An Inducible Phosphoenolpyruvate:Dihydroxyacetone Phosphotransferase System in *Escherichia coli*

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A phosphoenolpyruvate:dihydroxyacetone phosphotransferase was induced in *Escherichia coli* grown on dihydroxyacetone as sole carbon source or in its presence. This is the first example of a triose which can be acted upon by the membrane complex to provide a central intermediate in glycolysis. The presence of this system explains the ability of a mutant, in which the ATP-dependent glycerol kinase is genetically replaced by a glycerol:NAD 2-oxidoreductase, to grow on glycerol.

INTRODUCTION

Known bacterial phosphoenolpyruvate:sugar phosphotransferase systems transport only disaccharides, glycosides, hexoses, and hexitols. Free sugars are captured from the environment by these systems and translocated into the cell in a phosphorylated form. The translocation process can be described in a simplified manner as follows:

In the first reaction, the cytoplasmic enzyme I catalyses the transfer of the phosphoryl group from PEP to a histidine side chain of a small protein, HPr. In the subsequent reaction the phosphorylated small protein, P-HPr, donates its phosphoryl group via a transmembrane enzyme II complex to the entering sugar. Enzymes of type II are substrate specific and are generally induced only by their own substrates (Postma & Roseman, 1976). For the translocation of certain sugars, such as glucose, another sugar-specific carrier protein is required. This protein, factor III^{Glc}, has additional roles which include the regulation of adenylate cyclase activity: apparently the phosphorylated form of the factor is stimulatory and the free form is inhibitory (Feucht & Saier, 1980).

In a study on experimental evolution of glycerol utilization by *Escherichia coli*, the indigenous pathway (Lin, 1976) was first deleted in strain ECL204 by imposing double genetic blocks that abolished the respective activities of the ATP-dependent glycerol kinase and the membrane-associated aerobic sn-glycerol 3-phosphate dehydrogenase. Strain ECL424, in which an NAD-linked dehydrogenase served as the first enzyme for glycerol dissimilation, was then isolated (St. Martin *et al.*, 1977). This dehydrogenase of unknown background converted glycerol reversibly to DHA. Although not inducible by glycerol, the enzyme was produced at high level (Tang *et al.*,

Abbreviations: CAA, casein acid hydrolysate; DHA, dihydroxyacetone; HPr, histidine-containing protein; PEP, phosphoenolpyruvate.

1979, 1982a). Numerous attempts to demonstrate the presence of an ATP-dependent DHA kinase, however, were unsuccessful. That DHA indeed served as a metabolic intermediate was indicated by its initial excretion (reaching almost 0.5 mM in the culture medium) which was followed by its retrieval during the late phase of growth on glycerol (Tang *et al.*, 1982b). An alternative mechanism of DHA utilization must exist and is described in this report.

METHODS

Chemicals. Crystalline DHA, MOPS, and rabbit muscle L-lactate dehydrogenase (EC 1.1.1.27) were obtained from Sigma. Rabbit muscle glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) was purchased from Boehringer-Mannheim. All other chemicals were purchased as previously described (Forage & Lin, 1982) or were of reagent grade.

Bacterial strain and growth conditions. *Escherichia coli* strain ECL1 was subcloned from an alkaline phosphatase deletion mutant of *E. coli* K12 strain HfrC *phoA8 relA1 tonA22 T₂' λ⁺* (Hayashi *et al.*, 1964). Strain ECL204, which lacks glycerol kinase and aerobic G3P dehydrogenase activities (Richey & Lin, 1972), and strain ECL223, which lacks enzyme I activity (Solomon & Lin, 1972), were derived from strain ECL1. Strain ECL424, which utilizes glycerol by the NAD-linked dehydrogenase pathway, was derived from strain ECL204 (St. Martin *et al.*, 1977).

Bacteria were grown at 37 °C in 50 ml MOPS medium containing 1 mM-phosphate (Forage & Lin, 1982) and appropriate carbon and energy sources. For studies of enzyme induction CAA was added to 1% (w/v) and thiamin at 20 µg ml⁻¹ with or without the test carbohydrate at 20 mM. The effect of cAMP was tested at 5 mM. Cultures were incubated in 300 ml side arm flasks agitated at about 240 r.p.m. on a rotary shaker. For enzyme assays, cells from exponential growth phase were harvested by centrifugation at approximately 5000 g for 6 min and washed with a 'decryptification buffer' containing 5 mM-MgCl₂ and 0.1 M-sodium-potassium phosphate at pH 7.2 (Kornberg & Reeves, 1972a).

