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The Lactose Transporter from *Streptococcus thermophilus* is Activated by Phosphorylated IIA

Eric R. Geertsma, Ria H. Duurkens and Bert Poolman

Abstract

Lactose transport in *Streptococcus thermophilus* by the LacS protein is regulated at the level of transcription and translocation activity, reflecting the need to tune uptake to lactose metabolism. The carboxyl-terminal domain of LacS is homologous to IIA\textsuperscript{Glc} of the phosphoenolpyruvate-phosphotransferase system (PTS) and modulates the activity of the LacS carrier domain. PEP-dependent phosphorylation of LacS-IIA by the general energy coupling proteins of the PTS, Enzyme I and HPr, resulted in an increase in the rate of lactose counterflow. To determine whether unphosphorylated LacS-IIA inhibited, or the phosphorylated form stimulated lactose counterflow, a LacS-IIA truncation mutant of LacS was constructed. Detailed analyses of transport in whole cells and in proteoliposomes indicated that dephosphorylated LacS-IIA does not functionally interact with the carrier domain. Interaction of the phosphorylated form of LacS-IIA with the carrier appears to stimulate lactose counterflow transport. The proposed mode of regulation thus proceeds via a mechanism opposite to inducer exclusion, where transporters are inhibited by binding of the unphosphorylated form of IIA\textsuperscript{Glc}.

Introduction

Lactose is used by the lactic acid bacterium *Streptococcus thermophilus* as primary carbon- and energy source. The sugar is internalized via the lactose transporter LacS, which uses the electrochemical proton-gradient to accumulate the galactoside (30). *In vivo*, the proton-neutral exchange of the disaccharide lactose for galactose, end-product of the lactose metabolism, is the dominant reaction, enabling LacS to function as a net importer of the glucose moiety of lactose.

LacS is a member of the Major Facilitator Superfamily and more specifically of the GPH-family which contains members of both pro- and eukaryotic origin (84,93). LacS deviates from most GPH-family members by its multi-domain structure: it consists of a membrane-embedded carrier domain and a hydrophilic IIA domain, residing at the cytoplasmic face of the membrane (86).

The carrier domain catalyses the actual translocation reaction and forms a dimeric structure with subunits functionally interacting in the membrane (32,34,35,124). The IIA-domain is homologous to IIA\textsuperscript{Glc}-like domains of the PEP-PTS system (86) but is not essential for transport. Phosphorylation of the IIA-domain on His-552 by HPr(His\textasciitilde P) has been proposed to modulate the activity of the carrier domain (83,85).

The serine phosphorylated form of HPr, on the other hand, participates in the transcriptional control of *lacS*, which is additionally mediated by GalR and CcpA. GalR upregulates the *lacS* promoter (120), whereas CcpA represses transcription; HPr(Ser-P) acts as co-repressor of
CcpA. In the late exponential phase of growth, when the lactose intake decreases, the CcpA controlled transcriptional repression is relieved, as a result of a decrease in HPr(Ser-P), leading to an increase in the expression level of LacS.

The regulation of transport by the phosphorylation state of LacS-IIA contributes to a fast response of the cell to alterations in the external lactose concentration. Already after mid-exponential phase of growth, an increase in the level of HPr(His~P) and a decrease in HPr(Ser-P) results in an increased transport activity and an increase in the biosynthesis of LacS, thereby compensating for the decrease of substrate (lactose) and increase of inhibitory end-product (galactose) in the medium (42).

In Gram-negative bacteria, the activity of several non-PTS carbohydrate transporters is regulated by the phosphorylation state of IIA^{Glc} via the inducer exclusion mechanism (88). In the presence of a PTS substrate, IIA^{Glc} becomes dephosphorylated due to the transfer of the phosphoryl group to the PTS substrate. This dephosphorylated form of E. coli IIA^{Glc} interacts directly with its targets among which are the melibiose permease MelB (69), the lactose transporter LacY (74,107), the raffinose permease Rfb (112) and the ATPase MalK of the maltose ABC-transporter (19), thereby decreasing the uptake rates of the respective sugars. To determine whether the regulation of transport involves interaction of the unphosphorylated or phosphorylated form of LacS-IIA with the carrier domain, a C-terminal truncation of LacS was constructed that lacked the entire IIA domain. This deletion mutant provided kinetic evidence for a regulatory mechanism opposite to inducer exclusion type of regulation.

