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δ-Aminolevulic Acid Synthetase from Fetal Rat Liver: Studies on the Partially Purified Enzyme

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SUMMARY

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Hepatic δ-aminolevulinic acid (ALA) synthetase, the first and, in adults, the rate-limiting enzyme in the heme-biosynthetic pathway, was solubilized and purified 30-fold from 19-day fetal rat liver mitochondria. The properties of the partially purified enzyme were compared with those reported for ALA synthetase preparations from adult rat liver. Fetal and adult enzymes are similar in regard to substrate specificity, pH and temperature optima, and kinetic behavior, but differ substantially in terms of molecular weight, response to high cation concentrations, and regulatory properties. Unlike the adult enzyme, fetal ALA synthetase is not inhibited by the end product, hemin. These results suggest that regulatory differences in ALA synthetase at different stages of development may be due to variations in the biochemical properties of the enzyme in adult and fetal liver. The development of regulatory properties characteristic of the adult enzyme may occur concomitantly with mitochondrial maturation. Pharmacological alterations of the hepatocellular environment during gestation may affect the development of ALA synthetase as the rate-limiting enzyme in hepatic heme biosynthesis.

INTRODUCTION

Mammalian hepatic δ-aminolevulinic acid synthesis is the first and, in adults, the rate-limiting enzyme in the heme-biosynthetic pathway (1). It catalyzes the formation of δ-aminolevulinic acid from glycine and succinyl coenzyme A in the presence of pyridoxal 5′-phosphate. Studies performed in both *in vivo* (2–5) and on purified preparations of ALA[2] synthetase from the soluble (6–8) and mitochondrial (9, 10) fractions of adult rat liver have elucidated the kinetic, physical, and regulatory properties of this enzyme. ALA synthetase is thought to be synthesized in the endoplasmic reticulum and subsequently incorporated into the mitochondria (2, 3, 10). A variety of drugs and chemicals are known to increase the levels of ALA synthetase in both subcellular fractions of adult rat liver (3, 7, 11); this phenomenon is thought to underlie experimental porphyria in animals (1) and the exacerbation of acute intermittent porphyria in humans (12). ALA synthetase is regulated by the end product, heme, both by repression of

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[2] The abbreviations used are: ALA, δ-aminolevulinic acid; DTE, dithioerythritol; TCA, trichloracetic acid.

enzyme synthesis (13) and by feedback inhibition (6, 10).

Previous studies from this laboratory have shown that the activity of mitochondrial ALA synthetase in fetal rat liver is approximately 10 times that found in the adult (14). Fetal ALA synthetase activity declines to adult levels shortly after birth (15). During the fetal period ALA synthetase cannot be induced by chemicals which increase enzyme levels in the adult and is refractory to repression by the end product, heme (16, 17). In order to elucidate more clearly the properties of the fetal enzyme, as well as characterize the nature of the biochemical and regulatory differences in adult and fetal ALA synthetase, it was of interest to study the properties of the purified enzyme.

In this report a procedure is described for the purification of mitochondrial ALA synthetase from 19-day fetal rat liver which results in 30-fold purification of the enzyme. The properties of the partially purified enzyme were investigated and compared with those reported for ALA synthetase preparations from adult rat liver.

MATERIALS AND METHODS

Materials

Succinyl coenzyme A synthetase (succinate thiokinase, EC 6.2.1.4), pyridoxal 5'-phosphate, ATP, GTP, dithioerythritol, pyridoxamine 5'-phosphate, succinyl coenzyme A, bilirubin, cytochrome *c*, and coenzyme A were purchased from Sigma Chemical Company. Glycine, ALA, and heme as hemin were obtained from Calbiochem. [1-^{14}C]Glycine (44 mCi/mmole) was purchased from Schwarz/Mann. Lubrol-WX was purchased from General Biochemicals and was purified prior to use by the procedure of Nakao *et al.* (18). Sephadex G-25 and G-200 were obtained from Pharmacia, and Affi-Gel 10, from Bio-Rad Laboratories. Other chemicals were of reagent grade and were purchased from standard commercial sources.

Preparation of Animals

Date-bred Spraque-Dawley rats (CD strain) were obtained from the Charles River Breeding Company and were housed in individual cages with free access to food and water until the 19th day after breeding date. Animals were then killed by decapitation. Fetal livers from 50–70 rats were pooled for each experiment to yield approximately 60–70 g of tissue.

