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Lactose Transport System of *Streptococcus thermophilus*

FUNCTIONAL RECONSTITUTION OF THE PROTEIN AND CHARACTERIZATION OF THE KINETIC MECHANISM OF TRANSPORT*

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The kinetic mechanism of the lactose transport system of *Streptococcus thermophilus* was studied in membrane vesicles fused with cytochrome *c* oxidase containing liposomes and in proteoliposomes in which cytochrome *c* oxidase was coreconstituted with the lactose transport protein. Selective manipulation of the components of the proton (and sodium) motive force indicated that both a membrane potential and a pH gradient could drive transport. The galactoside/proton stoichiometry was close to unity. Experiments which discriminate between the effects of internal pH and ΔpH as driving force on galactoside/proton symport showed that the carrier is highly activated at alkaline internal pH values, which biases the transport system kinetically toward the pH component of the proton motive force. Galactoside efflux increased with increasing pH with a pK_a of about 8, whereas galactoside exchange (and counterflow) exhibited a pH optimum around 7 with pK_a values of 6 and 8, respectively. Imposition of ΔpH (interior alkaline) retarded the rate of efflux at any pH value tested, whereas the rate of exchange was stimulated by an imposed ΔpH at pH 5.8, not affected at pH 7.0, and inhibited at pH 8.0 and 9.0. The results have been evaluated in terms of random and ordered association/dissociation of galactoside and proton on the inner surface of the membrane. Imposition of ΔΨ (interior negative) decreased the rate of efflux but had no effect on the rate of exchange, indicating that the unloaded transport protein carries a net negative charge and that during exchange and counterflow the carrier recycles in the protonated form.

The lactose transport protein (LacS) of *Streptococcus thermophilus* is a polytopic membrane protein that traverses the cytoplasmic membrane most likely 12 times and contains a carboxyl-terminal hydrophilic extension of approximately 180 amino acids. The hydrophobic carrier domain of LacS is homologous to the melibiose carrier protein (MelB) of *Escherichia coli*, but, with the exception of a region between putative α-helices X and XI, LacS shares no similarity with the lactose transport protein (LacY) of *E. coli* (Poolman *et al.*, 1989, 1992). The carboxyl terminus of LacS is denoted IIA or enzyme III domain due to its similarity with IIA (enzyme III) protein domains of various phosphonenolpyruvate:sugar phosphotransferase systems (Poolman *et al.*, 1989, 1992).

The lactose transport system of *S. thermophilus* has been characterized at DNA level (Poolman *et al.*, 1989, 1990), and the role of the conserved (and other) histidine residues in the carrier and IIA domain has been assessed by biochemical characterization of site-directed mutants (Poolman *et al.*, 1992). The latter studies indicate that at least part of the galactoside recognition site of LacS, tentatively located between α-helices X and XI, may be similar to that of LacY. All these studies were performed with the cloned gene expressing LacS in *E. coli*. The level of expression of *lacS* in *E. coli* from its own promoter, however, is low in comparison with the expression level in *S. thermophilus*. Therefore, in the present study the mechanism of transport was analyzed using membrane vesicles isolated from *S. thermophilus* as starting material. In view of the similarity of LacS with MelB, which transports galactosides in symport with either protons, sodium, or lithium ions (Wilson and Wilson, 1987; Leblanc *et al.*, 1990), experiments were set up to determine the cation selectivity of LacS and to compare the transport of α-galactoside (melibiose) and β-galactoside (TMG,[1] lactose). Furthermore, in a recent report, dealing with a study of LacS in intact cells, it is concluded that LacS acts as a lactose/galactose antiporter (Hutkins and Ponne, 1991). The conclusion carries back to earlier observations that *S. thermophilus* only metabolizes the glucose moiety of lactose and that galactose is excreted into the medium stoichiometrically (Thomas and Crow, 1984). It has therefore been suggested that *in vivo* LacS may facilitate lactose/galactose exchange rather than lactose/cation symport (Poolman, 1990). In the present investigation, the different modes of facilitated diffusion mediated by the lactose transport protein of *S. thermophilus* have been analyzed *in vitro*. On basis of the effects of pH and membrane potential on the facilitated diffusion processes, a kinetic scheme of the translocation cycle of galactoside/proton symport and galactoside/galactoside exchange is proposed.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Preparation of Cell Suspensions —*S. thermophilus* A147 was grown semianaerobically in

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[1] The abbreviations used are: TMG, methyl-1-thio-β-D-galactopyranoside; Δp (or pmf), proton motive force ($\Delta\tilde{\mu}_H+/F$); $\Delta\tilde{\mu}_H+$, transmembrane electrochemical potential difference for protons; ΔpH, transmembrane pH gradient; ΔΨ, transmembrane electrical potential difference; Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Ches, 2[*N*-cyclohexylaminoethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*-*N*′-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; TMPD, *N*,*N*,*N*,*N*′-tetramethylphenylenediamine; TPP^+, tetramethylphosphonium ion.

Elliker broth (Elliker *et al.*, 1956) containing 0.5% beef extract and 0.2% lactose at 42 °C. Overnight cultures were harvested by centrifugation, washed twice, and resuspended to a final protein concentration of about 30 mg/ml in 50 mM potassium phosphate buffer, pH 6.5, containing 2 mM $MgSO_4$ (KPM).

Isolation of Membrane Vesicles and Membrane Fusion—Membrane vesicles of *S. thermophilus* were prepared as described previously (Otto *et al.*, 1982) with the following modifications: the culture was harvested during the exponential phase of growth (A_{660} of 0.6–0.8), the quantities of lysozyme, ribonuclease, and deoxyribonuclease were doubled, and the incubation steps were performed at 37 °C. Cytochrome *c* oxidase containing liposomes (22 nmol of heme *a*/100 mg of phopholipid) were prepared as described (Driessen and Konings, 1992) using L-α-phosphatidylethanolamine type IX obtained from *E. coli* (Sigma), which were further purified by aceton/ether washing (Kagawa *et al.*, 1973), and L-α-phosphatidylcholine type XI-E from fresh egg yolk (Sigma) in a ratio of 3:1 (weight/weight). Fusion between membranes vesicles and cytochrome *c* oxidase containing liposomes (10 mg of phospholipid/mg of vesicular protein) was performed by freeze/thaw sonication (Driessen and Konings, 1992). To form unilamellar vesicles, the thawed suspension was sonicated at 4 °C for 4 s with a macrotip at an output of 4 μm (peak to peak). By the same procedure, membrane vesicles were fused with liposomes devoid of cytochrome *c* oxidase. The fused membranes were collected by centrifugation (1 h at 185,000 × *g*, 4 °C) and suspended to a final protein concentration of about 15 mg/ml in 50 mM potassium phosphate (KP_i), pH 7.0, unless indicated otherwise.

