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PURIFICATION, GENERAL PROPERTIES AND TWO OTHER CATALYTIC ACTIVITIES OF α-KETOGLUTARATE:GLYOXYLATE CARBOLIGASE OF *MYCOBACTERIUM PHLEI*

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SUMMARY

1. An α-ketoglutarate:glyoxylate carboligase was purified 200- to 250-fold from the sonic extracts of *Mycobacterium phlei* by $(NH_4)_2SO_4$ fractionation, pH precipitation, starch block electrophoresis and column chromatography.

2. From studies on decarboxylation from ^{14}C-labelled substrates, the enzyme seemed to catalyze the condensation of α-ketoglutarate and glyoxylate to form α-hydroxy-β-ketoadipic acid in the presence of thiamine pyrophosphate. α-Hydroxy-β-ketoadipic acid was spontaneously decarboxylated to δ-hydroxylevulinic acid in the presence of acid.

3. The enzyme had an optimum pH of 6.3 in potassium phosphate buffer at 37°. K_m values for α-ketoglutarate and glyoxylate were 2.0 mM and 3.2 mM, respectively. The isoelectric point, determined by isoelectric focusing, was 5.6.

4. The enzyme activity was markedly activated by Mn^{2+} and was activated to a small extent by Mg^{2+}.

5. *p*-Chloromercuribenzene sulfonic acid, monoiodoacetic acid, EDTA and Zn^{2+} inhibited the enzyme activity.

6. The purified enzyme catalyzed α-ketoglutarate decarboxylase and α-ketoglutarate:acetaldehyde carboligase activities as well as α-ketoglutarate:glyoxylate carboligase activity.

INTRODUCTION

In 1966, MORIYAMA AND YUI[1] described an α-ketoglutarate:glyoxylate carboligase activity in a saprophytic mycobacterium, *Mycobacterium takeo*. The carboligase was partially purified from the particulate fraction of *M. takeo*. KOCH AND STOKSTAD[2] demonstrated this enzyme activity in rat liver mitochondria and suggested the formation of α-hydroxy-β-ketoadipic acid, which was then non-enzymically decarboxylated to δ-hydroxylevulinic acid. This enzyme activity has already been detected in a beef heart particulate fraction[3] and in a number of bacteria, such as *Escherichia coli*, *Micrococcus lysodeikticus*, *Bacillus subtilis* and *Rhodopseudomonas spheroides* (ref. 4 and unpublished results).

Recently, we have reported that δ-hydroxylevulinic acid, a reaction product of α-ketoglutarate:glyoxylate carboligase, is a competitive inhibitor of δ-aminoevulinic acid dehydratase and suggested the regulatory role of the carboligase activity on porphyrin synthesis in *Mycobacterium phlei*[5,6]. WANG *et al.*[7], however, reported the formation of δ-aminolevulinic acid from δ-hydroxylevulinic acid in the rat. They suggested that δ-hydroxylevulinic acid might be converted to α-ketoglutarate and δ-aminolevulinic acid *via* 4,5-dioxovalerate. On the other hand, SCHLOSSBERG *et al.*[8] suggested the formation of α,β-dihydroxy-γ-ketopimelic acid by the condensation of α-hydroxy-β-ketoadipic acid with glyoxylate in beef heart.

SCHLOSSBERG *et al.*[8] purified α-ketoglutarate:gloyxylate carboligase about 100-fold from beef heart and, during the purification procedures, α-ketoglutarate decarboxylase and α-ketoglutarate:acetaldehyde carboligase activities were parallel with that of α-ketoglutarate:glyoxylate carboligase. Thus, they suggested that all these three enzymic activities might be catalyzed by one enzyme.

The present paper describes the preparation of highly purified α-ketoglutarate:glyoxylate carboligase from the sonic extracts of *Mycobacterium phlei*. Some general properties and two other catalytic activities of the purified enzyme were studied.

