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Close Approximation of Putative α-Helices II, IV, VII, X, and XI in the Translocation Pathway of the Lactose Transport Protein of *Streptococcus thermophilus**

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The lactose transport protein (LacS) of *Streptococcus thermophilus* belongs to a family of transporters in which putative α-helices II and IV have been implicated in cation binding and the coupled transport of the substrate and the cation. Here, the analysis of site-directed mutants shows that a positive and negative charge at positions 64 and 71 in helix II are essential for transport, but not for lactose binding. The conservation of charge/side-chain properties is less critical for Glu-67 and Ile-70 in helix II, and Asp-133 and Lys-139 in helix IV, but these residues are important for the coupled transport of lactose together with a proton. The analysis of second-site suppressor mutants indicates an ion pair exists between helices II and IV, and thus a close approximation of these helices can be made. The second-site suppressor analysis also suggests ion pairing between helix II and the intracellular loops 6–7 and 10–11. Because the C-terminal region of the transmembrane domain, especially helix XI and loop 10–11, is important for substrate binding in this family of proteins, we propose that sugar and proton binding and translocation are performed by the joint action of these regions in the protein. Indeed, substrate protection of maleimide labeling of single cysteine mutants confirms that α-helices II and IV are directly interacting or at least conformationally involved in sugar binding and/or translocation. On the basis of new and published data, we reason that the helices II, IV, VII, X, and XI and the intracellular loops 6–7 and 10–11 are in close proximity and form the binding sites and/or the translocation pathway in the transporters of the galactosides-pentosides-hexuronides family.

The lactose transport protein, LacS, of *Streptococcus thermophilus* is a secondary transport system that belongs to the family of the galactosides-pentosides-hexuronides (GPH)[1] transporters (1). *In vivo*, LacS catalyzes a lactose/galactose exchange reaction, which is driven by the concentration gradients of both sugars across the membrane (2, 3). The LacS protein also catalyzes solute-H^+ symport, which is the proton motive force (Δp)-driven solute accumulation, but this transport mode is an order of magnitude slower than the exchange reaction (4). Kinetic analysis has shown that, not only in the solute-H^+ symport but also in the exchange mode of transport, protons participate in the translocation process (5).

When catalyzing exchange transport, sugar binding occurs in an alternating manner at the cytoplasmic and extracellular binding site. Specificity studies have revealed that the sugar binding site has a different architecture when exposed to the cytoplasmic or the extracellular face of the membrane (6). The intracellularly facing binding site has a high affinity for galactose through interactions with the C-2 and C-6 hydroxyl groups of the sugar; these hydroxyl groups do not participate in the binding of the sugar to the extracellularly facing binding site. Both binding sites and the translocation pathway are spacious in the C-1 to C-4 axes of the galactose moiety, because they are able to accommodate galactosides with large substitutions, especially the galactose C-1, *e.g.* trisaccharides or galactosides substituted with an aromatic ring are bound and transported.

In an effort to understand where the sites for substrate and cation binding are located in the members of the GPH family, several approaches, ranging from mutant isolation/selection to biophysical methods (*e.g.* 7, 8, 9, 10), have been used. The transmembrane or carrier domain of the proteins from the GPH family comprises 12 transmembrane-spanning α-helices. Comparison of the primary sequence of members of the GPH family has identified some general features. First, the putative α-helices II, IV, and IX have an amphipathic character suggesting interactions with both apolar/hydrophobic and polar/hydrophilic surfaces (1). The strongly hydrophilic character of one side of the transmembrane helices II and IV results from the presence of a number of conserved positively and negatively charged residues (see Fig. 1). These residues are thought to coordinate cation binding in the melibiose carrier (MelB) from *Escherichia coli* (1, 11, 12, 13). Second, the GPH family is characterized by a high sequence conservation in the loop between helices X and XI (see Fig. 1) (1). Electron spin resonance (ESR) studies have indicated that this region is not nearly as flexible as would be expected for such a large loop, and thus possibly it is located in the core of the protein (14). Moreover, a conserved residue Glu-379 in this loop is essential in coupling the transport of protons to the transport of sugar, because neutral substitution renders LacS unable to catalyze lactose-H^+ symport, whereas equilibrium transport is still catalyzed with wild type rates (5). Approximation of residue 373 in loop 10–11 within 15 Å from the C-1 atom of a galactose molecule in the binding site of LacS is apparent from solid-state nuclear magnetic resonance studies (14).

Obviously, proton and sugar transport by LacS are not separate events. Conformational coupling between sugar and pro-

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[1] The abbreviations used are: GPH, galactosides-pentosides-hexuronides; NEM, *N*-ethylmaleimide; Δp, proton motive force; PCR, polymerase chain reaction; TMG, methyl-β-D-thiogalactoside; DTT, dithiothreitol; MelB, melibiose carrier protein.

TABLE I
LacS mutants specified by the corresponding alleles in pSKE8hisC320A

Amino acid substitution(s)	Base pair substitution(s)
R64C	CGT → TGT
I65C	ATC → TGC
E67C	GAA → TGT
I70C	ATC → TGC
D71C	GAT → TGT
D133C	GAT → TGT
V134C	GTC → TGT
F135C	TTC → TGC
Y136C	TAC → TGC
K139C	AAA → TGT
D71C, R230A	GAT → TGT and CGT → GCT
D71C, R230D	GAT → TGT and CGT → GAT
D71C, L357F	GAT → TGT and CTT → TTT
D71C, R377H	GAT → TGT and CGT → CAT
D71C, R377C	GAT → TGT and CGT → TGT
D71C, R377D	GAT → TGT and CGT → GAT
D71C, D378N	GAT → TGT and GAT → AAT
D71C, D378C	GAT → TGT and GAT → TGT
R64C, D133N	CGT → TGT and GAT → AAT
R64C, D133C	CGT → TGT and GAT → TGT
R64C, D133R	CGT → TGT and GAT → CGT

ton binding/translocation will occur, and possibly the two ligands are transported through the same translocation pathway. By isolating and characterizing site-directed and second-site suppressor mutants, and by assessing site-directed modification of LacS in the presence and absence of sugar, we have obtained further information on the localization of regions and residues that are important for sugar and proton binding/translocation. An extended helix packing model is presented that brings together the catalytically important regions, that is, α-helices II and IV, loop region 10–11, and α-helix IX.