Solubilization and Reconstitution of the Lactose Transport Protein—Membrane vesicles were extracted consecutively with 5 M urea and 6% (weight/volume) sodium-cholate in 50 mM KP_i, pH 7.0 (Newman *et al.*, 1981). Membrane proteins (1 mg of protein) were solubilized with 1.25% (weight/volume) *n*-octyl-β-D-glucopyranoside (octyl glucoside) in the presence of 5 mg of a mixture of phosphatidylethanolamine/phophatidylcholine (3:1, weight/weight), 20% (volume/volume) glycerol and 50 mM KP_i, pH 7.0, in a final volume of 1.0 ml, unless indicated otherwise. The suspension was incubated for 30 min at 4 °C and then centrifuged at 185,000 × *g* (4 °C) for 1 h. The supernatant (1 ml) was removed and mixed with 0.25 ml of 20 mg/ml of PE/PC (3:1, weight/weight) in 4% (weight/volume) octyl glucoside and 0.25 ml of 50 mM KP_i, pH 7.0. The mixed micelle suspension was agitated by manual inversion of the tube and incubated for 10 min at 4 °C. Proteoliposomes were formed by detergent dilution (35-fold) into 50 mM KP_i, pH 7.0, or by detergent dialysis (against 500-fold volumes of 50 mM KP_i, pH 7.0) as described (In't Veld *et al.*, 1992).

Transport Assays—(i) Δp-driven Uptake. Fused membranes and proteoliposomes were diluted to a final protein concentration of about 0.25 mg/ml in KPM or in 25 mM potassium-Hepes, 25 mM potassium-Mes, 25 mM potassium-Pipes, 25 mM potassium-chloride containing 2 mM $MgSO_4$, (HMP buffer), pH 5.0–8.0, supplemented with 200 μM TMPD and 10 μM cytochrome *c*. The electron donor ascorbate (potassium-salt, 10 mM, final concentration) was added 2 min prior to the initiation of transport, and the incubation mixture was kept under continuous aeration. Transport was initiated upon addition of small aliquots of radiolabeled substrates. At given time intervals samples were withdrawn, diluted with 2 ml of ice-cold 0.1 M of lithium chloride, filtered immediately on 0.45-μm cellulose nitrate filters (Schleicher & Schüell GmbH, Dassel, Germany), and washed once with 2 ml of 0.1 M lithium chloride. Initial rates of uptake were determined from the amount of labeled substrate accumulated during the first 5 s. Transport assays were performed at 30 °C. Radioactivity was measured by liquid scintillation spectrometry.

(ii) Counterflow. Concentrated cell, membrane vesicle, fused membrane, or proteoliposome suspensions in KPM or HMP buffer were equilibrated in the presence of 2 mM TMG for 1 h at 42 °C. Aliquots (2 μl) were diluted 100-fold to final protein concentrations of 0.30 mg/ml (cells and membrane vesicles), 0.15 mg/ml (fused membranes), or 0.06 mg/ml (proteoliposomes) into KPM or HMP buffer of the indicated pH containing [^{14}C]TMG. Initial rates of counterflow were determined after 3 s of incubation. Experiments were performed at 25 °C and uptake was assayed by filtration as described above.

(iii) Efflux and exchange. Membrane preparations were equilibrated in the presence of 2 mM [^{14}C]TMG or 5 mM [^{3}H]melibiose at 42 °C for 1 h as described under Counterflow, except that membranes were diluted into buffer without (efflux) or with 2 mM of TMG or 5 mM of melibiose (equilibrium exchange). For efflux and exchange in the presence of artificially imposed diffusion potentials, the membranes were resuspended in the buffers specified below prior to loading with 2 mM [^{14}C]TMG or 5 mM [^{3}H]melibiose. To generate a ΔΨ, valinomycin was added to the membrane suspension to a final concentration of 2 nmol/mg of protein. The membranes were washed and resuspended in 120 mM KP_i or 120 mM sodium-phosphate (NaP_i), pH 6.5, containing 2 mM $MgSO_4$. Potassium-loaded membranes were diluted into the same buffer (no gradient) or in NaP_i buffer (ΔΨ, interior negative). Sodium-loaded membranes were diluted into the same buffer (no gradient) or in potassium-containing buffers (ΔΨ, interior positive). For efflux and exchange in the presence of a ΔpH, interior alkaline, membranes were resuspended in 100 mM potassium-acetate supplemented with 2 mM $MgSO_4$ and 20 mM KP_i, pH 6.5. Subsequently, the membranes were diluted into the same buffer (no gradient) or into 120 mM KP_i, pH 6.5, containing 2 mM $MgSO_4$ (ΔpH, interior alkaline). For efflux and exchange in the presence of a ΔpH, interior acid, membranes were equilibrated in the presence of 120 mM KP_i, pH 6.5, supplemented with 2 mM $MgSO_4$, and, subsequently, diluted 100-fold into 100 mM potassium-acetate, 20 mM KP_i, 2 mM $MgSO_4$, pH 6.5. For the generation of a Δp (interior alkaline and negative or interior acid and positive), the properly oriented potassium- and acetate-diffusion gradients were combined. To impose artificial diffusion potentials in the pH range of 5 to 9, the phosphate in the buffers specified above was replaced by similar concentrations of Pipes plus Ches. Efflux and exchange were assayed at 25–37 and 7–20 °C, respectively, as specified in the legends to figures.

