Isolation of the mature subunit of δ -aminolaevulinate synthase from embryonic chick liver

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We presented evidence indicating that the established procedure for purifying δ -aminolaevulinate (ALA) synthase from embryonic-chick liver yielded an enzyme with a partially degraded subunit of molecular weight 51 000 [Ades & Harpe (1981) *J. Biol. Chem.* **256**, 9329–9333]. We now report the purification from livers of porphyric embryos of a preparation of ALA synthase which consisted primarily of a 63 000-Da polypeptide and a component migrating as a smear of polypeptides with a minimum molecular weight of 52 000. Neither component could be recovered from liver mitochondria of normal embryos, where the amounts of ALA synthase were relatively low. The 52 000-Da component had been established to be the partially degraded subunit of the enzyme. Peptide-mapping analyses indicated that the 63 000- and the 52 000-Da components possessed significant structural homologies, and it was concluded that the 63 000-Da polypeptide represented the mature subunit of ALA synthase.

ALA synthase catalyses the first step of the pathway of haem biosynthesis (Granick & Sassa, 1971). In liver, the enzyme is located in the mitochondrial matrix (Patton & Beattie, 1973), but its subunit is synthesized on cytoplasmic polyribosomes (Sassa & Granick, 1970; Strand *et al.*, 1972). The biogenesis of ALA synthase and its regulation have been subjects of intense investigations, owing to the involvement of the enzyme with the onset of hepatic porphyrias (Maxwell & Meyer, 1978), as well as for determining the mechanism of translocation of the enzyme into mitochondria (Nakakuki *et al.*, 1980; Yamauchi *et al.*, 1980a,b; Kikuchi & Hayashi, 1981).

Although ALA synthase purified from embryonic-chick liver was reported to have a subunit molecular weight of 49 000–51 000 (Whiting & Granick, 1976; Ades & Harpe, 1981), we have demonstrated that the actual molecular weight of the subunit was significantly larger, being $65\,000 \pm 2000$ (Ades & Harpe, 1981). This was demonstrated indirectly by immunoreplicate-electrophoresis

analyses of SDS-solubilized mitochondria isolated from porphyric-chick embryos under conditions that minimized proteolytic activities (Ades & Harpe, 1981). We suggested then that the difference in subunit molecular weight between that of the isolated enzyme and that of the enzyme detected immunochemically in mitochondria was probably due to partial proteolytic degradation of the enzyme during purification (Ades & Harpe, 1981).

We now report that ALA synthase can indeed be purified from mitochondria of porphyric-chick embryos, with a 63 000-Da polypeptide constituting a major component of the preparation. Peptide-mapping analyses indicated that the lower-molecular-weight subunit of the purified enzyme has significant structural homology with the purified 63 000-Da polypeptide, directly proving our previous interpretation that the 49 000–51 000-Da subunit is a fragment of the 63 000-Da polypeptide.

Experimental

Purification of ALA synthase

Liver ALA synthase was purified from 19-day White Leghorn chick embryos pretreated with AIA + DDC by using a modification of the procedures of Whiting & Granick (1976). As detailed below, the enzyme was released from liver mitochondria by sonication and purified by gel-filtration

Abbreviations used: ALA synthase, δ -aminolaevulinate synthase (EC 2.3.1.37); AIA, allylisopropylacetamide; DDC, diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate; SDS, sodium dodecyl sulphate.

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chromatography, $(\text{NH}_4)_2\text{SO}_4$ fractionation, affinity chromatography on pyridoxamine 5'-phosphate linked to Sepharose 4B, and preparative isoelectric focusing. As the protein was susceptible to degradation, it was essential to perform the purification as rapidly as possible and to keep all samples between 0 and 4°C. The entire procedure could be completed in 8–9 days.

The activity of ALA synthase was determined by the colorimetric method of Sassa *et al.* (1979), with the assay being modified to include succinic thiokinase as described by Whiting & Granick (1976). Succinic thiokinase was purified from *Rhodopseudomonas spheroides* by the procedure of Burnham (1970). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin serving as a standard.

