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SOLUBILIZATION AND PARTIAL PURIFICATION OF MITOCHONDRIAL δ-AMINOLEVULINATE SYNTHASE FROM FETAL RAT LIVER

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Summary

A particulate preparation of δ-aminolevulinate synthase (EC 2.3.1.37) from fetal rat liver mitochondria has been rendered soluble by treatment with the non-ionic detergent Lubrol WX-4. A partial purification of the detergent-solubilized enzyme was achieved by gel filtration on Sephadex G-200. Unlike the adult mitochondrial enzyme, δ-aminolevulinate synthase from fetal liver is inhibited by NaCl but not by hemin.

The enzyme, δ-aminolevulinate synthase (succinyl-CoA:glycine succinyl transferase, EC 2.3.1.37) catalyzes the initial step in the heme biosynthetic pathway in mammals. δ-Aminolevulinate synthase is a mitochondrial matrix enzyme [1] and is considered to play an important role in the regulation of heme synthesis in the adult rat by catalyzing the rate-limiting step, the formation of δ-aminolevulinate [2]. Studies on the purification of mitochondrial δ-aminolevulinate synthase in rat liver have been hindered primarily due to difficulties encountered in solubilizing the particulate enzyme [1,3]. Previous studies from this laboratory [4] have shown that the activity of δ-aminolevulinate synthase in fetal rat liver mitochondria is at least 10 times that of the adult and does not decline to adult levels until shortly after birth. During the period of elevated activity, fetal δ-aminolevulinate synthase is refractory to induction by chemicals [5,6] and to repression by the end-product heme.[5]. Studies on the partially purified enzyme from fetal liver mitochondria were, therefore, desirable in order to more

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clearly identify differences in the biochemical properties of this enzyme, as compared with those of the adult and, hence, to attain better understanding of the regulation of hepatic heme synthesis during growth and hepatic development.

The present paper describes studies on the solubilization and stabilization of mitochondrial δ-aminolevulinate synthase from 19-day fetal rat liver and conditions giving a good yield and relatively high stability for further steps in purification. The efficacy of the solubilization procedure used in these studies is compared with those of other reported methods, and some of the properties of the partially purified fetal enzyme are described.

The materials used were purchased from the following sources. Succinyl-CoA synthetase (succinic thiokinase) (EC 6.2.1.4), pyridoxal 5′-phosphate, ATP, GTP, dithioerythritol, succinyl-CoA, bilirubin, cytochrome, coenzyme A (free acid), and Tergitol-NTX, an aliphatic detergent, from Sigma Chemical Co., St. Louis, U.S.A.; glycine, δ-aminolevulinate and hemin from Calbiochem, San Diego, U.S.A.; Sephadex G-200 from Pharmacia, Uppsala, Sweden; Triton X-100 from New England Nuclear, Boston, U.S.A.; and Lubrol WX-4 from General Biochemicals, Yellowspring, U.S.A. Lubrol was purified to remove ionic impurities by the procedure of Nakao et al. [7]. Date-bred Spraque—Dawley rats (C.D. strain) were purchased from Charles River Breeding Co., Boston, U.S.A. The pregnant rats were housed in individual cages with unlimited access to food and water. Fetuses were removed on the 19th day of gestation, and all subsequent operations were carried out between 0 and 5 °C. Fetal livers were pooled and homogenized in a Waring blender with 9 vol. of 0.25 M sucrose containing 50 mM Tris—HCl buffer, pH 7.5, 0.1 mM EDTA and 0.1 mM pyridoxal 5′-phosphate. Mitochondria were prepared as described previously [4]. The mitochondrial pellet was suspended in 50 mM Tris—HCl buffer, pH 7.5, containing 0.1 mM pyridoxal 5′-phosphate and 1 mM dithioerythritol. A 10% aqueous solution of Lubrol was added with stirring to achieve a final concentration of 0.6%, and the suspension was stirred for 1 h. A high speed supernatant was recovered by centrifugation at 105 000 × *g* for 1 h, in a Beckman ultracentrifuge. In experiments where other detergents were employed, Lubrol was replaced with the effective concentration of the desired detergent. In some experiments the mitochondrial suspension was subjected to sonication in a Branson sonifier at maximum power setting. Sonication was continued for four 15-s periods with an interval of 30 s between each burst of sound. The enzyme activity in the mitochondrial and 105 000 × *g* supernatant fractions was assayed by a previously described [4] modification of the method of Scholnick et al. [8], wherein NaCl was omitted from the incubation mixture and 1 mM dithioerythritol was substituted for β-mercaptoethanol. The subsequent fractions were assayed by determining the amount of δ-aminolevulinate formed in 30 min at 37 °C in a reaction mixture containing 50 mM Tris—HCl buffer, pH 7.5, 0.1 M glycine, $2 \cdot 10^{-4}$ M pyridoxal 5′-phosphate, $1.5 \cdot 10^{-4}$ M succinyl-CoA, 1 mM dithioerythritol and 0.2 ml of enzyme preparation in a final volume