(iv) Imposed Δp-driven uptake. For uptake driven by artificially imposed diffusion gradients (ΔΨ, interior negative; ΔpH, interior alkaline and Δp, interior negative and alkaline), membranes were treated as described under Efflux and Exchange except that loading with radiolabeled substrates was omitted. The membrane preparations were diluted 100-fold into buffers supplemented with the appropriate concentration of radiolabeled substrate. Uptake was assayed at 30 °C as described above.

Determination of the Electrical Potential Difference—The ΔΨ across the membrane (ΔΨ, inside negative) was determined from the distribution of the lipophilic cation TPP^+ using a TPP^+-selective electrode (Shinbo *et al.*, 1978). The ΔΨ was calculated from the steady state level of TPP^+ accumulation and was corrected for concentration-dependent binding of the probe to the membrane (Lolkema *et al.*, 1982).

Determination of the pH Gradient—The ΔpH across the membrane was determined from the fluorescence of pyranine (100 μM, final concentration) entrapped within fused membranes or proteoliposomes (Clement and Gould, 1981). External pyranine was removed by washing with the buffer indicated, and membranes were collected by centrifugation (185,000 × *g*, 45 min, 4 °C).

Determination of the Internal Volume—Trapped volume measurements were performed with the fluorophore calcein as described (Oko *et al.*, 1982). A value of 8 μl/mg of protein was determined for fused membranes. Alternatively, the specific internal volume was determined from the equilibration of [^{14}C]TMG in the absence of Δp. This method yielded values of 4.1, 8.7, and 70.3 μl/mg of protein for the membrane vesicles, fused membranes containing cytochrome *c* oxidase, and proteoliposomes, respectively.

Miscellaneous—Protein was determined by the method of Lowry *et al.* (1953) in the presence of 0.5% SDS (Dulley and Grieve, 1975) with bovine serum albumine as a standard. β-Galactosidase activity was determined from the hydrolysis of *ortho*-nitrophenyl-β-D-galactopyranoside (Citti *et al.*, 1965). Bovine heart cytochrome *c* oxidase was isolated according to described procedures (Yu *et al.*, 1975).

Chemicals—[D-Glucose-1-^{14}C]lactose (2.11 TBq/mol), [1-^{3}H]galactose (185 TBq/mol), and L-[^{14}C]alanine (6.6 TBq/mol) were obtained from the Radiochemical Centre Amersham, United Kingdom. [^{14}C] *methyl*-β-D-thiogalactopyranoside (1.85 TBq/mol) was obtained from Du Pont-New England Nuclear. D-[^{3}H]melibiose (0.12 TBq/mol) was a generous gift of Dr. G. Leblanc (Bassilana *et al.*, 1987). All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Membrane Vesicles, Fused Membranes, and Proteoliposomes—Membrane vesicles of lactose-grown *S. thermophilus* A147 were isolated by a protocol developed for *Lactococcus lactis* (Otto *et al.*, 1982). Although these membrane vesicles exhibited TMG counterflow activity, neither TMG nor alanine uptake driven by artificially imposed ion gradients could be demonstrated. Moreover, a large fraction of cytosolic pro-

teins (*e.g.* β-galactosidase) remained associated with the membranes which prevented the use of lactose as substrate in the transport assays. Fusion of the membrane vesicles with liposomes decreased the "leakiness" of the membranes (artificially imposed ion gradients were sustained for more than 2 min) and reduced the contamination with cytosolic enzymes. Fusion of the membrane vesicles with cytochrome *c* oxidase containing liposomes yielded membrane preparations in which, in the presence of the electron donor system ascorbate-TMPD-cytochrome *c*, proton motive force-driven uptake of TMG could be assayed (Fig. 1).

Alternatively, membrane vesicles were solubilized with *n*-octyl-β-glucopyranoside in the presence of phospholipids and glycerol. Reconstitution of membrane proteins was performed by detergent dilution or detergent dialysis. Both methods were equally efficient in reconstituting TMG (lactose, melibiose) and alanine transport activities (data not shown). Coreconstitution of the streptococcal membrane proteins with bovine heart cytochrome *c* oxidase enabled us to demonstrate Δp-driven lactose uptake in the proteoliposomes (not shown). Finally, efflux and exchange of galactosides were monoexponential and continued with pseudo-first order rate kinetics until nearly all radiolabel had disappeared from the membranes both in fused membranes and proteoliposomes. Treatment of the membranes with *p*-chloro-mercuribenzoic acid (100 μM, final concentration) resulted in inactivation of the lactose carrier, and these membranes displayed exit of TMG with a much lower rate constant (passive diffusion). The extent of release of sugars from the fused membranes and proteoliposomes with the carrier-mediated kinetics indicated that more than 95% of the membranes contained a lactose carrier molecule (data not shown).

Mechanism of Energy Coupling—The effect of ionophores and protonophores on the initial rate of TMG uptake, the steady state level of galactoside accumulation, and the magnitude of the components of the proton motive force were analyzed in membrane vesicles fused with cytochrome *c* oxidase containing liposomes (Fig. 1). Nigericin, which dissipates the ΔpH, decreased the initial rate of uptake and lowered the TMG accumulation level (Fig. 1*A*). Valinomycin, which dissipates the ΔΨ, had virtually no effect on the rate of uptake but lowered the steady state level of accumulation (Fig. 1*B*). The combination of valinomycin plus nigericin (Fig. 1*C*) and the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (not shown), which abolish the total Δp, reduced transport to equilibration levels. Monensin, which converts the ΔpH into a $\Delta\mu_{Na}{}^{+}$, lowered TMG transport in the presence of 5 mM sodium ions (not shown). The same TMG accumulation levels were reached irrespective of whether the ionophores (protonophores) were added prior to the initiation of transport or during the course of an experiment (indicated by *arrows*). Altogether, the results indicate that LacS-mediated TMG transport is coupled to the proton rather than to a sodium motive force. Similar conclusions were reached from the uptake of lactose (β-galactoside) and melibiose (α-galactoside) in proteoliposomes and fused membranes, respectively (data not shown).

