PURIFICATION AND SOME PROPERTIES OF δ-AMINOLEVULINATE(ALA) SYNTHETASE IN RABBIT RETICULOCYTES

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SUMMARY:
ALA synthetase has been purified approximately 4,400 fold from rabbit reticulocytes. The purified enzyme was demonstrated to be homogeneous by disc gel electrophoresis. The enzyme had a pH optimum of 7.6. Isoelectric point was 5.9. The enzyme required pyridoxal phosphate as a cofactor. Iron inhibited, but α,α'-dipyridyl neither inhibited nor activated the activity. Hemin inhibited ALA synthetase activity about 40% at the concentration of 10^{-5}M. Km for succinyl-CoA was 6.0×10^{-5}M and Km for glycine was 1.0×10^{-2}M.

ALA synthetase, the enzyme catalyzing the condensation of glycine and succinyl-CoA to form ALA, has been accepted as the rate controlling enzyme in heme biosynthesis(1-4) and large number of reports concerning this enzyme in Rhodopseudomonas spheroides(5-7), liver (1,2,8) and erythropoietic tissue(9,10,11) have been published. However, its purification has not yet been accomplished(5,8). This communication deals with the purification procedures and some properties of ALA synthetase in rabbit reticulocytes.

METHODS

Reticulocytosis was produced in New Zealand white rabbits(2-3kg) by acetylphenylhydrazine injection. Blood was collected from the femoral vein. About 200 ml of heparinized blood was washed twice with 0.9% saline and hemolyzed with 3 volumes of cold water for 10 min. After restoring isotonicity by adding 11.5% KCl solution, the hemolysate was centrifuged at 3000 xg for 10 min. The pellet, which was washed five times with 1.15% KCl solution by centrifuging

at 10,000 xg for 10 min, was then resuspended in the same KCl solution and further centrifuged at 700 xg for 10 min. The supernatant was centrifuged at 15,000 xg for 10 min. The pellet suspended in 40 ml of 1.15% KCl solution and sonicated for 3 min at maximum power(20 kc/s, 150 W) was centrifuged at 15,000 xg for 10 min to collect the pellet. After adding sodium deoxycholate(200 mg/10 ml pellet), the pellet was dissolved in five volumes of 1.15% KCl solution and well mixed. The mixture was centrifuged at 15,000 xg for 10 min. To 15 ml of the supernatant 3.0 g of solid ammonium sulfate was added. The precipitate was dissolved in a small volume of 0.05 M Tris buffer containing 0.1 mM pyridoxal phosphate. After dialyzing against the same buffer for several hours, the precipitate was removed by centrifugation. The supernatant was applied to gel filtration on Sephadex G-200(1.5 x 70 cm). The eluate containing the highest ALA synthetase activity was applied to an electrofocusing apparatus(110 ml column) using carrier ampholyte(pH 3-10). After electrofocusing at 5°C for 24 hours, the eluate containing the highest ALA synthetase activity was applied to gel filtration on Sephadex G-200. All the above procedures were carried out at 0-5°C. Except where otherwise stated, ALA synthetase activity was assayed by determining the amount of ALA formed at 37°C for 60 min in a reaction mixture* containing Tris buffer, pH 7.4, 0.4 μ moles; pyridoxal phosphate, 0.2 μ moles; glycine, 10 μ moles; succinyl-CoA 30 mμ moles in a final volume of 0.3 ml. The reaction was terminated by 0.5 ml of 5% trichloracetic acid. ALA formed was determined by reaction with modified Ehrlich's reagent. In some studies ALA was further characterized by column chromatography.

Protein concentration was determined by the method of Lowry et

*As EDTA and Mg^{++} decreased the enzyme activity by 10% at the concentration of 3 mM, we did not add them in the reaction mixture.

al. Protein concentration of the material containing carrier ampholyte was determined by the absorption at 280 mμ.

Table 1 Purification of ALA synthetase in rabbit reticulocytes

Purification step	Total protein (mg)	Specific activity (ALA mμ moles/60 min/mg protein)	Total activity (ALA mμ moles /60 min)	Yield (%)
Sonicated particle	1800	0.07	126	100
Extract by Na-deoxycholate	225	1.8	405	312
Ammonium sulfate fractionation	72	3.2	230	183
Sephadex G-200	18	10.8	194	154
Electrofocusing	0.6	104	62.4	50
Sephadex G-200	0.06	312	18.7	15

RESULTS AND DISCUSSION

Results of purification of ALA synthetase in rabbit reticulocytes are summarized in table 1. Because of the presence of succinyl-CoA deacylase in the sonicated particle, formation of ALA by this particle seems to be reduced. ALA synthetase has been purified approximately 4,400 fold. Main activity was found in the sonicated sediment. Most of ALA synthetase activity became extractable from this sediment by treating with sodium deoxycholate(see METHODS).

