Journal of Cellular Biochemistry 18:239-244 (1982)

Regulation of Lactose Transport by the Phosphoenolpyruvate-Sugar Phosphotransferase System in Membrane Vesicles of Escherichia coli

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Regulation of lactose uptake by the phosphoenolpyruvate-sugar phosphotransferase system (PTS) has been demonstrated in membrane vesicles of Escherichia coli strain ML308-225. Substrates of the phosphotransferase system inhibited D-lactate energized uptake of lactose but did not inhibit uptake of either L-alanine or L-proline. This inhibition was reversed by intravesicular (but not extravesicular) phosphoenolpyruvate. Lactose uptake was also inhibited by enzyme III^{glc} preparations that were shocked into the vesicles, and this inhibition was reversed by phosphoenolpyruvate. Intravesicular HPr and enzyme I stimulated methyl α-glucoside uptake but did not inhibit or stimulate lactose accumulation. Vesicles maintained at 0°C for several days partially lost 1) the ability to take up lactose, 2) the ability to accumulate PTS substrates, and 3) PTS-mediated regulation. Phosphoenolpyruvate addition restored all of these activities. These results support a mechanism in which the relative proportions of phosphorylated and nonphosphorylated forms of a phosphotransferase constituent regulate the activity of the lactose permease.

Key words: protein phosphorylation, regulation, allosteris regulation, protein effector, bacterial phosphotransferase system, sugar transport

The primary functions of the bacterial phosphoenolpyruvate-sugar phosphotransferase system (PTS) include the detection, transport, and phosphorylation of its sugar substrates [1]. In enteric bacteria, the PTS also regulates the activities of adenylate cyclase and certain carbohydrate permeases [1]. These regulatory func-

Abbreviations: PTS, phosphoenolpyruvate-sugar phosphotransferase system; HPr, heat-stable phosphoryl carrier protein of the PTS; enzyme III^{glc}, glucose specific enzyme III (free form); enzyme III^{glc}-P, glucose-specific enzyme III (phosphorylated form).

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Received July 25, 1981; accepted September 13, 1981.

0730-2312/82/1802-0239$02.00

tions allow the selection of PTS substrates over non-PTS carbohydrates for growth. For example, they allow the preferential utilization of glucose to lactose in Escherichia coli [1–5].

Involvement of the protein components of the PTS in these regulatory mechanisms has been established through 1) physiological and genetic manipulation of the activities of these proteins [1,6,7], 2) studies with a mutant strain bearing a heat-labile enzyme I [8], and 3) alteration of cellular phosphoenolpyruvate levels in cells capable of transporting this high-energy phosphoryl donor [9,10]. From the results of these studies, a model has been proposed to account for PTS-mediated regulation of non-PTS carbohydrate uptake [1,4]. Central to this scheme is the involvement of a regulatory protein, RPr, believed to be enzyme III^{glc}, which serves as a phosphorylated intermediate in the transfer of phosphate from phosphoenolpyruvate to glucose. In the presence of any PTS sugar, phosphate from enzyme III^{glc} is transferred through HPr to the sugar via EII^{glc}. The proposed model predicts that in the free (nonphosphorylated) form, enzyme III^{glc} interacts allosterically with non-PTS carbohydrate permeases, thereby inhibiting their activities. In the presence of sufficient phosphoenolpyruvate and in the absence of a PTS substrate, enzyme III^{glc} becomes phosphorylated and is unable to inhibit non-PTS permease activities.

In this report, we present preliminary biochemical evidence for the involvement of enzyme III^{glc} and other proteins of the PTS in this regulatory process. Membrane vesicles derived from E coli strain ML308-225 were used to determine the effects of PTS substrates and partially purified soluble proteins of the PTS on energized lactose uptake when these proteins were introduced into the intravesicular compartment by osmotic shock. The results support the proposed regulatory mechanism [1,4].

