Import of Rat Ornithine Transcarbamylase Precursor into Mitochondria: Two-Step Processing of the Leader Peptide

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Abstract. The mitochondrial matrix enzyme ornithine transcarbamylase (OTC) is synthesized on cytoplasmic polyribosomes as a precursor (pOTC) with an NH₂-terminal extension of 32 amino acids. We report here that rat pOTC synthesized in vitro is internalized and cleaved by isolated rat liver mitochondria in two temporally separate steps. In the first step, which is dependent upon an intact mitochondrial membrane potential, pOTC is translocated into mitochondria and cleaved by a matrix protease to a product designated iOTC, intermediate in size between pOTC and mature OTC. This product is in a trypsin-protected mitochondrial location. The same intermediate-sized OTC is produced in vivo in frog oocytes injected with in vitro-synthesized pOTC. The proteolytic processing of pOTC to iOTC involves the removal of 24 amino acids from the NH₂ terminus of the precursor and utilizes a cleavage site two residues away from a critical arginine residue at position 23. In a second cleavage step, also catalyzed by a matrix protease, iOTC is converted to mature OTC by removal of the remaining eight residues of leader sequence.

To define the critical regions in the OTC leader peptide required for these events, we have synthesized OTC precursors with alterations in the leader. Substitution of either an acidic (aspartate) or a "helix-breaking" (glycine) amino acid residue for arginine 23 of the leader inhibits formation of both iOTC and OTC, without affecting translocation. These mutant precursors are cleaved at an otherwise cryptic cleavage site between residues 16 and 17 of the leader. Interestingly, this cleavage occurs at a site two residues away from an arginine at position 15.

The data indicate that conversion of pOTC to mature OTC proceeds via the formation of a third discrete species: an intermediate-sized OTC. The data suggest further that, in the rat pOTC leader, the essential elements required for translocation differ from those necessary for correct cleavage to either iOTC or mature OTC.

Most mitochondrial proteins are encoded by nuclear genes and synthesized as larger precursors with amino-terminal amino acid extensions referred to as "pre-sequences" or "leader peptides" (reviewed in references 13 and 35). These extensions, characterized by the presence of basic amino acid residues and the absence of acidic amino acids, are themselves sufficient to direct uptake of the precursor proteins into mitochondria (10, 16, 18, 27, 39). Such uptake can occur posttranslationally (31) and often involves translocation across both the outer and inner membranes. Upon translocation, which requires an electrochemical potential across the inner mitochondrial membrane (7, 11, 19) and cytosolic nucleoside triphosphates (3, 9, 30), the NH₂-terminal extensions are processed by a chelator-sensitive protease(s) localized to the mitochondrial matrix (2, 5, 23–25).

The biogenesis of ornithine transcarbamylase (OTC),¹ a mitochondrial matrix protein of the urea cycle, conforms to this model (reviewed in reference 33). OTC is encoded in the nucleus, synthesized on free polysomes as a larger precursor (pOTC) bearing an NH₂-terminal leader peptide, and posttranslationally imported into mitochondria by a process requiring a mitochondrial membrane potential (4, 6, 19, 26). Processing of pOTC to its mature form requires a zinc-dependent protease(s) localized to the mitochondrial matrix (5, 23–25).

Previous studies of posttranslational uptake of pOTC into isolated mitochondria have shown the production of an OTC species, designated iOTC, intermediate in size between pOTC and mature OTC (1, 19, 20, 22–28). Some workers (1) have considered iOTC an in vitro artifact, while others (19, 25) have suggested that iOTC may represent a bona fide intermediate in the mitochondrial uptake pathway.

In this report, we present data showing that iOTC is formed within mitochondria during the course of normal biogenesis of OTC by a process that is dependent on a mitochondrial membrane potential, and is catalyzed by a mitochondrial matrix-localized protease. Further, using a tem-

¹ Abbreviations used in this paper: HMS, buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, pH 7.4; iOTC, intermediate ornithine transcarbamylase; OTC, ornithine transcarbamylase; pOTC, precursor ornithine transcarbamylase.
perature-shift protocol, we show unequivocally that iOTC is converted to OTC. To our knowledge, this is the first demonstration of such two-step cleavage for a matrix-localized mitochondrial enzyme. Finally, we have investigated the role of iOTC in the biogenesis of OTC by making amino acid substitutions in the leader peptide adjacent to the cleavage site that produces iOTC. Such substitutions abolish formation of both iOTC and OTC.