**Material and Methods**

**Bacterial strains**

E. coli MC1061 (130) and NM522/pAG3 (33) were cultivated at 37°C on Luria Broth under vigorous aeration. When appropriate the medium was supplemented with 50 µg/ml ampicillin or 50 µg/ml carbenicillin. S. thermophilus ST11(ΔlacS)/pGKHis (83) was grown semi-anaerobically on Elliker Broth (28) at 42°C supplemented with 5 µg/ml erythromycin, 1% lactose and 0.5% beef extract.

**Plasmid constructions**

DNA manipulations were done according to standard protocols. The Quickchange mutagenesis kit (Stratagene) was used to introduce site-directed mutations. All mutants were verified by DNA sequencing.

pBADlacSC320A – By substituting the Pro codon CCA for CCT, the internal NcoI site in lacS(C320A) on pSKE8EhisC320A (122) was removed, yielding pSKE8EhisC320ANL. The AatII-Xbal lacS-fragment of pSKE8N (66) was replaced by the Ncol-less AatII-Xbal lacS(C320A)-fragment from pSKE8EhisC320ANL, yielding pNlacSC320Ahis. The Ncol-Xbal fragment of pNlacSC320Ahis was transferred to pBAD/Myc-His B (Invitrogen), yielding pBADlacSC320A.

pSKlacSC320AΔIIA – The six nucleotides 3’ of the codon for Glu-474 in lacS(C320A) on pSKE8EhisC320A were replaced by a BamHI site, yielding pSKE8EhisC320A-BamHI-IIA. A 368 bp BamHI-XbaI fragment coding for a Factor Xa cleavage site and a hexa-His-tag was derived from pNZOpuAAHis (116) and ligated into the BamHI-XbaI digested vector, yielding pSKlacSC320AΔIIA.

pBADlacSC320AΔIIA – The AatII-Xbal fragment of pBADlacSC320A was exchanged for the AatII-Xbal fragment of pSKlacSC320AΔIIA, yielding pBADlacSC320AΔIIA.

pBADLacSC320AΔIIA-GFP – The 745 bp BclI-XbaI fragment of pNZOpuR-GFPuv (S.A. Henstra, unpublished data), containing the gene for the enhanced form of the Aequorea victoria green fluorescent protein (GFP), optimised for expression in E. coli (16), was ligated into BclI-XbaI restricted pBADsub1C320A (34), yielding pBADLacSC320AΔIIA-GFP containing an in-frame fusion of GFP to lacSΔIIA.

**Membrane vesicle preparation**

E. coli MC1061 cells harbouring pSKlacSC320AΔIIA or pSKE8EhisC320A were ruptured by a three-fold passage through a French pressure cell at 10,000 psi, and the inside-out membrane vesicles were collected as
described (34). Membrane vesicles were resuspended in 50 mM KPi, pH 7, frozen in liquid nitrogen and stored at -80°C. The protein concentration was determined using the DC Protein Assay (Bio-Rad).

**Protein purification**

All steps during the purification of the proteins were performed at 4°C unless indicated otherwise.