Assay of ALA Synthetase Activity

Fetal ALA synthetase at various stages of purification was assayed by one of the following methods, which were derived from procedures described by Scholnick *et al.* (7) and Whiting and Elliott (10). Modifications of these procedures were based on preliminary experiments for the determination of maximal fetal ALA synthetase activity at different stages of purification.

Method I. ALA synthetase activity in the mitochondrial and Lubrol-solubilized fractions was determined using an assay mixture containing a succinyl coenzyme A-generating system to compensate for the loss of succinyl coenzyme A by deacylase present in crude preparations (6). Thus enzyme activity was determined by measuring the amount of ALA formed at 37° in a reaction mixture containing sufficient succinyl coenzyme A synthetase to generate 1 μmole of succinyl coenzyme A in 30 min, 0.05 M Tris buffer (pH 7.5), 0.01 M $MgCl_2$, 0.1 M glycine, 0.01 M sodium succinate, 0.2 mM pyridoxal phosphate, 1 mM DTE, 5 mM EDTA, 60 μM coenzyme A, 3 mM ATP, 0.1 mM GTP, and 0.5 ml of the ALA synthetase preparation in a total volume of 2.5 ml. Reaction mixtures were shaken in a metabolic incubator for 30 min, and reactions were terminated by the addition of 0.5 ml of cold 10% trichloracetic acid solution. The ALA produced was converted to the 2-methyl-3-acetyl-4-propionic acid pyrrole by reaction with sodium acetate and acetylacetone, and was determined using modified Ehrlich's reagent (19) as previously described (14).

Method II. In fractions prepared subsequent to Sephadex G-200 chromatography succinyl coenzyme A was stable in the reaction medium. Therefore ALA synthetase was assayed by determining the amount of ALA formed at 37° in a reaction

mixture containing 0.05 M Tris buffer (pH 7.5), 0.1 M glycine, 0.1 mM pyridoxal phosphate, 0.15 mM succinyl coenzyme A, 1 mM DTE, and 0.2 ml of the enzyme preparation in a final volume of 1 ml. Reactions were terminated by addition of 0.25 ml of cold 10% TCA. The ALA formed was then determined as in method I.

Method III. For the purpose of establishing the stoichiometry between glycine utilized and ALA produced, an assay procedure utilizing [1-^{14}C]glycine as substrate was employed. The ALA synthetase activity was assayed by determining the amount of $^{14}CO_2$ formed at 37°. Reactions were carried out in plastic liquid scintillation counting vials fitted with wells attached to the shaft of a corked 18-gauge needle which was fitted through the lid of each counting vial, as previously described (20). Each well contained 0.2 ml of 10% NaOH solution. Into each vial was placed 0.8 ml of substrate solution containing 0.1 mM pyridoxal phosphate, 1 mM DTE, 0.1 M glycine, and 0.15 mM succinyl coenzyme A dissolved in 0.05 M Tris buffer (pH 7.5), together with 0.5 μCi of [1-^{14}C]glycine (50 μCi/ml, 44 mCi/mmole) and 0.2 ml of enzyme preparation. Incubations were allowed to proceed for 1 hr, and the reaction was terminated by injecting 0.25 ml of 10% TCA into the vial via the needle by means of a syringe. The cork was immediately replaced in the syringe hub to prevent escape of the CO_2. Vials were incubated for an additional hour to trap all the CO_2. Vials in which acid was added at the beginning of the experiment served as blanks. After the final incubation, vial caps containing $^{14}CO_2$ dissolved in NaOH were placed in glass counting vials containing 20 ml of toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation counting solution. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer with appropriate corrections for quenching. Under these conditions the amount of $^{14}CO_2$ produced was equivalent to the quantity of ALA formed in the reaction mixture, as determined by method II.

Protein Determinations

Protein concentrations were determined by the method of Lowry *et al.* (21), using bovine serum albumin (fraction V) as a standard. In chromatographic procedures protein elution profiles were determined by monitoring the optical density at 280 nm, using an Isco model UA-Z ultraviolet analyzer or a Beckman DUR spectrophotometer fitted with a Gilford absorbance recorder.

Preparation of Sephadex G-200

Sephadex G-200 was allowed to swell for several days in 0.05 M Tris buffer, pH 7.5. The slurry was then applied to a 2.6 × 40 cm column and equilibrated with 0.05 M Tris buffer, pH 7.5, containing 0.1 mM pyridoxal phosphate, 1 mM DTE, and 0.5% Lubrol. The void volume, as determined using blue dextran, was 61 ml.

