Ornithine transcarbamylase in liver mitochondria

Masataka Mori, Satoshi Miura, Tetsuo Morita, Masaki Takiguchi and Masamiti Tatibana
Dept. of Biochemistry, Chiba University School of Medicine, Inohana, Chiba, Chiba 280, Japan

Summary

Ornithine transcarbamylase (ornithine carbamoyltransferase, EC 2.1.3.3), the second enzyme of urea synthesis, is localized in the matrix of liver mitochondria of ureotelic animals. The enzyme is encoded by a nuclear gene, synthesized outside the mitochondria, and must then be transported into the organelle. The rat liver enzyme is initially synthesized on membrane-free polysomes in the form of a larger precursor with an amino-terminal extension of 3 400–4 000 daltons. In rat liver slices and isolated rat hepatocytes, the pulse-labeled precursor is first released into the cytosol and is then transported with a half life of 1–2 min into the mitochondria where it is proteolytically processed to the mature form of the enzyme. The precursor synthesized in vitro exists in a highly aggregated form and has a conformation different from that of the mature enzyme. The precursor has an isoelectric point (pI = 7.9) higher than that of the mature enzyme (pI = 7.2).

The precursor synthesized in vitro can be taken up and processed to the mature enzyme by isolated rat liver mitochondria. The mitochondrial transport and processing system requires membrane potential and a high integrity of the mitochondria. The transport and processing activities are conserved between mammals and birds or amphibians and is presumably common to more than one precursor. Potassium ion, magnesium ion, and probably a cytosolic protein(s), in addition to the transcarbamylase precursor and the mitochondria, are required for the maximal transport and processing of the precursor.

A mitochondrial matrix protease which converts the precursor to a product intermediate in size between the precursor and the mature subunit has been highly purified. The protease has an estimated molecular weight of 108 000 and an optimal pH of 7.5–8.0, and appears to be a metal protease. The protease does not cleave several of the protein and peptide substrates tested. The role of this protease in the precursor processing remains to be elucidated.

Rats subjected to different levels of protein intake and to fasting show significant changes in the level of enzyme protein and activity of ornithine transcarbamylase. The dietary-dependent changes in the enzyme level are due mainly to an altered level of functional mRNA for the enzyme. In contrast, during fasting, the increase in the enzyme level is associated with a decreased level of translatable mRNA for the enzyme.

Pathological aspects of ornithine transcarbamylase including the enzyme deficiency and reduced activities of the enzyme in Reye's syndrome are also described. A possibility that impaired transport of the enzyme precursor into the mitochondria leads to a reduced enzyme activity, is proposed.

Abbreviation

pOTC, precursor of ornithine transcarbamylase

Introduction

Ornithine transcarbamylase (ornithine carba-

Molecular and Cellular Biochemistry 49, 97–111 (1982). 0300-8177/82/0492-0097/$03.00

moyltransferase, EC 2.1.3.3) catalyzes the second step of urea biosynthesis in the liver of ureotelic animals (1). Reichard (2) isolated essentially pure ornithine transcarbamylase from rat liver. The bovine enzyme was purified to homogeneity by Marshall and Cohen (3–5) who established that the enzyme is a trimer of 108 000 daltons consisting of three identical subunits of 36 000 daltons. Molecular and catalytic properties of the bovine enzyme have been extensively studied (3–10). Subsequent preparations of pure ornithine transcarbamylase from rabbit liver (11), rat liver (12, 13), and human liver (14, 15) also proved to be trimers of approximately the same molecular weight and to consist of three identical subunits. The amino acid sequence of the NH_2-terminal region (24 amino acids) of the rat enzyme was determined (13). The rat enzyme can be purified to homogeneity by affinity chromatography with immobilized δ-N-(phosphonacetyl)-L-ornithine (16, 17), a potent transition state analog inhibitor of ornithine transcarbamylase (18–20).

Ornithine transcarbamylase is present almost exclusively in the liver mitochondria of ureotelic animals (21–23). Subfractionation of rat liver mitochondria showed that the enzyme is localized in the mitoplast (inner membrane plus matrix) (24) and in the mitochondrial matrix (25). A low but significant level of the enzyme is found in the intestinal mucosa of ureotelic animals (23, 26–28). The intestinal enzyme is also localized in the mitochondria (29).

Ornithine transcarbamylase is coded by a nuclear gene and synthesized on cytoplasmic 80S ribosomes. The newly synthesized enzyme must then traverse both the outer and inner mitochondrial membranes to achieve its final location in the mitochondrial matrix. Described herein are the mechanisms whereby extramitochondrially synthesized ornithine transcarbamylase is transported into the matrix of liver mitochondria. Regulation of the hepatic level of ornithine transcarbamylase and some pathological aspects of the enzyme are also given attention.

Cell-free synthesis of pOTC, the larger precursor of ornithine transcarbamylase

Cell-free synthesis of rat liver ornithine transcarbamylase was performed to elucidate the size of the primary translational product of the enzyme (30). Total RNA or poly(A)$^+$ H$^+$ RNA of rat liver was translated in a nuclease-treated rabbit reticulocyte lysate system in the presence of [^{35}S]methionine and the polypeptide synthesized was immunoprecipitated by using an antibody to the enzyme and protein A-bearing *Staphylococcus aureus* cells. The immunoprecipitates were subjected to SDS/10% polyacrylamide gel electrophoresis and fluorography (Fig. 1). The cell-free product (pOTC) migrated more slowly than did the subunit of the

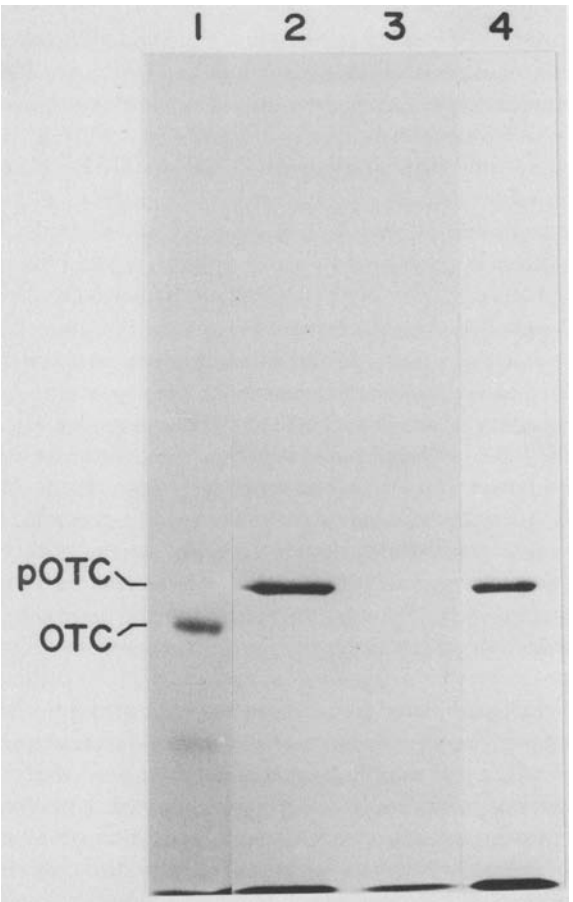


Fig. 1. SDS/polyacrylamide gel electrophoresis of the in vitro product (30). Total RNA (lanes 2 and 3) or poly(A)$^+$ RNA (lane 4) was translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. The in vitro product was isolated by immunoprecipitation and SDS/10% polyacrylamide gel electrophoresis and visualized by fluorography. In lane 3, anti-ornithine transcarbamylase was replaced by control IgG. Lane 1, mature ornithine transcarbamylase *(OTC)*.

mature enzyme. The apparent molecular weight of pOTC was 39 400, such being 3 400 daltons larger than the mature subunit (36 000 daltons). pOTC was identified as the primary translation product as follows. a) when the antibody was replaced by control IgG, no radioactive band was seen at the expected position; b) pOTC was synthesized in a wheat germ cell-free system as well as in a reticulocyte lysate system; c) mature ornithine transcarbamylase effectively competed with pOTC for the antibody; and d) pOTC yielded proteolytic fragments similar to those of the mature enzyme. Similar results were also reported by Conboy et al. (31). These results indicate that ornithine transcarbamylase is initially synthesized as a larger precursor (pOTC) which may be imported into mitochondria with proteolytic processing to the mature form of the enzyme.