*Enzyme I* – Purification of *B. subtilis* Enzyme I from *E. coli* NM522/pAG3 was done as described (41), with some modifications. Shortly, cells were washed and resuspended with 50 mM NaPi, pH 7, supplemented with 10% glycerol, 5 mM MgSO₄, 0.1 mg/ml RNase and DNase, and lysed by three passages through a French pressure cell as described under Membrane vesicle preparation. Whole cells and membrane fractions were removed by centrifugation (184k x g, 1 hr) and the supernatant was supplemented with 5 mM β-mercaptoethanol, 100 mM NaCl, 15 mM imidazole and the pH was adjusted to 8 with K₂HPO₄. Next, the cytosolic fraction was mixed with Ni-NTA resin, washed with 50 mM KPi, 100 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol (buffer A), pH 8, supplemented with 15 mM imidazole, and incubated for 1 hour with continuous mixing. The column was drained, washed with 10 volumes buffer A, pH 8, plus 15 mM imidazole, 10 volumes buffer A, pH 8, plus 25 mM imidazole, and eluted with buffer A, pH 7, plus 200 mM imidazole. Purified EI was extensively dialysed against 50 mM Tris, pH 7.4, plus 3 mM dithiothreitol, and subsequently aliquoted and stored at -80°C. The specific phosphorylation activity of Enzyme I was determined in a spectroscopic assay in which the formation of pyruvate from PEP upon phosphorylation of EI was coupled to the oxidation of NADH to lactate by lactate-dehydrogenase (42,129).

*HPr* – *S. thermophilus* ST11(∆lacS)/pGKHis cells were washed with 20 mM Tris-Cl, pH 8.5, resuspended to OD₆₆₀ = 75 and stored at -80°C. To maximise the yield of HPr, cells were lysed by osmotic cell lysis (30) and subsequent three-fold passage through a pressure cell at 1000 bar (12). The cytosolic fraction, obtained after removal of cell debris and membranes by centrifugation (184k x g, 1 hr), was extensively dialyzed (membrane cut-off of 3.5 kDa) to decrease the K₂SO₄ concentration to below 1 mM. A white precipitate that appeared was removed by centrifugation. The bulk of the lysozyme was removed by three subsequent incubations of the supernatant with 20 ml SP-sepharose for 1 hour while stirring. The filtrate was loaded onto a Q-sepharose column (2,5 x 30 cm), washed with 20 mM Tris-Cl, pH 8.5, and eluted by supplementing the wash solution with 80 mM NaCl. Peak fractions were pooled and concentrated on an Amicon cell with a YM3.5 membrane. Purified HPr was aliquoted and stored at -80°C. The specific phosphorylation activity of HPr was determined in a spectroscopic assay as described for EI.

*LacS* – LacS derivatives were purified from *E. coli* MC1061 membrane vesicles as described (35), but we stress that after solubilisation the insoluble material was removed by centrifugation (15 minutes, 267k x g).

**Whole cell transport assays**

*Cultivation* – All transport assays were done with *E. coli* MC1061 cells. Cultivation was started with a 1% (vol/vol) inoculum of an overnight culture and cells were grown until OD₆₆₀ = 0.5-0.6 was reached. Cultures were induced with the appropriate amount of arabinose and cultivation continued for 2.75 hrs. Cells were pelleted, washed twice with ice-cold 50 mM KPi, pH 7.1, plus 2 mM MgSO₄ (KPM pH 7.1), and resuspended to 36 mg protein/ml. Concentrated cell preparations were split and incubated at 4°C overnight.

*Transport assays* – Lactose transport was measured in dilute stirred cell suspensions at 30°C unless indicated otherwise. The reaction was quenched at different time-intervals by the addition of 2 ml ice-cold 0.1 M LiCl, followed by rapid filtration on 0.45 μm nitrocellulose filters (Schleicher & Schuell Inc.). Reaction tubes and filters were washed with another 2 ml ice-cold 0.1 M LiCl and the radioactivity associated with the filter was determined by liquid scintillation counting.

*Proton motive force (Δp)-driven lactose uptake* – Lactose accumulation in whole MC1061 cells was performed as described for DW2 cells (35).

