Vol. 105, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 15, 1982 Pages 1-7

PROCESSING OF PRE-ORNITHINE TRANSCARBAMYLASE REQUIRES A ZINC-DEPENDENT PROTEASE LOCALIZED TO THE MITOCHONDRIAL MATRIX

John G. Conboy, Wayne A. Fenton and Leon E. Rosenberg

Department of Human Genetics
Yale University School of Medicine
New Haven, Connecticut 06510

Received January 5, 1982

Proteolytic processing of the larger precursor of the rat liver mitochondrial matrix enzyme, ornithine transcarbamylase, has been studied in a cell free system using in vitro-synthesized precursor and various submitochondrial fractions. The protease responsible for cleavage to mature-sized enzyme fractionates with matrix marker enzymes. Maximal catalytic activity of this matrix protease requires approximately 0.1 mM Zn^{2+}, a concentration known to be in the physiological range for this divalent cation in intact mitochondria. Certain other divalent metal ions (Co^{2+} and Mn^{2+}) also stimulate this protease activity, while 1,10-phenanthroline, a divalent metal ion chelator, inhibits the protease. We conclude that proteolytic cleavage by a zinc-dependent protease in the mitochondrial matrix of rat liver is a required step in the conversion of pre-ornithine transcarbamylase to the mature subunit.

Biogenesis of the mitochondrial matrix enzyme, ornithine transcarbamylase (OTC[1]; EC 2.1.3.3), begins with synthesis on cytoplasmic ribosomes of a precursor (pre-ornithine transcarbamylase, or pOTC) which is ~4000 daltons larger than its corresponding mitochondrial subunit (1-3) by virtue of an NH_2-terminal extension of amino acids (4). Posttranslational uptake and proteolytic processing of the precursor by isolated rat liver mitochondria has recently been reported (2,5), opening the way to biochemical characterization of this "vectorial processing" mechanism in a simplified cell-free system.

Initial studies have suggested that there are two specific sites in pOTC which are susceptible to attack by mitochondrial protease(s) (5,6). Cleavage at one of these sites yields a product (iOTC) intermediate in size between pOTC and the mature subunit, while cleavage at the other site produces mature-sized subunit. Mori et al. (6) have reported previously some characteristics of a rat liver mitochondrial matrix activity which catalyzes cleavage of pOTC to iOTC. We report here our initial studies of

[1]Abbreviations used: pOTC, precursor of ornithine transcarbamylase subunit; iOTC, intermediate-sized form of the processed precursor; OTC, the subunit of ornithine transcarbamylase; SDS, sodium dodecyl sulfate.

0006-291X/82/050001-07$01.00/0

the protease activity which produces mature OTC subunits, including its submitochondrial localization and its metal ion requirements.

MATERIALS AND METHODS

Materials. L-[^{35}S]methionine (>600 Ci/mmol) was purchased from Amersham; inactivated Staphylococcus aureus cells were obtained from the Enzyme Center, Boston; and 1,10-phenanthroline was from Sigma. Other reagents used for translation and immunoprecipitation were those described previously (4).

Subfractionation of Mitochondria. Livers from male Sprague-Dawley rats (150-250 g) were used for preparation of intact mitochondria according to the method of Schnaitman and Greenawalt (7); the procedure described by Loewenstein et al. (8), which employs treatment of mitochondria with a dilute digitonin solution, was used to minimize contamination of the mitochondrial fraction by lysosomes. As indicated by assays of the lysosomal marker enzyme β-galactosidase, this procedure reduced lysosomal contamination by more than 99.8%. Mitochondria were separated into outer membrane, intermembrane space, and mitoplast fractions by the digitonin procedure (7), after which mitoplasts were further fractionated into inner membrane and matrix using Lubrol WX (7). Marker enzyme assays were performed as described (7).

Processing of pOTCase by Mitochondrial Fractions. Reaction mixtures contained 20 µl of translation mixture and 20 µl of mitochondria or fractions thereof, suspended in 220 mM mannitol/70 mM sucrose/2 mM Hepes, pH 7.4 at 4°C, in a final volume of 40-50 µl. Divalent metal ions (as the chloride salts) and 1,10-phenanthroline were added as indicated in the text and figure legends. Processing was performed by incubation at 26°C for 60 min; under these conditions, processing was linear with time for at least 30 min and with amounts of protein up to 400 µg. Samples were then diluted with 1.0 ml of 150 mM NaCl/10 mM EDTA/0.5% Triton X-100/2% (w/v) unlabeled methionine/0.25% SDS and immunoprecipitated as described (5).