Materials and Methods

Materials

Restriction enzymes and other DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). RNAsin was obtained from Promega Biotech (Madison, WI) and SP6 RNA polymerase was from New England Nuclear (Cambridge, MA). [35S]Methionine, [3H]Tyrosine, [3H]Proline, and [3H]Phenylalanine were obtained from Amersham Corp. (Arlington Heights, IL). Trypsin, soybean trypsin inhibitor 2,4-dinitrophenol (DNP), leupeptin, phenylmethylsulfonyl fluoride (PMSF), pepstatin A, hexokinase, and tosyl-lysine chloromethyl ketone (TLCK) were from Sigma Chemical Co. Rhodamine 6G was from Eastman Kodak Co. (Rochester, NY).

Methods

Construction of Plasmids. An Xba I-Sac II fragment of rat pOTC cDNA plasmid (21) and a Sac II-Pm I fragment of pOTC-1 (17) were ligated with Xba I/Pst I-cut SP65 plasmid (Promega Biotech). Subsequently, the full-length pOTC cDNA insert was isolated with minimal flanking sequence from SP65 by digestion with Msp I and inserted into the Acc I site in the polyclinier region of pGEM2 (Promega Biotech). The Al-20 construct was generated by cutting out a BamH I-BamH I fragment from the full-length pOTC cDNA insert and ligating the remaining plasmid. This construct lacks the codon for the initiator methionine at position 1 of precursor OTC. Therefore, the peptide translated from an mRNA transcribed from this construct is initiated at the next inframe codon for methionine, i.e., the codon corresponding to amino acid 21 of pOTC. Mutant pOTC constructs encoding leader peptides that have the wild type arginine residue at position 23 replaced with either glycine or aspartic acid were prepared by inserting a double-stranded oligonucleotide cassette (49 mer), with appropriately changed codons, between the unique Nco I site in the leader of pGEM-OTC and the Pvu II site at position I2 of mature OTC (21). The constructs were subsequently sequenced by the method of Sanger et al. (34) to ensure that the desired changes were made.

In Vitro Transcription and Translation. For transcription from the SP6 promoter, the plasmid DNA was linearized with Hha I downstream from the T7 promoter. Linearized plasmid DNA was then used in a transcription reaction containing 500 mM nucleotide triphosphates, 1 mM rNTP (r[2P]GTP, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM DTT, 30 U RNasin, and 40 U SP6 polymerase. The reaction was carried out for 60 min at 37°C. Aliquots of the transcription mixture were used directly in translation reactions. Cell-free translation using the rabbit reticulocyte lysate system was carried out as previously described (6).

Incubation of Translation Mixture with Isolated Mitochondria and with Matrix Fraction. Mitochondria were isolated from rat liver as previously described (6) and diluted with 220 mM mannitol, 70 mM sucrose, 2 mM Hepes (pH 7.4) (HBS buffer) to 40 mg/ml total protein. Aliquots of the mitochondrial suspension (20 μl) were incubated with 20 μl of the translation mixture containing mitochondrial concentrations of 20 μg/ml for 30 min at 27°C. Products were then analyzed by electrophoresis on SDS-PAGE as described (4). When indicated, mitochondrial import mixtures were separated by trypsin (final concentration 40 μg/ml) for 10 min on ice and then supplemented with soybean trypsin inhibitor at a final concentration of 100 μg/ml. In some experiments, mitochondrial import mixtures were separated by supernatant of the mitochondrial pellet by centrifugation in an Eppendorf microfuge (Brinkmann Instruments, Inc., Westbury, NY) for 5 min. Supernatants were removed and analyzed by electrophoresis on SDS-PAGE. Pellets were resuspended in mitochondrial isolation buffer (HBS) and then analyzed by SDS-PAGE. When indicated, 2,4-dinitrophenol (final concentrations indicated in figure legends) was added to 20 μl mitochondria (protein concentration 40 μg/ml) and the mixture incubated on ice for 10 min before the addition of the same volume of pOTC-containing translation mixture. When indicated, lanes were scanned with a scanning densitometer (Joyce, Loebel and Co., Ltd., Gateshead, England) and the peaks were integrated planimetrically.

A mitochondrial matrix fraction was prepared as described by Conboy et al. (5), diluted to 4 mg/ml protein concentration and supplemented with MnCl₂ and ZnCl₂ (0.1 mM final concentration). Wild type and mutant precursors synthesized in the E. coli cytoplasmic translation mixture were then added. When effect of protease inhibitors was being studied, the translation mixture was supplemented with an equal volume of a 100,000 g supernatant of reticulocyte lysate containing the inhibitor before addition to the matrix fraction. Reaction mixtures were incubated for 60 min at 27°C, at which time the reaction was stopped by addition of immunoprecipitation buffer (50 mM NaCl, 10 mM EDTA, 0.5% Triton X-100, 0.25 mM SDS, 2% methionine). Samples were then immunoprecipitated with anti-OTC antiserum and the immunoprecipitates analyzed by SDS-PAGE.

