Biosynthesis and Mitochondrial Processing of the β Subunit of Propionyl Coenzyme A Carboxylase from Rat Liver*

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Jan P. Kraus, Frantisek Kalousek, and Leon E. Rosenberg
From the Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

Propionyl-CoA carboxylase (ADP-forming) (EC 6.4.1.3), an oligomer of nonidentical subunits (αβ₄), has been localized to the mitochondrial matrix. As a first step in examining this enzyme's biogenesis, we have investigated in vitro the cell-free, rat liver RNA-directed synthesis of the β subunit, and its post-translational transport and processing by rat liver mitochondria. The β subunit is synthesized as a precursor ~7,500 daltons larger than its mature mitochondrial counterpart. The extension segment, comprising ~60 amino acids, is located at the NH₂ terminus of the precursor. Intact mitochondria translocate the precursor across both mitochondrial membranes, and a pre-pro-sequence localized to the mitochondrial matrix cleaves the precursor to a polypeptide identical in size and peptide composition to the mature β subunit.

The subunits of more than two dozen nuclear-coded mitochondrial enzymes have been shown recently to be synthesized on cytoplasmic polyribosomes as precursor polypeptides ~2,000 to ~10,000 daltons larger than their respective mature counterparts (see review in Ref. 1, 2). Post-translational uptake and processing of these precursors by isolated mitochondria has been demonstrated in some (1) but not all (2) cases.

The biogenesis of one such mammalian mitochondrial matrix enzyme, ornithine carbamoyltransferase (EC 2.1.3.3), has been investigated in detail in our laboratory (3-7) and in that of another group (reviewed in Ref. 8). Mammalian OTCase is a trimer of identical subunits. Its precursor is ~4,000 daltons larger than the corresponding mature subunit due to an NH₂-terminal extension sequence. This extension sequence is removed in two proteolytic steps during the complex, energy-dependent reaction sequence which results ultimately in translocation of the precursor across both mitochondrial membranes to the matrix, and in the assembly of the mature subunits to a functional trimer.

We have now begun to characterize the biogenesis of a second, similarly compartmentalized, but more structurally complicated mitochondrial matrix enzyme, propionyl-CoA carboxylase (propionyl-CoA:carbon-dioxide ligase (ADP-forming); EC 6.4.1.3). PCCase is a biotin-dependent enzyme composed of nonidentical subunits, i.e. an α subunit with Mₛ ~ 70,000 and a β subunit with Mₛ ~ 54,000. The enzyme has an oligomeric structure of αβ₄; each α subunit contains one covalently bound biotin prosthetic group (9).

Because inherited deficiency of PCCase activity in man results from mutations affecting the structure and function of either the α or β subunit and because all such deficiencies are inherited in an autosomal recessive fashion, it is clear that the genetic loci coding for α and β subunits are located in the nucleus (for reviews see Refs. 10 and 11). Thus, it follows that these polypeptides must be synthesized in the cytosol and transported across both mitochondrial membranes to the matrix. The present study of the biosynthesis and processing of the β subunit of PCCase was undertaken as a first step toward understanding the normal and pathologic regulation of this oligomeric enzyme's biogenesis.

**Experimental Procedures**

**Materials**—Calf liver tRNA was purchased from Boehringer-Mannheim; L-[¹⁴C]methionine (specific activity ~1000 Ci/mmol) was from Amersham.

**Antisera**—Antisera were raised in rabbits against isolated β subunits of human PCCase. PCCase, purified from human liver to homogeneity (9), was denatured with SDS. The α and β subunits were separated on an SDS-polyacrylamide gel and the β subunit was electroeluted from the gel using an ISCO Electrophoretic Concentrator (model 1750). Rats were injected with this antigen, and antisera were collected as described earlier (9).

**Post-translational Processing with Mitochondrial Fractions**—Intact rat liver mitochondria and subfractions thereof were prepared as described (6). Processing experiments were carried out as follows. The translation mixture was mixed 1:1 (v/v) with a buffer consisting of 2 mM Hepes, pH 7.4, 220 mM mannitol, 70 mM sucrose (HMS buffer), supplemented with cycloheximide (30 μg/ml), 2 mM EGTA, 4 mM MgCl₂, 4 mM ADP, 20 mM glutamate, and the appropriate mitochondrial fraction at 4 μg of protein/ml. Incubation was at 37 °C for 20 min. In experiments with intact mitochondria, the mitochondria were sedimented following incubation by centrifugation for 3 min at 7,000 × g, and the supernatant was transferred to a clean tube. After the mitochondria were rinsed with 300 μl of HMS buffer, the supernatant and pellet were each subjected to immunoprecipitation.

**Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis**—After translation and processing, all fractions were diluted 10-fold with 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 2% methionine, pH 7, and centrifuged for 15 min at 100,000 × g. Normal rabbit serum (10 μl) was added to the supernatants. After 30 min at room temperature, 100 μl of 10% (w/v) fixed Staphylococcus aureus cells (Bethesda Research Laboratories) were added, and the mixtures were incubated for 10 min and then centrifuged at 7,000 × g for 5 min. The samples which had not been processed with mitochondrial
fractations were adjusted with SDS to 0.1%. Antiserum (3-12 μl) was added to the supernatants and the mixtures were incubated overnight at 4°C. A second addition of S. aureus cells and subsequent washes were as described (5). Electrophoresis in SDS-9% polyacrylamide gel was performed as described by Laemmli (12), and the gel was fixed, dried, and fluorographed using Autoradiography (National Diagnostics).

Other Techniques—Homogeneous rat liver PCCase was labeled in vitro by reductive methylation (15) using [14C]formaldehyde (Amer- sham). For peptide mapping, regions containing the precursor of the β subunit of PCCase, its mitochondrially processed form, and an authentic α and β subunits of pure rat enzyme were excised from a frozen SDS-polyacrylamide gel subjected to digestion with S. aureus V8 protease as described by Cleveland et al. (14). Attempts to elute and digest the subunits from a dried gel processed for fluorography were unsuccessful. The digestion products were separated on an SDS-15% polyacrylamide gel and visualized by fluorography.

RESULTS

Specificity of the Antiserum—We have shown previously that β subunit-specific antiseraum interacts solely with rat liver polysomes containing the mRNA coding for the β subunit of PCCase, thus providing means for immunopurification of this mRNA species (15). We have also established that the anti-β subunit antiserum recognizes only free β chains, and does not react with either isolated α chains or the assembled αβi oligomers (results not shown).

Cell-free Translation of β Subunits of PCCase—Fig. 1, lane 2, shows an immunoprecipitate of a translation mixture reacted with anti-β subunit antiserum, while lane 3 shows the result of a control immunoprecipitation with normal rabbit serum. Comparison of lanes 2 and 3 reveals that the only specific immunoprecipitation product in lane 2 is the 61,500-dalton polypeptide, about 7,500 daltons larger than the mature β subunit (lane 1) obtained from radiolabeled homogeneous rat liver PCCase. This result indicates that the β subunit is synthesized on cytosolic polysomes as a precursor (pβPCCase) containing an amino acid extension of ~60 amino acid residues.

Processing of pβPCCase by Mitochondrial Fractions—In order to demonstrate that the above described pβPCCase can be converted to the mature β subunit, the following experiment was performed. Rabbit reticulocyte lysate translation system, programmed with rat liver mRNA in the presence of [35S]methionine, was used to prepare radiolabeled rat liver polyribosomes; after protein synthesis was halted, the translation mixture was exposed to intact rat liver mitochondria or to isolated submitochondrial fractions; then β subunit-specific immunoprecipitants were prepared. The results are summarized in Fig. 2. Lane 3 shows the supernatant of the translation mixture following processing with intact mitochondria. Nearly all of the pβPCCase (lane 2) has disappeared, suggesting that it has been taken up by the mitochondria. Lane 4 is the corresponding mitochondrial pellet containing, in addition to a trace of pβPCCase, a polypeptide indistinguishable in size from the mature β subunit (lane 1). An experiment to localize this processing activity is summarized in lanes 5-7 (Fig. 2). The mitochondria were stripped of the outer membrane and the intermembrane space by mild treatment with digitonin, and the remaining intact mitoplasts (containing the inner membrane and the matrix) were incubated with the translation mixture. All of the processed β subunit was recovered with the mitoplast pellet (compare lanes 4 and 5). When the mitoplast fraction was separated further into inner membrane and matrix, we found that the matrix fraction contained the proteolytic processing activity. This proteolytic activity was stimulated by the addition of Zn2+ (compare lanes 6 and 7). Interestingly, the sum of the precursor and the processed β subunit recovered when Zn2+ was not added (lane 6) was reduced compared to recovery seen in lane 7, possibly because the unprocessed pβPCCase was rapidly hydrolyzed by other proteases present in the matrix.