Spectrophotometric assay of enzymes II. Activities of enzymes II were assayed according to the procedure of Kornberg & Reeves (1972a). Toluene-treated cells were suspended in a reaction medium containing the carbohydrate substrate (1 mM), NADH, PEP, and lactate dehydrogenase. PEP, but neither NADH nor lactate dehydrogenase, could freely enter the treated cells, and phosphorylation of the carbohydrate substrate during translocation across the plasma membrane consumed this PEP. Pyruvate, the product, freely diffused into the medium and was reduced by lactate dehydrogenase at the expense of NADH, the concentration of which was followed by the decrease in absorbance at 340 nm. When substrate or PEP was limiting, a stoichiometric amount of NADH was oxidized (Kornberg & Reeves, 1972b). Enzyme activity is expressed in nmol of pyruvate released min⁻¹ (mg dry cells)⁻¹ at 30 °C. In the present study, omission of substrate, PEP, or lactate dehydrogenase gave low blank reaction rates (less than 4 units) indistinguishable from the rate observed when NADH alone was provided. ATP did not substitute for PEP. When strain ECL1 was induced in the ATP-dependent glycerol kinase, the pyruvate-releasing activity stimulated by glycerol was observed to be less than 2 units. This indicates that externally added PEP could not assure a continuous supply of ATP for the phosphorylation reaction.

Glycerol kinase. The activity was assayed by following the rate of [U-¹⁴C]glycerol phosphorylation as described by Forage & Lin (1982) and expressed as nmol min⁻¹ (mg protein)⁻¹ at 30 °C.

RESULTS

Growth

When tested with 20 mM-DHA as the sole source of carbon and energy in a low phosphate medium (1 mM), the wild-type strain ECL1 grew with a doubling time of 3 h, whereas the mutant strain ECL424 (in which glycerol kinase was replaced by an NAD-linked dehydrogenase) grew with a doubling time of 5 h. Growth rates on DHA cannot properly be studied in a medium containing a high concentration of phosphate, because the anion catalyses rapid formation of inhibitory concentrations of methylglyoxal (Riddle & Lorenz, 1968).

Kinetics of enzyme II for DHA

DHA-dependent release of pyruvate from PEP by toluene-treated cells (wild-type strain ECL1 induced with DHA) was linear with time (5 min) and the rate of pyruvate release was proportional to the dry weight of bacteria in the assay system (16 to 64 µg dry cells ml⁻¹). A plot of the reciprocal reaction rate as a function of the reciprocal of DHA concentration gave an apparent '*K_m*' for DHA of 0.1 mM. This value is rather high in comparison with the apparent *K_m* values of the other enzymes II: for example, 0.003 to 0.04 mM for mannitol, glucitol and galactitol (Lengeler, 1975); and 0.01 to 0.05 mM for glucose and mannose (Stock *et al.*, 1982).

Table 1. *Specific activities of enzymes II induced by growing strain ECL1 with their respective substrates*

All growth media contained acid hydrolysed casein 1% (w/v).

Compound added to CAA growth medium (20 mM)	Substrate for enzyme assay (1 mM)	Enzyme units
Arbutin (4-Hydroxyphenol- β-D-glucopyranoside)	Arbutin	0
D-Fructose	D-Fructose	120
D-Galactitol	D-Galactitol	0
D-Glucose	D-Glucose	77
D-Glucitol	D-Glucitol	36
D-Mannose	D-Mannose	79
Mannitol	Mannitol	120
N-Acetyl-D-glucosamine	N-Acetyl-D-glucosamine	82

However, the peculiarity appears not to be caused by the toluene treatment, because similarly treated cells gave an apparent K_m for glucose which was lower than that obtained from sonically disrupted cell preparations (Gachelin, 1969).