MATERIALS AND METHODS

Reagents

α-Ketoglutaric acid, L-glutamate dehydrogenase, β-NAD^+ and acetaldehyde were obtained from Sigma; sodium glyoxylate monohydrate, diphenyloxazole, creatine and naphthalene were from Merck; thiamine pyrophosphate, α-naphthol and bonito extract were from Wako Pure Chemical Industries, Ltd., Osaka; polypeptone was from Daigo Eiyo Kagaku Co., Ltd., Osaka; [^{14}C]glyoxylate and DL-[^{14}C]glutamate were from the Radiochemical Centre, Amersham; DEAE-cellulose was purchased from Serva. Diphenyloxazole, naphthalene and dioxane (Nakarai Chemicals Ltd., Kyoto) were of scintillator grade. All other chemicals used were of analytical grade.

Hydroxylapatite was prepared by the method of TISELIUS *et al.*[9].

Standard enzyme assay

The activity of α-ketoglutarate:glyoxylate carboligase was determined by measuring the amount of $^{14}CO_2$ evolved from [1-^{14}C]glyoxylate at 37°, after conversion of α-hydroxy-β-ketoadipic acid to δ-hydroxylevulinic acid (see RESULTS). Each Warburg flask contained, in the main compartment, 20 μmoles of [1-^{14}C]-glyoxylate ($1 \cdot 10^6$ disint./min), 10 μmoles of $MgSO_4$, 0.5 mg of thiamine pyrophosphate, 100 μmoles of potassium phosphate buffer, pH 6.5, and an appropriate amount of enzyme, in a total volume of 3.0 ml. A 1.5 cm × 2.0 cm filter paper equilibrated with 0.1 ml of 20% KOH was put into the centre well. The reaction was initiated by the addition of α-ketoglutarate (0.2 ml, 20 μmoles) from one side arm after 10 min preincubation, and was terminated by the addition of 0.1 ml of 4 M $HClO_4$ from the other side arm. After incubation for a further hour, the contents of the centre well were transferred to a vial containing 10 ml of dioxane scintillator cocktail (5 g PPO, 100 g napthalene and 1000 ml of dioxane). $^{14}CO_2$ evolved was measured in an Aloka Liquid Scintillation Counter 601. Under the assay conditions, reaction rates were constant with respect to time (at least 15 min) and were proportional to protein

concentration when $^{14}CO_2$ evolution was less than 0.1 μmole per min. A unit of enzyme activity is that amount of enzyme which evolves 1 μmole of $^{14}CO_2$ from [1-^{14}C]-glyoxylate in one min at 37°. Specific activity was defined as units of enzyme activity per mg of protein.

α-Ketoglutarate:acetaldehyde carboligase activity

The activity of α-ketoglutarate:acetaldehyde carboligase was determined by the method of BLOOM *et al.*[10] with a slight modification. Test tubes containing 20 μmoles of α-ketoglutarate, 100 μmoles of acetaldehyde, 10 μmoles of $MgSO_4$, 0.25 mg of thiamine pyrophosphate, 100 μmoles of potassium phosphate buffer, pH 7.6, and an appropriate amount of enzyme were incubated at 37°, in a total volume of 3.0 ml. The reaction was stopped by the addition of 0.5 ml of 4 M $HClO_4$. After deproteinization through a filter paper, 1 ml of the reaction mixture was mixed thoroughly with 3 ml of 1% creatine solution, and 1 ml of 5% α-napththol in 3.5 M NaOH was added. The mixture was allowed to stand at room temperature with occasional shaking. Absorbance at 530 nm was read after 120 min.

α-Ketoglutarate decarboxylase activity

The activity of α-ketoglutarate decarboxylase (α-ketoglutarate dehydrogenase, a member of the α-ketoglutarate dehydrogenase complex (EC 1.2.4.2.)) was determined by measuring the amount of $^{14}CO_2$ evolved from [1-^{14}C]α-ketoglutarate which was prepared from DL-[1-^{14}C]glutamic acid with glutamate dehydrogenase. The reaction mixture containing 30 μmoles of DL-[1-^{14}C]glutamic acid ($6 \cdot 10^6$ disint./min), 30 μmoles of β-NAD^+, 0.72 unit of L-glutamate dehydrogenase and 15 μmoles of potassium phosphate buffer, pH 7.5, was incubated for 20 min at room temperature. This reaction mixture, which should contain [1-^{14}C]α-ketoglutarate, was incubated in a Warburg flask with 10 μmoles of $MgSO_4$, 0.25 mg of thiamine pyrophosphate, 100 μmoles of potassium phosphate buffer ,pH 7.0, and the carboligase preparation, in a total volume of 3.0 ml. The reaction was stopped by the addition of 0.1 ml of 4 M $HClO_4$ from a side arm. After another 30 min incubation, the $^{14}CO_2$ evolved was measured as described for the determination of α-ketoglutarate:glyoxylate carboligase activity.