General Procedures. Cell-free protein synthesis was performed in a nuclease-treated reticulocyte lysate system (9), using rat liver polysomal RNA prepared as described (1). Protein determinations of mitochondrial fractions (10), SDS/polyacrylamide gel electrophoresis (11), and fluorography (12) of dried gels were done according to published procedures.

RESULTS

Theoretically, any of the four submitochondrial fractions (outer membrane, intermembrane space, inner membrane, and matrix) might contain the endoprotease(s) required to cleave the NH_2-terminal extension of pOTC. Therefore, we assayed each of these fractions (see Table I for marker enzyme distributions) for the presence of specific endoprotease activity using in vitro synthesized [^{35}S]pOTC as a substrate. As demonstrated earlier (5,6), intact mitochondria (Fig. 1a, lane 2) process pOTC at two different sites, yielding an intermediate-sized polypeptide (iOTC) and a mature-sized product (OTC). Initial attempts at assigning the activity responsible for formation of mature OTC to one of the mitochondrial fractions were unsuccessful; none of the isolated fractions, or combinations thereof, cleaved pOTC to the mature subunit (data not shown). However, when these fractions were assayed in the presence of zinc ions, the processing activity was recovered (Fig.

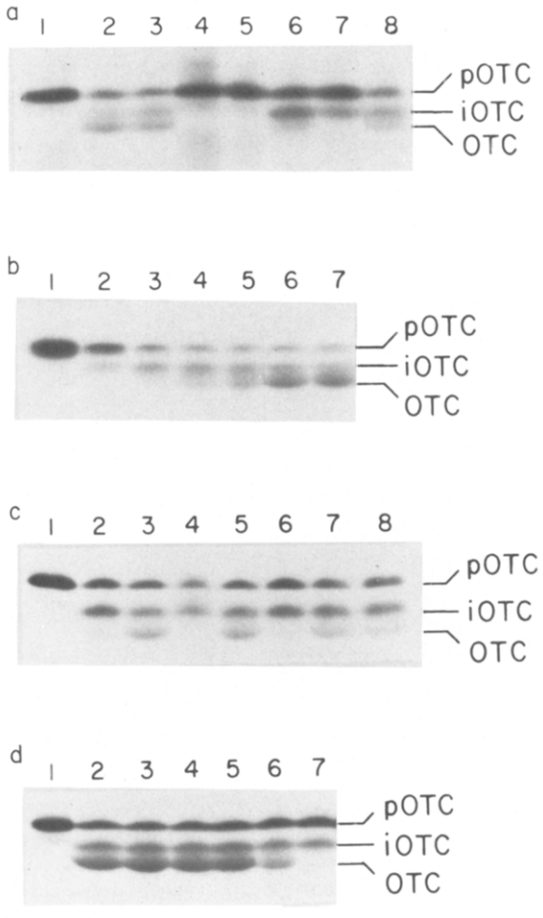

Fig. 1a. Distribution of the protease activity that converts pOTC to mature OTC among mitochondrial fractions. The indicated mitochondrial fractions were incubated with pOTC in the presence of 0.1 mM $ZnCl_2$ at 26°C for 60 min, after which immunoprecipitation, SDS/polyacrylamide gel electrophoresis, and fluorography were performed as described in "Methods". Only the relevant part of the gel is shown. Lane 1, no mitochondria added; lane 2, intact mitochondria, 120 µg protein; lane 3, intact mitoplasts, 120 µg protein; lane 4, 120 µg protein from outer membrane fraction; lane 5, 46 µg protein from intermembrane space fraction; lane 6, 120 µg protein from Lubrol WX-disrupted mitoplasts; lane 7, 120 µg protein from inner membrane fraction; lane 8, 120 µg protein from matrix fraction.

Fig. 1b. Zn^{2+} dependence of activity responsible for processing to mature OTC. Each processing reaction contained 400 µg matrix protein and Zn^{2+} at the indicated concentrations. Lane 1, no matrix added; lanes 2 and 3, no Zn^{2+} added; lane 4, 0.003 mM Zn^{2+}; lane 5, 0.01 mM Zn^{2+}; lane 6, 0.10 mM Zn^{2+}; lane 7, 0.30 mM Zn^{2+}. Lane 2 also contained 2 mM 1,10-phenanthroline.

Fig. 1c. Effect of other divalent metal ions on processing activity. Each processing reaction contained 400 µg of protein from a Lubrol WX-disrupted mitoplast fraction and the indicated metal ion at 0.1 mM. Lane 1, no mitoplast protein added; lane 2, no metal ion added; lane 3, Zn^{2+}; lane 4, Fe^{2+}, lane 5, Co^{2+}; lane 6, Mg^{2+}; lane 7, Mn^{2+}; lane 8, Ca^{2+}.