METHODS

[D-glucose-1-^{14}C]lactose was purchased from the Amersham Corp. [^{14}C]methyl α-glucoside was from New England Nuclear. Other compounds were of the highest purity available commerically. Protein concentrations were determined according to Lowry et al with bovine serum albumin as the standard [11]. Soluble PTS proteins, enzyme I, HPr, and enzyme III^{glc}, were purified from Salmonella typhimurium strain LJ144, which carries the E coli F′ 198 episome. This episome bears the pts and crr genes, and strain LJ144 possesses 4- to 6-fold increased activities of enzyme I, HPr, and enzyme III^{glc} [4]. HPr was purified by DEAE cellulose ion exchange and Biogel P-60 gel filtration chromatography as described previously [12]. Enzyme III^{glc} was purified by a previously reported procedure [13] modified as follows:

High-speed supernatants (120,000g, 120 min) of cell-free extracts of strain LJ144 were treated at 4°C with 30% (W/V) ammonium sulfate and the precipitate was removed by centrifugation at 20,000g for 20 min at 4°C. The supernatant was then brought to 80% ammonium sulfate, and the enzyme III^{glc}-containing precipitate was obtained by centrifugation as above. This preparation was dialyzed at 4°C against 10 mM Tris-HCl, pH 7.5, containing 0.5 mM dithiothreitol to remove salt and then applied to a DEAE cellulose column pre-equilibrated with the dialysis buffer. The column was washed and the enzyme III^{glc} subsequently eluted with two 1.5 column volume aliquots of equilibration buffer containing KCl at final

concentrations of 0.1 and 0.2 M, respectively. Enzyme III^{glc} activity eluted in the 0.2 M KCl wash. This fraction was concentrated and applied to a 5 × 88 cm BioRad P-60 column pre-equilibrated with 50 mM Tris HCl, pH 7.5, containing 1 mM EDTA and 0.5 mM dithiothreitol. Enzyme III^{glc} activity was eluted with this buffer, and fractions containing the activity were pooled and concentrated. This preparation was then dialyzed against 5 mM postassium phosphate, pH 7.4, containing 1 mM dithiothreitol and applied to a 10 × 0.6 cm BioRad HT hydroxyapatite column prepared by mixing 14 parts hydroxyapatite with 1 part cellulose (Whatman CF11) (to increase the flow rate), and equilibrated with the phosphate-dithiothreitol buffer. Proteins were eluted stepwise from the column with 5 and 55 mM potassium phosphate, pH 7.4. Enzyme III^{glc} activity eluted with 55 mM potassium phosphate wash. This material was redialyzed against 5 mM potassium phosphate buffer, pH 7.4, containing 1.0 mM dithiothreitol and applied to a fresh hydroxyapatite column. This column was eluted stepwise with 10, 20, 40, and 60 mM potassium phosphate buffer. Enzyme III^{glc} activity was recovered in the 40 mM potassium phosphate fraction. This preparation gave one major peak on sodium dodecyl sulfate polyacrylamide gel electrophoresis with an approximate molecular weight of 20,000 daltons and contained minor protein impurities. Enzyme I was purified by DEAE cellulose ion exchange and Biogel P-200 gel filtration chromatography [14]. Enzyme I and enzyme III^{glc} were concentrated by use of Aquacide (Calbiochem, type III), and HPr was concentrated by lyophilization. These proteins were dialyzed before use.

E coli strain ML308-225 was grown in Medium 63 without iron [6], supplemented with 0.5% D-glucose. The cultures were grown at 37°C with rotary shaking and harvested between the mid and late exponential growth phases. Methods for membrane vesicle preparation have been described previously [15–17]. Assay solutions for measuring lactose uptake contained at final concentrations: 0.1 M potassium phosphate buffer, pH 6.6, 20 mM D-lactate, and 0.2 to 0.5 mg of membrane protein. The uptake experiment was initiated by addition of [^{14}C]lactose (14.4 mCi/mmole) at a concentration of 0.4 mM. Other additions were as indicated. Assays were conducted at 25°C and terminated by 20-fold dilution of the vesicles in 0.1 M LiCl followed by rapid filtration on Millipore filters (0.45 μ pore size). The filters were then dried, and radioactivity was determined with a liquid scintillation counter. PTS proteins were shocked into vesicles by a modified heat and osmotic shock procedure [15]. Membrane vesicles in 0.1 M potassium phosphate buffer, pH 6.6, containing 1 mM dithiothreitol and maintained on ice were rapidly diluted into distilled water pre-equilibrated to 30°C. Soluble PTS proteins (previously dialyzed against 1 mM dithiothreitol to lower their salt content), phosphoenolpyruvate, and other additions were included in the distilled water. The shock solution was incubated at 30°C with shaking for 5 min after which the buffer concentration was restored to 0.1 M by addition of 1 M potassium phosphate, pH 6.6. The vesicles were stored on ice and used immediately.

