The Purification of Glycerate Kinase from *Hyphomicrobium* sp. and *Pseudomonas* AM1: Product Identification

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INTRODUCTION

Aerobic bacteria grown on methanol can utilize one of two pathways for carbon assimilation, either the serine pathway or the pentose phosphate cycle of formaldehyde fixation. Both *Pseudomonas* AM1 (Heptinstall & Quayle, 1970; Harder & Quayle, 1971) and *Hyphomicrobium* x and G (Harder & Attwood, 1973) utilize the serine pathway. During work on the carbon assimilation pathway of methanol-grown *Hyphomicrobium* sp. the relative rates of conversion of glycerate, 2-phosphoglycerate and 3-phosphoglycerate into phosphoenolpyruvate indicated that 2-phosphoglycerate was the product of the glycerate kinase. The conversion of 2-phosphoglycerate into 3-phosphoglycerate may be the first step in the biosynthesis of cellular material (Harder, Attwood & Quayle, 1973). Glycerate kinase has been purified from both methanol-grown *Hyphomicrobium* x and *Pseudomonas* AM1 and the product of the reaction identified.

METHODS

Organisms. The maintenance of *Hyphomicrobium* x and growth in liquid inorganic salts medium with methanol (0.5 %, v/v) was as described by Harder *et al.* (1973). *Pseudomonas* AM1 (NCIB9133) was maintained and grown in liquid culture as described by Salem, Hacking & Quayle (1973) using methanol (0.5 %, v/v) as carbon source.

Preparation of cell-free extracts. Bacteria were disrupted using an ultrasonic disintegrator (Harder *et al.* 1973). The supernatant fluids after centrifugation at 15000g for 15 min at 0 °C were used as the crude extracts.

Enzyme assays. Glycerate kinase (EC. 2.7.1.31) was assayed by the method described by Heptinstall & Quayle (1970). Phosphoglycerate mutase (EC. 2.7.5.3) was assayed by a modification of the method described in the Biochemica catalogue (1968), C. F. Boehringer und Soehn GmbH, Mannheim, Germany (Harder *et al.* 1973).

Protein determination. Protein was assayed by the Folin Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex) was used as the standard.

Purification. All buffers used during the purification contained mercaptoethanol (2 mM) and magnesium chloride (5 mM). The crude enzyme extract was brought to 30 % saturation with ammonium sulphate and allowed to stand at 0 °C for 30 min. The precipitate was collected by centrifugation at 15000g for 10 min and then discarded. The supernatant was then brought to 50 % saturation by a further addition of ammonium sulphate and allowed to stand as before. The precipitate was collected by centrifugation, resuspended in 50 mM-sodium phosphate buffer, pH 7.3, and applied to a Sephadex G-50 column (15 × 1.5 cm)

Table 1. *Purification of glycerate kinase from Hyphomicrobium x and Pseudomonas AM1*

Fraction	Total activity ($\mu\text{mol/min}$)	Total protein (mg)	Specific activity ($\mu\text{mol/min/mg protein}$)	Yield (%)	Purification
<i>Hyphomicrobium</i> extract					
Crude enzyme	6.9	94.0	0.07	100	—
50% $(\text{NH}_4)_2\text{SO}_4$ precipitate	4.0	25.5	0.16	58.3	2.3 $\times$
Sephadex G-50 filtration	3.0	15.0	0.20	43.5	2.9 $\times$
DEAE-cellulose chromatography	2.2	0.8	2.73	31.9	37.4 $\times$
<i>Pseudomonas</i> AM1 extract					
Crude enzyme	6.2	152.8	0.04	100	—
50% $(\text{NH}_4)_2\text{SO}_4$ precipitate	1.5	81.9	0.02	24.2	0.4 $\times$
Sephadex G-50 filtration	1.1	53.4	0.02	17.7	0.4 $\times$
DEAE-cellulose chromatography (mutase free)	1.3	5.3	0.24	21.0	5.9 $\times$
DEAE-cellulose chromatography (contaminated with mutase)	0.3	5.2	0.05	4.8	1.3 $\times$

equilibrated with 10 mM-sodium phosphate buffer, pH 7.0. The column was eluted and fractions (1.7 ml) were collected and monitored at 280 nm; the flow rate was 0.82 ml/min. Fractions showing absorbance at 280 nm were assayed for glycerate kinase activity. All tubes showing activity were bulked (10 ml) and placed at the top of a DEAE-cellulose DE-52 column (15 $\times$ 1.5 cm) equilibrated with 10 mM-sodium phosphate buffer, pH 7.0. The column was eluted using the same buffer containing a continuous linear gradient of sodium chloride (0 to 1 M). Fractions (4 ml) were collected and monitored as before for absorbance at 280 nm. The flow rate was 0.4 ml/min. Tubes were assayed for glycerate kinase activity. Those showing activity were then assayed for phosphoglycerate mutase activity.

