Hexokinase and Glucose–Phosphoenolpyruvate Phosphotransferase Synthesis in *Klebsiella aerogenes* Strains Growing in Continuous Culture

By IAN S. CARTER and ALASTAIR C. R. DEAN

Physical Chemistry Laboratory, University of Oxford, Oxford OX1 3QZ, U.K.

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Considerable differences in steady-state hexokinase specific activity were found in 16 N.C.I.B. strains of *Klebsiella aerogenes* grown in identical conditions in glucose-limited chemostats. Strain N.C.I.B. 8258 had no detectable activity, but its glucose–phosphoenolpyruvate phosphotransferase specific activity and that of the other strains were closely similar, and it is concluded that this phosphotransferase activity regulates the overall utilization of glucose, in which hexokinase plays no essential role. The hexokinase activity was subject to regulation by the availability of phosphorus, but this did not affect the glucose phosphotransferase activity. Lactose-grown organisms (including strain N.C.I.B. 8258) had no glucose phosphotransferase activity, but more than adequate hexokinase activity to phosphorylate the intracellularly liberated glucose.

Little information exists on the regulation of bacterial hexokinase. In *Pseudomonas aeruginosa* glucose was considered to be an inducer of the enzyme and citrate the precursor of a repressor (Ng & Dawes, 1967). In contrast, the enzyme in *Klebsiella aerogenes* PRL-R3 (N.C.I.B. 9901) appeared to be constitutive, since organisms grown in nutrient broth and in a minimal medium containing glucose as carbon source had the same activity (Kamel *et al.*, 1966). The nature and distribution of hexokinase in other classified strains is not known, and for this reason we have determined the steady-state hexokinase specific activities of 16 other N.C.I.B. strains of *K. aerogenes* growing in chemostat culture. Glucose–phosphoenolpyruvate phosphotransferase specific activities have also been determined in the 16 strains. With some exceptions (Kornberg, 1973), this enzyme system is generally absent from strict aerobes, but occurs in facultative anaerobes (Romano *et al.*, 1970), and as far as *K. aerogenes* is concerned has been detected in three unclassified strains (Kundig *et al.*, 1964; Tanaka & Lin, 1967) and in strain PRL-R3 (Hasson & Anderson, 1968).

Experimental

Organisms and culture

K. aerogenes N.C.I.B. 418 has been used in this laboratory for many years; the other N.C.I.B. strains were obtained from Torry Research Station, Aberdeen, Scotland, U.K. The strains were maintained by daily subculture under aerobic conditions at 37°C in either glucose- or lactose-limiting

medium, as appropriate, and were not used until they had received at least 20 subcultures. Chemostat culture was also carried out at 37°C, the cultures being aerated by passing 0.5 litre of air/min through the culture vessel (200 ml working volume). This maintained the O₂ partial pressure, measured with a New Brunswick O₂ probe, considerably in excess of the value [2 kPa (15 mmHg)] that Harrison & Pirt (1965) considered necessary for the balanced aerobic growth of *K. aerogenes* N.C.I.B. 418. The pH was maintained at 7.0 ± 0.05 with a Pye autotitrator. The chemostat and the general methods for its operation are described by Dean & Rogers (1967).

Solutions

Glucose-limited P_i medium contained per litre: FeSO₄·7H₂O, 0.32 mg; MgSO₄·7H₂O, 0.063 g; (NH₄)₂SO₄, 0.96 g; KH₂PO₄, 1.14 g; Na₂HPO₄·12H₂O, 6.13 g; glucose, 0.3 g. Glucose-limited glycerol 2-phosphate medium contained per litre: FeSO₄·7H₂O, 0.32 mg; MgSO₄·7H₂O, 0.063 g; KCl, 0.62 g; glycerol 2-phosphate [disodium salt hydrate (5½H₂O)], 0.67 g; (NH₄)₂SO₄, 0.96 g; Tris, 12.0 g; glucose, 0.3 g. The pH of the latter was adjusted to 7.0 with 2M-HCl. Lactose-limited medium contained 0.3 g of lactose/l, and for nutrient-limitations other than carbon-limitation the concentration of the appropriate rate-limiting nutrient was decreased to the value reported by Bolton & Dean (1972) and the glucose concentration was increased to 3 g/l. Phosphate buffer contained per litre: KH₂PO₄, 2.28 g; Na₂HPO₄·12H₂O, 12.3 g; MgCl₂·6H₂O, 0.1 g. Its pH was 7.1. Tris/HCl

