Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 514-522

THE TRANSPORT AND PROCESSING OF CARBAMYL PHOSPHATE SYNTHETASE-I IN MOUSE HEPATIC MITOCHONDRIA

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Received December 12, 1983

SUMMARY. Carbamyl phosphate synthetase-I (CPS-I)[2], purified from mouse hepatic mitochondria consists of electrophoretically homogeneous polypeptide species of 160 kilodaltons molecular weight (Kd). Monospecific antibody to CPS-I immunoprecipitated a putative precursor of 165Kd protein from in vitro translation products programmed with mouse liver free polysomes or poly(A) RNA. Isolated mitochondrial particles can take up and process pCPS-I into mature CPS-I of 160Kd in an in vitro transport system. The in vitro transport of CPS-I is energy dependent and requires intact mitochondria. The processing of pCPS-I appears to involve a single endoproteolytic cleavage.

CPS-I is an abundant hepatic tissue specific polypeptide of ureotelic species accounting for about 20% of the mitochondrial mass (1,2). CPS-I purified from rat, human, and amphibian sources consist of a single polypeptide species of 160Kd (1-6) which show extensive intraspecies, immunochemical cross-reactivity (6) suggesting a high degree of conservation. Using neonatal rat liver explants (7) and also rat hepatocytes (8), it has been shown that CPS-I is synthesized initially as a precursor of 165Kd on the cytoplasmic ribosomes which is converted to a 160Kd mature enzyme during or immediately after its transport into mitochondria. In spite of recent studies showing the efficient in vitro transport of a number of matrix proteins into mitochondria from animal cells (reviewed in 9, 10, and 11), previous attempts on the in vitro transport of CPS-I into rat liver mitochondria have been unsuccessful

[1] These results were presented in a thesis submitted to the graduate group of Biochemistry and Biophysics, University of Pennsylvania by NKB.

[2] Abbreviations used: CPS-I, carbamyl phosphate synthetase-I; pCPS-I, precursor of carbamyl phosphate synthetase-I; EDTA, Disodium ethylene diamine tetraacetate; CCCP, carbamyl cyanide m-chlorophenyl hydrazone; SDS, sodium dodecyl sulfate; PMSF, phenylmethyl sulfonyl fluoride; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-p-Tosylamino-2-phenylethylchloromethyl ketone.

0006-291X/84 $1.50

(7,11). An in vitro transport assay system is useful in the elucidation of molecular mechanisms involved in the unidirectional transport of tissue specific proteins such as CPS-I. We have, therefore, purified CPS-I from mouse liver mitochondria and studied its in vitro transport in mouse liver mitochondria.

EXPERIMENTAL PROCEDURES

Isolation of Mitochondria and Purification of CPS-I. For most of the experiments mitochondria were isolated from mouse liver using the sucrose-mannitol buffer as described before (12). For in vitro transport of protein, mitochondria were isolated using a modified Mannitol buffer (10 mM Hepes pH 7.4, 220 mM Mannitol, and 70 mM Sucrose). CPS-I was purified using a procedure previously described for rat liver CPS-I (13) and used for raising antibody in rabbits as described by Marshall and Cohen (14). Monospecific antibody was purified by antigen affinity chromatography (15).

In Vitro Protein Synthesis. Mouse liver poly(A) RNA and membrane free polysomes were translated in a mRNA dependent reticulocyte lysate system (16) using 1 uCi/ul ^{35}S methionine ($>$1000 Ci/mmol). Poly(A) RNA was isolated by poly(U)Sepharose chromatography method (17) and membrane free mouse liver polysomes were isolated using the high salt method of Shore et al. (13).

In Vitro Protein Transport into Mitochondria. The in vitro uptake of ^{35}S labeled proteins by isolated mitochondria was carried out by a modification of the procedure described by Conoboy et al. (18). A typical reaction mixture contained 25 ul reticulocyte lysate containing ^{35}S labeled in vitro translation products (3 to 4 x 10^6 cpm), 160 ug mitochondria isolated in the the modified Mannitol buffer and 60 ul modified Mannitol buffer containing 10 mM L-Methionine. The reaction was run at 27°C for 60 min. Unless otherwise mentioned, the samples were digested with 200 ug/ml of trypsin and chymotrypsin at 4°C for 90 min, mixed with 5 ul of protease inhibitor solution (1 mM PMSF, 10 mM ϵ-amino caproic acid, 3 mM EDTA, 10 ug/ml each of leupeptin, pepstatin, chymostatin, and antipain), and intact mitochondria were pelleted through a cushion of 1 M sucrose, 100 mM KCH_3COO, 20 mM Hepes (pH 7.4), and 0.1% bovine serum albumin by centrifugation at 130,000 x g for 45 min at 2-4°C. The mitochondrial pellet was washed twice with modified Mannitol buffer containing 25 mM EDTA and used for electrophoretic and immunochemical analyses.