Oocyte Microinjection Experiments. Female Xenopus laevis were obtained from the African Xenopus lab (Clarexich, Republic of South Africa), and were kept on a diet of beef liver and Nasco fog beetlie (Nasco Biologicals, Fort Atkinson, WI). Stage V-VI oocytes (8) were freed of follicular tissue by manual dissection, and were maintained in OR-2 saline (40). When indicated, mitochondrial import mixtures were treated with trypsin (500 μl M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% Na deoxycholate, and 0.1% NP-40, pH 7.4 (38), supplemented with 1 mM PMSF, 1 μM pepstatin A, and 2 mM leupeptin (lysis buffer).

The first cycle from the yield carrier OTC was 20-40%; the yield from repetitive cycles was 90-95%.

Results

Time Course of pOTC Import Suggests that iOTC Is an Intermediate

The time course of pOTC internalization was examined by incubating isolated rat liver mitochondria with pOTC-containing translation mixture at 27°C. At each time point an aliquot of the import mixture was removed and the products analyzed by SDS-PAGE. As shown in Fig. 1 A, incubation of the pOTC-containing translation mixture (lane 7) with mitochondria at 27°C leads to the conversion of pOTC into intermediate and mature-sized polypeptides. The amount of pOTC decreased steadily with time while the intensity of the mature OTC band increased steadily. Whereas no iOTC was detected at zero time, its intensity was maximal at 2 min of incubation and then decreased. Quantitation of this fluorograph by densitometric analysis is presented in Fig. 1 B. The observed kinetics are consistent with (but do not prove) a
precursor–product relationship between pOTC and iOTC and between iOTC and OTC.

Both iOTC and Mature OTC Are Localized within Mitochondria

Previous results on the posttranslational import of pOTC into isolated mitochondria have been interpreted to suggest that either iOTC is a bona fide intermediate in OTC biogenesis (19), or that iOTC is an artifact of the in vitro uptake systems (I). To examine these two general possibilities, we incubated mitochondria with pOTC-containing translation mixture at 27°C for 60 min, then separated the mitochondrial import mixture into mitochondrial pellet and supernatant fractions, and analyzed each by SDS-PAGE. As shown in Fig. 2 A, lane T, iOTC and mature OTC were produced during the incubation at the expense of pOTC (lane Tr). When this import mixture was centrifuged, only pOTC was found in the supernatant fraction (Fig. 2 A, lane S), while iOTC and OTC were found exclusively in the mitochondrial pellet (lane P) fraction. Some pOTC was recovered with the mitochondrial pellet as well; this could represent precursor either specifically bound to a surface receptor or nonspecifically adsorbed to the mitochondrial surface.

To determine the topology of iOTC, i.e., to test whether it is associated with the surface of the mitochondria or whether it has been translocated across the mitochondrial outer membrane, we tested its sensitivity to trypsin (Fig. 2 B). Translation mixture (Fig. 2 B, lane Tr) containing pOTC (and a small amount of A1-20 band which arises in the cell-free translation mixtures as a result of variable initiation at the internal methionine at position 21) was incubated with mitochondria; the total import mixture was then treated with trypsin and analyzed by SDS-PAGE (lanes T). Trypsin treatment resulted in total degradation of pOTC (and A1-20) and almost complete recovery of both iOTC and mature OTC. These data indicate that iOTC represents a mitochondrially associated, trypsin-protected species.

As mentioned earlier, Argan et al. (I) have dismissed iOTC as a nonmitochondrial in vitro product generated either by a “leaked” mitochondrial matrix protease or a contaminating protease of some other origin. They have presented evidence that, in their hands, iOTC was not localized within heart mitochondria but rather was recovered in the supernatant fraction of an import mixture. We do not find this to be the case when rat liver mitochondria are used in the import reaction. Indeed, as shown in Fig. 2, virtually all of the iOTC formed during a 60-min incubation at 27°C was associated with the mitochondrial pellet and was protected from trypsin digestion, indicating localization within the organelle. Therefore, we postulated that the discrepancy between our results and those of Argan et al. (I) might reflect differences in the intactness of mitochondria isolated from rat liver compared with those isolated from rat heart. It should be noted that preparation of heart mitochondria requires much longer homogenization of the tissue and hence could lead to production of more damaged mitochondria. To test this possibility, we exposed rat liver mitochondria to additional homogenization before using them in an import reaction. Such additional handling results in the isolation of mitochondria that leak, i.e., release some of their matrix content into the supernatant as evidenced by the release of significant amounts of mature OTC. Leaky mitochondria prepared in this way were incubated with translation mixture containing pOTC at 27°C. At selected time points, an aliquot of the import mixture was taken and the products were analyzed by SDS-PAGE. The results are shown in Fig. 3 A and closely match the time course of iOTC and OTC formation observed by Argan et al. (I). The amount of pOTC declined concomitantly with an increase in levels of iOTC and mature OTC. Comparison of this fluorograph to that in Fig. 1 A shows that the appearance of
The Journal of Cell Biology, Volume 105, 1987 2634

