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δ-AMINOLEVULINATE SYNTHASE ISOZYMES IN THE LIVER AND ERYTHROID CELLS OF CHICKEN

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SUMMARY: Antibodies raised against the purified chicken liver δ-aminolevulinate synthase showed a partial cross-reactivity with the chicken erythroid δ-aminolevulinate synthase. δ-Aminolevulinate synthase synthesized in vitro using polysomes from erythroid cells showed a subunit molecular weight of 55,000, whereas the enzyme synthesized in vitro using liver polysomes had a subunit molecular weight of 73,000. δ-Aminolevulinate synthase isolated from mitochondria of erythroid cells showed a molecular weight of 53,000, while the enzyme in liver mitochondria had a value of 65,000. These observations imply that the erythroid δ-aminolevulinate synthase differs from the hepatic enzyme.

δ-Aminolevulinate synthase is the first enzyme of the heme biosynthetic pathway in animals (1). Studies with chemically induced hepatic porphyria animals revealed that synthesis of δ-aminolevulinate synthase in the liver is subject to feedback regulation by heme at both the transcriptional (2-4) and translational (5-10) steps. Heme also inhibits the translocation of δ-aminolevulinate synthase from liver cytosol into mitochondria, thus giving rise to accumulation of a significant amount of the enzyme in the liver cytosol fraction in porphyria animals (11, 12). This would represent a unique feedback mechanism of heme biosynthesis acting on the translocation of δ-aminolevulinate synthase into mitochondria, the site of physiological functioning (10-12).

On the other hand, erythroid δ-aminolevulinate synthase has been shown not to be inducible by drugs that induce hepatic porphyria in animals (13-15) and moreover, the enzyme does not significantly accumulate in the cytosol fraction of erythroid cells when the cells were treated with hemin (16, 17). These observations suggest that the regulatory mechanism acting on δ-aminolevulinate synthase in erythroid cells may be different from that in the liver.

The present study aimed to clarify whether the hepatic and erythroid δ-aminolevulinate synthases are the same proteins or not, using a specific rabbit antiserum raised against a highly purified chicken liver δ-aminolevulinate synthase.

Abbreviations: AIA, allylisopropylacetamide; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Materials: L-[4,5-^{3}H]Leucine (147 Ci/mmol) and [^{14}C]-methylated proteins were obtained from Amersham International Ltd., Amersham. Microbial protease inhibitors (antipain, chymostatin, elastatinal, leupeptin and pepstatin) were from Peptide Institute, Osaka.

Conditioning of Chickens for Induction of Hepatic δ-Aminolevulinate Synthase and Preparation of Mitochondrial Fraction of Chicken Liver

White Leghorn chickens weighing 0.5 to 1.5 kg were used in all experiments. To induce hepatic δ-aminolevulinate synthase, two doses of DDC (suspended in corn oil; each dose, 400 mg/kg body weight) were administered subcutaneously at 0 h and 20 h of the experiment, and also two doses of AIA (dissolved in 0.9% NaCl; each dose, 250 mg/kg body weight) were administered subcutaneously at 20 h and 30 h. All chickens were killed at 40 h after the first administration of DDC.

Liver was homogenized in 9 volumes of 0.25 M sucrose containing 50 mM Tris-HCl buffer (pH 7.4), 0.1 mM pyridoxal phosphate, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 50 μg/ml each of all protease inhibitors, and the mitochondrial fraction was separated by centrifugation (9). The mitochondrial fraction was resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100, 0.1 mM pyridoxal phosphate, 0.1 mM dithiothreitol, and 100 μg/ml each of the protease inhibitors; the suspension was centrifuged at 150,000 xg for 90 min and the resulting supernatant was used as the mitochondrial extract.

Production of Anemia and Preparation of Mitochondrial Fraction of Erythroid cells

A mixture of acetylphenylhydrazine (dissolved in 0.9% NaCl; 20 mg/kg body weight) and phenylhydrazine hydrochloride (dissolved in 0.9% NaCl and neutralized; 30 mg/kg body weight) was injected intramuscularly into chickens. After a period of 60 h, blood was taken, erythroid cells were separated and hemolyzed, and the subsequent subcellular fractionation was carried out by the method of Guggenheim et al. (18). The mitochondrial extract was prepared in the same way as described above.