Other Procedures. Immunoprecipitation of ^{35}S labeled protein was carried out as described by Kessler (19) using Staphy-A-coated bacterial cells (IgG-Sorb, New England Biolabs) as immunoadsorbants. Protein samples including the immunoprecipitates were dissociated by heating at 90°C for 3 min in 20 to 50 ul modified Laemmli sample buffer (150 mM Tris-HCl pH 6.8, 6 mM EDTA, 10 mM B-mercaptoethanol, and 3% $NaDodSo_4$). Aliquots of clear supernatants were electrophoresed on 8% SDS-polyacrylamide gels and processed for fluorography using EN^{3}HANCE (New England Nuclear) as described before (12). Westernblot analysis was carried out as described by Burnette (20).

RESULTS AND DISCUSSION

With an objective to study the regulation of biosynthesis and mechanisms of transport of tissue specific proteins into mitochondria, we have purified a major hepatic mitochondrial enzyme CPS-I. Because of the labile nature of this enzyme, a rapid purification method described for the rat liver CPS-I was used with some modifications. As shown in Fig. 1A (lane 2), purified CPS-I consists of a nearly homogeneous species which comigrate with the major component of

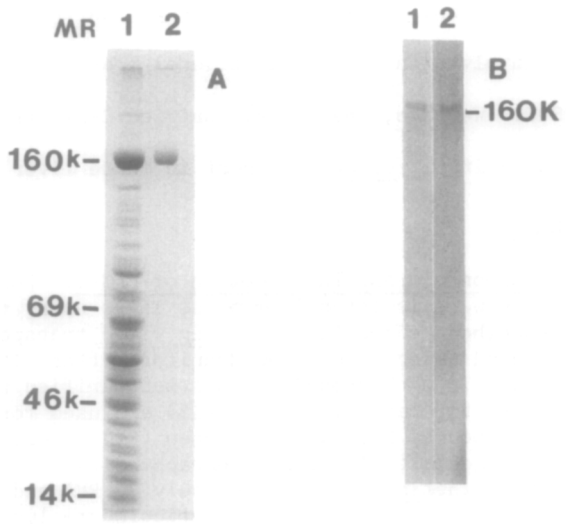

Figure 1: A. Electrophoretic Analysis of Purified CPS-I. Purified CPS-I was electrophoresed on 7.2% polyacrylamide-SDS gels and stained with Coomassie brilliant blue. Lane 1: mouse liver matrix protein (50 ug), and lane 2: purified CPS-I (10 ug).

B. Characterization of anti CPS-I-IgG. Mouse liver mitoplasts (30 ug) and purified mouse liver CPS-I (5 ug) were electrophoresed on 8% SDS-polyacrylamide gels. The proteins were electrophoretically transferred to cellulose nitrate filter and probed with anti CPS-I-IgG followed by ^{125}I protein A (S. aureus) as described by Burnette (20). An autoradiogram of the blot is shown. Lane 1: mosue liver mitoplasts, and lane 2: purified mouse liver CPS-I.

total mitoplast protein with a molecular weight of about 160Kd (Fig. 1A, lane 1). Thus, the purified CPS-I from mouse liver is over 90% pure and has a size identical to that of rat liver CPS-I. The anti CPS-I-IgG gave a single merging precipitin band when purified CPS-I and crude mitochondrial extracts were placed in side by side wells of Ouchterlony agar plates (results not shown) indicating its homogeneity. In Westernblot analysis, the anti CPS-I-IgG reacts with a single species of 160Kd polypeptide out of a large number of total mitoplast proteins (Fig. 1B, lane 1), which comigrates with purified CPS-I (lane 2). These results clearly indicate that the anti CPS-I-IgG is indeed monospecific.

