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SITE OF SYNTHESIS AND INTRACELLULAR TRANSPORT OF THE PRECURSOR OF MITOCHONDRIAL ORNITHINE CARBAMOYLTRANSFERASE

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SUMMARY

Ornithine carbamoyltransferase [EC 2.1.3.3] of rat liver, a mitochondrial matrix enzyme, is synthesized in a larger precursor form in cell-free protein-synthesizing systems. In cell-free translation programmed with polysomes or polysomal RNAs from rat liver, activity of mRNA coding for the precursor of the enzyme was seven to nine times higher in free polysomes than in membrane-bound polysomes. In rat liver slices, the pulse-labeled precursor of the enzyme was found to be present in a cytosol fraction and to rapidly disappear from the cytosol in pulse-chase experiments. These results indicate that ornithine carbamoyltransferase is initially synthesized on free polysomes as a larger precursor, which is then imported rapidly into mitochondria where it is processed to form the mature enzyme.

Ornithine carbamoyltransferase [EC 2.1.3.3], the second enzyme of urea biosynthesis, is localized in the matrix of liver mitochondria of ureotelic animals (1). The enzyme is a trimer of identical subunits, each of 35,300-39,600 daltons (2-7). The enzyme is coded by nuclear genes, synthesized on cytoplasmic ribosomes, and subsequently transported into the mitochondrial matrix. Conboy et al. (8) and we (9) showed that the enzyme was synthesized as a larger precursor (pOTC) which was 3,400-4,000 daltons larger than the mature subunit. We further showed that pOTC was transported into isolated mitochondria in association with post-translational processing to make the mature form of the enzyme (9). The processing appears to involve a neutral protease present in mitochondria (10).

Abbreviations: pOTC, precursor of ornithine carbamoyltransferase; SDS, sodium dodecyl sulfate.

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In this communication we report that pOTC can be detected in the cytosol of rat liver cells and is rapidly transported into mitochondria. The present study also shows that the enzyme is synthesized preferentially by free polysomes.

MATERIALS AND METHODS

Cell-Free Protein Synthesis——Free and membrane-bound polysomes were prepared from the liver of male Wistar rats (150-200 g) by the method of Ramsey and Steele (11). Total polysomal RNA was isolated from the polysomal pellets by the SDS-phenol method as described previously (12). RNA was estimated by using A_{260} (1 mg/ml) = 20. Polysomes or total polysomal RNA were translated in a nuclease-treated rabbit reticulocyte lysate with [^{35}S]methionine (about 400 μCi/ml) as described previously (12).

Immunoprecipitation——Immunoprecipitation was performed using antisera to bovine ornithine carbamoyltransferase (9) or to rat albumin and fixed Staphylococcus aureus cells essentially as described previously (9) in a solution (1.0 ml, pH 7.4) containing 10 mM Tris-HCl, 0.1% SDS, 0.1% Triton X-100 and 2 mM EDTA. The cells were washed three times with 1 ml of the same solution.

Experiments with Liver Slices——Livers from male Wistar rats (150-200 g) were sliced with a razor to a thickness of 0.5 mm. The slices (about 50 mg wet weight each) were incubated with [^{35}S]methionine (150 μCi/ml) under O_2 in scintillation vials at 37°C in a medium (1.0 ml, pH 7.6) described by Ries et al. (13) but modified as follows: 65 mM KCl, 3.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 71 mM NaCl, 6.2 mM sodium phosphate, and 19 amino acids except methionine (0.1 mM each).

Other Methods——SDS-polyacrylamide (10%) slab gel electrophoresis (14) and fluorography (15) were performed by the cited methods. The diphenyloxazole-impregnated gel strips containing radioactive polypeptides were cut out and counted for radioactivity in a toluene scintillant with an efficiency of about 77%. Trichloroacetic acid-insoluble radioactivity was measured as described (16).

Materials——Microbial protease inhibitors (antipain, leupeptin, chymostatin, and pepstatin) were obtained from the Peptide Institute (Osaka, Japan). Antiserum to rat albumin (rabbit) was from Cappel Laboratories, Pa., U.S.A. [^{35}S]Methionine (>800 Ci/mmol) was from New England Nuclear.

RESULTS AND DISCUSSION

Distribution of Albumin and pOTC Synthesis between Free and Membrane-bound Polysomes——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. Membrane-bound polysomes directed albumin synthesis much more actively than did free polysomes (Fig. 1A). Table I shows that membrane-bound polysomes incorporated 1.2% of the total trichloroacetic acid-insoluble radioactivity into albumin, whereas free polysomes incorporated only 0.06%, thus confirming the specificity of membrane-bound polysomes for the translation of secreted protein (17, 18).