buffer contained per litre: Tris, 6.06g; $MgCl_2$, 2.03g; and sufficient 2M-HCl to adjust the pH to 7.65, 8.0 or 8.2 at 20°C as necessary. The inorganic chemicals and the sugars were AnalaR (BDH Chemicals, Poole, Dorset, U.K.) grade, but the glycerol 2-phosphate (disodium salt) and the Tris were reagent grade obtained from BDH, who also supplied the glucose 6-phosphate dehydrogenase and the biochemicals used in the enzyme assays.

Enzyme assays

Glucose-phosphoenolpyruvate phosphotransferase activity was determined as described by Kornberg & Reeves (1972*a,b*) and hexokinase by the method of Bergmeyer *et al.* (1974), but with Tris buffer, instead of triethanolamine buffer, in the reaction mixture, since the former was used in the phosphotransferase assay. In our conditions maximum activity occurred at pH 8.2, which was also observed with a specific glucokinase in *Euglena gracilis* (Lucchini, 1971). Enzyme specific activities, determined from initial rates, are expressed as nmol of $NADP^+$ reduced/min per mg of protein. Protein was determined by the spectrophotometric method of Warburg & Christian (1941). Initial experiments determined the duration of the cellular-disruption procedures and also showed that halving or doubling the substrate concentration in the assays described below had no significant effect on enzyme specific activities. No activity was detected in either assay when cell preparations were heated to 100°C, or when intact cells or dialysed growth medium were used.

Glucose-phosphoenolpyruvate phosphotransferase. Bacteria, separated from the growth medium by centrifugation for 10 min at 8000g, were washed twice with phosphate buffer and resuspended in it at a biomass corresponding to 1.2 ± 0.1 mg of protein/ml. Then 2 ml of the suspension was cooled to 4°C and 0.1 ml of toluene/ethanol (1:9, v/v) was added with vigorous mixing for 2 min. A 0.1 ml sample of the resulting suspension was diluted with 2.9 ml of a reaction mixture containing $NADP^+$, $MgCl_2$, Tris/HCl buffer, pH 8.0, phosphoenolpyruvate and glucose at final concentrations of 0.2, 12, 50, 2 and 7.5 mM respectively, together with 1.2 units of glucose 6-phosphate dehydrogenase. The reaction was initiated by adding the glucose to the other constituents of the reaction mixture, which had reached thermal equilibrium at 25°C in a thermostatically controlled Unicam SP.500 spectrometer, followed by stirring with a plastic-coated glass rod, and the change in A_{340} was read.

Hexokinase. The separated organisms, after a wash with Tris/HCl buffer, pH 7.65, and resuspension

in it at a biomass corresponding to 2.0 ± 0.2 mg of protein/ml, were treated at 0°C for 6 min in an MSE-Mullard ultrasonic disintegrator. The reaction mixture (3 ml) contained 0.1 ml of cell preparation and 1.2 units of glucose 6-phosphate dehydrogenase together with $NADP^+$, $MgCl_2$, Tris/HCl buffer, pH 8.2, ATP and glucose at final concentrations of 0.2, 10, 50, 0.3 and 0.5 mM respectively. The reaction was again initiated by adding the glucose and its progress followed at 25°C as described above.

Results and Discussion

Hexokinase

Table 1 shows that the hexokinase specific activity of the 16 N.C.I.B. strains of *K. aerogenes* varied considerably from strain to strain, and ranged from no detectable activity (N.C.I.B. 8258) to an activity of 152 units (N.C.I.B. 8805) when grown at a dilution rate (D) of $0.5 h^{-1}$ in chemostats containing glucose-limited P_i medium. Our demonstration that a 4-fold variation in substrate concentration had no effect on the specific activity shows that V_{max} was measured in the assays rather than differences in K_m , and the absence of detectable activity from *K. aerogenes* N.C.I.B. 8258 clearly demonstrates that hexokinase plays little or no essential role in the metabolism of externally supplied glucose. Furthermore, irrespective of their hexokinase content, the 16 strains converted the carbon substrate into biomass with equal efficiency. The yields (g dry wt. of biomass/g of C utilized) were not significantly different at P 0.05 (mean 1.03, s.d. 0.025). The 16 strains also grew at the same rate in batch culture [μ_{max} , $1.26 \pm 0.070 h^{-1}$ (mean $\pm$ s.d.)], which agrees with the observation by Fraenkel *et al.* (1964) that a hexokinase-less mutant of *Escherichia coli* (strain FRI) grew normally on glucose.