Galactoside/Proton Stoichiometry—The galactoside/proton stoichiometry of LacS-mediated transport was determined from the steady state accumulation levels of melibiose and the magnitude of the components of the Δp. Melibiose was used as substrate in these experiments because the noncarrier-mediated flux of the disaccharide was at least 10-fold lower than that of TMG (not shown). In general, accumulation ratios predicted by the thermodymic equilibrium levels are difficult to reach for substrates with hydrophobic properties (Maloney and Wilson, 1973; Driessen *et al*, 1987). At pH 6.5 a Δp of −154 mV (ΔΨ of −130 mV and ZΔpH of −24 mV) was generated by ascorbate-TMPD-cytochrome *c* oxidation in the proteoliposomes, and this Δp did not depolarize upon addition of galactosides. Steady state melibiose accumulation levels ($melibiose_{in}/melibiose_{out}$) were reached after 30–40 min and depended on the external melibiose concentration. At thermodynamic equilibrium $\Delta\mu_{MEL}/F$ equals $n(-\Delta p)$, in which

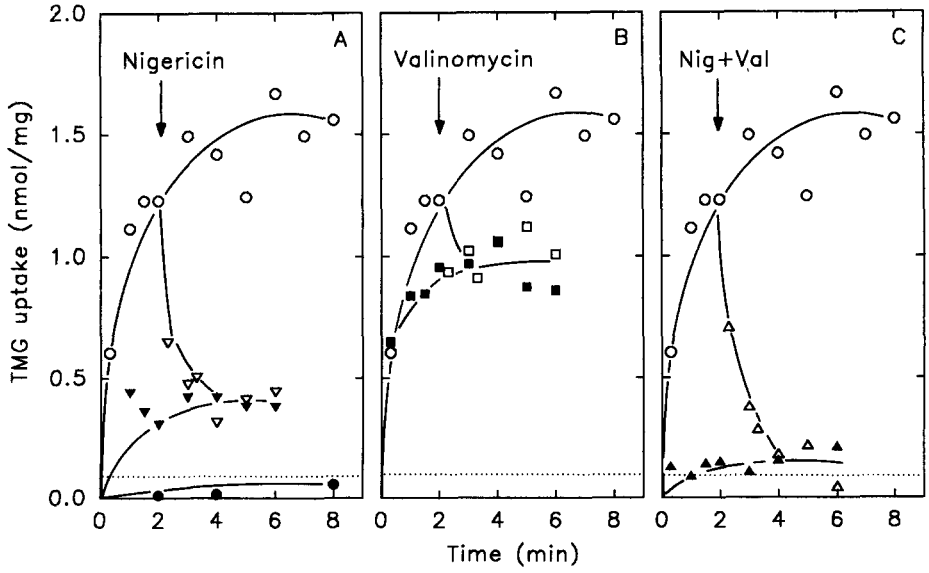

FIG. 1. **Effect of ionophores on TMG uptake in membrane vesicles fused with cytochrome *c* oxidase containing proteoliposomes.** [^{14}C]TMG (8.9 μM, final concentration) uptake by the fused membranes was assayed in 50 mM KP_i, pH 6.5, containing 2 mM $MgSO_4$ in the presence of the electron donor system ascorbate-TMPD-cytochrome *c*, and at a final protein concentration of 0.3 mg/ml (○). Nigericin (5 nM, final concentration) and valinomycin (50 nM, final concentration) were added together with the electron donor potassium-ascorbate (*closed symbols*) or at times indicated by the *arrows* (*open symbols*). *Panel A*, effect of nigericin (*downward triangles*). *Panel B*, effect of valinomycin (*squares*). *Panel C*, effect of valinomycin plus nigericin (*upward triangles*). TMG uptake in the absence of ascorbate (●) is shown in *panel A*. Equilibration levels are indicated by *dotted lines*.

$\Delta\mu_{MEL}/F$ represents the melibiose concentration gradient (in mV) and n the number of protons translocated in symport with melibiose. By extrapolating $\Delta\mu_{MEL}/F(-\Delta p)$ to an external melibiose concentration of zero, the melibiose/H^+ stoichiometry was estimated to be one (Fig. 2).

pH Dependence of Δp-driven Uptake—The effects of valinomycin and nigericin on the initial rate of TMG uptake and the components of the proton motive force were investigated further by titrating with the individual ionophores. As shown in Fig. 3*A*, nigericin lowered the ΔpH without having an effect on ΔΨ and decreased the initial rate of TMG uptake. Valinomycin, on the other hand, dissipated the ΔΨ and increased the ΔpH but had little or no effect on the initial rate of TMG uptake despite a significant drop in the total proton motive force (Fig. 3*B*). These data are most easily explained by assuming that LacS is activated at alkaline internal pH values (Fig. 3*C*). It was not possible to determine the pK_a of the internal pH dependence precisely since the internal pH could only be manipulated in a narrow range without causing major changes in the driving force (Δp) of the transport process.

The effects of pH on the initial rate of TMG uptake were further analyzed at external pH values of 5.0, 6.0, 7.0, and 8.0 while the internal pH was manipulated by the ionophores valinomycin or nigericin. The transport rates increased with increasing external pH when the internal pH was raised in parallel, whereas minor effects were observed at varying external pH while the internal pH was kept constant (Fig. 4). The total Δp varied somewhat nonsystematically with the external (and internal) pH, *i.e.* maximal and minimal values were reached at external pH values of 6.0 and 8.0, respectively (data not shown). Altogether, the complicated pH profiles as shown in Fig. 4 (and Fig. 3*C*) indicate that in the fused membranes protonation site(s) on the inner surface of the membrane control Δp-driven TMG uptake.

pH Profiles of Efflux, Exchange, and Counterflow—Efflux, equilibrium exchange, and counterflow were investigated at different pH values under conditions that Δp was zero. Concentrated membrane preparations (membrane vesicles or fused membranes) were equilibrated with 2 mM [^{14}C]TMG (efflux and exchange) or [^{12}C]TMG (counterflow) and then diluted rapidly 100-fold into the same buffer devoid of TMG (efflux) or with 2 mM unlabeled (exchange) or labeled (counterflow) TMG. To measure the exchange reaction accurately