EXPERIMENTAL PROCEDURES

Materials

D-[glucose-1-^{14}C]lactose (2.11 teslabecquerels/mol) was obtained from the Radiochemical Center, Amersham Pharmacia Biotech. Restriction enzymes, Pwo DNA polymerase, Triton X-100, and streptavidin-alkaline phosphatase conjugate were from Roche Molecular Biochemicals. Bacteriological media were from Difco. Hydroxylamine, 3-(*N*-maleimidopropinyl)biocytin (biotin-maleimide), and *N*-ethylmaleimide (NEM) were purchased from Sigma. Ni-NTA resin was from Qiagen, Inc. All other materials were of reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids

E. coli strains HB101 (15) and DW2 (16) were grown anaerobically in Luria broth (LB) at 37 °C or on MacConkey plates supplemented with 0.5% lactose. When appropriate, the medium was supplemented with 50 μg/ml ampicillin and/or 1 mM isopropyl-1-thio-β-D-galactopyranoside. pSKE8His-derived plasmids, encoding single cysteine mutants of LacS with a C-terminal 6-histidine tag, are listed in Table I. pSKE8his(C320A) was constructed by exchange of the *Nco*I/*Pst*I fragment from pGKhis(C320A) (4) to pSKE8his (4). Site-directed mutagenesis was performed by PCR using mutagenic primers and pSKE8his(C320A) DNA as template. Mutants are indicated by a letter-number-letter code, in which the first letter corresponds to the amino acid that is substituted and the second one indicates the substitution; the number corresponds to the position of the residue in wild type LacS. Mutations were verified by DNA sequencing of the entire genes.

Hydroxylamine Mutagenesis and Selection of Second-site Suppressor Mutants

Plasmid DNA of pSKE8his(C320A, R64C) and pSKE8his(C320A, D71C), 2.75 μg, in 55 μl of water, was incubated for 45 min at 65 °C with 65 μl of a fresh solution of NH_2OH in 100 mM sodium phosphate, pH 7.0, 200 mM NaCl plus 4 mM EDTA. To stop the reaction, a 20-μl sample was diluted into 15 μl of ice-cold 2.35 M NH_4Ac, and the DNA was precipitated with ethanol and dissolved in 4 μl of water. 2.5 μl was used to transform *E. coli* HB101, and cells were plated on MacConkey agar with 1% lactose. Red colonies appeared after overnight incubation. Notice that HB101/pSKE8his(C320A, R64C) and HB101/pSKE8his(C320A, D71C) gave rise to white colonies due to an inactive LacS protein (see "Results"). DNA was isolated from 50 red colonies, and used to retransform the HB101 cells. About 70% of the clones had retained the red phenotype on lactose-MacConkey agar, and DNA was isolated and subjected to DNA sequencing from 18 of these.

Transport Assays

Transport of [^{14}C]lactose was assayed at 30 °C using the rapid filtration technique (17). The transport reactions were stopped at different time intervals by dilution into 2 ml of ice-cold 0.1 M LiCl, rapid filtering on 0.45-μm cellulose nitrate filters (Schleicher & Schuell), and washing with another 2 ml 0.1 M LiCl.

Downhill Uptake—Lactose transport down the concentration gradient was measured in *E. coli* HB101 cells that were grown overnight in LB with 1 mM isopropyl-1-thio-β-D-galactopyranoside for maximal expression of β-galactosidase. The cells were washed twice in KPM (50 mM potassium phosphate, pH 7.0, with 2 mM $MgSO_4$) and resuspended to 30–40 mg/ml. Aliquots of 6 μl were diluted into 200 μl of KPM with 10 mM D-lithium lactate, and after 2 min of aeration, 50 μM [^{14}C]lactose was added to initiate the reaction.

Proton Motive Force (Δp)-driven Uptake—Δp-driven accumulation of lactose was measured in *E. coli* DW2 cells that were grown overnight on LB and washed twice with KPM. Aliquots of 6 μl cells (30–40 mg/ml) were diluted into 200 μl of KPM with 10 mM D-lithium lactate, and after 2 min of aeration, the uptake was started by the addition of 50 μmM [^{14}C]lactose.

Exchange and Efflux Down the Concentration Gradient—E. coli DW2 cells, grown overnight on LB and washed twice with KPM, were preloaded with [^{14}C]lactose by overnight incubation with 2.75 mM [^{14}C]lactose in KPM. Cells were de-energized by incubation with 30 mM sodium azide and 50 μM SF6847 for 2 h at room temperature. The uptake was started by dilution of 2 μl of 50 mg/ml cell suspension into KPM or KPM with 100 μM lactose for efflux and exchange, respectively.

Substrate Protection of NEM Inactivation of Single Cys LacS Mutants

Overnight cultures of *E. coli* HB101 were washed three times and resuspended in KPM to 30–40 mg/ml. To aliquots of 100-μl cell suspensions, 10 μl of 100 mM methyl-β-D-thiogalactoside (TMG) or 10 μl of buffer (control) was added. After 10 min of equilibration at 37 °C, freshly prepared NEM was added to a final concentration of 3 mM. After 30 min of incubation at room temperature, the reaction was stopped by the addition of 12 mM dithiothreitol (DTT). The cells were washed four times with 15 volumes of KPM. Lactose uptake down the concentration gradient was measured as described above.