of 1 ml. Reactions were terminated by addition of 0.25 ml 10% trichloroacetic acid solution. The δ-aminolevulinate formed was determined as previously described [4]. Gel filtration on a column of Sephadex G-200 (2.5 cm × 40 cm) equilibrated with 50 mM Tris—HCl buffer, pH 7.5, containing 0.1 mM pyridoxal 5′-phosphate, 0.5% Lubrol and 1 mM dithioerythritol was carried out at 4 °C. The exclusion volume for blue dextran was 61 ml. Protein concentrations were determined by the method of Lowry et al. [9].

TABLE I

ATTEMPTS AT SOLUBILIZING δ-AMINOLEVULINATE SYNTHASE FROM FETAL RAT LIVER MITOCHONDRIA

The enzyme activity was extracted from mitochondria using 50 mM Tris—HCl, pH 7.5, containing 0.1 mM pyridoxal 5′-phosphate and the following treatments. δ-Aminolevulinate synthase activity was determined as described in the text. Activity in untreated mitochondria is taken as 100%.

Treatment	δ-Aminolevulinate Synthase Activity in Soluble fraction (%)	Specific Activity (relative change)
None	0	—
Sonication only	0	—
Sonication in the presence of 1% Triton X-100	50	No Change
Sonication in the presence of 1% Triton X-100 + 1 mM Dithioerythritol	60	No Change
Sonication in the presence of 2 M LiCl + 5 mM Mg^{2+}	0	—
0.6% Lubrol + 1 mM Dithioerythritol	84	Increase
0.6% Lubrol + 1 mM Dithioerythritol + 0.25 M NaCl	0	—
0.6% Tergitol	33	Decrease

The procedures attempted at solubilizing fetal mitochondrial δ-aminolevulinate synthase are summarized in Table I. Initial attempts to disrupt mitochondria using conventional methods proved ineffective in recovering the enzyme activity in the 105 000 × *g* supernatant fraction. Freezing and thawing of mitochondria resulted in a total loss of activity. Lypholization and subsequent extraction of the mitochondria [1] was also unsuccessful. When the mitochondria were subjected to sonication, either in the presence or absence of high salt concentrations, all enzyme activity was lost. However, sonication in the presence of various detergents was partially successful. The best results were achieved when the mitochondrial pension was treated with 0.6% Lubrol by gentle stirring. This process released 75 to 85% of the activity in the solubilized form with a slight increase in the specific activity of the resultant enzyme fraction. Activity of the enzyme in the Lubrol-solubilized supernatant fraction was stable for at least 2 months at −20 °C.

