INHIBITORY EFFECT OF α-KETOGLUTARATE:GLYOXYLATE CARBOLIGASE ACTIVITY ON PORPHYRIN SYNTHESIS IN MYCOBACTERIUM PHLEI

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SUMMARY Effect of an α-ketoglutarate:glyoxylate carboligase on porphyrin synthesis was studied. δ-Hydroxylevulinic acid, a reaction product of the carboligase, inhibited porphyrin synthesis from δ-aminolevulinic acid in cell free extracts of Mycobacterium phlei. When the extracts were incubated with the substrates and the cofactors for the carboligase activity, remarkable inhibition of porphyrin synthesis was observed. The optimum pH of this inhibition was in accordance with that of the carboligase activity. When the 250-fold purified enzyme was added together with the substrates and the cofactors to the 50 to 80 % ammonium sulfate fraction of the cell free extracts, which was almost free from the carboligase activity, about 60 % inhibition was observed.

INTRODUCTION

An α-ketoglutarate:glyoxylate carboligase has been detected in Mycobacterium takeo, a saprophytic acid-fast bacterium, by Moriyama and Yui (2). The enzyme catalyzes the condensation of α-ketoglutarate and glyoxylate to produce δ-hydroxylevulinic acid.

Koch and Stokstad (1) have independently demonstrated that kind of enzyme in rat liver and postulated formation of α-hydroxy-β-ketoadipic acid, an intermediate, which is decarboxylated nonenzymatically to δ-hydroxylevulinic acid. Schlossberg et al. (6) substantiated their postulation and also confirmed the findings of Moriyama and Yui (2) with regard to the structure of δ-hydroxylevulinic acid.

Although partial purification and some characterizations of the carboligase have been done, the biological significance of the enzyme remains to be elucidated.

This paper describes the inhibitory effect of the carboligase activity on porphyrin synthesis in M. phlei.

METHODS

Cells of M. phlei (ATCC 19249) were cultured for 4 days as described in

the previous paper (2). Cell free extracts were prepared by twice centrifugation at 14,500 x g for 30 min of the sonically treated extracts.

The assay procedures of α-ketoglutarate:glyoxylate carboligase activity were described in the previous paper (2). The enzyme was purified 250-fold by ammonium sulfate fractionation, DEAE-cellulose column chromatography, zone electrophoresis and precipitation at pH 5.6 followed by hydroxylapatite column chromatography. The detailed procedures will be described elsewhere.

The amount of porphyrin formed was determined as follows. 1.5 x 18.0 cm test tubes containing 5 μmoles of δ-aminolevulinic acid, 100 μmoles of potassium phosphate buffer, pH 7.5 or 7.0, and varying amounts of the cell free extracts were incubated with shaking at 37°C for 20 hours, unless otherwise stated. The total volume was adjusted to 3.0 ml. The reaction was stopped by addition of five volumes of a mixture of acetic acid-ethyl acetate (1 : 3, v/v). After deproteinization through a filter paper, the ethyl acetate layer was washed thoroughly with distilled water. Porphyrin was extracted with 1.5 N HCl from the layer. The spectra from 380 to 430 mμ were recorded automatically by Hitachi spectrophotometer Model 124. An amount of porphyrin synthesized was determined by calculating the following equation (4):

$$OD_{corr.} = 2 \times OD_{406} - (OD_{380} + OD_{430})$$

The value of ODcorr. was used as an arbitrary unit of porphyrin synthesized.

RESULTS AND DISCUSSION

Nandi and Shemin (3) have shown that δ-aminolevulinate dehydratase of Rhodopseudomonas spheroides is competitively inhibited by levulinic acid. We confirmed that levulinic acid inhibits the porphyrin synthesis from δ-amino-

Table 1 Inhibition of porphyrin synthesis in the cell free extracts by addition of levulinic acid or δ-hydroxylevulinic acid.

		Inhibition (per cent of control)
Levulinic acid	3.3×10^{-2} M	29.6
	6.7×10^{-2} M	53.9
δ-Hydroxylevulinic acid	3.3×10^{-2} M	84.5
	6.7×10^{-2} M	95.1

Preincubation without δ-aminolevulinic acid was carried out for 60 min. The reaction was carried out at pH 7.5 with 1 ml of the cell free extracts (5.9 mg protein/ml) under the conditions in the text.

levulinic acid in cell free extracts of M. phlei. δ-Hydroxylevulinic acid which is also similar in structure to δ-aminolevulinic acid, inhibited porphyrin synthesis more strongly than levulinic acid (Table 1). As shown in Table 2, when the substrates and the cofactors for the carboligase were added to the cell free extracts, porphyrin synthesis was inhibited to the extent of 84 %. The inhibition was, however, 3, 19 or 11 % when α-ketoglutarate, glyoxylate or thiamine pyrophosphate (TPP) was added individually. When $MgSO_4$ alone was added, rather 26 % activation of porphyrin was observed. If a mere additive effect due to the combined addition of these four substances were expected, the net effect is calculated to be 7 % inhibition. 84 % inhibition rather suggests the fact that the substances were used as the substrates or

Table 2 Inhibition of porphyrin synthesis in the cell free extracts by addition of the substrates and cofactors for the carboligase.

