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HPr(His~P)-mediated Phosphorylation Differently Affects Counterflow and Proton Motive Force-driven Uptake via the Lactose Transport Protein of *Streptococcus thermophilus**

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The lactose transport protein (LacS) of *Streptococcus thermophilus* has a C-terminal hydrophilic domain that is homologous to IIA protein and protein domains of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS). The IIA domain of LacS is phosphorylated on His-552 by the general energy coupling proteins of the PTS, which are Enzyme I and HPr. To study the effect of phosphorylation on transport, the LacS protein was purified and incorporated into liposomes with the IIA domain facing outwards. This allowed the phosphorylation of the membrane-reconstituted protein by purified HPr(His~P) of *S. thermophilus*. Phosphorylation of LacS increased the V_{max} of counterflow transport, whereas the V_{max} of the proton motive force (Δp)-driven lactose uptake was not affected. In line with a range of kinetic studies, we propose that phosphorylation affects the rate constants for the reorientation of the ternary complex (LacS with bound lactose plus proton), which is rate-determining for counterflow but not for Δp -driven transport.

In *Streptococcus thermophilus*, lactose is taken up via the lactose transport protein LacS¹ in symport with a proton or in exchange for galactose. The lactose/galactose exchange reaction is the most relevant mode of transport as galactose is an end product of metabolism, and this reaction is more rapid than lactose/H⁺ symport. The LacS of *S. thermophilus* consists of a polytopic membrane-embedded translocator domain and a C-terminal hydrophilic IIA-like domain that has been shown to be phosphorylated on His-552 by HPr(His~P), a general energy coupling protein of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS, Refs. 1 and 2). By entrapping P-enolpyruvate, Enzyme I plus HPr, inside the lumen of membrane vesicles harboring LacS, for substrate levels below the apparent affinity constant (K_m) for transport, Δp -driven uptake

of lactose is partially inhibited upon phosphorylation of the protein (1).

In addition to histidine phosphorylation of HPr by Enzyme I with P-enolpyruvate as phosphoryl donor (3), HPr in Gram-positive bacteria can also be reversibly phosphorylated on a serine residue, yielding HPr(Ser-P), by a metabolite-activated ATP-dependent protein kinase and a HPr(Ser-P) phosphatase (4, 5, 6). Moreover, the doubly phosphorylated species, HPr(Ser-P/His~P), is also found in Gram-positive bacteria. The various species of HPr in *S. thermophilus* growing on the non-PTS sugar lactose have recently been quantified at different stages of growth (22). HPr(Ser-P) was found to be the dominant phosphorylated species in the exponential phase of growth, whereas HPr(His~P) dominated in the stationary phase. The transition from HPr(Ser-P) to HPr(His~P) paralleled the decrease in lactose and an increase in galactose concentration in the growth medium. Because of the decrease in lactose/galactose ratio in the medium, the transport capacity of the cell will decrease as growth proceeds. The apparent decrease in lactose transport capacity, however, is compensated by an increase in LacS expression levels, which is caused by the release of HPr(Ser-P)/CcpA-mediated inhibition of *lacS* transcription. The increase in HPr(His~P) at the late-exponential phase of growth paralleled an increase in the extent of phosphorylation of LacS, which could be another means to regulate the transport capacity of the cell. To obtain further insight into the regulation of lactose transport activity upon phosphorylation of LacS, we report here our studies with purified LacS reconstituted into proteoliposomes with the IIA domain facing outwards. To effectively phosphorylate LacS, the native HPr from *S. thermophilus* was purified and used as a phosphoryl donor.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions

Escherichia coli NM522/pAG3 (7) and M15/pAG4/pREP4 (8) were grown in Luria Broth supplemented with carbenicillin (50 μ g/ml) under vigorous aeration at 37 °C (9). When plasmid pREP4 was present, 50 μ g/ml kanamycin was added to the growth medium. Plasmid pAG3 and pAG4 carry the *ptsI* gene and the *hprK* gene of *Bacillus subtilis*, respectively, under control of the Taq promoter, and both genes are in frame with a sequence specifying an N-terminal His tag. Plasmid pREP4 (QIAGEN) carries the gene for the *lacI* repressor. For induction of gene expression, isopropyl- β -D-thiogalactopyranoside was added to the medium (1 mM) after the culture had reached an OD at 660 nm of 0.7. The cells were harvested after another 4 h of incubation. For large scale protein purification, the cells were grown in a 15-liter fermentor (Bio Bench ADI 1065; Applicon, Inc.) with the pH controlled at 7.0 and oxygen supply at 50% saturation.

S. thermophilus ST11(*AlacS*)/pGKHis (10) was grown semianaerobically at 42 °C in (B)elliker broth (10) supplemented with 0.5% beef extract, 20 mM lactose plus 5 μ g/ml erythromycin. pGKHis carries the

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This paper and the accompanying one are dedicated to the memory of Jonathan Reizer, from whom many studies on HPr-mediated regulation of metabolism have originated.

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¹ The abbreviations used are: LacS, lactose transport protein; PTS, phosphoenolpyruvate:carbohydrate phosphotransferase system; PAGE, polyacrylamide gel electrophoresis; Ni²⁺NTA, Ni-nitrilotriacetic acid, P-enolpyruvate, phosphoenolpyruvate; Pipes, 1,4-piperazinediethanesulfonic acid.