Preceding and subsequent studies have shown that most of the mitochondrial matrix (32–40), inner membrane (41, 42), intermembrane space (43), and outer membrane (44) proteins of higher animals as well as yeast and *Neurospora* mitochondrial proteins (see Refs. 45 and 46 for review) are synthesized in larger molecular weight forms with extrapeptides of 2 000–6 000 daltons (for exceptions see Refs. 47–51).

Site of pOTC synthesis

Free and membrane-bound polysomes from rat liver were separately incubated in a reticulocyte lysate system in the presence of [^{35}S]methionine and the products were tested for albumin and pOTC synthesis (52). Membrane-bound polysomes incorporated 1.2% of the total trichloroacetic acid-insoluble radioactivity into albumin (presumably pre-

Table 1. Synthesis of total protein, albumin, and pOTC by free and membrane-bound polysomes (52).

Expt.	Polysomes		Incorporation of [^{35}S]methionine into			Albumin/total protein (%)	pOTC/total protein (%)
	Type	Amount (A_{260}/ml)	Total protein[a] (dpm × 10^{-5})	Albumin (dpm)	pOTC (dpm)		
1	Free	8	8.8	503	385	0.063	0.042
		16	11.2	657	570	0.059	0.051
		24	14.8	844	580	0.057	0.039
						Mean = 0.060	*Mean* = 0.044
	Bound	8	11.8	9 780	64	0.83	0.005
		16	13.0	12 800	71	0.99	0.005
		24	10.0	16 400	51	1.64	0.005
						Mean = 1.15	*Mean* = 0.005
2	Free	10	7.6		244		0.032
		20	15.0		364		0.024
		30	18.7		546		0.029
							Mean = 0.028
	Bound	10	11.7		65		0.005
		20	19.3		68		0.004
		30	29.6		113		0.004
							Mean = 0.004

Rat liver free and membrane-bound polysomes prepared by the method of Ramsey and Steele (53), were translated in a rabbit reticulocyte lysate system. The in vitro products were isolated and visualized as described in Fig. 1. The gel strips containing radioactive polypeptides were cut out and counted for radioactivity.

[a] Total protein synthesis was corrected for endogenous protein synthesis without added polysomes (1.1×10^5 and 1.3×10^5 dpm in Experiments 1 and 2, respectively).

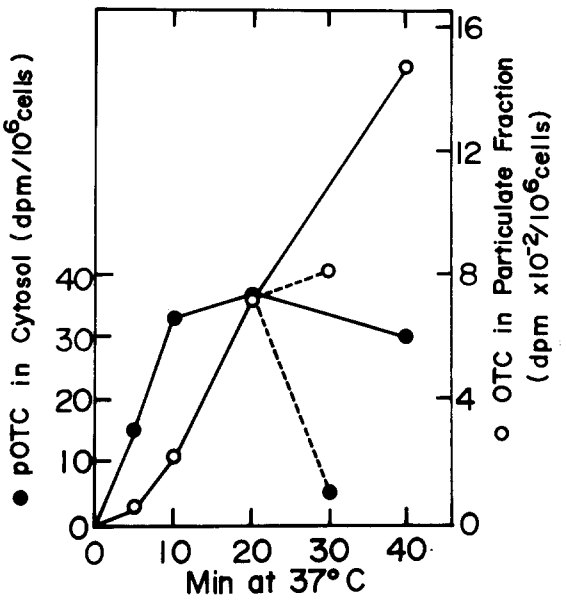


Fig. 2. Synthesis of pOTC and the mature enzyme in isolated hepatocytes (57). Hepatocytes (3.4×10^7 cells/ml) were incubated with [^{35}S]methionine (190 μCi/ml) at 37 °C. Radioactivities of pOTC in the cytosol fraction (●) and of the mature enzyme in the crude mitochondrial fraction (○) were measured. ----, data of the chase experiments.

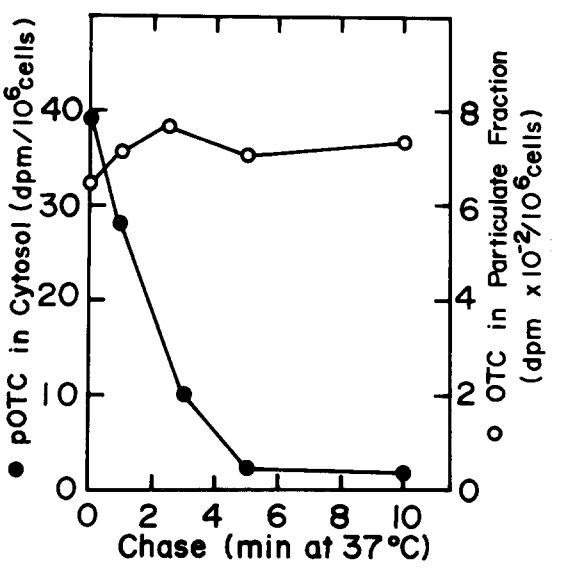


Fig. 3. Kinetics of pOTC disappearance from the cytosol in pulse-chase experiments (57). Hepatocytes (3.3×10^7 cells/ml) were pulsed with [^{35}S]methionine (170 μCi/ml) for 20 min at 37 °C and were chased. Radioactivities of pOTC in the cytosol fraction (●) and of the mature enzyme in the crude mitochondrial fraction (○) were measured.

proalbumin), whereas free polysomes incorporated only 0.06%, thus confirming the specificity of membrane-bound polysomes for the translation of the secreted protein (Table 1). In contrast, incorporation of radioactivity into pOTC was on the average eight times higher with free polysomes (0.044 and 0.028% of total protein in two separate experiments) than with membrane-bound polysomes (0.005 and 0.004% of total protein). Similar results were obtained when total RNAs from free polysomes and from membrane-bound polysomes were translated (52). These results indicate that ornithine transcarbamylase is synthesized preferentially, probably exclusively, on membrane-free polysomes. This is in accord with reports (34, 36, 50, 54) that other mitochondrial proteins of higher animals are synthesized on free polysomes, although some proteins may be synthesized on both free and membrane-bound polysomes (37, 55, 56).

Synthesis, intracellular transport, and processing of pOTC in rat liver slices and isolated hepatocytes

When rat liver slices were incubated with [^{35}S]methionine, pOTC was detected (52). Following subcellular fractionation, pOTC was found exclusively in the cytosol fraction. On the other hand, the labeled mature enzyme was found almost exclusively in a crude mitochondrial fraction. The specific radioactivity of pOTC in the cytosol fraction was much higher than that of the mature enzyme in the mitochondrial fraction. The pulse-labeled pOTC in the cytosol disappeared rapidly in pulse-chase experiments.

More detailed kinetic studies of the synthesis, intracellular transport, and processing of pOTC were performed with isolated rat hepatocytes (57). The hepatocytes were incubated with [^{35}S]methionine for various times and then fractionated into the cytosol and particulate (crude mitochondrial) fractions by the digitonin procedure of Zuurendonk and Tager (58). pOTC was found exclusively in the cytosol fraction, and the radioactivity in pOTC increased linearly for the first 10 min and then more slowly until a plateau was reached in 10–20 min of the pulse (Fig. 2). On the other hand, the labeled mature enzyme was found exclusively in the crude mitochondrial fraction. The radioactivity in the mature enzyme appeared after a lag time of a

few min and then increased almost linearly with time. The pulse-labeled pOTC disappeared almost completely from the cytosol in 10 min of the subsequent chase.

Kinetic aspects of pOTC disappearance in pulse-chase experiments are shown in Fig. 3. The pulse-labeled pOTC disappeared from the cytosol with an apparent half life of 1–2 min. The radioactivity of the mature enzyme showed a substantial increase during the chase.

These results indicate that ornithine transcarbamylase is initially synthesized as the larger precursor pOTC, exists in a cytosolic pool from which it is transported into mitochondria with a half life of 1–2 min and processed there to the mature enzyme, concomitantly with or immediately after the transport. We obtained very similar results for carbamyl phosphate synthetase I, the first enzyme of urea synthesis which also exists in the mitochondrial matrix (57). Raymond and Shore (59) reported a similar half life (about 2 min) of the synthetase in rat liver explants. These results are in sharp contrast to those reported by Yamauchi et al. (60) for δ-aminolevulinate synthetase of rat liver, another mitochondrial matrix enzyme. Under conditions where the enzyme is extensively induced, a large amount of the enzyme precursor accumulates in the cytosol fraction and is subsequently transferred to the mitochondria.

