CELL-FREE SYNTHESIS OF RAT LIVER δ-AMINOLEVULINATE SYNTHASE AND POSSIBLE OCCURRENCE OF PROCESSING OF THE ENZYME PROTEIN IN THE COURSE OF ITS TRANSLOCATION FROM THE CYTOSOL INTO THE MITOCHONDRIAL MATRIX

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1. Introduction

δ-Aminolevulinic acid (ALA) synthase in liver mitochondria can be increased markedly by the administration of porphyrinogenic drugs such as allylisopropylacetamide (AIA) and 3,5-dicarbethoxy-1,4-dihydrocollidine to animals, and under these conditions a considerable amount of ALA synthase also accumulates in the liver cytosol fraction [1]. The ALA synthase accumulating in the liver cytosol was shown to be a precursor in transit to the mitochondrial matrix [2]. The cytosolic ALA synthase has also been shown to be a dimer consisting of two identical subunits with min mol. wt 51 000 [1].

Here we attempted in vitro synthesis of ALA synthase in a reticulocyte lysate system with polysomes isolated from the liver of rats treated or untreated with AIA. The results indicated that functional mRNA for ALA synthase was actually increased in the liver of AIA-treated rat and free polysomes were the major site of ALA synthase synthesis. The ALA synthase synthesized in vitro had the same minimum molecular weight as that of the ALA synthase accumulating in the liver cytosol. On the other hand, the minimum molecular weight of the mitochondrial ALA synthase was found to be ~45 000 which is significantly smaller than that of the cytosolic ALA synthase. Possibly the cytosolic ALA synthase is subjected to processing in the course of its translocation into the mitochondrial matrix.

2. Materials and methods

2.1. *Conditioning of rats and preparation of polyribosomes*

Female Wistar strain rats (~150 g) were fasted for 24 h, then AIA (30 mg/100 g body wt) was injected subcutaneously and 12 h later, the same dose of AIA was again injected. Rats were killed by decapitation 3.5 h after the second AIA administration.

The livers were homogenized as in [3] in 4 vol. solution B (a mixture of 0.2 M sucrose, 0.1 M KCl, 5 mM $MgCl_2$, 0.02 M Tris–HCl (pH 7.5), 1 mM DTT and 0.5 mg heparin/ml and centrifuged at 20 000 × *g* for 10 min. For the preparation of total polysomes, the post-mitochondrial fraction was mixed with 0.1 vol. 10% Triton X-100 and 0.1 vol. 10% sodium deoxycholate, then layered over 5 ml 1.6 M sucrose cushion and centrifuged for 3 h at 200 000 × *g* in a RPR 50-2 Rotor of a Hitachi 55 P-7 ultracentrifuge. The pellet was suspended in solution A which is similar to solution B except for omission of heparin.

For the isolation of free and bound polysomes, the methods in [3,4] were used after slight modification. The post-mitochondrial fraction was layered over a discontinuous gradient consisting of 8 ml 0.3 M sucrose in solution B above, 3 ml 1.8 M sucrose in solution B, and centrifuged for 4 h at 200 000 × *g*. The pellet of free polysomes was suspended in solution A. The cloudy layer of bound polysomes at the lower gradient interphase was diluted with 2 vol. solution B, mixed with 0.1 vol. 10% Triton X-100 and 0.1 vol. 10% sodium deoxycholate, and layered over 1.6 M sucrose cushion. The pellet obtained after

a 3 h centrifugation at 200 000 × *g* was suspended in solution A.

