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δ-AMINOLEVULINIC ACID SYNTHETASE FROM THE PARTICULATE FRACTION OF LIVER OF PORPHYRIC RATS

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SUMMARY

1. δ-Aminolevulinic acid synthetase, the rate-limiting enzyme for haem biosynthesis, has been purified 11.2-fold in 26% yield from the particulate fraction of liver of porphyric rats.

2. Enzymatic activity is stable indefinitely at −150° and can be stabilized during purification with 0.1 mM pyridoxal phosphate or in the presence of 0.2 M potassium phosphate (pH 7.4).

3. The enzyme is inhibited 48% by $5 \cdot 10^{-5}$ M haemin. The inhibition is non-competitive with succinyl-CoA, but haemin is a mixed inhibitor with respect to glycine.

4. Despite the observation that 58% of the enzymatic activity does not precipitate upon centrifugation at 140 000 × *g* for 2 h, studies with gel filtration, polyacrylamide gel electrophoresis, and electronmicroscopy indicate that the enzymatic activity is contained in a large protein aggregate. The implications of this observation are discussed.

INTRODUCTION

δ-Aminolevulinic acid synthetase is the enzyme which catalyzes the reaction of glycine and succinyl-CoA to form δ-aminolevulinic acid, the first reaction which is specific for the biosynthesis of haem. The formation of δ-aminolevulinic acid is thought to be rate limiting for the synthesis of haem and marked increases in the rate of δ-aminolevulinic acid formation have been demonstrated in liver from animals with experimental porphyria and patients with acute intermittent porphyria.

δ-Aminolevulinic acid synthetase activity is found in the mitochondrial fraction of cells[1], probably in the matrix of the mitochondria[2]. In experimental porphyria, activity is also found in the cytosol[3]. This report will describe the partial purification of δ-aminolevulinic acid synthetase from the particulate fraction of liver from rats with experimental porphyria. The properties of the enzyme preparation indicate that despite treatment with detergent, the enzymatic activity remains part of a large protein aggregate.

EXPERIMENTAL PROCEDURE

Materials

Succinyl-CoA is prepared by the method of STADTMAN[4] immediately before use in the assay of δ-aminolevulinic acid synthetase. It is assumed that the concentration of succinyl-CoA is 80% of the starting CoA concentration.

Crystalline haemin is prepared by the method of LABBE AND NISHIDA[5] and dissolved before use by the method of BURNHAM AND LASCELLES[6]. *p*-Dimethylaminobenzaldehyde (Ehrlich's reagent) is obtained from Fisher and recrystallized from methanol–water. Allylisopropylacetamide is a gift from Hoffmann-La Roche, Inc. δ-Aminolevulinic acid–HCl, CoA, pyridoxal phosphate A grade and pyridoxal phosphate monohydrate, pyridoxal monohydrochloride, pyridoxamine dihydrochloride, and pyridoxamine phosphate A grade are from Calbiochem. Pyridoxal phosphate monohydrate is used throughout the purification procedure to stabilize the enzyme; pyridoxal phosphate A grade is used in the enzymatic assays. Desferal Mesylate (desferrioxamine) is a gift from CIBA. Sephadex G-25 coarse and G-200 are from Pharmacia, and Biogel 1.5 A and hydroxylapatite powder are from Bio-Rad; chromatographic columns are prepared from these materials according to the procedures outlined by the manufacturers. Calcium phosphate gel is from Bio-Rad and has a concentration of 48 mg of solid material per ml.

Enzyme purification

Sprague–Dawley male rats weighing from 120 to 150 g are treated with allylisopropylacetamide as described by MARVER *et al.*[7]. 12 h after the second injection, the rats are killed by decapitation. The livers are removed and chilled in a solution of 0.9% NaCl, 10 mM Tris–HCl (pH 8.0) and 0.5 mM EDTA. The following paragraphs describe a typical purification procedure.

All steps are carried out at 0–4°.

Step 1. Crude homogenate: 70 g, wet wt., of liver is homogenized in 210 ml NaCl–Tris–EDTA solution in a Thomas Teflon tissue grinder.

Step 2. Crude pellet: the homogenate is centrifuged at 4000 × *g* for 10 min and the supernatant is discarded. The pellet is suspended in about 200 ml NaCl–Tris–EDTA solution, and the suspension is again centrifuged and the supernatant discarded. The pellet is suspended in NaCl–Tris–EDTA solution to a total volume of 200 ml.