Substrate Protection of Biotin-maleimide Labeling of LacS Mutants Single Lys

The labeling with biotin-maleimide was performed as described in the previous paragraph, except that 950 μl of cell suspension, 400 μl of 250 mM lactose or buffer, and 45 μl of 100 mM biotin-maleimide in Me_2SO were used. The reaction was stopped by diluting the cells 40 times into KPM with 4 mM DTT. After washing with KPM, the cells were resuspended in 2 ml of KPM and disrupted by sonication. The cell debris was removed by centrifugation at 9000 × *g* for 10 min, after which the membranes were collected at 250,000 × *g* for 15 min. The membranes were solubilized in 0.5% Triton X-100, and LacS was purified using Ni^+-affinity chromatography as described previously, except that the column was washed with double the volume of wash buffer (4). The purified fractions were analyzed by immunodetection with antibodies raised against LacS and streptavidin-alkaline phosphatase conjugate.

Immunodetection of LacS

The amount of wild type and mutant LacS protein was estimated by immunodetection of LacS with antibodies raised against the IIA domain (18). Whole cell samples, prepared by boiling washed cell suspensions for 5 min in SDS-polyacrylamide gel electrophoresis sample buffer, or inside-out membrane vesicle samples (17) in SDS-polyacrylamide gel electrophoresis sample buffer were separated on a 12.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes by semi-dry electroblotting. Detection, using the Western-Light chemiluminescence detection kit with 3-(4-methoxyspiro{1,2-dioxetan-3.2′-

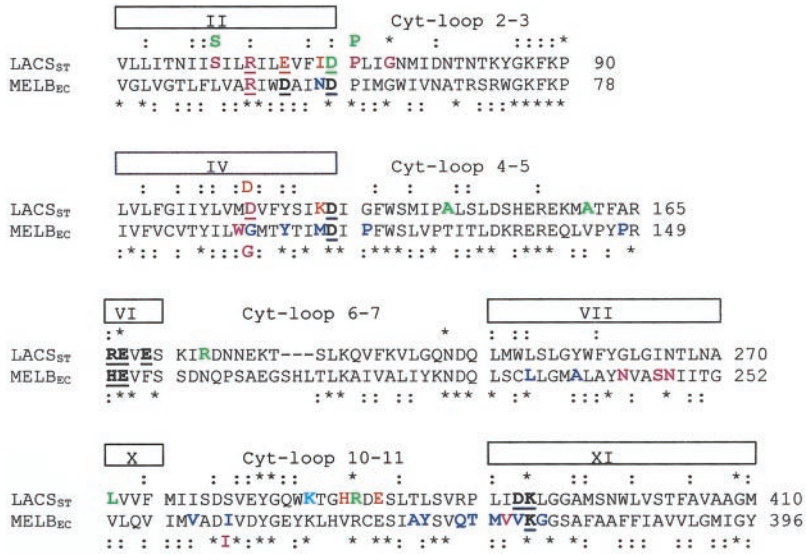

FIG. 1. **Sequence alignment of putative α-helices II, IV, VII, and XI and the loop regions 2–3, 4–5, 6–7, and 10–11 of LacS and MelB.** Charged residues within the putative α-helices are *underlined* and in *boldface* (33, 34); residues that were altered in LacS(R64C) (this study) or MelB(R52S) second-site suppressors (32) are in *pink*; residues that were altered in LacS(D71C) second-site suppressors are in *green*; residues important for sugar-H^+ symport in LacS are in *red* (this study; Refs. 5, 17); residues of which mutation resulted in an altered sugar or cation specificity in MelB are in *blue* (12, 25, 26); residue 373 of LacS, which is less than 15 Å from bound lactose, is in *light blue* (14). Similar (*colons*) or identical (*asterisks*) residues throughout the GPH family (1) or between MelB from *E. coli* and LacS from *S. thermophilus* are indicated in the *upper* and *lower lines*, respectively.

(5′chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate, disodium salt as substrate, was performed as described by Tropix, Inc.

RESULTS

Transport by Single Cysteine Mutants in Helices II and IV

The lactose transport protein (LacS) of *S. thermophilus* has several charged amino acids in the second and fourth transmembrane α-helix that are highly conserved within the GPH family (Fig. 1). To investigate the role of these charged amino acids in catalysis, each of these residues was replaced by cysteines or a charge-conserving amino acid, and the effect of the mutation on lactose transport was determined. Each of the mutants was made in the *lacS(C320A)* allele; the activity of LacS(C320A) was comparable to that of wild type LacS (Fig. 3*A*). Wild type LacS and the different mutants were expressed to similar levels as was determined by immunodetection of LacS in whole cell samples; typical examples are shown in Fig. 2. Because LacS(R64C) and LacS(D71C) were inactive, it was possible that these mutants were not assembled in the cytoplasmic membrane. Immunoblotting of inside-out membrane vesicles isolated from HB101 cells showed that LacS(R64C) and LacS(D71C) were present in the membrane at comparable levels as the wild type protein. Next, each of the mutants was characterized by assaying four modes of transport: [^{14}C]lactose uptake down the concentration gradient (downhill uptake), Δp-driven [^{14}C]lactose uptake, [^{14}C]lactose efflux down the concentration gradient, and [^{14}C]lactose/lactose exchange. Transport rates are presented as percentage of the transport rates catalyzed by wild type LacS, as expressed from pSKE8his (Fig. 3*A*).

