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PHOSPHOENOLPYRUVATE:FRUCTOSE PHOSPHOTRANSFERASE ACTIVITY IN WHOLE CELLS AND MEMBRANE VESICLES OF *ARTHROBACTER PYRIDINOLIS*

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SUMMARY

Mutants of *Arthrobacter pyridinolis* which were deficient in phosphoenolpyruvate:fructose phosphotransferase activity were studied using *in vitro* complementation assays. In this way the strains were divided into those deficient in the inducible membrane-bound component and into three groups which were deficient in different soluble components. One of the soluble components was inducible. Phosphoenolpyruvate:fructose phosphotransferase activity was also demonstrated in membrane vesicles prepared from fructose-grown *A. pyridinolis*. The activity was correlated with transport of fructose into the vesicles. Some kinetic parameters and temperature optima for the transport process in vesicles were studied.

INTRODUCTION

Arthrobacter pyridinolis is relatively restricted in its ability to use sugars as substrates for growth. Glucose, sucrose, and maltose can support growth, but only in the presence of certain Krebs cycle intermediates whose utilization is apparently coupled to glucose and α-glucoside transport[1]. L-Rhamnose and D-fructose are the only sugars found which can be used as growth substrates in the absence of any other carbon source. Fructose is first converted to fructose 1-phosphate by phosphoenolpyruvate:fructose phosphotransferase. Fructose 1-phosphate kinase then catalyzes the formation of fructose 1,6-diphosphate which enters the glycolytic pathway[2]. As in other organisms, the phosphotransferase system which catalyzes the first reaction of fructose metabolism in *A. pyridinolis* is associated with the uptake of the sugar, and contains both membrane-bound and soluble components[2]. Previous studies indicated that the membrane-bound component and at least one soluble component are inducible[2]. We now present evidence for a phosphotransferase system consisting of three soluble components, one of which is inducible, and an inducible membrane component. The activity of this phosphotransferase system has been characterized

in isolated membrane preparations of *A. pyridinolis* as had been done by Kaback[3] with a three-component phosphotransferase system from *Escherichia coli*.

MATERIALS AND METHODS

Bacteria and growth conditions

A. pyridinolis was grown, with shaking at 30 °C, in the mineral salts medium previously described[4]. Carbon sources were added to sterilized mineral salts medium from separate sterile solutions. Solid media were prepared by adding 0.85% (w/v) ionagar (Colab Inc.) to mineral salts medium. For routine maintenance cells were grown in peptone–yeast extract medium[2].

Chemicals

Fructose 1,6-diphosphate, isocitric acid, NADP ,phosphoenolpyruvate and penicillin were purchased from Sigma Chemical Company. Phosphohexose isomerase, glucose-6-phosphate dehydrogenase, and lysozyme were obtained from Boehringer, Mannheim. Deoxyribonuclease and ribonuclease were purchased from Worthington Biochemicals and ethylmethanesulfonate from Eastman Chemicals. [U-^{14}C]Fructose was obtained from Biochemical and Nuclear Corp. All other chemicals were obtained commercially at the highest purity available. The D-isomer of fructose was employed for all studies.

Enzyme assays

For preparation of cell-free extracts, cells were first washed in the buffer to be used in the assay, and were then broken by sonic disruption as described previously[4]. Phosphoenolpyruvate:fructose phosphotransferase activity was determined by the method of Tanaka *et al.*[5] using 2 mM fructose. Samples from the reaction mixture were spotted on diethylaminoethyl-cellulose discs[6]. The discs were washed exhaustively with water and then dried. The radioactivity was measured by scintillation counting. The assay was linear with both enzyme concentration and time in the ranges employed. Fructose-1,6-diphosphatase (EC 3.1.3.11) was determined by the method of Fraenkel and Horecker[7] and isocitric acid dehydrogenase (EC 1.1.1.42) by the method of Hanson and Cox[8]. The spectrophotometric assays were conducted using a Gilford Model 240 recording spectrophotometer at 26 °C. Protein was determined by the method of Lowry *et al.*[9] using lysozyme as a standard.