Affinity Chromatography

Pyridoxamine 5′-phosphate-linked Affi-Gel 10 (*N*-hydroxysuccinimide ester of Agarose gel in bead form with a 10-A arm length) was employed for specific absorption of fetal ALA synthetase. One gram of Affi-Gel 10 was suspended in 25 ml of 0.1 M phosphate buffer, pH 7.5, containing 35 mM pyridoxamine 5′-phosphate and was shaken at 4° for 19 hr. The gel was poured into a column (1 × 9 cm) and washed with 50 volumes of the cold buffer to remove ultraviolet-absorbing material. The gel was then equilibrated by washing with 3 volumes of 0.05 M Tris buffer, pH 7.5, containing 1 mM DTE.

RESULTS

In a previous study (14) it was shown that fetal hepatic ALA synthetase is synthesized in the endoplasmic reticulum of the fetal liver cell, but that activity is concentrated primarily in the mitochondria at levels approximately 10 times those found in the adult. ALA synthetase activity in the soluble fraction of the fetal liver cell is 20 times less than that of the mitochondrial fraction and, in contrast to

the adult enzyme, cannot be increased by inducing agents (14, 16). In order to study the properties of fetal ALA synthetase, therefore, it was necessary to purify the enzyme from the mitochondrial fraction. The following purification sequence was developed for ALA synthetase from 19-day fetal rat liver. The entire procedure was performed at 0–4°. A summary of all purification steps is shown in Table 1.

Purification of ALA Synthetase

Step I: Preparation of mitochondria. Fetal livers (60–70 g, wet weight) were pooled, washed, weighed, and homogenized in a Waring Blendor in 9 volumes of 0.25 M sucrose containing 0.02 M Tris-HCl buffer (pH 7.5), 0.1 mM EDTA, and 0.1 mM pyridoxal phosphate. The homogenate was centrifuged for 10 min at 600 × *g*, and the mitochondria were sedimented from the resulting supernatant solution by centrifugation at 9000 × *g* for 15 min. Mitochondria were washed twice by resuspension in the same solution and centrifuged. The washed pellet was suspended in 0.05 M Tris buffer, pH 7.5, containing 0.1 mM pyridoxal phosphate and 1 mM DTE so that each milliliter of suspension contained 3–5 mg of mitochondrial protein. The specific activity of the mitochondrial fraction was taken as 100%.

Step 2: Solubilization of fetal mitochondrial ALA synthetase. The most difficult problem associated with purifying fetal ALA synthetase was encountered in solubilizing the mitochondrial enzyme. A previous report from this laboratory (22) has described in detail the evaluation of various techniques utilized to solubilize this enzyme. Freezing and thawing, sonication, or lyophilization and subsequent extraction (10) were ineffective and often resulted in complete loss of enzyme activity. The best results were achieved when the mitochondrial suspension was treated with the non-ionic detergent Lubrol-WX. A 10% aqueous solution of Lubrol was added to the mitochondrial preparation with stirring to achieve a final concentration of 0.6%, and the suspension was stirred for 1 hr more. The mixture was then centrifuged at 105,000 × *g* for 1 hr. This treatment resulted in the release of 75–80% of the enzyme activity in the soluble fraction and 1.5–2-fold increase in specific activity.

Step 3: Sephadex G-200 chromatography. The solubilized material from step 2 was loaded onto a column (2.6 × 40 cm) of Sephadex G-200 equilibrated with 0.05 M Tris buffer, pH 7.5, containing 0.1 mM pyridoxal phosphate, 1 mM DTE, and 0.5% Lubrol. The enzyme was eluted in 5-ml fractions with the same buffer. ALA synthetase activity appeared as a single peak in fractions 24–28, as previously described (22). The most active fractions were pooled and either employed for further purification or stored at −20° for future enzyme studies. This step resulted in a further 5–6-fold increase in specific activity and a slight increase in the yield, possibly attributable to the removal of inhibitory material by column chromatography.