Product identification. The purified glycerate kinase free from phosphoglycerate mutase activity (1.3 ml) was incubated in the following mixture at 30 °C for 15 min: sodium phosphate buffer pH 7.3, 100 μmol ; ATP, 5 μmol ; glycerate, 4 μmol ; EDTA, 3 μmol ; and magnesium chloride, 30 μmol , in a total volume of 3.8 ml. The reaction was terminated by placing the tube in ice at 0 °C. Samples of the incubation mixture were taken for product identification by the enzymic method of Czok & Eckbert (1965). 2-Phosphoglycerate is converted to L-lactate by the addition of commercially purified enzymes. The NADH used in the production of lactate from pyruvate is followed spectrophotometrically at 340 nm.

The reaction mixture for product identification contains tris buffer pH 7.3, 50 μmol ; ADP, 0.5 μmol ; NADH, 0.15 μmol ; 2,3-diphosphoglyceric acid, 0.01 μmol ; sample and water to 1.0 ml. The extinction at 340 nm was recorded and then the linkage enzymes were added separately and in excess in the following order: lactic dehydrogenase, pyruvate kinase, phosphopyruvate hydratase and phosphoglycerate mutase. After each addition the total change in extinction at 340 nm was recorded.

Fig. 1. 280 nm absorption pattern of fractions eluted from DEAE cellulose columns. (a) Extract from methanol-grown *Hyphomicrobium* x. (b) Extract from methanol-grown *Pseudomonas* AM1. $\circ$, Kinase activity; Δ , phosphoglycerate mutase activity; broken line, extinction at 280 nm; solid line, sodium chloride gradient.

RESULTS AND DISCUSSION

The purification of the enzyme was followed by measuring the glycerate kinase activity at each purification step. A summary of the purification scheme is shown in Table 1. Only one peak of glycerate kinase activity was detected from the DEAE cellulose columns (Fig. 1a, b). All the tubes showing glycerate kinase were then assayed for phosphoglycerate mutase activity. The whole of the *Hyphomicrobium* glycerate kinase peak was free from

phosphoglycerate mutase activity. When *Pseudomonas* AM1 extracts were used the initial fractions of phosphoglycerate mutase activity were eluted together with the later fractions of glycerate kinase activity (Fig. 1*b*). In this case only the early fractions of glycerate kinase free from phosphoglycerate mutase were used for product identification.

The product of the glycerate kinase from both organisms was identified as 2-phosphoglycerate. No decrease in extinction at 340 nm was observed upon the addition of lactic dehydrogenase or pyruvate kinase. Thus pyruvate and phosphoenolpyruvate were not present in the product identification mixture. On the addition of phosphopyruvate hydratase a rapid decrease in extinction at 340 nm was observed, indicating the presence of 2-phosphoglycerate. No further reduction in optical density occurred upon the addition of commercially purified phosphoglycerate mutase. This was not due to the exhaustion of NADH or any other component of the product identification mixture, since the addition of 3-phosphoglycerate rapidly reduced the extinction further. Thus no 3-phosphoglycerate was originally present in the product identification mixture and the product of the glycerate kinase reaction was 2-phosphoglycerate. From the total change in extinction on the addition of each separate linkage enzyme the actual amount of product in the sample can be calculated. The total amount of 2-phosphoglycerate formed (0.49 $\mu\text{mol/ml}$ using *Hyphomicrobium* glycerate kinase and 0.23 $\mu\text{mol/ml}$ using *Pseudomonas* AM1 glycerate kinase) during the incubation period was seen to be consistent with the amount of product expected (0.66 $\mu\text{mol/ml}$ for *Hyphomicrobium* glycerate kinase and 0.36 $\mu\text{mol/ml}$ for *Pseudomonas* AM1 glycerate kinase) from the purified glycerate kinase activity added in that mixture and incubated for 15 min at 30 °C. The identification of 2-phosphoglycerate as the product of glycerate kinase agrees with that of the glycerate kinase in rat liver (Lamprecht, Heinz & Diamartstein, 1962) and in methanol-grown *Pseudomonas* MS (E. Bellion, personal communication) but differs from the glycerate kinase of glycerate adapted *E. coli* (Doughty, Hayashi & Guenther, 1966) where 3-phosphoglycerate was the identified product.

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