Growing *K. aerogenes* N.C.I.B. 418 at $D = 0.5 h^{-1}$ in glycerol 2-phosphate medium (glucose as carbon source) in conditions of carbon-, NH_4^+ -, K^+ -, Mg^{2+} -, SO_4^{2-} - and phosphate-limitation provided further evidence of the dispensability of hexokinase in the metabolism of externally supplied glucose. Hexokinase activity was only detected in phosphate-limited organisms. The specific activity was 62 units compared with an activity of 31 units found in glucose-limited P_i medium (Table 1) and an activity of 63 units in P_i -limited organisms in a further experiment. The pattern of behaviour contrasts with the constitutive nature of the enzyme in *K. aerogenes* PRL-R3 (see the introduction), and, as far as we are aware, our observation that hexokinase synthesis is subject to regulation by the availability of phosphorus has not been reported previously. The complete repression of hexo-

Table 1. *Hexokinase and glucose-phosphoenolpyruvate phosphotransferase specific activities of K. aerogenes strains*
The organisms were grown at $D = 0.5\text{h}^{-1}$ until steady-state conditions were reached in glucose-limited chemostats containing P_i as the phosphorus source. Enzyme activities are the means obtained by assaying six samples withdrawn from two chemostat cultures. In the hexokinase assays the s.e. of these means varied from 2.1 to 4.6 (mean 3.5) and in the phosphotransferase assays from 5.8 to 10.2 (mean 8.2). Specific activity is expressed as nmol of NADP^+ reduced/min per mg of protein.

Specific activity			Specific activity		
Strain	Hexokinase	Glucose-phosphoenolpyruvate phosphotransferase	Strain	Hexokinase	Glucose-phosphoenolpyruvate phosphotransferase
N.C.I.B. 418	31	175	N.C.I.B. 8595	17	160
N.C.I.B. 5938	109	169	N.C.I.B. 8793	82	171
N.C.I.B. 8017	22	180	N.C.I.B. 8805	152	176
N.C.I.B. 8021	65	176	N.C.I.B. 8806	145	168
N.C.I.B. 8152	71	180	N.C.I.B. 9024	104	161
N.C.I.B. 8153	77	175	N.C.I.B. 9025	87	166
N.C.I.B. 8258	0*	173	N.C.I.B. 9146	102	168
N.C.I.B. 8267	102	172	N.C.I.B. 9261	146	165

* An activity of less than approx. 2 units would not be detected in the assay procedure.

kinase synthesis when our strain (N.C.I.B. 418) was grown in medium containing an excess of glycerol 2-phosphate suggests that the organic phosphate is the repressor or is a precursor of it. The smaller degree of repression found when the medium contained P_i in excess of requirement might then be attributed to the production of a lower concentration of the repressor in these conditions. However, difficulties arise when the yields obtained in carbon-limited glycerol 2-phosphate medium are considered. These were abnormally high (1.72 and 1.74 units in replicate experiments) when calculated on the glucose present, but if the glycerol in the glycerol 2-phosphate is also used as a carbon source the values (1.05 and 1.06 units) fall within the range given above for glucose-limited P_i medium. A very low concentration of glycerol 2-phosphate remaining unconsumed, which would not be detected in yield determinations, might, however, be sufficient to repress hexokinase synthesis, and the existence of such residual concentrations of rate-limiting nutrients in chemostat culture is well established (Herbert *et al.*, 1956). Our cultures were definitely carbon-limited, since adding more glucose produced the expected increase in yield, and, if the mechanism proposed above operates, a higher residual concentration of glycerol 2-phosphate must remain in carbon-limited culture than in phosphate-limited culture. Further investigation is necessary, but it is clear that the P_i released in carbon-limitation is not the repressor, since the concentration in glucose-limited P_i medium is much higher and the repression less marked.