*Lactose counterflow transport* – Cells were incubated overnight in the presence of 10 mM lactose. The next day, cells were washed with KPM, pH 7.7, supplemented with 10 mM lactose and concentrated to 50 mg protein/ml. Cells were de-energized by incubation with 50 μM SF6847 plus 30 mM NaN₃ for 2 hours. Counterflow transport was started by 100-fold dilution of the cells into KPM, pH 7.7, yielding a final external ¹⁴C-lactose concentration of 100 μM.

*In vitro transport assays* - Membrane reconstitution - Membrane reconstitution of purified LacS and LacSΔIIA in liposomes, consisting of 3:1 ratio (wt/wt) of purified *E. coli* lipids and Egg PC, at a protein to lipid ratio of 141 pmol protein/mg lipid, was performed as described (66,124).
Orientation of reconstituted LacS\(\Delta\)IIA – Proteoliposomes of LacS\(\Delta\)IIA were washed with 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 5 mM CaCl\(_2\) (TNC), extruded through a 400 nm polycarbonate filter and concentrated to 1 mg LacS\(\Delta\)IIA/ml by centrifugation (267k \(\times\) g, 15 minutes, 10°C). 10 \(\mu\)l aliquots were diluted to 20 \(\mu\)l, mixed with 0.5 \(\mu\)l Factor Xa in 50% glycerol (Sigma) and incubated for 2-18 hours at room temperature. The untreated sample was incubated without Factor Xa; the control sample was incubated with Factor Xa in the presence of 1% Triton X-100. To quench the reaction, PMSF was added to a final concentration of 1 mM. Subsequently, SDS-PAGE sample buffer with elevated SDS concentration (1.2%) was added, and the samples were stored at -20°C. The increased SDS concentration prevented anomalous migration of the protein in the presence of a high lipid concentration. Subsequent SDS-PAGE, electrophoresis, automated immunodetection with a primary antibody directed against a hexa-His-tag (Amersham Pharmacia Biotech), and chemiluminescent detection was performed as described (35).

Transport assays – Lactose transport in proteoliposomes was done as described for whole cells. Phosphorylation of membrane reconstituted LacS was done as described (41).

Proton motive force (\(\Delta p\))-driven lactose uptake – Lactose transport driven by an artificial pH gradient and membrane potential was performed at 23°C as described (124), except that the samples were diluted 100-fold. Briefly, proteoliposomes were washed and resuspended in 20 mM KPi, pH 7.0, 100 mM potassium acetate plus 2 mM MgSO\(_4\). Samples were frozen in liquid nitrogen and slowly thawed at room temperature. After extrusion through 400 nm polycarbonate filters to obtain predominantly unilamellar vesicles, proteoliposomes were concentrated to approximately 15 \(\mu\)M protein (1.1 x 10\(^2\) mg lipid/ml). Transport was initiated by 100-fold dilution of aliquots into 120 mM Na-Pipes, pH 7.0, 2 mM MgSO\(_4\), 0.5 \(\mu\)M valinomycin and different concentrations of \(^{14}\)C-lactose.

Lactose counterflow transport – Lactose counterflow was done as specified earlier (125). Briefly, proteoliposomes were washed and resuspended in 50 mM KPi, pH 7.0, 2 mM MgSO\(_4\), plus 10 mM lactose. Samples were frozen in liquid nitrogen and slowly thawed at room temperature. After extrusion through 400 nm polycarbonate filters, proteoliposomes were concentrated to approximately 15 \(\mu\)M protein. Transport was initiated by 100-fold dilution of aliquots into 50 mM KPi, pH 7.0, 2 mM MgSO\(_4\) plus different concentrations of \(^{14}\)C-lactose.

Results

Deletion of LacS-IIA differently affects lactose counterflow and \(\Delta p\)-driven lactose uptake

To specify the mode of regulation by LacS-IIA, a truncated version of Cys-less LacS was constructed, in which the C-terminal 160 amino acids, corresponding to the IIA domain, were deleted. The C-terminus of this construct, designated LacS\(\Delta\)IIA, still comprised the linker region connecting the carrier and LacS-IIA domain and, additionally, a hexa-His tag. LacS\(\Delta\)IIA, expressed in \(E.\) coli MC1061, migrated on SDS-PAGE at approximately 40 kDa while LacS migrated around 55 kDa. Initial activity analyses were done in \(E.\) coli MC1061 in which the expression of LacS and derivatives was controlled by the AraC/PBAD system.

