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INCORPORATION OF CYTOSOL δ-AMINOLEVULINATE SYNTHASE OF RAT LIVER INTO THE MITOCHONDRIA IN VITRO

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Summary

When ^{125}I-labeled cytosol δ-aminolevulinate synthase was incubated in suspensions of rat liver mitochondria, the enzyme was incorporated into the mitochondira at the rate that was linear with time and with the [^{125}I]enzyme added. Subfractionation of the mitochondria using a digitonin technique revealed that the [^{125}I]enzyme was incorporated into the innermembrane-matrix fraction where endogeneous δ-aminolevulinate synthase is located.

It has been well established that the majority of the mitochondrial proteins are encoded by nuclear genes and synthesized on cytoplasmic ribsomes (1). Very little is yet known, however, about the mechanism by which cytoplasmically synthesized proteins are incorporated into the mitochondria. Kadenbach reported a transfer of proteins including cytochrome c from microsomes into mitochondria *in vitro* (2,3) but his interpretation of the data was questioned by Stratman *et al.* (4). Recently Marra *et el.* have presented evidence that mitochondrial aspartate aminotransferase, but not the cytoplasmic isoenzyme, passes from solution into the mitochondrial matrix *in vitro* and functions there (5).

δ-Aminolevulinate (ALA) synthase [EC2.3.1.37], first and rate-limiting enzyme in porphyrin biosynthesis, is one of the proteins that are synthesized on cytoplasmic ribosomes and incorporated into the mitochondria. When induced by the administration of porphyrinogenic drugs, ALA synthase of various animal livers accumulates in the cytosol (6-8). This cytosol enzyme consists of two catalytically inactive proteins and one catalytically active protein (9-12).

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The function of catalytically inactive proteins is yet unknown, though they can modulate the kinetic properties of the catalytically active protein (11). The mitochondrial enzyme is very similar to the catalytically active protein in the cytosol in molecular weight and some other properties (9-11), suggesting that only catalytically active protein may pass into the mitochondria. The present study, undertaken to test this possibilily using an in vitro system, provides the evidence that cytosol ALA synthase composed of three different proteins, but not the catalytically active protein alone, can be transferred from the solution into the isolated mitochondria.

Materials and Methods

Preparation of ^{125}I-labeled ALA synthase: Cytosol ALA synthase (complex form ALA synthase) and its catalytically active protein (stripped form ALA synthase) were purified from rat liver cytosol as described previously (11). The enzymes were labeled with ^{125}I using a chloramine-T method as described by Greenwood et al. (13). When kept frozed at -20°C, ^{125}I-labeled cytosol ALA synthase was active for 2 weeks in being transferred into the isolated mitochondria under the conditions described below.

Preparation of rat liver mitochondria and subfractionation into the mitochondrial outer membrane, intermembrane space and mitoplast: Livers of female rats weighing 150 to 170g which had been starved for 24 hr were homogenized in 9 volumes of medium A(0.25 M sucrose, 0.02 M Tris-HCl pH 7.6, 0.1 mM pyridoxal phosphate). The homogenate was centrifuged first at 600 x g for 10 min and then at 5,000 x g for 10 min. The resulting precipitate was washed once with medium A, and suspended in medium A to a final protein concentration of 17-20 mg/ml. Mitochondrial suspensions were kept on ice and used within 3 hr after preparation. The mitochondria were subfractionated into outer membrane, intermembrane space and mitoplast (inner membrane plus matrix) by a digitonin technique by Greenwalt (14). Kynurenine hydroxylase which is reported to be located in the outer membrane (15) was assayed by the method of Hayaishi (16). Mitochondrial sulfite oxidase, located in the intermembrane space (17), was assayed by measuring its sulfite-ferricyanide reductase activity as described by Cohen and Fridovich (18). Cytochrome c oxidase, a marker enzyme for the inner membrane, was assayed by measuring the loss in absorbance of reduced cytochrome c at 550 nm. Malate dehydrogenase in mitochondria is located in the matrix fraction and the activity was measured by the method of Ochoa (19). ALA synthase was assayed as described previously (7).

