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δ-AMINOLEVULINIC ACID DEHYDRATASE OF *MYCOBACTERIUM PHLEI*

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SUMMARY

δ-Aminolevulinic acid dehydratase (5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24) was purified 160-fold from crude extracts of *Mycobacterium phlei*. The pH optimum was 9.2; the K_m value for δ-aminolevulinic acid was $7.7 \cdot 10^{-5}$ M. Mg^{2+} was necessary for the maximum activity of the enzyme and EDTA inhibited the activity markedly. δ-Hydroxylevulinic acid, δ-hydroxy-γ-oxo-L-norvaline and levulinic acid inhibited the enzyme activity competitively.

Inhibitory effect of α-ketoglutarate:glyoxylate carboligase activity on δ-aminolevulinic acid dehydratase activity was studied using the cell free extracts of *M. phlei*.

INTRODUCTION

δ-Aminolevulinic acid dehydratase (5-aminolevulinate hydro-lyase (adding 5-aminolevulinate and cyclizing), EC 4.2.1.24) has been purified from various sources[1-9].

Although the thiol groups are known to be an essential requirement for the enzyme activity, other requirements seem to differ with enzyme sources. Enzyme preparations from *Rhodopseudomonas spheroides*[10] and *Nicotiana tabacum*[9] were both activated by Mg^{2+}. EDTA was a potent inhibitor of the enzyme from mammalian sources[1,3,7] except the mouse[4]. The enzyme from *R. spheroides* was insensitive to EDTA[2], and stimulatory effect of EDTA on the enzyme from mouse tissue was reported by COLEMAN[4]. Recently, NANDI *et al.*[8] and NANDI AND SHEMIN[11] have reported that the enzyme from *R. spheroides* has many characteristics of an allosteric enzyme, such as substrate cooperativity in the absence of K^+ and promotion of the enzyme association to an equilibrium mixture of monomer, dimer and trimer in the presence of K^+. K^+ was, however, not effective on the enzyme from a plant source[10,12] and that from ox liver[12].

Feed-back inhibition of this enzyme by heme also seems to depend upon the enzyme sources, *e.g.* inhibitory on the enzyme from *R. spheroides*[2,8] and from mammals[3,4], but not effective on the enzyme from *N. tabacum*[9].

NANDI AND SHEMIN[13] have reported that the enzyme from *R. spheroides* was

competitively inhibited by levulinic acid. We have reported that δ-hydroxylevulinic acid, a reaction product of α-ketoglutarate:glyoxylate carboligase, inhibited the porphyrin synthesis from δ-aminolevulinic acid in the cell free extracts of *Mycobacterium phlei*[14].

The present paper describes some general properties of δ-aminolevulinic acid dehydratase and some evidence for inhibitory effect of α-ketoglutarate:glyoxylate carboligase activity on the dehydratase activity of *M. phlei*.

MATERIALS AND METHODS

Materials

δ-Aminolevulinic acid hydrochloride was purchased from Schuhardt, Muenchen; recrystallized hemin was from Mann Research Laboratories, New York. Levulinic acid, thiamine pyrophosphate and bonito extract were from Wako Pure Chemical Industries, Ltd., Osaka; sodium glyoxylate was from Sigma Chemical Co.; α-ketoglutaric acid was from E. Merck; polypeptone was from Daigo Eiyo Kagaku Co., Ltd., Osaka; DEAE-cellulose was from Serva.

δ-Hydroxylevulinic acid was synthesized and supplied by courtesy of the Research Institute of Takeda Chemical Industries, Ltd., Osaka. δ-Hydroxy-γ-oxo-L-norvaline was a generous gift of Dr. Akira Miyake of the Institute. All other chemicals used were of analytical grade.

Hydroxylapatite was prepared by the method of TISELIUS *et al.*[15].

Methods

The activity of δ-aminolevulinic acid dehydratase was determined by the method of MAUZERALL AND GRANICK[16]. A 1.5 cm × 18.0 cm test tube containing 10 μmoles of δ-aminolevulinic acid neutralized by Tris, 10 μmoles of $MgSO_4$, 20 μmoles of β-mercaptoethanol, 100 μmoles of Tris–HCl buffer, pH 9.0, and enzyme was incubated at 37° unless otherwise stated. Total volume was 3.0 ml. The reaction was stopped by addition of 1 ml of a 20% trichloroacetic acid solution containing 0.1 M $HgCl_2$. After deproteinization through a filter paper, an equal volume of modified Ehrlich's reagent was added and the absorbance at 555 mμ was read after 15 min. $6.1 \cdot 10^4$ was used as the molecular extinction coefficient at 555 mμ. A unit of enzyme activity was defined as the amount of enzyme which produces 1 nmole of porphobilinogen in 60 min at 37°. Specific activity is the units of enzyme activity per mg of protein.

