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Kinetic characterization and regulation of phospho*enol*pyruvate-dependent methyl α-D-glucopyranoside transport by *Salmonella typhimurium* membrane vesicles

(phosphotransferase/toluene-treated vesicles/feedback inhibition/exchange transphosphorylation)

K. DAI-FANG LIU AND SAUL ROSEMAN

Department of Biology and the McCollum–Pratt Institute, The Johns Hopkins University, Baltimore, MD 21218

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ABSTRACT **Membrane vesicles from *Salmonella typhimurium* SB3507 were used to study the kinetics of methyl α-D-glucopyranoside (MeGlc) transport by the phospho*enol*pyruvate:glycose phosphotransferase system (PTS). During the first minute of phospho*enol*pyruvate-dependent MeGlc transport, two distinct rates were observed; an initial rapid rate, V_1 (V_{max}, 7.4–8.4 nmol·mg^{-1}·min^{-1}; K_m, 8.2–11.2 × 10^{-6} M), followed by a second slower rate, V_2 (V_{max}, 4–4.6 nmol·mg^{-1}·min^{-1}; K_m, 3.4–6.4 × 10^{-6} M). The change in rate occurred when the intravesicular MeGlc phosphate concentration was 0.2 mM or less, depending on the external MeGlc concentration. The rate-limiting component in MeGlc transport was found to be enzyme II-B^{Glc}, not phospho*enol*pyruvate uptake or the PTS proteins enzyme I, HPr, and III^{Glc}. The change from V_1 to V_2 thus suggests that the PTS is regulated in intact vesicles. However, this regulation was completely relieved by permeabilizing the vesicles with toluene. That is, the toluene-treated vesicles showed only V_1 for MeGlc phosphorylation. Evidence was obtained to show that pyruvate and its metabolic products generated by the vesicles exerted no effect on the rate of MeGlc transport. Furthermore, the result from a dual-label experiment excluded exchange transphosphorylation as the mechanism for regulating MeGlc transport by the vesicles. Possible mechanisms for regulation of the PTS are discussed.**

Sugar translocation in bacteria has been shown to be the rate-limiting step in the utilization of these compounds (1–4). We have been particularly interested in the phosphotransferase system (5, 6), a unique array of four proteins that catalyzes the phosphorylation and concomitant translocation of its sugar substrates across the bacterial membrane, with phospho*enol*pyruvate (*P-e*Prv) as the ultimate energy donor. Glucose, for example, is transported by the III^{Glc}/II-B^{Glc} system; the phosphoryl group is successively transferred from *P-e*Prv to two general proteins, enzyme I and HPr, to the sugar-specific phosphocarrier protein III^{Glc}, and thence to the sugar. During the last step, which is catalyzed by a specific membrane protein designated enzyme II-B^{Glc}, the sugar is phosphorylated and concomitantly transported across the membrane.

The present study was designed to provide information on the kinetics of the III^{Glc}/II-B^{Glc} system with a view to understanding the mechanism of regulation of this phosphotransferase system. To simplify these investigations we used the nonmetabolizable analogue methyl α-D-glucopyranoside (MeGlc), which, both *in vitro* and in whole cells, has been shown to be a unique substrate for the III^{Glc}/II-B^{Glc} system at micromolar concentrations (7, 8). In addition, bacterial membrane vesicles, closed systems devoid of most of the cytoplasmic constituents and thus considerably less complicated than whole cells, were used.

This report describes an initial examination of the kinetics of MeGlc transport by *Salmonella typhimurium* vesicles, the discovery of two distinct rates in the first minute of uptake, and attempts to elucidate the mechanism underlying this phenomenon.