Preparation of Polysomes

Liver polysomes were prepared as follows: Liver was homogenized in 9 volumes of 0.25 M sucrose containing 50 mM Tris-HCl buffer (pH 7.4), 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol and 4 mg/ml of heparin, and centrifuged at 15,000 xg for 10 min. The supernatant fluid was mixed with one-fourth volumes of 20% Triton X-100 and layered over 6 ml of 1.5 M sucrose containing the same additions as used for homogenization and then centrifuged at 150,000 xg for 180 min. Polysome pellets were rinsed and suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 25 mM KCl and 5 mM $MgCl_2$. Final polysome concentrations were 80 to 100 A_{260} units/ml. Erythroid cell polysomes were prepared as follows: Erythroid cells were lysed by addition of 2 volumes of deionized water and were gently stirred for 2 min. Isotonicity was restored by adding an equal volume of 40 mM Tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 10 mM $MgCl_2$, 2 mM dithiothreitol and 1 mg/ml of heparin, and the hemolysate was centrifuged at 15,000 xg for 10 min. Total polysomes were prepared from the resulting supernatant in the same way as for liver polysomes.

Cell-Free Synthesis of δ-Aminolevulinate Synthases

Ribonuclease-treated rabbit reticulocyte lysate (purchased from Amersham) was used. The reaction mixture contained, in a final volume of 100 μl, 20 μCi of [^{3}H]leucine, 0.1 mM pyridoxal phosphate, 100 μg/ml each of protease inhibitors, 1 to 5 A_{260} units of polysomes, and 60 μl of reticulocyte lysate. Incubation was carried out at 30°C for 60 min. To the cell-free protein synthesizing mixture, 50 μg of rabbit IgG specific to the hepatic δ-aminolevulinate synthase was added, and the mixture was kept at 4°C for 60 min. The immune complex formed was separated using the protein A-Sepharose (30 mg) method (19).

Labeling of Mitochondrial δ-Aminolevulinate Synthases

Erythroid δ-aminolevulinate synthase was labeled by incubating erythroid cells for 60 min at 37°C in the medium containing 2 mg/ml glucose, 5 mM KCl, 0.125 M NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 20 mM sodium phosphate buffer (pH 7.4), 8 μCi/ml [^{3}H]-leucine and 0.2 mM each of 19 other aminoacids. For labeling of hepatic δ-aminolevulinate synthase [^{3}H]-leucine (1 mCi/kg body weight) was administered intraperitoneally to the drug-treated chickens.

Preparation of Antibody

Chicken liver mitochondrial δ-aminolevulinate synthase was purified to apparent homogeneity by a method similar to that used for the rat liver cytosolic δ-aminolevulinate synthase (20); the data of the enzyme purification will appear elsewhere. IgG fractions of rabbit antiserum against the purified δ-aminolevulinate synthase were prepared as described previously (12).

RESULTS

Immunochemical Comparison of δ-Aminolevulinate Synthases from Chicken Liver and Chicken Erythroid Cells

Ouchterlony double-diffusion study demonstrated that the erythroid δ-aminolevulinate synthase was partially cross-reactive with antibodies against a chicken liver δ-aminolevulinate synthase (Fig. 1), indicating that the hepatic and erythroid enzymes share some of antigenic determinants. For closer examination of the immunoreactivity of the two δ-aminolevulinate synthases, an immunotitration experiment was performed, using 60 units of the respective enzymes. As can be seen from Fig. 2, the amount of IgG required to completely precipitate 60 units of the erythroid enzyme was about 6 fold larger than the amount required for the hepatic enzyme.

