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DIFFERENTIAL IMPORT AND PROCESSING OF THE PRECURSORS TO F_1-ATPase β-SUBUNIT AND ORNITHINE CARBAMYLTRANSFERASE BY LIVER, SPLEEN, HEART AND KIDNEY MITOCHONDRIA

Claude Côté and Diane Boulet

Lady Davis Institute for Medical Research
of the Sir Mortimer B. Davis - Jewish General Hospital,
3755 Cote Saint Catherine Road, Montreal, Quebec, Canada H3T 1E2

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Summary: The cytoplasmically made subunits 2 (β) and 3 (γ) of the H^+-ATPase from mammalian mitochondria are synthesized in vitro as larger polypeptides. In contrast, pre-cytochrome c could not, on the basis of its molecular weight, be distinguished from the mature polypeptide. This was shown by programming a reticulocyte lysate with rat heart RNA and immunoprecipitating the labeled translation products with polypeptide-specific antibodies. When a translated lysate containing the precursor to the β-subunit was incubated with isolated rat spleen mitochondria, it was converted to the mature subunit and was no longer susceptible to externally added trypsin. The conversion to the mature form occurred in the absence of protein synthesis. This post-translational maturation process of the β-subunit was more efficient when carried out with spleen or liver mitochondria than with heart or kidney mitochondria. The converse relative efficiency was observed when the processing of the precursor to ornithine carbamyltransferase by these mitochondria was examined. These results indicate that mitochondria do not discriminate against tissue-specific mitochondrial proteins. In addition, the observed varying degrees of efficiency of mitochondria from different tissues in importing and processing these two precursors suggest that the activity of precursor(s)-specific translocation-maturation systems varies between different types of mitochondria.

The majority of mitochondrial proteins are synthesized on cytoplasmic ribosomes in the form of larger molecular weight precursors which are post-translationally translocated into mitochondria and processed to their mature form within the organelle (1). Discriminatory import and processing of tissue-specific mitochondrial proteins by heterologous mitochondria was recently investigated (2). It was concluded that the spectrum of precursor(s)-specific receptors on the surface of mitochondria (3,4) and the specificity of the chelator-sensitive matrix protease involved in the maturation process of the precursors to mitochondrial proteins were characteristic of the mitochondrial translocation-maturation system of each tissue.

In the present study, we have compared the ability of mitochondria from four tissues to import and process the precursor to the β-subunit of F_1-F_o, a protein common to all mitochondria, and the precursor to ornithine carbamyltransferase (OCT),

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a tissue-specific protein localized in the matrix of liver mitochondria. The results indicate that the precursor of the β-subunit is preferentially imported by liver and spleen mitochondria while the OCT precursor is preferentially imported by heart and kidney mitochondria.

Experimental Procedures

The mitochondrial ATPase (F_1) from beef and rat heart mitochondria was isolated according to the procedure of Beechy et al. (5) as modified by Apps et al. (6). The subunits of BF_1 were isolated by preparative SDS-polyacrylamide gel electrophoresis. Antisera against the individual subunits of BF_1 were prepared by the general procedure described in Ref. 7. Specificity of the antisera and their ability to cross-react with the corresponding subunits of rat heart F_1 was determined by an immune-replica procedure involving the electrotransfer of the subunits from polyacrylamide slab gels to nitrocellulose paper (8) and immunodecoration by staining for peroxidase activity (9). Antibodies against horse heart cytochrome c were produced in rabbits and purified by immunoabsorption on an AffiGel-cytochrome c column (10). These antibodies were shown to cross react against rat heart cytochrome c (Sigma) by the immune replica technique described above (8,9). Antibodies against ornithine carbamyltransferase (OCT) and purified OCT were generously provided by Dr. G. Shore (McGill University, Montreal, Quebec). Cell-free synthesis was done for 90 min at 30°C in a nuclease-treated reticulocyte lysate (11) programmed with rat liver or rat heart RNA. Incorporation of [^{35}S]methionine into proteins was stopped by adding nonradioactive methionine (10 mM) and cycloheximide (100 μg/ml). The translated lysate was adjusted to 0.22 M mannitol, 0.070 M sucrose and 0.01 M EGTA and centrifuged at 140,000 g for 60 min, at 2°C.