trifugation into supernatant and pellet. Each fraction was then ana-
as described in Materials and Methods and then separated by cen-
tral homogenization and then incubated with translation mixture containing pOTC at 27°C for 60 min. An aliquot of the total import mixture was analyzed by SDS-PAGE (lane T). A second aliquot was supplemented with HMS and centrifuged. The supernatant was removed and analyzed by SDS-PAGE (lane S – trypsin). The mitochondrial pellet was resuspended in HMS and analyzed by SDS-PAGE (lane P – trypsin). A third aliquot was treated with trypsin as described in Materials and Methods and then separated by centrifugation into supernatant and pellet. Each fraction was then analyzed by SDS-PAGE (lanes S + trypsin and P + trypsin, respectively).

OTC is significantly delayed and reduced in the damaged mitochondria. The kinetics of iOTC appearance also show a dramatic difference: in “healthy” mitochondria (Fig. 1 A) iOTC appearance shows a discrete early peak; in leaky ones (Fig. 3 A), iOTC rises almost linearly with time from 0 to 20 min of incubation. The latter kinetics would be expected if pOTC was being cleaved in the supernatant by a released matrix protease. To test this hypothesis further, we incubated damaged mitochondria with pOTC-containing translation mixture at 27°C for 60 min and then separated the import mixture into supernatant and mitochondrial fractions. As shown in Fig. 3 B, lane T, iOTC and OTC were generated during the incubation. Upon separation, the majority of iOTC was recovered in the supernatant, the majority of OTC was recovered in the pellet, and pOTC was present in both fractions. After trypsin treatment, pOTC, OTC, and iOTC in the supernatant were completely degraded, while, in the pellet, some iOTC and OTC were protected but pOTC was not.

Based on these data, we conclude that the intermediate-sized OTC species produced by damaged mitochondria is, indeed, produced by a protease released from mitochondria and is not related to import. However, our data (Fig. 2) clearly show that under our conditions of import, the amount of such extramitochondrial iOTC produced is so low as to be undetectable, and that the iOTC which we observe is a mitochondrially located form.

Formation of iOTC Requires an Intact Mitochondrial Membrane Potential

Normal uptake of pOTC (as measured by formation of mature OTC) requires an intact mitochondrial membrane potential (19, 26). To test whether iOTC formation depends on the same uptake mechanism as formation of OTC, we incubated pOTC-containing translation mixture with mitochondria that had been treated with the uncoupler, DNP. In the presence of DNP, the electrochemical gradient across the mitochondrial inner membrane collapses, and ATP is degraded as the mitochondrial membrane ATPase is uncoupled from electron transport. In the presence of 50 or 100 μM DNP (Fig. 4, lanes 2 and 3, respectively), production of both iOTC and OTC was inhibited. This is further evidence that iOTC is formed during the course of normal uptake of pOTC into mitochondria because uncoupling of mitochondria in the in vitro uptake reaction should have no effect on the action of an extramitochondrial protease. In the presence of DNP, only small amounts of pOTC were recovered with the mitochondria (most of the input pOTC was present in the supernatant of an import mixture) and probably represents pOTC nonspecifically bound to the mitochondrial surface. This suggestion is based on our results (data not shown) that DNP prevents binding of pOTC to mitochondria in vivo. Trypsin treatment of a DNP-blocked import mixture led to complete degradation of pOTC (data not shown).

iOTC Can Be “Chased” into Mature OTC

As shown in Fig. 5, lane 1, incubation of an import mixture at 16°C results in formation of approximately equal amounts of iOTC and OTC. Therefore, we could address the question whether iOTC can be chased into mature OTC. Isolated mitochondria were incubated with pOTC-containing translation mixture for 30 min at 16°C (lane 1). The import mixture incubated at 16°C was supplemented with trypsin and incubated on ice for 10 min. At that time, ST1 was added and the sample was divided into two portions. One was analyzed by SDS-PAGE (lane 2), while the other was incubated at 27°C for 30 min and then analyzed by SDS-PAGE (lane 3). Densitometric tracings of the autoradiogram are shown below the photograph.

