Characterization of a protease apparently involved in processing of pre-ornithine transcarbamylase of rat liver

(Mitochondrial protease/precursor enzyme/import and processing/leupeptin inhibition)

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ABSTRACT The precursor of rat liver ornithine transcarbamylase (ornithine carbamoyltransferase; carbamoylphosphatet-ornithine carbamoyltransferase, EC 2.1.3.3) (pre-ornithine transcarbamylase), which was synthetized in a reticuloocyte lysate cell-free system, was converted to an apparently mature form of the enzyme by isolated rat liver mitochondria. The proteolytic processing involved two steps: (i) conversion of pre-ornithine transcarbamylase (38,400 daltons) to a product of about 37,000 daltons and (ii) further conversion to the apparently mature form of the enzyme (36,600 daltons). When mitochondria were subfractionated by digitonin treatment followed by sonication of a mitoplast fraction, the proteolytic activity catalyzing the first step was recovered mainly in a matrix fraction. Some activity was found in an intermembrane space fraction. The enzyme activity in the matrix fraction has an optimal pH at about 7.5. The activity was inhibited almost completely by 2 mM leupeptin and partly by 2 mM antipain but not significantly by other microbial protease inhibitors or serine protease inhibitors. It was inhibited strongly by 2 mM EDTA, 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetate, 2 mM p-chloromercuribenzenesulfonate, and 2 mM Hg(CH3COO)2 but not by N-ethylmaleimide or iodoacetamide. These results suggest that pre-ornithine transcarbamylase is first transported into the mitochondrial matrix and converted there to the mature form of the enzyme by a novel neutral protease(s).

Ornithine transcarbamylase (ornithine carbamoyltransferase; carbamoylphosphate:ornithine carbamoyltransferase, EC 2.1.3.3), which catalyzes the second step of urea synthesis, has been purified to homogeneity from bovine liver and extensively characterized (1–5). The enzyme has also been purified to homogeneity from rat (6–8) and human (9, 10) liver. The rat liver enzyme is a trimer of identical subunits, each of 36,600 (6), 39,600 (7), or 35,300 (8) daltons, and is localized in the mitochondrial matrix (11). Like other matrix enzymes (see refs. 12 and 13 for review), the transcarbamylase is coded by nuclear genes, synthesized on cytoplasmic ribosomes, and subsequently transported into mitochondria. Recently, we (14) and Conboy et al. (15) reported the in vitro synthesis of a precursor of the enzyme (pre-ornithine transcarbamylase or pOTCase) which is 3400–4000 daltons larger than the mature subunit. We (14) further showed that pOTCase was imported into mitochondria in vitro in association with posttranslational proteolytic processing of pOTCase to the apparently mature form of the enzyme.

In this communication we show that the processing of pOTCase of rat liver to the apparently mature form involves two successive proteolytic steps. Submitochondrial distribution and characterization of the protease catalyzing the first step of the processing are reported.

MATERIALS AND METHODS

Cell-Free Protein Synthesis. Total RNA of rat liver was prepared by phenol/NaDodSO4 extraction (16) and translated in a nucleo-treated reticuloocyte lysate system (17) as described (14, 16) except that the protease inhibitors were omitted from the reaction mixture. The translation mixture was used immediately after preparation or kept in small aliquots at −20°C.

Subfractionation of Mitochondria. Rat liver mitochondria were prepared by the method of Schnaitman and Greenawalt (18) except that bovine serum albumin was omitted from the isolation medium. Mitochondria were subfractionated into outer membrane, inner membrane plus matrix fractions by the digitonin procedure (18). Mitoplasts were suspended in 20 mM potassium Hepes, pH 7.4/1 mM dithiothreitol at a concentration of 38 mg of protein per ml, disrupted by sonication at 20 kHz for 2 min, and centrifuged at 105,000 × g for 60 min at 4°C (precipitate, inner membrane fraction; supernatant, matrix fraction). The matrix fraction was kept in small aliquots at −80°C. Protein of mitochondria and their subfractions was determined by the method of Clarke (19).

Preparation of Crude Lysosomal Extracts. A mitochondrially lysosomal fraction of rat liver prepared as described by Tsuji et al. (20) was subjected to osmotic shock by suspending the fraction in 25 mM sucrose. After standing for 20 min on ice, the suspension was centrifuged at 105,000 × g for 60 min. The pellet was washed with 0.1 M KCl; the supernatant and the washing were combined and used as crude lysosomal extracts (20). The extracts had acid phosphatase activity of 0.07 μmol/min per mg of protein when assayed with p-nitrophenylphosphate as substrate (20).