Since no significant amount of porphyrin was formed from porphobilinogen in crude extracts, no correction for this was applied.

Protein was determined by the method of LOWRY *et al.*[17] with bovine serum albumin as a standard, or spectrophotometrically.

RESULTS

Enzyme purification

Culture of M. phlei. Cells of *M. phlei* (ATCC 19249) were grown on glycerol broth which contained 10 g of bonito extract, 10 g of polypeptone, 3 g of NaCl and 30 ml of glycerol per l. The cells were harvested after 4 days at 37°. The cells collected

by Buchner funnel were washed thoroughly with 0.9% KCl solution and stored at −20° until used.

All subsequent procedures were carried out at 4°.

Preparation of cell free extracts. 20 g of the packed cells were suspended in 80 ml of 0.05 M potassium phosphate buffer, pH 7.5. The suspended cells were disrupted by sonication (9 kcycles, 30 min). Cell free extracts were prepared by centrifuging twice at 14 500 × *g* for 20 min.

$(NH_4)_2SO_4$ fractionation. Cell free extracts were centrifuged at 105 000 × *g* for 120 min. To the supernatant solution, solid $(NH_4)_2SO_4$ was added slowly with gentle stirring to give 30% saturation. After another 30 min stirring, the solution was centrifuged at 10 000 × *g* for 30 min. The supernatant solution was brought to 50% saturation with solid $(NH_4)_2SO_4$. After gentle stirring for another 30 min, the solution was centrifuged at 10 000 × *g* for 30 min. The precipitate obtained was dissolved in a minimum volume of 0.01 M potassium phosphate buffer, pH 6.5.

DEAE-cellulose column chromatography. The dissolved solution was dialyzed against 0.01 M potassium phosphate buffer, pH 6.5, and applied to a DEAE-cellulose column (8.0 cm × 50.0 cm) which was previously equilibrated with the same buffer. After washing the column with 0.01 M, 0.1 M and 0.2 M potassium phosphate buffer, pH 6.5, successively, δ-aminolevulinic acid dehydratase was eluted by 0.3 M potassium phosphate buffer, pH 7.5, containing 0.01 M β-mercaptoethanol.

Hydroxylapatite column chromatography. The eluate from DEAE-cellulose column was concentrated with the aid of a collodion bag and then dialyzed against 0.01 M potassium phosphate buffered saline, pH 7.5, containing 0.01 M β-mercaptoethanol. This enzyme solution was applied to a 1.0 cm × 20.0 cm column of hydroxylapatite which was previously equilibrated with the same buffered saline. Elution was carried out by a linear gradient of potassium phosphate buffer concentration from 0.01 to 0.1 M. The dehydratase came as a sharp peak at around 0.025 to 0.030 M potassium phosphate buffered saline, pH 7.5, containing 0.01 M β-mercaptoethanol (Fig. 1). The results of the typical enzyme purification are summarized in Table I.

Properties of enzyme

pH optimum. The pH optimum was around 9.2 in glycine buffer. As shown in

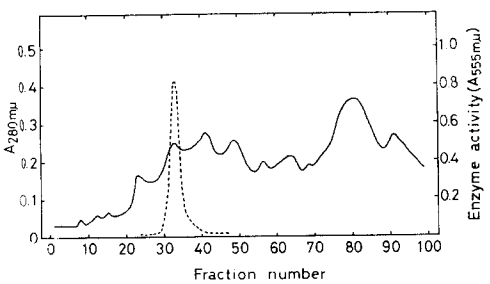

Fig. 1. Elution profile of the dehydratase from hydroxylapatite column. The dehydratase was eluted with a linear gradient of 0.01 to 0.1 M potassium phosphate buffered saline, pH 7.5, containing 0.01 M β-mercaptoethanol. Column size, 1.0 cm × 20 cm; fractions, 3.4 ml each; total volume, 400 ml. 0.2 ml of each fraction was assayed under the conditions described in the text. ———, absorbance at 280 mμ; – – – – –, absorbance at 555 mμ.

TABLE I

PURIFICATION OF δ-AMINOLEVULINIC ACID DEHYDRATASE OF *M. phlei*

Fraction	*Total protein (mg)*	*Specific activity (units/mg)*	*Total activity (units)*
1. Sonicated extracts	8364	4.4	36 900
2. 30–50% $(NH_4)_2SO_4$ fraction	3657	6.5	23 900
3. DEAE-cellulose column chromatography	103	70.1	7 225
4. Hydroxylapatite column chromatography	5.6	704	3 940

Fig. 2, the enzyme was much more active in Tris–HCl buffer. Therefore, enzyme activity was determined using Tris–HCl buffer, pH 9.0, in all subsequent experiments unless otherwise stated.