Figure 3. Import of pOTC by damaged mitochondria. (A) Isolated mitochondria were subjected to additional homogenization (see text for details) and then incubated with translation mixture containing pOTC (lane T) at 27°C. At each time point a 5-μl aliquot of the import mixture was removed and analyzed by SDS-PAGE. (B) Isolated mitochondria were subjected to additional homogenization and then incubated with translation mixture containing pOTC at 27°C for 60 min. An aliquot of the total import mixture was analyzed by SDS-PAGE (lane T). A second aliquot was supplemented with HMS and centrifuged. The supernatant was removed and analyzed by SDS-PAGE (lane S – trypsin). The mitochondrial pellet was resuspended in HMS and analyzed by SDS-PAGE (lane P – trypsin). A third aliquot was treated with trypsin as described in Materials and Methods and then separated by centrifugation into supernatant and pellet. Each fraction was then analyzed by SDS-PAGE (lanes S + trypsin and P + trypsin, respectively).

Figure 4. DNP inhibits formation of iOTC. DNP was added to isolated mitochondria at a concentration of 50 or 100 μM. After 10 min on ice, pOTC-containing translation mixture was added and the import mixture incubated at 27°C for 30 min. Mitochondria were isolated by centrifugation and analyzed by SDS-PAGE. Lane 1, no DNP; lane 2, 50 μM DNP; lane 3, 100 μM DNP.

Figure 5. Conversion of iOTC into mature OTC. Isolated mitochondria were incubated with pOTC-containing translation mixture for 30 min at 16°C (lane 1). The import mixture incubated at 16°C was supplemented with trypsin and incubated on ice for 10 min. At that time, ST1 was added and the sample was divided into two portions. One was analyzed by SDS-PAGE (lane 2), while the other was incubated at 27°C for 30 min and then analyzed by SDS-PAGE (lane 3). Densitometric tracings of the autoradiogram are shown below the photograph.
Matrix fraction cleavage of pOTC: effect of inhibitors. pOTC-containing translation mixture was supplemented with ZnCl₂ (0.1 mM), MnCl₂ (0.1 mM), and 100,000 g supernatant of reticulocyte lysate containing specific inhibitor. Mitochondrial matrix fraction was added to 4 mg/ml final protein concentration, and the mixture was incubated at 27°C for 60 min (lanes 3–8) or at 16°C for 30 min (lane 2). The reaction was stopped by adding immunoprecipitation buffer. The OTC species were immunoprecipitated and the immunoprecipitates analyzed by SDS-PAGE. Lane 1, pOTC-containing translation mixture plus matrix fraction incubated at 16°C; lane 2, pOTC-containing translation mixture; lane 3, pOTC-containing translation mixture plus matrix fraction incubated at 16°C; lane 4, as in lane 3 plus leupeptin (2 mg/ml); lane 5, as in lane 3 plus pepstatin A (2 mg/ml); lane 6, as in lane 3 plus 1,10-phenanthroline (5 mM); lane 7, as in lane 3 plus rhodamine 6G (40 μg/ml); lane 8, as in lane 3 plus 50 U/ml hexokinase and 0.1 M glucose.

Figure 6. Matrix cleavage of pOTC: effect of inhibitors. pOTC-containing translation mixture was supplemented with ZnCl₂ (0.1 mM), MnCl₂ (0.1 mM), and 100,000 g supernatant of reticulocyte lysate containing specific inhibitor. Mitochondrial matrix fraction was added to 4 mg/ml final protein concentration, and the mixture was incubated at 27°C for 60 min (lanes 3–8) or at 16°C for 30 min (lane 2). The reaction was stopped by adding immunoprecipitation buffer. The OTC species were immunoprecipitated and the immunoprecipitates analyzed by SDS-PAGE. Lane 1, pOTC-containing translation mixture; lane 2, pOTC-containing translation mixture plus matrix fraction incubated at 16°C; lane 3, pOTC-containing translation mixture plus matrix fraction incubated at 27°C; lane 4, as in lane 3 plus leupeptin (2 mg/ml); lane 5, as in lane 3 plus pepstatin A (2 mg/ml); lane 6, as in lane 3 plus 1,10-phenanthroline (5 mM); lane 7, as in lane 3 plus rhodamine 6G (40 μg/ml); lane 8, as in lane 3 plus 50 U/ml hexokinase and 0.1 M glucose.

