Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

5. PURIFICATION AND PROPERTIES OF GLYOXYLIC DEHYDROGENASE*

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An enzyme, glyoxylic dehydrogenase, which catalyses a coenzyme A (CoA)- and triphosphopyridine nucleotide (TPN)-dependent oxidation of glyoxylate to oxalate

was discovered in extracts of oxalate-grown *Pseudomonas oxalaticus* by Quayle, Keech & Taylor (1960, 1961). The enzyme was partially purified and some of its properties were studied. It was suggested that oxalyl-coenzyme A was an intermediate in the reaction

and that reactions (2) and (3) were reversible.

Evidence was presented which indicated that the reversal of glyoxylic dehydrogenase, i.e. the reductive synthesis of glyoxylate from oxalyl-coenzyme A, occupies a key role in microbial synthesis of cell constituents from oxalate. The enzyme is under adaptive control, being synthesized by *P. oxalaticus* during growth on oxalate, but is absent during growth on other substrates.

This paper records a purification of the enzyme obtained from *P. oxalaticus*, with procedures of ammonium sulphate precipitation, gel absorption and ion-exchange chromatography, and an examination of its properties.

* Part 4: Quayle, Keech & Taylor (1961).

MATERIALS AND METHODS

Special chemicals. Coenzyme A (75% pure) was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., TPN from C. F. Boehringer und Söhne, Mannheim, Germany, and sodium glyoxylate (95% pure) was a gift from Dr I. Zelitch.

Growth of the organisms. *P. oxalaticus* was grown in liquid culture (10 l. batches) in a growth medium consisting of sodium oxalate and mineral salts, harvested by centrifuging and stored at -15° . The experimental details have been reported by Quayle & Keech (1960). *Pseudomonas* OD1, obtained from the National Collection of Industrial Bacteria (Culture no. 8735), was grown on malate and oxalate as described by Jayasuriya (1955).

Purification of glyoxylic dehydrogenase

Step 1: preparation of bacterial extract. Frozen bacteria (32 g., wet wt.) were crushed in a Hughes (1951) press at -25° and then mixed with 250 ml. of 0.03 M-sodium phosphate-potassium phosphate buffer (pH 7.6) at 2° containing approx. 2 mg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.). The resulting extract was centrifuged at 16 000 g for 20 min. and the solid residue discarded. All subsequent operations were performed at 2° .

Step 2: treatment with protamine sulphate. To the supernatant solution (210 ml.) was added, with stirring, 135 mg. of protamine sulphate. The curdy precipitate was centrifuged down and discarded.

Step 3: ammonium sulphate precipitation and dialysis. Ammonium sulphate (62.5 g.) was added with stirring to the supernatant solution (205 ml.) to give 40% saturation. The precipitated protein was separated by centrifuging and dissolved in 0.03 M-sodium pyrophosphate buffer (pH 8.5) to a final volume of 10.6 ml. The supernatant solution (220 ml.) was brought to 60% saturation by the addition of ammonium sulphate and the precipitated protein was dissolved in pyrophosphate buffer to a final volume of 9.2 ml. Finally, the ammonium sulphate concentration in the resulting supernatant solution (235 ml.) was increased to 80% and the precipitated protein was dissolved to a volume of 7.6 ml. in pyrophosphate buffer. The pH was maintained at 7.0-7.2 throughout the precipitation procedure by occasional addition of 0.1 N-NaOH. The protein fraction precipitating between 40 and 60% saturation was dialysed for 1.5 hr. against 3 l. of 0.03 M-sodium pyrophosphate buffer (pH 7.8). This solution (11.7 ml.) was stored overnight at -15° .

Step 4: alumina Cy-gel adsorption. The dialysed fraction was diluted to 114 ml. with water and the enzyme adsorbed on to Cy-gel by treatment with 0.34 vol. of gel (30.6 mg./ml.). The gel was separated by centrifuging and the adsorbed enzyme was eluted in four successive extractions with 30 ml. of 0.01 M-sodium phosphate-potassium phosphate buffer (pH 7.5). The four eluates were combined, giving a total volume of 113 ml.

Step 5: concentration of enzyme by ammonium sulphate precipitation, followed by dialysis. To the combined eluates ammonium sulphate was added to 80% saturation. The precipitated protein was separated by centrifuging and dissolved to a final volume of 9.6 ml. in 3 mM-2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7.3). The solution was dialysed against 3 l. of the same buffer for 2 hr.

