Partial Purification of Glycerate Kinase from *Flavobacterium* sp.

By ANDREW WILLETTS

Department of Biological Sciences, University of Exeter, Exeter EX4 4QG

(Received 6 March 1978)

INTRODUCTION

A *Flavobacterium* sp. (NCIB 11171) grown on ethylene glycol oxidized the diol to glyoxylate which was further metabolized to pyruvate by the glycerate pathway (Child & Willetts, 1978). However, it was not established whether the reaction product of glycerate kinase in ethylene glycol-grown NCIB 11171 was 3-phosphoglycerate, as in glycerate-grown *Escherichia coli* operating a restricted Embden–Meyerhof–Parnas pathway (Doughty *et al.*, 1966), or 2-phosphoglycerate, as in methanol-grown *Hyphomicrobium* x (Harder *et al.*, 1973; Hill & Attwood, 1974, 1976a), methanol-grown *Pseudomonas* AM1 (Hill & Attwood, 1974, 1976a) and methylamine-grown *Pseudomonas* MA (Newaz & Hersh, 1975) operating the serine pathway.

This communication describes the partial purification of glycerate kinase from ethylene glycol-grown NCIB 11171 and demonstrates indirectly that the product of the reaction is 2-phosphoglycerate.

METHODS

Micro-organism maintenance and culture. The maintenance and growth of NCIB 11171 in ethylene glycol minimal medium was as previously described (Child & Willetts, 1978).

Preparation of cell extracts. Bacteria were suspended in 0.1 M-potassium phosphate buffer, pH 7.0, and disrupted ultrasonically; the supernatant after centrifugation (0° C, 10000 g, 15 min) was used as the cell extract (Child & Willetts, 1978).

Enzyme assays. Glycerate kinase (EC 2.7.1.31), phosphopyruvate hydratase (EC 4.2.1.11), phosphoglycerate mutase (EC 2.7.5.3) and the conversion of glycerate to phosphoenolpyruvate were assayed as described by Harder *et al.* (1973). 3-Phosphoglycerate kinase (EC 2.7.2.3) was assayed by the method of Roustan *et al.* (1973).

Protein determination. Protein was assayed by a Folin-Ciocalteu method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Partial purification. All procedures were carried out at 0 °C unless otherwise stated.

Step 1. Ammonium sulphate fractionation. Cell extract from ethylene glycol-grown cells was adjusted to 30% saturation with ammonium sulphate, allowed to stand for 30 min and the precipitated protein was removed by centrifugation (0 °C, 15000 g, 15 min). The supernatant was adjusted to 50% saturation with ammonium sulphate, allowed to stand for 40 min and then centrifuged as above. The resultant pellet of precipitated protein was washed twice in 0.1 M-potassium phosphate buffer, pH 7.0, and resuspended in the minimum volume of buffer.

Step 2. Bio-Gel P300 gel filtration. The 50% ammonium sulphate precipitate in buffer (3.5 ml, protein content 10.42 mg ml⁻¹) was applied to a Bio-Gel P300 column (35 × 2.5 cm) and eluted with 0.1 M-potassium phosphate buffer, pH 7.0, by upward-flow gel filtration. The flow rate was 0.15 ml min⁻¹. Fractions (4.7 ml) were collected, monitored at 253.7 nm with an LKB Uvicord I UV absorptiometer and assayed for glycerate kinase, phosphoglycerate mutase and 3-phosphoglycerate kinase activities. Specific activity measurements of the peak fractions (Fig. 2) showed approximately 100-fold purification of glycerate kinase, 125-fold purification of phosphoglycerate mutase and 160-fold purification of 3-phosphoglycerate kinase relative to crude extract. Active fractions were stored at -20 °C if not used immediately.

Indirect determination of reaction product of partially purified glycerate kinase. The NADH-dependent reduction of pyruvate resulting ultimately from the phosphorylation of glycerate (Fig. 1) was assayed spectrophotometrically at 340 nm. Partially purified glycerate kinase (2.0 ml, 4 mg protein), free from phosphoglycerate mutase activity, was incubated for 30 min at 25 °C in a reaction mixture (final vol. 4.0 ml) con-

Fig. 1. Possible pathways for the metabolism of glycerate.

taining: 50 μmol potassium phosphate buffer, pH 7.0; 10 μmol ATP; 10 μmol DL-glyceric acid; 5 μmol EDTA; and 10 μmol MgCl_2 . The identity of the resultant phosphorylated glycerate product was established by assaying samples of the incubation mixture in a reaction mixture (final vol. 1.0 ml) containing: 50 μmol Tris buffer, pH 7.3; 1 μmol ADP; 0.2 μmol NADH; and 0.01 μmol 2,3-diphosphoglyceric acid. The absorbance at 340 nm was determined and then successively redetermined after the sequential addition of 50 μg lactate dehydrogenase, then 20 μg pyruvate kinase, then 20 μg phosphopyruvate hydratase and then 1.0 ml partially purified phosphoglycerate mutase (1 mg protein).

Chemicals. DL-Glyceric acid, 2,3-diphosphoglyceric acid, 3-phosphoglyceric acid, 2-phosphoglyceric acid, lactate dehydrogenase (ex rabbit muscle), pyruvate kinase (ex rabbit muscle) and phosphopyruvate hydratase (ex rabbit muscle) were obtained from Boehringer.

RESULTS AND DISCUSSION

Comparison of the specific activities of glycerate kinase [0.010 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$], phosphoglycerate mutase [0.004 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] and phosphopyruvate hydratase [0.021 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] in cell extracts of ethylene glycol-grown NCIB 11171 suggests that the conversion of glycerate into phosphoenolpyruvate [0.009 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$] proceeds via 2-phosphoglycerate but not 3-phosphoglycerate during operation of the glycerate pathway. The equivalent activities in acetate-grown cell extracts of NCIB 11171 were 0, 0.002, 0.014 and 0 $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$, respectively.