A Mitochondrial Matrix Fraction Cleaves pOTC to iOTC

To further characterize the pOTC to iOTC cleavage, we incubated pOTC with an isolated mitochondrial matrix fraction, prepared as described by Conboy et al. (5). The matrix fraction cleaved pOTC to both iOTC and OTC (Fig. 5, lanes 2 and 3). In an attempt to selectively inhibit iOTC formation, we tested a number of protease inhibitors for their effect on the matrix-catalyzed cleavage. The protease inhibitor pepstatin A (1 mg/ml) and the chelating agent 1,10-phenanthroline (5 mM), each inhibited formation of mature OTC without substantially inhibiting iOTC formation (Fig. 6, lanes 5 and 6). Leupeptin (2 mg/ml) reduced formation of both iOTC and OTC (Fig. 6, lane 4), while rhodamine 6G and ATP depletion had no effect (Fig. 6, lanes 7 and 8). Significantly, we did not find an inhibitor that specifically inhibited production of iOTC while allowing OTC production. Because we could build up intermediate under conditions (16°C incubation) in which little OTC was produced (Fig. 6, lane 2), we used this system to produce sufficient iOTC to define the sequence of the NH₂ terminus of the iOTC peptide.

iOTC Is Produced by Cleavage within the pOTC Leader Sequence

To establish that the apparent difference in molecular weight between iOTC and either pOTC or mature OTC reflects cleavage of a part of the pOTC leader and not some modification merely altering electrophoretic mobility on SDS-PAGE, we sequenced the amino terminus of iOTC. Precursor OTC was synthesized in a reticulocyte lysate in the presence of [³²P]tyrosine or [³²P]proline. The translation mixture was incubated with a mitochondrial matrix fraction at 16°C (under conditions which favor iOTC production) and then immunoprecipitated with anti-OTC antiserum. The immunoprecipitated material was eluted from protein A-Sepharose, dialyzed, and subjected to automated Edman degradation (Fig. 7). [³²P]Tyrosine and [³²P]proline were chosen because these amino acids are found in the region of the pOTC leader expected to be retained in iOTC. Importantly, they are not found in the amino-terminal portion of the leader; thus, no label would be released from any residual pOTC in the immunoprecipitate. Radioactivity released during Edman degradation of [³²P]tyrosine-labeled material was detected in cycle 3; when material was radiolabeled with [³²P]proline, radioactivity was detected in cycle 6. The numbering of each cycle of Edman degradation was checked by analyzing amino acids released during each cycle from carrier mature OTC and comparing them with the sequence of mature OTC. In the cycle in which [³²P]tyrosine was released, cold valine was detected and in the cycle containing [³²P]proline, cold lysine was present. Comparison with the amino acid sequence deduced from the cDNA sequence (21; see Fig. 7) shows, therefore, that the cleavage site used in production of iOTC is between residues 24 (asparagine) and 25 (phenylalanine) of the leader.

iOTC Is Formed In Vivo

The data presented thus far define parameters of iOTC formation and conversion to OTC by isolated rat liver mitochondria. To eliminate the possibility that generation of iOTC...
represents an artifactual (but mitochondrial) cleavage due to the disruption of mitochondria during their isolation, we examined the import of rat pOTC in an intact cell, i.e., in the frog oocyte. This experimental system eliminates the possible mitochondrial damage which might occur during liver homogenization and fractionation. Translation mixtures containing either pOTC or Δ1-20 pOTC were injected into oocytes. Since Δ1-20 pOTC is neither taken up by rat liver mitochondria nor cleaved (22; and Fig. 2), this mutant polypeptide acts as a control in the oocyte system. After injection, the cells were incubated for 120 min at 19°C and then lysed by addition of lysis buffer (see Materials and Methods) containing protease inhibitors. The lysates were immunoprecipitated and analyzed by SDS-PAGE. As shown in Fig. 8, pOTC, iOTC and OTC could be detected in oocytes injected with pOTC-containing translation mixture while only the starting material, Δ1-20 pOTC, was seen in oocytes injected with Δ1-20 pOTC-containing translation mixture. Recent results with OTC precursors expressed in vivo in transfected HeLa cells have also revealed an intermediate during import of human OTC in vivo (data not shown).

**Figure 8.** Intermediate OTC is formed in vivo. Frog oocytes were injected with translation mixture containing either pOTC or Δ1-20 OTC and incubated at 19°C for 120 min. Oocytes were then lysed in the presence of proteolytic inhibitors, the lysates were immunoprecipitated, and the immunoprecipitates were analyzed by SDS-PAGE. Lanes 1 and 2, immunoprecipitated polypeptides from cells injected with pOTC or Δ1-20 OTC-containing translation mixtures, respectively.

Mutations Introduced Adjacent to iOTC Cleavage Site Abolish Production of Normal iOTC and OTC

As a further test of the importance of iOTC in the production of OTC, we attempted to disrupt the cleavage site between position 24 and 25 by introducing novel amino acids into the pOTC sequence. We chose as our target the arginine at position 23 of the leader for several reasons: (a) an abundance of arginine residues is the one common feature of all mitochondrial leader peptides sequenced thus far; (b) while characterizing a partially purified, leader peptide–cleaving mitochondrial protease, Miura et al. (23) suggested that most leaders that are cleaved by a chelator-sensitive matrix protease have a basic amino acid residue at position −2 or −3 relative to the cleavage site; and (c) substitution of a glycine for arginine 23 of the human pOTC leader abolishes cleavage of that precursor to mature OTC (15).