Disc electrophoresis

Polyacrylamide disc gel electrophoresis of native enzyme was carried out essentially according to the method of DAVIS[11]. The enzyme sample containing glycerol was layered directly on the separating gel. Electrophoresis of sodium dodecyl sulfate-treated enzyme was carried out according to the method of DUNKER AND RUECKERT[12].

Isoelectric focusing

Isoelectric focusing was carried out according to the LKB manual with the LKB-Prodokter preparative electric focusing column (110-ml capacity). The enzyme sample was prepared by dialysis against 1% carrier ampholyte, pH 4.0 to 6.0. Sucrose was used as the stabilizing medium.

Protein determination

Protein was determined spectrophotometrically or by the method of LOWRY *et al.*[13] with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

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 were collected by a Buchner funnel, were washed thoroughly with 0.9% KCl solution and stored at −20° until used. Approx. 10 to 15 g of packed cells were obtained per l of medium. All subsequent procedures were carried out at 4°.

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

$(NH_4)_2SO_4$ fractionation. To the cell-free extracts, solid $(NH_4)_2SO_4$ (144 g/l) was added slowly with gentle stirring to give 25% saturation. After another 30 min stirring, the solution was centrifuged at 10 000 × *g* for 30 min. To the supernatant solution, solid $(NH_4)_2SO_4$ (125 g/l) was added to give 45% saturation. After another 30 min gentle stirring, 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 about 10 l of 0.01 M potassium phosphate buffer, pH 6.5, and then the enzyme was centrifuged at 105 000 × *g* for 120 min. The yellow supernatant solution was applied to a DEAE-cellulose column (8 cm × 50 cm) which was previously equilibrated with the same buffer. After washing the column with 0.01 M and 0.1 M potassium phosphate buffer, pH 6.5, successively, α-ketoglutarate:glyoxylate carboligase was eluted by 0.2 M potassium phosphate buffer, pH 6.5.

pH precipitation. The pooled eluate from a DEAE-cellulose column was concentrated with the aid of a collodion bag and then dialyzed against about 10 l of 0.01 M acetate buffer, pH 5.6, for 1 to 2 days. The solution was centrifuged at 10 000 × *g* for 20 min. The precipitate obtained was dissolved in a minimum volume of sodium phosphate buffer, $I = 0.1$, pH 7.5, and then dialyzed against the same buffer.

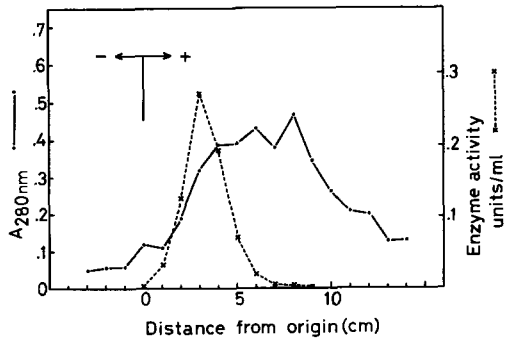

Fig. 1. Zone electrophoretic pattern of α-ketoglutarate:glyoxylate carboligase after precipitation at pH 5.6. 5.7 ml of enzyme solution (93 mg protein) were applied to a starch trough, 1.5 cm × 13 cm × 25 cm, 2 mA/cm² for 50 h.