A detailed study of the effectiveness of Lubrol in solubilizing the enzyme from fetal liver mitochondria was next undertaken. Table II summarizes the effects of varying the Lubrol concentration as well as the time of exposure on the recovery of the enzyme in the soluble (105 000 × *g* supernatant) fraction. Optimal solubilization was achieved when the ratio of mito-

TABLE II

EFFECT OF VARYING LUBROL CONCENTRATION AND TIME OF EXPOSURE ON SOLUBILIZATION OF FETAL RAT LIVER MITOCONDRIAL δ-AMINOLEVULINATE SYNTHASE

Mitochondria were suspended in 50 mM Tris—HCl, pH 7.5, containing 1 mM dithioerythritol and 0.1 mM pyridoxal 5′-phosphate at a protein concentration of about 8.0 mg/ml prior to detergent treatment. The enzyme activity in the 105 000 × *g* supernatant was assayed as described in the text. An enzyme unit is defined as the formation of 1 nmole δ-aminolevulinate per 30 min at 37 °C.

Lubrol Concn (%)	Ratio of Lubrol to Protein Concn	Exposure Time (min)	Enzyme Activity in Soluble Fraction (%)	Protein in Soluble Fraction (%)	Specific Activity (units/mg protein)
0.0 (control)	0	—	0	0	—
0.6	0.75	30	28.0	50.0	0.56
0.6	0.75	60	59.6	54.7	1.10
0.6 + 0.25 M NaCl	0.75	60	0	54.7	0
1.0	1.25	60	36.8	55.3	0.64

chondrial protein to detergent concentration was 0.75, and when the extraction was allowed to proceed for one hour. Increasing the concentration of Lubrol, however, resulted in poorer yield of the enzyme activity although a higher percentage of the total protein from mitochondria was rendered soluble. This fact presumably may be due to removal of stabilizing phospholipid from the enzyme on prolonged exposure to the detergent. When the extraction was carried out using 0.6% Lubrol in the presence of 0.25 M NaCl, no measurable activity was recovered in the soluble fraction. This observation is in contrast to the results obtained with the adult enzyme. The soluble cytosol δ-aminolevulinate synthase described by Scholnick et al. [8] also requires 0.25 M NaCl for maximal activation. High concentrations of NaCl, i.e. up to 0.8 M, were also required to render the adult mitochondrial enzyme soluble in the presence of a reducing agent and to maintain it in the soluble form [1]. The apparent loss of activity of fetal δ-aminolevulinate synthase may be due to the inhibition of the enzyme by Na^+, as indicated in Table III. An 80% inhibition was noted when 0.25 M NaCl was included in the incubation mixture. Alternatively, inactivation may be the consequence of an ionic strength effect.

TABLE III

EFFECT OF Na^+ ON THE ACTIVITY OF δ-AMINOLEVULINATE SYNTHASE FROM FETAL RAT LIVER MITOCHONDRIA

The most active fractions following chromatography on a Sephadex G-200 column were pooled and assayed for enzyme activity either in the absence or presence of NaCl at various concentrations as indicated. δ-Aminolevulinate Synthase activity was assayed as described in the text.

[NaCl] (mM)	δ-Aminolevulinate Synthase Activity (Units)	Inhibition (%)
0	24.56	0
25	20.80	15.0
50	17.40	27.8
100	8.00	67.4
250	4.90	80.0

Further fractionation of the detergent-solubilized enzyme, employing $(NH_4)_2 SO_4$ precipitation, was not successful. This fact may be due to similar inactiviating or inhibitory properties of NH_4^+. Recent experiments in this laboratory have indicated that inhibition of fetal δ-aminolevulinate synthase by cations is not restricted to Na^+ and NH_4^+, but can be seen with other mono- and divalent cations, as well.