RESULTS

In preliminary experiments, the D-lactate-stimulated uptake of lactose into membrane vesicles derived from the glucose grown E coli strain ML308-225 cells was found to be inhibited by methyl α-glucoside, a nonmetabolizable glucose

analogue. Fructose and mannitol inhibited lactose accumulation in vesicles prepared from fructose and mannitol grown cells, respectively. Intravesicular phosphoenolpyruvate (5 mM) reversed the inhibitory effects of methyl α-glucoside, mannitol, and fructose. The glucoside did not inhibit D-lactate-stimulated L-alanine or L-proline uptake (data not shown). These results indicated that the PTS did not interfere with generation of the proton motive force [1].

To determine the regulatory roles of the various PTS proteins, they were shocked into the intravesicular compartment. Table I summarizes the effects of the various proteins on methyl α-glucoside uptake and phosphorylation. In the presence of phosphoenolpyruvate, HPr and enzyme III^{glc} stimulated methyl α-glucoside accumulation slightly and markedly stimulated sugar phosphorylation. In combination, enzyme I, HPr, and enzyme III^{glc} stimulated phosphorylation more than did any one of the individual proteins (Table I).

Enzyme I and HPr neither inhibited nor stimulated lactose uptake (data not shown). Inhibition of lactose uptake by enzyme III^{glc} preparations occurred in the absence of exogenous phosphoenolpyruvate, but the inhibitory effect was abolished when 10 mM phosphoenolpyruvate was present (Fig. 1). The enzyme III^{glc} preparation exerted its inhibitory effect only when it was present intravesicularly (Fig. 2). With lower levels of phosphoenolpyruvate (which did not completely overcome the inhibitory action of intravesicular enzyme III^{glc}), enzyme I, and HPr facilitated relief from inhibition by the phosphate donor (data not shown). These results suggest that enzyme I and HPr function in the phosphorylation of RPr, and that enzyme III^{glc} is this proposed regulatory protein.

Membrane vesicles derived from E coli strain ML308-225 cells and maintained on ice for several days exhibited poor lactose transport. Vesicles concurrently lost the ability to accumulate appreciable methyl α-glucoside, and in these vesicle preparations, methyl α-glucoside no longer inhibited lactose uptake (Table II). All three functions were restored by intravesicular phosphoenolpyruvate (Table II). Other

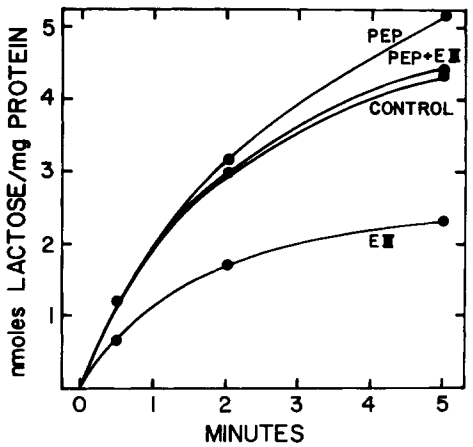

Fig. 1. Regulation of lactose uptake by enzyme III^{glc} and phosphoenolpyruvate in membrane vesicles from E coli strain ML308-225 cells. Lactose uptake assays were conducted as described in the text. The final concentration of phosphoenolpyruvate in the reaction mixture was 10 mm. Control vesicles underwent the shock treatment with no additions. Abbreviations: EIII, enzyme III^{glc}; PEP, phosphoenolpyruvate.