After substitution of the residues Arg-64 and Asp-71 by cysteines, transport of lactose was no longer observed (Fig. 3*A*). The charge-conserving mutations R64K and D71E rendered the proteins partially active in all four modes of transport (not shown), from which we conclude that a positively charged residue at position 64 and a negatively charged one at position 71 are essential for transport activity. Cysteine substitution of the residues Glu-67 and Asp-133 abolished Δp-driven uptake completely but did not effect the exchange and downhill uptake. Strikingly, the E67C and D133C mutants showed a more than 4-fold increase in the rate of lactose efflux. The K139C mutant displayed transport properties similar to those of E67C and D133C, although the downhill uptake and exchange reactions were significantly reduced; the efflux and exchange transport of the parent allele and K139C are shown in somewhat more detail in Fig. 3*B*. Cysteine substitution of Ile-70 did not affect exchange and efflux and uptake down the concentration gradient, but largely impaired Δp-driven uptake. Contrary to the substitutions of the heretofore described highly conserved residues, cysteine substitution of residues on the hydrophobic faces of helices II and IV, *e.g.* Ile-65, Val-134, and Phe-135 did not have an effect on any of the transport modes. Altogether, we conclude that the residues Arg-64 and Asp-71 in helix II are essential for transport, whereas Glu-67, Ile-70, Asp-133, and Lys-139 are needed for the coupled transport of a sugar molecule together with a proton.

FIG. 2. **Expression levels of LacS in** *E. coli* HB101. The expression levels of various mutants was analyzed by immunoblotting of total cell lysates. *Lane 1*, LacS(D71C, R377H); *lane 2*, LacS(D71C, R377D); *lane 3*, LacS(D71C, R377C); *lane 4*, LacS(R64C, D133C); *lane 5*, LacS(K139C); *lane 6*, LacS(D71C, R230C); *lane 7*, LacS(D71C, R230D); *lane 8*, LacS(C320A); *lane 9*, LacS(D378C); *lane 10*, LacS(R64C); *lane 11*, LacS(D71C, L357F); *lane 12*, LacS(D71C, D378D); *lane 13*, LacS(D71C); *lane 14*, LacSwt.

Second-site Suppressor Mutants

Second-site suppressor mutations can yield information about close approximation of residues within the protein, because the defect of the primary mutation can be restored by substitution of one or more neighboring residues (19, 20). This genetic technique can be particularly powerful when functional ion pairs within the protein are involved (21–23). Two mutants in helix II, *i.e.* LacShis(R64C) and LacShis(D71C), were used for selection of second-site suppressors, because these displayed a white phenotype on lactose-MacConkey plates. Consistent with the transport data (Fig. 3*A*), the wild type and all other mutants had a red (or pink, K139C) colony phenotype. Transport positive colonies turn red on the indicator plates as a result of acidification of the medium after lactose fermentation. Hydroxylamine-mutagenized plasmid DNA (pSKE8his(C320A, R64C) or pSKEhis(C320A, D71C)) was transformed to HB101, and cells were plated on lactose-MacConkey agar. DNA was isolated from 50 red colonies and used to retransform HB101 cells. About 70% of the clones had retained the red phenotype and from 18 highly red colonies, 8 suppressors from pSKE8his(R64C) and 10 from pSKE8his(D71C), DNA was isolated and the entire *lacS* genes were sequenced. The second-site suppressors had on average two mutations of which some were silent (Table II). All mutations are C → T or G → A

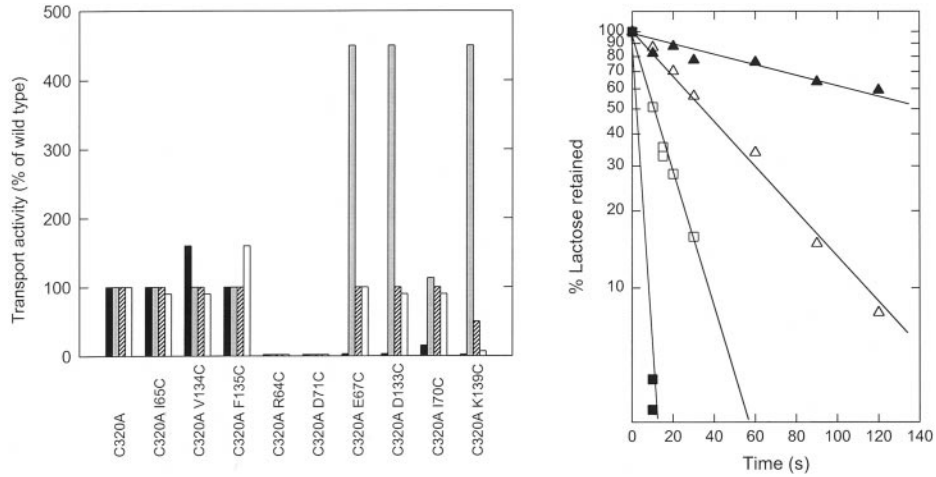

FIG. 3. **Transport activities of cysteine-less and single cysteine mutants of LacS.** *A*, the initial rates of transport are shown as a percentage of the initial transport rate catalyzed by wild type LacS; 100% corresponds to 4, 2.5, 50, and 7.5 nmol/min × mg of protein for downhill uptake, efflux down the concentration gradient, exchange, and Δp-driven uptake, respectively. Plasmid pSKE8his with the indicated mutations was used to transform *E. coli* HB101 or DW2. Downhill [^{14}C]lactose uptake (*white bars*) was measured in *E. coli* HB101 cells in which the intracellular concentration of lactose remains low due to the expression of β-galactosidase. Efflux down the concentration gradient (*gray bars*), exchange (*striped bars*), and Δp-driven uptake (*black bars*) were measured in *E. coli* DW2, which has a chromosomal deletion in the *lacZ* gene allowing lactose to accumulate inside the cell. Downhill and Δp-driven uptake of [^{14}C]lactose were assayed for different time intervals in pre-energized cells; the reaction was started by the addition of 50 μM [^{14}C]lactose. Efflux and exchange were measured in de-energized cells that were preloaded with 2.75 mM [^{14}C]lactose. The exit of [^{14}C]lactose was assayed in the absence (efflux) and presence (exchange) of 100 μM lactose in the external buffer. *B*, exit of [^{14}C]lactose from *E. coli* cells expressing LacS(C320A) (*closed symbols*) and LacS(K139C) (*open symbols*) under conditions of [^{14}C]lactose/lactose exchange (*squares*) and efflux of [^{14}C]lactose down the concentration gradient (*triangles*).

substitutions as is expected from the mechanism of hydroxylamine mutagenesis (24).