Rat heart mitochondria were freshly prepared as described in (12). Rat liver, kidney and spleen mitochondria were prepared by the method of Greenwalt (13). Freshly prepared mitochondria were resuspended in a medium containing 10 mM HEPES, pH 7.4, 220 mM mannitol, 70 mM sucrose, 10 mM succinate, 2.5 mM K_2HPO_4, pH 7.4, 0.15 mM ADP and 1 mM DTT. The in vitro import assay contained 50 μl of translated reticulocyte lysate (≅2 x 10^6 CPM of protein-bound ^{35}S) and 100 μg of mitochondrial protein in a final volume of 100 μl. The mixture was incubated for 60' at 30°C and then chilled on ice. For protease treatment, one-half of the mixture was treated with trypsin (100 μg/ml); after 30 min at 0°C, trypsin activity was arrested by the addition of 1 mg/ml of soybean trypsin inhibitor and mitochondria were then reisolated by centrifugation (15,000 g, 3 min); supernatants and pellets were subjected to immunoprecipitation.

Ornithine carbamyltransferase was immunoprecipitated from the supernatant and the mitochondrial pellet as described in (12). When radioactive OCT was immunoprecipitated from liver mitochondria, an excess of antisera was used to obviate the competition from unlabeled OCT present in the matrix.

Published methods were used for preparing rat liver RNA using the SDS/phenol-chloroform-isoamyl alcohol extraction procedure (14), for preparing rat heart RNA using guanidine thiocyanate-cesium chloride procedure as described (15,16), for measuring protein (17), for immunoprecipitation of F_1 subunits (18), for solubilization of immunoadsorbed antigens (18), for SDS-polyacrylamide gel electrophoresis (19), and for fluorography (20).

Results

In order to investigate the synthesis of subunits 2 (β) and 3 (γ) of the H^+-ATPase complex, a nuclease-treated reticulocyte lysate was programmed with total rat heart or liver RNA; these RNA preparations stimulated the incorporation of [^{35}S]methionine into proteins 5-to-7 fold and 20-to-25 fold, respectively. Figure 1 shows that subunits

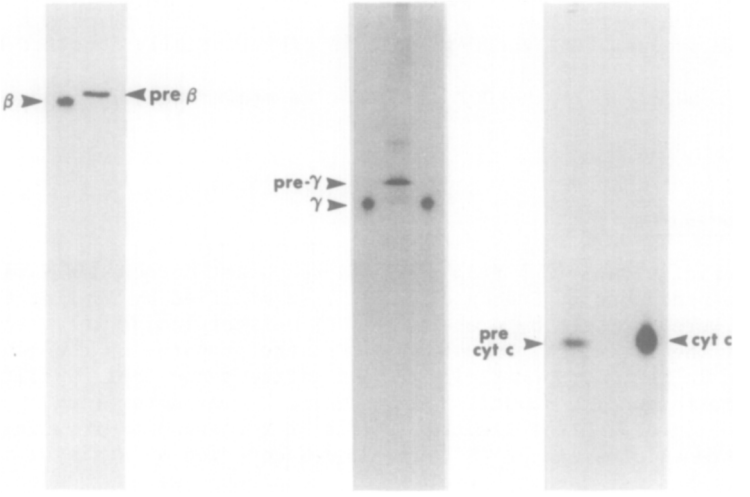

Fig. 1. Subunits β and γ of the F_1-ATPase complex are made as larger precursors in a reticulocyte lysate programmed with rat heart RNA; apocytochrome c immunopurified from that lysate comigrates with the holocytochrome - see «Experimental Procedures» for details. Left, ^{125}I-labeled mature β-subunit and pre-β-immunoprecipitated from a reticulocyte lysate. Middle, ^{125}I-labeled mature γ-subunit and pre-γ-immunoprecipitated from a translated lysate. Right, the precursor to cytochrome c immunoprecipitated from a translated lysate and ^{125}I-labeled rat heart cytochrome c. A fluorogram from a dried 14% polyacrylamide slab gel is shown.

2 (β) and 3 (γ) of F_1-ATPase complex made in vitro migrate more slowly than the radioactive «mature» subunits. The difference in molecular weight is about 2,000 and 6,000 for β and γ respectively. In contrast, the precursor to cytochrome c could not, on the basis of its molecular weight, be distinguished from the mature form of the cytochrome

In vitro synthesized precursor to the F_1 β-subunit is imported into and processed by mitochondria in the absence of protein synthesis

A translated lysate programmed with rat liver RNA was incubated with spleen mitochondria. Half of the mixture was subsequently incubated with trypsin to digest ^{35}S-labeled polypeptides accessible to the added protease. The other half served as the control. The mitochondria were pelleted by centrifugation; the supernatant and the pelleted mitochondria were checked by immunoprecipitation and sodium-dodecyl sulfate PAGE for the presence of the precursor and the mature form of the F_1 β-subunit. The results presented in Fig. 2 show that the mitochondrial pellet of the control sample (no protease treatment) contains the larger and the mature forms of the subunit (track 3). In contrast, the mitochondrial pellet of the protease-treated