The biosynthesis of CPS-I was studied in a mRNA dependent reticulocyte lysate system. As shown in Fig. 2, the polypeptides synthesized under these in vitro conditions resolve into a relatively large number of components in the size range of 10 to 200Kd. Immunoprecipitation of total translation products with pre-immune sera yields essentially no detectable bands (lane 4)

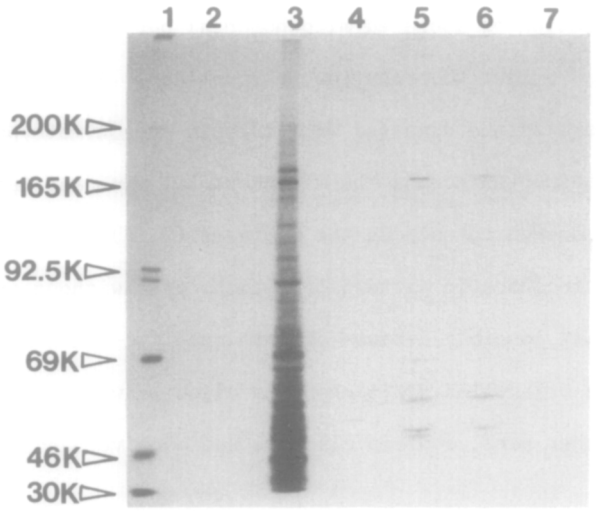

Figure 2: Identification of Putative Precursor of CPS-I in Cell Free Translation Products. The mosue liver free polysomes (200 OD/ml at 260 nm) were translated in a rabbit reticulocyte lysate system using 1 uCi/ul ^{35}S methionine. Aliquots (30 ul) were used for immunoprecipitation with anti CPS-I-IgG. Both the immunoprecipitate and the in vitro translation products (0.5 ul) were electrophoresed on SDS-polyacrylamide gels and processed for fluorography. Lane 1: ^{14}C standard markers, lane 2: in vitro translation products without added free polysomes, lane 3: in vitro translation products with added free polysomes, lane 4: immunoprecipitate using pre-immune sera, lane 5: immunoprecipitate using anti CPS-I-IgG, and lanes 6 and 7: same as lane 5 except that 10 and 30 ug of purified CPS-I, respectively, were added during immunoprecipitation.

while the anti CPS-I-IgG yields a major polypeptide of 165Kd (lane 5). This polypeptide is about 5Kd larger than the purified CPS-I. The 165Kd polypeptide as well as some of the lower molecular weight polypeptides seen in lane 5 are partly (lane 6) and totally (lane 7) competed out by 10 ug and 30 ug, respectively, of purified CPS-I suggesting that the lower molecular weight polypeptides seen in the immunoprecipitate (lane 5) are either incomplete chains or degraded CPS-I polypeptides. The 165Kd species could be detected in the in vitro translation products regardless of whether mouse liver total RNA, poly(A) RNA, or polysomes were used as the source of mRNA. Experiments in our laboratory (not presented here) using intact hepatocytes have shown that the 165Kd species is turned over rapidly with a $t_{\frac{1}{2}}$ of less than 5 min (21) indicating that the 165Kd species is the true precursor of mouse liver CPS-I. In this respect, the biosynthetic mode of mouse liver CPS-I appears to be nearly identical to that reported for the rat CPS-I system (7,8).

The transport of pCPS-I was studied using a reconstituted system which involves the incubation of isolated mitochondrial particles with reticulocyte lysate containing ^{35}S labeled precursor polypeptides. After incubation, mitochondria were separated and digested with trypsin and chymotrypsin to distinguish proteins transported across the mitochondrial membrane from those non-specifically adhered to outside the membrane (9,18). As shown in Fig. 3 (lane 2), the electrophoretic pattern of mitochondria without proteolytic digestion is complex possibly because of both specific and non-specific binding of proteins to the organelles. Mitochondria digested with trypsin and chmyotrypsin, on the other hand, contain discrete radioactive bands (see lane 3).