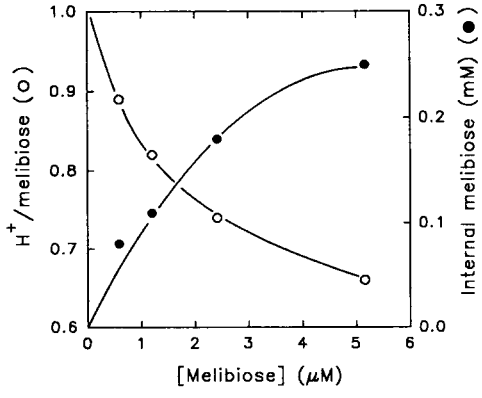

FIG. 2. **Melibiose/proton stoichiometry in proteoliposomes.** Melibiose uptake by proteoliposomes (see "Experimental Procedures") was assayed in 50 mM KP_i, pH 6.5, containing 2 mM $MgSO_4$, and supplemented with the electron donor system ascorbate-TMPD-cytochrome *c*. Melibiose uptake was assayed for 60 min, and the steady state accumulation levels were estimated. In parallel experiments the magnitude of ΔΨ and ΔpH was determined. The apparent melibiose/proton stoichiometry was calculated from the melibiose concentration gradient ($\Delta\mu_{MEL}/F$) and the Δp ($=\Delta\Psi - Z\Delta pH$).

the temperature of the assay medium was set at 7 °C; efflux was assayed at 25 °C. As shown in Fig. 5*A*, the rate of TMG efflux increased with increasing pH with a pK_a of about 8.2. Under conditions that an equimolar concentration of TMG was present externally, exit of [^{14}C]TMG (equilibrium exchange) displayed an optimum at pH 7.2 (apparent p*K* values of 6.2 and 8.2). TMG counterflow exhibited a pH dependence similar to that of exchange (data not shown). Although exchange is one to two orders of magnitude faster than efflux around neutral pH, both activities become very similar at pH 10. Efflux and exchange of melibiose displayed pH profiles similar to that of TMG both in membrane vesicles (Fig. 5*B*) and in fused membranes (not shown), except that differences in rates of efflux and exchange were less pronounced than with TMG.

Effect of Membrane Potential and Internal pH on Efflux and Exchange—Galactoside/proton symport involves the net translocation of a charge (proton) across the membrane. Consequently, the reorientation of either the ternary carrier-galactoside-proton complex and/or the unloaded carrier should involve net movement of charge. Since exchange is more rapid than efflux in the pH range of 5 to 9, the rate-determining step for TMG and melibiose efflux down a concentration gradient could involve a reaction associated with the return of the unloaded carrier to the inner surface of the membrane. If net movement of charge is involved in this step, the membrane potential should affect efflux. Membrane potential (inside negative relative to outside) was imposed by means of a valinomycin-mediated potassium diffusion gradient (see "Experimental Procedures"). Since passive fluxes of melibiose are less manifest than those of TMG, the α-digalactoside was used as substrate in the following experiments. The results presented in Fig. 6 show that efflux of melibiose from fused membranes was retarded by a membrane potential (interior negative) at pH 5.8 (*panel A*), pH 7.0 (*panel C*), pH 8.0 (*panel E*) and pH 9.0 (not shown). Equilibrium exchange of melibiose, on the other hand, was not affected by the membrane potential at any pH tested (Fig. 6 *B*, *D*, and *F*, not shown). These results are consistent with a translocation cycle for efflux in which a negative charge moves to the inside during the reorientation of unloaded binding sites. Since exchange is unaffected by ΔΨ it is unlikely that any of the translocation intermediates of this reaction carries a net charge.

To discern external and internal pH effects in the pH dependences of the facilitated diffusion reactions (Fig. 5), efflux and exchange of melibiose were assayed at pH 5.8, 7.0, 8.0, and 9.0 in the presence of a pH gradient (inside alkaline relative to outside). To raise the internal pH an outwardly directed acetate diffusion gradient was imposed (see "Experimental Procedures"). As shown in Fig. 6, in the presence of a ΔpH the rate of efflux was reduced at all pH values tested. The relative effect of the ΔpH on the rate of melibiose efflux increased with increasing pH. Equilibrium exchange of melibiose was enhanced by ΔpH (inside alkaline) at pH 5.8 (Fig. 6*B*), not significantly affected at pH 7.0 (Fig. 6*D*), and retarded at pH 8.0 (Fig. 6*F*) and 9.0 (not shown). These results clearly indicate that the rates of efflux and exchange are differently affected by pH and that the observed pH dependence of exchange (Fig. 5) could be due to changes in the internal pH.

DISCUSSION

The kinetic mechanism of the lactose transport protein (LacS) of *S. thermophilus* has been analyzed in fused membranes and in proteoliposomes. The effects of ionophores and