Properties of pOTC

As described above, pOTC, the precursor of ornithine transcarbamylase, has an extrapeptide extension of 3 400–4 000 daltons (30, 31). It is apparent that pOTC plays a key role in the transfer of the enzyme into the mitochondria. Kraus et al. (61) have recently shown that the peptide extension is located at the NH_2 terminus of pOTC. To gain insight into the possible role(s) of the NH_2-terminal extension of pOTC in its transport into the mitochondria, we examined the properties of the precursor.

Hydrophobicity of pOTC

Hydrophobicity of pOTC and the mature enzyme was examined by hydrophobic chromatography with octyl-Sepharose and phenyl-Sepharose (62). The elution profile of pOTC from both gels was practically identical to that of the mature enzyme.

A high isoelectric point of pOTC

pOTC synthesized in vitro and the mature enzyme prepared by incubating pOTC with isolated rat liver mitochondria (see below), were subjected to isoelectric focusing (62). The mature enzyme was focused as a single protein at pH 7.2, which is in accord with the pI value reported for the purified rat enzyme (13). On the other hand, pOTC was focused at about pH 7.9. We also found that several basic proteins such as histones and protamines strongly inhibit the uptake and processing of pOTC by isolated mitochondria (62). These results suggest that the basic nature of the precursor, and probably that of the extension, is required for interaction of the precursor with the mitochondria. The results are in accord with the recent report of Anderson (63), who showed that probable precursors for several mitochondrial proteins of human cells are quite basic. It is conceivable that the interaction between positively charged peptide extension and negatively charged proteins or phospholipids of the mitochondrial outer membrane facilitates the specific binding of the precursors to the organelle.

Aggregation states of pOTC

Rat liver ornithine transcarbamylase and its precursor pOTC were subjected to sucrose gradient centrifugation (64). The mature enzyme sedimented with an $s_{20,\omega}$ value of 6.0 S, which agrees with the previously reported value (12) and corresponds to the trimeric form. On the other hand, pOTC synthesized in a rabbit reticulocyte lysate system sedimented more broadly with a peak $s_{20,\omega}$ value of 14 S (Fig. 4). pOTC synthesized in another cell-free protein synthesizing system derived from wheat germ also sedimented with a similar $s_{20,\omega}$ value. In the same experiments, the larger precursor of carbamyl phosphate synthetase I sedimented with an $s_{20,\omega}$ value of 13 S (Fig. 4), which is larger than that of the mature enzyme (7.8 S).

Aggregation states of pOTC were further analyzed by gel filtration (64). Mature ornithine transcarbamylase was eluted with an apparent M_r of 110 000 from a Sephacryl S-200 column (Fig. 5). On

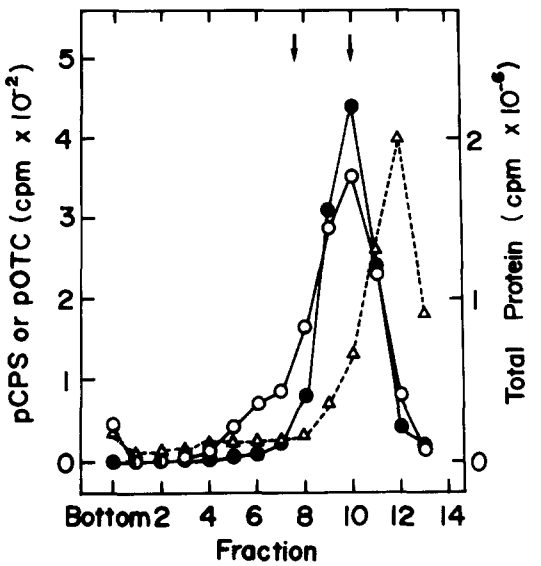


Fig. 4. Sucrose gradient centrifugation of pOTC and carbamyl phosphate synthetase precursor (64). The postribosomal supernatant of the cell-free translated mixture was centrifuged at 20 °C at 60 000 rpm for 90 min on a linear 5 to 20% sucrose gradient (4.8 ml) in a Hitachi 65P ultracentrifuge using an RPS 65TA rotor. Fractions were collected from the bottom of the centrifuge tube and subjected to immunoprecipitation, SDS-gel electrophoresis, and fluorography. Radioactivities of pOTC (○) and carbamyl phosphate synthetase I precursor (pCPS, ●) were measured. △, Trichloroacetic acid-insoluble radioactivity. Two downward arrows show the positions of protocatechuate 3,4-dioxygenase (19.4 S) and catalase (11.3 S).

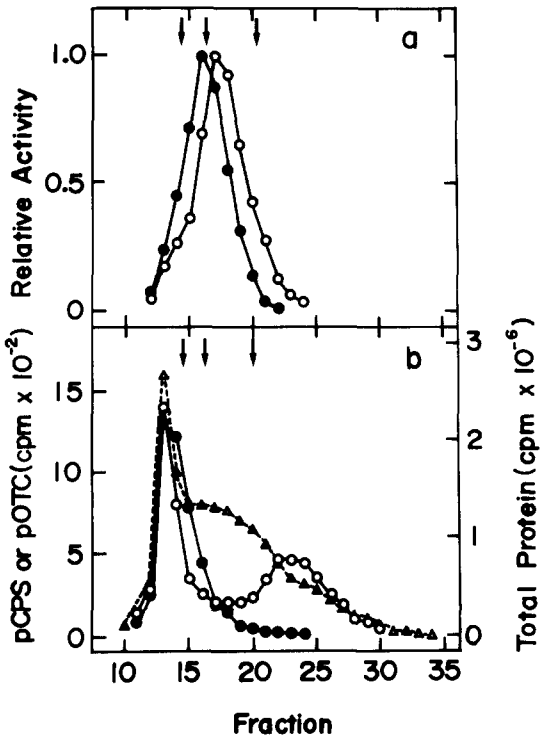


Fig. 5. Sephacryl S-200 chromatography of pOTC, carbamyl phosphate synthetase I precursor, and the mature enzymes (64). The purified mature enzymes (*a*) and the postribosomal supernatant of the cell-free translated mixture (*b*) were layered on a column (1.24 × 25 cm) of Sephacryl S-200 equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.2 M NaCl and 1 mM dithiothreitol. Elution was carried out at 4 °C with the same buffer and fractions of 0.92 ml were collected. In *a*, ornithine transcarbamylase (○) and carbamyl phosphate synthetase I (●) activities were measured. In *b*, trichloroacetic acid-insoluble radioactivity (△) and radioactivities of pOTC (○) and carbamyl phosphate synthetase I precursor (pCPS, ●) were measured as in Fig. 4. Three downward arrows show the positions of catalase (247 000), alcohol dehydrogenase (141 000), and hemoglobin (65 000).

the other hand, about half of pOTC was eluted in the void volume and the remaining portion was eluted broadly with a peak corresponding to an apparent M_r of 40 000. In the same experiments, carbamyl phosphate synthetase I was eluted with an apparent M_r of 150 000, whereas more than half of its precursor was eluted in the void volume (Fig. 5). These results indicate that the precursors synthesized in vitro form aggregates larger than those of the respective mature enzymes, under the conditions employed. It is likely that these aggregates are homo-oligomers rather than complexes with other proteins because pOTC synthesized in two different cell-free systems sedimented as a similar aggregate in a sucrose gradient. It remains to be elucidated whether or not extramitochondrially synthesized pOTC and carbamyl phosphate synthetase I precursor actually exist in aggregated forms in living cells. Zimmermann and Neupert (65) showed that *Neurospora* ADP/ATP carrier, a mitochondrial inner membrane protein, synthesized in vitro is present as an oligomeric complex, although this protein is not synthesized as a larger molecule.

From these results it is clear that the precursors have conformations different from those of the respective mature enzymes. This conclusion has been supported by our recent findings that pOTC is much more susceptible to proteases than is the mature enzyme (unpublished results). The data of Kraus et al. (61) who found differences in antigenicity between pOTC and the mature subunit in ionic detergents, are compatible with our conclusion.