Steps of the purification. (a) Embryos (18 days) were each given 2 mg of AIA + 4 mg of DDC to raise the starting amounts of the enzyme (Whiting & Granick, 1976; Ades & Harpe, 1981). The drugs were dissolved in 0.1 ml of dimethyl sulphoxide and injected into the fluid surrounding each embryo. At 18 h after administration of the drugs, livers were removed from 168 embryos and rinsed in 0.15 M-NaCl. The pooled livers were homogenized in 0.25 M-sucrose / 1 mM-EGTA / 0.01 mM-pyridoxal phosphate / 10 mM-Tris/HCl, pH 7.4, in a final volume of 500 ml. The homogenate was centrifuged at 500 g for 10 min in a Sorvall SS34 rotor. The nuclear pellets were resuspended in 200 ml of the buffer and centrifuged once more at 500 g for 10 min. The postnuclear supernatants were combined and centrifuged at 17000 g for 15 min. The crude mitochondrial pellets were suspended in 250 ml final volume with the sucrose buffer and centrifuged at 500 g for 10 min to pellet contaminating nuclei. The supernatant was centrifuged at 7700 g for 15 min and the mitochondrial pellets were suspended once more in 175 ml of the sucrose buffer and pelleted by centrifugation at 7700 g for 15 min. The pellets were frozen in liquid nitrogen and stored at -70°C until used.

(b) To purify the enzyme, the mitochondrial pellets were thawed and suspended in 12 ml of sonication buffer (0.05 M-Tris/HCl/0.01 mM-pyridoxal 5'-phosphate/1 mM-dithiothreitol, pH 7.6). The enzyme was released from mitochondria by sonication with four 30 s bursts at maximum output with a Sonifier Cell Disruptor (Heat Systems-Ultrasonics Inc., Long Island, NY, U.S.A.) fitted with the 0.5-in (1.27 cm) probe. The resulting homogenate was centrifuged at 100000 g for 90 min in a Beckman type 40 rotor. After centrifugation, the supernatant was kept and the pellets were resuspended in 12 ml of sonication buffer. The resulting suspension was sonicated once more and centrifuged at 100000 g for 90 min as described above. The supernatant from the

second centrifugation was pooled with that from the first centrifugation and the pellets were discarded.

(c) The pooled supernatant was adjusted to 0.8 M-NaCl and 1.0 mM-dithiothreitol, then loaded on a gel-chromatography column (5.0 cm × 80 cm) packed with Sephadex G-150 (Pharmacia Fine Chemicals, Uppsala, Sweden); the gel had been equilibrated with elution buffer (50 mM-Tris/HCl/0.5 M-NaCl/0.2 mM-dithiothreitol/0.2 mM-pyridoxal 5'-phosphate/0.1 mM-EDTA, pH 7.6). The column was eluted with the elution buffer and 11.5 ml fractions were collected. The fractions eluted from the column were assayed for ALA synthase activity, and those containing the higher activities were pooled.

(d) $(\text{NH}_4)_2\text{SO}_4$ was added to the pooled fraction to 85% saturation (559 g/litre) with constant stirring over a 20 min period, and proteins were allowed to precipitate during an additional period of 20 min. The precipitated material was collected by centrifugation at 27000 g for 20 min in the SS34 rotor. Most of the precipitate was dissolved in 15 ml of buffer A (0.02 M-Tris/HCl/80 mM-NaCl/0.1 mM-EDTA/0.1% Triton X-100/1 mM-dithiothreitol, pH 7.4) and left overnight on ice. The remaining insoluble material was removed by centrifugation at 12000 g for 15 min in the SS34 rotor. The supernatant was kept.

(e) The supernatant from the last step was loaded on an affinity-chromatography column (2.5 cm × 20 cm) consisting of pyridoxamine 5'-phosphate covalently linked to Sepharose 4B and equilibrated with buffer A. [The affinity gel had been prepared by coupling CNBr-activated Sepharose 4B (Cuatrecasas, 1970) with 6-aminoheptanoic acid (Barry & O'Carra, 1973); pyridoxamine phosphate was then linked to the hydrocarbon arm with a carbodi-imide (Miller *et al.*, 1972)]. After the addition of the sample, the column was washed with buffer A until proteins with no affinity for the gel were eluted as one peak. Proteins bound to the gel (which included most of the ALA synthase originally loaded) were then eluted with buffer A containing 10 mM-pyridoxal 5'-phosphate. Fractions (9 ml) were collected throughout the steps. The fractions containing the higher activities of ALA synthase were pooled.