MATERIALS AND METHODS

Materials. MeGlc, D-(−)-3-phosphoglyceric acid (grade II, disodium salt), and *P-e*Prv (potassium salt) were purchased from Sigma. The pH of *P-e*Prv solutions was adjusted to neutral with potassium bicarbonate. Nutrient broth and Casamino acids were purchased from Difco. Hydrofluor obtained from National Diagnostics (Somerville, NJ) was used to determine radioactivity in aqueous samples, and Research Products International (Elk Grove Village, IL) Scintillator concentrated liquid scintillator (containing 1.25 g/liter of 2,5-diphenyloxazole, 2.5 g/liter of dimethyl-1,4-bis[5-phenyl(oxazolyl)]benzene) diluted 1:23 with toluene obtained from Research Products International was used to determine radioactivity on dried filters. Glass fiber filters, Whatman GF/F, were from Thomas. All other chemicals were of reagent grade from standard sources. Methyl α-D-[U-^{14}C]-glucopyranoside and methyl α-D-[U-^{3}H]glucopyranoside were synthesized from D-[U-^{14}C]glucose (1–5 mCi/mmol; 1 Ci = 37 GBq; Amersham) and D-[2-^{3}H(N)]glucose (14.1 Ci/mmol; New England Nuclear), respectively (9). Both labeled glucosides were free of the β-anomer.

Bacterial Strain and Medium. *S. typhimurium* strain SB3507 (*ara-9 pts*$^{+}$ *trpB223*) was obtained from P. E. Hartman. Cells were grown in minimal salt medium, medium 63 M (10), which was supplemented with 1% Casamino acids (acid-hydrolyzed casein with vitamins; Bacto Casamino Acids, Difco) and tryptophan (20 μg/ml). The carbon source in the growth medium was 0.2% D-glucose.

Preparation of Vesicles. Membrane vesicles were prepared according to a modification of the procedure of Kaback (11) with further improvements (unpublished data). Protein concentrations were determined by a modified microbiuret procedure (12) with bovine serum albumin as standard.

Transport Experiments. To follow transport of MeGlc by membrane vesicles, two measurements were made. One, designated "uptake," was to determine the total amount of MeGlc accumulated inside the vesicles; the other, designated "phosphorylation," was to measure the total amount of methyl α-D-

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Abbreviations: PTS, phospho*enol*pyruvate:glycose phosphotransferase system; *P-e*Prv, phospho*enol*pyruvate; MeGlc, methyl α-D-glucopyranoside; MeGlc-*P*, methyl α-D-glucopyranoside 6-phosphate.

glucopyranoside 6-phosphate (MeGlc-*P*) present inside and outside the vesicles. The temperature and the amount of vesicle protein used in the transport experiments are indicated in the figure legends. The final reaction mixture in a 30-ml glass beaker coated with bovine serum albumin contained 50 mM potassium phosphate buffer (pH 6.6) and 10 mM $MgSO_4$. Vesicles resuspended in the reaction medium were incubated for 3 min either in the presence or absence of 5 mM *P-e*Prv by swirling at 110 rpm in a water bath. The reaction was started by addition of Me[^{14}C]Glc. Aliquots of 100 μl were taken at various times to determine "uptake" and "phosphorylation" as described (11). Net uptake and net phosphorylation are presented—i.e., values were corrected for any uptake or phosphorylation in the absence of added *P-e*Prv, which was equivalent to ≈0.5 nmol of MeGlc-*P* per mg of vesicle protein.

Toluene Treatment. Vesicles (0.5 ml; membrane protein concentration, 10 mg/ml) were pipetted into a 13 × 100 mm test tube containing 3 μl of toluene at room temperature, shaken vigorously with a Vortex mixer for 10 sec, and stored on ice. The toluene-treated vesicles were gently mixed by hand before an aliquot was withdrawn. All experiments performed with toluene-treated vesicles were completed within 5 hr after treatment.

RESULTS

Kinetic Properties of MeGlc Transport by *S. typhimurium* Vesicles. To examine the kinetics of MeGlc transport, it was necessary to ensure that the *S. typhimurium* vesicles were not leaky. In each experiment we established that the vesicles were intact by showing that all of the intravesicular radioactivity was MeGlc-*P* and that the total MeGlc-*P* formed during transport was retained by the vesicles.