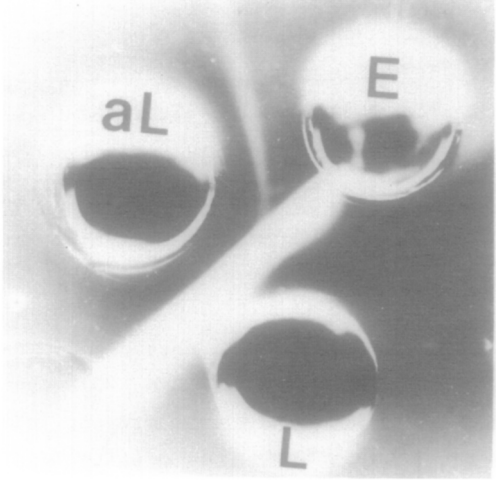

Fig. 1. Ouchterlony double diffusion analysis of hepatic δ-aminolevulinate synthase and erythroid δ-aminolevulinate synthase. aL, 10 μl of rabbit antiserum raised against purified chicken liver mitochondrial δ-aminolevulinate synthase; L, 50 units of purified liver mitochondrial δ-aminolevulinate synthase; E, 25 units of mitochondrial δ-aminolevulinate synthase from erythroid cells.

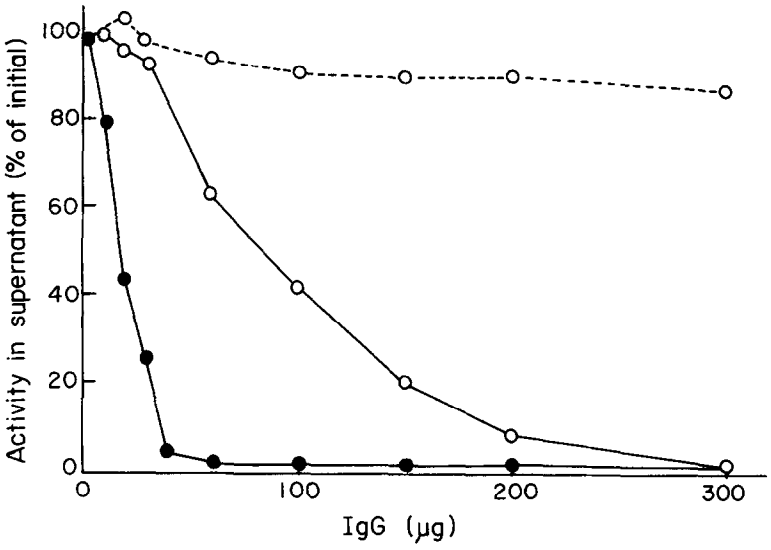

Fig. 2. Immunotitration experiments. Purified liver mitochondrial δ-aminolevulinate synthase (60 units) was mixed with the indicated amounts of hepatic δ-aminolevulinate synthase-specific IgG (●——●). Partially purified erythroid cell mitochondrial δ-aminolevulinate synthase (60 units) was also mixed with the indicated amounts of the hepatic enzyme-specific IgG (o——o) or control IgG (o---o). After 12 h, the mixtures were centrifuged and the supernatants obtained were assayed for δ-aminolevulinate synthase.

Comparison of Molecular Weights of Hepatic δ-Aminolevulinate Synthase and Erythroid δ-Aminolevulinate Synthase

Hepatic δ-aminolevulinate synthase was labeled *in vivo* by injection of [^{3}H]-leucine and was isolated from the mitochondrial extract by immunoprecipitation with IgG specific to the chicken liver δ-aminolevulinate synthase. The enzyme synthesized *in vitro* using total liver polysomes was also immunoprecipitated with IgG against the liver δ-aminolevulinate synthase. The molecular weights of the immunoprecipitated δ-aminolevulinate synthases were estimated by means of SDS-polyacrylamide slab gel electrophoresis followed by fluorography, and the values of 65,000 (Fig. 3, lane 2) and 73,000 (Fig. 3, lane 1) were obtained for the mitochondrial enzyme and the *in vitro* synthesized enzyme, respectively. Ades and Harpe (24, 25) reported that the embryonic chick liver δ-aminolevulinate synthase present in mitochondria had a subunit molecular weight of 63,000 and that the enzyme appeared to be synthesized as a precursor of 75,000 daltons. More recently, Borthwick *et al.* (26) reported that the δ-aminolevulinate synthase purified from chick embryo liver and the enzyme synthesized *in vitro* had the subunit molecular weights of 68,000 and 74,000, respectively. The differences in apparent molecular weights of δ-aminolevulinate synthases in these and our studies seem to be due to differ-