Peptide Mapping—Thus far we have shown that pβPCCase and its processed form specifically interact with anti-β subunit antiserum and that the mitochondrially processed form of the precursor is indistinguishable in size from the in vivo synthesized β subunit of rat liver PCCase. Additional evidence that the 61,500-dalton polypeptide is a true β subunit precursor came from examination of a one-dimensional peptide map (Fig. 3). The mitochondrially processed β subunit (lane 1), the putative precursor (lane 2), and authentic [14C]formaldehyde-labeled β subunit (lane 3) were partially digested with S. aureus V8 protease and electrophoresed. Striking similarity in the peptide patterns for all three proteins was observed. For comparative purposes, a one-dimensional peptide map of the α subunit is also shown (lane 4), which produced a distinctly different pattern.

Fig. 2. Uptake and processing of β subunit precursor by rat mitochondria and mitochondrial subfractions. Post-translational incubations with mitochondrial fractions (lanes 3-7) were at 37°C for 20 min followed by immunoprecipitation with anti-β subunit antiserum in the absence of SDS. Only the relevant portion of the gel fluorogram is shown. Lane 1, mobility of β subunit of homogeneous rat liver PCCase; lane 2, mobility of pβPCCase; lane 3, 7,000 × g supernatant after incubation of translation mixture with intact mitochondria; lane 4, mitochondrial pellet corresponding to above supernatant; lane 5, incubation of translation mixture with mitoplasts; lane 6, incubation of translation mixture with mitochondrial matrix fraction; lane 7, same as lane 6 except that Zn2+ was added to 0.1 mM final concentration.

W. Fenton and L. Rosenberg, unpublished results.
electrophoresed in SDS-15% polyacrylamide gel. The fluorogram of the dried gel is shown.

dially processed cut out, and digested with 0.2 pdPCCase:

as described under “Experimental Procedures.” Following localization by fluorography of adjacent lanes, the appropriate hands were with intact mitochondria.

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N-formyl[^5S]methionyl-tRNA,”“. Products immunoprecipitated with anti-[@

PCCase antiserum were visualized by fluorography for I,

pppcc -

unlabeled methionine and 7[^3S]methionine-labeled subunit precursor synthesized under these conditions are labeled exclusively at their NH2 termini (5, 16, 17). To determine whether the amino acid extension is located at the NH2 termini of the β precursor, NH2-terminally labeled polypeptides were processed with mitochondria in a manner identical to that used for polypeptides labeled internally with[^5S]methionine (e.g. Fig. 2, lanes 2 and 4). As shown in Fig. 4 (lane 2), labeling in the presence of N-formyl[^5S]methionyl-tRNA[^Met] followed by immunoprecipitation also yields a precursor 7,500 daltons larger than the authentic β subunit (lane 1). When the translation mixture was processed with mitochondria, no radiolabel was recovered with the mitochondrial pellet (lane 3), suggesting that the NH2-terminal label had been lost with the amino acid extension during processing. We conclude that translation of the mRNA coding for the β subunit of PCCase begins with those residues comprising the amino acid extension of the precursor and proceeds to those amino acid residues ultimately found in the mature β subunit sequence.

**DISCUSSION**

We have shown that the β subunit of PCCase is synthesized as a precursor containing an NH2-terminal amino acid extension; the overall M, of the cytosolic precursor is ~7,500 daltons larger than its mature mitochondrial counterpart. This difference, representing ~60 amino acids, is one of the largest thus far observed for a cytosolic precursor of a mitochondrial protein. Only precursors of adrenodoxin (extension M, ~ 8,000) (18) and possibly of β-aminolevulinate synthase (extension M, ~ 5,000–10,000) (19, 20) have been found to have as large amino acid extensions. More than two dozen precursors of mitochondrial proteins contain amino acid extensions in the range of 2,000–10,000 daltons (1, 2). It is becoming increasingly clear that there is no relation between the size of the amino acid extension (“signal peptide”) and the size of the mature polypeptide. Thus, a polypeptide as large as carbamyl phosphate synthetase (M, = 160,000) is synthesized with a relatively short extension of ~5,000 daltons (21, 22), while a small polypeptide such as adrenodoxin (M, = 12,000) is translated with an amino acid extension of ~8,000 daltons (18). There must be other, as yet obscure, determinants of the length and charge of the amino acid extension required to present a tertiary structure of the precursor compatible with its solubility in the cytosol (23, 24), its recognition by mitochondrial membranes, and its cleavage by a mitochondrial protease(s).