Step 3. Deoxycholate suspension: 4 ml 10% sodium deoxycholate are added with vigorous stirring for 20 min. The suspension is centrifuged at 13 000 × *g* for 20 min and the pellet, including a loosely packed layer, is discarded.

Step 4. $(NH_4)_2SO_4$ precipitation: the suspension is put through a 4.5 cm × 50 cm Sephadex G-25 (coarse grade) column prepared and developed with a buffer of 0.02 M Tris–HCl (pH 8.0) and 0.5 mM EDTA. The yellow, protein-containing fraction is collected in 300 ml, and 3 ml of 0.01 M pyridoxal phosphate is added immediately. 30 ml neutralized 10% streptomycin sulfate is added and the resulting suspension is stirred for 15 min and centrifuged at 13 000 × *g* for 15 min. The pellet is discarded and the supernatant made to 60% saturation with $(NH_4)_2SO_4$. After stirring ⌜for⌝ ⌜10 min,⌝ ⌜the suspension is centrifuged at 13 000 × *g* for 15 min and the supernatant discarded. The pellet is dissolved

in a total volume of 75 ml with 50 mM Tris–HCl (pH 8.0) and 0.1 mM pyridoxal phosphate–0.5 mM EDTA solution. The solution is made to 40% saturation with $(NH_4)_2SO_4$ and is stirred and centrifuged again. The pellet is dissolved in 0.02 M Tris–HCl (pH 7.4) and pyridoxal phosphate–EDTA solution to a final volume of 40 ml.

Step 5. Calcium phosphate gel elution: the solution is found to contain 2.44 g protein. 36 ml $CaPO_4$ gel (gel/protein = 0.7) is added with stirring for 10 min and the suspension is then centrifuged at 3000 × *g* and the supernatant discarded. The gel is suspended in 37.5 ml 0.12 M potassium phosphate (pH 8.0) and pyridoxal phosphate–EDTA solution, stirred for 15 min, and again centrifuged. The gel is washed 3 more times in an identical manner and the four washes are combined.

Step 6. Hydroxylapatite elution: the combined washes are diluted with 150 ml pyridoxal phosphate–EDTA solution and then put through a 1.5 cm × 25 cm hydroxylapatite column prepared in 0.05 M potassium phosphate (pH 7.4) and pyridoxal phosphate–EDTA solution. The column is developed with 40 ml 0.075 M potassium phosphate (pH 7.4) and pyridoxal phosphate–EDTA solution and then with 0.175 M potassium phosphate (pH 7.4) and pyridoxal phosphate–EDTA solution. Addition of the latter solution results in the gradual elution of a distinct yellow band, which is collected as 60 ml of eluate. This solution is made 50% saturated in $(NH_4)_2SO_4$, centrifuged, and the supernatant discarded. The pellet is suspended in a total volume of 7.5 ml with 0.02 M Tris–HCl (pH 7.4) and pyridoxal phosphate–EDTA solution.

δ-Aminolevulinic acid synthetase assay

Activity is determined by minor modification of the method of MARVER *et al.*[7]. The incubation mixture consists of 100 μmoles of glycine, 0.5 μmole of EDTA, 100 μmoles Tris–HCl (pH 7.4), 0.5 μmole pyridoxal phosphate, and succinyl-CoA as and preparations of δ-aminolevulinic acid synthetase indicated below, in a final volume of 1 ml. Mixtures are incubated at 37° for 30 min. Units given are nmoles δ-aminolevulinic acid produced per min. Deoxycholate treatment removes interfering activities and δ-aminolevulinic acid produced in the incubation mixtures can be determined directly without further purification with ion exchange chromatography. The reaction product was identified as δ-aminolevulinic acid by paper chromatography by the method of URATA AND GRANICK[8].

The concentration of succinyl-CoA required for optimum enzymatic activity varies with the state of purification of δ-aminolevulinic acid synthetase. 0.2 mM succinyl-CoA is used for Step 2 and 0.4 mM succinyl-CoA for the other steps of the purification procedure.

