Transport of Proteins into Hepatic and Nonhepatic Mitochondria: Specificity of Uptake and Processing of Precursor Forms of Carbamoyl-Phosphate Synthetase I†

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ABSTRACT: An in vitro system reconstituted with mouse liver polysome translation products was used to study the nature of polypeptide species imported into mitochondria from different mouse tissues such as liver, kidney, brain, and heart, as well as from Ehrlich ascites, Novikoff hepatoma, and Morris hepatoma 3924A tumor lines. Mouse hepatic mitochondria import a number of proteins including 160-kilodalton (kDa) carbamoyl-phosphate synthetase I (CPS-I). Two other proteins of 63 and 57 kDa of unknown function are also imported as major components by mouse liver mitochondria. Under these in vitro conditions, however, mitochondria from non-CPS-I expressing tissues such as brain, kidney, and heart failed to import and process the precursor forms of CPS-I (pCPS-I). Furthermore, mitochondria from three different tumor lines (Novikoff hepatoma, Morris hepatoma, and Ehrlich ascites) containing negligible CPS-I activity were also unable to import and process pCPS-I to any significant level. Similarly, the 63-kDa protein was selectively transported into liver and kidney mitochondria and also into Ehrlich ascites mitochondria at reduced levels, but not into mitochondria from heart and brain. Nevertheless, the 57-kDa protein and a number of proteins of <45 kDa are transported efficiently by all of the mitochondrial types studied. These results provide evidence for tissue- or cell-specific selectivity at the mitochondrial membrane level for the transport of some proteins. The transports of 63- and 57-kDa proteins are differentially inhibited by mouse liver mitochondrial matrix and membrane fractions, respectively. Furthermore, mitochondrial membrane fraction from liver, but not from heart, inhibits the transport of pCPS-I into mouse liver mitochondrial particles. These results suggest that the inability of nonhepatic and tumor cell mitochondria to transport pCPS-I may be due to lack of specific membrane recognition sites.

The majority of mitochondrial proteins are imported into the organelles by a posttranslational mechanism (Hallermayer et al., 1977; Harmey et al., 1977; Schatz, 1979; Neupert & Schatz, 1981; Sabatini et al., 1982; Schatz & Butow, 1983; Hay et al., 1984). These imported proteins are processed to mature subunits and assembled in four different intramitochondrial compartments, i.e., the outer membrane, the intermembrane space, the inner membrane, and the matrix space. The exact mechanism by which these proteins are channeled into various intramitochondrial compartments is not clearly understood (Schatz & Butow, 1983; Hay et al., 1984). Recent experiments of Douglas et al. (1984) and Horwich et al. (1984), however, suggest that the structural information contained in the precursor polypeptide itself plays an important role in the accurate delivery of proteins to the mitochondrial compartment. Furthermore, in the case of proteins belonging to the mitochondrial inner membrane and matrix space, it has been demonstrated that the N-terminal portion of the precursor polypeptide termed "presequence" or "N-terminal extension" is essential for the transport process (Maccacchini et al., 1979b; Schatz, 1979; Schatz & Butow, 1983). It is widely believed that specific interaction of the precursor species possibly involving the presequence with receptor-like components on the mitochondrial surface (Korb & Neupert, 1978; Teintze et al., 1982; Gasser et al., 1982; Ohashi et al., 1982; Schatz & Butow, 1983; Hay et al., 1984) is an obligatory step in the unidirectional translocation of polypeptides across the mitochondrial membrane.

Because of their tissue-specific functional heterogeneity, mammalian mitochondria offer useful systems to understand the molecular mechanisms involved in the acquisition and functional maturation of polypeptides. A number of studies have suggested little or no tissue-specific selectivity at the organelle level for the transport of proteins under both in vitro and in vivo conditions (Miura et al., 1982, 1983; Argan et al., 1983; Horwich et al., 1984). For example, the precursor forms of the hepatic mitochondria specific enzyme OCT[1] are efficiently transported to mitochondria from non-OCT expressing tissues, such as heart and kidney, and processed into proteins electrophoretically similar to the native enzyme (Argan et al., 1983; Shore et al., 1983; Miura et al., 1983). Furthermore, introduction of cloned OCT cDNA, containing the SV40 regulatory sequences, into non-OCT expressing HeLa cells results in the accumulation of OCT-like protein and associated enzyme activity in the mitochondrial fraction (Horwich et al., 1984). In contrast to these observations suggesting no apparent discrimination at the level of transport, recent experiments of Matocha & Waterman (1984) showed that only mitochondria from adrenal cells, but not from heart and liver, take up and process the precursor forms of cytochrome P-450 scc under in vitro conditions. Recently, we have shown that precursors

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TPCK, L-1-(*p*-tosylamino)-2-phenylethyl chloromethyl ketone; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; CPS-I, carbamoyl-phosphate synthetase I (ammonia) (EC 6.3.4.16); pCPS-I, precursor of carbamoyl-phosphate synthetase I; LES, Lettre Ehrlich ascites mouse tumor cells; OCT, ornithine carbamoyltransferase; pOCT, precursor of ornithine carbamoyltransferase.