Membrane vesicles prepared from cells induced for *P-e*Prv permease (13) were used to study *P-e*Prv-dependent MeGlc transport. The initial rates of uptake and phosphorylation by membrane vesicles were determined at five concentrations of MeGlc (Fig. 1). In all cases, an initial rapid rate, designated V_1, changed to a second slower rate, designated V_2. The time at which the rate changed from V_1 to V_2 appeared to depend on the concentration of MeGlc used. These results suggested that the change in rate might be related to the intravesicular MeGlc-

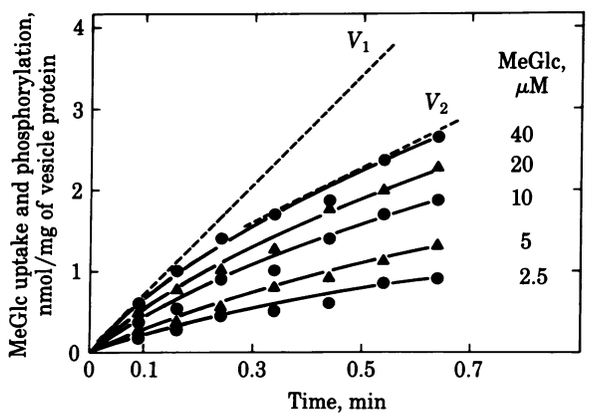

FIG. 1. Initial uptake and phosphorylation of MeGlc by *S. typhimurium* vesicles. The assay mixture (total vol, 1 ml) containing 5 mM *P-e*Prv and 1 mg of vesicle protein was first incubated at 30°C for 3 min. Me[^{14}C]Glc (specific activity, 4.1 mCi/mmol), at the indicated concentrations, was added to start the reaction. Independent and duplicate assays were carried out to determine uptake and phosphorylation. Only uptake data are shown (●, ▲) because each phosphorylation curve was coincident with its respective uptake curve. Tangent lines (---) are drawn along one uptake curve to indicate the change in rate from V_1 to V_2.

P level. Using an average vesicular volume of 5 μl per mg of vesicle protein (11), the change took place when the concentration of MeGlc-*P* was <0.2 mM. Both V_1 and V_2 exhibited typical Michaelis–Menten kinetics. The kinetic constants were determined independently with several different vesicle preparations. The apparent K_m and V_{max} values for V_1 were 8.2–11.2 × 10^{-6} M and 7.4–8.4 nmol·mg^{-1}·min^{-1}, respectively; for V_2, 3.4–6.4 × 10^{-6} M and 4–4.6 nmol·mg^{-1}·min^{-1}, respectively.

Determination of the Rate-Limiting Component in *P-e*Prv-Dependent MeGlc Transport by *S. typhimurium* Vesicles. As mentioned in the Introduction, sugar transport mediated by enzyme II-B^{Glc} requires *P-e*Prv, as well as enzyme I, HPr, and III^{Glc} of the phospho*enol*pyruvate:glycose phosphotransferase system (PTS). Therefore, the availability of *P-e*Prv and the levels of the three soluble PTS proteins in the vesicles could directly affect the rate of sugar transport. To understand why the rates changed from V_1 to V_2 (Fig. 1), it was essential to determine the rate-limiting component in MeGlc transport by the vesicles.

We first examined the kinetics of *P-e*Prv transport by the vesicles with the use of [^{14}C]*P-e*Prv. The apparent K_m and V_{max} values for *P-e*Prv uptake were 7.25 × 10^{-3} M and 385 nmol·mg^{-1}·min^{-1}, respectively. Thus, the rate of *P-e*Prv transport by the vesicles was at least 20-fold greater than that of MeGlc transport. Furthermore, there was no detectable metabolism of *P-e*Prv by the vesicles (data not shown), suggesting that intravesicular *P-e*Prv remained as *P-e*Prv. Therefore, we concluded that MeGlc transport by the vesicles was not limited by the supply of *P-e*Prv.