Uptake and processing of pOTC by isolated mitochondria

Reconstitution experiments in vitro using the cell-free synthesized pOTC and isolated mitochondria should provide a basis for elucidation of the mitochondrial transport of the precursor and its processing. The first successful reconstitution experiments were apparently done in our laboratory (30, 66–68).

pOTC synthesized in vitro was converted to an apparently mature form of the enzyme by isolated rat liver mitochondria (Fig. 6). In addition to the mature enzyme, a 37 000-dalton product was formed. This product is mentioned below. The processed product (mature form) was recovered exclusively in the sedimented mitochondria and was not extractable with 1 M KCl. The processed product could be extracted with digitonin. The concentration of digitonin required was higher than that for the intermembrane space enzyme, adenylate kinase, but lower than that for endogenous ornithine transcarbamylase, which is localized in the matrix space (67). These results indicate that pOTC is transported deeply into the isolated mitochondria, although not completely to the matrix space, in association with the proteolytic processing to the mature enzyme. The uptake and processing of pOTC by the isolated mitochondria was not affected by the mature enzyme (30). The transport of pOTC into the mitochondria was confirmed by the finding that the processed product was no longer susceptible to externally added proteases (30, 68). These results have been confirmed by Conboy et al. (61, 69).

Further studies have shown that a portion of the processed product is recovered in the matrix fraction, whereas the remaining portion remains associated with the membrane fraction (unpublished results). From these results it appears that the processing of pOTC occurs during and not after the transport and that the processed product which remains associated with the membrane(s) is further transported into the matrix space. The processed product recovered from the matrix fraction sedimented with an $s_{20,\omega}$ value of 6.7 S, which is close to that of the mature enzyme (67). This would infer that the newly transported and processed product was assembled almost completely to the active trimer form.

Energy dependency of pOTC transport

Energy requirement for the mitochondrial transport and processing of pOTC was studied using the in vitro reconstituted system. Effects of uncouplers, ionophores, and respiratory inhibitors on pOTC transport and processing (assayed by pOTC processing) are shown in Table 2 (see also Refs. 67 and 68). Uncouplers such as dinitrophenol and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and ionophores such as valinomycin and nonactin strongly inhibited the transport-processing of pOTC. The inhibition by FCCP could not be restored by the addition of ATP plus oligomycin; under the conditions in which ATP can enter the mitochondria and be utilized in the ATP-dependent reactions. On the other hand, neither KCN, NaN_3, antimycin A nor oligomycin had any marked

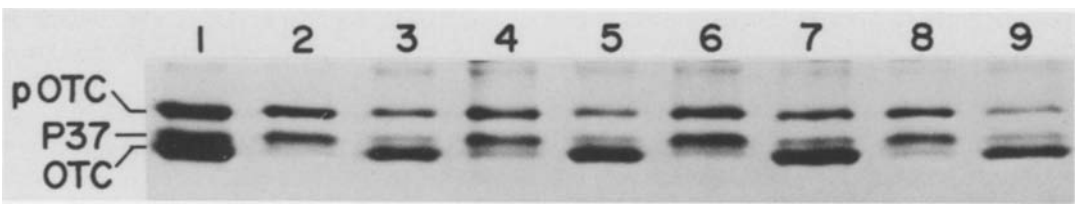


Fig. 6. Uptake and processing of pOTC by isolated rat liver mitochondria (67). Postribosomal supernatant (50 μl) of the cell-free translated mixture was incubated with rat liver mitochondria (50 μg of protein, 2 μl) for 60min at 25 °C. After incubation, 0.45 ml of buffered 0.25 M sucrose containing no additions (lanes 2 and 3), 0.3 M KCl (lanes 4 and 5), 1 M KCl (lanes 6 and 7), or 0.05 mg/ml of digitonin (lanes 8 and 9) was added. The supernatant (lanes 2, 4, 6, and 8) and the pelleted mitochondria (lanes 3, 5, 7, and 9) were analyzed separately. Lane 1, the whole reaction mixture was analyzed without fractionation. *OTC*, ornithine transcarbamylase; P37, 37 000-dalton product.

Table 2. Effects of uncouplers, ionophores, and respiratory inhibitors on uptake-processing of pOTC by isolated mitochondria.

Additions	Inhibition[a]
Dinitrophenol (0.1 mM)	++
FCCP (1 μM)	++
FCCP, MgATP (2 mM) & oligomycin (5 nmol/mg protein)	++
Valinomycin (10 nM)	++
Nonactin (0.1 μM)	++
KCN (5 mM)	-
NaN_3 (5 mM)	±
Antimycin A (5 μg/mg protein)	±
Oligomycin (5 nmol/mg protein)	-
KCN & oligomycin	++
Antimycin & oligomycin	++

[a] ++, strongly inhibitory
+, moderately inhibitory
±, not inhibitory or slightly inhibitory
-, not inhibitory.

effect. However, the transport-processing was strongly inhibited when both KCN or antimycin A and oligomycin were added. Under these conditions, formation of membrane potential by electron transport as well as by ATP in the matrix space would be inhibited. These results suggest strongly that the membrane potential is the direct energy donor of the transport-processing of pOTC. The transport step rather than the processing step is presumably energy-dependent, because pOTC was recovered almost exclusively in the medium after incubation with the mitochondria in the presence of the uncouplers or ionophores. Neupert et al. (70) came to the same conclusion, as based on experiments with mitochondrial proteins of *Neurospora*. The data of Nelson and Schatz (71) that the transfer of yeast mitochondrial protein precursors or their processing is dependent on ATP in the matrix may have to be re-evaluated.

Potassium ion, magnesium ion, and a high molecular weight component(s) are required for pOTC transport

Mitochondrial uptake and processing of pOTC in the reconstituted system in vitro was usually performed by incubating 50 μl of the cell-free translated mixture containing labeled pOTC (or its postmicrosomal supernatant) with isolated mitochondria in a total volume of 52–54 μl (66–68). When a small volume (less than 10 μl) of the cell-free translated mixture containing the same amount of labeled pOTC, was incubated with the mitochondria in 50 μl of an isotonic sucrose medium, little processing of pOTC was observed. We have found that at least three components contained in the cell-free translation mixture in addition to pOTC, are required for the optimal transport and processing of pOTC. One of the factors is potassium ion. The optimal concentration is around 120 mM. Potassium ion cannot be replaced by sodium ion. The second factor is magnesium ion; its optimal concentration is about 1.6 mM. Other divalent metal ions tested were ineffective. The third factor(s) is a non-dialyzable one(s) contained in the reticulocyte lysate. Dialyzed reticulocyte lysate stimulated pOTC processing several fold. This component(s) was completely inactivated by heat treatment for 2 min at 100 °C. Dialyzed rabbit erythrocyte lysate was also effective while hemoglobin was not. Properties of the component(s) and its mode of action remain to be elucidated.

Specificity of mitochondrial transport and processing

We showed that the mitochondria from rat non-hepatic tissues such as kidney, spleen and ascites cells, all of which lack ornithine transcarbamylase, are able to take up pOTC and to process it to the mature form of the enzyme (67, 68). We have further shown that the liver mitochondria of a pigeon, a uricotelic animal which have no ornithine transcarbamylase, can also take up pOTC and process it to the mature enzyme (unpublished results). These results indicate that pOTC can be transported into mitochondria and processed to the mature enzyme by a transport-processing system which is physiologically not that for pOTC, and that the transport-processing system is probably common to more one precursor. The question as to whether or not the system is common to all matrix proteins, and even to inner membrane proteins remains to be elucidated. Zimmermann et al. (72), in their studies on the transport of mitochondrial proteins into *Neurospora* mitochondria, reported that cytochrome *c* and two other inner membrane proteins (AD-

P/ATP carrier and subunit 9 of the oligomycin-sensitive ATPase) have different import pathways into mitochondria.

The mitochondrial transport and processing activities are highly conserved

Rat liver pOTC synthesized in vitro was taken up and processed to an apparently mature form of the enzyme by mouse liver mitochondria (68). Rat liver pOTC was also taken up and processed to the mature form of the enzyme by pigeon liver and frog liver mitochondria (unpublished results). Thus, the mitochondrial transport and processing activities are conserved in both the mammalian and bird or amphibian systems. Attempts to demonstrate the uptake and processing of the rat liver pOTC by yeast mitochondria have not been successful.

Processing of pOTC and a mitochondrial protease

Effects of protease inhibitors on pOTC processing by isolated mitochondria

Effects of various protease inhibitors on the conversion of pOTC to the mature enzyme by isolated mitochondria are shown in Table 3. The conversion was strongly inhibited by chymostatin and *N*-tosyl-L-phenylalanine chloromethylketone, inhibitors of chymotrypsin-like proteases. The inhibition by *o*-phenanthroline and EDTA appears to be due to inhibition of pOTC transport rather than to that of pOTC processing, because as shown above, magnesium ion is required for the transport of pOTC into the mitochondria. *p*-Aminobenzamidine which was reported to inhibit the processing of carbamyl phosphate synthetase I precursor in rat liver explants (59), was not inhibitory. Attempts to accumulate pOTC and the carbamyl phosphate synthetase I precursor in isolated hepatocytes by chymostatin, *N*-tosyl-L-phenylalanine chloromethylketone, leupeptin or globomycin have not been successful.

