We have characterized further the biogenesis in vitro of ornithine transcarbamylase, a homotrimeric mitochondrial matrix enzyme synthesized in the cytoplasm as a larger precursor. When cell-free translation mixtures containing the ornithine transcarbamylase precursor (40 kDa) were chromatographed on Bio-Gel P-200 columns, all of the precursor eluted as aggregates or complexes with molecular weights >200 kDa. None of the precursor bound to a ligand affinity column containing \( \delta-N-(\text{phosphonoacetyl})\)-L-ornithine (\( \delta\)-PALO), a transition-state analog and competitive inhibitor of carbamyl phosphate binding, which recognizes native ornithine transcarbamylase. In contrast, a significant portion of the labeled mature-sized subunits, formed when intact mitochondria processed the precursor, bound specifically to the \( \delta\)-PALO column, were eluted by carbamyl phosphate, and chromatographed on a Bio-Gel P-300 column with a mobility identical to that of native, trimeric ornithine transcarbamylase. No such binding to \( \delta\)-PALO was observed for the mature-sized monomer or dimer, or for the intermediate-sized ornithine transcarbamylase polypeptide. Moreover, processing by a mitochondrial matrix fraction failed to yield trimeric enzyme, despite prodigious amounts of mature-sized monomers. We conclude that \( \delta\)-PALO recognizes only trimeric ornithine transcarbamylase composed of mature-sized subunits and that such trimers can be assembled in vitro by intact mitochondria following translocation and proteolytic processing.

Ornithine transcarbamylase (ornithine carbamoyltransferase; EC 2.1.3.3), a major hepatic mitochondrial matrix enzyme of ureotelic animals (1, 2), has been isolated from the liver of several mammalian species (cow, rat, and human) as a homotrimer consisting of 36–39-kDa subunits (3–7). The subunit is encoded by a gene on the X chromosome (8–10), synthesized in the cytosol, and transported across both mitochondrial membranes to the matrix.

In vitro biogenesis of the rat ornithine transcarbamylase subunit has been studied extensively in our laboratory and that of others (11–22). The subunit is synthesized on free polyribosomes (11) as a larger precursor of \( \sim 40 \) kDa (12, 13) bearing an NH\(_2\)-terminal extension of \( \sim 4 \) kDa (14). The precursor, whose cytosol half-life is only 1–2 min (11, 15), is taken up by intact mitochondria post-translationally and processed to the mature-sized subunit (16, 17).

During this in vitro translocation and processing, which require, respectively, energy generated by the electrochemical potential across the inner mitochondrial membrane (18, 19) and a Zn\(^{2+}\)-dependent matrix protease (20), a protein intermediate in size (\( \sim 37 \) kDa) between the precursor and the mature-sized subunit is consistently observed (14, 16–19). The biologic significance of this intermediate-sized polypeptide, however, has been challenged (21).

We now report data concerning the last step in this pathway of biogenesis, namely assembly of the subunits into homotrimers. Using an affinity column (6) containing a transition-state analog of the ornithine transcarbamylase reaction, \( \delta\)-PALO,\(^1\) we show that a significant fraction of the mature-sized subunits formed during uptake and processing by intact mitochondria in vitro are assembled into trimers indistinguishable in size from active ornithine transcarbamylase.

**EXPERIMENTAL PROCEDURES**

**Sources of Materials**—\( \text{L-}{\[^{35}\text{S}]}\text{Methionine} (>800 \text{ Ci/mmol}) \) was purchased from Amersham; \( \text{L-}{\[^{3}\text{H}]\text{ornithine}} (2.1 \text{ Ci/mol}) \) and \( \text{EN'HANCE} \) were from New England Nuclear; \( \alpha-N\)-carbobenzoxy-L-ornithine was obtained from Vega Biochemicals; the triethyl ester of phosphonoacetic acid was from Aldrich; Bio-Gel A-0.5m, Bio-Gel P-200, and Bio-Gel P-300 were from Bio-Rad; 2,4-dinitrophenol, carbamyl phosphate, and Hepes were obtained from Sigma. Formalin-fixed *Staphylococcus aureus* cells were from Bethesda Research Laboratories. Rabbit serum was purchased from Accurate Chemicals. All reagents used for cell-free translation, mitochondrial processing, and SDS-10% polyacrylamide gel electrophoresis were obtained as described previously (17). The following homogeneous proteins, used as molecular weight standards, were prepared or purchased as described previously: rat liver ornithine transcarbamylase (6); rat liver pro-ponyl-CoA carboxylase (23); Escherichia coli seryl-tRNA synthetase (24); E. coli arginyl-tRNA synthetase (24); and beef heart cytochrome c (24).