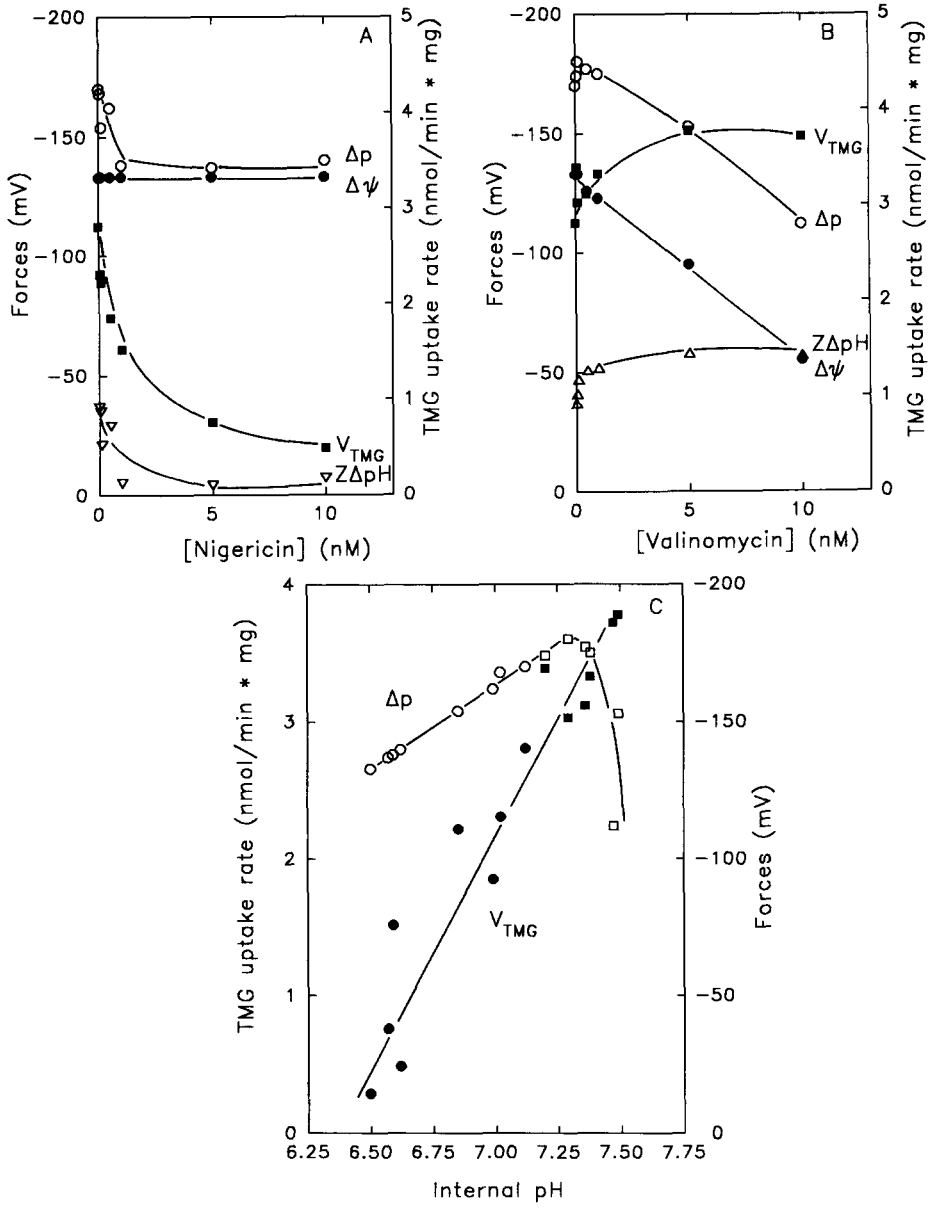

FIG. 3. **Effect of increasing concentrations of ionophores on TMG uptake and the magnitude of the components of the Δp, and the internal pH dependence of the initial rate of TMG uptake.** Fused membranes were resuspended in HMP buffer, pH 6.5, and energized by ascorbate-TMPD-cytochrome *c* oxidation. Increasing concentrations nigericin (*panel A*) and valinomycin (*panel B*) were added. Conditions were similar to those described in the legend to Fig. 1 except that the final protein concentration was 0.24 mg/ml. The initial rate of TMG uptake was estimated from the amount of label accumulated over 5 s, and the magnitudes of the components of the Δp were estimated as described. *Panel C*, The dependence of the initial rate of TMG uptake on the internal pH (data were taken from *panels A* and *B*). For comparison the magnitude of the Δp is also shown.

uncouplers on Δp-driven galactoside uptake indicate that transport proceeds in symport with a proton. Despite the similarities in the primary structure of LacS and MelB (Poolman *et al.*, 1989; 1992), the LacS carrier protein shows no substrate-dependent cation selectivity. By contrast, the melibiose carrier protein cotransports α-galactosides (and galactose) with H^+, Na^+, and to a lesser extent Li^+, whereas β-galactosides are transported equally well with Na^+ and Li^+ but not with H^+ (Wilson and Wilson, 1987; Leblanc *et al.*, 1990). In fact, the functional characteristics of LacS resemble more those of the lactose carrier protein (LacY) of *E. coli* than those of MelB (see below).

Activation/inhibition of transport activity by pH can be envisaged in terms of two types of proton-binding sites (Poolman *et al.*, 1987). The first type involves binding/release of the symported proton and can be interpreted as catalytic site. The second type of proton-binding site is not directly involved in the catalytic mechanism but affects transport allosterically. When LacS facilitates Δp-driven uptake the galactoside and proton are released on the inner surface of the membrane. Since Δp-driven TMG uptake is stimulated at alkaline internal pH values one could argue that under these conditions the translocation cycle is rate-limited by the release of the catalytic proton on the inner surface of the membrane. In fact, the effect of a large drop in the ΔΨ on the rate of Δp-driven uptake can be compensated by a relatively minor increase in the ΔpH (internal pH) (Figs. 1 and 3). Notice that a lowering of ΔΨ does result in a lower level of TMG uptake (Fig. 1*B*), indicating that the steady state level of galactoside accumulation is coupled to Δp. Thus, the ΔpH component of the Δp not only acts as a driving force for transport but also affects transport by influencing the equilibrium between the protonated and deprotonated forms of the carrier protein or the rate of proton transfer from the protein to the solvent on the inner surface of the membrane (Fig. 7). An increase in ΔpH (or internal pH) would shift the equilibrium to the unprotonated form of the carrier protein, and as a result the influx of TMG is accelerated.

For efflux down a concentration gradient, a proton and galactoside molecule have to be bound by the carrier protein on the inside, and both have to be released on the outside. The inhibition of efflux by a ΔpH (interior alkaline) is in

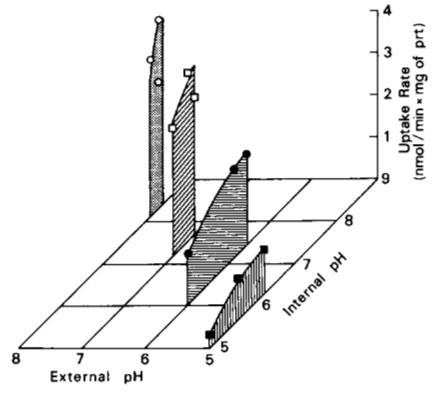

FIG. 4. **Effect of external and internal pH on the initial rate of TMG uptake.** Conditions were similar to those described in the legend to Fig. 3 except that HMP buffers at pH 5.0 (■), 6.0 (●), 7.0 (□), and 8.0 (○) were used and the final protein concentration was 0.25 mg/ml. The components of the Δp were estimated in the absence and presence of valinomycin (50 nM, final concentration) or nigericin (5 nM, final concentration), and the effect of these manipulations on the initial rate of [^{14}C]TMG (8.9 μM, final concentration) uptake were analyzed. The initial rate of TMG uptake as a function of external and internal pH is shown; effect of pH on the components of Δp is not shown.