Fig. 1d. 1,10-phenanthroline inhibits Zn^{2+}-stimulated processing. Processing reactions contained 340 µg matrix protein, 0.1 mM Zn^{2+}, and the indicated concentrations of 1,10-phenanthroline. Lane 1, no matrix added; lane 2, no 1,10-phenantroline added; lane 3, 0.02 mM; lane 4, 0.06 mM; lane 5, 0.2 mM; lane 6, 0.6 mM; lane 7, 2 mM.

1a). This experiment shows, first, that virtually all of the activity present in intact mitochondria (lane 2) is recovered in the mitoplast fraction (lane 3); outer membrane and intermembrane space fractions have little or no activity (lanes 4 and 5, respectively). Further fractionation of the mitoplasts shows that, of the mature OTC-producing protease activity present in solubilized mitoplasts (lane 6), >90% is recovered in the matrix fraction (lane 8), as determined by scanning densitometry of the fluorograph. The small portion of activity recovered in the inner membrane fraction (lane 7), ∿5-10% of the activity in solubilized mitoplasts, can be attributed to contamination of this fraction by matrix proteins (see Table I).

In contrast, the distribution of iOTC-producing protease activity among the mitochondrial compartments is distinctly different (Fig. 1a). Although this activity is also localized to the mitoplasts (lane 3), it fractionates approximately equally between the inner membrane (lane 7) and the matrix (lane 8) fractions. In this case, the inner membrane activity cannot be completely attributed to contamination by soluble matrix proteins (see Table I).

Fig. 1b shows that the activity in the matrix which produces mature OTC is dependent on the concentration of Zn^{2+} present in the assay mixture. Barely detectable in the absence of added Zn^{2+} (lane 3), the production of mature OTC is stimulated in a concentration-dependent fashion (lanes 4-6) by the addition of Zn^{2+} up to 0.1 mM. Increasing the Zn^{2+} concentration to 0.3 mM has little additional effect (lane 7). Conversely, chelation of Zn^{2+} by addition of 1,10-phenanthroline (2 mM) leads to complete suppression of this activity (lane 2). It should be noted that the activity of the protease

TABLE I

Distribution of Marker Enzymes in Submitochondrial Fractions

Fraction	Enzyme Activity*			
	Monoamine oxidase	Adenylate kinase	Cytochrome c oxidase	Glutamate dehydrogenase
Mitochondria	100	100	100	100
Outer membrane	32	4.5	15	1
Intermembrane space	48	81	4	11
Mitoplast	10	<1	67	86
Inner membrane	8	<1	67	33
Matrix	<1	<1	<1	57

*Expressed as percent of activity in unfractionated mitochondria. Marker enzymes used: monoamine oxidase, outer membrane; adenylate kinase, intermembrane space; cytochrome c oxidase, inner membrane; glutamate dehydrogenase, matrix. Total marker enzyme recovery in subfractions ranged from 82% for cytochrome c oxidase to 98% for glutamate dehydrogenase. Protein content of fractions (expressed as percent of total mitochondrial protein) was as follows: outer membrane, 5.9; intermembrane space, 12.1; inner membrane, 33.4; matrix, 48.0.

which produces iOTC is not significantly affected by these changes in Zn^{2+} concentration.

The ability of other divalent metal ions to stimulate the processing activity leading to formation of mature OTC is shown in Fig. 1c. Besides Zn^{2+} (lane 3), Co^{2+} (lane 5) is also very effective in activating this enzyme when each ion is tested at 0.1 mM. Mn^{2+} (lane 7) and Ca^{2+} (lane 8) are less effective at this concentration, while Mg^{2+} (lane 6) has little stimulatory ability. Interestingly, Fe^{2+} appears to inhibit this processing activity (lane 4).

Fig. 1d confirms the divalent metal ion dependence of the protease which produces mature subunits. Processing was catalyzed by a matrix fraction supplemented with 0.1 mM Zn^{2+}; increasing concentrations of 1,10-phenanthroline were added. Little or no inhibition of processing is seen until the 1,10-phenanthroline concentration is raised to 0.6 mM (lane 6); a further increase to 2 mM suppresses activity almost entirely (lane 7). Although production of iOTC is not decreased in this experiment, higher concentrations of 1,10-phenanthroline (6 mM) do partially inhibit the enzyme responsible for its formation (results not shown).