The enzyme at this stage of purity was stable for up to 2 weeks at 4° and for at

TABLE 1

Purification of ALA synthetase from fetal rat liver

Step and fraction	Total activity[a]	Total protein	Specific activity	Yield
	units	*mg*	*units/mg protein*	%
1. Mitochondria	1825	590	3.1	100
2. Lubrol (105,000 × *g*) supernatant	1405	280	5.0	77
3. Sephadex G-200	1510	84	18.0	83
4. Affinity chrmatography	372	4.1	92.0	20

[a] A unit of enzyme activity is defined as the formation of 1 nmole of ALA in 30 min at 37°. ALA synthetase was assayed as described in MATERIALS AND METHODS.

least 2 months at −20° and was free of deacylase activity, present in earlier fractions. Enzyme activity could therefore be determined in this and subsequent steps by method II. Attempts to concentrate fetal ALA synthetase after column chromatography either by $(NH_4)_2SO_4$ fractionation or by membrane dialysis, using an Amicon dialyzer equipped with an XM50 Diaflow ultrafilter, resulted in the loss of all enzyme activity.

Step 4: Affinity chromatography. The active fraction from gel filtration was desalted on a column of Sephadex G-25 equilibrated with 0.05 M Tris buffer, pH 7.5, containing 1 mM DTE. The eluate, containing the apoenzyme free of pyridoxal phosphate, was then loaded onto a column (1 × 9 cm) of Bio-Rad Affi-Gel 10 coupled with pyridoxamine 5'-phosphate, prepared as described in MATERIALS AND METHODS. The column was washed twice with 25 ml of the above buffer, once with 20 ml of 0.05 M Tris buffer, pH 7.5, containing 1 mM DTE and 10 mM pyridoxal phosphate, and once with 20 ml of 0.05 M Tris buffer containing 1 mM DTE, 10 mM pyridoxal phosphate, and 0.5 M NaCl. ALA synthetase was then eluted in 5-ml fractions with 0.05 M Tris buffer, pH 7.5, containing 1 mM DTE, 0.5 M NaCl, and 50 mM pyridoxal phosphate. ALA synthetase was eluted as a single, symmetrical peak of activity. This procedure resulted in a 30-fold increase in specific activity and an over-all yield of approximately 20%. It was later discovered that both NaCl and pyridoxal phosphate, in high concentrations, are inhibitory to fetal ALA synthetase. Therefore the values for the recovery of ALA synthetase after affinity chromatography are probably conservative estimates. Results of recent experiments also indicate that recovery may be substantially improved by using KCl in place of NaCl in the elution buffer.

In control experiments which were designed to test the operation of the affinity chromatography principle in the purification of fetal ALA synthetase by this method the enzyme preparation was passed through a column of Affi-Gel 10 which had not previously been linked with pyridoxamine 5'-phosphate. In this case no enzyme activity could be recovered in any of the fractions collected.

Properties of Fetal ALA Synthetase

The enzyme was relatively unstable after purification by affinity chromatography, as compared with the preparation following gel filtration. Storage of the 30-fold purified enzyme for 7 days at −20° resulted in a loss of 50% of the initial enzyme activity, whereas 100% of the activity of the preparation from gel filtration was retained under these conditions. Because of the greater stability of the latter fraction the enzyme preparation from step 3 was used for most studies. However, similar results were achieved in studies using the enzyme prepared through step 4.

Fetal ALA synthetase showed a pH optimum at 7.5 when assayed using either 0.05 M Tris or 0.1 M potassium phosphate buffer. A 50% reduction in enzyme activity was observed at pH 6.8 and 8.2. Fetal ALA synthetase has a temperature optimum of 37° and was irreversibly inactivated when incubated at temperatures above 50° for 30 min.

In a previous report from this laboratory (22) it was shown that fetal ALA synthetase is inhibited in the presence of high NaCl concentrations. This observation contrasts with those reported for ALA syn-

TABLE 2

Effects of high salt concentrations on fetal ALA synthetase activity

ALA synthetase purified through step 3 was assayed by method II in a reaction medium containing the indicated concentration of the particular salt. Similar results were obtained using ALA synthetase purified through affinity chromatography (step 4). The actual control value of ALA synthetase activity was 23.1 nmoles/mg of protein in 30 min.

Salt concentration	Inhibition			
	NaCl	KCl	$CaCl_2$	$MgCl_2$
mM	%	%	%	%
10	26	0	17	20
20	33	0	34	42
100	55	28	44	70
200	80	36	79	90

thetase purified from both mitochondrial (10) and soluble (7) fractions of adult rat liver, both of which required the presence of high NaCl concentrations for activation and stabilization. Table 2 shows that the inhibitory effects of high salt concentrations on fetal ALA synthetase are not restricted to Na^+ but are common to other cations as well, including K^+, Ca^{++}, and Mg^{++}. The percentage inhibition produced by these cations increased in proportion to their concentration in the reaction mixtures; the order of inhibition was $Mg^{++} > Na^+ \geq Ca^{++} > K^+$.