2.2. *Cell-free protein synthesis in rabbit reticulocyte lysate system*

The rabbit reticulocyte lysate was prepared as in [5]. Cell-free protein synthesis was done essentially as in [3], using 200 μg creatine phosphokinase/ml and 8 mM creatine phosphate as an energy generating system. The incubation mixture also contained 10 μg/ml each of leupeptin, antipain, chymostatin, pepstatin and elastatinal to prevent possible digestion by proteases. The reaction was carried out at 30°C for 60 min using 0.5 mg (as RNA) polysomes and 50 μCi L-[4,5-^{3}H]leucine (67 Ci/mmol), in 1 ml final vol. which contained 0.4 ml reticulocyte lysate. The reaction was stopped by chilling, and an appropriate amount of a mixture of EDTA, KCl and Triton X-100 dissolved in Tris–HCl buffer (pH 7.5) was added to the reaction mixture final conc. 15 mM, 750 mM and 1%, respectively, with the aim to release the ^{3}H-labeled nascent peptides from polysomes. This mixture was centrifuged at 150 000 × *g* for 30 min. An aliquot (0.7 ml) of the resulting supernatant (1.8 ml) was mixed with an ALA-synthase-specific antibody (6 mg IgG) [2] and 750 units unlabeled ALA synthase partially purified from the liver cytosol fraction of AIA-treated rats, and left at 2°C for 24 h. Immunoprecipitates formed were collected and dissolved in 0.25 ml buffer for sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [2]. Aliquots (0.1 ml each) of the sample were subjected to SDS–polyacrylamide gel electrophoresis and analyzed for incorporation of radioactivity as in [2].

3. Results

3.1. *Synthesis of ALA synthase in vitro with total liver polysomes from AIA-treated and untreated rats*

With either polysomes (0.5 mg RNA) from AIA-treated or untreated rats, the activities of protein synthesis as measured by incorporation of [^{3}H]leucine into whole protein as in [2] were quite similar, and protein synthesis proceeded linearly for 60 min. At 60 min incubation, immunoreactive proteins were isolated from the reaction mixtures with an ALA synthase-specific antibody and analyzed. As shown in fig.1, incorporation of radioactivity into immunoprecipitated proteins was much higher when polysomes from AIA-treated rats were employed. This result indicates that the amount of mRNA for ALA synthase was increased in the liver of AIA-treated rats.

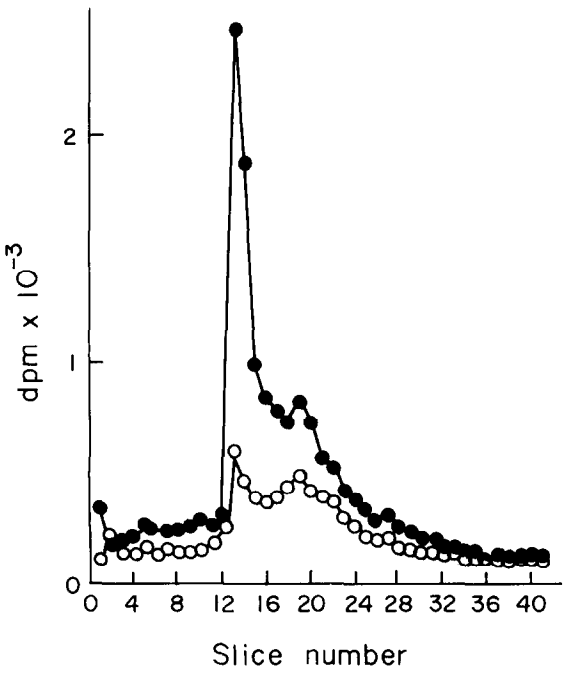

Fig.1. Synthesis of ALA synthase in vitro with total liver polysomes. Gels (0.6 × 7 cm) were frozen and cut into 1.5 mm segments and assayed for radioactivity. (•) With the liver polysomes from AIA-treated rat; (○) with polysomes from control rat untreated with AIA.

3.2. *Synthesis of ALA synthase in vitro with free and bound polysomes prepared from the liver of AIA-treated rats*

With either the free or bound polysomes (0.5 mg RNA), the protein synthetic activities as measured by incorporation of radioactivity into whole protein were not significantly different. As shown in fig.2, however, the ability of free polysomes to specifically synthesize ALA synthase was remarkably higher than that of the bound polysomes, suggesting that ALA synthase is synthesized predominantly on free polysomes.