**General Procedures**—Cell-free protein synthesis was performed in a nuclease-treated, rabbit reticulocyte lysate system using rat liver polysomal RNA as described (12). Post-translational processing of the ornithine transcarbamylase precursor by intact and broken mitochondria was carried out as noted previously (17, 20), as was processing in the presence of 2,4-dinitrophenol (19). Protein concentration (25) and SDS-10% polyacrylamide gel electrophoresis (26) were done according to published procedures.

**Immunoprecipitation**—Cell-free translation products or products obtained after processing with intact mitochondria were diluted with 1–2 ml of 150 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 2% (w/v) unlabeled methionine, and 0.25% SDS, pH 7.0. A preincubation with 5 μl of normal rabbit serum for 30 min at room temperature followed by the addition of 50 μl of 10% (w/v) fixed *S. aureus* cells for 10 min preceded centrifugation at 100,000 × g for 15 min. Incubation of supernatants with rabbit anti-rat ornithine transcarbamylase antiserum, a second addition of *S. aureus* cells, and subsequent washes were as described (17).

**Synthesis and Coupling of \( \delta\)-PALO**—The procedure of Hoogenraad (27) was followed for \( \delta\)-PALO synthesis and coupling to epoxy-activated Sepharose 6B, using either \( \alpha-N\)-carbobenzoxy-L-ornithine or copper \( \text{[G-}{\[^{3}\text{H}]\text{ornithine}} \) as starting materials.

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\(^1\) The abbreviations used are: \( \delta\)-PALO, \( \delta-N-(\text{phosphonoacetyl})\)-L-ornithine; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Affinity Chromatography—Samples (0.2–1.2 ml) of cell-free translation mixture, or cell-free translation mixture containing intact or broken mitochondria, were diluted with 10 mM Hepes buffer, pH 7.4, 1 mM 2-mercaptoethanol, and 0.5% Triton X-100. After 30 min, samples were applied to a δ-PALO/Seapharose 6B ligand affinity column (0.7 × 5 cm) which had the capacity to bind more than 1 mg of homogeneous ornithine transcarbamylase at the flow rate employed by 2 ml/h. After adsorption, the flow rate was increased to 10 ml/h. The column was washed with 20 ml of the above Hepes buffer without carbamyl phosphate as described (6), but without KCl. The affinity columns were regenerated with 0.2 M NaOH and re-equilibrated with 10 mM Hepes, pH 7.4, 1 mM 2-mercaptoethanol.

Gel Filtration—Samples of cell-free translation mixture, or eluates from the δ-PALO column, were chromatographed on either Bio-Gel A-0.5m, Bio-Gel P-300, or Bio-Gel P-200 columns (1 × 46 cm) eluted with 10 mM Hepes, pH 7.4, 0.5 M NaCl, and 1 mM 2-mercaptoethanol. Rat ornithine transcarbamylase, purified rabbit IgG, E. coli seryl-tRNA synthetase, E. coli arginyl-tRNA synthetase, ovalbumin, and beef heart cytochrome c were used as molecular weight standards. Samples containing cell-free translation products and intact mitochondria were treated with Triton X-100 (0.5%) before chromatography. Fractions of 0.75 ml were collected.

RESULTS AND DISCUSSION

Physical State and δ-PALO Binding Characteristics of the Ornithine Transcarbamylase Precursor—When the products of cell-free translation were applied to a P-200 gel filtration column and the eluted fractions were immunoprecipitated with anti-ornithine transcarbamylase antiserum and electrophoresed, the pattern shown in Fig. 1 was obtained. As expected, only a single major band corresponding in mobility to the ornithine transcarbamylase precursor was noted. None of the precursor, however, chromatographed as a monomer (fractions 38–42). Rather, virtually all precursor eluted at the void volume, indicating a molecular size >200 kDa, and implying aggregation of the precursor. Since no labeled band other than that of the precursor was consistently observed, it seems likely that such aggregates are either homo-oligomers or complexes of the precursor with unlabeled proteins present in the lysate. These results are in agreement with those reported by Miura et al. (28) using sucrose gradient centrifugation and Sepacryl S-200 chromatography. Although there is no data demonstrating that such aggregation occurs in intact cells, it may be of interest that in vitro aggregation of precursors for several other mitochondrial proteins has been observed. These include carbamyl phosphate synthetase I (28), malate dehydrogenase (29), and the adenine nucleotide carrier (29, 30).