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of the hepatic tissue specific enzyme carbamoyl-phosphate synthetase I (pCPS-I) are imported and processed into mature enzyme by hepatic mitochondria under in vitro conditions (Bhat & Avadhani, 1984). With a view to further understand the transport of tissue-specific proteins, in this paper we have compared the nature of polypeptides transported into hepatic and nonhepatic mitochondria and report a discriminatory transport and processing of the hepatic mitochondria specific enzyme CPS-I.

Experimental Procedures

Isolation of Mitochondria and Submitochondrial Fractions. Mitochondria were isolated from mouse liver, brain, kidney, heart, and LES cells by differential centrifugation in a modified sucrose–mannitol buffer system (220 mM D-mannitol, 70 mM sucrose, 20 mM Hepes, 3 mM Tris, pH 7.4) as described before (Bhat et al., 1982). Mitoplasts were prepared by the digitonin fractionation method (Schneitman & Greenawalt, 1968; Bhat et al., 1982). Both mitochondria and mitoplasts were washed twice with mitochondrial isolation buffer and used for the in vitro uptake studies. The inner membrane and matrix fractions were prepared from mitoplasts by hypotonic swelling followed by sonication (Pedersen et al., 1978). Briefly, mitoplasts were suspended in 10 mM Tris-HCl (pH 8.5) at 30 mg/mL and incubated at 4 °C for 15 min. The suspension was pulse-sonicated twice (in Branson sonifier at 20% output) for 30 s. The matrix fraction was separated from the inner membrane fraction by centrifugation at 160000*g* for 60 min at 4 °C. The inner membrane fraction was washed once with 10 mM Tris-HCl (pH 8.5) and pelleted as described above. Both the inner membrane and the matrix fractions were stored in 100-μL aliquots at −70 °C. The extent of cross-contamination in the inner membrane and matrix fractions was estimated by assaying CPS-I activity and cytochrome oxidase activity (Pedersen et al., 1978), respectively. Microsomes and cytosol fractions were prepared from postmitochondrial supernatant by differential centrifugation (Niranjan & Avadhani, 1980).

Enzyme Purification and Antibody Preparation. CPS-I was purified from mouse liver mitochondrial matrix, as described previously (Bhat & Avadhani, 1984). Purified CPS-I was used to raise antibody in rabbits, and monospecific anti-CPS-I–IgG was prepared by antigen affinity chromatography (Bhat & Avadhani, 1984). Antibody to rat liver CPS-I was a generous gift of Dr. Gordon Shore, McGill University, Montreal, Canada.

Isolation of Free Polysomes. Free polysomes from mouse liver were isolated according to the method of Raymond & Shore (1981). The buffer system for polysome isolation consisted of 250 mM sucrose, 50 mM Tris–acetate (pH 8.5), 10 mM $Mg(CH_3COO)_2$, 150 mM $K(CH_3COO)$, and 7 mM 2-mercaptoethanol. Polysome pellets were rinsed with, and suspended in, buffer containing 150 mM $K(CH_3COO)$, 20 mM Hepes (pH 7.6), 5 mM $Mg(CH_3COO)_2$, and 0.1 mM dithiothreitol at a final concentration of 10 mg/mL (11 OD units at 260 nm = 1 mg) and stored in liquid N_2.

Posttranslational Uptake of Proteins into Mitochondria. Polysomes were translated in micrococcal nuclease treated rabbit reticulocyte lysate prepared according to Pelham & Jackson (1976), with optimal concentrations of K^+ (120–125 mM) and Mg^{2+} (1.4–1.6 mM) ions in the presence of 1 μCi/μL [^{35}S]-L-methionine (>1000 Ci/mmol). After 60 min of incubation, protein synthesis was terminated by mixing with 300 μg/mL cycloheximide and 3 mg/mL unlabeled L-methionine. Aliquots were used for the uptake studies as required. One milliliter of in vitro translation products with added free polysomes contained 150 × 10^6 trichloroacetic acid insoluble ^{35}S protein cpm. The in vitro uptake of translation products into mitochondria or mitoplasts was carried out by a modification of the procedures described for yeast (Maccacchini et al., 1979a,b; Gasser et al., 1982) and *Neurospora crassa* (Zimmerman et al., 1979). A typical reaction mixture contained 25 μL of reticulocyte lysate containing translation products [(3–4) × 10^6 cpm], 160–200 μg of mitochondria or mitoplasts, and 10 mM methionine in 60 μL of modified sucrose–mannitol buffer. The reaction was carried out at 27 °C for 60 min as required. At the end of incubation, samples were chilled and treated with 200 μg/mL each of trypsin and chymotrypsin and mixed end-over-end at 4 °C for 120 min. After the protease treatment, samples were mixed with 5 μL of protease inhibitor solution A (1 mM PMSF, 10 mM ε-aminocaproic acid, 3 mM EDTA, 10 mg/mL each of leupeptin, pepstatin, chymostatin, and antipain, 0.1 mg/mL each of TPCK and TLCK, and 0.25 mg/mL aprotinin), and intact mitochondria were pelleted through a sucrose cushion (1 M sucrose, 0.1 M $K(CH_3COO)$, 0.02 M Hepes, pH 7.4, 1% bovine serum albumin, and protease inhibitor solution A) by centrifugation at 130000*g* for 45 min at 2 °C (McAda & Douglass, 1982). In some experiments, mitochondria were pelleted through a sucrose cushion as described above without protease treatment. The pellets were suspended in modified sucorse–mannitol buffer containing 25 mM EDTA, 2 mM methionine, and protease inhibitor solution A and were pelleted at 13000*g* for 10 min at 2 °C. Mitochondria were washed twice with the same medium, and pellets were lysed in 100 μL of buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mM PMSF, 1% Triton X-100, 0.1% $NaDodSO_4$, and 0.5% sodium deoxycholate. Aliquots of each sample were used for $NaDodSO_4$–PAGE, for immunoprecipitation, or for determination of trichloroacetic acid insoluble protein cpm as described before (Bhat & Avadhani, 1984). In experiments involving the effects of inhibitors and of subcellular fractions, mitochondria were first incubated with these agents for 10 min and then mixed with reticulocyte lysate containing in vitro translation products and incubated further for 60 min at 27 °C. After the incubation, mitochondria were pelleted and processed as described above.