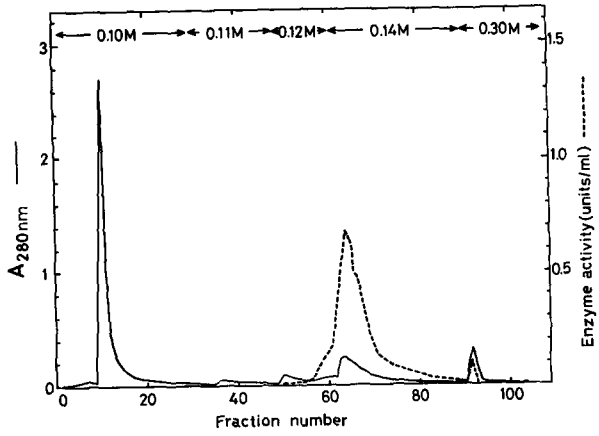

Fig. 2. Elution profile of α-ketoglutarate:glyoxylate carboligase from hydroxylapatite column. Pooled enzyme (about 40 mg of protein) after zone electrophoresis was applied to a 1 cm × 20 cm hydroxylapatite column. The column was washed with the indicated concentration of potassium phosphate-buffered saline, pH 6.5. 5.6-ml fractions were collected.

Zone electrophoresis. The enzyme solution was then applied to zone electrophoresis (starch block electrophoresis). The method described by KUNKEL[14] was employed with a starch block equilibrated with sodium phosphate buffer, $I = 0.1$, pH 7.5. Electrophoresis was carried out at constant current (2 mA/cm^2) for 45 to 55 h. About a 100-mg batch of enzyme solution was applied to a 1.5 cm × 13 cm × 25 cm starch block. After electrophoresis, the block was sectioned at 1-cm intervals and the segments were extracted twice with 10 ml of 0.01 M potassium phosphate-buffered saline, pH 6.5, and the extracts of each segment were filtered through a filter paper. At this stage, the enzyme solution was colorless and clear. The electrophoretic pattern of the enzyme was as shown in Fig. 1.

Hydroxylapatite column chromatography. The extracts were concentrated with the aid of a collodion bag and dialyzed against 2 l of 0.01 M potassium phosphate-buffered saline, pH 6.5. The enzyme solution was applied to a 1 cm × 20 cm hydroxylapatite column which had previously been equilibrated with the same buffered saline. After washing the column with 0.01 M, 0.1 M, 0.11 M and 0.12 M potassium phosphate-buffered saline, pH 6.5, successively, the carboligase was eluted with 0.14 M potassium phosphate-buffered saline, pH 6.5 (Fig. 2). The concentration of the buffered saline which elutes α-ketoglutarate:glyoxylate carboligase from hydroxyl-

TABLE I

TYPICAL PURIFICATION OF α-KETOGLUTARATE:GLYOXYLATE CARBOLIGASE OF *M. phlei*

Fraction	*Total protein (mg)*	*Specific activity (units/mg)*	*Total activity (units)*
1 Sonic extracts	16 736	0.009	148
2 25–45% $(NH_4)_2SO_4$ fraction	5 754	0.022	126
3 DEAE-cellulose column chromatography	792	0.091	72
4 Precipitation at pH 5.6	373	0.134	550
5 Zone electrophoresis	42	0.680	29
6 Hydroxylapatite column chromatography	7.8	2.21	17.2

apatite column seems to differ with each hydroxylapatite preparation. In one case, for example, the carboligase was eluted with 0.115 M to 0.12 M potassium phosphate-buffered saline, pH 6.5. In every case, however, the purity of the enzyme preparation was almost the same: about 200- to 250-fold purification from the sonic extracts was attained.

The typical purification data are summarized in Table I.

Purity of the enzyme preparation

The enzyme preparation eluted from hydroxylapatite column was homogeneous on Sephadex G-200 gel filtration and isoelectric focusing (Fig. 3), but polyacrylamide