Processing of pOTCase by Mitochondria and Their Subfractions. The reaction mixture contained 20 μl of translation mixture and 30 μl of mitochondria or their subfractions suspended in 20 mM potassium Hepes, pH 7.4/0.25 M sucrose/1 mM dithiothreitol in a final volume of 50 μl. After incubation at 25°C, 1 ml of 10 mM Tris-HCl, pH 7.4/0.1% NaDodSO4/2 mM EDTA/0.5 mM antipain/0.5 mM leupeptin/0.5 mM chymostatin/0.5 mM pepstatin and 26 μg of affinity-purified anti-bovine ornithine transcarbamylase (14) were added to the reaction mixture and the mixture was kept for 20 min at 25°C. Then, fixed Staphylococcus aureus cells (Cowan I strain, 100 μl of a 10% suspension) were added, and the cells were washed and pOTCase and its products were extracted as described (14).
Materials. Microbial protease inhibitors antipain, leupeptin, chymostatin, and pepstatin were obtained from Peptide Institute (Osaka, Japan). Elastatinal, phosphoramidon, amastatin, and bestatin were provided by T. Aoyagi (Institute of Microbial Chemistry, Tokyo), and aprotinin was from Mochida Pharmaceutical Co. (Tokyo). Fixed S. aureus cells were prepared by a modification (16) of the procedure described by Kessler (26).

RESULTS

Incorporation of [35S]methionine into pOTCase by nuclease-treated reticulocyte lysate programmed with total RNA from rat liver was allowed to proceed for 90 min at 25°C. When the reaction mixture containing labeled pOTCase was subsequently incubated with a mitochondrial preparation of rat liver and the immunoprecipitates were analyzed by NaDodSO4/polyacrylamide gel electrophoresis and fluorography, the amount of labeled pOTCase (39,600 daltons) was reduced and two radioactive polypeptides appeared; one was about 37,000 daltons and the other was at the position of the mature subunit of the enzyme (36,000 daltons) (Fig. 1). Kinetic studies showed a precursor-product relationship between the 37,000-dalton product and the apparently mature form of the enzyme. These results, as well as our previous observation (14), indicate that the processing involves two steps: (i) conversion of pOTCase to a product of 37,000 daltons and (ii) further conversion to the apparently mature form of the enzyme. Inclusion of anti-or-
nithine transcarbamylase in the reaction mixture resulted in a complete inhibition of the processing. The processing was posttranslational, because similar conversion was observed when postribosomal supernatant of the translation mixture was incubated with a mitochondrial preparation. No such conversion was observed when pOTCase was incubated with a microsomal fraction or a cytosol fraction as shown previously (14).

In order to exclude the possibility that the "processing" activity was associated with lysosomes contained in the mitochondrial fraction, pOTCase was treated with crude lysosomal extracts (Fig. 2). pOTCase was degraded with increasing amounts of the extracts, but no limited proteolysis was seen. Thus, it was concluded that the processing activity was associated with mitochondria.

Rat liver mitochondria were fractionated into the four fractions by digitonin treatment and by sonication of the low-speed pellet. Assay of marker enzymes in the subfractions showed that the fractions (mitoplast, outer membrane, intermembrane space, inner membrane, and matrix) were reasonably enriched with the respective components (Table 1). The proteolytic activity of the first three subfractions catalyzing the conversion of pOTCase to the 37,000-dalton product was highest in the mitoplast fraction (Fig. 3). Some activity was seen in the intermembrane space fraction and little activity in the outer membrane fraction. When the mitoplast fraction was further subfractionated, most of the processing activity was recovered in the matrix fraction. These results indicate that the processing activity is located mainly in the mitochondrial matrix.

We found that, in contrast to the processing by isolated mitochondria, inclusion of anti-ornithine transcarbamylase in the assay mixture did not inhibit the proteolytic conversion of pOTCase by the matrix fraction and was effective in preventing degradation of pOTCase and its 37,000-dalton product. The conversion in the presence of the antibody increased with

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Submitochondrial distribution of protease activity involved in the processing of pOTCase. Twenty microliters of a translation mixture containing about 1000 cpm of pOTCase was incubated for 30 min with submitochondrial fractions as follows. (A) Lane 1, none; lanes 2, 4, 10, 50, and 200 ng of outer membrane fraction, respectively; lanes 5 and 6, 11 and 68 ng of intermembrane space fraction, respectively; lanes 7 and 8, 10 and 50 ng of mitoplast fraction, respectively. (B) Lane 5, none; lanes 1 and 2, 10 and 50 ng of inner membrane fraction, respectively; lanes 3 and 4, 10 and 50 ng of matrix fraction, respectively. Immunoprecipitation was performed with 13 ng of anti-ornithine transcarbamylase and 50 ng of S. aureus cells, and the immunoprecipitates were analyzed by NaDodSO4/10% polyacrylamide gel electrophoresis and fluorography. Portions of the fluorograms are shown. 37, 37,000-dalton product.