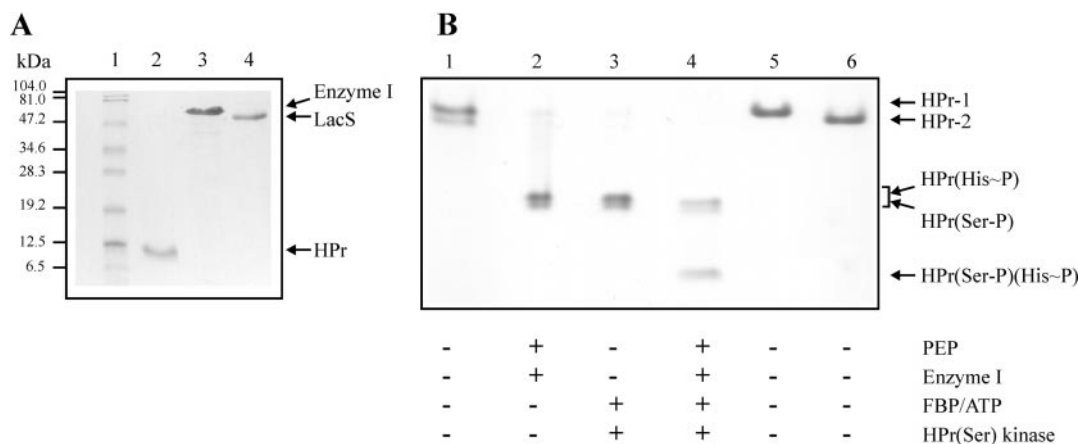


FIG. 1. Protein purification and characterization of HPr from *S. thermophilus*. A, Coomassie Brilliant Blue-stained SDS-PAGE gel (15% polyacrylamide). Lane 1, protein markers; lane 2, HPr purified from *S. thermophilus* (~7 µg of protein); lane 3, Enzyme I-His₆ purified from *B. subtilis* (~2 µg of protein); lane 4, LacS-His₆ purified from *S. thermophilus* (~2 µg of protein). B, Coomassie Brilliant Blue-stained non-denaturing PAGE gel (15% polyacrylamide). The presence of HPr (3 µg), Enzyme I (0.5 µg) plus 5 mM P-enolpyruvate (PEP), and/or HPr(Ser) kinase (0.5 µg) plus 10 mM FBP and 5 mM ATP is indicated below the figure. The phosphorylation reactions were carried out at 37 °C for 10 min in a total volume of 20 µl and a buffer composition of 50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol plus 10 mM MgSO₄. Phosphorylation of HPr by HPr(Ser) kinase and Enzyme I was carried out sequentially; HPr was first incubated with HPr(Ser) kinase plus 10 mM FBP and 5 mM ATP for 10 min, after which Enzyme I plus 5 mM P-enolpyruvate were added, and the incubation was continued for another 20 min. Lanes 1 to 4, HPr (both forms); lane 5, HPr-1; and lane 6, HPr-2.

lacS gene in frame with a sequence specifying a C-terminal His tag under control of the *lacS* promoter. For large scale protein purification, the cells were grown in a 15-liter fermentor with pH controlled at 6.8.

Isolation of Membranes

Membrane vesicles of *S. thermophilus* were isolated as described (11) with the following modifications: the cell wall was digested with 10 mg/ml lysozyme, and DNase and RNase were added to final concentrations of 100 µg/ml each. Membrane preparations were stored in liquid nitrogen.

Protein Purification

HPr—Cells of *S. thermophilus* ST11(Δ lacS)/pGKH_{is} were lysed in a buffer containing 20 mM Tris-HCl, pH 8.5, 10 mM MgSO₄, following the procedure described under "Isolation of Membranes." The cytosolic protein fraction was collected after removal of cell debris and membranes (48,200 × g; 4 °C). NaCl was added to a final concentration of 70 mM, and the sample was loaded onto a S-Sepharose column (fast flow) to remove the lysozyme. Both the S- and DEAE-Sepharose columns used hereafter were equilibrated with 20 mM Tris-HCl, pH 8.5, plus 70 mM NaCl. The flow-through of the S-Sepharose column was loaded onto a DEAE-Sepharose fast flow column (1.6 × 40 cm, Amersham Pharmacia Biotech). Again the flow-through was collected and concentrated by ultrafiltration in an Amicon cell with an YM1 membrane (*M_w*, 1,000 cut-off value). The salt concentration was lowered to ≤15 mM NaCl by adding 100 mM Tris-HCl, pH 8.5, and the protein sample was applied onto a MonoQ column (HR16/10 Amersham Pharmacia Biotech) that was equilibrated with 20 mM Tris-HCl, pH 8.5. After washing with 5 column volumes of the same buffer, proteins were eluted with a 15–80 mM NaCl gradient (240 ml) at a flow rate of 2 ml/min. HPr eluted at approximately 55 mM NaCl. Fractions containing HPr were pooled, desalted on a PD10 column (Amersham Pharmacia Biotech) against 50 mM Tris-HCl, pH 7.4 and concentrated to about 5 mg/ml by ultrafiltration in an Amicon cell with an YM1 membrane. Purified HPr samples were stored at –80 °C.