Fetal ALA synthetase was found to have an absolute requirement for the substrates glycine and succinyl coenzyme A and for the cofactor pyridoxal phosphate. No ALA was formed when any of these substances was omitted from the reaction mixture. The apparent K_m value for glycine, as determined from a double-reciprocal plot, was 1.7×10^{-2} M. The stoichiometric correlation of the utilization of glycine with the formation of ALA was confirmed using method II to measure ALA synthesis and method III to measure [1-^{14}C]-glycine utilization. In a typical experiment 43.8 nmoles of CO_2 were produced, corresponding to 32.4 nmoles of ALA synthesized.

The apparent K_m value for succinyl coenzyme A was 8×10^{-5} M, and that for pyridoxal phosphate was 6×10^{-6} M. Pyridoxal phosphate caused inhibition of enzyme activity at concentrations greater than 1×10^{-4} M (Fig. 1).

Fetal ALA synthetase was sensitive to inhibition by Pb^{++} within a range of concentrations from 0.1 to 2 mM. The inhibition appeared to be competitive, as shown in Fig. 2. A concentration of 750 μM lead acetate was shown in a preliminary experiment to produce 50% inhibition of fetal ALA synthetase. A K_i for lead of 1.9×10^{-6} M was calculated from the data shown in Fig. 2. In addition to Pb^{++}, Hg^{++} also produced marked inhibition of the enzyme. A concentration of 100 μM $HgCl_2$ in the reaction medium was required to produce 50% inhibition, whereas 200 μM $HgCl_2$ resulted in 100% inhibition of fetal ALA synthetase activity.

It is well known that ALA synthetase from adult rat liver is regulated by the end product, heme, both by repression (13) and by feedback inhibition (6, 10). Since previous studies from this laboratory have demonstrated that fetal ALA synthetase is refractory to repression by heme *in vivo* (14, 16), it was of particular interest to determine the direct effects of heme on the partially purified enzyme (Table 3). It was most interesting to observe that heme did not inhibit fetal ALA synthetase in concentrations up to 200 μM, a concentration which has been shown to produce 90% inhibition of the adult enzyme (6). In fact, heme seemed to produce a substantial

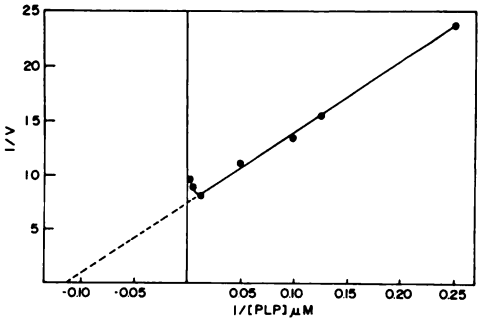

FIG. 1. *Double-reciprocal plot showing apparent K_m of fetal ALA synthetase for pyridoxal 5'-phosphate (PLP)*

The assay was performed by method II with varying concentrations of pyridoxal phosphate. The cofactor was removed from the ALA synthetase prior to assay by passage of the enzyme preparation through a column of Sephadex G-25. The apparent K_m was calculated to be 6×10^{-6} M.

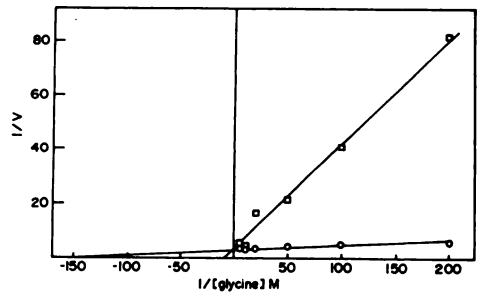

FIG. 2. *Double-reciprocal plot showing inhibition of fetal ALA synthetase by 750 μM lead acetate at varying concentrations of glycine*

The assay was performed by method II. □, lead acetate; ○, glycine.