Step 6: ion-exchange chromatography. A slurry of 5 g. of diethylaminoethylcellulose (DEAE-cellulose, Whatman DE50) in 3 mM-tris buffer (pH 7.3) was poured into a chromatographic column (2.5 cm. $\times$ 25 cm.) and this was washed with 1 l. of the same buffer. The dialysed extract was diluted with an equal volume of water and was applied to the column, which was subsequently washed with 200 ml. of the same buffer. The enzyme is not eluted under these conditions. The column was then eluted with a solution of tris buffer-potassium chloride in which the potassium chloride formed a gradient of increasing concentration.

Fig. 1. Elution of glyoxylic dehydrogenase from DEAE-cellulose column. For conditions, see text. O, Glyoxylic-dehydrogenase activity (units/ml.); ●, glyoxylic-dehydrogenase specific activity (units/mg. of protein).

This was formed by drawing the column eluent from 600 ml. of 3 mM-tris buffer (pH 7.3) connected by siphon tube to 600 ml. of M-potassium chloride. The levels of solution in both flasks dropped at the same rate throughout. The flask containing the tris buffer was stirred continuously. Fractions of eluate (approx. 6.5 ml.) were collected automatically, at a flow rate of 40–50 ml./hr. Under these conditions the glyoxylic dehydrogenase was eluted mainly in fractions 49–54 (Fig. 1). These were combined and the resulting solution (41 ml.) was stored as separate 2 ml. samples at -15° .

A summary of the purification procedure is given in Table 1.

Assay of glyoxylic-dehydrogenase activity

The enzyme was assayed in a Cary recording spectrophotometer by measuring the rate of TPN reduction in the following reaction mixture, at 25° . The complete system, in a 1.5 ml. silica cell (light-path, 1 cm.), consisted of 27 μ moles of sodium pyrophosphate buffer (pH 8.6), 2.5 μ moles of cysteine, 5 μ moles of sodium glyoxylate, 0.2 μ mole of CoA, 0.2 μ mole of TPN, enzyme extract and water to 1 ml. CoA and TPN were omitted from the blank cell. The reaction was started by addition of the TPN and the rate of its reduction was measured as ΔE at 340 m μ over 3 min. The initial rate was proportional to the amount of enzyme used. Cysteine was included in the reaction mixture as its presence enhanced the linearity of the rate of TPN reduction with time at the higher reaction rates. One unit of enzyme activity is defined as the amount of enzyme which catalyses the reduction of 1 μ mole of TPN in 1 min. under the conditions of assay. Specific activity is expressed as units/mg. of protein.

Protein determination. Protein was estimated by the method of Folin & Ciocalteu (1927).

RESULTS

Stability and pH optimum. The activity of glyoxylic dehydrogenase rapidly diminishes in crude cell-free extracts and losses of up to 70% of

Table 1. *Purification of glyoxylic dehydrogenase*

For experimental details see the Materials and Methods section.

Step	Vol. (ml.)	Units/ml.	Concn. of protein (mg./ml.)	Specific activity (units/mg. of protein)	Total units	Yield (%)
1 Supernatant obtained from crude extract after centrifuging	210	1.87	6.4	0.29	395	100
2 Supernatant obtained after removal of protamine sulphate precipitate by centrifuging	205	1.93	6.3	0.30	395	100
3 Fraction precipitated by ammonium sulphate (40–60%)	9.2	40.5	66	0.61	373	94
4 Combined eluates obtained from Cy-gel adsorption, after dialysis	121	2.28	1.37	1.7	273	69
5 Precipitation of protein by ammonium sulphate (80%) followed by dialysis of dissolved precipitate	9.6	15.1	11	1.4	145	37
6 Pooled selected fractions after column chromatography	41	1.0	0.24	4.2*	41	10

* The peak of activity was 7.2.

the activity have been observed in 6 hr. at 0° (Quayle *et al.* 1961). Considerable losses of enzyme activity at various stages of purification were commonly encountered, but these were kept to a minimum by carrying out the initial purification procedures as quickly as possible. Purification resulted in much greater stability (Table 2).

The pH optimum for oxidation of glyoxylate by the partially purified enzyme is 8.6 (Quayle *et al.* 1961) and this figure is not altered on further purification of the enzyme.

Absence of interfering enzymes. The glyoxylic dehydrogenase was free of any detectable reduced pyridine nucleotide-oxidase activity, pyridine nucleotide-linked glyoxylic acid-reductase or glycollic acid-dehydrogenase activities, or of glyoxylic acid-carboligase activity (Krakow, Hayashi & Barkulis, 1959).