The IIA domain of LacS, expressed as soluble protein (IIA\(^{\text{LacS}}\)), can be phosphorylated \textit{in vitro} and \textit{in vivo} by HPr(His\(^{\text{~P}}\)) from \(E.\) coli (43), suggesting that the membrane-associated LacS-IIA domain might also be phosphorylated in \(E.\) coli cells. To determine whether the phosphorylation state of LacS varies as a function of the expression level, e.g., by titrating out HPr and knowing that the phosphoryl transfer between HPr and LacS is extremely slow, we determined the initial uptake rates in cells induced with different concentrations of L-arabinose. Both lactose counterflow and \(\Delta p\)-driven lactose uptake (Fig. 1) were measured (Fig. 2), since these transport modes respond differently to LacS-IIA phosphorylation (41). For LacS, the ratio of the activities of both modes of transport was constant over a broad range of inducer concentrations, suggesting that the initial transport rates were only governed by the concentration of the LacS protein in the membrane. Also, the ratio between the initial rates of \(\Delta p\)-driven lactose transport and lactose counterflow by LacS\(\Delta\)IIA was constant in this inducer
Regulation of LacS

range, but differed significantly from the ratio observed for LacS. The initial rates of \(\Delta p\)-driven lactose transport were equal for LacS and LacS\(\Delta II A\), but the initial rate of counterflow transport by LacS\(\Delta II A\) was only half that of LacS. As lactose counterflow is more responsive to the phosphorylation state of LacS-IIA than \(\Delta p\)-driven lactose uptake (41), the deletion of LacS-IIA most likely decreases the initial rate of lactose counterflow, whereas it has no effect on \(\Delta p\)-driven transport.

![Diagram of counterflow and \(\Delta p\)-driven transport](image)

**Figure 1. Schematic representation of counterflow and \(\Delta p\)-driven transport.** E reflects the unliganded protein, \(ESH\) the fully liganded protein with both the substrate and the proton binding site occupied. S and H represent the substrate (a galactoside) and the proton, respectively. The subscripts out and in refer to the location of the binding sites, extracellular and cytoplasmic, respectively. In the wildtype protein, both the fully liganded and unliganded binding sites can reorient. A, lactose counterflow transport. A substrate is bound on the outside (step 1), the binding sites reorient (step 2) and the substrate is released on the inside (step 3). Next, another substrate is bound on the inside (step 3') and reorientation to the extracellular site takes place (step 2'). Upon release of the substrate (step 1'), another round of translocation can start. Note that the counterflow mode of transport is overall an electroneutral process. The reorientation of the liganded carrier (step 2 and 2') is proposed to be accelerated upon phosphorylation of LacS-IIA and be responsible for the increased lactose counterflow rate of phosphorylated LacS. B, \(\Delta p\)-driven lactose transport. Similar steps (no. 1, 2, and 3) as described for counterflow transport take place, but upon release of the substrate on the inside (step 3), substrate is not bound on the trans side. Rather, the unliganded binding site reorients to the outside (step 4) and initiates another round of translocation.

**Substitution of the II A domain by GFP differently affects lactose counterflow and \(\Delta p\)-driven lactose uptake**

To exclude the possibility that the decreased rate of LacS\(\Delta II A\) counterflow transport was caused by an aspecific global rather than regulatory effect of the deletion of the 17 kDa cytoplasmic domain, the 26 kDa *Aequorea victoria* green fluorescent protein (GFP) was fused to the C-terminus of LacS\(\Delta II A\)(C320A). This 79 kDa protein, designated LacS\(\Delta II A\)-GFP, was expressed and correctly inserted into the membrane as shown by fluorescent confocal imaging (unpublished data). The initial rates of counterflow and \(\Delta p\)-driven lactose transport by
LacS\(\Delta\)IIA-GFP were equal to LacS\(\Delta\)IIA (Fig. 3), suggesting that the transport activity of the carrier domain is specifically affected by the removal of the IIA-domain.