Immunoprecipitation. Samples for immunoprecipitation were prepared as follows: (a) mitochondria were solubilized in immunoprecipitation buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA fortified with protease inhibitor solution A (2 mM PMSF, 50 mM ε-aminocaproic acid, 1 μg/mL each of leupeptin, pepstatin, antipain, and chymostatin, and 10 μg/mL each of TPCK and TLCK), 20 mM methionine, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% $NaDodSO_4$, and 0.02% NaN_3; (b) in vitro translation products were diluted 4-fold with buffer containing 100 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 20 mM methionine, protease inhibitor solution A (as described above), and 0.04% NaN_3. In both cases, insoluble materials were removed by centrifugation at 233000*g* for 2–3 h at 2 °C. The supernatants were used for immunoprecipitation as described by Kessler (1975). The immunoprecipitates bound to *Staphylococcus aureus* cell suspension (IgGSorb, Enzyme Center) were pelleted through a sucrose cushion (1 M sucrose, 0.025 M Tris-HCl, pH 7.4, 5 mM EDTA, 0.5% Triton X-100, and protease inhibitor solution A) to remove nonspecific contaminants. The IgGSorb pellet was washed 3 times with buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 0.1% $NaDodSO_4$, and 0.02% NaN_3. The pellets were suspended in 50 μL of modified Laemmli (1970) sample buffer (150 mM

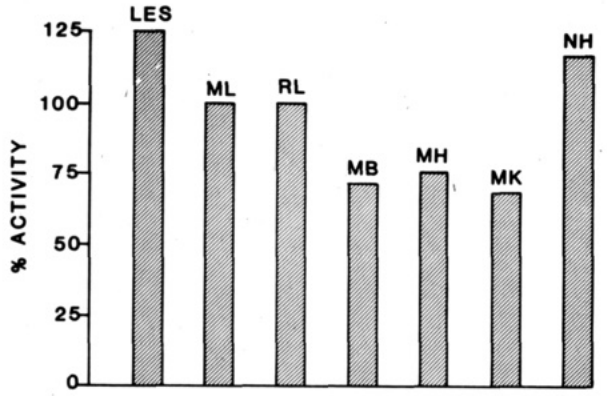

FIGURE 1: Relative activity of hepatic, nonhepatic, and tumor mitochondria for in vitro uptake. The polypeptide uptake activity into different mitochondria was determined by incubating mouse liver free polysome directed in vitro translation products (4.0×10^6 cpm) with mitochondria (2 mg/mL) from hepatic, nonhepatic, and tumor cells. The details of incubation, protease digestions, and separation of mitochondria through a sucrose cushion were as described under Experimental Procedures. The mitochondrial pellet was lysed, and aliquots were used for the determination of hot trichloroacetic acid insoluble ^{35}S-labeled protein cpm. The extent of uptake in mouse liver mitochondria (8.0×10^5 cpm/mg) was taken as 100% activity. LES, Les mitochondria; ML, mouse liver mitochondria; RL, rat liver mitochondria; MB, mouse brain mitochondria; MH, mouse heart mitochondria; MK, mouse kidney mitochondria.