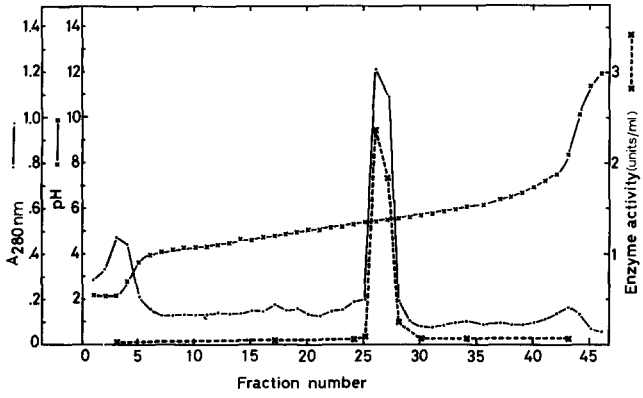

Fig. 3. Isoelectric focusing of purified α-ketoglutarate:glyoxylate carboligase. The eluate from a hydroxylapatite column was concentrated with the aid of a collodion bag giving 4.5 ml of solution containing 6 mg of protein and applied to a preparative electric focusing column (110-ml capacity). Enzyme solution was dialyzed against 1% carrier ampholyte, pH 4.0 to 6.0, for several hours before application to the column. Electrolysis was carried out at constant voltage (300 V) for 45 h at 4°. 2.0-ml fractions were collected after electrolysis and the pH of each fraction was determined. 1.0 ml of 0.05 M potassium phosphate buffer, pH 6.5, was added to each fraction before protein determination and enzymic assay.

disc gel electrophoresis showed one minor protein band which migrated slower than the major protein band (Fig. 4). This minor band was supposed to be an aggregate of the major protein band, because the minor band disappeared completely after sodium dodecyl sulfate treatment of the enzyme preparation.

Properties of the enzyme

Requirements of the enzyme. Requirements of purified α-ketoglutarate:glyoxylate carboligase of *M. phlei* are shown in Table II. KOCH AND STOKSTAD[2] reported 58% activity without addition of thiamine pyrophosphate. No activity was observed with our present enzyme preparation, when thiamine pyrophosphate was omitted from the reaction mixture. This difference might be due to the degree of purity. Mg^{2+} was necessary for the maximum activity.

pH optimum. The pH optimum of the purified enzyme was around 6.3 in potassium phosphate buffer (Fig. 5), which is in good agreement with that of the rat liver enzyme[2].

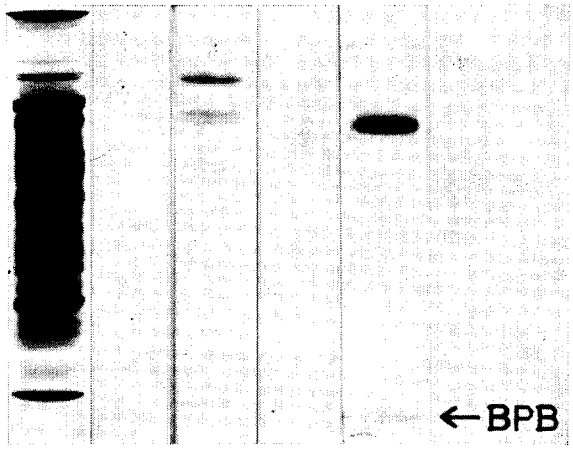

Fig. 4. Polyacrylamide-gel electrophoresis at stages in the purification of α-ketoglutarate:glyoxylate carboligase. Migration was toward the bottom (anode). BPB, bromophenol blue. From left to right, the gels contained aliquots from Steps 3, 5 and 6.

TABLE II

REQUIREMENTS OF THE ENZYME

15 μg of the purified enzyme were assayed as described in the text with the indicated changes.

System	*% Control activity*
Complete	100
α-Ketoglutarate omitted	0
Thiamine pyrophosphate omitted	0
$MgSO_4$ omitted	71

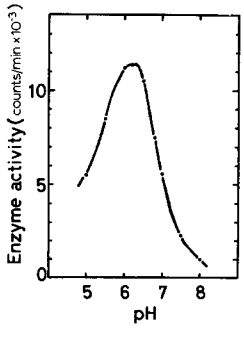

Fig. 5. Effect of pH on the purified carboligase activity. The enzyme was assayed in potassium phosphate buffer of the indicated pH.

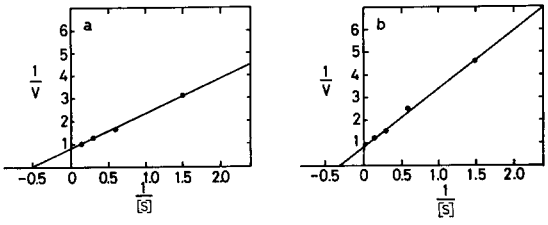

Fig. 6. Double reciprocal plots of initial velocity and substrate concentration. v, velocity of enzyme activity expressed by μmoles CO_2 evolved; $[S]$, concentration of substrate (mM). (a) α-Ketoglutarate was the variable substrate. (b) Glyoxylate was the variable substrate.