Analytical methods

Protein is determined by the biuret method or by absorption at 280 nm. Biuret determinations of crude preparations are not always reproducible and these values are taken as rough approximations. Lipid content is determined by the method of FOLCH *et al.*[9]. Succinic dehydrogenase is assayed by the method of HAYASHI *et al.*[3], and cytochrome oxidase activity is determined by the method of COOPERSTEIN AND LAZAROW[10]. Acrylamide gel electrophoresis is carried out by the methods of MAIZEL[11]. Specimens of the enzyme preparation from Step 6 are negatively stained for electron-microscopy by mixing with an equal volume of 1% phosphotungstic acid (pH 7.0).

RESULTS

As shown in Table I, δ-aminolevulinic acid synthetase has been purified about 11-fold in 26% yield. The purification procedure is rapid and reproducible. More complex methods have resulted in from 20- to 40-fold purification, but the yield of enzyme is very low and the results inconsistent. No further purification could be achieved in a reproducible manner with ion-exchange chromatography, treatment with heat, acetone, or acid, modification of the $(NH_4)_2SO_4$ fractionation, gravimetric methods, or gel filtration.

TABLE I

PURIFICATION OF δ-AMINOLEVULINIC ACID SYNTHETASE

Fraction	*Total enzyme activity (units × 10^{-3})*	*Total protein (g)*	*Specific activity (units/mg)*	*Yield (%)*	*Purification (-fold)*
1. Crude	1.08	18.9	0.057	100	1.0
2. Crude pellet	1.46	14.0	0.11	136	1 8
3. Deoxycholate	1.15	7.4	0.16	107	2.7
4. $(NH_4)_2SO_4$	0.46	2.4	0.20	43	3.4
5. CaPO gel	0.40	0.9	0.45	37	7.8
6. Hydroxylapatite	0.28	0.44	0 64	26	11.2

The enzyme is stable indefinitely at −150° to −195° (liquid nitrogen). δ-Aminolevulinic acid synthetase activity declines 35% in 24 h at 4° in 0.01 M potassium phosphate (pH 7.4), but no enzymatic activity is lost if 0.1 mM pyridoxal phosphate is added or if the concentration of buffer is increased to 0.2 M potassium phosphate. At 37°, the enzyme is inactivated rapidly, but pyridoxal phosphate, EDTA, and glycine can protect the enzyme as shown in Table II.

TABLE II

PROTECTION OF ENZYMATIC ACTIVITY

Preincubation of 0.70 unit of δ-aminolevulinic acid synthetase (1.09 mg protein) is carried out at 37° for 15 min in the complete assay mixture given in the text, except for the omissions indicated in the table and succinyl-CoA. The omitted components and succinyl-CoA are added at the end of the preincubation and the enzymatic activity determined.

Omission	*Activity (%)*
None	100
Glycine, EDTA, pyridoxal phosphate	24
EDTA, pyridoxal phosphate	11
Glycine, pyridoxal phosphate	59
Glycine, EDTA	70
Pyridoxal phosphate	57
EDTA	84
Glycine	94
No preincubation	98

Conditions for enzyme assay

Purified preparations of δ-aminolevulinic acid synthetase have an absolute requirement for glycine ($K_m = 2.5 \cdot 10^{-3}$ M) and succinyl-CoA ($K_m = 2 \cdot 10^{-4}$ M) for the synthesis of δ-aminolevulinic acid. Linear Lineweaver–Burk plots are obtained for these two substrates; there is no inhibition by high concentrations of succinyl-CoA in purified δ-aminolevulinic acid synthetase although enzyme from Steps 1 and 2 of the purification procedure is inhibited by excess succinyl-CoA.

An absolute requirement for pyridoxal phosphate cannot be demonstrated, probably because δ-aminolevulinic acid synthetase is purified in the presence of this cofactor in order to stabilize the enzyme. Despite Sephadex G-25 gel filtration of the enzyme preparation, without added pyridoxal phosphate the formation of δ-aminolevulinic acid proceeds at 64% of the rate in the presence of optimum concentrations of the cofactor (0.5 mM). Pyridoxamine phosphate, but neither pyridoxal monohydrochloride nor pyridoxamine dihydrochloride, can replace pyridoxal phosphate as a cofactor in the incubation mixture and as a stabilizer of δ-aminolevulinic acid synthetase. The addition of 0.5 mM or higher concentrations of EDTA to the reaction mixture stimulates δ-aminolevulinic acid formation 1.5- to 4-fold.

TABLE III

EFFECT OF HAEMIN ON δ-AMINOLEVULINIC ACID SYNTHETASE ACTIVITY

Haemin is added to the assay mixture to give the final concentration indicated. 0.67 unit of δ-aminolevulinic acid synthetase (1.05 mg protein) is used.