Mutagenesis

Cells were treated with ethylmethanesulfonate as previously described[4]. For enrichment of mutants that were negative for growth on fructose, the mutagenized cells were incubated overnight in mineral salts medium plus fructose (0.05 M) and 100 μg of pencillin G/ml. The cells were then washed with mineral salts medium and plated on peptone–yeast extract medium. Colonies were replicated onto fructose plates. Strains that failed to grow on the fructose plates were isolated from the peptone–yeast extract medium replicate. Those that exhibited growth in liquid medium containing malate but no growth in fructose medium were further characterized as described under Results.

Preparation of membrane vesicles

Spheroplasts were prepared from wild type cells grown to the logarithmic phase (20–22 h) in mineral salts medium *plus* 0.05 M fructose. After centrifugation of the culture at 16 000 × *g*, the pellet was washed with 10 mM Tris (pH 8.0) and was resuspended in 30 mM Tris (pH 8.0) containing 20 mM $MgSO_4$ and 10% mannitol. The cells were then incubated at 30 °C. Lysozyme was added to a final concentration of 250 μg/ml for 2 min. Spheroplasts were then isolated and membrane vesicles were prepared according to the method of Kaback[10] with the following modifications: 10% mannitol was used for osmotic stabilization instead of 20% sucrose; and lysis of the spheroplasts was carried out at 30 °C instead of 37 °C. The membranes, at a concentration of 1.0 mg protein/ml, were finally resuspended in 0.1 M potassium phosphate buffer (pH 6.6) and were frozen and stored in liquid N_2.

Transport and enzyme assays using membrane vesicles

Fructose transport by isolated membrane preparations was assayed using a filtration assay with a reaction mixture containing [^{14}C]fructose (usually at 40 μM, 25 μCi/ml), vesicles, buffer, and other additions as described by Kaback[3] except that: the reaction mixture for experiments on temperature dependence contained 25 μl of vesicles in a total volume of 50 μl; the reactions were terminated by a 25-fold dilution (or 40-fold dilution in experiments on temperature dependence) with 0.5 M LiCl; and the filters used were from Matheson–Higgins Co. (0.45 μm). The radioactivity of the filters was measured by scintillation counting. Each set of samples was corrected for a control as described by Kaback[3].

For assays of enzymatic activities in the membrane preparations, a sample of vesicles was first diluted to twice the original volume in the buffer to be used in the assay. The vesicles were then subjected to sonic disruption for 30 s.

RESULTS

Characterization of mutants by in vitro complementation

Approximately fifty mutants were isolated which were unable to grow on fructose, but were capable of growth on malate. Extracts were prepared from each mutant after growth of the cells on glutamate (50 mM) *plus* fructose (50 mM). The extracts were assayed for phosphoenolpyruvate:fructose phosphotransferase activity both alone and in the presence of fractions from glutamate *plus* fructose-grown wild type cells. These fractions were prepared by centrifugation of the wild type extract at 150 000 × *g* for 2 h. The fraction designated "supernatant" was the supernatant obtained after this centrifugation. The fraction designated "pellet" was obtained by resuspending the pellet from this centrifugation in a volume of 0.05 M Tris (pH 7.6) equal to the initial volume of extract employed. In some experiments, the pellet was washed several times with buffer, but these washings did not change the results significantly. As shown in Table I, both the supernatant and pellet fractions of the wild type cells contained appreciably less activity when assayed individually, than did whole extract. A combination of the two fractions resulted in restoration of activity to a level even higher than that of the original extract. The results of complementation experiments with three different mutant strains and the wild type fractions are also shown in Table I. Extracts of all three mutant strains showed con-

TABLE I

In vitro COMPLEMENTATION OF EXTRACTS OF PHOSPHOTRANSFERASE-DEFICIENT MUTANTS WITH FRACTIONS FROM WILD TYPE *A. pyridinolis*

Assays for phosphoenolpyruvate: fructose phosphotransferase activity were conducted on extracts or fractions from glutamate (0.05 M) + fructose (0.05 M)-grown cells in a final volume of 1.0 ml containing 0.05 ml of each fraction or extract. The wild type extract, supernatant and pellet fractions contained 9.8, 4.8 and 5.5 mg of protein/ml, respectively. The extracts of AP100, AP133, and AP243 contained, respectively, 3.2, 2.9 and 7.7 mg of protein/ml.