Enzyme I—Enzyme I from *B. subtilis* was purified from *E. coli* NM522/pAG3. Cells were harvested by centrifugation, washed twice with 50 mM sodium phosphate (NaPi), pH 7.0 and resuspended in 50 mM NaPi, pH 7.0, plus 10% (w/v) glycerol. After breaking the cells with a French pressure cell (20,000 psi), the lysate was incubated with DNaseI/RNaseI (0.1 mg/ml) in the presence of 10 mM MgSO₄ for 30 min at 37 °C. The cytosolic protein fraction was collected after centrifugation for 15 min at 40,000 × g. NaCl and imidazole were added to the supernatant to final concentrations of 100 and 30 mM, respectively. Enzyme I-His₆ was purified by Ni-nitrilotriacetic acid (Ni²⁺-NTA) affinity chromatography as described by Galinier *et al.* (7). The fractions containing Enzyme I-His₆ were pooled and desalted on a PD10 column against 50 mM Tris-HCl, pH 7.4. Purified Enzyme I samples were stored at –80 °C.

HPr(Ser) Kinase—HPr(Ser) kinase from *B. subtilis* was purified from *E. coli* M15/pAG4/pREP4. All purification steps are the same as described for Enzyme I purification.

LacS—Solubilization, purification, and membrane reconstitution of LacS were performed as described by Knol *et al.* (10, 12). Briefly, right-side-out membrane vesicles (5 mg/ml) of *S. thermophilus* ST11(Δ lacS)/pGKH_{is} were solubilized on ice for 20 min with 0.5% Triton X-100 in 15 mM imidazole, pH 8.0, 100 mM NaCl plus 10% (w/v) glycerol. Further steps were the same as described previously. To obtain proteoliposomes with the IIA domain facing outwards, the reconstitution was performed with Triton X-100-treated preformed liposomes that were composed of *E. coli* lipids and L- α -phosphatidylcholine from egg yolk in a ratio of 3:1 (w/w) (12). LacS was incorporated into the liposomes at a protein to lipid ratio of 1:100 (w/w). The proteoliposomes were resuspended in 50 mM potassium phosphate, pH 7.0, and 2 mM MgSO₄ (KPM buffer) with or without 10 mM lactose if not indicated otherwise and frozen in 1-ml aliquots in liquid nitrogen.

Transport Assays in Proteoliposomes

All transport assays were carried out with mild magnetic stirring at 30 °C. The transport reactions were stopped at different time intervals by dilution of the samples with 2 ml of 100 mM LiCl and rapid filtering on 0.45-µm cellulose nitrate filters (Schleicher & Schuell GmbH, Dassel, Germany). Radioactivity was measured by liquid scintillation spectrophotometry after dissolving the filters in 2 ml of scintillation fluid (Emulsifier Scintillator Plus[®], Packard Inc).

Lactose Counterflow—Proteoliposomes in KPM plus 10 mM lactose were allowed to thaw slowly at room temperature after which they were extruded through a 400-nm polycarbonate filter to convert the membranes into unilaminar vesicles. Proteoliposomes were collected by centrifugation (20 min at 280,000 × g, 15 °C) and resuspended in KPM plus 10 mM lactose to about 1.3 mg/ml LacS. Aliquots of 2-µl (or 1-µl) proteoliposome suspensions (~1 mg/ml) were diluted into 200 µl of KPM containing 3.6 µM [¹⁴C]lactose. When necessary, different concentrations of unlabeled lactose were added to the KPM buffer to increase the external lactose concentration. The components needed to phosphorylate LacS were added to the assay buffer and to the concentrated proteoliposomes 5 min prior to the initiation of the uptake assay as described below.

Δ p-driven Uptake— Δ p-driven lactose uptake was performed as described by Foucaud and Poolman (13). Proteoliposomes were prepared in 20 mM potassium phosphate, pH 7.0, 100 mM potassium acetate (KAc) plus 2 mM MgSO₄ as described above. Aliquots of 2-µl proteoliposome suspensions (~1 mg/ml LacS) were diluted into 200 µl of 120 mM NaPipes, pH 7.0, 2 mM MgSO₄, 1 µM valinomycin plus 3.6 µM [¹⁴C]lactose and different concentrations of cold lactose.

Phosphorylation of membrane-reconstituted LacS protein was effected by incubation of the proteoliposomes (LacS concentration of ~1 mg/ml, which is ~14 µM) with Enzyme I purified from *B. subtilis* (1 µM),