(f) The pooled fractions from the affinity-chromatography step were concentrated by chromatography on hydroxyapatite. The fractions were passed through a column (1 cm × 5 cm) packed with Bio-Gel HT (Bio-Rad Laboratories, Richmond, CA, U.S.A.), equilibrated with buffer HA (20 mM-potassium phosphate/0.01 mM-pyridoxal 5'-phosphate/1 mM-dithiothreitol, pH 7.6). The column was washed with 3 vol. of buffer HA, and then ALA synthase was eluted from the column as one sharp peak with 0.4 M-potassium phosphate, pH 7.6. The fractions containing the enzyme were pooled and

dialysed for 20 h against 2 litres of buffer HA. The sample was kept for the isoelectric-focusing step of the purification.

(g) Isoelectric focusing was performed in a 110 ml column (LKB 8100 Ampholine electrofocusing column) in a linear gradient of 5–50% (w/v) sucrose prepared in 0.15 M-dithiothreitol. The gradient contained also 3.0% Ampholines with a working pH range from 6 to 8 (Bio-Lyte 6/8, obtained from Bio-Rad Laboratories). Halfway during the formation of the gradient, the sample of partially purified ALA synthase, supplemented with Ampholine to a concentration of 3.0%, was added to the gradient maker into the chamber containing the dense sucrose solution, then formation of the gradient was continued. The cathode solution was 0.25 M-NaOH and the anode solution was 60% (w/v) sucrose in 0.25 M-H₃PO₄. Focusing was done at 5 W for 36 h, and then the gradient was fractionated into 2.5 ml fractions. The pH and ALA synthase activity of each fraction were determined immediately. The fractions containing the higher activities of ALA synthase were pooled.

(h) The pooled fractions were dialysed against 3 litres of buffer HA for 20 h. The dialysed samples were loaded on a hydroxyapatite column prepared as described above (step f), then ALA synthase was eluted from the column with 0.4 M-potassium phosphate, pH 7.6. The fractions (0.5 ml) containing ALA synthase activity were pooled and dialysed overnight against 1 litre of buffer HA. The dialysed sample represented the purified enzyme.

Radioiodination of ALA synthase

Purified ALA synthase was radioiodinated with the Enzymobead reagent (immobilized lactoperoxidase and glucose oxidase, obtained from Bio-Rad, Rockville Center, NY, U.S.A.). Approx. 50–100 μ g of protein in 50 μ l of 0.2 M-sodium phosphate, pH 7.4, was added to 50 μ l of the hydrated Enzymobead reagent together with 1 mCi of Na¹²⁵I (New England Nuclear, Boston, MA, U.S.A.) and 25 μ l of 1% β -D-glucose. The mixture was incubated for 20 min at room temperature. The radioiodinated protein was then separated from free ¹²⁵I and the Enzymobead reagent by gel filtration on Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), on a 0.7 cm $\times$ 30 cm column eluted with 0.02 M-sodium phosphate, pH 7.4, containing 0.1 mg of cytochrome c/ml. Typically, a specific radioactivity of 2 μ Ci/ μ g of protein was obtained by this procedure.

Gel electrophoresis

Electrophoretic analyses of proteins were performed on 0.15 cm-thick slabs of 10% polyacrylamide gels in the presence of SDS in the system of Laemmli (1970). Proteins were stained with

Coomassie Blue R by standard methods. The standards used and their respective molecular weights were: bovine serum albumin, 68 000; ovalbumin, 46 000; carbonic anhydrase, 30 000; cytochrome c, 12 400.

Two-dimensional gel electrophoresis was done by the method of O'Farrell (1975) by using carrier ampholytes with a working pH range of 4–9 (Bio-Rad Laboratories, Richmond, CA, U.S.A.).