Molecular weight of this enzyme was estimated to be approximately 200,000 by its elution behavior on Sephadex G-200 column.

Electrofocusing using carrier ampholyte(pH 3-10) gives a very sharp peak of ALA synthetase activity as shown in figure 1. Isoelectric point was 5.9. The portion containing the highest ALA synthetase activity was applied to gel filtration on Sephadex

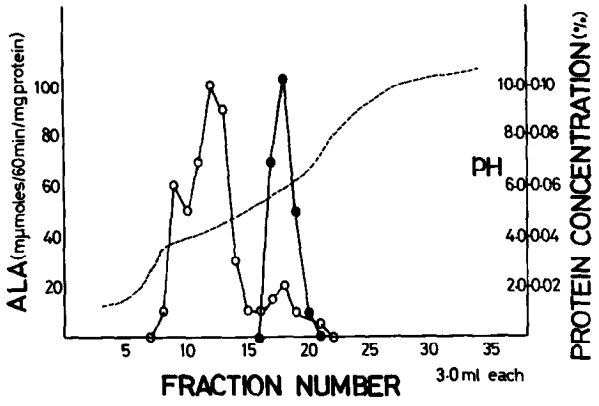

Figure 1 Electrofocusing of ALA synthetase
110 ml column with 1% carrier ampholyte(pH 3-10) was used. Sample volume was 6.0 ml of the eluate from Sephadex G-100 column. The separation was carried out for 24 hours at 5°C. protein concentration; o——o ALA synthetase activity; ●——● pH;.....

G-200. Eluate from the column containing the highest ALA synthetase activity was concentrated 10 fold by ultrafiltration, and the concentrated enzyme was subjected to disc gel electrophoresis on acrylamide gel. Only one sharp band was observed as shown in figure 2. No enzyme activity was seen anywhere along the gel because the gel system inhibited the enzyme activity. However, this band is considered to be the enzyme itself, because above purification procedure gives as much as 4,400 fold purification and eluate from the second gel filtration gives parallel distribution between protein concentration and the enzyme activity.

Purified enzyme has a pH optimum of 7.6. Several other properties are shown in figure 3. Km for succinyl-CoA was 6.0×10^{-5} M at the glycine concentration of 30 mM, and Km for glycine was 1.0×10^{-2} M at the succinyl-CoA concentration of 100 μM. Omission of pyridoxal phosphate decreased the activity by 40%..

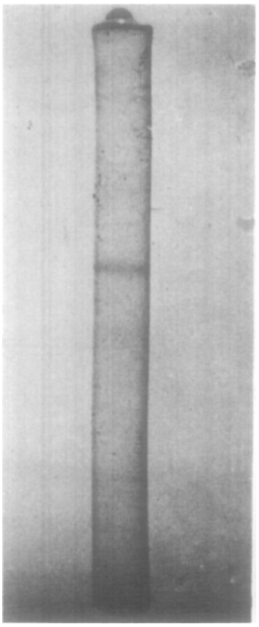

Figure 2 Polyacrylamide disc gel electrophoresis of the purified ALA synthetase
Enzyme preparation was subjected to disc gel electrophoresis with 7.5% polyacrylamide gel and Tris buffer, pH 8.9. The separation was carried out for 1 hour at room temperature with a constant current of 3 mA per tube. Protein was stained with Amido Schwarz. The direction of migration was toward the top of the gel.

SH compounds, especially CoA, inhibited the activity. N-ethylmaleimide, a SH alkylating agent, also inhibited the activity. Iodoacetamide, on the other hand, enhanced the enzyme activity.

Iron has been considered to have some effect on the induction and activity of ALA synthetase. Stein et al(16) reported that ferric citrate exerted a pronounced synergic effect on the induction of hepatic ALA synthetase produced by allylisopropylacetamide(16). On the other hand, some workers reported that ferrous iron activated ALA synthetase activity in chicken erythrocytes(19,11,15). In this experiment iron inhibited the activity of purified ALA synthetase. Moreover α,α'-dipyridyl did not exert any effect on the activity of