To determine whether such oligomeric or complexed pre-ornithine transcarbamylase had a carbamyl phosphate binding site which could be recognized by δ-PALO, we applied to a δ-PALO ligand affinity column 380 μl of translation mixture containing ~3 × 10^6 cpm of ornithine transcarbamylase precursor to which 10 μg of homogeneous rat ornithine transcarbamylase had been added as carrier. Whereas the carrier enzyme was specifically bound and eluted with carbamyl phosphate with 80% recovery, no labeled ornithine transcarbamylase precursor was detected, either when the eluate was subjected to SDS-polyacrylamide gel electrophoresis directly (Fig. 2, lane 3) or when the eluate was immunoprecipitated with anti-ornithine transcarbamylase antiserum prior to electrophoresis (data not shown). In fact, virtually all precursor was found in the unadsorbed fraction (lane 2).

Assembly of Newly Processed Mature Ornithine Transcarbamylase Subunits—When we repeated the experiment just described under conditions shown previously to permit post-translational processing of ornithine transcarbamylase precursor to its mature size by intact rat liver mitochondria (17), however, very different results were noted (Fig. 2). When we applied this entire mixture to the δ-PALO column (lane 4), all precursor, all intermediate-sized material, and ~60% of mature-sized ornithine transcarbamylase polypeptides did not bind (lane 5). SDS-polyacrylamide gel electrophoresis of the material eluted with carbamyl phosphate, representing ~40% of the mature-sized polypeptides, revealed only a single band identical in size to the mature subunit, even without immunoprecipitation (lane 6). An identical result was obtained when ornithine transcarbamylase proteins were recovered from this eluate by immunoprecipitation prior to electrophoresis.

To determine whether the mature-sized ornithine transcarbamylase eluted from the δ-PALO column represented monomeric or oligomeric material, aliquots of the eluate were chromatographed on Bio-Gel P-300 (Fig. 3). Only a single

![Fig. 1. Aggregation of ornithine transcarbamylase precursor. Aliquots of cell-free translation mixture were chromatographed on Bio-Gel P-200 column prior to immunoprecipitation, SDS-10% polyacrylamide gel electrophoresis, and fluorography. The molecular weight of proteins in each fraction (top) was estimated based on the following markers: propionyl-CoA carboxylase, 500 kDa; ornithine transcarbamylase, 108 kDa; seryl-tRNA synthetase, 100 kDa; arginyl-tRNA synthetase, 73 kDa; cytochrome c, 12 kDa. Every other fraction was immunoprecipitated as shown by the bottom numbers. The lane shown at the far right contains unfractonated, immunoprecipitated translation mixture. See "Experimental Procedures" for details of gel filtration and immunoprecipitation. For details of cell-free translation and electrophoresis, see Refs. 17 and 25. pOTC, ornithine transcarbamylase precursor.](http://www.jbc.org/)

![Fig. 2. Binding to δ-PALO ligand affinity column of newly processed, mature-sized ornithine transcarbamylase subunits. Fluorogram of dried gel after SDS-10% polyacrylamide gel electrophoresis is shown. 380 μl each of translation mixture (lanes 1–3), or of translation mixture incubated with intact mitochondria for 60 min (lanes 4–6) were applied on two identical δ-PALO/Seapharose 6B columns. After extensive washing, the adsorbed material was eluted with carbamyl phosphate. The original translation mixtures (1.8 μl) (lanes 1 and 4) and the material (lanes 2 and 5) which failed to adsorb to the δ-PALO column and which was concentrated to original volume (380 μl) were immunoprecipitated prior to electrophoresis. Carbamyl phosphate eluates (lanes 3 and 6) were electrophoresed after lyophilization without immunoprecipitation. In order to apply to lane 3 of the gel the maximal amount of radioactive material, the lyophilized sample was dissolved in 40 μl of distilled water and 20 μl was electrophoresed. The material applied in lane 6 was dissolved in the original volume of translation mixture (380 μl) and 6 μl was electrophoresed. See "Experimental Procedures" for details of affinity chromatography and immunoprecipitation. pOTC, ornithine transcarbamylase precursor; iOTC, 37-kDa ornithine transcarbamylase intermediate; OTC, mature-sized ornithine transcarbamylase subunit.](http://www.jbc.org/)
FIG. 3. Assembly of newly processed ornithine transcarbamylase subunits to trimers. Eluate from δ-PALO-Sepharose 6B column was applied to Bio-Gel P-300 column with several molecular weight markers and homogeneous rat liver ornithine transcarbamylase. Individual fractions were assayed for ornithine transcarbamylase activity and for [35S]methionine (right ordinate). Molecular weight marker elution pattern is shown on left ordinate. 1, purified rabbit IgG; 2, serine-tRNA synthetase; 3, arginyl-tRNA synthetase; 4, ovalbumin. OTC, ornithine transcarbamylase. See “Experimental Procedures” for details of column chromatography and enzyme assays.