Peptide mapping

Peptide mapping of polypeptides by limited proteolysis was done by the method of Cleveland *et al.* (1977). Typically, a sample of a radioiodinated preparation of purified ALA synthase containing 2×10^6 c.p.m. of ¹²⁵I was mixed with 5 μ g of carrier ALA synthase and the mixture was subjected to polyacrylamide-gel electrophoresis in the presence of SDS. After electrophoresis, the proteins in the gel were stained with Coomassie Blue R, then destained briefly (for a total of 1 h) as described by Cleveland *et al.* (1977). The stained bands of interest were sliced from the gel, then incubated at room temperature for 30 min in sample buffer (0.125 M-Tris/HCl/2 mM-EDTA/0.1% SDS, pH 6.8). The gel used to separate the peptide fragments consisted of a 1.5 mm-thick slab; the stacking gel was 5 cm long and the separating gel was 17 cm (15% polyacrylamide). Each slice containing a polypeptide to be mapped was loaded on to a slot of the stacking gel, topped with 20 μ l of sample buffer containing 20% (v/v) glycerol and 0.0001% Bromophenol Blue and by 10 μ l of sample buffer containing 10% glycerol, 0.0001% Bromophenol Blue and the proteinase used to cleave the polypeptide in the gel. Electrophoresis was performed at room temperature, and the current was turned off for 30 min when the Bromophenol Blue dye was at the bottom of the stacking gel. At the end of this period, electrophoresis was continued at 6°C. At the end of electrophoresis, the gels were fixed in 10% acetic acid for 20 min and dried under vacuum on filter paper. The ¹²⁵I-labelled peptide fragments were detected by fluorography with DuPont Cronex X-ray film (E. I. DuPont de Nemours and Co., Wilmington, DE, U.S.A.) and an intensifying screen (DuPont Cronex Lightning-Plus AE).

Sources of materials

AIA was a gift from Hoffmann-LaRoche, Nutley, NJ, U.S.A. DDC was obtained from Aldrich, Milwaukee, WI, U.S.A.

Results

In a previous paper (Ades & Harpe, 1981), we presented evidence indicating that the procedure established by Whiting & Granick (1976) for the

purification of embryonic-chick liver ALA synthase yielded an enzyme with a partially degraded subunit. The procedure involved the purification of the enzyme from sonic extracts of liver mitochondria isolated from 19-day chick embryos pretreated with the porphyrogenic agents AIA and DDC; these two drugs induced the synthesis of ALA synthase, resulting in a 200–300-fold increase in the starting amounts of the enzyme in liver.

In attempting to purify the undegraded form of ALA synthase, our main modification was to use mitochondria less contaminated by lysosomes as the starting material. The procedure for preparing mitochondria described in the Experimental section differs from the method described by Whiting & Granick (1976) and yields a mitochondrial fraction significantly less contaminated by lysosomes as assessed by the specific activities of acid phosphatase measured in the two preparations. For practical considerations regarding the minimal amount of starting material necessary for the purification, gradient-purified mitochondria were not used. A special effort also was made to perform the purification as rapidly as possible, to minimize the chances of protein degradation. We elected not to include proteinase inhibitors in the purification in order to minimize the variables in the procedure, especially since the nature of the proteolytic activity involved in the degradation of the enzyme was not known. Data on the recovery of ALA synthase after each step of the purification are presented in Table 1. The purified enzyme had a specific activity of 50 nmol of ALA produced/min per mg of protein at 35°C, similar to values previously reported (Whiting & Granick, 1976; Ades & Harpe, 1981). This preparation consisted of three major components (Fig. 1, slot B): a polypeptide migrating as a sharp band with an apparent molecular weight of 63 000, a component migrating as a broad band with a minimal molecular weight of 52 000, and a minor polypeptide with an apparent molecular weight of 47 000. Densitometer scans of the gel indicated that

the 63 000-Da and the 52 000-Da components constituted 22% and 63% of the preparation respectively. The 52 000-Da component co-migrated with the degraded subunit of ALA synthase, purified

Fig. 1. *Compositions of preparations of purified ALA synthase*

A sample of each of the indicated preparations was analysed by polyacrylamide-gel electrophoresis in the presence of SDS and stained for proteins. (A) ALA synthase purified from chick embryos as described by Whiting & Granick (1976); (B) ALA synthase purified by using the minor modifications described in the text; (C) the composition of the corresponding fraction of liver shown in slot (B) but obtained from livers of normal embryos. The positions of molecular-weight standards are shown to the right.