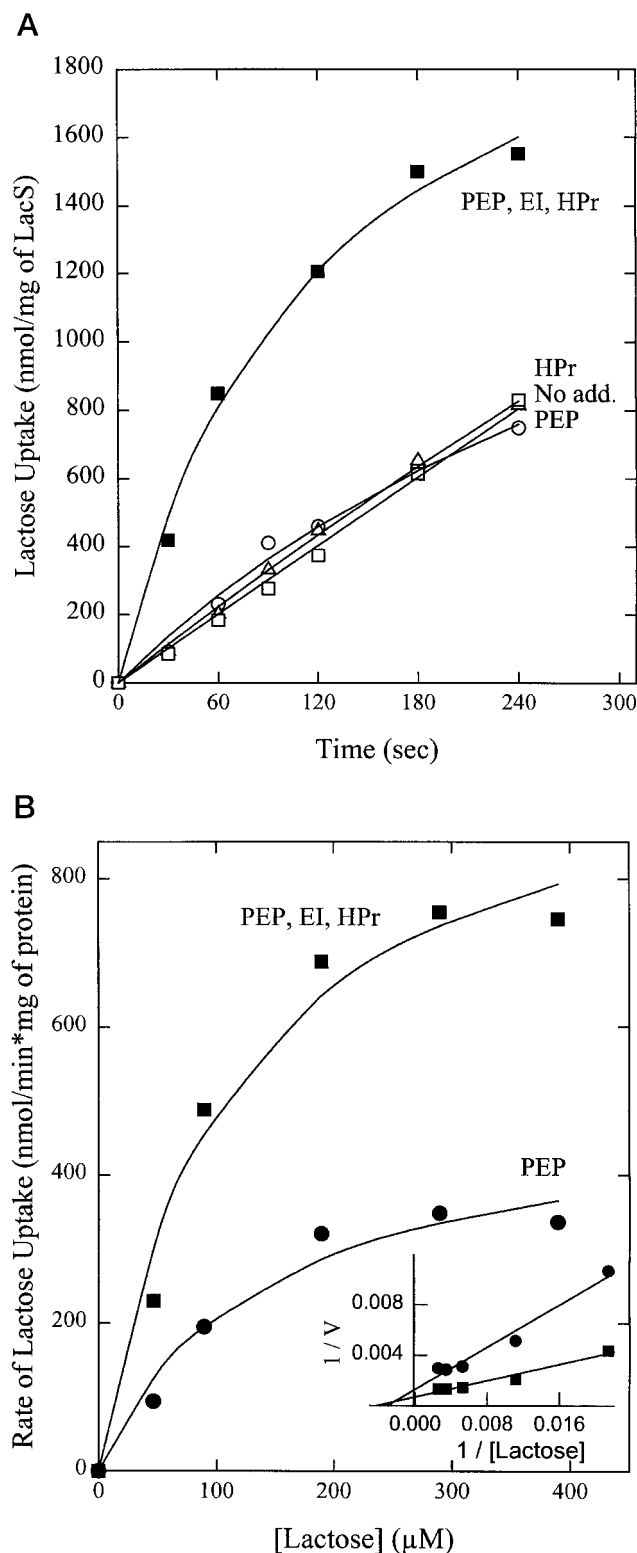


FIG. 2. Effect of LacS phosphorylation on lactose counterflow. Proteoliposomes containing LacS were equilibrated with 10 mM lactose in KPM, pH 7.0. Prior to the uptake assay, the proteoliposomes were incubated for 5 min with P-enolpyruvate (5 mM, PEP), Enzyme I (1 μ M, EI), and/or HPr (25 μ M) as indicated. The final LacS concentration was 1 mg/ml (14.2 μ M). At time zero, 1 μ l of proteoliposomes suspension was diluted into 209 μ l of KPM plus [14 C]lactose and containing P-enolpyruvate, Enzyme I, and/or HPr at concentrations of 5 mM, 1 μ M, and 10 μ M, respectively. A, time course of lactose counterflow. The final lactose concentration in the external medium was 90 μ M. B, kinetics of lactose counterflow; the external lactose concentrations varied from 47–390 μ M. Counterflow was measured under conditions where P-enolpyruvate, Enzyme I, and HPr (■) or only P-enolpyruvate (●) was present.

HPr purified from *S. thermophilus* (25 μ M) plus P-enolpyruvate (5 mM). For the control experiments, one or more of these components was omitted from the mixture. Incubations were performed in KPM plus 10 mM lactose for lactose counterflow or in 20 mM KPi, pH 7.0, 100 mM KAc plus 2 mM MgSO₄ for Δ p-driven uptake of lactose. After 5 min of incubation at room temperature, the proteoliposomes were diluted 100-fold into KPM containing 3.6 μ M [14 C]lactose (for lactose counterflow) or into 120 mM NaPipes, pH 7.0, 2 mM MgSO₄, 1 μ M valinomycin plus 3.6 μ M [14 C]lactose (for Δ p-driven lactose uptake) containing Enzyme I, HPr, and/or P-enolpyruvate at concentrations of 1 μ M, 10 μ M, and 5 mM, respectively. Adjustments were made for buffer components because of dilution as a result of Enzyme I, HPr, and/or P-enolpyruvate additions.

Phosphorylation Assays

Phosphorylation State of LacS by Pyruvate Burst—To determine the fraction of LacS with the IIA domain facing outwards, proteoliposomes (1–2 μ M) were incubated with 5.3 μ M [14 C]P-enolpyruvate, 0.6 μ M Enzyme I plus 1.7 μ M HPr in the presence or absence of 0.5% (w/v) *n*-dodecyl- β -D-multoside. Following the addition of [14 C]P-enolpyruvate, [14 C]pyruvate is formed in amounts equivalent to the quantities of Enzyme I, HPr plus LacS. In the absence of DDM, only inside-out reconstituted LacS is phosphorylated, whereas in the presence of DDM all LacS is phosphorylated. The pyruvate burst experiments were carried out in 50 mM KPi, pH 7.0, 5 mM MgCl₂ plus 5 mM dithiothreitol at 30 $^{\circ}$ C, and [14 C]pyruvate determination and [14 C]P-enolpyruvate synthesis were performed as described by Robillard and Blaauw (14).