DISCUSSION

In this report we present initial studies of a mitochondrial protease which plays a critical role in processing pOTC to its mature subunit. First, our mitochondrial fractionation experiments indicate that the mature OTC subunit-producing protease fractionates with matrix enzymes (Fig. 1a and Table I). This localization suggests that processing of pOTC to mature subunits occurs after transport of at least part of the precursor across the inner mitochondrial membrane to the matrix.

Further studies of this matrix protease show, most importantly, that it requires approximately 0.1 mM Zn^{2+} for maximal catalytic activity (Fig. 1b). Decreasing the concentration of Zn^{2+}, either by dilution (as occurs during preparation of the matrix fraction) or by chelation (with 1,10-phenanthroline, Fig. 1d), leads to a loss of activity. Direct measurements of metal ions in mitochondria have indicated that the concentration of Zn^{2+} is approximately 0.1 mM (13), strongly supporting the idea that this zinc requirement is physiologically significant. Among the other divalent metal ions tested *in vitro*, Co^{2+} stimulates activity as well as Zn^{2+} does; Mn^{2+} is somewhat less stimulatory (Fig. 1c). However, these ions are present at much lower concentrations in intact mitochondria (13), and are therefore less likely to play an important role in the processing of mitochondrial precursor proteins *in vivo*.

This zinc-dependent protease appears to be distinct from the protease activity described by Mori *et al.* (6) that cleaves pOTC to the intermediate-

sized protein. Besides the obvious difference in specificity of cleavage site, our experiments demonstrate that these two protease activities are distributed differently among the submitochondrial fractions (Fig. 1a), and that they have strikingly different cation requirements (Fig. 1b and 1c). The existence of two specific endoproteases raises the possibility that pOTC is processed to mature subunits in two successive proteolytic steps, as proposed by Mori et al. (6). While this hypothesis may be correct, alternative explanations, including the possibility of artifactual cleavage in the cell free system, have not been ruled out. Further studies with purified enzymes and purified substrates may be required to obtain definitive evidence on this matter.

It is now apparent that many, perhaps most, cytoplasmically synthesized mitochondrial proteins are made as larger precursors which require proteolytic processing at some point during their biogenesis (14,15). Characterization and purification of the processing enzymes which cleave these precursors will aid in the delineation of the number and specificity of processing pathways which exist in the mitochondrion. Recent reports indicate that at least two cytoplasmically synthesized precursors of yeast mitochondrial proteins are cleaved by yeast mitochondrial proteases which resemble in two key features the zinc-dependent protease described here (16,17): both proteases were localized to the matrix fraction and both were inhibited by chelating agents (1,10-phenanthroline and EDTA) specific for divalent metal ions. Further studies will be required to determine the relationship of these yeast proteases to the zinc-dependent protese that cleaves pOTC in our mammalian system.

ACKNOWLEDGEMENT

This work was supported by grant AM 09527 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

REFERENCES

1. Conboy, J.G., Kalousek, F. and Rosenberg, L.E. (1979) Proc. Natl. Acad. Sci. (USA) 76, 5724-5727.
2. Mori, M., Miura, S., Tatibana, M. and Cohen, P.P. (1980) J. Biochem. (Tokyo) 88, 1829-1836.
3. Morita, T., Mori, M., Tatibana, M. and Cohen, P.P. (1981) Biochem. Biophys. Res. Commun. 99, 623-629.
4. Kraus, J.P., Conboy, J.G. and Rosenberg, L.E. (1981) J. Biol. Chem. 256, 10739-10742.
5. Conboy, J.G. and Rosenberg, L.E. (1981) Proc. Natl. Acad. Sci. (USA) 78, 3073-3077.
6. Mori, M., Miura, S., Tatibana, M. and Cohen, P.P. (1980) Proc. Natl. Acad. Sci. (USA) 77, 7044-7048.
7. Schnaitman, C. and Greenawalt, J.W. (1968) J. Cell Biol. 38, 158-175.
8. Loewenstein, J., Scholte, H.R. and Wit-Peeters, E.M. (1970) Biochim. Biophys. Acta 223, 432-436.

9. Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.
10. Clarke, S. (1976) J. Biol. Chem. 251, 950-961.
11. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
12. Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88.
13. Thiers, R.E. and Vallee, B.L. (1957) J. Biol. Chem. 226, 911-920.
14. Schatz, G. (1979) FEBS Lett. 103, 203-211.
15. Neupert, W. and Schatz, G. (1981) Trends Biochem. 6, 1-4.
16. Boehni, P., Gasser, S., Leaver, C. and Schatz, G. (1980) in Structure and Expression of the Mitochondrial Genome (Kroon, A.M. and Saccone, C., eds.), pp 423-433, North-Holland, Amsterdam.
17. McAda, P.C. (1981) Fed. Proc. 40, 1659.