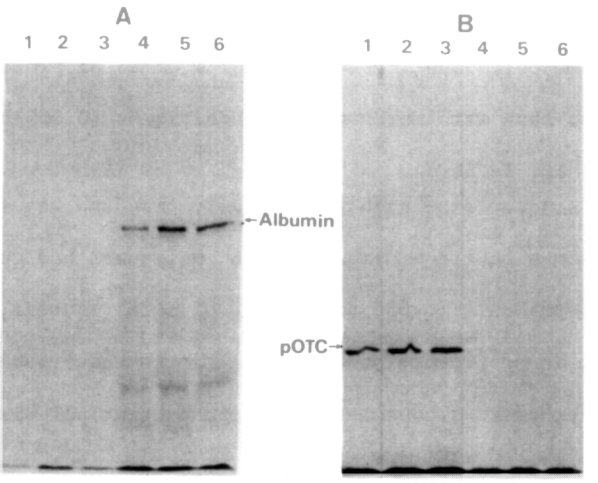

Fig. 1. Synthesis of albumin (A) and pOTC (B) by free and membrane-bound polysomes. Rat liver free (lanes 1-3) and membrane-bound (4-6) polysomes were translated in a rabbit reticulocyte lysate system (50 µl) as described in Materials and Methods with polysomal concentration of 8 (1 and 4), 16 (2 and 5), or 24 A_{260} units/ml (3 and 6). In A, 2 µl of the translation mixture was subjected to immunoprecipitation with 5 µl of albumin antiserum and 100 µl of 10% S. aureus cells; in B, 45 µl was subjected to immunoprecipitation with 5 µl of ornithine carbamoyltransferase antiserum. The immunoprecipitates were subjected to SDS gel electrophoresis and fluorography (2-days exposure at -80°C).

Table I. Synthesis of total protein, albumin, and pOTC by free and membrane-bound polysomes

Expt.	Polysomes		Incorporation of [^{35}S]methionine into			Albumin/total protein (%)	pOTC/total protein (%)
	Type	Amount (A_{260}/ml)	Total protein[a] (dpm x 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	0.044
	Bound	8	11.8	9780	64	0.83	0.005
		16	13.0	12800	71	0.99	0.005
		24	10.0	16400	51	1.64	0.005
						Mean=1.15	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

Experiment 1 is the same as that in Fig. 1. [a] Total protein synthesis was corrected for endogenous protein synthesis without added polysomes (1.1 x 10^5 and 1.3 x 10^5 dpm in Experiments 1 and 2, respectively).

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) (Fig. 1B, Table I).

Total RNA was isolated from each type of polysomes and was translated. RNAs from free polysomes and from membrane-bound polysomes had quite different capacities for pOTC synthesis (0.035 and 0.005% of total protein, respectively) (Fig. 2, Table II). These proportions were similar to those observed for polysome-directed synthesis. These results indicate that pOTC is synthesized preferentially, probably exclusively, by free polysomes. This is in accord with previous reports (19-21) that other mitochondrial matrix enzymes of higher animals are synthesized by free polysomes, although some matrix enzymes may be synthesized preferentially by membrane-bound polysomes (22).

Detection of pOTC in Liver Slices——When rat liver slices were incubated with [^{35}S]methionine the putative precursor of ornithine carbamoyltransferase (pOTC) was detected (Fig. 3A). The apparent molecular weight of this putative precursor was the same as that obtained by *in vitro* translation of mRNA (9) or of polysomes (see above). Following subcellular fractionation, pOTC was found exclusively in a cytosol fraction and the radioactivity in pOTC increased with time up to 30 min. In a crude mitochondrial fraction the radiolabeled mature form of the enzyme appeared in 10 min of incubation and increased with time. The radiolabeled enzyme found in the cytosol fraction at 30 min of incubation was probably due to leakage from mitochondria during tissue homogenization and subsequent fractionation, because 5-10% of the enzyme activity was recovered in the soluble fraction under similar conditions. In fact, staining of the gel with Coomassie blue showed that the ornithine carbamoyltransferase protein was found in the cytosol as well as in the crude mitochondrial extracts and that the enzyme protein in the two fractions had apparently the same specific radioactivity (data not shown). On the other hand, no protein band was detected by staining at the position of the radiolabeled pOTC. Thus, the specific radioactivity of pOTC was much higher than that of the mature enzyme.

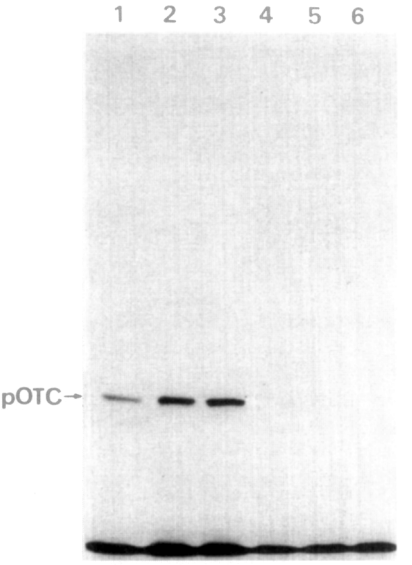

Fig. 2. Synthesis of pOTC by total RNA from free and membrane-bound polysomes. Experimental conditions were the same as described in Fig. 1B except that RNAs from free (lanes 1-3) or membrane-bound (4-6) polysomes were translated with RNA concentrations of 0.4 (1 and 4), 0.8 (2 and 5), or 1.2 mg/ml (3 and 6).