The levels of PTS-soluble proteins were estimated by rocket immunoelectrophoresis. The intravesicular concentrations of HPr and III^{Glc} were determined to be 34.5 and 8.2 μM, respectively. Both HPr and III^{Glc} were apparently present in excess in the vesicles because their respective K_m values for enzyme I and enzyme II-B^{Glc} have been estimated as 5 × 10^{-6} M and 3 × 10^{-6} M (14, 15). The specific activity of enzyme I in the vesicles, 0.35 nmol·mg^{-1}·min^{-1}, was 10-fold higher than that of enzyme II-B^{Glc}. Therefore, we conclude that MeGlc transport by the vesicles is rate-limited by enzyme II-B^{Glc} of the PTS.

Possible Regulatory Mechanisms for MeGlc Transport by *S. typhimurium* Vesicles. The results described above clearly show that neither *P-e*Prv transport nor the levels of enzyme I, HPr, or III^{Glc} in the vesicles affect the initial transport of MeGlc. Because there was a change in the rate from V_1 to V_2, the PTS must be subject to regulation in intact vesicles. To gain information on the nature of the regulatory species, vesicles were treated with toluene, which permeabilized the membranes selectively to low molecular weight solutes. As shown in Fig. 2, toluene-treated vesicles were fully active in phosphorylating MeGlc; thus, the soluble PTS proteins were not lost or inactivated by the toluene treatment. Equally important, the change in rate from V_1 to V_2 seen in intact vesicles was not observed in toluene-treated vesicles. Only one rate of sugar phosphorylation, similar to V_1 of sugar transport with intact vesicles, was obtained. One interpretation of these results is that the inhibitory species formed in intact vesicles is a low molecular weight compound (or compounds) that is lost from toluene-treated vesicles into the medium.

Two low molecular weight solutes, pyruvate and MeGlc-*P*, are formed intravesicularly during transport of sugars by the PTS and are therefore potential feedback inhibitors that would be lost from the vesicles by toluene treatment. As indicated above, the change from V_1 to V_2 occurs when the MeGlc-*P* concentration in the vesicles is ≈0.2 mM. Consistent with this interpretation, Fig. 3 shows that only one rate, V_2, was ob-

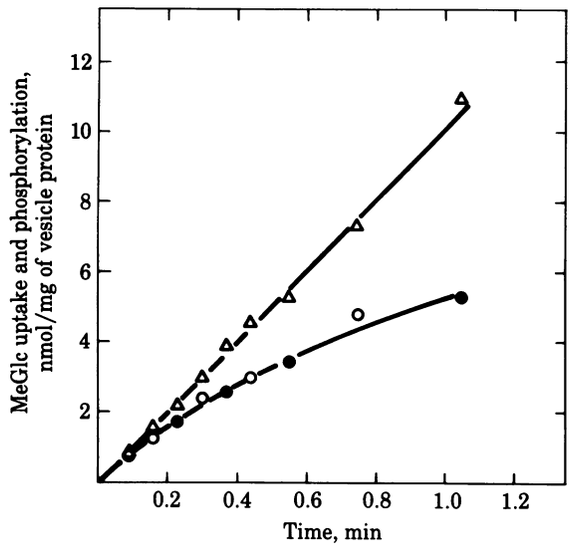

FIG. 2. Initial uptake and phosphorylation of MeGlc by intact and toluene-treated vesicles. Vesicles were treated with 0.6% toluene. The assay mixture (total vol, 1.5 ml) containing 5 mM *P-e*Prv and 1.5 mg of vesicle protein was incubated at 25°C for 3 min. The reaction was started by adding Me[^{14}C]Glc at a final concentration of 40 μM (specific activity, 2.5 mCi/mmol). For intact vesicles, alternate aliquots were taken to determine uptake (●) and phosphorylation (○). For toluene-treated vesicles (△), only phosphorylation was measured because no uptake was observed (data not shown).

served when the vesicles were loaded with ≈0.35 mM MeGlc-*P* (^{3}H-labeled) and pyruvate prior to adding MeGlc (^{14}C-labeled) to the vesicles. When higher concentrations of sugar phosphate and pyruvate were first loaded into the vesicles, the degree of inhibition was even greater (data not shown).