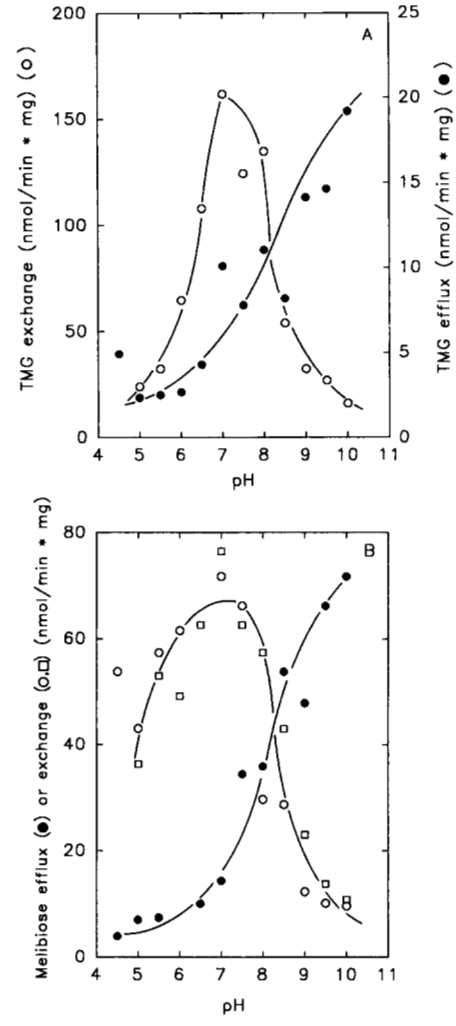

FIG. 5. **Effect of pH on efflux and equilibrium exchange of TMG (*panel A*) and melibiose (*panel B*) in membrane vesicles.** Experiments were performed in 30 mM potassium-citrate, 30 mM KP_i, 30 mM potassium-Ches, 2 mM $MgSO_4$ of the indicated pH, supplemented with valinomycin (50 nM) plus nigericin (5 nM), and at a final protein concentration of 0.3 mg/ml as described under "Experimental Procedures." The release of [^{14}C]TMG and [^{3}H]melibiose from the membranes in the assays of efflux and exchange was monoexponential and first order rate constants ($k = \ln 2/t_{1/2}$) could be estimated. The rates of efflux and exchange were obtained by multiplying k with the internal galactoside concentration (2 mM or 8.2 nmol/mg of protein for TMG; 5 mM or 20.5 nmol/mg of protein for melibiose). Efflux and equilibrium exchange of TMG were assayed at 25 and 7 °C, respectively; efflux and equilibrium exchange of melibiose were performed at 35 and 15 °C, respectively. For exchange of melibiose the results of two independent experiments (○, □) are shown.

accordance with a lower rate of protonation on the inner surface of the membrane or a decrease in the relative abundance of the protonated carrier intermediate. Under conditions that the internal and external pH are equal (Fig. 5), an increase in pH enhances the release of the proton on the outside but at the same time decreases the protonation of the carrier on the inside. The observed increase in the rate of TMG (and melibiose) efflux with increasing pH should therefore be the resultant of two opposite pH effects, one being exerted on the inner and the other on the outer surface of the membrane. Consequently, a decrease in the rate of efflux is ultimately expected with increasing pH, *i.e.* at higher pH values than used in these experiments.

When saturating amounts of galactosides are present both on the inside and the outside of the membrane, *i.e.* in the exchange and counterflow experiments, the rates of radiolabel equilibration are faster than the rates of efflux and exhibit a pH optimum. Above pH 7 the rate of exchange decreases sigmoidally with a pK_a of about 8, and below pH 7 the exchange activity decreases with a pK_a of about 6. To discriminate between external and internal pH effects, the rates of exchange have been estimated at fixed external pH values and in the presence and absence of an imposed ΔpH (Fig. 6). An increase in internal pH (ΔpH) at external pH > 7 inhibits the rate of melibiose exchange, whereas a similar pH stimulates exchange at external pH < 7. These observations indicate that (de)protonation steps on the inner surface of the membrane affect the rate of galactoside exchange. To interpret the effects of pH on LacS-mediated galactoside efflux and exchange, the reactions involved in the translocation are schematically represented (Fig. 7). In this scheme the ternary carrier-galactoside-proton (CLH) complex is formed on the inner surface of the membrane through the binding of either galactoside first and proton last (*bold type letters*) or proton first and galactoside last, the ternary complex reorients binding sites, and the galactoside and proton are released on the outside in unspecified order. From this point on efflux and exchange differ. During efflux the unloaded carrier protein reorients its binding sites, whereas during exchange the carrier cycle proceeds in the opposite direction ("backward reaction"), *i.e.* another galactoside molecule and proton bind, the ternary complex reorients its binding sites, and the proton and galactoside are released in the internal medium. In this scheme protonation and deprotonation steps (*step 2*) take place during exchange on the inner surface of the membrane if the ternary complex is formed through the binary CL^- complex (Fig. 7, *steps 1* and *2*). If the exchange pathway proceeds via the binary CH complex the galactoside may associate/dissociate (*step 2'*) without association/dissociation of the proton (*step 1'*) (Fig. 7). Since an imposed ΔpH has converse effects on the rate of exchange at pH < 7 and pH > 7, one could argue that exchange is rate-limited at "high" internal pH by the protonation of the carrier molecule ("forward reaction") and at low internal pH by the deprotonation (backward reaction). The resultant of the two opposing (internal) pH effects may lead to an optimum in the pH depend-