Effect of Mg^{2+} and EDTA. Mg^{2+} was necessary for the maximum activity of the enzyme. EDTA was a potent inhibitor of the enzyme. Almost complete inhibition

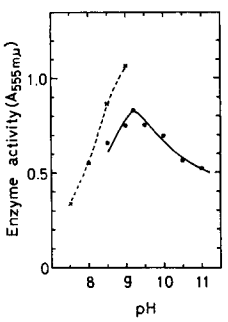

Fig. 2. Effect of pH on the dehydratase activity. 120 μg of the enzyme preparation purified only through DEAE-cellulose column chromatography was used. Enzyme activity was determined in Tris-HCl buffer (×– – – – – –×) or in glycine buffer (●———●).

was obtained by addition of 6.7 μM EDTA, and the inhibition could be recovered by addition of Mg^{2+} (Table II).

Effect of some analogues of δ-aminolevulinic acid. NANDI AND SHEMIN[13] reported that levulinic acid is a competitive inhibitor of δ-aminolevulinic acid dehydratase of *R. spheroides*. We confirmed their observations using the enzyme from *M. phlei* (Fig. 3). δ-Hydroxylevulinic acid which is a reaction product of α-ketoglutarate: glyoxylate carboligase, and δ-hydroxy-γ-oxo-L-norvaline, an antituberculous amino acid, were also inhibitors of the dehydratase (Table III). The kinetics of these inhibitors were also of competitive nature (Fig. 4).

Effect of hemin and K^+. Almost no effect of hemin up to 1.0 mM on the present enzyme preparation was observed. K_2SO_4 was not stimulatory but slightly inhibitory at high concentration; *e.g.* at $1.7 \cdot 10^{-2}$ M, about 38% inhibition was observed.

Effect of α-ketoglutarate:glyoxylate carboligase activity on the dehydratase activity. Inhibitory effect of the carboligase activity on the dehydratase activity was examined in cell free extracts of *M. phlei*. As shown in Table IV, when the substrates

TABLE II

EFFECT OF Mg^{2+} AND EDTA

Each tube contained 30 μg of enzyme, 10 μmoles of δ-aminolevulinic acid, 20 μmoles of β-mercaptoethanol, 100 μmoles of Tris-HCl buffer, pH 9.0, and the indicated amount of $MgSO_4$ and/or EDTA.

Addition	*Enzyme activity (%)*
None	100
0.33 mM $MgSO_4$	141
1.0 mM $MgSO_4$	146
3.3 mM $MgSO_4$	175
1.7 μM EDTA	76
3.3 μM EDTA	27
6.7 μM EDTA	8
0.01 mM EDTA	6
0.01 mM EDTA + 0.33 mM $MgSO_4$	135

and cofactors for the carboligase were added to the cell free extracts, the dehydratase activity was inhibited to the extent of 88%. The inhibition was, however, 12, 13 and 15% when α-ketoglutarate, glyoxylate or thiamine pyrophosphate was added independently. When $MgSO_4$ alone was added, rather 24% activation of the dehydratase

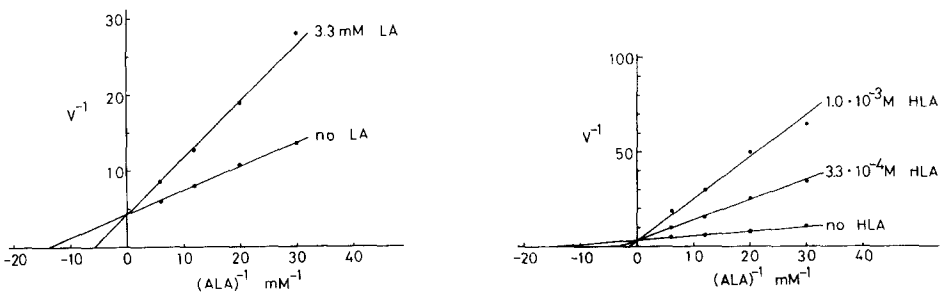

Fig. 3. Double reciprocal plots of initial velocity and δ-aminolevulinic acid concentration in the absence and presence of levulinic acid (LA). 30 μg of enzyme was assayed under the conditions described in the text. ALA, δ-aminolevulinic acid; v, velocity of enzyme activity expressed by absorbance at 555 mμ.