HPr Phosphorylation—Phosphorylation of HPr by Enzyme I or HPr(Ser) kinase was carried out as described in Ref. 7. A typical assay consisted of 3 μ g of HPr purified from *S. thermophilus* in 50 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol plus 10 mM MgSO₄ in a total volume of 20 μ l, which was incubated for 10 min at 37 $^{\circ}$ C with 0.5 μ g of Enzyme I purified from *B. subtilis* plus 5 mM P-enolpyruvate or with 0.5 μ g of HPr(Ser) kinase purified from *B. subtilis* plus 10 mM fructose 1,6-bis phosphate (FBP) and 5 mM ATP. HPr phosphorylation was analyzed by separating the different species of HPr on non-denaturing PAGE (15% polyacrylamide), and the proteins were detected by Coomassie Brilliant Blue staining.

Miscellaneous

SDS-PAGE and Coomassie Brilliant Blue staining of gels were performed as described previously (2). The concentration of purified HPr and LacS was determined spectrophotometrically at 280 nm, using molar extinction coefficients of 1.4×10^4 M⁻¹ cm⁻¹ for HPr and of 7.6×10^4 M⁻¹ cm⁻¹ for LacS. In the case of LacS, corrections were made for free and bound Triton X-100 by determining the absorbance at 280 and 290 nm and using experimentally determined A_{280}/A_{290} ratios for the protein in Triton X-100 or free detergent. Protein concentrations of membrane vesicles, Enzyme I and HPr(Ser) kinase were determined by the D_c Protein Assay (Bio-Rad) using bovine serum albumin as standard. Analysis of amino acid composition was performed on an Applied Biosystems 476A sequencer by the Biotechnology Laboratory (N.A.P.S.), University of British Columbia, Canada.

Materials

D-[glucose-1- 14 C]Lactose (2.11 TBq/mol) was obtained from the radiochemical center, Amersham Pharmacia Biotech, United Kingdom. Ni²⁺-NTA resin was from Qiagen, Inc.; Bio-Beads SM-2 and Bio-Spin columns were from Bio-Rad Laboratories, Inc.; S- and DEAE-Sepharose fast flow resins, PD-10 and MonoQ columns (HR 16/10), and Triton X-100 were from Amersham Pharmacia Biotech. Protein concentration was performed in concentration cells and centrifrons from Amicon, Inc.; total *E. coli* lipids and egg yolk L- α -phosphatidylcholine were obtained from Avanti Polar Lipids. All other materials were reagent grade and obtained from commercial sources.

RESULTS

Protein Purification and Characterization of HPr from *S. thermophilus*—To study P-enolpyruvate-dependent Enzyme I/HPr-mediated regulation of the lactose transport protein of *S. thermophilus*, Enzyme I of *B. subtilis* and HPr and LacS of *S. thermophilus* were purified to homogeneity (Fig. 1A). Enzyme I and LacS were purified by metal affinity chromatography

The data were fitted to the Michaelis-Menten equation and replotted as Lineweaver-Burk (inset).

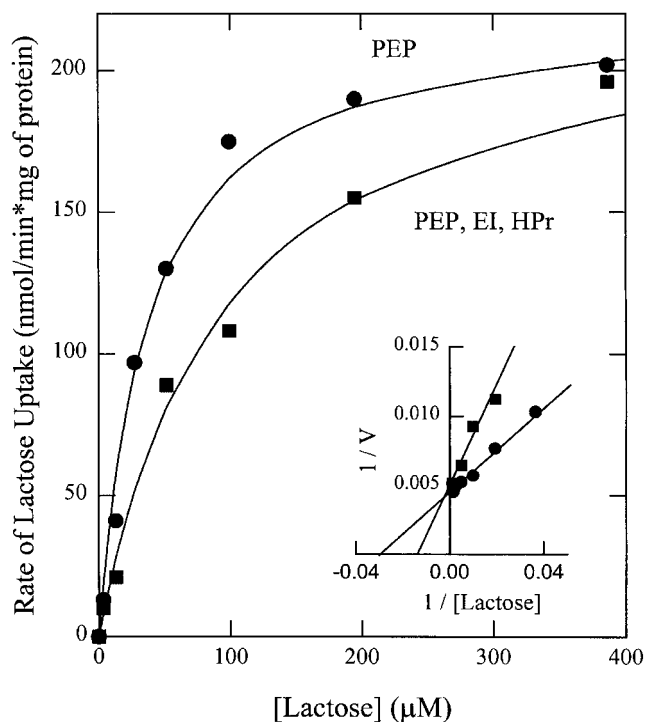


FIG. 3. **Effect of LacS phosphorylation on the kinetics of lactose/H⁺ symport.** Proteoliposomes containing LacS were equilibrated with 20 mM potassium phosphate, pH 7.0, 100 mM KAc plus 2 mM MgSO₄. Prior to the uptake assay, the proteoliposomes (final LacS concentration of 1 mg/ml) were incubated for 5 min with P-enolpyruvate (5 mM, *PEP*), Enzyme I (1 μM, *EI*), and/or HPr (25 μM) as indicated. At time zero, 2 μl of proteoliposome suspension was diluted into 212 μl of 120 mM NaPipes pH 7.0, 2 mM MgSO₄, 1 μM valinomycin plus [¹⁴C]lactose and containing P-enolpyruvate, Enzyme I, and/or HPr at concentrations of 5 mM, 1 μM, and 10 μM, respectively. The final lactose concentration in the external medium varied from 3.6–404 μM. The data were fitted to the Michaelis-Menten equation and replotted as Lineweaver-Burk (*inset*).

whereas HPr was purified by a combination of anion and cation exchange chromatography. The yield of HPr was 1.1 mg of protein from 1 liter of *S. thermophilus* harvested at an OD₆₆₀ of 2.