Strain	*Fraction*	*Addition*	*nmoles fructose 1-phosphate formed/ min per reaction mixture*
Wild type	Whole extract	—	1.93
	Supernatant	—	0.87
	Pellet	—	0.52
	Supernatant + pellet	—	3.89
AP133	Whole extract	—	0.46
	Whole extract	Wild type pellet	2.87
	Whole extract	Wild type supernatant	1.56
AP100	Whole extract	—	0.43
	Whole extract	Wild type pellet	1.19
	Whole extract	Wild type supernatant	3.34
AP243	Whole extract	—	0.52
	Whole extract	Wild type pellet	0.83
	Whole extract	Wild type supernatant	1.81
	Whole extract	AP100 whole extract	2.45

siderable activity alone, as did extracts from uninduced glutamate-grown wild type cells (see legend to Table II), although none of the mutant strains showed growth on fructose. AP133 showed an increase in activity in the presence of either wild type pellet or wild type supernatant; the increase observed with addition of wild type pellet was much greater than that observed upon addition of supernatant. AP100 and AP243, on the other hand, were complemented by wild type supernatant to a much greater extent than by wild type pellet. On this basis, AP133 was presumed to be deficient in a membrane-bound (pellet) component of the phosphotransferase system while AP100 and AP243 were presumed to be deficient in a soluble (supernatant) component. All the fructose-negative mutants were examined in this way. About fifteen strains fell into one of the two categories just described. Extracts from another group of strains showed slightly increased activity with both wild type supernatant and pellet, each wild type fraction causing the same increment in activity. The rest of the mutants were not complemented *in vitro* by either of the wild type fractions.

Experiments were then conducted to elucidate groups among those mutants presumed to be deficient in a supernatant component. Various pairs of extracts from appropriate mutant strains were assayed together for phosphotransferase activity, and the results were compared to those obtained with each strain alone. The data for one complementing pair of mutants, AP100 and AP243, are shown in Table I. Extracts from noncomplementing mutants exhibited no more activity when assayed together than the sum of the individual activities of the two extracts. On the basis of the results of the complementation experiments between pairs of mutants, the strains were readily divisible into groups. Three groups of supernatant mutants, *i.e.* strains with defects in three different soluble components, were found and designated Groups A, B, and C. Complementation experiments between mutants in the mem-

TABLE II

DETERMINATION OF WHICH SUPERNATANT COMPONENTS ARE CONSTITUTIVE

Phosphoenolpyruvate: fructose phosphotransferase activity was assayed in a total final volume of 1.0 ml containing 0.05 ml of each extract or fraction used. Whole extracts of AP194, AP243, AP253, and glutamate-grown wild type cells were employed. These extracts contained 6.8–8.1 mg of protein/ml. The extract from the glutamate grown wild type cells formed 0.32 nmoles of fructose 1-phosphate/min per reaction mixture when assayed alone. The supernatant fraction from glutamate + fructose-grown wild type cells was used. This fraction contained 4.8 mg of protein/ml, and formed 0.71 nmole of fructose 1-phosphate/min per reaction mixture when assayed alone.

Group	*Strain*	*Wild type supernatant or extract added*	*nmoles fructose 1-phosphate formed/min per reaction mixture*
A	AP 194	None	0.56
		Fructose + glutamate	1.78
		Glutamate	0.55
B	AP243	None	0.52
		Fructose + glutamate	1.81
		Glutamate	1.47
C	AP253	None	0.58
		Fructose + glutamate	2.12
		Glutamate	1.62

brane-bound component, AP133, AP358 and AP353, suggested that these mutants may fall into two groups, but these results were based on an insufficiently large number of mutants for a definitive conclusion.