Concentration of haemin (M)	*Inhibition (%)*
$2.0 \cdot 10^{-4}$	93
$1.5 \cdot 10^{-4}$	89
$1.0 \cdot 10^{-4}$	81
$7.5 \cdot 10^{-5}$	65
$5.0 \cdot 10^{-5}$	48
$3.0 \cdot 10^{-5}$	34
$1.0 \cdot 10^{-5}$	3

The enzymatic assay is linear with the quantity of the enzyme preparation added from 0.12 to 2.0 units of δ-aminolevulinic acid synthetase. The assay is linear with time for 15 min; there is a slight decrease in the rate of δ-aminolevulinic acid formation from 15 to 30 min, but 30 min is used as the incubation time for assays of enzyme from Steps 4–6 in order to increase the sensitivity of the assay. The pH optimum for the reaction is from 7.0 to 8.5 in either Tris or phosphate buffer. The rate of δ-aminolevulinic acid synthesis doubles from 30° to 40°.

Effect of haemin

Haemin inhibits the formation of δ-aminolevulinic acid as shown in Table III. Kinetic studies indicate that haemin is a non-competitive inhibitor with respect to succinyl-CoA; as shown in Fig. 1, however, haemin is a mixed inhibitor with respect to glycine. Preincubation of enzyme and haemin in the presence of the complete incubation mixture for assay except for succinyl-CoA and glycine, at 37° for 15 min,

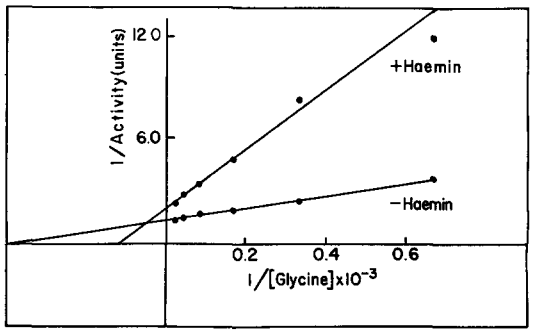

Fig. 1. Lineweaver–Burk plot of the effect of varying concentrations of glycine on the enzymatic activity in the presence and absence of haemin. Standard assay conditions are used as described in the text with 0.73 unit of δ-aminolevulinic acid synthetase (1.14 mg protein) and $5 \cdot 10^{-5}$ M haemin.

has no effect on the percent inhibition by haemin. $Fe(NH_4)_2(SO_4)_2$ and $FeCl_3$ are not significantly inhibitory at a concentration of $1.0 \cdot 10^{-4}$ M, and $1.6 \cdot 10^{-4}$ M desferrioxamine has no effect on the reaction.

Physical properties

Ultracentrifugation of an enzyme preparation from Step 6 gives the results shown in Table IV. Most of the enzymatic activity remains in the supernatant; despite this observation, however, there is evidence that δ-aminolevulinic acid synthetase activity is associated with a large protein complex.

Over 90% of the protein and enzymatic activity in these preparations is excluded from Sephadex G-200 and Bio-Rad 1.5A columns. Furthermore, only a trace of the protein enters a 3.5% polyacrylamide gel during disc gel electrophoresis at pH 8.8. If the enzyme preparation is heated at 100° for 1 min with 1% sodium dodecyl sulfate and is then subjected to electrophoresis in a 5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate, numerous protein bands can be demonstrated within the gel.

Results of studies of enzyme from Step 6 by electron microscopy reveal the presence of many amorphous particles of different sizes, averaging about 50 mM in diameter. These particles disappear when the enzyme preparation is treated with 1% sodium dodecyl sulfate.

TABLE IV

ULTRACENTRIFUGATION OF δ-AMINOLEVULINIC ACID SYNTHETASE

24 mg of the enzyme preparation from Step 6 in 2 ml of a solution of 0.9% saline, 20 mM Tris–HCl (pH 8.0), and pyridoxal phosphate–EDTA solution is centrifuged at 140 000 × *g* for 2 h. The pellet is suspended in 1 ml of 20 mM Tris–HCl (pH 8.0) and pyridoxal phosphate–EDTA solution..