TABLE 3

Effects of heme on adult and fetal ALA synthetase activity

Heme as hemin was dissolved in a minimal volume of 0.01 N NaOH and adjusted to pH 7.5 with 0.01 N HCl. ALA synthetase activity was assayed by method II in a reaction mixture containing the indicated concentration of hemin. Flasks in which 0.05 M Tris buffer, pH 7.5, was substituted for the enzyme preparation were used to correct for the colorimetric contribution of hemin to the spectrophotometric assay. Actual control values of ALA synthetase activities were 26.4 and 2.15 nmoles/mg of protein in 30 min for fetal and adult enzymes, respectively.

Heme concentration	ALA synthetase activity	
	Fetal	Adult
μM	*% control*	
1	100	100
10	111	75
50	116	61
100	123	55
200	150	

increase in enzyme activity at higher concentrations in the reaction mixture. At 200 μM, heme produced an increase in fetal ALA synthetase activity of 150% of the control value. Similar results were observed when fetal ALA synthetase was incubated with cytochrome *c*. On the other hand, bilirubin produced no alteration of fetal ALA synthetase activity when incubated with the enzyme preparation at concentrations ranging from 1 to 200 μM. A comparison of the effects of heme on both adult and fetal rat liver mitochondrial ALA synthetase is also presented in Table 3. For these experiments ALA synthetase was solubilized with Lubrol from the mitochondrial fraction of adult rat liver in a manner identical with that described for the fetal enzyme. In contrast to observations made with regard to the fetal enzyme, hemin produced a progressive inhibition of adult ALA synthetase activity.

The approximate molecular weight of fetal ALA synthetase was estimated to be 47,000, using molecular sieve chromatography on Sephadex G-200 (Fig. 3). Sucrose density gradient centrifugation was utilized as an alternative means of estimating the molecular weight of the fetal enzyme. As determined by this method, the molecular weight of fetal ALA synthetase was estimated to be approximately 50,000.

DISCUSSION

Numerous reports regarding the regulation of hepatic ALA synthetase in mammalian liver have appeared in recent years (2, 3, 13, 14, 23–25). Studies involving purified enzyme preparations from subcellular fractions of adult rat liver (6, 10) have provided some of the most convincing evidence regarding the intracellular distribution, the molecular properties, and the regulatory mechanisms of hepatic ALA synthetase in adult mammals. The present study is the first known report of the properties of ALA synthetase purified from fetal rat liver mitochondria.

Several similarities in the properties of adult and fetal ALA synthetase are indicated by these studies. Both soluble (7) and mitochondrial (10) adult enzyme preparations, like the fetal enzyme, have an absolute requirement for the substrates glycine and succinyl coenzyme A and for the cofactor pyridoxal phosphate. Very little ALA synthetase activity was observed in the absence of any of these substances. The K_m values for these compounds and the pH and temperature op-

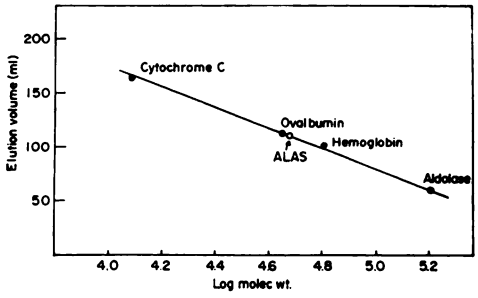

FIG. 3. *Estimation of molecular weight of fetal ALA synthetase (ALAS) by elution from Sephadex G-200*

The elution buffer was 0.05 M Tris, pH 7.5, containing 1 mM DTE, 0.1 mM pyridoxal phosphate and 0.5% Lubrol. Five-milliliter fractions were collected. Cytochrome *c* (mol wt 12,270), ovalbumin (45,000), horse hemoglobin (64,500), and aldolase (158,000) were used as marker proteins. Blue dextran 2000 was used to determine the exclusion volume (61 ml). The elution volumes of the marker proteins were determined by measuring the absorbance at 280 nm.

tima are also similar. It is interesting that ALA synthetase in both adult and fetal rat liver have high K_m values for glycine, in the range of 10 mM. This fact may be of particular interest in regard to the regulation of heme biosynthesis in fetal liver, since measurements recently made in this laboratory indicate that the fetal hepatic glycine concentration does not exceed the K_m value of ALA synthetase for this substrate.

On the other hand, fetal ALA synthetase appears to differ significantly from the adult preparations in the presence of high salt concentrations, in terms of the estimated molecular weight, and, perhaps more importantly, in response to the end product, heme. Developmentally related variations in enzyme biochemistry or in mitochondrial function may account for the observed differences.