In order to allow manipulation of the LacS-IIA phosphorylation state, further analyses were done in vitro. Since full-length LacS is reconstituted in liposomes in the inside-out orientation (66), and the affinities of the outward and inward facing lactose-binding sites differ approximately one order of magnitude, it was important to determine the orientation of membrane-reconstituted LacS\(\Delta\)IIA. LacS\(\Delta\)IIA was reconstituted as described (66,124) and the orientation in the membrane was assessed by determining the accessibility of the Factor Xa cleavage site, which precedes the C-terminal hexa-His tag of LacS\(\Delta\)IIA. Proteolysis by Factor Xa detaches the His-tag from the protein. Separation of the protein on SDS-PAGE and subsequent immunoblotting with anti-His antibody gives an estimate of the amount of inside-out-oriented protein. Equal amounts of protein were analyzed and immunoblotting showed that the untreated protein had a high reactivity towards the anti-His antibody, whereas detergent-solubilized and Factor Xa-treated LacS\(\Delta\)IIA proteoliposomes gave no signal (Fig. 4). The LacS\(\Delta\)IIA proteoliposomes, treated with Factor Xa only, showed very low reactivity with the antibody; only after long exposure a faint band became visible (Fig. 4, bottom box, lane 2). The reactivity of Factor Xa-treated LacS\(\Delta\)IIA in intact proteoliposomes was only a little more intense than detergent-solubilized and Factor Xa-treated LacS\(\Delta\)IIA, indicating that LacS\(\Delta\)IIA was incorporated in the lipid vesicles predominantly in an inside-out orientation, similar to the full-length LacS protein, allowing a direct comparison of both species.

Figure 2. Initial rates of lactose transport by LacS and LacS\(\Delta\)IIA at different inducer concentrations. E. coli MC1061 cells were induced with 1 \(\times\) 10\(^{-5}\), 1 \(\times\) 10\(^{-4}\), or 1 \(\times\) 10\(^{-3}\)% (wt/vol) of L-arabinose and cells were prepared as described under Material and Methods. White and grey bars represent initial uptake rates of LacS and LacS\(\Delta\)IIA, respectively. Open and hatched bars reflect \(\Delta\)p-driven and counterflow lactose transport, respectively.

**LacS\(\Delta\)IIA is membrane-reconstituted in the inside-out orientation**

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The transport kinetics of LacSΔIIA mimic that of dephosphorylated LacS

To avoid differences in reconstitution efficiency due to variations in detergent concentration, purified LacS and LacSΔIIA were brought to the same molar concentration before being mixed with the Triton X-100-titrated liposomes. For transport driven by the proton motive force (Δp), the proteoliposomes were equilibrated with 20 mM KPi, pH 7.0, 100 mM potassium-acetate
plus 2 mM MgSO_4. The artificial Δp was formed by diluting the proteoliposomes at time zero 100-fold into 120 mM Na-Pipes, pH 7.0, 2 mM MgSO_4, plus 0.5 µM valinomycin. Purified and membrane-reconstituted LacS was in the dephosphorylated state since the addition of an excess of HPr from *S. thermophilus*, which accelerates IIA^LacS dephosphorylation (43), had no effect on counterflow and Δp-driven lactose transport (results not shown). To phosphorylate LacS, proteoliposomes were incubated with purified EI from *B. subtilis* and HPr from *S. thermophilus* in the presence of PEP (41). In correspondence with earlier observations, Δp-driven lactose transport was slightly inhibited in the phosphorylated full length LacS protein due to a small decrease in affinity (Fig. 5A). The rates of Δp-driven lactose transport by LacS^ΔIIA fitted best with the activity of dephosphorylated LacS.