The arginine at position 23 of the rat pOTC leader was changed to either glycine or aspartic acid by standard methods of in vitro site-directed mutagenesis. The mutant precursors were synthesized in the rabbit reticulocyte lysate system, and the translation products were incubated with isolated mitochondria. As shown in Fig. 9 A, both precursors were cleaved to a novel OTC species, whose gel mobility was intermediate between pOTC and the “Δ1-20” band. In the case of the asp 23 substitution, the novel cleaved form was the only “new” product detected after incubation of the mutant precursor with mitochondria. Whereas the novel cleavage product was also the predominant product formed from the gly 23 mutant precursor, longer exposures of the fluorograph revealed weak bands corresponding to iOTC and OTC. Since inhibition of OTC production corresponds in each case with inhibition of iOTC production, we conclude that the production of OTC is tightly linked to the production of iOTC.

**The Novel Cleavage Form Is the Product of Mitochondrial Import and a Matrix Protease**

We incubated both the asp 23 and the gly 23 mutant precursors with isolated mitochondria to establish whether the formation of the novel cleavage products reflected import of the mutant precursor and cleavage by a matrix protease. Because analogous results were obtained for asp 23 and gly 23 mutations adjacent to the position 23 residue of the OTC leader, we concluded that the formation of the novel cleavage products either reflected import of the novel precursors or cleavage by a matrix protease.
tants, only the data for asp 23 are shown. Asp 23 pOTC-containing translation mixture was incubated with mitochondria (Fig. 9B, lane 3) and then proteinase K was added to the reaction mixture (Fig. 9B, lane 4). As expected for an imported protein, the new product is protected from externally added protease, while most of the precursor and the unimportable Δ1-20 are degraded.

The results of incubation of asp 23 precursor with mitochondria in the presence of DNP are presented in Fig. 9B, lane 2. No cleavage to the novel product was observed in the presence of inhibitor, as would be expected if cleavage follows the normal uptake which is dependent on a mitochondrial membrane potential.

To further characterize the cleavage of the mutant rat precursors, we incubated asp 23 and gly 23 pOTCs with a soluble matrix fraction from rat liver mitochondria. As shown in Fig. 9C, asp 23 precursor was cleaved by the soluble matrix fraction to a product migrating with mobility identical to the product seen after incubation with intact mitochondria. Analogous results were obtained for gly 23 pOTC (data not shown).

Identification of the “Cryptic” Cleavage Site

Because we were interested in the novel cleavage site that is used in the gly 23- and asp 23-substituted rat OTC precursors, we used the matrix fraction to prepare [3H]phenylalanine- and [35S]methionine-labeled asp 23 and gly 23 cleavage products for radiosequencing. We used either [3H]phenylalanine or [35S]methionine in the translation mixture, reasoning that these labels would be close to the amino terminus of an intermediate species which was apparently longer than the Δ1-20 internal initiation product. As shown in Fig. 10, label from [3H]phenylalanine-labeled material was recovered in cycle 9, while label from [35S]methionine-labeled material was recovered in cycles 1 and 5. This maps the asp 23 novel cleavage site between residues 16 and 17 of the pOTC leader. The methionine label released in cycle 1 is from the pOTC and Δ1-20 which remain in the sequenced sample (see Fig. 9C for an example of the material that was sequenced). We have also determined that the major product formed during incubation of gly 23 substituted pOTC with mitochondria is cleaved between residues 16 and 17 (data not shown).

Discussion

During the nearly ten years that the biogenesis of OTC has been investigated, a rat OTC species (iOTC) intermediate in size between the precursor and the mature form of OTC has been observed repeatedly in this and in a number of other laboratories. The precise significance of this molecule, however, has not been defined. While some (19, 25) have proposed that iOTC represents a bona fide intermediate in the mitochondrial import pathway of OTC, others (1) have concluded that the appearance of iOTC is due to artificial cleavage of pOTC and is not part of the physiologic pathway of mitochondrial import and cleavage. In this report we present data that define iOTC as a true intermediate in the mitochondrial import pathway of OTC. This conclusion is based upon the following findings: (a) iOTC produced in an import reaction containing undamaged mitochondria is recovered in the mitochondrial pellet; (b) iOTC produced at 27°C is trypsin protected, suggesting intramitochondrial localization; (c) iOTC can be converted directly to mature OTC; (d) iOTC is not produced in the presence of an uncoupler of oxidative phosphorylation, indicating that cleavage occurs only after pOTC is internalized by mitochondria; (e) iOTC is produced in intact cells, thereby excluding the possibility that its appearance is due to damaged mitochondria or to some other artifact inherent in the in vitro protocols; and (f) substitutions near the iOTC cleavage site inhibit formation of both iOTC and mature OTC.