The specific interaction of the detergent, Lubrol, and the particulate δ-aminolevulinate synthase has permitted us to attempt further purification of the solubilized enzyme using chromatography on a Sephadex G-200 column with good retention of enzyme activity. A typical fractionation is shown in Table IV. This procedure afforded a 5-fold purification of the solubilized enzyme. The apparent increase in the yield after step 2 may be due to the removal of inhibitory material by column chromatography on Sephadex G-200. The eluting buffer requires the presence of 0.5% Lubrol, as indicated above, in order to prevent aggregation of the enzyme with the

TABLE IV

FRACTIONATION OF δ-AMINOLEVULINATE SYNTHASE FROM FETAL LIVER MITOCHONDRIA

Experimental procedures are described in the text.

Step	Fraction	Specific Activity (units/mg protein)	Total Activity (units)	Yield (%)
1	Mitochondria	3.6	1827	100.0
2	Lubrol (105 000 × *g*) supernatant	5.2	1401	76.5
3	Sephadex G-200	18.6	1517	82.5

other proteins in the solubilized fraction (Fig. 1). When the elution was carried out using buffer devoid of detergent, the enzyme was eluted as broad disperse bands of activity. In contrast, reaggregation of the solubilized adult mitochondrial enzyme was prevented by having a high concentration of salt and reducing agent in the eluting buffer [1].

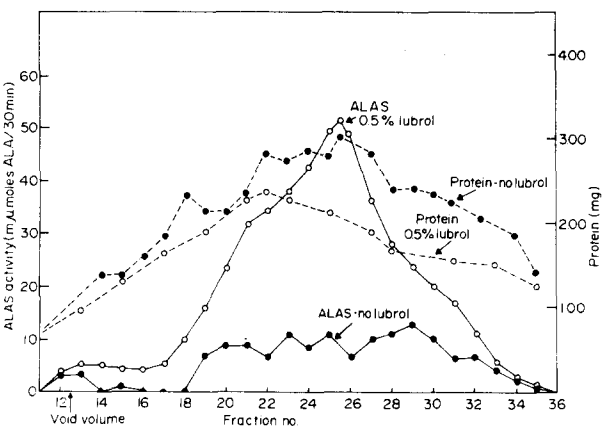

Fig. 1. Behavior of detergent-solubilized enzyme on Sephadex G-200. 50 ml of 105 000 × *g* supernatant fraction (of Fraction 2. Table IV) were applied to a 40 cm × 2.5 cm column of Sephadex G-200 and eluted at 4 °C with 50 mM Tris—HCl buffer (pH 7.5) containing 0.1 mM pyridoxal 5′-phosphate and 1 mM dithioerythritol. In experiments where Lubrol was used the elution buffer contained 0.5% Lubrol, and the column was pre-washed with the elution buffer containing 0.5% Lubrol before applying the sample. Fractions of 5 ml were collected. 0.2 ml of each fraction was assayed for enzyme activity as described in the text. Protein was determined on aliquots of 0.1 ml of each fraction. ALAS, δ-aminolevulinate synthase.

It was most interesting to note that, unlike the adult enzyme [1,10], neither the Lubrol-solubilized supernatant nor the Sephadex G-200 fractions of fetal δ-aminolevulinate synthase was inhibited by hemin within a range of concentrations from $1 \cdot 10^{-6}$ M to $1 \cdot 10^{-4}$ M. Similar results were seen when bilirubin and cytochrome *c* were substituted for hemin in the reaction mixture. These results are consistent with the observation [5] that fetal δ-aminolevulinate synthase is refractory to inhibition by hemin in vivo and suggest that a biochemical modification of the enzyme may occur during development which determines its susceptibility to inhibition by hemin.

In conclusion, the present studies have described the solubilization and partial purification of mitochondrial δ-aminolevulinate synthase from fetal rat liver. Solubilization of the particulate enzyme with high retention of activity has been, perhaps, the most difficult problem to overcome in investigating the biochemical characteristics of this enzyme. These studies suggest that fetal δ-aminolevulinate synthase possesses properties which are significantly different from those of the adult enzyme, and which may account for differences in the regulation of hepatic heme synthesis at different stages of development.

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