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**Figure 5. Lactose transport by LacS^ΔIIA and (un)phosphorylated LacS in proteoliposomes.** Initial rates of Δp-driven lactose transport (A) and lactose counterflow (B) were plotted and fitted to the Michaelis-Menten equation. Note that the transport rates are expressed as mol lactose internalized / mol transporter * min (= min^-1).* Black and white symbols represent transport rates of LacS and LacS^ΔIIA, respectively. Triangles and circles indicate phosphorylated and unphosphorylated LacS. Solid lines show the Michaelis-Menten fit of the LacS samples while the fit for LacS^ΔIIA is represented by a dotted line.
For lactose counterflow transport, the proteoliposomes were equilibrated with 10 mM lactose, concentrated and diluted 100-fold into buffer containing tracer amounts of $^{14}$C-lactose, yielding a final external lactose concentration of approximately 100 µM. In contrast to the small inhibitory effect of phosphorylation on $\Delta p$-driven lactose transport, lactose counterflow was highly stimulated with phosphorylated LacS (Fig. 5B). The kinetics of lactose counterflow transport by LacS$\Delta$IIA were similar to the kinetics of dephosphorylated LacS. Taken together, the combined data of both $\Delta p$-driven lactose uptake and lactose counterflow convincingly demonstrate that LacS$\Delta$IIA mimics the transport characteristics of LacS in the dephosphorylated state.

Discussion

The lactose transport capacity in *Streptococcus thermophilus* is regulated at the transcriptional level by GalR and HPr(Ser-P)-mediated CcpA repression, and at the level of the LacS activity by HPr(His~P)-mediated phosphorylation of the LacS-IIA domain. This regulatory network allows the tuning of transport to the metabolism of lactose and the energy status of the cell and avoids an overcapacity of transport, which can ultimately lead to decreased growth rates and lower cell density as has been show in a *ccpA* disruption strain (114).

In this study, the regulation of the lactose transport activity of the membrane-embedded carrier domain of LacS by the LacS-IIA domain was examined. Previous studies on the role of the IIA domain of LacS had already shown that: 1) the LacS-IIA domain is not essential for translocation (83) 2) LacS is phosphorylated on His-552 by HPr(His~P) (43,86) and 3) the LacS-IIA domain modulates the activity of the carrier domain. However, it was not established whether regulation took place via interaction of the carrier domain with phosphorylated LacS-IIA, dephosphorylated LacS-IIA, or both LacS-IIA species.

To determine which scenario was applicable, a C-terminal 160 amino acid truncation mutant, LacS$\Delta$IIA, was constructed. In *E. coli* MC1061, transport by LacS$\Delta$IIA was compared to LacS and LacS$\Delta$IIA-GFP. Counterflow transport by LacS proceeded at approximately twice the rate of $\Delta p$-driven lactose transport, while both LacS$\Delta$IIA and LacS$\Delta$IIA-GFP showed similar rates for both modes of transport. The activities of LacS$\Delta$IIA and LacS$\Delta$IIA-GFP were equal to $\Delta p$-driven transport by LacS. Since both the LacS-IIA deletion and substitution mutant displayed similar transport characteristics, the presence or absence of a C-terminal soluble domain does not seem to affect the activity of the carrier domain. We therefore propose that the LacS-IIA domain must physically interact with the carrier domain in order to exert its regulatory role. Based on the *in vitro* transport data, showing that LacS$\Delta$IIA indeed mimics unphoshorylated LacS, the transport characteristics of LacS in *E. coli* cells suggest that the protein is phosphorylated under these conditions.