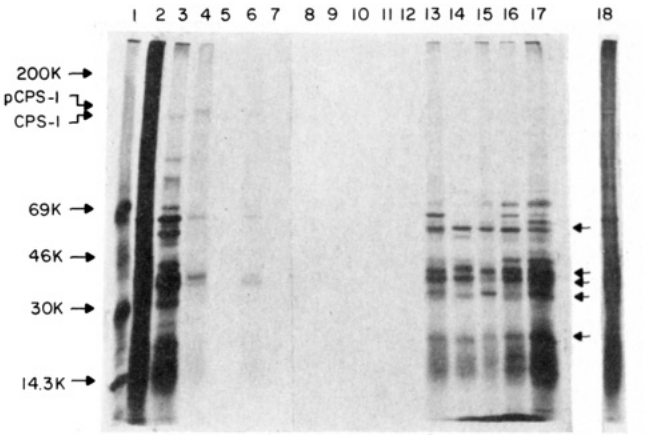

FIGURE 2: Electrophoretic comparison of polypeptides transported in vitro into mitochondria from hepatic, nonhepatic, and tumor cells. In vitro incubation was carried out as described in Figure 1. Portions of protease-digested mitochondria (40 μg) or mitochondrial proteins immunoprecipitated with anti-CPS-I–IgG were analyzed by 7.2% NaDodSO$_4$–PAGE. In cases where polysome translation products were incubated without added mitochondria, the protein samples were used for either electrophoresis or immunoprecipitation, without digestion with proteases. After electrophoresis, the gel was stained with Coomassie brilliant blue, destained, and processed for fluorography as described under Experimental Procedures. Lanes 1–17 were exposed to X-ray film for 4 days, and lane 18 was exposed for 12 h. (Lane 1) ^{14}C-Labeled molecular weight markers; (lane 2) mouse liver free polysome translation products; (lane 3) in vitro incubated mouse liver mitochondria; (lane 4) polysome translation products incubated as in lane 3 without added mitochondria immunoprecipitated with anti-CPS-I–IgG; (lane 5) same as in lane 4 except that 10 μg of purified CPS-I added during immunoprecipitation; (lane 6) sample as in lane 3 immunoprecipitated with anti–CPS-I–IgG; (lane 7) same as in lane 6 except that 10 μg of purified CPS-I added during immunoprecipitation; (lanes 13–17) 40 μg of in vitro incubated mitochondria from mouse kidney, mouse brain, mouse heart, Novikoff hepatoma, and LES, respectively; (lanes 8–12) proteins immunoprecipitated from 400 μg each of in vitro incubated kidney, brain, heart, Novikoff hepatoma, and LES mitochondria, respectively, with anti-CPS-I–IgG; (lane 18) a shorter exposure (12 h) of lane 2 to X-ray film.

Tris-HCl, pH 6.8, 6 mM EDTA, 6% w/v NaDodSO$_4$, 20% v/v glycerol, 10% v/v 2-mercaptoethanol, and 0.001% w/v Bromophenol blue) and boiled for 3 min at 95 °C. Aliquots of supernatant were analyzed by NaDodSO$_4$–PAGE or used for the determination of hot trichloroacetic acid insoluble protein cpm.

Other Procedures. Protein content was estimated by the method of Lowry et al. (1958). Details of the NaDodSO$_4$–PAGE and fluorography were as described before (Bhat et al., 1982; Bhat & Avadhani, 1984).

RESULTS

In Vitro Transport of Polypeptides into Mitochondria. To determine the qualitative and quantitative nature of protein transport into mitochondria from different tissues, we have studied the uptake of mouse liver free polysome directed in vitro translation products into mitochondria from hepatic and nonhepatic tissues (i.e., brain, kidney, and heart) as well as from Lettre Ehrlich ascites tumor cells and Novikoff hepatoma cells. The relative amounts of labeled polypeptides (total radioactivity per milligram of mitochondrial protein) taken up by mitochondria from different tissues are presented in Figure 1. It is seen that the uptake activity is similar in mitochondria from both mouse and rat hepatic tissues, whereas the uptake activity is 20–30% higher in mitochondria from LES and Novikoff hepatoma tumor cells. Although not shown, mitochondria from another rat hepatic tumor, i.e., Morris hepatoma 3924A, take up relatively high levels of radiolabeled polypeptides comparable to LES. On the other hand, mitochondria from mouse brain, kidney, and heart show considerably lower uptake activities compared to those of hepatic mitochondria.

Tissue Specificity in the Transport of Polypeptides. The electrophoretic patterns of [^{35}S]methionine-labeled polypeptides imported into mitochondria from various mouse tissues and tumor cells are shown in Figure 2. As shown in lane 3, mouse liver mitochondria take up a number of heterogeneous-size proteins including a large species of 160 kDa. As previously reported from our laboratory (Bhat & Avadhani, 1984), this 160-kDa species is the mature form of CPS-I since it is quantitatively immunoprecipitated with anti-CPS-I–IgG. Also, this immunoprecipitable 160-kDa polypeptide is about 5 kDa smaller than the putative pCPS-I immunoprecipitated from total polysome translation products (lane 4). The immunoprecipitates from both total polysome translation products (lane 4) and in vitro incubated mitochondria (lane 6) also contain a number of smaller polypeptides that are effectively competed out by purified CPS-I (lanes 5 and 7), suggesting that they represent incomplete polypeptide chains or proteolytic products (Bhat & Avadhani, 1984). Due to extreme sensitivity of CPS-I to proteolytic attack (Clarke, 1976; Shore et al., 1979), various in vitro incubations tend to cause degradation of these large molecules. In all of the experiments included in this paper, therefore, we have used immunocompetition with purified 160-kDa CPS-I as a criterion to determine that the radiolabeled polypeptides of >20–100 kDa immunoprecipitable with anti-CPS-I–IgG are derived from CPS-I or pCPS-I.

The ^{35}S-labeled mouse liver free polysome translation products imported by kidney (lane 13), heart (lane 14), brain (lane 15), Novikoff hepatoma (lane 16), and Ehrlich ascites tumor cell (lane 17) mitochondria under the in vitro conditions indicate distinct qualitative differences. For example, the 160-kDa species immunoprecipitable with anti-CPS-I–IgG (lanes 3 and 6) is not imported at detectable levels by all of the three nonhepatic mitochondria, Novikoff hepatoma mitochondria, and also Ehrlich ascites mitochondria. Similarly, use of even 10 times the amount of in vitro incubated mitochondria as that of liver mitochondria (lane 6) for immuno-