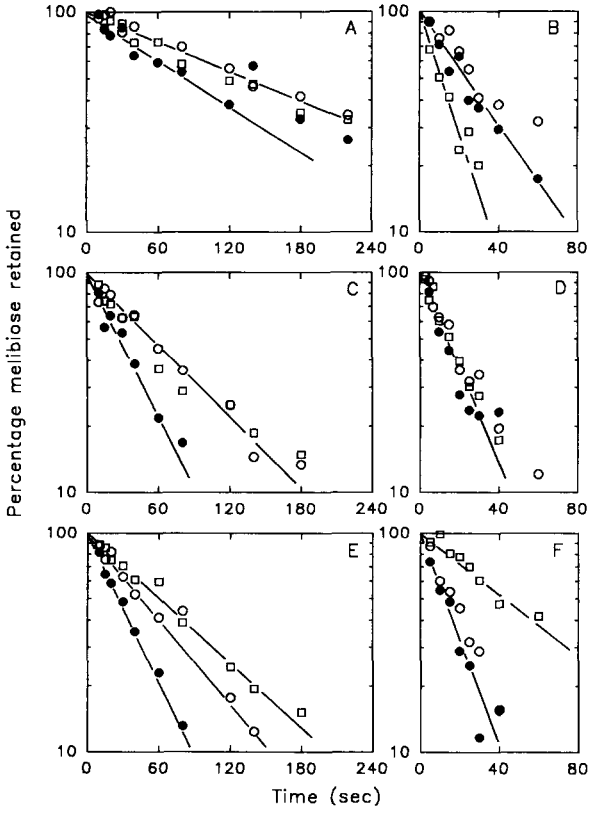

FIG. 6. **Effect of ΔΨ and ΔpH on melibiose efflux and exchange.** Fused membranes were resuspended in 20 mM potassium-Pipes, 20 mM potassium-Ches, 100 mM potassium-acetate, 2 mM $MgSO_4$ of the indicated pH, and supplemented with 5 mM [^{3}H] melibiose. Following equilibration, the membranes were collected by centrifugation and resuspended to final protein concentrations of about 15 mg/ml. To initiate efflux and exchange the concentrated membrane suspensions were rapidly diluted 100-fold into the appropriate buffers to create no diffusion gradient (●), ΔΨ (interior negative, ○) or ΔpH (interior alkaline, □) (see "Experimental Procedures"), and devoid of melibiose (efflux, *panels A, C,* and *E*) or supplemented with 5 mM melibiose (equilibrium exchange, *panels B, D,* and *F*). The medium pH was 5.8 (*panels A* and *B*), 7.0 (*panels C* and *D*) or 8.0 (*panels E* and *F*). Efflux and exchange were assayed at 37 and 20 °C, respectively.

ence of exchange. Assuming ordered binding with proton first and galactoside last (*steps 1′* and *2′*), exchange may be inhibited by high internal pH, due to a lowering of the concentration of the binary CH intermediate, but should not be affected by a low internal pH. In that case one has to invoke additional regulatory (allosteric) (de)protonation steps to explain the pH dependence of the rate of exchange. In conclusion, by interpreting the pH profiles in terms of catalytic (de)protonation steps the results are consistent with a mechanism in which the binding of the galactoside occurs first and proton last. The observed pH dependences of the rates of efflux and Δp-driven uptake do not allow a distinction to be made between an ordered or random binding mechanism.

Since the rate of efflux is affected by imposed ΔΨ, whereas the rate of exchange is not, the unloaded carrier protein most likely carries a negative charge (C^- in Fig. 7). Finally, the effects of ΔpH and ΔΨ on efflux and exchange indicate that the components of Δp affect different steps in the translocation process, and, as a consequence, a unique relationship between the rate of uptake and Δp upon selective manipulation of either ΔΨ or ΔpH is not to be expected.

The translocation scheme put forward for LacS-mediated galactoside transport differs from that proposed for the LacY

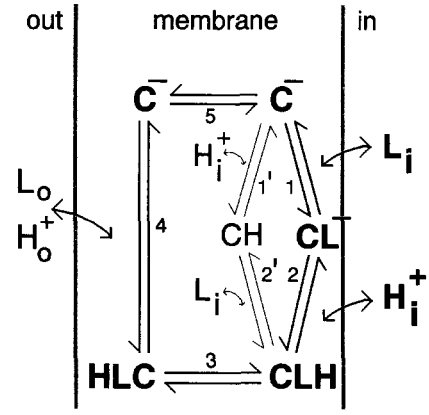

FIG. 7. **Schematic representation of reactions involved in LacS-mediated galactoside transport.** *C*, H^+, and *L* represent the carrier protein, proton, and ligand (galactoside), respectively. In the model ordered binding and release of substrate and proton (substrate first, proton last (*bold type letters*), proton first, galactoside last) on the inner surface of the membrane are indicated. The order of binding and release on the outer surface of the membrane are not specified. The negative charge carried by the unloaded carrier and binary carrier-ligand complex is indicated.

protein of *E. coli*. These differences have for the greater part their origin in the pH dependence of the exchange reaction catalyzed by LacS which is claimed to be independent of pH in case of LacY (Kaczorowski and Kaback, 1979; Kaczorowski *et al.*, 1979; Garcia *et al.*, 1983; Viitanen *et al.*, 1983; Kaback, 1990), although other studies indicate that pH effects can also be observed during LacY-mediated exchange (Wright, 1986; Page, 1987). The effect of ΔΨ on the rate of galactoside efflux, and the lack of a ΔΨ effect on rate of exchange (Fig. 6) are similar for LacS and LacY.

It has been suggested that lactose uptake by *S. thermophilus* is facilitated by a galactose/lactose antiporter (Hutkins and Ponne, 1991). These studies were performed in whole cells in which the lactose taken up is immediately hydrolyzed and the galactose, used to preload the cells, is slowly metabolized,[2] which complicates the analysis of the transport mechanism. The evidence presented in this paper indicates that the lactose transport protein catalyzes lactose (galactoside)/proton symport as well as homologous and heterologous exchange. The requirement for an alkaline internal pH for maximal lactose/proton symport activity and the observation that the maximal rate of exchange is at least 10-fold higher than the maximal rate of Δp-driven uptake supports the suggestion that the lactose transport system may predominantly catalyze lactose/galactose exchange under physiological conditions (Poolman *et al.*, 1989, 1990). Notice that *S. thermophilus* usually grows between pH 7 and 5, and, depending on how well the organism can regulate the intracellular pH Δp-driven lactose uptake may be far too low to meet the observed lactose utilization rates (Poolman, 1990).

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