The N terminus of purified HPr from *S. thermophilus* was analyzed, and the sequence of the first 51 amino acids was determined: ¹MASKDFHIVAETGIHARPATLLVQTASKFASDITLEYKKGKAVNLSKIMGVM⁵¹. This amino acid sequence is identical to the N-terminal sequence of HPr from *Streptococcus mutans* and *S. salivarius* except for the glutamate residue at position 36, which is variable among HPr proteins purified from Gram-positive bacteria (15, 16). The predicted Enzyme I and HPr(Ser) kinase phosphorylation sites, His-15 and Ser-46, are both present in the HPr protein from *S. thermophilus*. As anticipated and shown in Fig. 1B, purified HPr was phosphorylated by Enzyme I in the presence of P-enolpyruvate (*lane 2*) and by HPr(Ser) kinase in the presence of ATP (*lane 3*). Phosphorylation of both the histidine residue and the serine residue (HPr(Ser-P)(His~P)) was observed when P-enolpyruvate/Enzyme I and ATP/HPr(Ser) kinase were present together (Fig. 1B, *lane 4*).

The purification procedure yielded two forms of HPr, HPr-1 and HPr-2, which had different mobilities in Tris-containing non-denaturing PAGE gels, and both were phosphorylated by Enzyme I as well by HPr(Ser) kinase (Fig. 1B). HPr-1 was separated from HPr-2 as the two HPr forms eluted from the Mono-Q column at different NaCl concentrations, that is about 50 and 60 mM, respectively (Fig. 1B, *lanes 5 and 6*). Analysis of the N-terminal amino acid sequence of both HPr forms re-

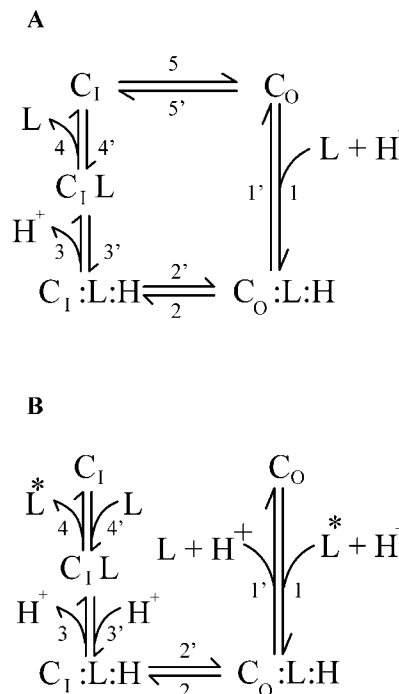


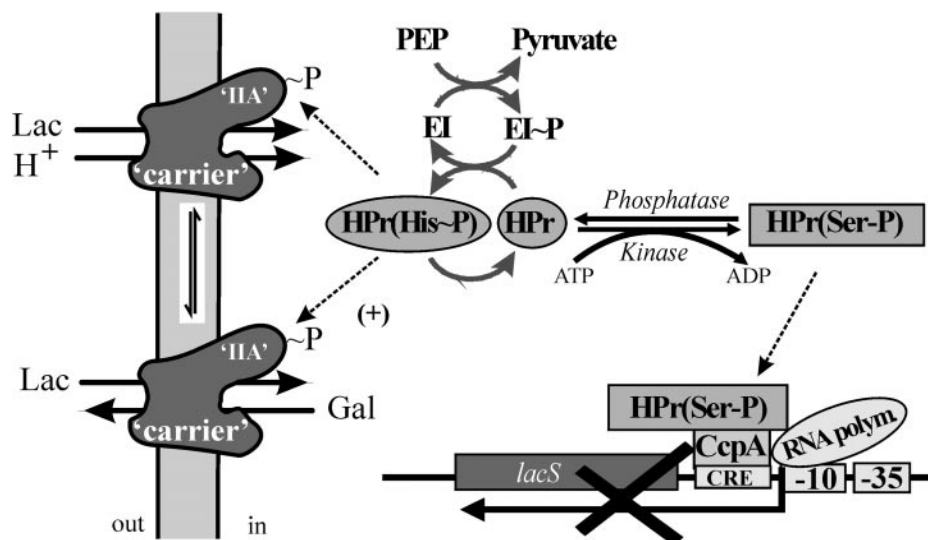
FIG. 4. **Kinetic scheme for LacS-mediated transport.** Kinetic scheme for Δp -driven uptake (A) and lactose counterflow (or exchange) (B). C_1 and C_0 represent the inward and outward facing conformation, respectively, of the carrier in the membrane in the *in vivo* situation. L and H^+ refer to lactose and proton, respectively. In B, the asterisks are used to discriminate the forward-half of the counterflow reaction cycle from the backwards-half. The (simulated) rate constants of the individual steps are given in Ref. 17.

vealed that HPr-1 differed from HPr-2 by the absence of the N-terminal methionine residue. The physiological relevance of this N-terminal methionine cleavage of HPr is unclear as the functional properties (degree of phosphorylation in growing cells, kinetics of phosphorylation) of HPr-1 and HPr-2 were identical (data not shown). The ratio of HPr-1/HPr-2 in *S. thermophilus* growing exponentially on lactose was 3.4 and decreased to 3.0 in stationary phase cells. When the cells were grown on the PTS sugar sucrose, the ratio of HPr-1/HPr-2 was 2.2 irrespective of the phase of growth.