Fig. 4. Double reciprocal plots of initial velocity and δ-aminolevulinic acid concentration in the absence and presence of δ-hydroxylevulinic acid. 44 μg of enzyme was assayed under the conditions described in the text. ALA, δ-aminolevulinic acid; v, velocity of enzyme activity expressed by absorbancy at 555 mμ; HLA, δ-hydroxylevulinic acid. Almost the same result was obtained with δ-hydroxy-γ-oxo-L-norvaline.

activity was observed. 88% inhibition obtained by the simultaneous addition of these four compounds suggests that these substances must have been used as the substrates and cofactors by the carboligase which was contained in the cell free extracts.

TABLE III

EFFECT OF δ-AMINOLEVULINIC ACID ANALOGUES

Each tube contained 15 μg of enzyme and the indicated compounds. Experimental procedures are described in text.

Analogue tested	*Concn. (mM)*	*Inhibition (%)*
δ-Hydroxylevulinic acid	0.67	37
	3.3	76
	10	94
δ-Hydroxy-γ-oxo-L-norvaline	0.67	17
	3.3	36
	10	59
Levulinic acid	10	0
	20	1
	33	29

DISCUSSION

The pH optimum of 9.2 observed in our experiment is a little lower than that of yeast[5] and a little higher than that of *R. spheroides* enzyme[8], but much higher than that of the mammalian enzyme[1,4,7] and a plant enzyme[9]. In general, the pH optimum of the enzyme from microorganisms seems to be higher than that of the animal and the plant.

The K_m value for δ-aminolevulinic acid in this study ($7.7 \cdot 10^{-5}$ M, Figs. 3 and 4) is lower than those reported by all other investigators, *e.g.* $1.4 \cdot 10^{-4}$ M for the ox liver enzyme[7] or $1.0 \cdot 10^{-3}$ M for *R. spheroides* (calculated from ref. 11).

Mg^{2+} was the absolute requirement for the dehydratase of *N. tabacum*[9]. This was the case for the present enzyme preparation. Mg^{2+} was necessary for the maximum activity of the dehydratase and EDTA was inhibitory. These results suggest that Mg^{2+} is absolutely necessary for the activity and EDTA might chelate endogenous Mg^{2+}.

TABLE IV

INHIBITION OF THE DEHYDRATASE ACTIVITY IN THE CELL FREE EXTRACTS BY ADDITION OF THE SUBSTRATES AND COFACTORS FOR THE CARBOLIGASE

Preincubation without δ-aminolevulinic acid was carried out for 60 min. Each tube contained 10 μmoles of δ-aminolevulinic acid, 20 μmoles of β-mercaptoethanol, 100 μmoles of potassium phosphate buffer, pH 7.0, 1 ml of the dialyzed cell free extracts (6.5 mg protein/ml) and the indicated substances.

Addition	*System*				
α-Ketoglutarate, 0.67 mM	+	−	−	−	+
Glyoxilate, 0.67 mM	−	+	−	−	+
$MgSO_4$, 3.3 mM	−	−	+	−	+
Thiamine pyrophosphate, 0.17 mg/ml	−	−	−	+	+
Inhibition (%)	11.8	31.0	−24.0	14.8	88.2

Recently we have reported the inhibitory effect of α-ketoglutarate:glyoxylate carboligase activity on porphyrin synthesis of *M. phlei* and postulated that a reaction product, δ-hydroxylevulinic acid, might inhibit δ-aminolevulinic acid dehydratase competitively[21]. We confirmed this suggestion by demonstrating a direct inhibitory effect of δ-hydroxylevulinic acid on the partially purified dehydratase (Table III, Fig. 4). Inhibition of the dehydratase activity by the substrates and cofactors for the carboligase was observed in the cell free system of *M. phlei* (Table IV). In addition this inhibition was very marked at neutral pH where the carboligase activity is high, and almost no inhibition was observed at 9.0 where the dehydratase is very active and the carboligase is almost inactive (not shown). These findings are very suggestive that α-ketoglutarate:glyoxylate carboligase might be playing a regulatory role on δ-aminolevulinic acid dehydratase by producing δ-hydroxylevulinic acid, which is a competitive inhibitor of the dehydratase. However, the role of this carboligase on the regulation of porphyrin synthesis inside the cell is yet to be assessed.