Further characterization of mutants deficient in supernatant components

Previous work had indicated that the membrane-bound component(s) and at least one soluble component of the phosphotransferase system in *A. pyridinolis* were inducible[2]. In order to elucidate the inducible *vs* the constitutive nature of the three soluble components, a member of each of the groups of supernatant mutants was complemented with an extract of glutamate-grown wild type cells. The results were compared with complementation data for these same strains with the supernatant fraction from glutamate *plus* fructose-grown wild type cells. As shown in Table II, both AP243 and AP253 showed almost as much activity when assayed together with extract from glutamate-grown wild type as when assayed together with supernatant from glutamate *plus* fructose-grown wild type. Thus the components which are deficient in these two mutants were presumed to be constitutive. On the other hand, AP194 showed no difference in activity when assayed in the presence or absence of extract from glutamate-grown wild type; addition of supernatant from glutamate *plus* fructose-grown wild type, however, caused a significant increase in activity. AP194 was presumed to be deficient in an inducible component of the phosphotransferase system. Similar experiments with other strains in the three groups were consistent with the conclusion that Group A mutants were deficient in an inducible soluble component and that Groups B and C mutants were deficient in constitutive soluble components of the phosphotransferase system.

Properties of membrane vesicles prepared from fructose-grown wild type cells

The preparations of membrane vesicles contained no whole cells, and fewer than one unlysed spheroplast per oil immersion field (800-fold magnification) as

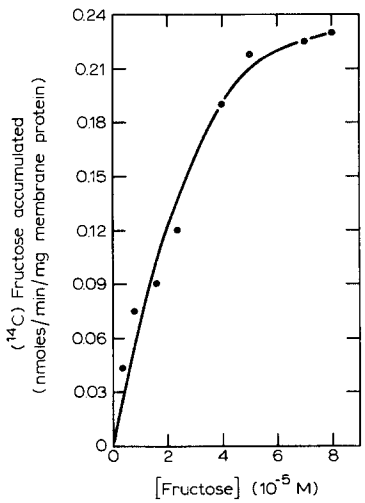

Fig. 1. The effect of phosphoenolpyruvate concentration on the initial rate of fructose uptake into vesicles. Transport was assayed at 38 °C as described under Materials and Methods using varying concentrations of phosphoenolpyruvate. The reactions were terminated 10 min after the addition of [^{14}C]fructose.

observed with the phase-contrast microscope. The structures seen in electron micrographs of thin sections of vesicles supported the view that the vesicles were closed membranous sacs. Sonicated vesicle preparations were assayed for isocitrate dehydrogenase and fructose-1,6-diphosphatase. While crude extracts of whole cells contained high levels of these cytoplasmic enzymes (0.72 and 0.3 I.U./mg protein, respectively), the vesicles contained no detectable activity of either enzyme. In addition, the fact that membrane vesicles could not use 2-phosphoglycerate as a substitute for phosphoenolpyruvate in support of fructose transport (see below), indicated that the membrane preparations had no enolase activity.

Phosphoenolpyruvate-dependent fructose transport in the membrane vesicles

In the presence of phosphoenolpyruvate at 35 °C, membrane vesicles took up

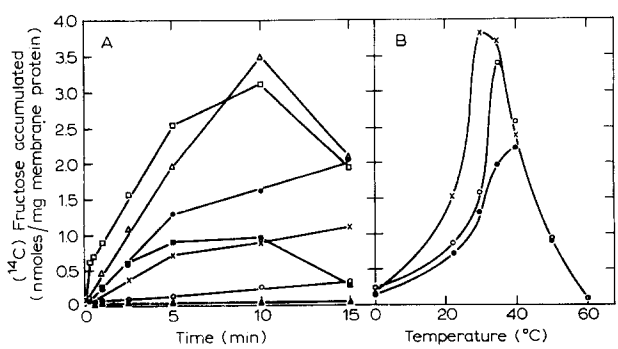

Fig. 2. The effect of temperature on the time course of fructose uptake by vesicles (A) ○—○, 0 °C; ×—×, 22 °C; ●—●, 30 °C; △—△, 35 °C; □—□, 40 °C; ■—■, 45 °C; ▲—▲, 50 °C. (B) ●—●, 5 min; ○—○, 10 min; ×—×, 20 min.