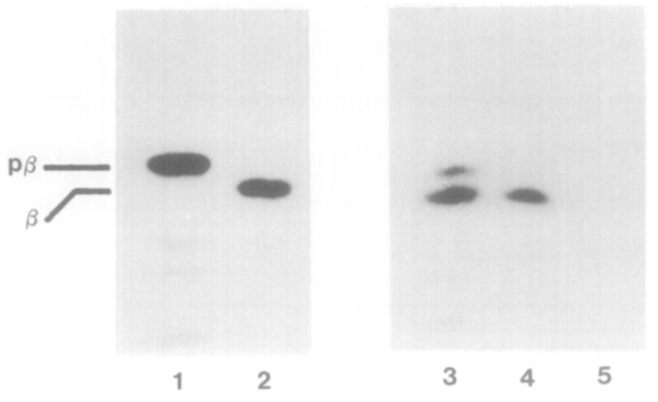

Fig. 2. Import and processing of F_1-β-subunit precursor by isolated mitochondria: the F_1-β-subunit precursor is cleaved and rendered protease-resistant during incubation with rat spleen mitochondria. Isolated spleen mitochondria (1.0 mg/ml) were incubated (60' at 30°C with ^{35}S-methionine-labeled translation products from a lysate programmed with rat liver RNA (50 µl, ≅2 x 10^6 cpm of protein-bound ^{35}S); maturation and uptake of the F_1-β-subunit precursor was checked by protease treatment, immunoprecipitation, SDS-polyacrylamide gel electrophoresis and fluorography (see Experimental Procedures). A photograph of the fluorogram is shown: 1) F_1-β-subunit precursor; 2) precursor (pβ) processed to its mature size by a matrix fraction from liver mitochondria; 3) F_1-β-subunit precursor and mature form recovered from mitochondria not treated with protease; 4) F_1-β-subunit recovered from mitochondria treated with protease; 5) supernatant after protease treatment.

aliquot (track 4) contained only the mature form of the subunit; the precursor (pβ) was also absent from the supernatant (track 5). These results suggest that the precursor is outside the mitochondria, while the mature form is protected and is apparently inside the mitochondrial membranes.

Recently, a zinc-dependent endoprotease was localized to the mitochondrial matrix and was shown to have as its distinctive property a highly specific processing activity on precursors to mitochondrial proteins (21-24). We checked if a component of the matrix compartment could process the precursor to the β-subunit as follows: an aliquot of the translated lysate was incubated for 60 min at 30°C with a liver mitochondrial matrix fraction (2 mg protein/ml) prepared as in Ref. 26. The sample was subsequently solubilized in SDS and processed for immunoprecipitation as described above. The results presented in Fig. 2, lane 2 show that the F_1-β-subunit precursor is completely processed to its mature form by the matrix fraction. This process is inhibited by EGTA (10 mM) and by O-phenanthroline (2 mM) (not shown). No intermediate form of the precursor can be detected suggesting that its maturation is a single-step event. The

radiolabeled «mature» polypeptide was shown to comigrate with the stained β-subunit from a chemically purified F_1 preparation (not shown).

Preliminary results indicated that heart mitochondria had, under various experimental conditions, a limited capacity for the uptake and processing of the precursor to the F_1-β-subunit. This prompted us to test the efficiency of the translocation-maturation process by mitochondria from other tissues. We compared the ability of fou types of mitochondria to import and process the precursor of a universal component of the inner membrane, i.e., F_1-β-subunit, to that of importing and processing a tissue-specific component of the matrix compartment, i.e. ornithine carbamyltransferase. An aliquot of a translated lysate was incubated with either heart, liver, spleen or kidney mitochondria. Mitochondria were reisolated by centrifugation and resuspended in homogenizing buffer; one-half was immunoprecipitated with anti-OCT antibodies; the other half was similarly processed with anti-F_1-β-subunit antibodies. The efficiency of importing and processing the two precursors by these four mitochondria preparations is estimated from the quantity of the mature products associated with the mitochondrial pellets; this represents the fraction of these precursors initially present in the lysate which has been converted to their mature forms and is reflected by the intensity of the band on the fluorogram representing the mature form of the polypeptides. The results presented in Fig. 3 indicate that a greater fraction of the