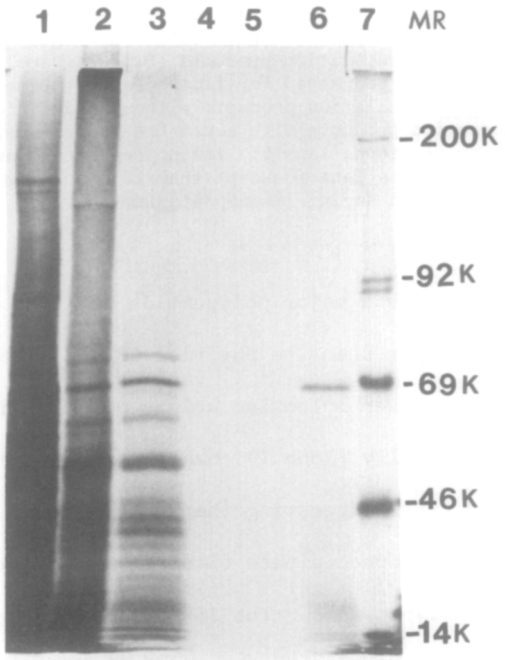

Figure 3: In Vitro Transport of Proteins into Isoalted Mouse Liver Mitochondria. Transport assays using ^{35}S labeled in vitro translation products were run in 85 ul volumes as described under Experimental Procedures. At the end of incubation, mitochondria were separated and processed for electrophoresis either before or after digestion with trypsin/chymotrypsin. Dissociated mitochondrial proteins (100 ug each) were electrophoresed and fluorographed as described in Fig. 2. Lane 1: 2 ul of free polysomal in vitro translation products, lane 2: in vitro incubated mitochondria without proteolytic digestion, lane 3: in vitro incubated mitochondria after proteolytic digestion, lane 4: same as in lane 3 except that mitochondria were lysed with 0.5% Triton X-100 before protease treatment, lane 5: same as in lane 3 except that in vitro translation products were predigested with trypsin/chymotrypsin for 20 min at 2-4° C, lane 6: same as in lane 3 except 12 uM CCCP was added during in vitro incubation, and lane 7: ^{14}C standard markers.

These include a minor band of 160Kd and a number of bands of varied intensities ranging from > 10Kd to about 70Kd. Disruption of mitochondrial membranes by treatment with Triton X-100 renders all of these polypeptides sensitive to proteases (lane 4), confirming that these polypeptides are indeed localized intramitochondrially. Furthermore, negligible amounts of ^{35}S labeled polypeptides are imported when proteolytically degraded in vitro translation products are used for uptake (lane 5) indicating that intact, undegraded polypeptides are essential for the transport. The transport of 160Kd protein, as well as almost all other proteins excepting a protein in the molecular weight range of 67Kd, is completely inhibited by CCCP (lane 6), an uncoupler of oxidative phosphorylation, suggesting the energy dependence of transport.

We have used anti CPS-I-IgG as a discriminating reagent to demonstrate that the 160Kd protein, transported in vitro into mitochondria, is indeed CPS-I. As shown in Fig. 4, lanes 2 and 3 represent electrophoretic patterns of polypeptides associated with in vitro incubated mitochondria before and after proteolytic digestion, respectively. Lane 4 represents the immunoprecipitate, using anti CPS-I-IgG, of reticulocyte lysate translation mixture incubated under the normal reaction conditions without added mitochondria. Nearly 40% of the radioactivity is detected in the pCPS-I band even after 60 min incubation. The remaining 60% of the counts, including a major species of about 45Kd, probably represents the degradation products and/or incomplete chains since they are all effectively competed out by purified CPS-I (lane 5). Immunoprecipitation of postmitochondrial supernatant from a complete incubation mixture (with added mitochondria) also shows the presence of significant levels of pCPS-I indicating that mitochondria, or associated enzymes, do not cause exaggerated degradation of pCPS-I (lane 9). Immunoprecipitation of proteins from in vitro incubated mitochondria without protease digestion (such as the one presented in lane 2) yields both the 165Kd pCPS-I and 160Kd mature CPS-I in addition to a number of low molecular weight degradation products (see lane 6) all of which are competed out by purified CPS-I (lane 7). The immunoprecipitation of proteins from protease digested mitochondria, on