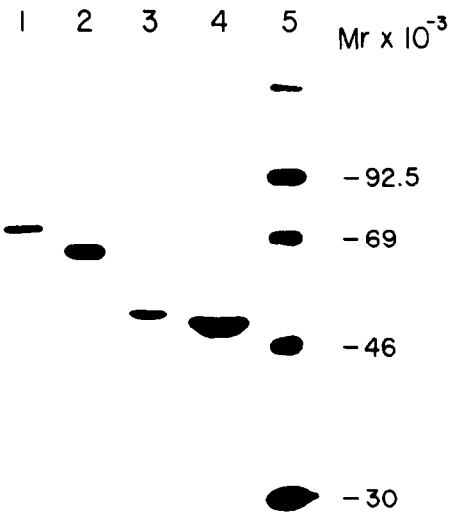

Fig. 3. Comparison of molecular weights of mitochondrial δ-aminolevulinate synthases and in vitro synthesized δ-aminolevulinate synthases of liver and erythroid cells. Labeled δ-aminolevulinate synthases were immunoprecipitated and analyzed by SDS-10% polyacrylamide gel electrophoresis according to Laemmli (21). The location of radioactive bands was determined by fluorography. Lane 1, hepatic enzyme synthesized in vitro; lane 2, liver mitochondrial enzyme; lane 3, erythroid enzyme synthesized in vitro; lane 4, mitochondrial enzyme from erythroid cells; lane 5, [^{14}C]-methylated marker proteins: phosphorylase b (92,500) bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000).

ences in electrophoretic conditions and marker proteins used in respective studies.

Erythroid cells prepared from chickens which had been treated with phenylhydrazine and acetylphenylhydrazine were incubated in a medium containing [^{3}H]-leucine, and the enzyme in the mitochondrial extract of the erythroid cells was immunoprecipitated with a hepatic δ-aminolevulinate synthase-specific IgG. Then, the immunoprecipitates were analyzed by the combination of SDS-polyacrylamide slab gel electrophoresis and fluorography. The molecular weight of the mitochondrial enzyme thus examined was about 53,000 (Fig. 3, lane 4). On the other hand, when the enzyme synthesized in vitro using polysomes isolated from erythroid cells was analyzed in a similar way, the newly synthesized enzyme showed a molecular weight of about 55,000 (Fig. 3, lane 3), which is about 2,000 larger than the value for the mitochondrial enzyme.

DISCUSSION

Several investigators suggested that the regulatory mechanism acting on erythroid δ-aminolevulinate synthase may be different from that for the hepatic δ-aminolevulinate synthase (13-15). In addition, Bishop et al. suggested the existence of δ-aminolevulinate synthase isoenzymes on the basis of dif-

ferences in kinetic and ligand-binding properties observed between the enzymes isolated from erythroid and non-erythroid tissues (17). Immunodiffusion and immunotitration experiments in the present study provided convincing evidence that in the chicken the erythroid δ-aminolevulinate synthase and the hepatic δ-aminolevulinate synthase are different tissue-specific entities. Furthermore, the erythroid δ-aminolevulinate synthase synthesized in vitro was about 18,000 daltons smaller than the hepatic enzyme synthesized in vitro. These observations suggest that the mRNA for erythroid δ-aminolevulinate synthase differs from the mRNA for the hepatic δ-aminolevulinate synthase. However, it is not clear at present whether the hepatic and erythroid ALA synthases are specified by the two independent genomic sequences. It is interesting in this connection to note that Young et al. reported that a single mouse α-amylase gene specifies two different tissue-specific mRNAs (27).

Our experiments also demonstrated that both hepatic and erythroid δ-aminolevulinate synthases are made as larger precursors which are transported into mitochondria in association with proteolytic processing. However, the cleaved polypeptide (Mr = 8,000) of the hepatic δ-aminolevulinate synthase, which seems to be a signal sequence, is larger than that (Mr = 2,000) of the erythroid enzyme. This would imply that the hepatic enzyme and the erythroid enzyme have different signal sequences which are needed for the translocation of respective enzymes across the mitochondrial membranes.

The finding that the erythroid δ-aminolevulinate synthase differs from the hepatic δ-aminolevulinate synthase in both immunoreactivity and molecular size would provide a clue for understanding the different features in hepatic and erythropoietic porphyrias.

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