BIOSYNTHESIS OF RAT LIVER CARBAMOYLPHOSPHATE SYNTHETASE I ON CYCLOHEXIMIDE-SENSITIVE RIBOSOMES

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1. Introduction

Mitochondrial ribosomes appear to synthesize < 10% of total mitochondrial protein [1]. The remaining portion of the protein is synthesized extramitochondrially and transferred into the mitochondria in subsequent steps. Mitochondrial–cytoplasmic cooperation has been well documented in yeast and *Neurospora crassa* for the biosynthesis and assembly of subunits of mitochondrial inner membrane enzymes (cytochrome oxidase, oligomycin-sensitive ATPase and cytochrome *b*) [1]. However, there is little information concerning the biosynthesis and translocation of mitochondrial matrix proteins. In this case, a precursor must be transported across the inner membrane. Investigation of this mechanism may be important for the understanding of mitochondrial biogenesis.

Carbamoylphosphate synthetase I (CPS-I [ATP: carbamate phosphotransferase(dephosphorylating) EC 2.7.2.5]), the enzyme in the initial step of urea synthesis, is localized in the mitochondrial matrix [2]. In ureotelic liver, CPS-I is the main protein of mitochondrial matrix and represents > 40% of the total matrix protein [3]. Furthermore, this enzyme is markedly induced either by dietary protein [4] or by administration of glucagon [5]. If CPS-I is synthesized on extramitochondrial ribosomes, this enzyme may be most suitable for the study of translocation of mitochondrial matrix protein.

To initiate this study, the intracellular site of biosynthesis of this enzyme was investigated. Taking advantage of the selective inhibition by cycloheximide, it was found that the biosynthetic site of rat liver CPS-I is extramitochondrial ribosomes.

2. Materials and methods

Wistar rats (120–130 g) from Shizuoka Lab. Animals Agricultural Co. were used. Ornithine carbamoyltransferase was purified from bovine liver as in [6]. Commercial sources of reagents were as follows: cycloheximide from P-L Biochemical Inc.; L-[U-^{14}C]-leucine and NCS-solubilizer from Amersham; *N*-acetyl-L-glutamate from Sigma. All other chemicals were of analytical grade.

CPS-I and protein were assayed as in [7].

In order to purify CPS-I from one rat, the procedure in [7] was modified as follows. Liver (1 g) was used for the particle fractionation, the cetyltrimethylammonium chloride-extraction, and the first ammonium sulfate fractionation. For the DEAE–Sephadex chromatography, a 2 ml column (0.95 × 2.8 cm) was used. The enzyme was eluted from this column by stepwise elution with KCl between 30–50 mM which was dissolved in 'buffer A_1'. Because the purity of these enzyme preparations was sufficient for the purpose of our investigation (see section 3), further steps in the procedure [7] were omitted.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was performed with the use of the discontinuous Laemmli buffer system [8]. A slab gel with the dimension of 11 cm (migration distance) × 16 cm and 1.0 mm thick was used. The gel was stained for 20 min with 0.1% Coomassie blue R-250 in 50% trichloroacetic acid and destained in 7% acetic acid.

To study the effect of cycloheximide on the biosynthesis of CPS-I, a normal rat was injected intraperitoneally with various amounts of cycloheximide

dissolved in 0 9% NaCl After 25 min, rat was injected with 4 55 μCi/100 g body wt [^{14}C]leucine (0 035 μCi/μmol) intraperitoneally The rat was killed by decapitation 35 min later and the carcass was cooled immediately in ice cold water Two pieces of liver weighing 1 g were removed and one was used for purification of the enzyme The other piece of liver was used to measure incorporation of [^{14}C]leucine into mitochondrial and cytoplasmic protein For this purpose, this piece was homogenized in 9 ml medium containing 0 25 M sucrose, 5 mM Tris–HCl (pH 7 4) and 0 1 mM EDTA with aid of a Potter-Elvehjem homogenizer at a speed of 50 rev /min with 3 strokes The homogenate was centrifuged at 500 × g for 10 min and the pellet was discarded Mitochondrial and cytoplasmic fractions were obtained by centrifugation of the supernatant layer at 9000 × g for 10 min Mitochondria were further fractionated into acid-soluble and acid-insoluble membraneous fractions by centrifuging at 90 000 × g for 60 min, after incubation in 1 4% acetic acid for 30 min at 0°C [9]

For measurement of [^{14}C]leucine incorporation, protein from each fraction was sedimented in 5% trichloroacetic acid, washed 2 times with 5% trichloroacetic acid and once with ethanol ether chloroform (2 2 1) mixture and dissolved in 1 ml NCS-solubilizer Radioactivity was counted in toluene-based scintillator with Aloka LSC 673 scintillation spectrometer

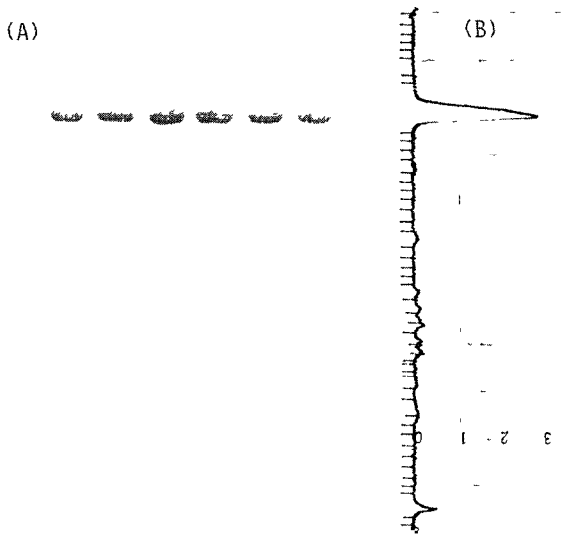

Fig 1 SDS–gel electrophoresis of CPS-I purified individually from 6 rats CPS-I was purified individually from 1 g liver pieces from 6 rats as in section 2 An aliquot of purified enzyme corresponding to 25 μg protein was mixed with equal volume of a medium containing 0 125 M Tris–HCl (pH 6 8), 4% SDS, 20% glycerol and 10% 2-mercaptoethanol This sample was heated for 3 min in boiling water before electrophoresis (A) Electrophoretogram of purified enzyme preparations from six rats (B) A typical densitogram scanned with Toyo Densitorol DMU-33C using a 560 nm filter