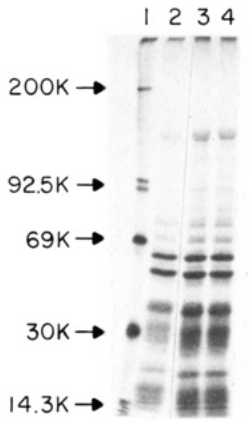

FIGURE 3: Effects of mouse brain and kidney mitochondria on the uptake of pCPS-I and the 63-kDa protein by hepatic mitochondria. In vitro incubation was carried out as described in Figure 1, with 2 mg/mL mouse liver mitochondria, with or without added mouse brain or mouse heart mitochondria (0.6 mg/mL in each case). Mitochondria from a total of 25 μL of reaction mixture were digested with protease, pelleted through sucrose, solubilized in $NaDodSO_4$ containing buffer, and electrophoresed on an 8% polyacrylamide gel as described under Experimental Procedures. (Lane 1) ^{14}C-labeled molecular weight marker; (lane 2) in vitro incubated mouse liver mitochondria; (lane 3) same as in lane 2, with added mouse brain mitochondria; (lane 4) same as in lane 2 with added mouse heart mitochondria.

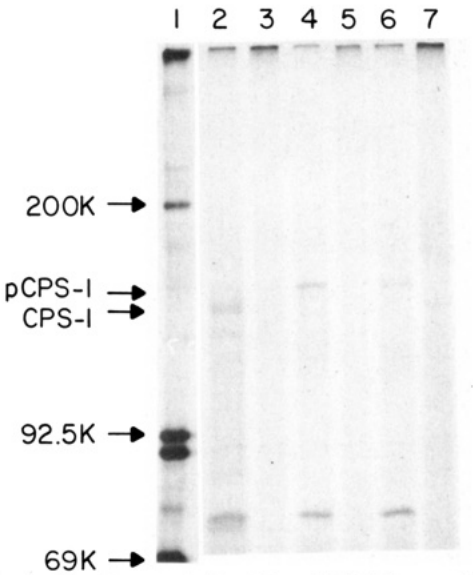

FIGURE 4: In vitro transport of rat liver CPS-I into rat liver and hepatoma mitochondria. Rat liver free polysome translation products were incubated with rat liver and rat hepatoma mitochondria as described in Figure 1. The mitochondrial pellet was protease-digested and used for immunoprecipitation with anti-CPS-I–IgG, whereas the postmitochondrial supernatant fractions were immunoprecipitated without protease treatment. (Lane 1) ^{14}C-labeled markers; (lanes 2, 3, and 5) immunoprecipitates of in vitro incubated rat liver mitochondrial protein (50 μg), Novikoff hepatoma mitochondrial protein (200 μg), and Morris hepatoma mitochondrial protein (200 μg), respectively; (lanes 4 and 6) immunoprecipitates of 100-μL postmitochondrial supernatant fractions from Novikoff hepatoma and Morris hepatoma 3924A mitochondrial incubations; (lane 7) immunoprecipitate of 40 μg of mouse liver mitochondria.

precipitation with anti-CPS-I–IgG does not yield a detectable 160-kDa band in all of these five cases (lanes 8–12), suggesting that nonhepatic mitochondria as well as the tumor mitochondria used in the present experiments are unable to transport CPS-I under the in vitro conditions. In addition, a 63-kDa protein, which is a major imported species in hepatic mitochondria (lane 3), is also imported at varied levels by kidney, Novikoff hepatoma, and LES mitochondria (lanes 13, 16, and 17). However, this protein is not imported at any significant level by heart and brain mitochondria (lanes 14 and 15). In contrast to a possible tissue specificity for the import of 160-kDa CPS-I and 63-kDa species of unknown function, polypeptides such as the 57 kDa and 43 kDa, as well as a number of other small molecular weight proteins as indicated by the arrows (see Figure 2), are transported into mitochondria of all species studied.

Although not shown, postmitochondrial supernatant fractions of in vitro incubations with kidney, brain, heart, Ehrlich ascites, and Novikoff hepatoma mitochondria contain distinct 165-kDa pCPS-I immunoprecipitable with anti-CPS-I–IgG. These results indicate that the inability of the three nonhepatic and two tumor cell mitochondria to import significant levels of CPS-I is not due to rapid degradation of precursor molecules during in vitro incubation. Furthermore, as shown in Figure 3, addition of mouse brain or heart mitochondria to the in vitro reaction mixture did not lower the levels of uptake of 160-kDa CPS-I and the 63-kDa protein by mouse liver mitochondria (lanes 3 and 4), suggesting that the undetectable levels of transport of these proteins into nonhepatic mitochondria are not due to the presence of some unknown inhibitory factors.

The inability of Novikoff hepatoma mitochondria to transport pCPS-I was further verified with Morris hepatoma 3924A. Since both of these tumor lines are of rat hepatic origin, in vitro translation products directed by rat liver free polysomes were used in this experiment. It is seen from Figure 4 (lanes 2 and 7) that both rat liver and mouse liver mitochondria are able to import and process pCPS-I into 160-kDa CPS-I. In contrast, mitochondria from both Novikoff hepatoma (lane 3) and hepatoma 3924A (lane 5) are unable to import immunodetectable levels of pCPS-I, although postmitochondrial supernatant fractions from these incubations contain significant levels of pCPS-I (lanes 4 and 6). These results indicate that mitochondria from Novikoff hepatoma and hepatoma 3924A, which lack significant CPS-I activity (Lawson et al., 1975), have lost the ability to import CPS-I.