3.3. *Comparison of the sizes of subunits of the cytosolic ALA synthase, the ALA synthase synthesized in vitro, and the mitochondrial ALA synthase*

Immunoprecipitate of ^{3}H-labeled ALA synthase synthesized in vitro was mixed with immunoprecipi-

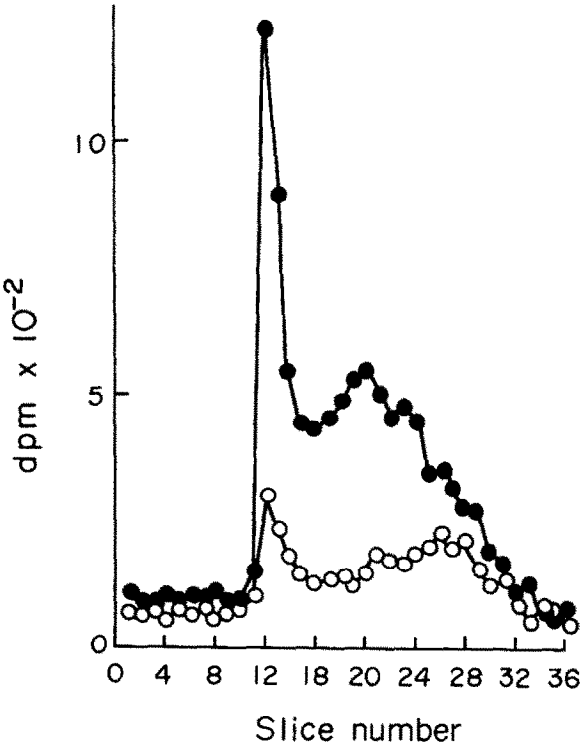

Fig.2. Comparison of activities of free and bound polysomes for synthesis of ALA synthase. Liver polysomes were prepared from AIA-treated rat. Other conditions and procedures were as in fig.1 and section 2. (•) With free polysomes; (○) with bound polysomes.

tate of ^{14}C-labeled cytosolic ALA synthase prepared in vivo by injection of [^{14}C]leucine into AIA-treated rat [2] and the mixture was subjected to SDS–polyacrylamide gel electrophoresis. As can be seen from fig.3A, the peaks of both ^{3}H and ^{14}C radioactivities appeared at the same position on the gel, indicating that the subunit molecular weight of the ALA synthase synthesized in vitro is equal to that of the cytosolic ALA synthase.

On the other hand, when the mixture of immunoprecipitates of the ^{14}C-labeled cytosolic ALA synthase and the ^{3}H-labeled mitochondrial ALA synthase was subjected to SDS–polyacrylamide gel electrophoresis, the major portion of the ^{3}H-labeled mitochondrial ALA synthase was found to have a significantly higher mobility than that of the ^{14}C-labeled cytosolic ALA synthase (fig.3B). It would mean that the mitochondrial ALA synthase has a smaller minimum molecular weight as compared with that of the cytosolic ALA synthase.

A more precise examination of the minimum molecular weights of ^{3}H-labeled ALA synthase synthesized in vitro, ^{3}H-labeled cytosolic ALA synthase and ^{3}H-labeled mitochondrial ALA synthase was