3 Results

Here, biosynthesis of CPS-I was measured by incorporation of [^{14}C]leucine into purified enzyme preparations from individual animals In order to confirm the reproducibility of the purification and the purity of enzyme preparations, purifications from 6 animals were performed Enzyme preparations with specific activity of 20 9 ± 1 31 (mean ± SD) were obtained This value corresponds to 81 6% of that in [7] SDS-gel electrophoretograms of these preparations (fig 1) show the purity and the reproducibility of the purification Densitometry of these electrophoretograms indicated 87 2 ± 4 9% purity for these preparations

Figure 2 shows the effect of cycloheximide on the incorporation of [^{14}C]leucine into proteins of various fractions Administration of cycloheximide at < 0 2 mg/100 g body wt resulted in nearly complete inhibition (94%) of [^{14}C]leucine incorporation into the cytoplasmic fraction Parallel inhibition was also observed in the acid-soluble mitochondrial fraction These facts indicate that proteins in these fractions are synthesized in cycloheximide-sensitive microsomal ribosomes On the other hand, incorporation into the acid-insoluble membraneous fraction of mitochondria was less sensitive to cycloheximide This fact implies that the acid-insoluble fraction of mitochondria is rich in protein synthesized on cycloheximide-insensitive mitochondrial ribosomes The inhibition of the incorporation into this fraction by low dose of cycloheximide may correspond to protein which is synthesized on extramitochondrial ribosomes and transferred into membrane of mitochondria It is probable that the additional inhibition in this fraction at higher

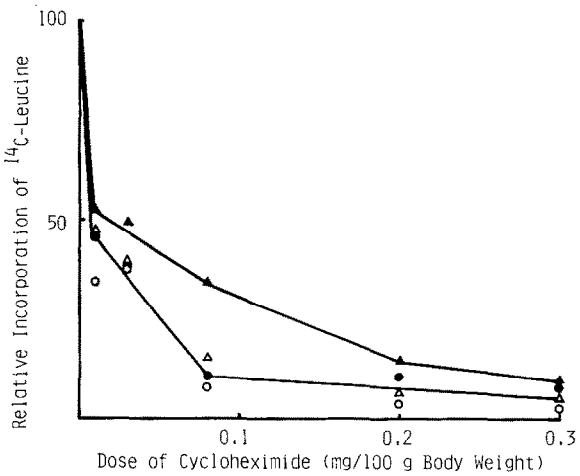

Fig.2. Inhibition of [^{14}C]leucine incorporation by cycloheximide into CPS-I and protein in 3 subcellular fractions. Rat was injected intraperitoneally with various amounts of cycloheximide. After 25 min, 4.55 μCi [^{14}C]leucine was injected intraperitoneally. The rat was killed 35 min later. CPS-I (●) was purified from 1 g liver from each rat. [^{14}C]Leucine incorporated into 17.7 units of the enzyme was counted. From a separate 1 g piece of each liver, the cytoplasmic fraction (△), acid-insoluble membraneous (▲) and acid-soluble (○) fractions of mitochondria were prepared as in section 2. [^{14}C]Leucine incorporated into each fraction, corresponding to 0.25, 0.4 and 0.4 g liver, respectively, was determined. The averaged count in CPS-I from 3 rats which were not injected with cycloheximide was 12.9 dpm/unit. The averaged count was: 60 100 dpm/g liver; for the cytoplasmic fraction was 4120 dpm/g liver for the acid-insoluble membraneous fraction of mitochondria; 8450 dpm/g liver for the acid-soluble mitochondrial fraction.

dose of cycloheximide (⩾ 0.1 mg/100 g body wt) is a reflection of the interdependence between cytoplasmic and mitochondrial protein synthesis [1].

Incorporation into CPS-I, purified from each rat injected various amounts of cycloheximide, was inhibited in a similar manner as that of the cytoplasmic and the acid-soluble mitochondrial fractions. If CPS-I is synthesized on mitochondrial ribosomes, [^{14}C]leucine incorporation must be less sensitive to cycloheximide than the incorporation into the acid-insoluble membraneous fraction, the protein of which may originate both from microsomal and mitochondrial ribosomes. Based on these facts, it may be concluded that CPS-I is synthesized on cycloheximide-sensitive microsomal ribosomes and translocated into mitochondrial matrix.

4. Discussion

In another study of the biosynthesis of mitochondrial matrix enzyme a precursor of glutamate dehydrogenase was shown [10] in the microsomal fraction [10]. This precursor of glutamate dehydrogenase was characterized and an investigation of the intracellular translocation of a mitochondrial enzyme precursor begun [11,12]. However, CPS-I may be a more suitable enzyme for this purpose. In ureotelic liver, this enzyme is the main protein of mitochondrial matrix [3]. Moreover, CPS-I is induced either by dietary protein intake [4] or by administration of glucagon [5]. This is a great advantage for the study of the translocation of mitochondrial matrix enzyme. It is possible that translocation as well as biosynthesis of CPS-I may be regulated under these conditions. Our results provide important information for the study of intracellular translocation of CPS-I.

Acknowledgements

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