Table 3. Effects of protease inhibitors on pOTC processing by isolated rat liver mitochondria.

Strong inhibition
Chymostatin (0.5 mM)
N-Tosyl-L-phenylalanine chloromethylketone (0.5 mM)
N-Tosyl-L-lysine chloromethylketone (2 mM)
o-Phenanthroline (2 mM)
Moderate inhibition
Leupeptin (2 mM)
EDTA (2 mM)
Globomycin (50 μg/ml)
No or slight inhibition
Antipain (2 mM)
Elastatinal (2 mM)
Bestatin (2 mM)
Amastatin (2 mM)
E-64 (2 mM)
Phosphoramidone (2 mM)
Diisopropylfluorophosphate (2 mM)
Phenylmethylsulfonyl fluoride (2 mM)
p-Aminobenzamidine (2 mM)
EGTA (2 mM)
Aprotinin (2 mg/ml)

Mitochondria of a high integrity are required for pOTC processing

Rat liver mitochondria were treated with varying amounts of digitonin, and the release of mitochondrial enzymes and pOTC processing activity of the treated mitochondria were measured (68, Fig. 7).

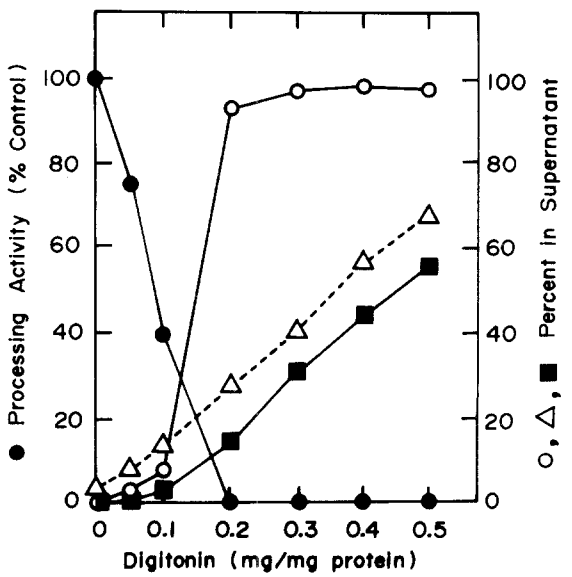


Fig. 7. Processing of pOTC by digitonin-treated mitochondria (68). Rat liver mitochondria (17 mg protein/ml) were treated with various concentrations of digitonin for 10 min at 25 °C. Activities of adenylate kinase (O) and ornithine transcarbamylase (■), and protein (△) in the supernatant and the pelleted mitochondria were measured. pOTC processing by digitonin-treated mitochondria (●) was measured as in Fig. 6.

Adenylate kinase, an intermembrane space enzyme, was released almost completely at a digitonin concentration of 0.2 mg/mg protein. Ornithine transcarbamylase which is localized in the matrix, was released gradually by higher concentrations of the detergent. On the other hand, the ability of mitochondria to convert pOTC to the mature enzyme was decreased with low concentrations of digitonin, and was lost completely at 0.2 mg/mg protein of the detergent. The concentration of the detergent required for inactivation of the processing activity was similar or somewhat lower than that required for the release of adenylate kinase. Similar results were obtained with mitochondria disrupted mechanically by sonication (68). An extremely high sensitivity of the mitochondrial processing activity to both digitonin treatment and sonication suggests that a high integrity of the mitochondria, including the outer membrane or intermembrane fluid or both, is required for the transport and processing of pOTC. It is likely that when the mitochondrial integrity is affected, the pOTC transport is impaired and the precursor is not accessible to the protease responsible for the processing.

We obtained results indicating that a high integrity of mitochondrial membranes, in addition to the structural integrity, is required for the transport and processing of pOTC. pOTC processing by isolated mitochondria was strongly inhibited by membrane-perturbing reagents such as polymixin B (0.1 mg/mg protein), flufenamic acid (0.1 mM), chlorpromazine (0.1 mM), dibucaine (1 mM), and reserpine (0.1 mM) (73). These reagents are known to interact with phospholipids. Therefore, it is likely that perturbation of membrane phospholipid structure impairs mitochondrial uptake and processing of pOTC.

A mitochondrial protease that cleaves pOTC

When the rat liver mitochondria were disrupted extensively by sonication, the activity to form the mature enzyme was lost and the activity to form the 37 000-dalton product became more prominent (66, 68) (Fig. 8). Similar results were obtained when the mitochondria were disrupted by 1% Triton X-100 or 1% deoxycholate. These results were confirmed by Kraus et al. (61). Subfractionation of the mitochondria revealed that the protease catalyzing the conversion of pOTC to the 37 000-dalton product is localized mainly in the matrix and partly in the intermembrane space (66). To understand the physiological role of this protease and of the 37 000-dalton product, the protease was purified 140-fold from the matrix fraction of rat liver (74). The protease had an estimated molecular weight of 108 000 and an apparent pI of 5.5. Mature ornithine transcarbamylase did not inhibit the cleavage of pOTC by the protease and presumably it cleaves a specific site on the extrapeptide of pOTC. This has been demonstrated by Kraus et al. (61). The protease has an optimal pH of 7.5–8.0 when pOTC is the substrate (66).

The protease was inhibited strongly by 1 mM leupeptin, 2 mM *o*-phenanthroline, 2 mM EDTA, 1 mM zincon, 2 mM *p*-chloromercuriphenyl sulfonate, and 2 mM Hg $(CH_3COO)_2$, and moderately by 2 mM antipain, 1 mg/ml of aprotinin, 2 mM EGTA, and 2 mM $Pb(CH_3COO)_2$. Other inhibitors including chymostatin, *N*-tosyl-L-phenylalanine chloromethylketone, *N*-tosyl-L-lysine chlorometh-

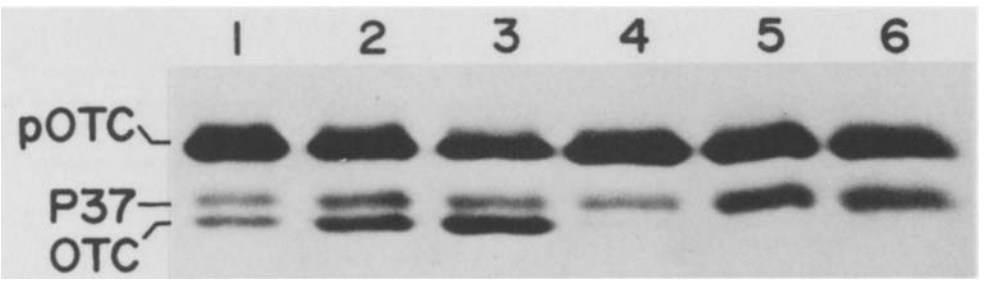
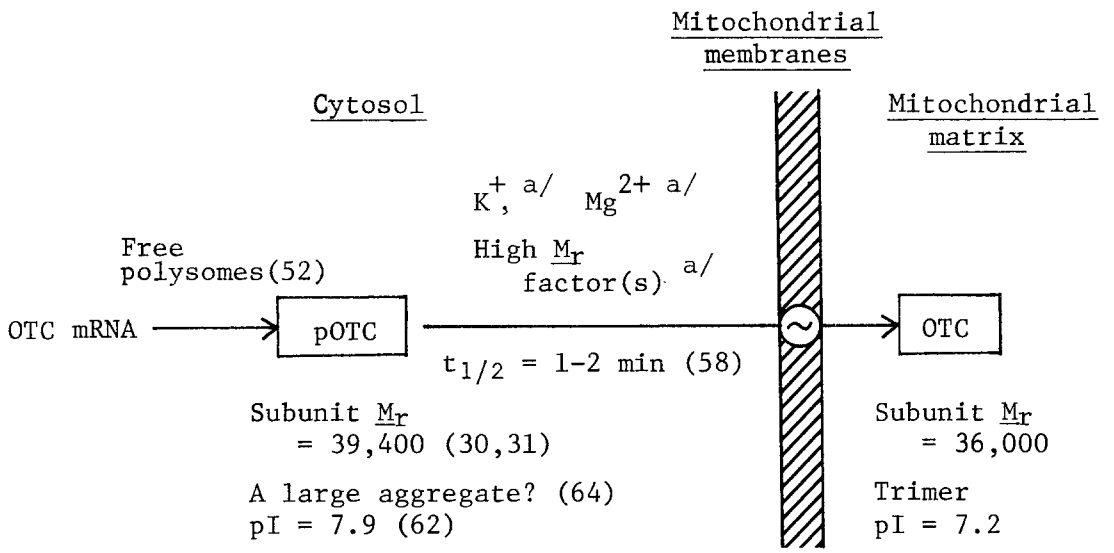


Fig. 8. Formation of the 37 000-dalton product by disrupted mitochondria. The cell-free translated mixture containing labeled pOTC was incubated with 10, 40, and 100 μg of intact mitochondria (lanes 1–3, respectively) or 10, 40, and 100 μg of sonicated mitochondria (lanes 4–6, respectively) for 60 min at 25 °C. The products were isolated and analyzed as in Fig. 6.