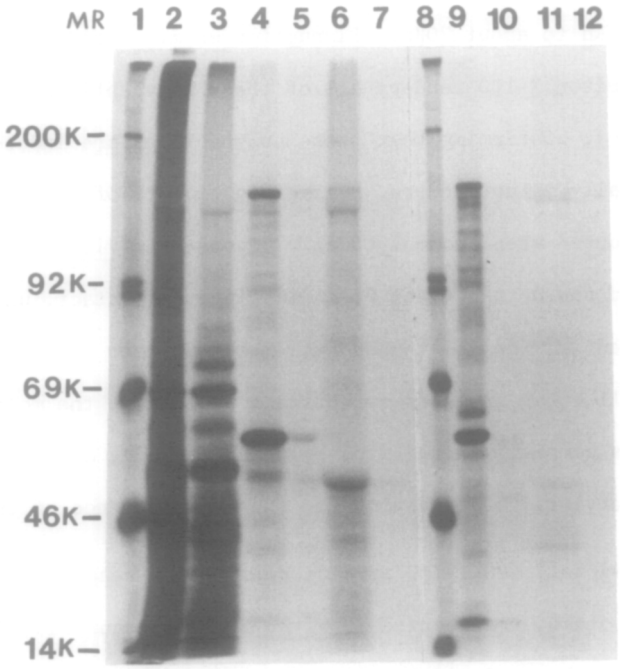

Figure 4: In Vitro Transport and Processing of CPS-I Using Isolated Mitochondria. In Vitro transport experiments were run as described in Fig. 3 and both the mitochondrial pellet and postmitochondrial supernatant fractions were immunoprecipitated with anti CPS-I-IgG. Other details were as described under Experimental Procedures. Lanes 1 and 8: ^{14}C standard markers, lane 2: in vitro incubated mitochondria without protease digestion, lane 3: same as in lane 2 after protease digestion, lane 4: immunoprecipitate of reticulocyte lysate incubated in vitro without added mitochondria, lane 5: same as in lane 4 except that 5 ug of purified CPS-I was added during the immunoprecipitation, lane 6: immunoprecipitate of mitochondrial protein without protease digestion, lane 7: same as in lane 6 but immunocompeted with 10 ug purified CPS-I, lane 9: immunoprecipitate of postmitochondrial supernatant from a complete incubation mixture, lane 10: same as in lane 9 but immunocompeted with 5 ug of purified CPS-I, lane 11: immunoprecipitate of proteins from mitochondria digested with proteases, and lane 12: same as in lane 11 but immunocompeted with 10 ug purified CPS-I.

the other hand, yields only 160Kd band along with some low molecular weight degradation products, without any detectable 165Kd protein (lane 11). The CPS-I and pCPS-I bands from the gel in Fig. 5 were excised and assayed for radioactivity in order to quantitate the % of transport. The immunoprecipitate in lane 4 contained 482 ^{35}S cpm in the pCPS-I band while that in lane 9 contained 360 cpm. The mature CPS-I in lane 11 contained 160 cpm. These results indicate that only about 20-25% of the 165Kd pCPS-I detectable in the incubation reaction is transported into mitochondria under these in vitro conditions. Considering that only about 40% of the immunoprecipitable ^{35}S cpm is present in intact

pCPS-I after 60 min incubation, the actual % of pCPS-I transported may be in the range of 8 to 12%. We would also like to point out that in repeated attempts we have been unable to demonstrate any significant transport/processing of pCPS-I into mitochondria from non-hepatic tissues such as heart, kidney, and brain (unpublished results, ref. 21), suggesting that CPS-I transport is tissue specific

The results presented here indicate that pCPS-I, associated with mitochondria, are localized outside the membrane while the processed CPS-I is localized intramitochondrially in a manner resistant to proteolytic digestion. It has been suggested that binding or precursor molecules to specific sites termed "receptor sites" located on mitochondrial outer membrane or at the junctions of inner and outer membranes may be a prerequisite step for the transport (22). It remains to be seen in pCPS-I binding involves such "receptor sites".

The in vitro system described here for the transport of CPS-I offers many advantages for determining the informational requirements for the protein transport,such as the topogenic sequences (23), receptor sites (22) or the role of "extra sequences" in the precursor molecule. Previous attempts to demonstrate the in vitro transport of CPS-I have been unsuccessful probably because this protein presents several problems uncommon to other matrix proteins which have been shown to be actively transported under in vitro conditions (for a review see 10, 11, and references therein): (i) CPS-I is probably the largest protein transported into mitochondria and its size itself may present a considerable barrier; (ii) it is an abundant enzyme with a very slow turnover rate of 9½ d (1) and therefore it may be transported at very low levels; and (iii) under in vitro conditions both CPS-I and pCPS-I are highly labile and only a fraction of input pCPS-I may be available for the transport under these conditions. Considering all these factors, the relatively low levels of transport of CPS-I observed in the present study may reflect the inherent transport characteristics of this enzyme. In conclusion, we have presented evidence that significant levels of pCPS-I is transported in mouse liver mitochondria in vitro and processed into mature 160Kd CPS-I. The processing appears to involve a single step endo-

proteolytic cleavage of a 5Kd peptide since no other intermediary size species between pCPS-I and mature CPS-I are detected.

Acknowledgements. This research was supported in part by NSF grant PCM 80-22646. The authors are thankful to Dr. Gordon Shore for discussion and advise on the purification of CPS-I and to Nina Leinwand for helping with the preparation of this manuscript.

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