Second-site Suppressors of LacS(R64C)—Mutations suppressing the defect in R64C were found in the portion of the gene that corresponds to the N-terminal region of the protein, *i.e.* α-helix II (S61F, P72L), cytoplasmic loop 2–3 (G75S), and α-helix IV (D133N). Although clearly red on lactose-MacConkey agar, downhill lactose uptake catalyzed by these suppressors was less than 15% of the wild type activity, indicating a poor K_m and/or V_{max} (Fig. 4; data not shown). Neither of the suppressor mutations restored the Δp-driven lactose accumulation capacity of the wild type (not shown). Regained activity in LacS(R64C, D133N) suggests that in the wild type LacS the opposite charges at positions 64 and 133 are stabilized by the formation of an ion pair, indicating a close approximation of helices II and IV. Consistent with this suggestion is the observation that the residues 133 and 64 are located at approximately the same height in the membrane. An unpaired charge on Asp-133 would thus inactivate the carrier protein, and neutralization of this residue restores activity. Along similar lines of reasoning one could explain the S61F suppressor mutation. The aromatic side chain of Phe-61, which is located one helix turn above Arg-64, might lower the polarity of the environment around the carboxylate Asp-133, and thereby increase its pK_A. The unpaired charge of the carboxylate of Asp-133 in LacS(R64C, S61F) would thus be neutralized at the prevailing pH.

To obtain further evidence for the hypothesis of the ion pair between Arg-64 and Asp-133, the double mutant LacS(R64C, D133C) was constructed. As anticipated, the transport activity and phenotype on lactose-MacConkey agar was similar to that of LacS(R64C, D133N) (Fig. 4). When the residues 64 and 133 are in close proximity, replacement of Asp-133 for an arginine might well be tolerated in the R64C background. This is indeed the case, LacS(R64C, D133R) shows 40% downhill transport activity compared with wild type LacS (Fig. 4). The P72L and G75S suppressor mutations are less easily explained, but might relate to a different position of helix II in the membrane, *e.g.* a position that places the unpaired charge at Asp-133 in a less unfavorable environment.

TABLE II
Second-site suppressors of LacS D71C and LacS R64C

Mutations[a]	Location of suppressor mutation	Activity[b]
		%
R64C suppressor mutations		
S61F (4*)	**Helix II**	15
S61F P72L	**Helix II**, end of helix II	15
D133N	**Helix IV**	15
G75S (2*)	Loop 2–3	15
D71C suppressor mutations		
P72S	End of helix II	15
S61F, P72L, T81I	Helix II, end of helix II, loop 2–3	15
G94S, S302L	Helix III, helix VIII	40
A161T	Loop 4–5	30
A149V	Loop 4–5	40
R230C	**Loop 6–7**	60
R230C, G546K, G572S	**Loop 6–7** en IIA domain	60
F261L, **L357F**, T411I	Helix VII, **helix X**, loop 11–12	30
L357F, T411I	**Helix X**, loop 10–11	30
R377H, D378N	**Loop 10–11**	60

[a] The mutations indicated in *bold* are responsible for the gain of transport function as was shown after construction of single-site suppressors by site-directed mutagenesis (Fig. 4).

[b] The downhill transport activity, presented as a percentage of the transport activity of wild type LacS, was measured as described in the legend to Fig. 3. The error in the measurements is ±5%.

Second-site Suppressors of LacS (D71C)—In contrast to the mutations suppressing the R64C substitution, those suppressing D71C were found in different regions of the LacS molecule. Most active was the triple mutant LacS(D71C, R377H, D378N) with two mutations in inter-helix loop 10–11. This mutant catalyzed downhill lactose uptake at more than 60% the rate catalyzed by the wild type protein. It did not catalyze significant Δp-driven uptake as was the case for the other D71C suppressors. To dissect which of the mutations, R377H or D378N, suppressed the defect of D71C, the corresponding "single" mutants LacS(D71C, R377H), LacS(D71C, D378N), LacS(D71C, R377C), LacS(D71C, R377D), and LacS(D71C, D378C) were constructed by site-directed mutagenesis. The

transport data clearly indicate that the Arg-377 substitution is responsible for the gain of function (Fig. 4).

The second most active D71C suppressors (60% of the wild type) were LacS(D71C, R230C) and LacS(D71C, R230C, G546K, G572S). The R230C substitution is responsible for the restored activity, because the G546K and G572S mutations in the regulatory (IIA) domain of LacS did not alter uptake rate. Site-directed substitution of Arg-230 for an alanine or an aspartate also restored activity in the D71C mutant. The second-site suppressors LacS(L357F, T411I) and LacS(F261L, L357F, T411I) catalyzed downhill uptake at a rate that is 30% of the wild type. They have the mutation L357F in common, which is located in helix X. Because the double mutant LacS(D71C, L357F) constructed by site-directed mutagenesis showed a similar transport activity, we conclude that the Leu to Phe substitution at position 357 restored the transport activity (Fig. 4).

The second-site mutations P72S, A161T, A149V, and (S61F, P72L, T81I) are all located in the intracellular half of helix II or the intracellular loops 2–3 and 4–5. This is in accordance with a close approximation of helices II and IV, which was already concluded from the R64C suppressors. Strikingly, some of the mutations suppressing the D71C mutation are the same or similar to the ones suppressing the R64C mutation, *e.g.* the suppressor mutations S61F and/or P72L are found in the following combinations (R64C, S61F, P72L), (D71C, S61F, P72L, T81I), and (D71C, P72S).

The suppressor analysis together with the measurements of the constructed site-directed mutants reveals that all relevant pairs of mutations, except one, are located in the intracellular halves of helices II, IV, and X or the intracellular loops 2–3, 4–5, 6–7, and 10–11. The strongest indications for close approximation are found for helices II and IV (R64C, D133N), helix II and loop 6–7 (D71C, R230C), helix II and loop 10–11 (D71C, R377H), because these involve pairs of amino acids of opposite charge.