Glucose-phosphoenolpyruvate phosphotransferase

The synthesis of this enzyme was examined in all the conditions in which hexokinase activities were determined. The specific activities of the 16 N.C.I.B. strains grown in glucose-limited P_i medium ranged from 160 to 180 units, and an activity of 173 units was found for strain N.C.I.B. 8258, in which hexokinase could not be detected (Table 1). Furthermore, changing from phosphate-limitation to phosphate-excess in glycerol 2-phosphate medium, which completely repressed hexokinase synthesis in strain N.C.I.B. 418, had a negligible effect on the phosphotransferase. The specific activities in phosphate-, carbon-, NH_4^+ -, K^+ -, Mg^{2+} - and SO_4^{2-} -limited organisms were 169, 179, 163, 172, 175 and 173 units respectively. Similarly, in phosphate-limitation in P_i medium the specific activity was 165 units compared with 175 units in glucose-limitation. These activities (including those in Table 1) are not significantly different at $P=0.05$ [171 ± 5.81 (mean $\pm$ s.d.)].

The glucose-phosphoenolpyruvate phosphotransferase specific activity increased linearly with increasing D values. The mean activity of 171 units reported above was obtained at $D = 0.5\text{h}^{-1}$, and in further experiments with strains N.C.I.B. 5938, 8258 and 8805 mean specific activities of 86 units (s.d. 4.00) and 272 units (s.d. 7.65) were found at $D = 0.25$ and 0.75h^{-1} respectively. Herbert & Kornberg (1976) observed a similar linear relationship between D and the rate of uptake of [^{14}C]glucose

when strains K21t and B11 of *E. coli* were grown in glucose-limited chemostats. Our results also confirm their conclusion that it is the glucose-phosphoenolpyruvate phosphotransferase activity (which was equated with the rate of glucose uptake) that sets the pace of overall glucose utilization. A calculation shows that, if the phosphotransferase system phosphorylates all the glucose required to maintain growth, a minimum activity of 280 units is necessary at $D = 0.5\text{h}^{-1}$. Our mean specific activity of 171 units applies to organisms grown at 37°C but assayed at 25°C , and increasing the assay temperature to 37°C in experiments with strains N.C.I.B. 8017 and 8153 increased the specific activity to 332 and 324 units respectively, values that are close to the calculated requirement.

Lactose-utilizing organisms

The specific activity of glucose-phosphoenolpyruvate phosphotransferase in organisms utilizing lactose as sole carbon source was less than 4 units, which is the limit of detection in our assay, but their hexokinase specific activities increased to a constant value [196 ± 3.13 units (mean $\pm$ s.d.)]. This was established by growing strains, derived from N.C.I.B. strains 418, 8017, 8153, 8258 and 9261 by subculture in lactose medium, in lactose-limited chemostats at $D = 0.5\text{h}^{-1}$. Their growth rates in batch culture in lactose medium [$\mu_{\text{max.}} 1.26 \pm 0.081\text{h}^{-1}$ (mean $\pm$ s.d.)] were not significantly different from those of the parent strains in glucose medium, showing that the absence of phosphotransferase has not impaired growth rate. In agreement with the conclusions of Anderson & Wood (1969), these results strongly suggest that hexokinase phosphorylates the glucose liberated intracellularly from lactose and the phosphorylating potential available is more than adequate. An activity of 140 units is required and our mean specific activity of 196 units was doubled when the assays were carried out at 37°C . That this function is not unique to hexokinase is, however, shown by the persistence of phosphotransferase activity in a lactose-grown mutant of *E. coli*, which lacked both hexokinase and a functional galactose-catabolic pathway, but its growth rate was 20% lower than that of an isogenic strain containing hexokinase (Curtis &

Epstein, 1975). Nevertheless, the function of hexokinase in the metabolism of externally supplied glucose remains obscure, particularly in view of the wide variation in activity we observed, and, in the absence of further evidence, it is not immediately apparent why the glucose-scavenging activity commonly attributed to this enzyme (Anderson & Wood, 1969) should vary so much in closely related strains grown in identical conditions.

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