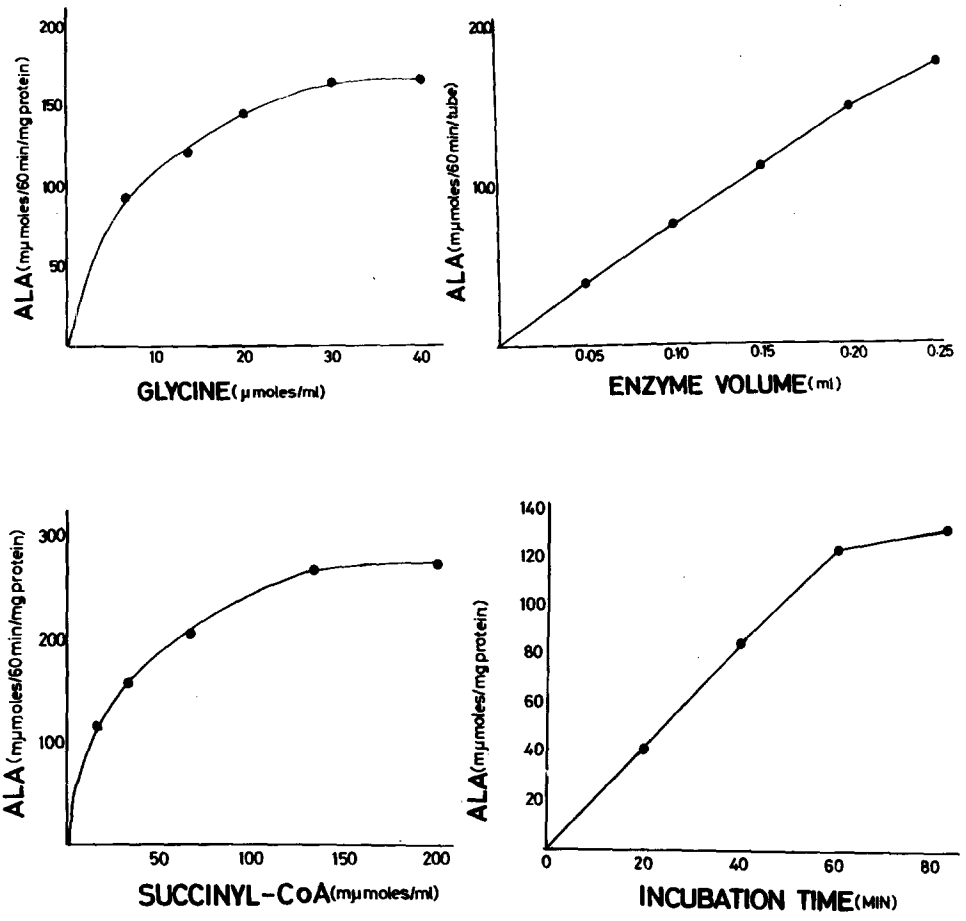

Figure 3 Effect of glycine concentration, enzyme volume, succinyl-CoA concentration, and incubation time on the ALA synthetase activity

this enzyme. From this result, we conclude that ALA synthetase in rabbit reticulocytes does not require iron for its activity.

Purified enzyme loses almost of its activity when stored at 4°C overnight, but when frozen overnight it loses about 15% of its activity in the presence of 0.1 mM pyridoxal phosphate and 0.05 M Tris buffer(pH 7.4).

Many workers reported that heme represses ALA synthetase in liver (3,12,13,14). On the other hand, Burnham and Lascelles demonstrated

Table 2 Effect of SH compounds and iodoacetamide on the purified ALA synthetase activity in rabbit reticulocytes

Additions	Concentration (M/L)	Activity (% of control)
None	10^{-3}	100
CoA	10^{-3}	6
GSH	10^{-3}	53
β-Mercaptoethanol	10^{-3}	63
Cysteine	10^{-3}	41
N-Ethylmaleimide	10^{-3}	29
Iodoacetamide	10^{-3}	109
Iodoacetamide	10^{-2}	145

These substances and Fe compounds(table 3) did not interfere With the color development of ALA pyrrole at these concentrations.

Table 3 Inhibitory effect of hemin and iron on the purified ALA synthetase activity in rabbit reticulocytes

Additions	Concentration (M/L)	Inhibition (%)
Hemin	10^{-4}	63
Hemin	10^{-5}	37
$FeSO_4$	10^{-4}	32
$FeSO_4$	10^{-5}	21
$FeCl_3$	10^{-4}	41
$FeCl_3$	10^{-5}	22
α,α'-dipyridyl	10^{-3}	0
α,α'-dipyridyl	10^{-4}	0

that heme inhibited ALA synthetase activity in Rhodopseudomonas spheroides(5). Although Karibian et al proved that the pathway from glycine to ALA in rabbit reticulocytes was inhibited by hemin, the inhibition occured at too high concentration of hemin(9).

Recently, Scholnick et al reported that 5×10^{-5} M of heme

inhibited the activity of partially purified ALA synthetase in cytosol of liver by 30%(8). However, many workers reported that hemin did not inhibit ALA synthetase activity in liver homogenate or mitochondria at the physiologic concentration(3,12,14). In this experiment hemin inhibited the activity of purified ALA synthetase in rabbit reticulocytes about 40% at the concentration of 1×10^{-5} M. This inhibition is marked as compared with the results reported earlier. This differences may be due to the difference of the purity of the enzyme, or to the difference of the nature of the enzyme between liver and erythropoietic tissue. Our present result seems to indicate that the endproduct inhibition is one of the controlling factors in heme synthesis in rabbit reticulocytes.

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