polysome preparation used might be damaged. However, this explanation appears unlikely because the same polysome preparation was found to produce precursor with an even lower yield of import and
Fig. 6. Sucrose gradient centrifugation of pre-mAspAT and newly processed mAspAT. (The postribosomal supernatant of a reticulocyte lysate (A), or the postmitochondrial supernatant of CCCP-treated chicken embryo fibroblasts (B) each containing labeled pre-mAspAT, or the supernatant of a sonicated homogenate of untreated chicken embryo fibroblasts containing newly processed mAspAT (C) were centrifuged on linear sucrose gradients for 20 h. (For details on the preparation of samples A, B, and C, see “Experimental Procedures.”) After centrifugation, 5 fractions of 1 ml each from the bottom part of the gradient and 12 fractions of 0.5 ml each from the top part of the gradient (A) or 22 fractions of 0.5 ml each (B and C) were collected and analyzed by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and fluorography. D, the bands of the fluorograms of A, B, and C were scanned densitometrically. The readings were plotted against the corresponding Mr values. ●, pre-mAspAT synthesized in a reticulocyte lysate (A); ○, pre-mAspAT synthesized in chicken embryo fibroblasts (B); △, newly processed mAspAT from chicken embryo fibroblasts (C). Five downward arrows show the positions of the Mr markers: cytochrome c (13,000); peroxidase (40,000); mAspAT (90,000); lactate dehydrogenase (140,000); catalase (240,000). Mr values >240,000 were estimated by extrapolation. The marker proteins, dissolved in 150 mM potassium chloride, 10 mM Tris chloride (pH 7.4) were centrifuged in separate tubes in the same run. Their positions were determined by the absorbance of the eluted fractions at 280 nm and when plotted against the Mr values gave straight lines. If mAspAT and peroxidase were added to a postribosomal supernatant from a reticulocyte lysate or to a postmitochondrial supernatant from homogenized chicken embryo fibroblasts and centrifuged in parallel to the Mr marker proteins, their positions in the gradients were found to be unchanged as determined by measuring their enzymatic activities.

processing in other reticulocyte lysates. The finding that precursor from chicken embryo fibroblasts after homogenization of the cells was neither imported into isolated mitochondria nor processed by a mitochondrial extract also might indicate that under cell-free conditions the precursor is subject to post-translational alterations prohibiting its import and processing. Such alterations could include covalent modifications, complexation with other cellular constituents, or denaturation. Denatured precursors have been reported not to be processed (18).

The studies on the aggregation state of the precursor showed that pre-mAspAT in both a reticulocyte lysate and a postmitochondrial supernatant from chicken embryo fibroblasts is not a homogeneous species. Centrifugation and gel filtration experiments indicate that pre-mAspAT may exist as high (Mr > 300,000) and low Mr aggregates. With both methods the apparent Mr of the low Mr form corresponds with the Mr determined for mature mAspAT. Thus, the low Mr material may represent a dimeric form of pre-mAspAT although an association of the precursor with other proteins cannot be excluded.

For several other mitochondrial precursor proteins, Mr values ranging from that of a monomer up to 500,000 have been found. Each protein under a given set of conditions yielded a characteristic aggregation profile. On gel filtration analysis, the bulk of the precursors of the β-subunit of yeast F1-ATPase (34), of rat liver ornithine transcarbamylase (35), of Neurospora ATP/ADP carrier (12), and of rat liver adenine nucleotide carrier (36) behaved as high Mr aggregates. On centrifugation the precursors of rat liver ornithine transcarbamylase and carbamoyl phosphate synthetase (35) as well as the precursor of subunit 9 of Neurospora ATPase (37) showed broad Mr profiles ranging from monomer up to high Mr aggregates. For the precursors of rat liver mAspAT (38) and
malate dehydrogenase (39) \( M \) values only slightly larger than those for the mature dimeric form of the corresponding enzyme have been determined by gel filtration. The marked variation in the aggregation profile of a given precursor under different conditions of analysis on the one hand and among different precursor proteins on the other suggests that the aggregation state depends critically on the experimental conditions, e.g. duration of experiment, temperature, availability of cytosolic factors, or ionic conditions. In the case of pre-\( \text{mAspAT} \), gel filtration which was performed in the short time of 12 min (elution time of high \( M \), aggregates) and at 20 °C favored the detection of high \( M \), aggregates, while after centrifugation for 20 h at 2 °C, the ratio between high and low \( M \), material was shifted towards the latter.

In conclusion, all experimental evidence including the present data indicate that mitochondrial precursor proteins are present in the cytosol as low \( M \), forms (homo- or hetero-oligomers) and as high \( M \), forms. The formation of aggregates may protect the precursors against proteolysis (41), or in analogy to the signal recognition particle for secretory proteins (41) to be involved in the specific translocation of the precursor from its site of synthesis to the mitochondrial outer membrane. In vitro import assays such as that established in this study will allow us to address this question experimentally.

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