Table 1. *Recovery of ALA synthase from livers of 168 chick embryos after the various steps of purification*
One unit of enzyme catalyses the formation of 1.0 nmol of δ -aminolaevulinate/h at 35°C. Letters in parentheses refer to the various steps of purification of ALA synthase outlined in the Experimental section.

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Liver (original)	—	14 700	198 000	13.5	100
Soluble extracts from mitochondria (b)	30	590	58 300	98.8	29
Gel chromatography (c)	185	166	39 200	236	20
Concentration with NH_4SO_4 (d)	15	136	31 800	234	16
Affinity chromatography (e)	38	38.1	21 500	564	11
Concentration on hydroxyapatite (f)	3.0	31.4	17 600	560	8.9
Isoelectric focusing (g)	30	3.0	8700	2900	4.4
Final preparation (h)	3.0	2.3	6950	3000	3.5

as described previously (Fig. 1, slot A). Neither the 63 000-Da nor the 52 000-Da component could be recovered from liver mitochondria of normal embryos, where the starting amounts of the enzyme were 0.5% of those of the porphyric embryos used in the actual purification of the enzyme (Ades & Harpe, 1981); only the 47 000-Da polypeptide, previously shown to be a contaminant (Ades & Harpe, 1981), could be detected (Fig. 1, slot C).

The electrophoretic mobility of the 52 000-Da component under denaturing conditions suggested that it might have consisted of polypeptides with a heterogeneous composition, ranging in molecular weights from 52 000 to 57 000. Analysis of the preparation of ALA synthase by the two-dimensional gel-electrophoresis method of O'Farrell (1975) did not resolve this component into distinct subspecies. In a similar but more informative approach, electrophoretic analyses and ALA synthase activity determinations were performed on fractions from the sucrose gradient used in the isoelectric-focusing step for the purification of the enzyme. Over 80% of the ALA synthase activity in the gradient was recovered in fractions between pH 7.0 and 8.2. The distribution of the 63 000-Da polypeptide (with pI 7.2; Fig. 2, peak a) in the gradient was quite similar to that of the 52 000-Da component (with pI 7.5; Fig. 2, peak b). As evident from the data shown in Fig. 2, the 52 000-Da component could not be resolved into distinct polypeptides in these analyses either, and the distribution of this component clearly coincided with the distribution of ALA synthase activity in the gradient.

The fact that the 63 000-Da polypeptide was recovered from liver mitochondria of porphyric embryos, but could not be recovered by the same procedure from liver mitochondria of normal embryos, raised the possibility that the 63 000-Da polypeptide represented the mature subunit of ALA synthase, with the 52 000-Da component representing the partially degraded subunit. This possibility was tested by peptide-mapping analyses by using the limited-proteolysis method of Cleveland *et al.* (1977). In these analyses, the molecular-weight distribution of the fragments resulting from the proteolytic digestion of the 63 000-Da component was compared with the distribution of fragments originating from the digestion of the 52 000–57 000-Da smear. A sample of the preparation of purified ALA synthase was radiolabelled with ^{125}I , then the labelled 63 000-Da polypeptide and the 52 000–57 000-Da smear were purified in gel slices after their electrophoretic separations on polyacrylamide gels in the presence of SDS; each component was then digested with either trypsin or chymotrypsin and the resulting fragments were compared. The results are shown in Fig. 3. In each case the molecular-weight

Fig. 2. Distributions of proteins and of ALA synthase activity from the preparative isoelectric-focusing column