Inhibitors of pOTC transport-processing

1. Dinitrophenol, FCCP (but not KCN) (67)
2. Valinomycin, nonactin a/
3. Histones, protamines (62)
4. Membrane-perturbing agents a/ (73)
5. Chymostatin, TPCK, TLCK a/
6. o-Phenanthroline a/

Fig. 9. Schematic representation of the synthesis, intracellular transport and processing of ornithine transcarbamylase.
[a] This paper. OTC, ornithine transcarbamylase; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; TLCK, *N*-tosyl-L-lysine chloromethylketone; ~ membrane potential.

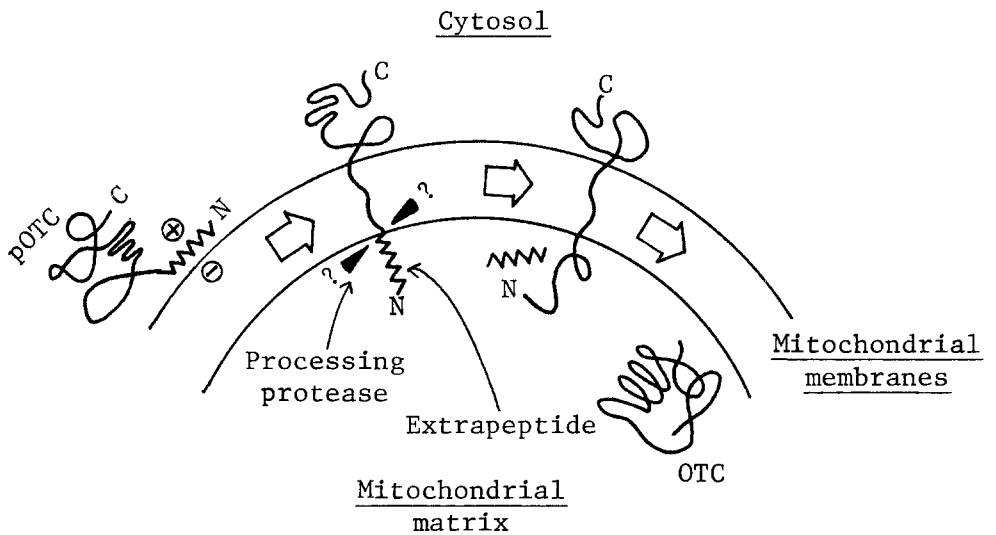


Fig. 10. Proposed mechanism of pOTC transport into mitochondria. Basic nature and probably that of the peptide extension, appears to facilitate the binding of the precursor with the mitochondria. Processing of pOTC occurs during rather than after the transport by the processing protease which is presumably localized in the matrix space or the inner membrane of the mitochondria. OTC, ornithine transcarbamylase.

ylketone were either not inhibitory or were only slightly inhibitory. Thus, the pattern of inhibition of the protease differs from that of the pOTC processing by the isolated mitochondria (Table 3). The possibility that the mitochondrial protease catalyses the first of the two steps in processing of pOTC (66), should be reevaluated.

The purified protease did not cleave any of the protein and peptide substrates tested, including the precursors of secretory proteins containing signal peptides (74) and did not cleave the precursor of carbamyl phosphate synthetase I, under the conditions employed. However, the protease converted the precursor of serine: pyruvate transaminase (38) to an apparently mature form of the enzyme (unpublished results). This protease may catalyze the processing of some matrix protein precursors containing that of serine: pyruvate transaminase. Böhni et al. (74a) have identified and partially purified a mitochondrial protease from yeast which is very similar to the rat liver mitochondrial protease described herein. They claimed that the yeast protease is responsible for the processing of yeast mitochondrial protein precursors to the mature polypeptides.

All the results described above are summarized in Figs. 9 and 10.

Ornithine transcarbamylase level in liver mitochondria

The mitochondrial level of ornithine transcarbamylase will depend on the relative rates of synthesis and degradation of the enzyme. The effects of dietary protein (75) and hormones (76) on levels of ornithine transcarbamylase and other urea cycle enzymes have recently been reviewed (see also Refs. 77–81, 81a). Schimke showed that the activities of enzymes involved in urea biosynthesis increased coordinately as dietary protein intake increased (82–84). While rats fed a protein-free diet showed a decrease in the enzyme levels, starvation led to an increase (83). Similar adaptations to dietary protein intake were reported for primates (85). Induction of the urea cycle enzymes of rat liver by amino acids has recently been studied by Snodgrass and Lin (86). The dietary protein-dependent changes in the activities of the enzymes are due to changes in concentration of the enzyme proteins (87–90). Nicoletti et al. (91), using a double isotope technique estimated the half life of carbamyl phosphate synthetase I in rats to be in the order of 7.7 days on a normal diet, 3.3 days on a high protein diet, and 4.6 days on a protein-free diet. Studies with *Rana catesbeiana* liver preparations indicated a half life of carbamylphosphate synthetase I in the order of 3.5 and 2.3 days, respectively, in the absence and presence of thyroxine (92). In a similar amphibian system, the half life of ornithine transcarbamylase was estimated to be of the order of 4 days (76). Such studies on rat liver ornithine transcarbamylase have not been documented.

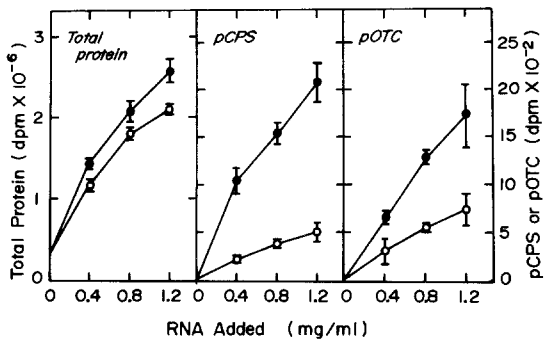


Fig. 11. Effects of dietary protein content of translatable mRNA levels for total protein, carbamyl phosphate synthetase I precursor, and pOTC (89). Cell-free translation in a reticulocyte lysate system was performed with various concentrations of total hepatic RNA isolated from rats fed a 5% (○) or 60% (●) casein diet for 8 days. Each point represents the mean value of two animals, with the bars indicating the range.

With the recent development of techniques for cell-free synthesis of ornithine transcarbamylase and carbamyl phosphate synthetase I, it has been possible to study some of the molecular mechanisms of the enzyme synthesis. Effects of dietary protein on the translatable mRNAs for the two mitochondrial urea cycle enzymes were studied in the rat (89) and the results are shown in Fig. 11. Translatable levels of hepatic mRNAs for the two enzymes were found to be higher in rats fed a high protein diet than in those fed a low-protein diet. The differences in mRNA levels correlated closely with both the levels of enzyme activity and enzyme protein. These results indicate that the dietary protein-dependent changes in the levels of carbamyl phosphate synthetase I and ornithine transcarbamylase are due mainly to changes in the levels of translatable mRNAs for these enzymes. In contrast, during fasting, the increase in levels of these enzymes was associated with a decrease in levels of

translatable mRNAs for the enzymes (89). It thus appears that the increase in levels of the two enzymes in liver from fasted rats is the result of a decreased rate of degradation of these enzymes rather than the result of an enhanced rate of synthesis. Induction of tadpole liver carbamyl phosphate synthetase I by thyroxine was shown to be associated with an increased level of mRNA for the enzyme (93, 94).