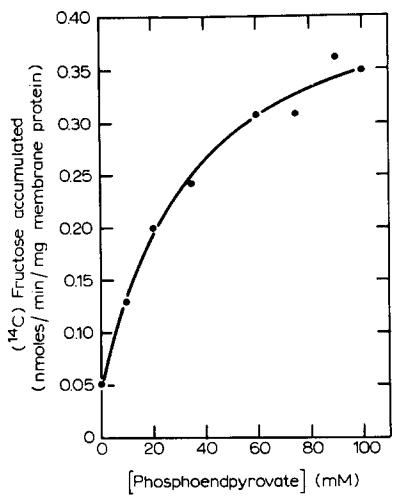

Fig. 3. Kinetics of fructose uptake by isolated membrane preparations. Vesicles were assayed for phosphoenolpyruvate: fructose phosphotransferase activity as described under Materials and Methods at 32 °C with varying concentrations of [^{14}C]fructose. Incubations were terminated 10 min after addition of the sugar substrate.

0.35 nmole of fructose/min per mg of protein. In the absence of phosphoenolpyruvate or when nucleotide triphosphates or 2-phosphoglycerate are substituted for phosphoenolpyruvate, no more than 0.05 nmoles of fructose were taken up per min per mg of protein. In Fig. 2, the relationship between phosphoenolpyruvate concentration and the initial rate of fructose uptake is shown. The uptake of fructose by the vesicles increased rapidly with increasing concentrations of phosphoenolpyruvate from 0 to 20 mM, and then continued to increase less rapidly up to 100 mM. In all subsequent experiments phosphoenolpyruvate was employed at a concentration of 100 mM.

The effect of temperature on the time course of accumulation of fructose by the vesicles in the presence of phosphoenolpyruvate is shown in Figs 3A and 3B. A slow steady accumulation was observed at 0 °C, and increasingly rapid rates of accumulation were found at 22 °C and 30 °C over a 15-min period. At higher temperatures, 35 °C–45 °C, fructose was accumulated for 10 min, after which there was an apparent loss of fructose from the vesicles. No accumulation was observed at 50 °C. As depicted in Fig. 3B, the smaller the time period used to measure fructose accumulation (over a 5 to 20-min range), the higher the temperature optimum for uptake.

The effect of fructose concentration on the initial rate of its uptake by vesicles was then determined at 32 °C in the presence of 100 mM phosphoenolpyruvate (Fig. 4). Uptake showed Michaelis-Menten kinetics; a K_m of $1.5 \cdot 10^{-5}$ M was calculated from a Lineweaver–Burk plot of the data in Fig. 4.

DISCUSSION

In spite of the leakiness of phosphotransferase system mutants of *A. pyridinolis*, which is similar to that observed with such mutants in other species[11], *in vitro* complementation experiments have provided a means of defining categories of mutations in this system. Thus the phosphotransferase system of *A. pyridinolis* appears, as sug-

gested previously[2], to resemble those of *Staphylococcus aureus*[12] and *Aerobacter aerogenes*[13] in possessing three soluble components, two of which are constitutive and one of which is inducible. Presumably one of the constitutive components is Enzyme I and the other is a phosphocarrier protein, although this question has not been approached in the present study. The inducible soluble component would be analogous to the Factor III in other species. The possibility that the inducible membrane-bound component of the phosphotransferase system consists of more than one component would also be consistent with work in other bacteria[14].

While it is useful to distinguish between membrane-bound and soluble components in studies of the components of the phosphotransferase system, this distinction appears to be a very relative one in view of the activity of this system in membrane vesicles .The properties of the activity in vesicles are similar to those described by Kaback[3] in his studies of *E. coli* vesicles. Phosphoenolpyruvate is required for significant uptake. As in *E. coli*, high concentrations of phosphoenolpyruvate are required, perhaps reflecting a relative impermeability of the membranes to this compound. In *A. pyridinolis* the concentration of fructose supporting a half-maximal rate of uptake is approximately four times higher than in *E. coli*. This is consistent with the difference between the growth rates of the two organisms on fructose. The experiments on the effect of temperature suggest that while increasing temperature up to 40 °C increases the initial rate of hexose uptake by the vesicles, as the temperature is raised above 30 °C, the vesicles become leaky.

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