Effects of Submitochondrial Fractions on the in Vitro Transport of Proteins. The effects of mitochondrial membrane and the matrix fractions on the transport of tissue-specific proteins such as CPS-I, the 63-kDa species, and also the general proteins transported into all of the mitochondrial types (57 kDa, 43 kDa, etc.) were studied in order to determine the nature and location of recognition signals for the transport process. The inner membrane preparations, here after termed membrane fraction, used in these experiments contained significant (10–15%) contaminating outer membrane fraction, but negligible matrix-specific enzyme CPS-I. Similarly, the matrix fraction contained less than 1% of inner membrane specific marker enzyme cytochrome *c* oxidase. The inability of mitochondrial inner membrane and matrix fractions to convert pCPS-I to 160-kDa CPS-I was tested with mouse liver free polysome translation products as the source of precursor proteins. As shown in Figure 5, mouse liver mitochondrial membrane fraction is unable to process pCPS-I to a significant level (lanes 5 and 7), whereas matrix fractions from both mouse liver and rat hepatoma 3924A mitochondria were able to convert significant amounts of pCPS-I to 160-kDa mature CPS-I (lanes 3 and 4). The immunoprecipitates in these two lanes also contain a number of minor protein bands that may be attributed to the proteolytic activity known to be present in the mitochondrial matrix fraction (Desautels & Goldberg, 1982). The results on the conversion of pCPS-I to CPS-I by the matrix protein are in agreement with previous studies in yeast, *Neurospora*, and rat liver (McAda & Douglas, 1982; Miura et al., 1982; Morita et al., 1982; Cerletti et al., 1903;

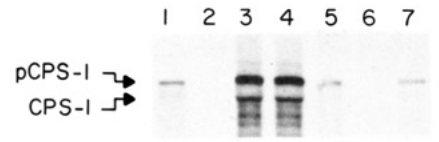

FIGURE 5: In vitro processing of pCPS-I by hepatic submitochondrial fractions. The membrane and matrix fractions were isolated from digitonin-treated mouse and rat liver mitochondria. ^{35}S-Labeled mouse liver polysome translation products (205000*g* supernatant) were incubated with either mitochondrial membrane (2.2 mg of protein/mL of translation product) or matrix (2.2 mg of protein/mL) at 30 °C for 30 min. The control polysomes (5×10^4 cpm) and polysomes treated with mitochondrial membrane (5×10^4 cpm) or matrix (5×10^5 cpm) fractions were immunoprecipitated with anti-CPS-I–IgG and electrophoresed on 7.2% NaDodSO$_4$–PAGE. (Lane 1) Control polysome translation products; (lane 3) translation products incubated with mouse liver matrix; (lane 4) translation products incubated with hepatoma 3924A mitochondrial matrix; (lane 2) duplicate sample from lane 3 immunoprecipitated in the presence of 10 μg of purified CPS-I; (lanes 5 and 7) translation products treated with mouse liver mitochondrial membrane; (lane 6) identical sample as in lane 5 immunoprecipitated in the presence of 10 μg of purified CPS-I.

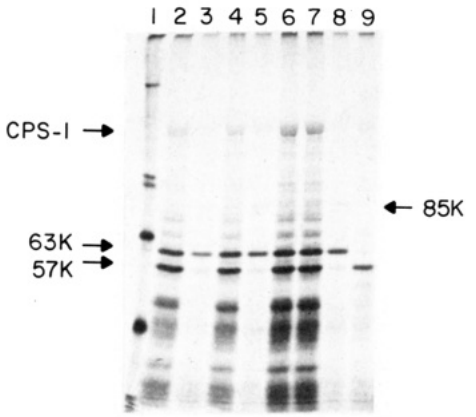

FIGURE 6: Effects of mouse liver submitochondrial fractions on the in vitro transport of proteins into mouse liver mitochondria. The effects of the membrane and matrix fractions from mouse liver mitoplasts on the in vitro uptake of polypeptides into mouse liver mitochondria were studied as described under Experimental Procedures. Protein (100 μg), solubilized from protease-digested mitochondria, was analyzed on 7.2% NaDodSO$_4$–PAGE and subjected to fluorography. (Lane 1) ^{14}C-Labeled standard markers; (lanes 2, 4, 6, and 7) mitochondria incubated for 15, 30, 45, and 60 min in the absence of added matrix fractions; (lanes 3, 5, and 8) mitochondria incubated for 15, 30, and 60 min in the presence of added matrix fraction (1.5 mg of protein/mL of reaction volume); (lane 9) mitochondria incubated for 60 min in the presence of added membrane fraction (1.5 mg of membrane protein/mL of reaction volume).

Schmidt et al., 1983; Bhoni et al., 1983) systems, which demonstrated the occurrence of "processing enzyme" activity in the mitochondrial matrix fraction. Furthermore, the results also show that the inability of hepatoma 3924A and Novikoff hepatoma mitochondria to import CPS-I is not due to lack of processing activity.