TABLE I. Stimulation of Methyl α-Glucoside Uptake and Phosphorylation by Membrane Vesicles From E coli Strain ML308-225*

Additions	Uptake	Methyl α-glucoside phosphorylation (nmoles/min/mg protein)
None	0.15	0.2
PEP	0.60	2.7
PEP + enzyme I	ND[a]	2.2
PEP + enzyme III	0.64	21
PEP + HPr	0.72	19
PEP + HPr + enzyme III	0.76	31
PEP + HPr + enzyme I	ND	18
PEP + HPr + enzyme III + enzyme I	ND	39

*Experimental procedures were as described in the text. Phosphoenolpyruvate (PEP), when present, was added to a final concentration of 5 mM.
[a]ND, not determined

TABLE II. Activation of Lactose Uptake and PTS-Mediated Regulation by Phosphoenolpyruvate*

Vesicle preparation	Methyl α-glucoside (5mM)	Phosphoenolpyruvate (5 mM)	Lactose uptake (nmoles/min/mg protein)	Methyl α-glucoside uptake (nmoles/min/mg protein)
Fresh vesicles	–	–	2.9	0.1
	+	–	1.8	–
	–	+	3.1	0.3
	+	+	2.4	–
3-day-old vesicles	–	–	0.2	0.04
	+	–	0.2	–
	–	+	1.1	0.2
	+	+	0.3	–

*Methyl α-glucoside, when present, was added extravesicularly. Phosphoenolpyruvate was shocked into the vesicles as described under Methods. Lactose uptake assays were conducted as described in the text. Methyl α-glucoside uptake assays were performed as previously described [15].

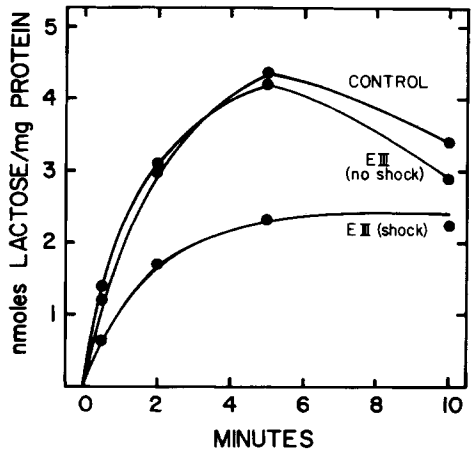

Fig. 2. Effects of intravesicular and extravesicular enzyme III^{glc} on lactose uptake in membrane vesicles from E coli strain ML308-225 cells. Lactose uptake assays were conducted as described under Methods. Enzyme III^{glc} was added to the vesicle preparation either before subjecting the vesicles to osmotic shock (EIII, shock) or after subjecting the vesicles to the shock procedure (EIII, no shock). Enzyme III^{glc} was not added to the control vesicles.

potential phosphate donors tested, including glucose-6-phosphate and ATP, failed to restore these activities although 2-phosphoglycerate had a mild restorative effect (data not shown).

DISCUSSION

In this report, we have shown that PTS-mediated regulation of the lactose permease can be demonstrated in E coli membrane vesicles. The enzyme III^{glc}, when shocked into membrane vesicles, was shown to markedly affect the regulation of lactose uptake (Figs. 1, 2), although it exerted minimal effects on methyl α-glucoside uptake (Table I). This observation presumably results from the fact that membrane-associated proteins are primarily responsible for PTS-mediated uptake in E coli vesicles [18,19], while soluble enzyme III^{glc} presumably mediates regulation [1,2]. Most significantly, a central regulatory role for enzyme III^{glc} is suggested by the biochemical data reported in this communication. The modulatory mechanism controlling the regulatory activity of enzyme III^{glc} appears to involve its phosphorylation since phosphoenolpyruvate reversed both methyl α-glucoside and enzyme III^{glc} promoted inhibition of lactose uptake. A role of enzyme I and HPr in the phosphorylation of the protein was supported by the observation that these proteins facilitated reversal of the inhibitory effect by low concentrations of phosphoenolpyruvate. The mechanisms by which phosphoenolpyruvate stimulated lactose uptake and restored PTS-mediated regulation of lactose uptake in aged vesicles have yet to be elucidated.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant 1 RO1 AI 14176-01 from the National Institute of Allergy and Infectious Diseases and a postdoctoral research fellowship F32 AI 05749-01 to S.S.D. from the National Institute of Allergy and Infectious Diseases.

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