K_m values. K_m values for α-ketoglutarate and glyoxylate calculated from double reciprocal plots of substrate concentration and initial velocity, were 2.0 mM and 3.2 mM, respectively, in the presence of thiamine pyrophosphate, Mg^{2+} and saturated concentration of cosubstrate (Fig. 6).

TABLE III

EFFECT OF DIVALENT CATIONS AND EDTA

Each vessel contained 15 μg of enzyme, 20 μmoles of α-ketoglutarate, 20 μmoles of [1-^{14}C]glyoxylate ($1 \cdot 10^6$ disint./min), 0.5 mg of thiamine pyrophosphate, 100 μmoles of Tris–maleate buffer, pH 6.5, and the indicated cations or EDTA.

Addition (1.0 mM)	*% Control activity*
None	100
$MgSO_4$	131
$MnSO_4$	275
$CaCl_2$	100
$ZnSO_4$	3
EDTA	7

Effect of divalent cations and EDTA. Mn^{2+} and Mg^{2+} activated the enzyme activity whereas Ca^{2+} had no effect. Mn^{2+} was more effective than Mg^{2+} in Tris–maleate buffer, but Mn^{2+} caused unfavorable precipitates in phosphate buffer. Because we used phosphate buffer throughout the purification procedures, Mg^{2+}

TABLE IV

EFFECT OF SH GROUP AND SH INHIBITORS

15 μg of enzyme were assayed as described in the text with the changes indicated.

Addition	*% Control activity*
None	100
Glutathione (3.3 mM)	110
2,3-Dimercaptopropanol (3.3 mM)	78
β-Mercaptoethanol (3.3 mM)	116
p-Chloromercuribenzene sulfonic acid (1.0 mM)	0
Monoiodoacetic acid (3.3 mM)	17
N-Ethylmaleimide (3.3 mM)	110

TABLE V

EFFECT OF $HClO_4$ ON $^{14}CO_2$ EVOLUTION

[1-^{14}C]α-Ketoglutarate was prepared from DL-[1-^{14}C]glutamic acid as described for the assay of α-ketoglutarate decarboxylase activity. Incubation was carried out as described in the text with the changes indicated.

Labelled substrate	*System*	$^{14}CO_2$ *(counts/min)*
[1-^{14}C]α-ketoglutarate	$-HClO_4$	45 682
	$+HClO_4$	45 453
[1-^{14}C]Glyoxylate	$-HClO_4$	11 447
	$+HClO_4$	157 713

was chosen for the standard assay system. Zn^{2+} and EDTA were potent inhibitors of the enzyme at concentration of 1 mM. The results are summarized in Table III. These data suggest that the carboligase of *M. phlei* contains Mn^{2+} or Mg^{2+}, and that EDTA might chelate these endogenous metal ions. Further investigations on enzyme structure and the role of these metal ions for the enzyme activity are awaited.

Effect of sulfhydryl reagents. *p*-Chloromercuribenzene sulfonic acid and monoiodoacetic acid inhibited the enzyme activity, but *N*-ethylmaleimide had no effect. 2,3-Dimercaptopropanol, reduced glutathione and β-mercaptoethanol were not activators of the enzyme (Table IV).