It is of interest to compare the properties of pdPCCase with those of pOTCase. The amino acid extension is located at the NH2 termini of both precursors, in agreement with data for several yeast mitochondrial proteins shown to be translated with NH2-terminal amino acid extensions (25). The pOTCase appears to be processed in two steps: first, to an intermediate by a protease localized either in the mitochondrial inner membrane and/or in the matrix; second, to a mature size subunit by a Zn2+-dependent protease localized to the matrix (6, 8). In contrast, we found no suggestion of an intermediate processing step for pdPCCase; the Zn2+-dependent protease in the mitochondrial matrix fraction appeared to remove the entire 7,500-dalton extension, yielding a polypeptide indistinguishable in size and in peptide pattern from an authentic β subunit purified from rat liver. Although all of our data, particularly that obtained with N-formyl[^5S]methionyl-tRNA[^Met] (Fig. 4), are compatible with the thesis that only NH2-terminal processing of pdPCCase occurs intramitochondrially, COOH-terminal processing has not been rigorously excluded. Interestingly, recent results from our laboratory demonstrate convincingly that COOH-terminal processing of

**Fig. 3. Peptide patterns of β subunit precursor synthesized in vitro, of its mature counterpart processed with isolated mitochondria, and of authentic pure mature β and α subunits. Radiolabeled precursor and mature forms of β subunit were obtained as described under “Experimental Procedures.” Following localization by fluorography of adjacent lanes, the appropriate bands were cut out, and digested with 0.2 pdPCCase; lanes 3 and 4, [14C]formaldehyde-labeled β and α subunits of pure PCCase, respectively.**

**Fig. 4. In vitro synthesis and processing of β subunit precursor labeled exclusively at NH2 terminus. pdPCCase was synthesized in 1.5 ml of translation mixture in which[^5S]methionine was replaced by 45 nmol of unlabeled methionine and 7 × 10⁴ dpm of N-formyl[^5S]methionyl-tRNA[^Met]. Processing, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis of N-formyl[^5S]methionyl-labeled polypeptides was performed as described under “Experimental Procedures.” Products immunoprecipitated with anti-β PCCase antiserum were visualized by fluorography for 28 days. Lane 1, α and β subunits of pure PCCase; lane 2, pdPCCase labeled at NH2 terminus; lane 3, pdPCCase labeled at NH2 terminus after incubation with intact mitochondria.**

**Position of the Amino Acid Extension within the pdPCCase—Cell-free protein synthesis was carried out in the rabbit reticulocyte system programmed with rat liver polysomal RNA in the presence of N-formyl[^5S]methionyl-tRNA[^Met] as the sole radioactive substrate. The polypeptides synthesized under these conditions are labeled exclusively at their NH2 termini (5, 16, 17). To determine whether the amino acid extension is located at the NH2 terminus of the β precursor, NH2-terminally labeled polypeptides were processed with mitochondria in a manner identical to that used for polypeptides labeled internally with[^5S]methionine (e.g. Fig. 2, lanes 2 and 4). As shown in Fig. 4 (lane 2), labeling in the presence of N-formyl[^5S]methionyl-tRNA[^Met] followed by immunoprecipitation also yields a precursor 7,500 daltons larger than the authentic β subunit (lane 1). When the translation mixture was processed with mitochondria, no radiolabel was recovered with the mitochondrial pellet (lane 3), suggesting that the NH2-terminal label had been lost with the amino acid extension during processing. We conclude that translation of the mRNA coding for the β subunit of PCCase begins with those residues comprising the amino acid extension of the precursor and proceeds to those amino acid residues ultimately found in the mature β subunit sequence.**
pOTCase does not occur during formation of its mature subunit (26). Further, we have observed that pOTCase reacted with anti-@ antisera readily in the presence of the negatively charged detergent SDS, whereas this detergent prevented interaction between the mature subunit and the antisera. Similar findings, using higher concentrations of SDS, were noted for pOTCase and its mature subunit (5).

Because of the strict specificity of the p subunit antisera for free @ chains and its lack of recognition of the assembled enzyme, we can conclude that the immunoprecipitated processed p subunits represent a pool of free @ chains in the mitochondrial matrix. This may occur either because they are made in excess of N subunits or because they are vectorially processed in greater numbers than @ subunits under these conditions.

The availability, in our laboratory, of nearly homogeneous mRNAs coding for precursors of OTCase and of the @ subunit of PCCase (15) has already simplified cloning of OTCase cDNA (26). Synthesis of such cloned cDNAs which, in turn, will permit prediction of the amino acid sequences characteristic of the precursor peptides and protease cleavage site(s), should now be possible for pPCCase as well.

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