Maximal stimulation of the soluble form of adult ALA synthetase was achieved in the presence of 0.25 M NaCl (7), whereas the adult mitochondrial preparation required up to 0.8 M NaCl for solubilization and stabilization (10). In contrast, 0.2 M NaCl, $MgCl_2$, or $CaCl_2$ produced 80-90% inhibition of fetal ALA synthetase, whereas no inhibition occurred with 0.2 M Tris buffer. The specific inactivation of fetal ALA synthetase at high cation concentrations, unlike the adult enzyme, suggests that this enzyme may possess a different molecular configuration from that of the adult. The mechanism of this inactivation had yet to be determined, but may involve a shift of the tautomeric form of the enzyme, a change of hydration state, or some other process which results in alteration of biochemical behavior.

The estimated molecular weight of 47,000–50,000 for fetal ALA synthetase is relatively low in comparison with that proposed for the solubilized adult mitochondrial preparation (77,000) (10). Previous studies have shown that both adult (2, 3) and fetal (14) ALA synthetase are synthesized in the endoplasmic reticulum prior to incorporation into the mitochondria, and it is proposed that the mitochondria provide a locus for the formation of the physiologically active enzyme (26). The observed differences in the molecular weight, as well as in other properties of the solubilized adult and fetal enzymes, therefore might be explained in terms of differences in mitochondrial function at the two stages of development. Fetal mitochondrial biogenesis occurs at twice the rate observed in adults, and the morphological development of intramitochondrial structures, such as cristae and inner membranes, does not occur until after birth (27, 28). Thus the orientation of a functional ALA synthetase within the mitochondrial matrix, and the development of susceptibility of the enzyme to end product inhibition such as that seen in the adult, may not occur in the functionally immature fetal mitochondria. Such a situation could partially account for the observed diffferences in the properties of the isolated mitochondrial enzyme preparations. This hypothesis is consistent with the correlation of the appearance of morphologically distinct intramitochondrial membranes with the change in the properties of fetal ALA synthetase to those of the adult enzyme (17).

The mechanism of the apparent stimulatory effect of heme on fetal ALA synthetase activity remains to be determined. This phenomenon, however, is consistent with results previously observed *in vivo* (14) and appears to represent, at least in part, a direct effect of heme on the enzyme itself. It has been postulated that heme may stimulate a general increase in protein synthesis in mammalian cells (29) and may play a facilitatory role in fetal mitochondrial biogenesis (30). The present studies suggest that stabilization of ALA synthetase in fetal liver cells may represent one mechanism through which this is accomplished.

The inhibition of fetal ALA synthetase by lead acetate occurs at concentrations (0.0 – 1 mM) which have been reported to have no effect on the partially purified adult enzyme (6). Variations in the extent of purification of the two preparations could account for these differences. Alternatively, the isolation of fetal ALA synthetase from the mitochondria as a relatively small, unassociated molecule might afford a greater accessibility of sulfhydryl groups

at the reactive site of the enzyme to lead, and hence result in a greater sensitivity to inhibition by such compounds. The apparent competitive interaction of lead and glycine with regard to fetal ALA synthetase is a rather unique observation and is the subject of ongoing investigations in this laboratory. From the data calculated from Fig. 2 one might conclude that the nature of this interaction involves a more complicated phenomenon than mere complex formation between lead and glycine. Since the K_i value for lead ions is of the order of 10^{-6} M, whereas the K_m value for glycine is approximately 10^{-2} M, one would expect that a considerably higher lead ion concentration would be required to produce inhibition by complexation with glycine than was observed in these studies. Reversal of lead inhibition of fetal ALA synthetase by amino acids other than glycine has not yet been demonstrated.

In conclusion, the present studies suggest that fetal mitochondrial ALA synthetase is a relatively small but functionally active molecule, which is similar to the adult enzyme in regard to its enzymatic properties but is subject to regulatory influences which are quite distinct from those of the adult. These differences are reflected in the behavior of the isolated enzyme preparations. It is suggested that changes in the molecular configuration of the fetal enzyme occur concomitantly with mitochondrial maturation, at which time the development of regulatory properties characteristic of the adult enzyme occurs. Although the nature of these changes has yet to be determined, it is postulated that physiological or pharmacological alterations in the hepatocellular environment during gestation may be of particular importance in the development of ALA synthetase as the rate-limiting enzyme in hepatic heme biosynthesis.

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