Pathological aspects of ornithine transcarbamylase

Ornithine transcarbamylase deficiency

A number of cases of hyperammonemia associated with ornithine transcarbamylase deficiency have been reported (see Ref. 95 for a review) and X-linkage of the enzyme was presented (96–98). We carried out studies on molecular properties of the enzyme in a patient with the antibody to the enzyme (99). The liver from the patient and from the controls contained similar amounts of protein cross-reactive with the antibody. However, the patient's liver contained an inactive form of the enzyme in addition to an active form of the enzyme. This may be considered to reflect the X-linkage of the enzyme at the molecular level. A slight increase of ornithine transcarbamylase protein in sparse-fur mice with ornithine transcarbamylase deficiency was shown by immunotitration (17). Quite recently, Briand et al. (100) studied the liver of 16 male patients by immunoassay. In several who died in the neonatal period and in whom there was no residual enzymatic activity, cross-reacting material was not detectable. These results suggest that a gene mutation can alter the rate of the synthesis, or degradation, or both of the enzyme protein, in addition to kinetic and molecular properties. The possibility that the extramitochondrially synthesized precursor of a variant enzyme cannot enter the mitochondria and is degraded in the cytosol, remains to be tested.

Reduced activity of ornithine transcarbamylase in Reye's syndrome

Reye's syndrome is often associated with hyperammonemia. Both mitochondrial urea cycle enzymes, ornithine transcarbamylase and carbamyl phosphate synthetase I, reportedly have reduced activities in crude liver homogenates prepared from patients with this syndrome (101–105). Normal kinetic properties of ornithine transcarbamylase in Reye's syndrome have been reported in many cases (103, 104), although in one case (101) abnormal kinetic properties were reported. Pierson et al. (14) purified ornithine transcarbamylase from normal human liver and the liver of a patient with Reye's syndrome, and found no difference in molecular and kinetic properties between the two enzymes. The molecular basis for the reduction in the activities is unclear. Since mitochondrial damage has been a consistent finding in the livers of Reye's syndrome patients (105), it seems plausible that leakage of the enzymes from the mitochondria or impaired transport of the enzyme precursors into the mitochondria leads to reduced enzyme activities. The latter possibility warrants attention since a high integrity of mitochondrial structure as well as of mitochondrial membranes is required for the transport of ornithine transcarbamylase precursor (pOTC) into mitochondria (68, 73; see above).

Acknowledgements

We thank Drs P. P. Cohen (University of Wisconsin) and F. Ikeda (this laboratory) for their participation in part of the original work presented in this paper, M. Ohara for assistance with preparation of the manuscript, and Miss K. Shinya for secretarial services. The original work was supported in part by Scientific Research Grants from the Ministry of Education, Science and Culture of Japan.

References

1. Grisolia, S. and Cohen, P. P., 1952. J. Biol. Chem. 198: 561–571.
2. Reichard, P., 1957. Acta Chem. Scand. 11: 523–536.
3. Marshall, M. and Cohen, P. P., 1972. J. Biol. Chem. 247: 1641–1653.
4. Marshall, M. and Cohen, P. P., 1972. J. Biol. Chem. 247: 1654–1668.
5. Marshall, M. and Cohen, P. P., 1972. J. Biol. Chem. 247: 1669–1682.
6. Marshall, M. and Cohen, P. P., 1977. J. Biol. Chem. 252: 4276–4286.

7. Marshall, M. and Cohen, P. P., 1980. J. Biol. Chem. 255: 7287–7290.
8. Marshall, M. and Cohen, P. P., 1980. J. Biol. Chem. 255: 7291–7295.
9. Marshall, M. and Cohen, P. P., 1980. J. Biol. Chem. 255: 7296–7300.
10. Marshall, M. and Cohen, P. P., 1980. J. Biol. Chem. 255: 7301–7305.
11. Ono, K., Okano, S. and Ito, Y., 1972. Seikagaku (in Japanese) 44: 479.
12. Clarke, S., 1976. Biochem. Biophys. Res. Commun. 71: 1118–1124.
13. Lusty, C. J., Jilka, R. L. and Nietsch, E. H., 1979. J. Biol. Chem. 254: 10030–10036.
14. Pierson, D. L., Cox, S. L. and Gilbert, B. E., 1977. J. Biol. Chem. 252: 6464–6469.
15. Kalousek, F., François, B. and Rosenberg, L. E., 1978. J. Biol. Chem. 253: 3939–3944.
16. Hoogenraad, N. J., Sutherland, T. M. and Howlett, G. J., 1980. Anal. Biochem. 101: 97–102.
17. Briand, P., Cathelineau, L., Kamoun, P., Gigot, D. and Penninckx, M., 1981. FEBS Lett. 130: 65–68.
18. Mori, M., Aoyagi, K., Tatibana, M., Ishikawa, T. and Ishii, H., 1977. Biochem. Biophys. Res. Commun. 76: 900–904.
19. Hoogenraad, N. J., 1978. Arch. Biochem. Biophys. 188: 137–144.
20. Penninckx, M. and Gigot, D., 1978. FEBS Lett. 88: 94–96.
21. Grisolia, S., Koritz, S. B. and Cohen, P. P., 1951. J. Biol. Chem. 191: 181–187.
22. Caravaca, J. and Grisolia, S., 1960. J. Biol. Chem. 235: 684–693.
23. Raijman, L., 1974. Biochem. J. 138: 225–232.
24. Schnaitman, C. and Greenawalt, J. W., 1968. J. Cell Biol. 38: 158–175.
25. Gamble, J. G. and Lehninger, A. L., 1973. J. Biol. Chem. 248: 610–618.
26. Lowenstein, J. M. and Cohen, P. P., 1956. J. Biol. Chem. 220: 57–70.
27. Hall, L. M., Johnson, R. C. and Cohen, P. P., 1960. Biochim. Biophys. Acta 37: 144–145.
28. Jones, M. E., Anderson, A. D., Anderson, C. and Hodes, S., 1961. Arch. Biochem. Biophys. 95: 499–507.
29. Uchiyama, C., Mori, M. and Tatibana, M., 1981. J. Biochem. (Tokyo) 89: 1777–1786.
30. Mori, M., Miura, S., Tatibana, M. and Cohen, P. P., 1980. J. Biochem. (Tokyo) 88: 1829–1836.
31. Conboy, J. G., Kalousek, F. and Rosenberg, L. E., 1979. Proc. Natl. Acad. Sci. USA 76: 5724–5727.
32. Shore, G. C., Carignan, P. and Raymond, Y., 1979. J. Biol. Chem. 254: 3141–3144.
33. Mori, M., Miura, S., Tatibana, M. and Cohen, P. P., 1979. Proc. Natl. Acad. Sci. USA 76: 5071–5075.
34. Yamauchi, K., Hayashi, N. and Kikuchi, G., 1980. FEBS Lett. 115: 15–18.
35. Sonderegger, P., Jaussi, R. and Christen, P., 1980. Biochem. Biophys. Res. Commun. 94: 1256–1260.
36. Sakakibara, R., Huynh, Q. K., Nishida, Y., Watanabe, T. and Wada, H., 1980. Biochem. Biophys. Res. Commun. 95: 1781–1788.
37. Nabi, N. and Omura, T., 1980. Biochem. Biophys. Res. Commun. 97: 680–686.
38. Oda, T., Ichiyama, A., Miura, S., Mori, M. and Tatibana, M., 1981. Biochem. Biophys. Res. Commun. 102: 568–573.
39. Ades, I. Z. and Harpe, K. G., 1981. J. Biol. Chem. 256: 9329–9333.
40. Aziz, L. E., Chien, S. M., Patel, H. V. and Freeman, K. B., 1981. FEBS Lett. 133: 127–130.
41. Schmerlzer, E. and Heinrich, P. C., 1980. J. Biol. Chem. 255: 7503–7506.
42. DuBois, R. N., Simpson, E. R., Tuckey, J., Lambeth, J. D. and Waterman, M. R., 1981. Proc. Natl. Acad. Sci. USA 78: 1028–1032.
43. Ono, H. and Ito, A., 1982. J. Biochem. (Tokyo) 91: 117–123.
44. Shore, G. C., Power, F., Bendayan, M. and Carignan, P., 1981. J. Biol. Chem. 256: 8761–8766.
45. Schatz, G., 1979. FEBS Lett. 103: 203–211.
46. Neupert, W. and Schatz, G., 1981. Trends Biochem. Sci. 6: 1–4.
47. Zitomer, R. S. and Hall, B. D., 1976. J. Biol. Chem. 251: 6320–6326.
48. Zimmermann, R., Paluch, U. and Neupert, W. 1979. FEBS Lett. 108: 141–146.
49. Zimmermann, R., Paluch, U., Sprinzl, M. and Neupert, W., 1979. Eur. J. Biochem. 99: 247–252.
50. Matsuura, S., Arpin, M., Hannum, C., Margoliash, E., Sabatini, D. D. and Morimoto, T., 1981. Proc. Natl. Acad. Sci. USA 78: 4368–4372.
51. Mihara, K. and Blobel, G., 1980. Proc. Natl. Acad. Sci. USA 77: 4160–4164.
52. Morita, T., Mori, M., Tatibana, M. and Cohen, P. P., 1981. Biochem. Biophys. Res. Commun. 99: 623–629.
53. Ramsey, J. C. and Steele, W. J., 1976. Biochemistry 15: 1704–1712.
54. Raymond, Y. and Shore, G. C., 1979. J. Biol. Chem. 254: 9335–9338.
55. Kawajiri, K., Harano, T. and Omura, T., 1977. J. Biochem. (Tokyo) 82: 1403–1416.
56. Northemann, W., Schmerzer, E. and Heinrich, P. C., 1981. Eur. J. Biochem. 119: 203–208.
57. Mori, M., Morita, T., Ikeda, F., Amaya, Y., Tatibana, M. and Cohen, P. P., 1981. Proc. Natl. Acad. Sci. USA 78: 6056–6060.
58. Zuurendonk, P. F. and Tager, J. M., 1974. Biochim. Biophys. Acta 333: 393–399.
59. Raymond, Y. and Shore, G. C., 1981. J. Biol. Chem. 256: 2087–2090.
60. Yamauchi, K., Hayashi, N. and Kikuchi, G., 1980. J. Biol. Chem. 255: 1746–1751.
61. Kraus, J. P., Conboy, J. G. and Rosenberg, L. E., 1981. J. Biol. Chem. 256: 10739–10742.
62. Miura, S., Mori, M., Morita, T. and Tatibana, M., 1982. Biochem. Int. 4: 201–208.
63. Anderson, L., 1981. Proc. Natl. Acad. Sci. USA 78: 2407–2411.
64. Miura, S., Mori, M., Amaya, Y., Tatibana, M. and Cohen, P. P., 1981. Biochem. Int. 2: 305–312.
65. Zimmermann, R. and Neupert, W., 1980. Eur. J. Biochem. 109: 217–229.