Incorporation of cytosol ALA synthase into the mitochondria: ^{125}I-Labeled cytosol ALA synthase or its catalytically active protein (specific activity, 11,000 and 9,000 cpm/μg, respectively) in 10 μl of 0.9% NaCl containing 0.01 M potassium phosphte buffer pH 7.2 was added to 1 ml of mitochondrial suspension (170-200 μg as proteins) in medium A. The incubations were carried out at 37°C for 10 min except otherwise described. After the incubations, the suspensions were rapidly centrifuged for 10 min at 10,000 x g at 2°C. The resulting precipitates were resuspended in medium A and centrifuged down. This washing was repeated three times.

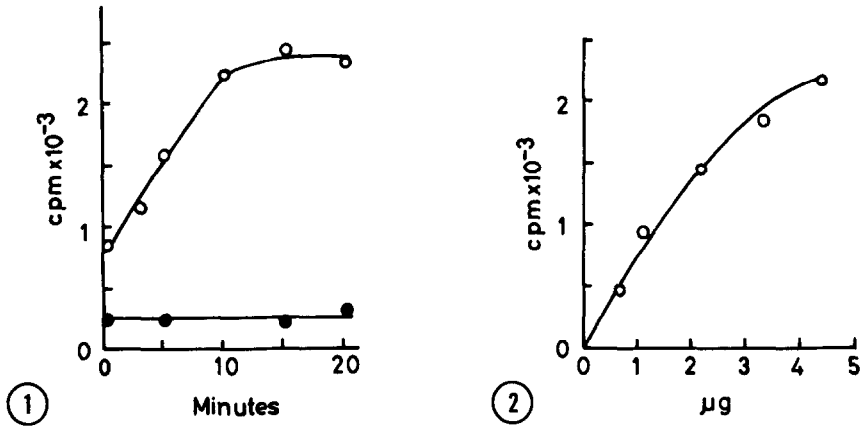

Fig.1. Time course of radioactivity accumulation in the mitochondria. Mitochondrial suspensions contained ^{125}I-labeled cytosol ALA synthase (23,000 cpm) (o—o) or catalytically active protein (19,000 cpm) (•—•). Other experimental conditions are described in Materials and Methods except that the incubation times were varied.

Fig.2. Radioactivity accumulation in the mitochondria as a function of added ^{125}I-labeled cytosol ALA synthase. Abscissa; the amount of ^{125}I-labeled cytosol ALA synthase, ordinate; the increase of radioactivity in the mitochondria during the 10-min incubation.

Other methods: Sodium dodecyl sulfate gel electrophoresis was performed by the method of Weber and Osborn with a 5% polyacrylamide gel (20) except that the sample was denatured according to the method of Palmiter et al. (21) and dialyzed against 0.02 M sodium phosphate buffer (pH.7.2) containing 0.1% sodium dodecyl sulfate before the electrophoresis. The gel was cut into 1.6-mm slices for counting. Protein was estimated by a biuret method with a bovine serum albumin as a standard. The radioactivity of ^{125}I was counted in Searle auto-well gamma counter 1185 with a counting efficiency of 84%.

Results

Figure 1 shows the time course of ALA synthase incorporation into the mitochondria. When ^{125}I-labeled cytosol ALA synthase was incubated in suspensions of mitochondria, the radioactivity in the mitochondria increased linearly until 10 min and reached a maximum which was about 10% of that of added [^{125}I]enzyme. A separate experiment has shown that mitoplasts also took up the [^{125}I]enzyme at the rate similar to that obtained with intact mitochondria (data not presented). With the ^{125}I-labeled catalytically active protein

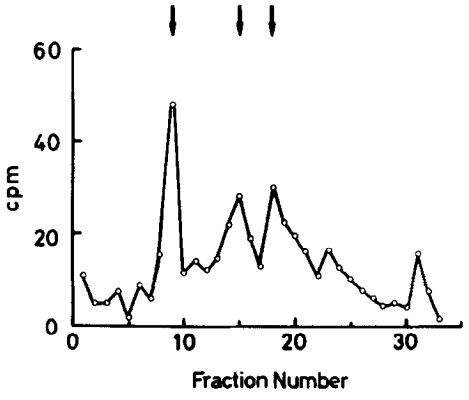

Fig.3. Sodium dodecyl sulfate gel electrophoresis of mitochondria incubated with ^{125}I-labeled cytosol ALA synthase. Experimental conditions are described in Materials and Methods except that the incubation time was 5 min. The position of front of dye (Coomassie blue) was at fraction 34.

component of cytosol ALA synthase,however, no accumulation of the radioactivity in the mitochondria was observed (Fig.1).