Experiments were then conducted to determine whether the inhibitor was pyruvate, but the following lines of evidence in-

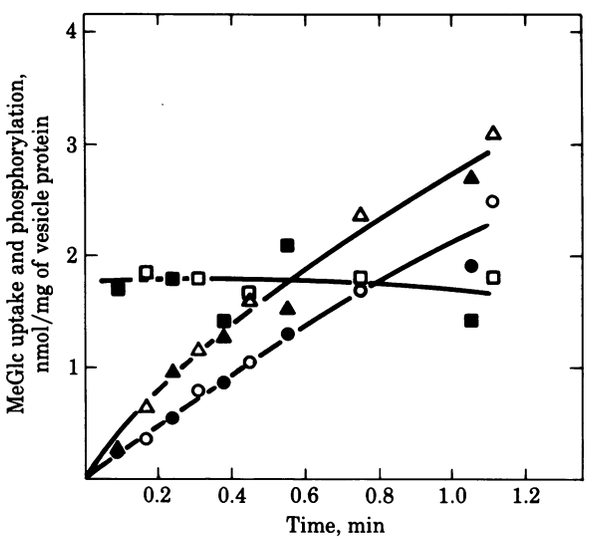

FIG. 3. Initial uptake and phosphorylation of Me[^{14}C]Glc by vesicles preloaded with Me[^{3}H]Glc-*P*. The assay mixture (total vol, 1.5 ml) containing 1.5 mg of vesicle protein, 5 mM *P-e*Prv, and 5 μM Me[^{3}H]Glc (specific activity, 2.2 mCi/mmol) was first incubated for 3 min at 26°C. At time zero, Me[^{14}C]Glc at a final concentration of 40 μM (specific activity, 1.9 mCi/mmol) was added. Both uptake (solid symbols) and phosphorylation (open symbols) were determined. ^{14}C label is indicated by ●——○, and ^{3}H label by ■——□. The control experiment (▲——△) was conducted in a similar fashion except that Me[^{3}H]Glc was omitted from the assay mixture.

dicate that neither pyruvate nor the catabolite(s) derived from pyruvate is the inhibitory species. (*i*) No significant inhibition of MeGlc transport by the vesicles was observed when sugar transport was conducted in the presence of pyruvate at concentrations ranging from 5 μM to 20 mM. (*ii*) Vesicles were found to take up pyruvate actively, suggesting that the lack of inhibition of sugar transport by pyruvate is not due to a failure of pyruvate to reach the interior of the vesicles where the regulatory site of the PTS is presumably located. (*iii*) Vesicles were found to metabolize pyruvate at a rapid rate. We therefore concluded that neither pyruvate nor its metabolic product(s) inhibits MeGlc transport by the vesicles under these conditions.

The available data therefore suggested that the intravesicular sugar phosphate was the feedback inhibitor of the PTS, and this inhibition resulted in the change in rate from V_1 to V_2 and ultimately to an even greater inhibition as more sugar phosphate accumulated inside the vesicles. This idea was tested directly by adding MeGlc-*P* to toluene-treated vesicles. Inhibition of sugar phosphorylation was in fact observed at high concentrations of MeGlc-*P* but, at the concentrations discussed above, for instance at 0.5 mM, the toluene-treated vesicles were not at all inhibited (data not shown). This result argues against the idea that MeGlc-*P* is a feedback inhibitor of the PTS, but we note that toluene-treated vesicles may behave differently with respect to regulation of this type (see *Discussion*).

One possible mechanism, aside from feedback inhibition, for MeGlc-*P* to regulate sugar transport is by exchange transphosphorylation. It has been reported that enzyme II-B^{Glc} in *S. typhimurium* catalyzes a direct transfer of the phosphoryl group from glucose 6-phosphate to MeGlc (16). If such an exchange reaction occurred in vesicles, it would not result in a net accumulation of sugar phosphate inside the vesicles, and competition between exchange transphosphorylation and *P-e*Prv-dependent sugar transport would result in an apparent inhibition of the latter process. However, the result from a dual-label experiment shown in Fig. 3 clearly shows that vesicles preloaded with Me[^{3}H]Glc-*P* do not exchange internal ^{3}H-labeled sugar for external Me[^{14}C]Glc and, furthermore, that the preloaded vesicles transported the ^{14}C-labeled sugar only at the second rate (V_2). Thus, exchange transphosphorylation does not cause the change in rate from V_1 to V_2 during initial transport of MeGlc by *S. typhimurium* vesicles.