66. Mori, M., Miura, S., Tatibana, M. and Cohen, P. P., 1980. Proc. Natl. Acad. Sci. USA 77: 7044–7048.
67. Mori, M., Morita, T., Miura, S. and Tatibana, M., 1981. J. Biol. Chem. 256: 8263–8266.
68. Morita, T., Miura, S., Mori, M. and Tatibana, M., 1982. Eur. J. Biochem. 122: 501–509.
69. Conboy, J. G. and Rosenberg, L. E., 1981. Proc. Natl. Acad. Sci. USA 78: 3073–3077.
70. Neupert, W., Freitag, H., Janes, M., Schleyer, M., Schmidt, B. and Teintze, M., 1981. Abstracts of the meeting supported by Japan-USA cooperative scientific program "Biogenesis of membranes and cell organelles". pp. 29–31 (Kyoto, Japan).
71. Nelson, N. and Schatz, G., 1979. Proc. Natl. Acad. Sci. USA 76: 4365–4369.
72. Zimmermann, R., Henning, B. and Neupert, W., 1981. Eur. J. Biochem. 116: 455–460.
73. Mori, M., Miura, S., Morita, T. and Tatibana, M., 1982. In: Urea Cycle Diseases (Lowenthal, A. and Mori, A., eds.), Plenum, New York, New York, in press.
74. Miura, S., Mori, M., Amaya, Y. and Tatibana, M., 1982. Eur. J. Biochem. 122: 641–647.
74a. Boehni, P., Gasser, S., Leaver, C. and Schatz, G., 1980. In: The Organization and Expression of the Mitochondrial Genome (Kroon, A. M. and Saccone, C., eds.) pp. 423–433, Elsevier North-Holland, Amsterdam.
75. Aebi, H., 1976. In: The Urea Cycle (Grisolia, S., Báguena, R. and Mayor, F., eds.) pp. 275–296, Wiley, New York, New York.
76. Cohen, P. P., 1981. Current Topics in Cellular Regulation 18: 1–19.
77. Snodgrass, P. J., Lin, R. C., Müller, W. A. and Aoki, T., 1978. J. Biol. Chem. 263: 2748–2753.
78. Gebhardt, R. and Mecke, D., 1979. Eur. J. Biochem. 97: 29–35.
79. Lamers, W. H., 1980. Ph. D. Thesis, University of Amsterdam, Amsterdam.
80. Palekar, A. G., Collipp, P. J. and Maddaiah, V. T., 1981. Biochem. Biophys. Res. Commun. 100: 1604–1610.
81. Christowitz, D., Mattheyse, F. J. and Balinski, J. B., 1981. Enzyme 26: 113–121.
81a. Gautier, C. and Vaillant, R., 1981. Biochem. Biophys. Res. Commun. 98: 51–57.
82. Schimke, R. T., 1962. J. Biol. Chem. 237: 459–468.
83. Schimke, R. T., 1962. J. Biol. Chem. 237: 1921–1924.
84. Schimke, R. T., 1963. J. Biol. Chem. 238: 1012–1018.
85. Nazum, C. T. and Snodgrass, P. J., 1971. Science 172: 1042–1043.
86. Snodgrass, P. J. and Lin, R. C., 1981. J. Nutr. 111: 586–601.
87. Schimke, R. T., 1964. J. Biol. Chem. 239: 3808–3817.
88. Tsuda, M., Shikata, Y. and Katunuma, T., 1979. J. Biochem. (Tokyo) 85: 699–704.
89. Mori, M., Miura, S., Tatibana, M. and Cohen, P. P., 1981. J. Biol. Chem. 256: 4127–4132.
90. Murakami, A., Kitagawa, Y. and Sugimoto, E., 1981. Agric. Biol. Chem. 45: 1899–1900.
91. Nicolleti, M., Guerri, C. and Grisolia, S., 1977. Eur. J. Biochem. 75: 583–592.
92. Shambaugh, G. E., III, Balinski, J. B. and Cohen, P. P., 1969. J. Biol. Chem. 244: 5295–5308.
93. Mori, M., Morris, S. M. and Cohen, P. P., 1979. Proc. Natl. Acad. Sci. USA 76: 3179–3183.
94. Pouchelet, M. and Shore, G. C., 1981. Biochim. Biophys. Acta 654: 67–76.
95. Shih, V. E., 1978. In: The Metabolic Basis of Inherited Diseases (Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S., eds.) pp. 362–386, McGraw-Hill, New York, New York.
96. Short, E. M., Conn, H. O., Snodgrass, P. J., Campbell. A. G. M. and Rosenberg, L. E., 1973. N. Engl. J. Med. 288: 7–12.
97. DeMars, R., LeVan, S. L., Trend, B. L. and Russel, L. B., 1976. Proc. Natl. Acad. Sci. USA 73: 1693–1697.
98. Ricciuti, F. C., Gelehrter, T. D. and Rosenberg, L. E., 1976. Am. J. Hum. Genet. 28: 332–338.
99. Mori, M., Uchiyama, C., Miura, S., Tatibana, M. and Nagayama, E., 1980. Clin. Chim. Acta 104: 291–299.
100. Briand, P., François, B., Rabier, D. and Cathelineau, L., 1982. Biochim. Biophys. Acta 704: 100–106.
101. Thaler, M. M., Hoogenraad, N. J. and Boswell, M., 1974. Lancet 2: 438–440.
102. Brown, T., Hug, G., Bove, K., Brown, H. and Lanski, L., 1974. Lancet 2: 716–717.
103. Sinatra, F., Yoshida, T., Applebaum, M., Mason, W., Hoogenraad, N. J. and Sunshine, P., 1975. Pediatr. Res. 9: 829–833.
104. Brown, T., Hug, G., Lanski, L., Bove, K., Scheve, A., Ryan, M., Brown, H., Schubert, W. K., Partin, J. C. and Lloyd-Still, J., 1976. N. Engl. J. Med. 294: 861–867.
105. Thaler, M. M., 1978. In: Reye's syndrome II (Crocker, J. F. S., ed.) pp. 115–138, Grune and Stratton, New York, New York.

Received 20 April 1982