As shown in Figure 6, addition of mouse liver matrix results in the inhibition of total uptake of ^{35}S-labeled polypeptides by mouse liver mitochondria (lanes 3, 5, and 8) as against steadily increasing uptake up to 60 min of incubation by control mitochondria without added matrix (lanes 2, 4, 6, and 7). The inhibition by matrix fraction is reflected in terms of a drastically reduced number of polypeptide components and also a lowered intensity of the majority of the polypeptides (Figure 6), including the CPS-I and 57-kDa species. The observed inhibition of in vitro transport by the matrix may be due to processing and removal of N-terminal portions of the precursor molecule, which is essential for the transport (Schatz & Butow, 1983; Hay et al., 1984), or segments of mature

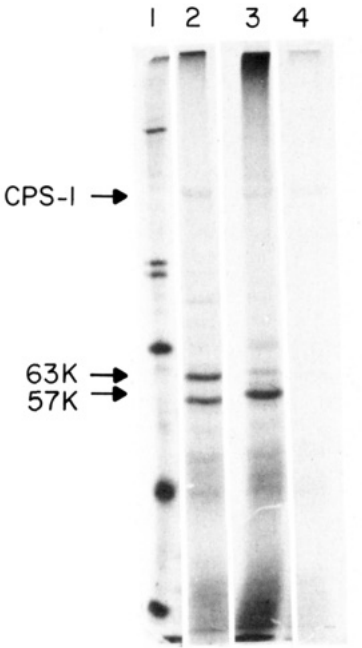

FIGURE 7: Effects of mouse heart mitochondrial membrane on the in vitro uptake of polypeptides by mouse liver mitochondria. Mouse liver mitochondria were incubated with mouse liver free polysome translation products in the presence or absence of heart mitochondrial membrane fraction. Other details are as in Figure 6. Mitochondrial protein (60 μg) was analyzed by 7.2% NaDodSO$_4$–PAGE and fluorography. (Lane 1) ^{14}C-Labeled molecular weight markers; (lane 2) control mouse liver mitochondria; (lane 3) mouse liver mitochondria with added heart mitochondrial membrane; (lane 4) duplicate sample as in lane 3 immunoprecipitated with anti-CPS-I–IgG.

polypeptides present in the matrix fraction may compete for the same precursor binding site as shown for crytochrome *c* (Zimmerman & Neupert, 1980; Matsuura et al., 1981). It is also seen from Figure 5 that the polypeptide species least affected by the matrix fraction is the 63-kDa protein, whose intensity steadily increased with time (lanes 3, 5, and 8). Surprisingly, addition of mouse liver mitochondrial membrane also severely inhibited (80%) the transport of most of the proteins including CPS-I, the 63-kDa protein, and all of the proteins of <50 kDa (see Figure 6, lane 9). In contrast to the inhibition caused by the matrix fraction (lane 8), however, the membrane fraction had the least effect on the transport of 57-kDa protein (see lane 9). Although not shown, mouse heart matrix fraction inhibits the transport of almost all of the proteins excepting the 57-kDa species to varied degrees, as shown for the liver matrix. However, a somewhat different picture emerges with the addition of the membrane fraction from mouse heart mitochondria. As seen from Figure 7 (lanes 2 and 3), mouse heart membrane had little effect on the transport of 160-kDa CPS-I and 57-kDa species, although it inhibited the transport of a number of other proteins including the 63-kDa protein. These results show that the mitochondrial matrix fractions inhibit the transport of 57-kDa polypeptides. Although the membrane fractions from both of the sources inhibit the transport of 63-kDa protein, commonly taken up by mitochondria from all tissues, only the liver mitochondrial membrane inhibits the transport of hepatic mitochondrial specific enzyme CPS-I.

Discussion

Recent studies in our laboratory showed that mouse liver mitochondria can import and process a number of proteins including pCPS-I under in vitro conditions (Bhat, 1982; Bhat & Avadhani, 1983, 1984). The in vitro transport of almost

all of the proteins was energy-dependent since uncouplers of oxidative phosphorylation like carbamoyl cyanide *m*-chlorophenylhydrazine inhibit the uptake (Bhat & Avadhani, 1983, 1984). In the present study, this in vitro system was exploited to verify if there is any tissue-specific discrimination for the in vitro transport of proteins at the organelle level. The in vitro system used in this study is specific in that the patterns of polypeptides taken up by mitochondria from a given tissue type are reproducible. Nevertheless, between experiments there is 10–30% variation in the relative intensities of bands particularly in the high molecular weight range of >85 kDa. As pointed out before (Bhat & Avadhani, 1984), the extent of pCPS-I transport is considerably improved by using optimal in vitro incubation conditions (Bhat & Avadhani, 1984) and freshly isolated polysomes and mitochondria. All of the polypeptides transported in vitro into mouse liver mitochondria comigrate with mitochondrial proteins labeled under in vivo conditions (Bhat, 1982), suggesting that the in vitro transport system is specific.