Fraction	*Activity (units)*	*Specific activity (units/mg)*	*Yield (%)*
Step 6 enzyme	9.17	0.38	100
Supernatant	5.33	0.40	58
Pellet	1.95	0.26	21

There are no detectable levels of the mitochondrial enzymes cytochrome oxidase or succinate dehydrogenase associated with partially purified δ-aminolevulinic acid synthetase, although these activities are readily demonstrable in preparations from Steps 1 and 2. Lipid is 15–20% by weight of the material in Steps 1–3; following $(NH_4)_2SO_4$ precipitation in Step 4, lipid content is less than 1% by weight.

DISCUSSION

Early studies of δ-aminolevulinic acid synthetase from higher organisms used whole crude homogenates[7], particulate fractions[12], or intact mitochondria[1,13]. Interpretation of data obtained in these studies is difficult because of variables like permeability factors, membrane structure, and the requirement for other enzymes such as a succinyl-CoA generating system.

We have attempted to obtain a soluble, purified preparation of δ-aminolevulinic acid synthetase to investigate the mechanism of action of this enzyme. Treatment of the particulate fraction of porphyric rat liver with deoxycholate yields an enzyme preparation in which most of the enzymatic activity remains in suspension after centrifugation at 140 000 × *g* for 2 h. Efforts to purify further δ-aminolevulinic acid synthetase from this preparation have given disappointing results. Only 11.2-fold overall purification could be achieved.

Studies of the physical state of the enzyme preparation explain the difficulty in purification. Failure of an enzyme to sediment at 100 000 × *g* for 1 or 2 h has often been taken as evidence that the enzyme is soluble. Several criteria, however, suggest that δ-aminolevulinic acid synthetase is not in free solution but is part of a complex containing numerous proteins.

Exclusion of the enzyme from gel filtration columns and 3.5% polyacrylamide gel, together with the formation of numerous protein bands upon sodium dodecyl sulfate treatment with subsequent gel electrophoresis, supports this hypothesis. Studies with the electronmicroscope indicate that material in the enzyme preparation forms large aggregates. Although the size of the aggregates observed may be an artifact of the staining procedure, the presence of aggregates and their disappearance with sodium dodecyl sulfate treatment are in agreement with the evidence from the studies with polyacrylamide gel electrophoresis.

A tendency for the δ-aminolevulinic acid synthetase from the cytosol of porphyric rats to aggregate has been observed[14], and an estimated molecular weight of 600 000 *g* for the enzyme from cytosol has been reported[15]. Molecular weights varying from 115 000 *g* (ref. 15) to over 380 000 *g* (ref. 16) have been suggested in preliminary reports of the enzyme from particulate preparations. These studies have been based on sucrose gradient and gel filtration experiments with enzyme preparations which have not been well characterized by gel electrophoresis or analytical ultracentrifugation.

The absence of significant amounts of lipid and of some of the mitochondrial enzymes from the partially purified enzyme preparations suggests that these complexes are not intact portions of mitochondria. It seems likely that the aggregates are formed as a result of hydrophobic interactions among a group of proteins including δ-aminolevulinic acid synthetase following their release from mitochondria.

If δ-aminolevulinic acid synthetase is contained within a complex, the active site of the enzyme may be partially blocked, an hypothesis which would account for the high K_m values observed for substrates of the reaction and the high concentration of haemin required for inhibition of δ-aminolevulinic acid synthetase activity. Studies with the enzyme from cytosol[17] and from bone marrow mitochondria[16] have yielded similar K_m values and similar levels of haemin are required for inhibition of activity.

The failure of iron salts and of a compound that chelates iron to affect the enzymatic activity when they are added in a concentration of $1 \cdot 10^{-4}$ M indicates that the inhibition observed with haemin cannot be solely due to the iron contained in haemin. Previous studies demonstrating stimulation of δ-aminolevulinic acid synthetase by iron in high concentrations[18,19] were not repeated with this enzyme preparation because of interference with the colorimetric assay by these concentrations of iron. The mechanism of the mixed inhibition of δ-aminolevulinic acid synthetase by haemin with respect to glycine is not clear; because of the physical state of the enzyme, it is difficult to interpret the data from studies of kinetic properties.

It is hoped that the stable, partially purified preparation of δ-aminolevulinic acid synthetase described in this paper will provide a starting point for attempts to separate the enzyme from the protein complex. Achievement of this separation would permit further purification of δ-aminolevulinic acid synthetase and study of its mechanism of action.

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