As for the regulation of porphyrin synthesis, δ-aminolevulinic acid synthase has long been believed to be playing an important role. Recently, however, NANDI *et al.*[8,11] reported some evidence that δ-aminolevulinic acid dehydratase might be the second point of the regulation of porphyrin synthesis in *R. spheroides*. They reported that the enzyme activity *versus* substrate concentration plot shows a sigmoidal response. In addition, this sigmoidality becomes hyperbolic saturation curve in the presence of K^+, Li^+ or Rb^+. Direct conformational changes by K^+ which promotes association of the enzyme to an equilibrium mixture of monomer, dimer and trimer, was observed. Low concentration of hemin or protoporphyrin inhibited the dehydratase activity[8]. Thus the dehydratase of *R. spheroides* exhibits many of the characteristics of an allosteric enzyme which might play some role in the regulation of metabolism. The enzyme from wheat leaves[10,12] and ox liver[12], however, exhibits no allosteric effect by addition of K^+. Recently, SHETTY AND MILLER[12] reported that the dehydratases from these two sources were activated by PO_4^{3-}. In our present study, the dehydratase activity of *M. phlei* was neither dependent on the addition of K^+ nor was inhibited by hemin. These results suggest that there might exist species differences in the regulation mechanism of porphyrin synthesis. Regulatory role of α-ketoglutarate:glyoxylate carboligase activity on this regulation suggested above, is very interesting because the carboligase activity has already been detected in animals[18,19] and in a variety of bacteria (refs. 20, 21 and unpublished observation).

δ-Hydroxy-γ-oxo-L-norvaline, which is also similar in structure to δ-aminolevulinic acid, is an antituberculous amino acid isolated from the culture broth of *Streptomyces akiyoshiensis*[22]. Although the amino acid has been reported to be effective only against strains of human and bovine tubercle bacilli, we found that this compound inhibits the dehydratase activity of a saprophytic acid-fast bacillus, *M. phlei*.

Competitive nature of inhibition on δ-aminolevulinic acid dehydratase caused by δ-hydroxylevulinic acid and δ-hydroxy-γ-oxo-L-norvaline might suggest the formation of a Schiff-base enzyme–substrate intermediate as NANDI AND SHEMIN[13] have reported using another competitive inhibitor, levulinic acid.

REFERENCES

1 A. M. C. BATTLE, A. M. FERRAMOLA AND M. GRINSTEIN, *Biochem. J.*, 104 (1967) 244.
2 B. F. BURNHAM AND J. LASCELLES, *Biochem. J.*, 87 (1963) 462.
3 P. CLASSINO, D. BOSIGNORE AND C. CARTASEGNA, *Biochem. J.*, 101 (1966) 550.
4 D. L. COLEMAN, *J. Biol. Chem.*, 241 (1966) 5511.
5 O. L. C. BARREIRO, *Biochim. Biophys. Acta*, 139 (1967) 479.
6 D. DOYLE AND R. T. SCHIMKE, *J. Biol. Chem.*, 244 (1969) 5449.
7 K. D. GIBSON, A. NEUBERGER AND J. J. SCOTT, *Biochem. J.*, 61 (1955) 618.
8 D. L. NANDI, K. F. BAKER-COHEN AND D. SHEMIN, *J. Biol. Chem.*, 243 (1968) 1224.
9 A. S. SHETTY AND G. W. MILLER, *Biochem. J.*, 114 (1969) 331.
10 D. L. NANDI AND E. R. WAYGOOD, *Can. J. Biochem.*, 45 (1967) 327.
11 D. L. NANDI AND D. SHEMIN, *J. Biol. Chem.*, 243 (1968) 1231.
12 A. S. SHETTY AND G. W. MILLER, *Biochim. Biophys. Acta*, 185 (1969) 458.
13 D. L. NANDI AND D. SHEMIN, *J. Biol. Chem.*, 243 (1968) 1236.
14 H. YAMASAKI AND T. MORIYAMA, *Biochem. Biophys. Res. Commun.*, 38 (1970) 638.
15 A. TISELIUS, S. HJERTEN AND O. LEVIN, *Arch. Biochem. Biophys.*, 65 (1956) 132.
16 D. MAUZERALL AND S. GRANICK, *J. Biol. Chem.*, 219 (1956) 435.
17 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
18 J. KOCH AND E. L. R. STOKSTAD, *Biochem. Biophys. Res. Commun.*, 23 (1966) 585.
19 M. A. SCHLOSSBERG, D. A. RICHERT, R. J. BLOOM AND W. W. WESTERFELD, *Biochemistry*, 7 (1968) 333.
20 T. MORIYAMA AND G. YUI, *Biken J.*, 9 (1966) 263.
21 H. YAMASAKI AND T. MORIYAMA, *Biochem. Biophys. Res. Commun.*, 39 (1970) 790.
22 K. KANAZAWA, K. TSUCHIYA AND T. ARAKI, *Am. Rev. Respirat. Diseases*, 81 (1960) 924.