When liver slices were incubated for 30 min with [^{35}S]methionine and subsequently chased in the presence of excess, unlabeled methionine, pulse-labeled pOTC disappeared from the cytosol compartment within 10 min of the subsequent chase (Fig. 3B). However, a concomitant increase of radioactivity

Table II. Synthesis of total protein and pOTC by polysomal RNAs.

RNA		Incorporation of [^{35}S]methionine into		pOTC/total protein (%)
Source	Amount (mg/ml)	Total protein (dpm x 10^{-5})	pOTC (dpm)	
Free polysomes	0.4	13.5	446	0.033
	0.8	23.4	840	0.036
	1.2	25.2	895	0.036
				Mean = 0.035
Bound polysomes	0.4	14.2	82	0.006
	0.8	15.4	78	0.005
	1.2	20.6	104	0.005
				Mean = 0.005

The experiment is the same as that in Fig. 2. [a/] Total protein synthesis was corrected for endogenous protein synthesis without exogenous RNA (2.7 x 10^5 dpm).

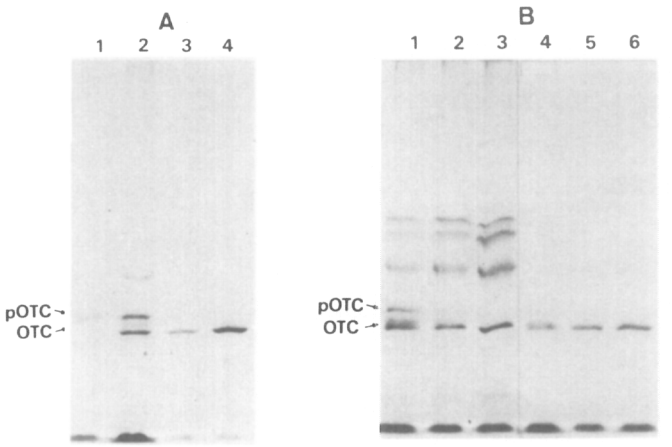

Fig. 3. Detection of pOTC in the cytosol of the liver cells. (A) Two liver slices (about 50 mg wet weight each) were incubated with [^{35}S]methionine, one for 10 (lanes 1 and 3) and the other for 30 min (2 and 4). Each slice was homogenized in 0.5 ml of 10 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate/0.1 M KCl/0.25 M sucrose/1 mM antipain/1 mM leupeptin/1 mM chymostatin/1 mM pepstatin (pH 7.4), and a 6,000 x g (10 min) precipitate and a 105,000 x g (30 min) supernatant were prepared. The 6,000 x g precipitate was extracted with 0.5 ml of 10 mM potassium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate/0.5% Triton X-100/the four protease inhibitors (pH 7.4) and centrifuged at 105,000 x g for 20 min to remove insoluble materials. The whole samples of the cytosol fractions (lanes 1 and 2) were subjected to immunoprecipitation using 20 µl of ornithine carbamoyltransferase antiserum and 200 µl of 10% S. aureus cells, SDS gel electrophoresis, and fluorography (7-days exposure), whereas only one-fifth of the crude mitochondrial extracts (lanes 3 and 4) were processed as above to immunoprecipitate quantitatively the newly synthesized carbamoyltransferase plus the enzyme already present. (B) Three liver slices (about 50 mg wet weight each) were incubated with [^{35}S]methionine for 30 min and then chased for 0 (lanes 1 and 4), 10 (2 and 5), or 30 min (3 and 6) in the medium containing 5 mM unlabeled methionine. Each slice was processed as in A. Lanes 1-3, cytosol fractions; 4-6, crude mitochondrial extracts. OTC, ornithine carbamoyltransferase.

in the mitochondrial mature enzyme could not be determined because of the large amount of the radioactive enzyme already present. The radioactive bands observed between pOTC and the mature enzyme (Fig. 3A, lane 2; lanes 1 and 4) could not be identified.

The present study, together with previous reports (8-10), provides supporting data for the proposal that ornithine carbamoyltransferase is initially synthesized outside the mitochondria in a larger precursor form (pOTC) which is then transported into mitochondria in association with post-translational proteolytic processing. The extramitochondrial pool of pOTC is small and it is rapidly transferred to mitochondria. Similar findings were reported for

rat liver carbamoyl-phosphate synthetase I [EC 6.3.4.16], another mitochondrial matrix enzyme (19). These results imply that a mechanism similar to that proposed by Schatz (23), based on studies on synthesis of mitochondrial enzymes in yeast, also operates in higher animals.

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