Phosphorylation of Purified LacS and Membrane-reconstituted LacS—Pyruvate-burst experiments were used to evaluate whether or not membrane-reconstituted LacS was phosphorylated by HPr(His~P). The previously established unidirectional reconstitution of the LacS protein in liposomes (10, 12) was confirmed by the pyruvate-burst assay (data not shown). In fact, the amount of [¹⁴C]pyruvate produced was maximal and not significantly different in the presence or absence of detergent. This shows that >95% of all LacS molecules are incorporated with the IIA domain facing outwards and are phosphorylated by HPr(His~P).

Effect of Phosphorylation on Lactose Counterflow—The effect of phosphorylation on the activity of LacS protein was studied for both lactose counterflow and lactose/H⁺ symport modes of transport. For counterflow activity, the proteoliposomes were equilibrated with 10 mM lactose and diluted into a buffer with tracer amounts of radiolabeled lactose. This resulted in a rapid and transient accumulation of [¹⁴C]lactose, of which the kinetics of uptake over the first 4 min is shown (Fig. 2A). When the proteoliposomes were pre-incubated with P-enolpyruvate, HPr, or a combination of these components, the lactose uptake via LacS was similar to that of control samples. On the contrary, when LacS was pre-incubated with P-enolpyruvate and Enzyme I plus HPr, a condition that results in phosphorylation of

FIG. 5. Regulation of lactose transport in *S. thermophilus*. Schematic representation of HPr(His~P)-mediated phosphorylation of LacS that stimulates (+) lactose transport activity and HPr(Ser-P)/CcpA-mediated regulation of *lacS* transcription. *EI*, kinase, phosphatase, *cre*, and *RNA polym.* represent Enzyme I, HPr(Ser) kinase, HPr(Ser) phosphatase, catabolite responsive element, and RNA polymerase, respectively. *PEP*, P-enolpyruvate; *Gal*, galactose.



the transport protein, the lactose uptake via LacS was increased by approximately 3-fold. These data indicate that HPr(His~P)-mediated phosphorylation of LacS stimulates the counterflow activity of LacS.

The apparent stimulation of LacS counterflow activity was studied in more detail by analyzing the transport reaction as a function of the external lactose concentration (Fig. 2B). The initial rates of lactose uptake were measured under conditions where LacS was phosphorylated or not phosphorylated. The maximal uptake rate (V_{max}) was increased by a factor of 2, whereas the affinity constant for transport was somewhat lower when LacS was in the phosphorylated state.

Effect of Phosphorylation on Δp -driven Lactose Uptake—The effect of phosphorylation on the Δp -driven lactose uptake was studied in proteoliposomes in which an artificial membrane potential was generated by means of a valinomycin-mediated K^+ diffusion potential and a pH gradient was generated by an outward-directed acetate diffusion gradient. LacS was phosphorylated by pre-incubation of the proteoliposomes with P-enolpyruvate, Enzyme I plus HPr. The control sample contained only P-enolpyruvate (unphosphorylated LacS). The corresponding compounds were also present in the buffer in which the transport reaction was assayed. Fig. 3 shows the initial lactose uptake rates at different lactose concentrations for both conditions. Upon phosphorylation of LacS, the Δp -driven lactose uptake rate at low lactose concentrations was somewhat decreased, which is in agreement with earlier findings (1). The V_{max} of uptake was similar for phosphorylated and unphosphorylated LacS. The data indicate that HPr(His~P)-mediated phosphorylation of LacS results in a 2-fold increase in the apparent affinity constant (K_m^{app}) for Δp -driven lactose transport.

DISCUSSION

In this study, we report on the regulation of the lactose transport protein of *S. thermophilus* through HPr(His~P)-mediated phosphorylation. The regulation was studied *in vitro* using purified Enzyme I and HPr and proteoliposomes in which the LacS protein was present in the inside-out orientation. This *in vitro* membrane system is most suitable for our studies as phosphorylation of LacS could easily be manipulated by the addition of P-enolpyruvate, Enzyme I, and/or HPr to the outside medium. As the proteoliposomes are well sealed and maintain ion-gradients over long periods of time, it was possible to measure accurately Δp -driven lactose uptake as well as lactose counterflow.

Kinetic analysis of the two transport modes showed that the V_{max} of lactose counterflow was increased by a factor of 2 upon phosphorylation of LacS, whereas the apparent inhibition of Δp -driven uptake appears to be because of an increase of the affinity constant (K_m) for uptake. The latter data are consistent with earlier observations in which Δp -driven lactose uptake was studied in hybrid membrane vesicles in which P-enolpyruvate, Enzyme I, plus HPr were present internally and externally and in which a proton motive force was generated by oxidation of cytochrome *c* via cytochrome *c* oxidase. These experiments were carried out at low lactose concentrations ($6 \mu M$) that are far below the K_m of transport. As the hybrid membrane vesicles are relatively leaky, it was at that time not possible to perform kinetic analysis of artificial ion gradients driven uptake or counterflow type of transport. These studies have now become possible through development of a more defined and well sealed proteoliposomal system.