A sample enriched for ALA synthase obtained from the affinity-chromatography step of the purification was mixed into the sucrose gradient of a preparative isoelectric-focusing column (total volume 100 ml) under the conditions described by Whiting & Granick (1976). After focusing, the gradient was fractionated into 2.5 ml samples, and 40 μl portions from each sample were analysed by polyacrylamide-gel electrophoresis in the presence of SDS to determine the polypeptide compositions of the samples; portions from each sample were also analysed for ALA synthase activity. Densitometer scans at 546 nm are shown of slots on the polyacrylamide gel stained for proteins, and all slots shown were scanned at the same sensitivity. The numbers of the fractions from the focusing column whose scans are represented are indicated (to the left of each scan) together with the specific activities (act.) of ALA synthase (nmol/h per ml) recovered in the individual fractions and the pH of each fraction. The positions of the 63 000-Da (a), the 52 000–57 000-Da (b) and the 47 000-Da (c) components recovered in the purified enzyme preparation are indicated. The top of the gel is indicated by the arrow.

Fig. 3. Peptide-mapping analyses of the 63000-Da polypeptide and of the 52000-Da smear recovered in purified ALA synthase

The 63000-Da polypeptide and the 52000–57000-Da smear were purified in gel slices from a sample of ^{125}I -labelled ALA synthase preparation after electrophoresis on a polyacrylamide gel in the presence of SDS. Slices containing each labelled polypeptide were loaded on to a second gel and electrophoretically eluted into the second gel in the presence of a proteinase in order to generate the proteolytic fragments under the conditions described in the text. The fragments resulting from the digestions of each of the iodinated polypeptides were detected by fluorography after electrophoresis, and slots from the exposed X-ray films are shown above. The digestions were done either with 20 μg of trypsin (Tryp.) or with 20 μg of α -chymotrypsin (Chym.), as indicated. (A) Fragments from the 52000–57000-Da smear; (B) fragments from the 63000-Da polypeptide.

distributions of the smaller fragments (representing the more completely digested peptides) clearly co-migrated, indicating that a structural homology existed between the 52000-Da component and the 63000-Da polypeptide. These findings indicated that the 63000-Da polypeptide was indeed the mature subunit of ALA synthase and supported the conclusion in our previous paper (Ades & Harpe, 1981) that the 52000-Da component was a collection of fragments produced during purification of the enzyme.

Discussion

We previously presented evidence (Ades & Harpe, 1981) indicating that the molecular weight of the mature subunit of ALA synthase of embryonic-chick liver was significantly greater than that of the subunit recovered from the enzyme purified by the method of Whiting & Granick (1976). This evidence was indirect and obtained from immunoreplicate-electrophoresis analyses in which the subunit of the enzyme was identified in SDS-solubilized liver mitochondria from porphyric embryos. The results indicated that the molecular weight of the undegraded subunit was 65000 ± 2000 . In the present paper we report the purification of ALA synthase consisting of two major components: a smear of polypeptides with molecular weights ranging from 52000 to 57000, previously established to represent the partially degraded ALA synthase (Ades & Harpe, 1981), and a 63000-Da polypeptide. Two sets of observations indicated that the 63000-Da protein was the mature subunit of ALA synthase: first, the protein could not be purified from mitochondria of normal embryos, where the amounts of ALA synthase were 0.5% of those in mitochondria of the porphyric embryos normally used in the purification; and second, peptide-mapping analyses indicated that structural homologies existed between the 63000-Da polypeptide and the proteins in the 52000–57000-Da smear.

We and others have shown (Ades & Harpe, 1981; Brooker *et al.*, 1980) that the subunit of ALA synthase of embryonic-chick liver is synthesized as a 70000–75000-Da polypeptide in cell-free translation systems stimulated with mRNA from livers of porphyric embryos. Those results, together with those in the present paper, suggest that, like a number of mitochondrial proteins (Maccacchini *et al.*, 1979; Harmey & Neupert, 1979; Raymond & Shore, 1979; Mori *et al.*, 1979; Conboy *et al.*, 1979; Lewin *et al.*, 1980; Mihara & Blobel, 1980), the subunit of ALA synthase is synthesized as a larger precursor form (70000–75000-Da) which becomes processed to the mature form (63000-Da) at some point during its translocation into mitochondria.

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