Substrate specificity. Glyoxylic dehydrogenase is specific for glyoxylate: no activity was observed with formaldehyde, acetaldehyde, glycolaldehyde, pyruvate, oxaloacetate, α -oxoglutarate or hydroxypyruvate. The enzyme is specific for TPN; no activity was observed with DPN. The requirement for CoA cannot be met with other thiol-containing compounds such as cysteine, glutathione, thioethanolamine or pantetheine.

Activators. No metal-ion requirement by the enzyme has been found. Its activity is not altered by the presence of 5 mM-ethylenediaminetetraacetate in the complete reaction system.

Inhibitors. No inhibition of the enzyme activity was observed when the following compounds were added to the standard assay system at a final concentration of 1 mM: formaldehyde, glyoxal, acetaldehyde, glycolaldehyde, pyruvate, oxaloacetate, α -oxoglutarate, glycollate, hydroxypyruvate, glycinate, 3-phosphoglycerate, malate.

It had previously been noted (Quayle *et al.* 1961) that the reaction catalysed by glyoxylic dehydrogenase was markedly inhibited if tris buffer was used in place of pyrophosphate buffer. This effect has also been observed with the purified enzyme

and it is found that the extent of the inhibition increases with time of preincubation of the enzyme in tris buffer (0.03 M, pH 8.6) at room temperature.

Kinetic properties. The effect of varying the concentration of glyoxylate, TPN and CoA, singly, on the initial rate of reduction of TPN catalysed by glyoxylic dehydrogenase was studied. In each case the relationship followed that predicted by the Michaelis-Menten equation. Plots of the reciprocal of the reaction velocity against the reciprocal of

Fig. 2. Lineweaver-Burk plot of the effect of glyoxylate concentration on the initial rate of TPN reduction in the presence of constant amounts of TPN and CoA, catalysed by glyoxylic dehydrogenase. The complete system, contained in a 1 ml. quartz cell, consisted of 21 μmoles of sodium pyrophosphate buffer (pH 8.6), 3.5 μmoles of cysteine, 0.8 μmole of CoA, 0.8 μmole of TPN, 0.1 ml. of enzyme, glyoxylate and water to 1 ml. The blank cell lacked CoA and TPN. Reaction was started by addition of TPN.

Fig. 3. Lineweaver-Burk plot of the effect of TPN concentration on the initial rate of TPN reduction in the presence of constant amounts of glyoxylate and CoA, catalysed by glyoxylic dehydrogenase. Experimental conditions were as described under Fig. 2 except that 2 μmoles of glyoxylate were present in the reaction system.

Table 2. Stability of glyoxylic dehydrogenase in pooled fractions from diethylaminoethylcellulose column

Results are expressed as percentages of the initial activity of the enzyme.

Temp.	Time of standing	Enzyme activity (%)
-15°	3 weeks	100
0	5 hr.	100
	24 hr.	48
25	5 hr.	45
	24 hr.	1.5
51-52	2 min.	0

the substrate concentration (Lineweaver & Burk, 1934) are shown in Figs. 2 and 3. The K_m values at pH 8.6 and 25° for glyoxylate and TPN are 5.7×10^{-4} M and 3.4×10^{-5} M respectively.

Under conditions in which the enzyme, glyoxylate and TPN were in excess, while CoA was limiting, it was observed that the rate of TPN reduction showed little diminution even after the reduction of five equivalents of TPN for each equivalent of CoA initially present. Such behaviour was also found with the partially purified enzyme (Quayle *et al.* 1961). It was suggested that this was due to an enzymic or chemical hydrolysis of oxalyl-CoA as in equation (4). This question has not been clarified in the present study. Acetyl-CoA is stable in 30 mM-pyrophosphate buffer (pH 8.6) in the presence of the glyoxylic dehydrogenase, as judged by its extinction at 232 m μ . Hence, the postulated breakdown of oxalyl-CoA must be due either to its much greater instability, compared with acetyl-CoA, or to an enzymic breakdown, such as the action of a deacylase which was not removed by the enzyme-purification procedure and which does not attack acetyl-CoA. Chemical instability of oxalyl-CoA at pH 8.6 would not be unexpected in view of the well-known lability of oxalic esters, e.g. the methyl and ethyl oxygen esters are hydrolysed in cold water without addition of alkali. Although a linear Lineweaver-Burk plot was obtained when the concentration of CoA was varied in the presence of excess amounts of TPN and glyoxylate (Fig. 4), the significance of the K_m so obtained (1.8×10^{-4} M) will depend on whether the breakdown of oxalyl-CoA is enzymic or not. If it is enzymic, then this K_m will vary with

Fig. 4. Lineweaver-Burk plot of the effect of CoA concentration on the initial rate of TPN reduction in the presence of constant amounts of glyoxylate and TPN, catalysed by glyoxylic dehydrogenase. Experimental conditions were as described under Fig. 2 except that 2 μ moles of glyoxylate were present in the reaction system.

the relative proportions of the deacylase- and glyoxylic-dehydrogenase enzymes in any particular preparation.