Specific induction of enzyme II for DHA

Eight different enzymes II have been reported for *E. coli*. They are encoded by the genes *bgIC*, *fruA*, *gatA*, *glcA*, *gutA*, *manA*, *mtlA*, and *nagE* and act respectively on β-glucosides (such as arbutin), D-fructose, D-galactitol, D-glucose, D-glucitol, D-mannose, D-mannitol, and N-acetyl-D-glucosamine as characteristic substrates. All of these enzymes act on more than one metabolizable substrate, although some of the secondary substrates are not inducers (see for example Lengeler *et al.*, 1981). Significant DHA-phosphorylating activity (24 units) was found in cells of strain ECL1 only when grown in the presence of the triose. In a parallel test in which lactate dehydrogenase was replaced by rabbit muscle glycerol 3-phosphate dehydrogenase to assay the DHA phosphate formed, 80% of the pyruvate released was accounted for by the phosphorylated triose. Cells grown in the presence of D-fructose, D-glucose, D-glucitol, D-mannose, D-mannitol, or N-acetyl-D-glucosamine showed phosphotransferase activity for the corresponding substrate (Table 1), but not for DHA (data not shown). Neither the *bgIC* nor the *gatA* system was inducible in this *E. coli* strain. The enzyme II responsible for vectorial phosphorylation of DHA is therefore distinct from all the previously known enzymes II in *E. coli*.

Activities of enzyme II for DHA in E. coli mutants

When glycerol was added as a carbon and energy source for the wild-type strain ECL1 and for strain ECL204, the enzyme II for DHA was not significantly induced. In contrast, the mutant strain ECL424 that relied on the NAD-linked glycerol dehydrogenase for glycerol utilization produced 25 units of enzyme II activity for DHA. On the other hand, when DHA was added as a carbon and energy source, all three strains were fully induced in enzyme II for DHA (22 to 24 units). However strain ECL223, lacking enzyme I of the phosphotransferase system, was unable to phosphorylate DHA when grown with either glycerol or DHA.

Since lack of enzyme I activity interferes with the induction of enzymes by increasing the severity of catabolite repression (attributed to the inhibition of adenylate cyclase by the non-phosphorylated form of factor III^{Glc}) and repression can be counteracted by exogenous cAMP (Pastan & Perlman, 1969), the possibility that the absence of the DHA phosphorylating activity in strain ECL223 grown in the presence of the inducer was the result of strong catabolite repression could be tested. Table 2 shows that whereas the induction of the ATP-dependent glycerol kinase was prevented by the enzyme I defect and partly overcome by the provision of cAMP, the DHA phosphorylating activity remained undetectable in cells grown even in the

Table 2. *Induction of enzyme II for DHA and glycerol kinase in the wild-type strain and in the mutant lacking enzyme I*

Casein hydrolysate was added at 1% (w/v) and thiamin at $20 \mu\text{g ml}^{-1}$ to all growth media. Glycerol and DHA were each added at 20 mM and cAMP at 5 mM.

Strain	Compound added to CAA growth medium	Activity of enzyme II for DHA	Activity of glycerol kinase
ECL1	None	0	43
	Glycerol + DHA	24	380
	Glycerol + DHA + cAMP	24	470
ECL223	None	0	45
	Glycerol + DHA	0	46
	Glycerol + DHA + cAMP	0	130

Fig. 1. A postulated scheme for glycerol dissimilation with DHA as an intermediate by strain ECL424.

presence of both DHA and cAMP. Thus the phosphorylation of DHA seemed to depend directly upon an intact phosphotransferase system, as one would expect.

DISCUSSION

Growth on DHA has also been observed with *Klebsiella pneumoniae* strain 1033 [previously *K. aerogenes* (Magasanik *et al.*, 1953; Lin *et al.*, 1960)], *K. pneumoniae* strain NCIB418 (Forage & Lin, 1982), and *Salmonella typhimurium* (Gutnick *et al.*, 1969). In the *K. pneumoniae* strains, an ATP-dependent DHA kinase was shown to be necessary for growth. However, one would predict on phylogenetic grounds that *S. typhimurium* uses a PEP-phosphotransferase system for the utilization of DHA.

The existence of an enzyme II for DHA explains how the *E. coli* mutant, strain ECL424, is able to exploit an NAD-linked dehydrogenase, instead of an ATP-dependent kinase, for growth on glycerol. However, the way in which the mutant utilizes DHA as an intermediate in glycerol dissimilation might not be straightforward. The excretion of DHA during the initial phase of growth on glycerol (Tang *et al.*, 1982*b*) and the likelihood that substrates of the phosphotrans-

ferase system are acted on more readily when presented extracellularly (Kaback, 1968; Delobbe *et al.*, 1976; Lengeler, 1976) suggest that a large fraction of the DHA produced from glycerol escaped from the cytoplasm (through a route yet to be defined), only to be immediately or eventually recaptured (Fig. 1). If so, in the evolution of strain ECL424 which regained the ability to grow on glycerol, nature responded to the artificial selective pressure with an unorthodox mechanism.

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