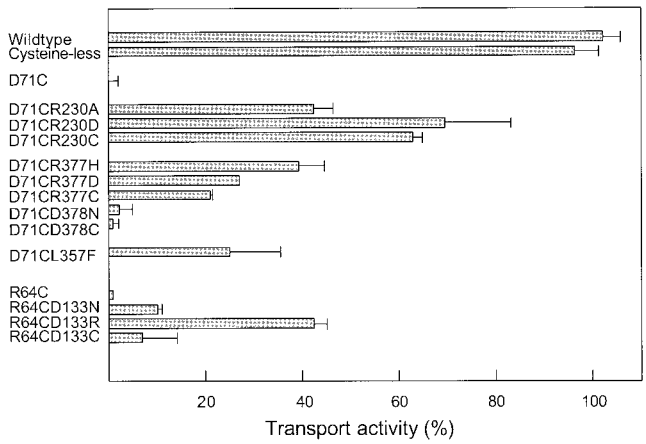

FIG. 4. **Downhill lactose uptake of site-directed double mutants of LacS.** Indicated is the initial rate of [^{14}C]lactose uptake as a percentage of the wild type activity as measured in *E. coli* HB101 cells (100% is 4 nmol/min × mg of protein). Uptakes were performed as described in the legend to Fig. 3.

Substrate Protection of Maleimide Labeling of Single Cysteine Mutants in Helices II and IV

The N-terminal region of LacS has been proposed to be part of the actual cation binding site on the basis of conservation of charged residues, amphipathicity of helices II and IV, mutant analysis, and analysis of MelB fusions (1). The mutational analysis described here confirms that this region is indeed important for proton binding and/or coupling. This region, however, has never been directly associated with sugar binding and/or translocation. The proximity relations found for helices II, IV, X, and loop 10–11 suggest that helices II and IV are also involved in sugar binding and/or translocation.

Substrate protection of chemical modification is a means of showing that a specific region in the protein is directly involved in binding or at least conformationally coupled to the binding of substrate. Upon alkylation of the cysteines at position 67 in helix II and 133 in helix IV with NEM, downhill uptake is inhibited (Fig. 5, *B* and *C*). The presence of a saturating concentration of nonmetabolizable substrate, thiomethylgalactose (used at a concentration of at least 10 times the apparent K_m of the low affinity site) (6), protects Cys-67 and Cys-133 from labeling by NEM (Fig. 5, *B* and *C*). The control experiment shows that downhill uptake of LacShis(C320A) is not affected by the incubation with NEM or TMG (Fig. 5*A*).

For the interpretation of the second-site suppressor mutants, it is important to establish the nature of the transport-negative phenotype of the R64C and D71C mutants. Because a direct binding assay is not available (K_D of lactose binding is in the millimolar range (14)), biotin-maleimide modification of the cysteines in these mutants was determined in the presence and absence of a saturating concentration of lactose (at about 10 times the apparent K_m of the low affinity site). After labeling, LacS was purified by Ni^+-affinity chromatography, and the

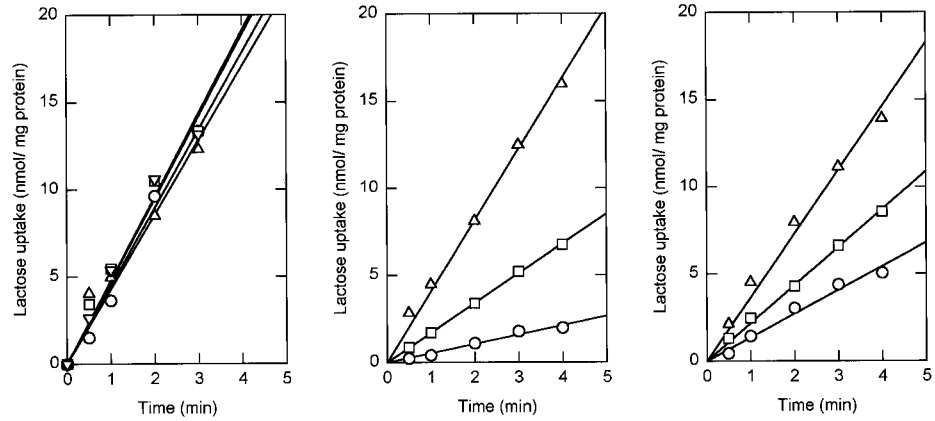

FIG. 5. **Substrate protection of NEM inactivation of Cys-67 in helix II, and Cys-133 in helix IV.** Overnight cultures of *E. coli* HB101 cells expressing LacS(C320A) (*A*), LacS(E67C) (*B*), and LacS(D133C) (*C*) were washed three times in KPM and resuspended in KPM (△ and ○) or KPM with 10 mM TMG (▽ and □). After 10 min of equilibration at 37 °C, 3 mM NEM (○ and □) or buffer (△ and ▽) was added. After 30 min of incubation at room temperature, 12 mM DTT was added and the cells were immediately washed four times in 15 volumes of KPM. [^{14}C]Lactose uptake down the concentration gradient was measured in *E. coli* HB101 as described in the legend to Fig. 3. ○, activity after NEM labeling in the absence of TMG; □, activity after NEM labeling in the presence of TMG; ▽, activity after pre-incubation with TMG; △, control.