![Fig. 4](https://example.com/fig4.png)

**Fig. 4.** Effect of pH on the processing activity. Ten microliters of a translation mixture was incubated with a matrix fraction (20 μg of protein) for 60 min in the presence of 13 ng of anti-ornithine transcarbamylase. Buffers used were potassium 2-(N-morpholino)-ethanesulfonate (pH 6.5), potassium Hepes (pH 7.0-8.0), and Tris-HCl (pH 8.5 and 9.0) (100 mM each). After incubation, the reaction mixture was subjected to immunoprecipitation, NaDodSO4/10% polyacrylamide gel electrophoresis, and fluorography. Gels were cut into strips and radioactivity in the bands of pOTCase and the 37,000-dalton product (37) was measured. A portion of the fluorogram is shown at the top of the figure. ○, pOTCase plus 37,000-dalton product; ●, 37,000-dalton product.
amounts of the matrix fraction up to 20 μg of protein and with time up to 60 min at 25°C (data not shown).

The effect of pH on the processing activity of the matrix fraction is shown in Fig. 4. The activity was maximal at about pH 7.5, decreased at pH 6.5, but did not significantly decrease up to pH 9.0.

The effect of various protease inhibitors on the processing activity of the matrix fraction is shown in Fig. 5. The activity was inhibited almost completely by 2 mM leupeptin (inhibiting trypsin, plasmin, papain, and cathepsin B) and by 2 mM antipain (inhibiting trypsin, papain, and cathepsins A and B), but not by chymostatin (inhibiting chymotrypsins, papain, and cathepsins A, B, and D), pepstatin (inhibiting pepsin, cathepsin D, and renin), elastatinal (inhibiting elastase), phosphoramidone (inhibiting thermolysin and collagenase), bestatin (inhibiting aminopeptidases), or amastatin (inhibiting aminopeptidases). (For review of these microbial protease inhibitors see refs. 27 and 28.) Leupeptin partly inhibited the activity at a concentration of 0.5 mM but did not significantly inhibit the activity at a concentration of 0.1 mM (data not shown). The activity was inhibited strongly by 2 mM EDTA, 2 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N',N'-tetraacetate, and antipain (1 mg/ml) (inhibiting trypsin, chymotrypsin, plasmin, kallikrein, and cathepsin D); weakly by 2 mM diisopropyl fluorophosphatase, 2 mM tosyllysine chloromethyl ketone, and 2 mM tosylphenylalanine chloromethyl ketone; and not at all by soybean trypsin inhibitor or phenylmethylsulfonyl fluoride. It was strongly inhibited by 2 mM p-chloromercuriphenylsulfonate and 2 mM Hg(CH₃COO)₂; the inhibition by p-chloromercuriphenylsulfonate was reversed by 4 mM dithiothreitol. However, the enzyme was not inhibited by N-ethylmaleimide or iodoacetamide.

Specificity of the proteolytic activity of a matrix fraction was investigated as follows. A translation mixture containing pOTCase was incubated with a matrix fraction, and immunoprecipitation and washings were performed in 1% Triton X-100 in an attempt to retain polypeptides other than pOTCase and its product. The limited proteolysis of pOTCase to the 37,000-dalton product proceeded with time (Fig. 6). On the other hand, other polypeptides were degraded gradually and no limited proteolysis was observed. The proteolytic activity that degraded practically all peptides in a nonlimited fashion...
was contained in the reticulocyte lysate as well as in the mitochondrial matrix fraction (Fig. 6). These results indicate that the “processing” activity is fairly specific for pOTCase.

**DISCUSSION**

It is now apparent that a number of mitochondrial proteins of higher animals (14-16, 29, 30) as well as of yeast (21, 31, 32) and *Neurospora* (33, 34) are initially synthesized as larger precursors. The precursor of ornithine transcarbamylase (pOTCase) (14) and those of other enzymes (16, 21, 32) are converted posttranslationally to the apparently mature form of the enzymes by isolated mitochondria (14, 21, 32) or a mitochondrial membrane preparation (16). The present results show that the processing of pOTCase by isolated mitochondria involves two successive proteolytic steps. The proteolytic enzyme catalyzing the first step of the processing was recovered mainly in the mitochondrial matrix fraction. Thus, it is likely that pOTCase (or its extrapeptide region) first enters the matrix and is converted there to the mature form of the enzyme. However, some processing activity appears to be located also in the intermembrane space; the activity is probably not due to the leakage from the matrix, because little ornithine transcarbamylase activity was recovered from the intermembrane space fraction. Anti-ornithine transcarbamylase inhibited the processing of pOTCase by isolated mitochondria probably by preventing the entry of pOTCase into the organelle.

The protease in the matrix fraction described here is apparently different from any of the well-characterized proteases with respect to sensitivity to protease inhibitors and subcellular localization. The enzyme is different from a mitochondrial protease described by Aoki (35), which is located on the mitochondrial inner membrane of bone marrow cells and sensitive to disopropylfluorophosphatase and elastatin. To our knowledge, the enzyme described by Aoki is the only mitochondrial protease that has been highly purified and well characterized. Furthermore, the protease described here appears to catalyze the limited proteolysis of pOTCase in a highly specific manner.

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