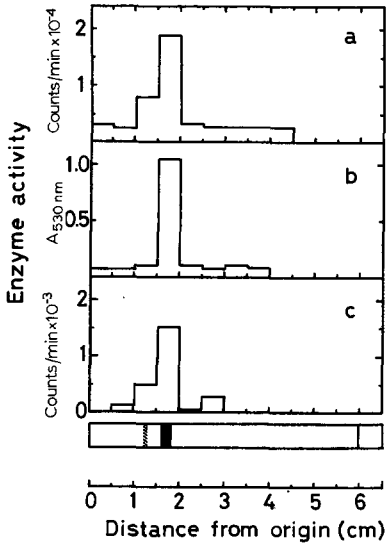

Fig. 7. Three enzymic activities of the purified enzyme. After polyacrylamide disc gel electrophoresis (2 mA per tube), gels were sliced into 5-mm sections. These sections were suspended in respective reaction mixture by means of a small glass homogenizer. The migration pattern of the protein is shown at the bottom. The band which appeared at 6 cm from origin is bromophenol blue which was used as a tracking dye (see Fig. 4). (a) α-Ketoglutarate:glyoxylate carboligase activity: About 200 μg of the purified enzyme were applied to a column. Each section was assayed as described in the text except that 0.16 μmole of [1-^{14}C]glyoxylate ($1 \cdot 10^6$ disint./min) was used. Incubation was carried out for 12 h. (b) α-Ketoglutarate:acetaldehyde carboligase activity: About 200 μg of the purified enzyme were applied to a column. Enzymic assay was carried out as described in the text for 6 h. (c) α-Ketoglutarate decarboxylase activity: 300 μg of the purified enzyme were applied to a column and enzymic assay was carried out as described in the text for 10 h.

Reaction mechanism. KOCH AND STOKSTAD[2] suggested that α-ketoglutarate:glyoxylate carboligase catalyzes the condensation of α-ketoglutarate and glyoxylate to form an unstable intermediate, α-hydroxy-β-ketoadipic acid, which might be decarboxylated non-enzymically to δ-hydroxylevulinic acid in the presence of acid. We confirmed these findings with highly purified enzyme preparations. As shown in Table V, $^{14}CO_2$ evolution from [1-^{14}C]glyoxylate increased more than 14 times after the addition of perchloric acid whereas $^{14}CO_2$ evolution from [1-^{14}C]α-ketoglutarate (prepared from [1-^{14}C]glutamic acid) did not increase. These results suggest that α-ketoglutarate:glyoxylate carboligase catalyzes the condensation of these two substrates with the decarboxylation from α-ketoglutarate, and then the decarboxylation from glyoxylate proceeds non-enzymically in the presence of acid to produce δ-hydroxylevulinic acid.

$$HOOC{-}CH_2{-}CH_2{-}CO{-}\overset{*}{C}OOH + CHO{-}\dot{C}OOH \xrightarrow{\text{Thiamine pyrophosphate, } Mg^{2+}} HOOC{-}CH_2{-}CH_2{-}CO{-}CH(OH){-}COOH + \overset{*}{C}O_2$$

$$\xrightarrow{H^+} HOOC{-}CH_2{-}CH_2{-}CO{-}CH_2OH + \dot{C}O_2$$

α-Ketoglutarate decarboxylase and α-ketoglutarate:acetaldehyde carboligase activity of α-ketoglutarate:glyoxylate carboligase. Fig. 7 shows α-ketoglutarate:glyoxylate carboligase, α-ketoglutarate decarboxylase and α-ketoglutarate:acetaldehyde carboligase activities of the purified enzyme after polyacrylamide disc gel electrophoresis. After electrophoresis, the gels were sliced into 5-mm sections and were assayed individually for respective enzymic activities. Each section was homogenized thoroughly before enzymatic assay by means of a small glass homogenizer. One of the columns was stained to visualize the migration pattern of the enzyme. As shown in Fig. 7, all of these three enzyme activities were in the major protein band.

These results show that one enzyme (or possibly, an enzyme complex) has three different catalytic activities, those of α-ketoglutarate:glyoxylate carboligase, α-ketoglutarate decarboxylase and α-ketoglutarate:acetaldehyde carboligase. SCHLOSSBERG *et al.*[8] have already suggested that these three enzyme activities might be catalyzed by one enzyme. Our findings described here provide much more direct evidence that the enzyme which catalyzes the condensation of α-ketoglutarate and glyoxylate to form α-hydroxy-β-ketoadipic acid is also responsible for single decarboxylation from α-ketoglutarate (α-ketoglutarate dehydrogenase, a member of α-ketoglutarate dehydrogenase complex) and for the condensation of α-ketoglutarate and acetaldehyde to form δ-hydroxy-γ-ketohexanoic acid.

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