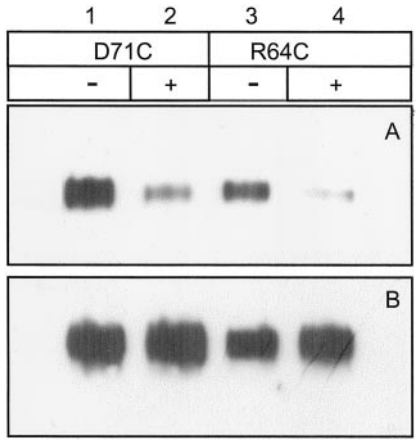

FIG. 6. **Substrate protection of biotin-maleimide labeling of LacS(R64C) and LacS(D71C).** *E. coli* HB101 cells expressing LacS(D71C) (*lanes 1* and *2*) or LacS(R64C) (*lanes 3* and *4*) were labeled with biotin-maleimide in the absence (*lanes 1* and *3*) or presence (*lanes 2* and *4*) of 70 mM lactose. LacS was purified and analyzed by immunoblotting with anti-LacS antibody to detect the total amount of LacS (*B*), and a streptavidin-alkaline phosphatase conjugate was used to detect the fraction of biotinylated LacS (*A*).

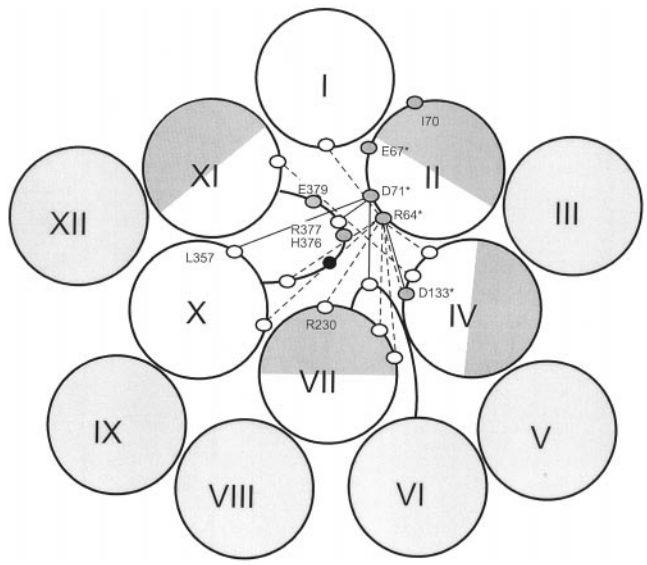

FIG. 7. **Helix packing model of the carrier domain of the lactose transport protein of** *S. thermophilus. Continuous lines* connect residues that were identified by second-site suppressor analysis in LacS, *i.e.* R64C-D133N (between helices II and IV), D71C-R230C (between helix II and loop 6–7), D71C-R377H (between helix II and loop 10–11), and D71C-L357F (between helices II and X) (this study). *Dashed lines* connect residues that were identified by second-site suppressor analysis in MelB, *i.e.* R52S-D19G (between helices II and I), R52S-W116R and R52S-G117D (between helices II and IV), R52S-S247R, R52S-N244S, and R52S-N248K (between helices II and VII), R52S-T338R (between helices II and X), R52S-I352V (between helix II and loop 10–11), and D124S-V375A (between helices IV and XI) (30–32). The *black circle* in loop 10–11 indicates the position of residue 373, which is located within 15 Å from bound galactose (14). *Stars* indicate residues where substrate binding and/or translocation can protect for site-directed cysteine modification in LacS (this study). Residues that are involved in coupling sugar and proton translocation in LacS (R64C, E67C, I70C, D71C, D133C, H376Q, and E379Q/A/D) are indicated in *gray* (this study; Refs. 5, 17). The hydrophobic faces of amphipathic helices II, IV, and XI (1) and the face of helix VII that harbors residues important for the specificity of proton and sugar binding in MelB are *shaded* (32).

amount of biotinylated LacS was determined with streptavidin-alkaline phosphatase. The substrate protection of biotin-maleimide labeling of LacS(R64C) and LacS(D71C) demonstrates that lactose is still bound by the mutants (Fig. 6). Equivalent concentrations of glucose, which is not a substrate of LacS, did not inhibit labeling of the cysteine residues with biotin-maleimide (not shown). Because there are at least two binding conformations in LacS, one facing the extracellular side and one facing the cytoplasm, we cannot exclude the possibility that only one of the binding conformers is intact, and that the other one is restored in the suppressor mutants. It is, however, more likely that the suppressor mutations relieve a defect in the translocation step, rather than in the binding of lactose. Finally, the substrate protection of the labeling of the cysteines at positions 64, 67, 71 (helix II), and 133 (helix IV) indicated that the hydrophilic faces of these transmembrane segments participate in ligand binding.

DISCUSSION

The aim of this paper was to localize the regions and residues in the lactose transport protein of *S. thermophilus* that are important for sugar and proton binding and translocation. From the analysis of the site-directed mutants and second-site suppressors, and from the assessment of substrate protection of site-directed modification, we conclude that the helices I, II, IV, VII, X, and XI and the intracellular loops 6–7 and 10–11 are involved in proton and sugar transport and propose a model for the helix packing in the LacS protein.

Mutagenesis of the (conserved) charged residues in helices II and IV showed that a basic residue at position 64 and an acidic one at 71 are essential for transport. Importantly, the capacity to bind is retained in LacS(R64C) and LacS(D71C) as was shown from labeling studies with biotin-maleimide in the presence and absence of lactose. The fact that none of the D71C or R64C suppressors has regained the capacity to catalyze Δp-driven uptake indicates that the energy-coupling mechanism is much more sensitive to (small) structural changes in the protein than sugar binding and translocation. Consistent with this notion are the observations that residues Glu-67 and Ile-70 in helix II and Asp-133 in helix IV are important for the coupled transport of the sugar together with a proton (Δp-driven uptake), but not essential for translocation *per se*. Mutants carrying a single cysteine substitution at these positions catalyze downhill uptake and exchange at wild type rates. Very similar observations have been made for a number of substitutions in the inter-helix loop 10–11 (5, 17). Furthermore, in MelB aspartate 55 and asparagine 58, equivalent to positions 67 and 70 in LacS, are required for coupling of TMG transport to sodium as a cation. Binding of α- and β-galactosides, on the other hand, still occurs but now independent of sodium ions (25, 26).