How can one explain the different effects of LacS phosphorylation on Δp -driven uptake and counterflow activity? According to the kinetic model for lactose transport via LacS (13, 17), lactose counterflow (or exchange) proceeds via binding and release of ligands (lactose and H^+) at the inner and outer surface of the membrane, and reorientation of the binding sites via the ternary complex (C:L:H); steps 2 and 2' in Fig. 4B. The reorientation of the loaded binding sites is rate-determining under conditions of lactose counterflow (or exchange, Ref. 17). Δp -driven lactose uptake (Fig. 4A) proceeds via ligand binding at the outer surface (step 1), reorientation of the binding site (step 2), release of ligands at the inner surface (steps 3 and 4), and reorientation of the unloaded binding site (step 5). This latter step is much slower than the reorientation of the ternary complex (step 2) in the counterflow reaction, and consequently lactose counterflow (or exchange) transport is faster than Δp -driven uptake. We now propose that phosphorylation affects the rate constants for the reorientation of the ternary complex, which is rate-determining for counterflow but not for Δp -driven uptake. This step is accelerated upon phosphorylation and, as a result, the counterflow activity is increased. Phosphorylation of LacS has no effect on the V_{max} of Δp -driven uptake as this rate is largely controlled by the reorientation of the unloaded binding site of LacS (Fig. 4, step 5). At this point it is not possible to assign the K_m shift to a particular step(s) in the catalytic cycle. Finally, we emphasize that the counterflow transport reported here is equivalent to lactose/galactose exchange *in vivo* and that this reaction and not the Δp -driven

uptake is most relevant in lactose (glycolysing)-metabolizing cells of *S. thermophilus*. We thus conclude that HPr(His~P)-mediated phosphorylation of LacS evokes maximal activity of the lactose transport protein *in vivo* by increasing the V_{\max} of the lactose/galactose exchange reaction.

In this work, we also report on the presence and purification of two forms of HPr in *S. thermophilus*. Both HPr forms have similar biochemical properties with respect to phosphorylation by Enzyme I or HPr(Ser-P) kinase and their ability to phosphorylate LacS. HPr-1 differed from HPr-2 by the absence of the N-terminal methionine residue, and HPr-1 was the dominant form when *S. thermophilus* ST11(Δ lacS)/pGKhis cells were grown on lactose. The presence of different forms of HPr has thus far only been reported for species that belong to the *Streptococcus* or *Lactococcus* genus (18).

Ye *et al.* (19, 20) reported that the lactose/H⁺ and glucose/H⁺ symporters of *Lactococcus brevis* are regulated by allosteric interaction with HPr(Ser-P). We also tested whether or not the LacS protein was affected by HPr(Ser-P). Up to 5 times excess of HPr(Ser-P) over LacS did not have a specific effect on counterflow activity or Δ p-driven uptake. Also the HPr(S46D) mutant that mimics HPr(Ser-P) because of its negative charge at residue 46 did not affect the Δ p-driven lactose uptake.² In addition, we tested whether purified IIA^{LacS} or purified LacS in the detergent-solubilized state and immobilized to a Ni²⁺-NTA resin was able to specifically retard the migration of HPr or HPr(Ser-P). Despite the testing of various experimental parameters, we never observed any interaction between IIA or LacS and HPr or HPr(Ser-P).³ We thus have no indication whether the LacS protein is regulated allosterically by HPr(Ser-P).

In *E. coli* the lactose/H⁺ symport protein (LacY) is regulated by IIA^{Glc} protein. Allosteric interaction of the unphosphorylated form of IIA^{Glc} resulted in an inhibition of lactose uptake, whereas phosphorylated IIA^{Glc} did not interact with LacY (23, 24, 25). Future studies have to establish whether it is the phosphorylated form of IIA^{LacS} that stimulates or the unphosphorylated IIA^{LacS} that inhibits the translocation reaction mediated by the carrier domain of the LacS protein.

Overall, the data indicate that HPr must be in the histidine-phosphorylated state for maximal activity of the lactose transport system of *S. thermophilus*. This condition is met in cells at the late-exponential and stationary phase of growth (22). The transition from HPr(Ser-P) to HPr(His~P) parallels a decrease in lactose and an increase in galactose concentration in the growth medium. Because the K_m^{out} for lactose is higher than that for galactose (21), the lactose transport capacity will decrease as lactose decreases and galactose accumulates in the

medium. As depicted in Fig. 5, we propose that when lactose uptake becomes limiting for growth, *S. thermophilus* increases the concentration of the LacS protein by relieving HPr(Ser-P)/CcpA-mediated catabolite repression of *lacS* transcription, and increasing the activity of the LacS protein by HPr(His~P)-mediated phosphorylation. The regulation is such that *lacS* transcription and LacS activity are maximal when the concentration of HPr(Ser-P) is low and HPr(His~P) is high. This dual regulation causes the lactose transport capacity of *S. thermophilus* to become attenuated when physiological conditions result in a shift from HPr(Ser-P) to HPr(His~P).

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² B. Poolman and J. Reizer, unpublished results.

³ M. G. W. Gunnewijk and B. Poolman, unpublished results.