As illustrated in Fig. 2, the radioactivity accumulated in the mitochondria was nearly proportional to the labeled cytosol ALA synthase added into the suspension.

The incubated mitochondria were subjected to the sodium dodecyl sulfate gel electrophoresis to ascertain whether the radioactivity found in the mitochondria was actually derived from cytosol ALA synthase. As shown in Fig.3, three major peaks (indicated by arrows in the figure) and two minor peaks (fractions 23 and 31) of radioactivity were resolved. The radioactivity found in three major peaks amounted to 60% of the total recovered from the gel. Molecular weights of these three peaks were estimated to be about 130,000, 80,000 and 50,000. These values correspond well to those of the protein components of the purified cytosol ALA synthase (11). The nature of two other small peaks is currently unknown.

Table 1 represents the distribution of the radioactivity, endogeneous ALA synthase, and the marker enzymes in three submitochondrial fractions; outer membrane, intermembrane space, and mitoplast. These data show that three

Table 1. Intramitochondrial Distribution of Radioactivity, ALA synthase, and Marker Enzymes

	Whole mitochondria	Mitoplast	Intermembrane space	Outer membrane	Recovery
Protein	100	61.7	20.1	13.2	95
^{125}I	100 (41)	93 (62)	7.8 (15.8)	16 (50)	117
ALA synthase	100 (3.5)	113 (6.4)	0 (0)	5.7 (1.1)	119
Cytochrome c oxidase	100 (707)	71 (840)	2 (71.6)	17 (932)	90
Malate dehydrogenase	100 (1,560)	82 (2,130)	21 (1,680)	0 (0)	103
Sulfite oxidase	100 (65.9)	0 (0)	91 (307)	3 (1.8)	94
Kynurenine hydroxylase	100 (1.14)	24 (0.47)	33 (1.92)	41 (3.63)	98

Mitochondria (0.2 mg as protein) incubated with the ^{125}I-labeled cytosol ALA synthase (46,000 cpm) were mixed with carrier mitochondria (100 mg as protein), treated with digitonin (0.12 mg digitonin per mg of mitochondrial protein), and subfractionated. The enzyme activity of ^{125}I-labeled ALA synthase was calculated to be less than 0.3% of the total found in the whole mitochondria. Values are expressed as per cent activity of the total in the whole mitochondria and those in the parentheses are specific activity as expressed cpm per mg of protein for ^{125}I, nmoles of ALA formed per hr per mg of protein for ALA synthase, and nmoles of the product formed per min per mg of protein for the marker enzymes.

submitochondrial fractions were reasonably well separated although a considerable amount of kynurenine hydroxylase was recovered in the intermembrane space possibly owing to the solubilization of the enzyme by digitonin (16). Over 90% of the radioactivity was recovered in the mitoplast fraction where virtually all the ALA synthase activities were also found, indicating that most, if not all, radioactivity found in the mitochondria was not simply adsorbed on the outer surface but incorporated into the innermembrane–matrix fraction where ALA synthase is destined to be settled.

Discussion

The present study has shown that the cytosol ALA synthase of rat liver, but not the catalytically active protein component of the enzyme, can be incorporated into the innermembrane-matrix fraction of isolated mitochondria

where ALA synthase functions physiologically. This suggests that the catalytically inactive protein components of cytosol ALA synthase may play an important role in the transfer of the enzyme into the mitochondria, although the possibility can not be excluded that during the iodination procedure the catalytically active protein was modified to such an extent that no incorporation into the mitochondria was observed. Chloramine-T is reported to cause an inactivation of some enzymes (22, 23).

It is known that hepatic plasma membranes have specific receptors for the proteins to be incorporated into the cells (24). It seems unlikely, however, that there exists a specific receptor for the ALA synthase on the outer surface of the mitochondria since the mitoplast which is devoid of outer membrane can also incorporate the ^{125}I-labeled cytosol ALA synthase.

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