Radioisotopically labeled peak was observed, its mobility matching exactly that of enzymatically active carrier ornithine transcarbamylase.

The combined data from Figs. 2 and 3 indicate that newly processed ornithine transcarbamylase subunits are assembled to their final trimeric form by intact mitochondria in vitro. Thus, they extend the earlier results of Mori et al. (18) who showed that sucrose gradient centrifugation of mitochondrialy translated protein mixture identified a product which moved to a position “close to that” of the mature enzyme. To exclude the unlikely possibility that assembly occurred during affinity chromatography rather than prior to it, the experimental sequence was reversed. Processed translation products were first chromatographed on a Bio-Gel A-0.5m column, then pooled fractions were applied to the δ-PALO affinity column. As seen in Fig. 4, immunoprecipitation of the Bio-Gel column fractions yielded a complex pattern. As expected, the precursor was observed only in oligomeric form (fractions 14–17). Whereas intermediate- and mature-sized subunits were found in most fractions, nearly half of the mature-sized polypeptides were concentrated in fractions 19–23, i.e. in the region occupied by trimeric ornithine transcarbamylase (108 kDa). When these Bio-Gel column fractions were combined into four pools (fractions 14–18, 19–23, 24–28, and 35–40), applied to the δ-PALO column, eluted with carbamyl phosphate, and electophoresed, a mature-sized labeled band was seen only in the pool obtained from fractions 19–23 (data not shown). Most of the mature-sized polypeptides which did not bind to the δ-PALO column had a molecular weight slightly larger (~140 kDa) than trimeric ornithine transcarbamylase. This result not only confirms that in vitro assembly to trimers has occurred in mitochondria, but it also emphasizes that the δ-PALO column fails to bind monomeric, dimeric, or aggregated mature-sized ornithine transcarbamylase subunits. Because prior work from other laboratories has indicated that each mature subunit of ornithine transcarbamylase contains a carbamyl phosphate binding site (31–33), it would appear from our results that such sites are recognized by δ-PALO only in the conformation achieved in the trimeric form. It should be noted that the apparent molecular weight of the aggregated precursor decreased to ~170 kDa when the translation mixture was processed by intact mitochondria (Fig. 4). We currently have no explanation for this phenomenon.

Several negative results also bear mention. When posttranslational processing was carried out in the presence of 2,4-dinitrophenol, an inhibitor which blocks formation of mature subunit and leads to accumulation of the intermediate (18, 19), no binding to δ-PALO was observed, even when volumes of applied translation mixture were 30 times larger than those used in Fig. 2, lane 6. Similary, the intermediate-sized ornithine transcarbamylase formed by a mitochondrial matrix fraction in the absence of Zn2+ (20) did not bind to δ-PALO. These findings demonstrate that removal of a significant portion of the NH2-terminal extension fails to “uncover” the carbamyl phosphate binding site recognized by δ-PALO. Finally, when mature-sized subunits were formed by a matrix fraction in the presence of Zn2+ (20) rather than by intact mitochondria, no assembly to trimer was noted. This may mean that a critical concentration of mature monomers, essential for trimer formation, is achieved only when the ornithine transcarbamylase precursor is processed by intact mitochondria and not under the more dilute conditions which prevail when a soluble matrix fraction is employed. Alternatively, the three-dimensional structure of the intact mito-
chondrion may provide, through its membranes and spaces, a highly organized scaffold which permits newly imported, processed subunits to recognize and assemble with their identical counterparts. It is also possible that passage through the mitochondrial membrane(s) imparts a new three-dimensional structure to the mature subunit which is required for assembly.

Acknowledgments—We thank Wayne Fenton for valuable discussions and Marilyn Feldman for secretarial expertise.

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