α-ketoglutarate	6.7×10^{-3} M	+	-	-	-	+
glyoxylate	6.7×10^{-3} M	-	+	-	-	+
$MgSO_4$	3.3×10^{-3} M	-	-	+	-	+
TPP	0.17 mg/ml	-	-	-	+	+
% inhibition		3	19	-26	11	84

Preincubation without δ-aminolevulinic acid was carried out for 60 min. The reaction was carried out at pH 7.0 with 1 ml of the cell free extracts (5.9 mg protein/ml).

cofactors by the carboligase contained in the cell free extracts, resulting the production of δ-hydroxylevulinic acid, an inhibitor of porphyrin synthesis. Another circumstantial evidence supporting this suggestion is the fact that the maximum inhibition was observed at pH 6.5, which was the optimum pH for the carboligase activity of M. phlei (Fig. 1).

In order to demonstrate the direct inhibitory effect of the carboligase activity, we tried to obtain the porphyrin forming enzyme system free from the carboligase activity. Cell free extracts were fractionated with ammonium sulfate. Most of the activities of both the carboligase and porphyrin forming enzymes from δ-aminolevulinic acid were found in the fraction which was

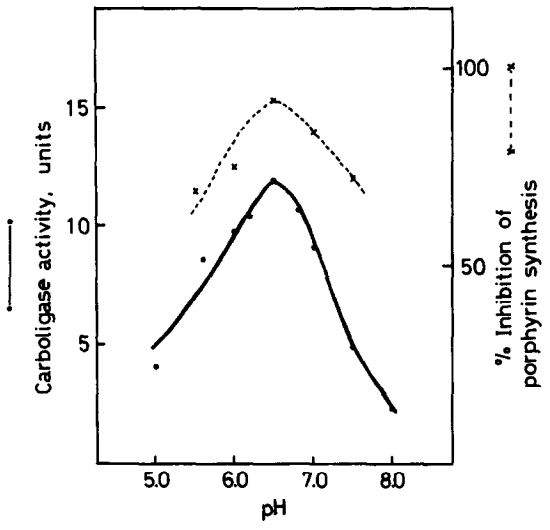

Figure 1 Effect of pH on the inhibition of porphyrin synthesis in the cell free extracts by addition of the substrates and cofactors for the carboligase. The reaction mixture was adjusted to the indicated pH by potassium phosphate buffer. The conditions of the reaction are same as described in Table 2.

Table 3 Ammonium sulfate fractionation of the cell free extracts.

Fraction	carboligase activity %	porphyrin forming activity %
0 - 30 % $(NH_4)_2SO_4$	12.8	8.5
30 - 50 "	85.6	74.4
50 - 80 "	1.6	17.1

precipitated at the salt saturation of 30 to 50 % (Table 3). The 50 - 80 % ammonium sulfate fraction, however, included about 17 % of the total activity of the porphyrin forming enzymes, whereas almost no carboligase activity was detected. To this fraction was added α-ketoglutarate, glyoxylate, $MgSO_4$ and TPP. Only 26 % inhibition of porphyrin synthesis from δ-aminolevulinic acid was observed. When the 250-fold purified carboligase (Fig. 2) was added to the mixture, the inhibition increased: 58.5 and 63.3 % inhibition were observed when 4.4 μg and 8.8 μg of the purified carboligase preparation per ml of the reaction mixture were added respectively (Table 4).

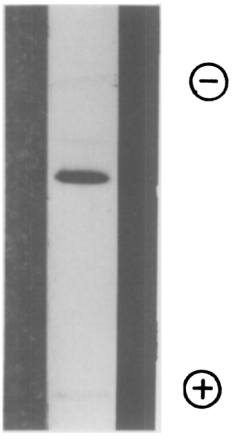

Figure 2 Polyacrylamide gel electrophoresis of 250-fold purified carboligase preparation. 22 μg of protein was applied. Electrophoresis was carried out according to the method of Ornstein and Davis (4).

Table 4 Inhibition of porphyrin forming activity in the 50 to 80 % ammonium sulfate fraction by addition of the purified carboligase.

α-ketoglutarate	6.7×10^{-3} M	-	+	-	+	+
glyoxylate	6.7×10^{-3} M	-	+	-	+	+
$MgSO_4$	3.3×10^{-3} M	-	+	-	+	+
TPP	0.17 mg/ml	-	+	-	+	+
carboligase	4.4 μg/ml	-	-	+	+	-
	8.8 μg/ml	-	-	-	-	+
% inhibition		0.0	25.9	0.0	58.5	63.3

5.4 mg protein of 50 to 80 % ammonium sulfate fraction was used. The reaction was carried out as described in Table 2.

These results provide the evidences that the carboligase activity in the cells has the inhibitory effect on porphyrin synthesis. This inhibitory effect suggests the facts that the carboligase activity might be playing an important role for the regulation of porphyrin synthesis from δ-aminolevulinic acid by producing δ-hydroxylevulinic acid which may inhibit δ-aminolevulinate dehydratase competitively as does levulinic acid.

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