The results presented in Figures 1 and 2 reveal several qualitative and quantitative differences in the in vitro uptake of polypeptides by mitochondria from different tissues. In the present study we have focused special attention to hepatic mitochondria specific enzyme CPS-I and two other proteins of 63 kDa and 57 kDa, which represent the major imported species in mouse liver mitochondria (see Figure 2). Although the functional nature of 63- and 57-kDa species remains unknown, experiments on the subfractionation of metabolically labeled hepatic mitochondria (Bhat, 1982) indicate that the 63-kDa protein is an inner membrane *ectopic* protein, while the 57-kDa species is a soluble matrix protein. The results presented in this paper indicate that pCPS-I is transported at significant levels into both mouse and rat hepatic mitochondria but not into mitochondria from CPS-I nonexpressing tissues like brain, heart, and kidney (Figure 2). Furthermore, Ehrlich ascites tumor cell mitochondria, which show 20–30% higher in vitro transport activity (Figure 1), fail to import detectable levels of pCPS-I (see Figure 2). A distinct 160-kDa ^{35}S-labeled protein can be immunoprecipitated from 40 μg of in vitro incubated mouse liver mitochondria (Figure 2, lane 6) with anti-CPS-I–IgG. However, use of even 10 times the amounts of in vitro incubated mitochondria (400 μg) from brain, heart, and kidney, as well as Ehrlich ascites cells, does not yield an immunodetectable CPS-I band. It is also surprising that mitochondria from two different rat hepatomas, i.e., Novikoff hepatoma and Morris hepatoma 3924A, are unable to transport detectable pCPS-I (Figure 4). Both of these tumor lines contain negligible CPS-I activity (Lawson et al., 1975), and also, one of them, i.e., hepatoma 3924A, has been shown to contain undetectable CPS-I mRNA (Ryall et al., 1984). Nevertheless, the matrix fractions from these sources contain enzyme activity to process pCPS-I to CPS-I. The precise reasons for the inefficient uptake of pCPS-I by these hepatoma mitochondria remain unknown at this time, although in vitro competition experiments of the type shown in Figures 6 and 7 (results not presented) appear to implicate an altered membrane property as the possible cause.

The tissue specificity for the in vitro transport of certain proteins like CPS-I was further supported by observation that a 63-kDa polypeptide, which is imported as a major component by liver and kidney mitochondria and as a minor component by Ehrlich ascites mitochondria, is not imported at a significant level by heart and brain mitochondria. In contrast, however, a matrix protein of 57 kDa is transported efficiently in all of the mitochondrial types studied. The results of these experiments indeed demonstrate that there is tissue-specific discrimination at the level of mitochondrial membranes for the transport of certain proteins. In principle, these observations on tissue specificity are in agreement with the results of Matchoa & Waterman (1984) showing the in vitro transport of precursor forms of cytochrome P-450 scc only by adrenal cortex mitochondria but not by mitochondria from heart and liver.

It has been suggested that mitochondrial membranes contain "receptor"-like binding sites (Henning et al., 1983; Hay et al., 1984). Some studies have also shown that specific binding of precursor proteins to the membrane "receptor sites" is an obligatory step for the transport process (Zwizinski et al., 1983; Chien et al., 1984). Though the precise location of the receptors remains unknown, studies of Neupert and his colleagues and Schatz and his colleagues (Henning et al., 1983; Daum et al., 1982) have indicated that they may be located on the inner and outer membrane junctions. Initial experiments in our laboratory showed that digitonin-treated mitochondria containing about 10–15% outer membrane fragments were able to transport nearly 85–90% of the proteins (including CPS-I) transported by mitochondria with intact outer membrane (Bhat, 1982). We have, therefore, used the membrane and matrix fractions from digitonin-treated mitochondria in competition experiments aimed at understanding the molecular basis of the observed tissue specificity for the transport of CPS-I and the 63-kDa protein. Specific comparisons and observations emerging from these competition experiments can be summarized as follows. (1) The results showing that the membrane fraction from mouse liver but not from mouse heart mitochondria inhibit or compete for the in vitro transport of pCPS-I into hepatic mitochondria suggest that hepatic mitochondrial membrane may contain sites for binding pCPS-I. Similarly, although not shown here, membrane fractions from both of the hepatoma mitochondria did not inhibit the pCPS-I transport into hepatic mitochondria, suggesting the possible loss of binding sites in these tumor cells. (2) The results allow an interesting comparison between the mode of import of pCPS-I and the 57-kDa species. Although both of these are matrix proteins, the transport of CPS-I but not that of 57-kDa species is inhibited by the liver mitochondrial membrane fraction. Since CPS-I is a hepatic mitochondrial specific polypeptide and the 57-kDa protein is common to all mitochondrial types studied, these results raise the distinct possibility that separate modes of recognition may be involved in the transport of these proteins. (3) Our results also suggest that the transport of the other tissue-specific proteins (63-kDa species) may involve a somewhat different mechanism as that of CPS-I since its transport is inhibited by membrane fractions of both liver and heart mitochondria. (4) The differential effects of inner membrane and matrix fractions on the transport of 63- and 57-kDa species also suggest that these two proteins may be transported by two independent mechanisms.

In summary, results presented in this paper show that there is tissue-specific discrimination at the mitochondrial level for the in vitro transport of at least two proteins, i.e., CPS-I and a 63-kDa protein of unknown function. The inability of nonheaptic and tumor cell mitochondria to import pCPS-I may be due to the possible absence of proper binding sites in these organelles. The requirement for a unique mitochondrial membrane site as indicated above may be the special feature of the pCPS-I transport system and may not be applicable to the transport of other tissue-specific proteins like pOCT, which is efficiently imported into mitochondria from non-OCT-ex-

pressing tissues like kidney (Miura et al., 1982) and heart (Argan et al., 1983). Further, in support of previous findings in *Neurospora* mitochondria (Zimmerman et al., 1981), our results also suggest the possibility that multiple mechanisms may exist for the transport of proteins into animal cell mitochondria.

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