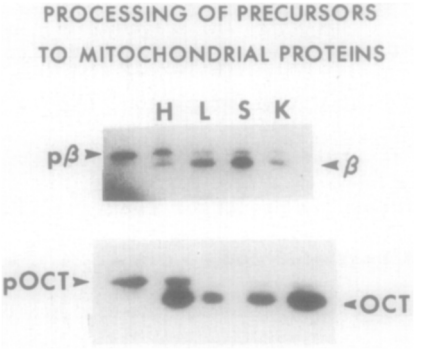

Fig. 3. Import and processing of F_1-β-subunit and ornithine carbamyltransferase (OCT) by mitochondria isolated from heart, liver, spleen and kidney mitochondria. Aliquots (100 µl, ≅4 x 10^6 cpm of protein-bound ^{35}S) of ^{35}S-methionine-labeled products translated in a reticulocyte lysate programmed with rat liver RNA were added to intact mitochondria, incubated at 30°C for 60 min; immunoprecipitation, SDS-polyacrylamide gel electrophoresis and fluorography were performed as described in «Experimental Procedures». A photograph of the fluorogram is shown. Precursor (P) and mature forms recovered in heart (H), liver (L), spleen (S), and kidney (K) mitochondria.

β-subunit precursor is processed by spleen and liver mitochondria than by heart and kidney mitochondria. Immunoprecipitation of pβ from the supernatants of these reaction mixtures revealed that the quantity of unprocessed precursors in that fraction (i.e. intensity of the band on the fluorogram) was inversely related to that of the mature plus precursor forms in the pellets, i.e. kidney > heart > liver > spleen (not shown). These results suggest that differential processing of this precursor by these mitochondria is not an apparent observation reflecting instead different extents of degradation of these polypeptides by these mitochondrial preparations. In the case of OCT, mitochondria from heart and kidney import and process a greater fraction of its precursor than liver or spleen mitochondria. The quantity of precursors shown in the left lanes (pβ and pOCT) represents one third of the quantity used in the import assay.

Discussion

The results of a recent report (25) indicated that precursors to two polypeptides of the mammalian cytochrome b-c_1 complex differ significantly in size from those reported for the corresponding subunits of yeast and Neurospora b-c_1 complexes suggesting possible functional differences in the processing mechanism of that complex between higher and lower eucaryotic cells.

The present data indicate that two major subunits of rat F_1-F_o synthesized on cytoplasmic ribosomes are made as a larger form in vitro. We have also observed that the size of the additional peptide present in the precursor of the γ-subunit is much larger than that in the β-subunit (6,000 vs 2,000) and that the precursor to rat heart cytochrome c cannot, on the basis of its molecular weight, be distinguished from the mature form of the cytochrome. The precursor to βF_1 is imported post-translationally and processed to its mature form via a single step event by a metal-dependent protease located in the mitochondrial matrix. These results confirm and extend those previously reported on the biogenesis of the F_1 subunits and cytochrome c from yeast (24,26-28) and Neurospora (29,30), suggesting a functional similarity between the mechanisms of import and processing of these components of the inner membrane in higher and lower eucaryotes.

In the present communication, we describe the import and processing of a universal polypeptide of the mitochondrial inner membrane. We observed that heart and kidney mitochondria have a limited capacity to import the precursor to the β-subunit of the proton-ATPase. However, such mitochondria were actively importing and processing the precursor to ornithine carbamyltransferase, a tissue-specific mitochondrial protein, thus suggesting the existence of precursor(s)-specific pathways for the import of distinct or group of precursors to mitochondrial proteins. This proposition is substantiated by the fact that the F_1-β-subunit precursor is preferentially imported by spleen and liver mitochondria while the OCT precursor is preferentially imported by heart and kidney mitochondria. The fact that the precursor to OCT, an enzyme found in liver mitochondria, is imported by all four types of mitochondria suggests 1) that mitochondria from different tissues do not discriminate against tissue specific mitochondrial proteins (2,31), and 2) that the import pathways can translocate and process more than one precursor. The fact that pβ and pOCT are imported and processed with varying degrees of efficiency by different types of mitochondria, suggests that the activity of precursor(s)-specific import systems varies between mitochondria from different tissues. It is unlikely that this result is due to differing degrees of functional integrity of these different mitochondria preparations since the efficiencies of conversion of these precursors to their mature forms by these mitochondria are inversely related. While it is likely that these conclusions also hold true for other mitochondrial proteins (2), whether or not they reflect the characteristics and the properties of the in vivo process remains to be documented. Control of the activity of the import pathways could be a pivotal component of a post-translational regulatory system of mitochondrial biogenesis in animal tissues.

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