In the mutants E67C, D133C, and K139C, and to a lesser extent I70C, the uncoupled phenotype coincides with an increase in the efflux rate. How can this gain of efflux activity be explained? In a transport protein catalyzing the coupled transport of a solute (S) together with a proton (H), the fully loaded (ESH) and the empty carrier (E) reorient their binding sites upon translocation of a solute plus proton from out to in (uptake) or in to out (efflux). The coupling efficiency decreases when also the binary states of the carrier, ES or EH, are able to reorient their binding sites (for a full account, see Ref. 27). These so-called ES and EH leaks frequently occur (or become manifest) when one or more critical residues are substituted. The rate-determining step in efflux down the concentration gradient by the LacS protein is the reorientation of the empty carrier ($E^{out} \rightarrow E^{in}$). The increased efflux rate together with wild type exchange and facilitated influx rates can be explained when the reorientation $EH^{out} \rightarrow EH^{in}$ has become faster than the reorientation of the empty carrier in the wild type (EH leak). In principle, the increased efflux rate can also be explained by an ES leak type, but then one not only needs to invoke an ES leak

pathway but also must assume that the reorientation of the empty carrier from out to in is faster than in the wild type ($E^{out}_{mutant} \rightarrow E^{in}_{mutant}$ faster than $E^{out}_{wt} \rightarrow E^{in}_{wt}$).

Substrate protection of inactivation by alkylation of Cys-64, Cys-67, Cys-71 (helix II), and Cys-133 (helix IV) shows that these regions are not only involved in proton coupling but also conformationally active upon sugar binding or even directly interacting with bound sugar. The observation that the helices II and IV are involved in both cation and sugar binding can be explained by conformational coupling of sugar and proton binding or even structural overlap between the binding sites for sugar and proton. Similarly, the loop between helices 10 and 11 has been implicated in sugar binding (14, 28) as well as proton binding (5, 17). The observation that the majority of the MelB mutants isolated on the basis of TMG resistance, Li^+ resistance, or Li^+ dependence, but also site-directed mutants in helices I, II, and IV, exhibit simultaneous alterations in cation and substrate recognition (12, 25, 26) may be interpreted as an indication for structural overlap of the proton and sugar binding sites. Evidence for conformational coupling in MelB comes from (i) proteolytic studies showing that cleavage in inter-helix loop 4–5 is dependent on both cation and sugar binding (29) and (ii) fluorescence studies showing that the polarity of the environment of the fluorescent substrate changes in a sodium-dependent fashion (9).

All second-site suppressor mutants that cure the transport-negative phenotype of LacS(R64C) and LacS(D71C), except one, are located in the intracellular halves of helices II, IV and X or the intracellular loops 2–3, 4–5 and 6–7 and 10–11. The strongest indications for neighboring residues are found in helices II and IV (R64C, D133N), helix II and loop 6–7 (D71C, R230C), helix II and loop 10–11 (D71C, R377H), because these involve pairs of amino acids of opposite charge. On the basis of the second-site suppressor analysis and published data, we propose a model for the packing of the transmembrane-spanning helices of LacS in the membrane (Fig. 7). In this model the helices I, II, IV, VII, X, and XI are in close proximity and form at least part of the binding site and translocation pathway of the transport protein. Because there are no data, neither for LacS nor any other member of the GPH family, that assumes a role in catalysis for helices III, V, VI, VIII, IX, and XII, they have been designated as a second ring outside the core of the protein.

The helices II and IV are located next to each other and form part of the translocation pathway for several reasons: (i) these helices have a highly conserved amphipathic and substitution pattern within the GPH family (1); (ii) several conserved charged residues located in helices II and IV are important for coupled transport and/or cation binding in LacS (this study); (iii) the helices II and IV either interact directly with the sugar-substrate, or at least their conformation changes upon sugar binding (this study); (iv) second-site suppressor analysis has indicated that Arg-64 in helix II and Asp-133 in helix IV possibly form an ion pair (this study).

The loop between helices X and XI has been given a location in the core of the protein, because (i) solid-state nuclear magnetic resonance data have indicated that the residue Lys-373 in this loop is located less than 15 Å from the C-1 of bound galactose (14), (ii) a possible ion pair between Arg-377 with Asp-71 in helix II was found in the second-site suppressor analysis (this study), and (iii) ESR experiments have shown that the loop has a flexibility that is more in accordance with a location in the core of the protein than with a cytoplasmic location (14).

The intracellular loop 6–7 was placed in the proximity of helices II and IV, because an arginine to cysteine mutation in this loop can restore activity of the D71C mutant. Putative α-helices X and XI and the loop between X and XI are proposed to be part of the translocation pathway on the basis of the second-site suppressor found between helices II and X (this study) and because helix XI has a conserved amphipathic character (1).

The model is in accordance with, and accommodates, all published data obtained for other members of the GPH family. The most relevant published data are summarized here. Second-site suppressor analysis in MelB (30–32) suggests interactions between helix II and helices IV, VII, and X, and inter-helix loop 10–11 (Fig. 7, *dotted lines*). Additional suppressor pairs were found between helices IV and XI, and helices II and I. Moreover, the mutations that alter the sugar and/or cation recognition of MelB, as isolated by selection for TMG resistance or Li^+ resistance, all lie in the regions depicted here as the core of the carrier protein (25, 26). In some cases the same residues are found as second-site suppressors and as mutations changing the sugar-specificity, *e.g.* I352V in loop 10–11 of MelB. The specificity mutations found in helix VII of MelB reside on the same face of the helix as the suppressor mutations (32). Evidence for relatively close distance of the N- and C-terminal regions also comes from fluorescence resonance energy transfer experiments, where the tryptophans in inter-helix loop 2–3 and helix IX of MelB were estimated to be 20 and 14 Å away from the bound substrate, respectively (9, 10).

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