In the course of investigating regulation of the PTS, free sugar and *P-e*Prv were found to exert opposite effects on sugar transport by *S. typhimurium* vesicles (data not shown). Inhibition of MeGlc transport by micromolar concentrations of MeGlc was observed with both intact and toluene-treated vesicles, indicating that sugar substrates are exceedingly potent inhibitors of the PTS. On the other hand, stimulation of V_1 of MeGlc transport was noted by increasing the length of preincubation of vesicles with *P-e*Prv (V_2 did not increase). It is not understood how the PTS is regulated by *P-e*Prv or free sugar, but tentative interpretations of these intriguing observations are proposed below.

DISCUSSION

Two distinct rates are present during the initial transport of MeGlc by *S. typhimurium* vesicles (Fig. 2). From kinetic studies of *P-e*Prv transport and quantitative analysis of intravesicular levels of enzyme I, HPr, and III^{Glc}, enzyme II-B^{Glc} was found to be the rate-limiting component in the transport process. Therefore, the change in the initial rate from V_1 to V_2 suggests that II-B^{Glc} of the PTS is subject to stringent regulation. A similar rapid decline in the rate of MeGlc uptake by whole cells of *S. typhimurium* (8) and *Staphylococcus aureus* (17) has been

reported. An immediate question is how sugar transport mediated by the PTS is regulated. Although we do not yet have the answer to this important question, data presented in this report rule out several mechanisms.

The regulatory species appear to be small molecules because the change in rate of MeGlc transport was completely relieved by permeabilizing the vesicles with toluene (Fig. 2). The lack of inhibition of MeGlc transport by pyruvate, in addition to the very active uptake and metabolism of pyruvate by intact vesicles, rules out the regulatory role of pyruvate or its metabolic products on the PTS in this vesicle system. Kornberg (18) has suggested that pyruvate or a metabolite formed from pyruvate inhibits sugar translocation by the PTS in whole cells.

The role of hexose phosphates in regulating sugar transport in bacteria has been controversial. Our data show that when the vesicles have accumulated 0.2 mM MeGlc-*P* or less, there is a change from V_1 to V_2 in the rate of transport. The dual-label experiment (Fig. 3) indicates that this change in rate does not result from exchange transphosphorylation between external free sugar and internal sugar phosphate. It is not certain, however, whether sugar phosphate regulates the PTS via feedback inhibition, because hexose phosphates at 0.5 mM did not inhibit the rate of MeGlc phosphorylation by toluene-treated vesicles. It is possible that toluene uncouples the PTS from regulation normally existing in intact vesicles. Alternatively, a regulator, yet to be identified, may be generated by intact vesicles during the initial stage of sugar transport and this regulator, being a low molecular weight compound, is lost from toluene-treated vesicles. Consistent with our observations with toluene-treated vesicles, a lack of apparent regulation of the PTS was noted in cell-free extracts and with purified proteins (19).

The stimulation by *P-e*Prv and inhibition by MeGlc of sugar transport by *S. typhimurium* vesicles is intriguing. There is no doubt that this observation further reflects the complexity of the PTS. It is possible that phosphorylation of the PTS proteins by *P-e*Prv results in association of enzyme I, HPr, and III^{Glc} with the membrane component, enzyme $II\text{-}B^{Glc}$, whereas dephosphorylation of the PTS by free sugars causes dissociation of such a functional complex. Alternatively, the process of phosphorylation and dephosphorylation of the PTS may result in reversible conformational changes of the PTS complex between states that exhibit different degrees of efficiency.

It is not unlikely that intact cells use more than one mechanism, possibly many, to regulate the PTS, because the rate of sugar transport and the intracellular concentrations of sugar phosphates are extremely important to cell survival. The results reported here show that the PTS is subject to stringent regulation and that the regulatory mechanisms may be complex.

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