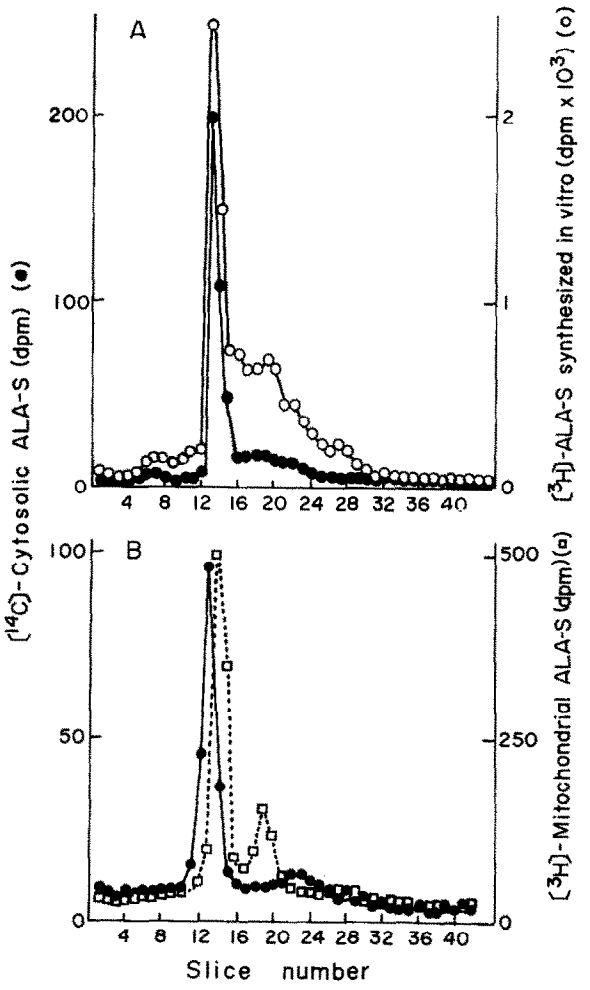

Fig.3. Comparison of minimum molecular weights of the cytosolic, mitochondrial, and in vitro synthesized ALA-synthases. (A) Comparison of the ^{3}H-labeled cytosolic ALA synthase (8000 dpm) (•) and the in vitro synthesized ^{14}C-labeled ALA synthase (800 dpm) (○). ALA-S stands for ALA synthase. (B) Comparison of the ^{14}C-labeled cytosolic ALA synthase (300 dpm) (•) and the ^{3}H-labeled mitochondrial ALA synthase (1500 dpm) (□). ^{3}H-Labeled mitochondrial ALA synthase was prepared in vivo by injection of [^{3}H]leucine into AIA-treated rat. The mitochondria were prepared as in [2] and ^{3}H-labeled ALA synthase in a sonic extract of the mitochondria was immunoprecipitated. In preparation of the mitochondrial ALA synthase, the whole procedures from homogenization of the liver to the immunoprecipitation of the enzyme were carried out in the presence of 10 μg/ml each of 5 kinds of the microbial protease inhibitors as in section 2.2. For other details see text.

performed, using [^{14}C]methylated myosin (mol. wt 200 000), phosphorylase B, (92 500), bovine serum albumin (69 000), ovalbumin (46 000), carbonic anhydrase (30 000) and lysozyme (14 500) as marker proteins and assuming their molecular weights as indicated in parentheses, respectively, according to the information from Radiochemical Center, Amersham, from where the marker proteins were obtained: Individual immunoprecipitates of ^{3}H-labeled ALA synthases were mixed with a mixture of ^{14}C-labeled marker proteins and subjected to SDS–polyacrylamide gel electrophoresis separately. The data indicated that the ALA synthase synthesized in vitro and the cytosolic ALA synthase had apparently the same minimum mol. wt 51 000, whereas that of the mitochondrial ALA synthase was ~45 000.

4. Discussion

This study sho' that functional mRNA for ALA synthase is increase. in the liver of AIA-treated rat which shows a higher activity of ALA synthase than the AIA-untreated rat and that ALA synthase is synthesized predominantly, if not exclusively, on free polysomes in the rat liver. The minimum molecular weight of ALA synthase synthesized in vitro was apparently equal to that of the cytosolic ALA synthase. However, the mitochondrial ALA synthase was found to have a significantly smaller minimum molecular weight as compared with that of the cytosolic ALA synthase. Also the two subunits of the mitochondrial ALA synthase appeared to have the same molecular size as judged from the results on SDS–polyacrylamide gel electrophoresis (fig.3B). These observations strongly suggest that the ALA synthase synthesized on free polysomes is released into the cytosol fraction and then is translocated into the mitochondrial matrix possibly being accompanied with processing of both subunits of the cytosol-form of the enzyme in the course of translocation. A similar situation has been reported for other mitochondrial matrix enzymes in the rat liver; ornithine transcarbamoylase [6,7] and carbamoyl phosphate synthetase I [8–10]. It should be noted, however, that mitochondrial fractions from rat liver are usually contaminated by lysosomes which would release their proteolytic enzymes upon sonication and this would raise the possibility that the mitochondrial enzyme becomes artifactually processed during the preparation of the enzyme. To minimize this possibility, we used in the present study microbial protease inhibitors throughout the whole procedure for the preparation of immunoprecipitate of the ^{3}H-labeled mitochondrial ALA synthase (see legend to fig.3). Still some uncertainty would remain with respect to the intactness of the enzyme preparation obtained.

ALA synthase synthesized in vitro with polysomes from drug-treated chick embryo liver had minimum mol. wt 70 000 [11] and ALA synthase with the same molecular weight could also be found in the cytosol fraction of chick embryo liver, while the mitochondrial ALA synthase showed minimum mol. wt 49 000 [11]. In our study with rat liver, however, the minimum molecular weight obtained for the cytosolic ALA synthase as well as in vitro synthesized ALA synthase was 51 000. This discrepancy remains to be examined.

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