Conclusive evidence on the mechanism of regulation of LacS-IIA came from *in vitro* studies. Full length LacS was membrane-reconstituted in a predominantly inside-out orientation. Penetration of the large hydrophilic LacS-IIA domain through the Triton X-100-treated bilayer is energetically unfavourable and this has been proposed to be the ground for the inside-out orientation of LacS (66). It has now been shown that the absence of the LacS-IIA domain yields proteoliposomes in which the carrier domain is in the same orientation as the full-length LacS protein, suggesting that the orientation is not determined by the large hydrophilic LacS-IIA domain but by the membrane-embedded carrier domain itself. Since the affinities of both
binding sites of LacS differ approximately one order of magnitude, the identical orientation of LacS and LacSΔIIA in proteoliposomes enabled a direct comparison of both proteins in vitro. In vitro analyses of the effect of phosphorylation of LacS on the translocation of lactose indicated that the rate of Δp-driven lactose transport of phosphorylated LacS is somewhat decreased compared to the unphosphorylated species. The $V_{\text{max}}$ of lactose counterflow transport of phosphorylated LacS, on the other hand, was increased 4-6 times. Both observations are in agreement with previous studies (41,83). The stimulation of lactose counterflow was explained by assuming an increase in rate constants for the reorientation of the ternary enzyme-substrate-proton complex (Fig. 1) upon phosphorylation (41). This isomerisation of the ternary complex has been proposed to be the rate-limiting step in lactose counterflow transport (82). Although Δp-driven lactose transport also comprises a reorientation step of the ternary complex, the $V_{\text{max}}$ for this mode of transport was unaffected, which agrees with the idea that this step is not rate-determining for Δp-driven uptake.

The results obtained thusfar are summarized in a working model (Fig. 6). For both lactose counterflow and Δp-driven lactose transport, the kinetics of translocation of unphosphorylated LacS and LacSΔIIA are very similar, suggesting that the LacS-IIA domain does not functionally interact with the carrier domain in its unphosphorylated state. The stimulation of lactose counterflow upon phosphorylation of LacS-IIA by HPr(His~P) is most likely caused by a direct interaction of the phosphorylated IIA domain with the carrier domain. The regulation by LacS-IIA takes place intramolecularly as inferred from the analysis of tandem fusion proteins consisting of a LacSΔIIA subunit fused to the N-terminus of a LacS subunit (34). These covalent dimers contained only one LacS-IIA domain per dimer and either the first or the second carrier domain was inactivated by mutation. Only tandem constructs in which the LacS-IIA domain was attached to a functional carrier showed an increase in counterflow transport

![Figure 6. A model of the interactions between the carrier and IIA domain of LacS.](image)

The membrane-embedded carrier domain of LacS is represented by a white box to which the soluble IIA domain (represented by a white oval) is coupled via a flexible linker. The phosphorylation state of LacS-IIA is represented by X and P, reflecting the unphosphorylated and the phosphorylated state, respectively. The two-way arrows indicate lactose counterflow transport and their size reflects the rate of transport. A, upon phosphorylation of the IIA domain by HPr(His~P), the IIA domain interacts with the carrier domain; B, this interaction accelerates the counterflow transport. The other subunit of the dimer (depicted in grey) is not affected by the opposing IIA domain. For clarity only one phosphorylated subunit per dimer is shown.
relative to Δp-driven transport, indicating that LacS-IIA regulation takes place within one subunit. The proposed mode of regulation differs from the mechanism of inducer exclusion in *E. coli*. Transporters like LacY and MelB are inhibited by the dephosphorylated form of IIAGlc, which is homologous to the LacS-IIA domain. Since structural changes upon phosphorylation of IIAGlc are limited to small shifts (<1.5 Å) of active site residues (79) and the interaction surfaces of IIAGlc with HPr, IIBGlc, glycerol kinase and LacY largely overlap (13,81,105), it seems likely that the LacS carrier domain interacts with the equivalent interface on the LacS-IIA domain. Most likely, specific contact points at the interaction surface on the cytoplasmic face of the carrier domain of LacS and the LacS-IIA domain govern the opposite mode of regulation.

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