Occurrence of the enzyme in Pseudomonas OD 1. Glyoxylic-dehydrogenase activity (0.91 μ mole of TPN reduced/min./mg. of protein under the standard assay conditions) was found in oxalate-grown *Pseudomonas OD 1*, whereas extracts of the same organism grown on malate showed an activity of 0.16 μ mole of TPN reduced/min./mg. of protein.

DISCUSSION

The present study shows that glyoxylic dehydrogenase is an enzyme specific for the oxidation of glyoxylate in the presence of TPN and CoA. It is thus similar to the acetaldehyde dehydrogenase discovered in *Clostridium kluyveri* by Burton & Stadtman (1953) and subsequently found in *Escherichia coli* by Dawes & Foster (1956). This enzyme catalyses the reaction:

It is freely reversible, with a value of -4.2 kcal. for $\Delta G'$ at pH 7.0 and 25° as determined from equilibrium measurements.

The question of the reversibility of glyoxylic dehydrogenase is an important one in connexion with the synthesis of cell constituents from oxalate. From the free-energy data of Burton (1961) it can be calculated that the free-energy change $\Delta G'$ for reaction (1) is -8.1 kcal. Assuming a value of -9 kcal. for $\Delta G'$ of reaction (6) (Burton, 1957)

it follows that $\Delta G'$ of reaction (7) is $+0.9$ kcal.:

Thus on thermodynamic grounds reaction (7) would be expected to be freely reversible. Indirect evidence indicated this to be the case (Quayle *et al.* 1961). Authentic oxalyl-CoA has not, as yet, been synthesized in substrate amounts and in its absence a direct demonstration of reversibility of the purified enzyme cannot be made. The occurrence of side reactions, e.g. reaction (6), precludes a measurement of the reaction equilibrium.

The suggestion that the enzyme plays a key role in microbial growth on oxalate is further supported by its presence in oxalate-grown *Pseudomonas OD 1* and its decrease in activity to one-sixth when the organism is grown on malate.

SUMMARY

1. A purification of glyoxylic dehydrogenase, obtained from oxalate-grown *Pseudomonas oxalaticus*, is described. The purification involves ammonium sulphate precipitation, gel absorption and ion-exchange chromatography.

2. The pH optimum for the coenzyme A- and triphosphopyridine nucleotide-linked oxidation of glyoxylate to oxalate is 8.6.

3. The glyoxylic dehydrogenase is free of the following enzymic activities: reduced pyridine nucleotide oxidase, pyridine nucleotide-linked glyoxylic acid reductase or glycollic acid dehydrogenase, and glyoxylic acid carboligase.

4. The enzyme is specific for both triphosphopyridine nucleotide and coenzyme A. Diphosphopyridine nucleotide will not replace the former nor will cysteine, glutathione, thioethanolamine or pantetheine replace the latter. In the presence of triphosphopyridine nucleotide and coenzyme A the enzyme will not oxidize formaldehyde, acetaldehyde, glyceraldehyde, pyruvate, oxaloacetate, α -oxoglutarate or hydroxypyruvate. The presence of any of these compounds at 1 mM concentration does not inhibit the oxidation of glyoxylic acid catalysed by the enzyme.

5. No metal-ion requirement for the action of glyoxylic dehydrogenase has been found.

6. K_m values of 5.7×10^{-4} M and 3.4×10^{-5} M, at pH 8.6 and 25°, have been found for glyoxylate and triphosphopyridine nucleotide respectively. Coenzyme A is required for the oxidation in catalytic quantities.

7. The free-energy changes involved in the oxidation of glyoxylate are discussed.

8. Glyoxylic dehydrogenase has also been found in extracts of oxalate-grown *Pseudomonas* OD1. This activity is decreased to one-sixth when the same organism is grown on malate.

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Glutamic-Oxaloacetic Transaminase of Cauliflower

1. PURIFICATION AND SPECIFICITY

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Although the scope of the transamination reaction in extracts of higher plants has been amply demonstrated (Wilson, King & Burris, 1954), little information is available on the enzymology of plant

transaminases. Cruickshank & Isherwood (1958) reported the partial purification of glutamic-oxaloacetic and glutamic-pyruvic transaminases from wheat-germ without giving details of the purification obtained; although the two activities were not separated, evidence was obtained which indicated that different enzymes were responsible for the two activities, as is the case in animal

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