The production of intermediates in the biogenesis of mitochondrial enzymes has been noted before. Partially cleaved forms of yeast cytochrome b2 (29, 32) (a soluble enzyme of the intermembrane space), cytochrome c1 of yeast (12, 29) and Neurospora (36), Fe/S protein of ubiquinol-cytochrome c reductase (14) (proteins located in the inner membrane and exposed to the intermembrane space), and ATPase subunit 9 (37) (inner membrane protein) have been demonstrated in vitro and in vivo. These precursors are first cleaved within the leader to an intermediate form by a matrix-located protease, then cleaved again by a protease located either in the matrix (37) or in the intermembrane space (29, 32). The two-step processing which has been described for the precursors of yeast cytochrome b2 and the Fe/S protein of Neurospora has been shown to be important in determining the sub mitochondrial localization of these enzymes. After translocation, cytochrome b2 precursor is cleaved to produce an intermediate containing a domain spanning the inner mitochondrial membrane and majority of the protein protruding into the intermembrane space. In the second cleavage step, the membrane domain is removed, resulting in the release of soluble enzyme into the intermembrane space. The Fe/S protein precursor is translocated entirely into the matrix where the extreme NH2 terminus of the leader peptide is

Figure 10. Sequence analysis of novel OTC species. Amino acid sequence of pOTC deduced from the cDNA sequence (21). Arrow indicates location of the cleavage site to produce novel OTC species. Positions of labeled amino acids, phenylalanine (triangle) and methionine (diamond), are indicated. Mutant pOTC (asp 23) was synthesized in reticulocyte lysate in the presence of [35S]methionine (left) or [3H]phenylalanine (right). Precursor was cleaved by a matrix fraction as shown in Fig. 9C, the radiolabeled proteins were prepared for sequencing as described in Materials and Methods and subjected to Edman degradation.
cleaved. A remaining hydrophobic portion of the leader peptide then acts as a "signal peptide" and directs the transfer of the mature portion of the protein back across the inner membrane. Once transfer is complete, a protease located outside the inner mitochondrial membrane removes the remaining portion of the leader.

To our knowledge this is the first report describing a matrix enzyme precursor that is cleaved twice during its biogenesis. Why a second cleavage event has evolved (or persisted) in the biogenesis of OTC is unclear. Since both cleavage events occur in the mitochondrial matrix, the portion of the leader peptide left on iOTC is probably not a subunit of a more general mitochondrial protease. In a recent paper, Nguyen et al. (25) have shown that deletion of residues 22-30 of this same leader peptide in rat pOq~ leader adjacent to the iOTC cleavage site inhibits other, more general, mitochondrial protease.

A different, chelator-insensitive, mitochondrial protease that might allow time for interaction with additional subunits in order to facilitate trimer formation. (c) A second proteolytic event may permit the formation of precise OTC NH2 terminus by trimming of iOTC. The ensuing delay in folding of the translocated protein might allow time for interaction with additional subunits in order to facilitate trimer formation. (c) A second import channel might have arisen which is associated with a different, chelator-insensitive, mitochondrial protease that produces only intermediate from pOTC. Subsequent conversion of this iOTC to OTC would then require cleavage by another, more general, leader peptide protease.

We have shown that certain amino acid substitutions in the rat pOTC leader adjacent to the iOTC cleavage site inhibit formation of iOTC and mature OTC. These substitutions allow translocation of the altered precursors but lead to formation of a novel form of OTC cleaved between residues 16 and 17 of the leader. In a recent paper, Nguyen et al. (28) have shown that deletion of residues 22-30 of this same precursor has similar effects: translocation of the altered precursor without cleavage to mature OTC, and inefficient cleavage of the translocated precursor at a new site within the leader peptide.

Several explanations for the effect of position 23 substitution and deletion of position 23 on formation of mature OTC can be put forward. If iOTC is an obligate precursor to OTC, then interfering with iOTC cleavage would also inhibit OTC production. Alternatively, the critical peptide structure determined by arginine 23 may prevent both iOTC and OTC cleavage sites to the active site or sites of one or more matrix proteases. In this second case, the critical conformation must be maintained after removal of amino acid residues 1-24, because we have clearly demonstrated that iOTC formed in isolated mitochondria can be cleaved to OTC.

Each of the above models makes specific predictions regarding the number and specificities of mitochondrial matrix proteases. We are currently isolating the protease responsible for each of the steps of pOTC conversion to OTC in order to define whether formation of iOTC is an obligatory step in OTC production.

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