Glycerate kinase, phosphoglycerate mutase and 3-phosphoglycerate kinase from ethylene glycol-grown NCIB 11171 appear to be separate single protein moieties as assessed by gel filtration on Bio-Gel P300 (Fig. 2), although the initial fractions of phosphoglycerate mutase activity were eluted together with the later fractions of glycerate kinase activity. The product of purified glycerate kinase (fraction no. 20, Fig. 2) was indirectly confirmed as 2-phosphoglycerate. The failure of the successive addition of lactate dehydrogenase and pyruvate kinase to promote any decrease in absorbance at 340 nm indicated that neither pyruvate nor phosphoenolpyruvate were initially present in the product identification reaction mixture. The presence of 2-phosphoglycerate was confirmed by the rapid decrease in absorbance at 340 nm observed after the subsequent addition of phosphopyruvate hydratase. The failure of the further addition of partially purified phosphoglycerate mutase (fraction no. 26, Fig. 2) to promote any change in the absorbance at 340 nm indicated that no 3-phosphoglycerate, the product of glycerate kinase in glycerol-grown *E. coli* (Doughty *et al.*, 1966), was present

Fig. 2. Separation and partial purification of enzymes involved in ethylene glycol metabolism by *Flavobacterium* sp. NCIB 11171. An extract of ethylene glycol-grown cells, after fractionation with $(\text{NH}_4)_2\text{SO}_4$, was chromatographed on Bio-Gel P300 and fractions were assayed for glycerate kinase, phosphoglycerate mutase and 3-phosphoglycerate kinase activities as described in Methods. Protein was detected automatically by measuring absorbance at 253.7 nm with an LKB Uvicord I UV absorptiometer. Enzyme activities were found only in the fractions shown. ○, Phosphoglycerate mutase activity; ●, glycerate kinase activity; □, 3-phosphoglycerate kinase activity; ---, absorbance at 253.7 nm. All enzyme activities are expressed as $\mu\text{mol min}^{-1} (\text{mg protein})^{-1}$.

in the product identification reaction mixture. A decrease in absorbance at 340 nm consequent on the addition of 10 μmol 3-phosphoglycerate established the competence of the product identification reaction mixture to detect endogenous 3-phosphoglycerate and thereby implied that 2-phosphoglycerate was the reaction product of glycerate kinase in ethylene glycol-grown NCIB 11171. The absence of any detectable phosphoglycerate mutase activity from the commercial preparations of lactate dehydrogenase, pyruvate kinase and phosphopyruvate hydratase used in the assays supported the suggestion that 2-phosphoglycerate was the reaction product of glycerate kinase in ethylene glycol-grown NCIB 11171. The absence of 3-phosphoglycerate kinase (fraction no. 15 to 19, Fig. 2) from the partially purified glycerate kinase and phosphoglycerate mutase preparations used in the assays decreased the possibility that the 3-phosphoglycerate kinase plus glyceraldehyde-3-phosphate dehydrogenase-dependent metabolism of 3-phosphoglycerate to glyceraldehyde 3-phosphate via 1,3-diphosphoglycerate interfered with the observed activities as in crude cell-free extracts of *Hyphomicrobium* x (Hill & Attwood, 1976b). The phosphoglycerate mutase present in ethylene glycol-grown NCIB 11171 may serve as the first enzyme in the pathway of gluconeogenesis operating during growth on the aliphatic glycol: an equivalent role for the enzyme has been suggested in methanol-grown *Hyphomicrobium* x (Harder *et al.*, 1973).

REFERENCES

- CHILD, J. & WILLETS, A. (1978). Microbial metabolism of aliphatic glycols. Bacterial metabolism of ethylene glycol. *Biochimica et biophysica acta* **538**, 316–327.
- DOUGHTY, C. C., HAYASHI, J. A. & GUENTHER, H. L. (1966). Purification and properties of D-glycerate 3-kinase from *Escherichia coli*. *Journal of Biological Chemistry* **241**, 568–572.
- HARDER, W., ATTWOOD, M. M. & QUAYLE, J. R. (1973). Methanol assimilation by *Hyphomicrobium* sp. *Journal of General Microbiology* **78**, 155–163.
- HILL, B. & ATTWOOD, M. M. (1974). The purification of glycerate kinase from *Hyphomicrobium* sp. and *Pseudomonas* AM1: product identification. *Journal of General Microbiology* **83**, 187–190.
- HILL, B. & ATTWOOD, M. M. (1976a). Purification and characterization of phosphoglycerate mutase from methanol-grown *Hyphomicrobium* x and *Pseudomonas* AM1. *Journal of General Microbiology* **96**, 185–193.
- HILL, B. & ATTWOOD, M. M. (1976b). The effect of adenosine triphosphate on phosphoglycerate mutase activity from *Hyphomicrobium* x and *Pseudomonas* AM1 grown on reduced one-carbon compounds. *Journal of General Microbiology* **97**, 335–338.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- NEWAZ, S. S. & HERSH, L. B. (1975). Reduced nico-

- tinamide adenine dinucleotide-activated phosphoenolpyruvate carboxylase in *Pseudomonas* MA: potential regulation between carbon assimilation and energy production. *Journal of Bacteriology* **124**, 825–833.
- ROUSTAN, C., BREVET, A., PRADEL, L. A. & VON THOAI, N. (1973). Yeast 3-phosphoglycerate kinase. Interaction of the enzyme with substrates studied by partial isotope exchange and difference spectrophotometry. *European Journal of Biochemistry* **37**, 248–255.