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

Received for publication, February 17, 2000, and in revised form May 10, 2000

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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* (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-
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-1-14C]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-maleimidopropyl)biotin (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 100 μg/ml ampicillin and/or 1 mM isopropyl-1-thio-D-galactopyranoside (IPTG) for expression of *lacZ* or 4 mM IPTG for expression of *lacZ* regulated by the P75 promoter. Overnight cultures of *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-C(320A, 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 [14C]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 of 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 MgSO4) and resuspended to 30–40 mg/ml. Aliquots of 6 μl were diluted into 200 μl of KPM with 10 mM β-lactam, and after 10 min of aeration, the uptake was started by the addition of 50 μM [14C]lactose.

**Proton Motive Force (ΔpH)-driven Uptake**—3p-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 β-lactam, and after 10 min of aeration, the uptake was started by the addition of 50 μM [14C]lactose.

**Exchange and Efflux Down the Concentration Gradient—E. coli DW2 cells, grown overnight on LB and washed twice with KPM, were preloaded with [14C]lactose by overnight incubation with 2.75 μM [14C]lactose in KPM. Cells were de-energized by incubation with 30 mM sodium azide and 10 μM SF6847 for 2 h at room temperature. The uptake was started by dilution of 2 μl of 50 μM 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-n-thiogalactoside (MTG) 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 850 μl of cell suspension, 400 μl of 250 mM lactose or buffer, and 45 μl of 100 mM biotin-maleimide in Me2SO 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 Ni2⁺-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-methoxyxynpropyl)1,2-dioxetan-3,2-
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-helices 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. 3A). 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), \(\Delta p\)-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. 3A).

After substitution of the residues Arg-64 and Asp-71 by cysteines, transport of lactose was no longer observed (Fig. 3A). The charge-conserving mutations R64K and D71E rendered transport of lactose no longer observed (Fig. 3A).

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. 3A), 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. Because the mutants Arg-64 and Asp-71 by cysteines, transport of lactose was no longer observed (Fig. 3A). The charge-conserving mutations R64K and D71E rendered transport of lactose no longer observed (Fig. 3A).

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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 \( V_{\text{max}} \) (Fig. 4; data not shown). Neither of the suppressor mutations restored the \( \Delta p \)-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 inactive 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, 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 inactive 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 \( pK_a \).

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, D133L) 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.

| Mutations | Location of suppressor mutation | Activity |
|-----------|--------------------------------|---------|
| S61F (4*) | Helix II | 15 |
| S61F P72L | Helix II, end of helix II | 15 |
| D133N | Helix IV | 15 |
| G75S (2*) | Loop 2–3 | 15 |

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 \( \Delta p \)-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

![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 \( \Delta p \)-driven uptake, respectively. Plasmid pSKE8his with the indicated mutations was used to transform E. coli HB101 or DW2. Downhill \([14C]lactose\) uptake (white bars) was measured in E. coli HB101 cells in which the intracellular concentration of lactose remains low due to the expression of \( \beta \)-galactosidase. Efflux down the concentration gradient (gray bars), exchange (striped bars), and \( \Delta p \)-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 \( \Delta p \)-driven uptake of \([14C]lactose\) were assayed for different time intervals in pre-energized cells; the reaction was started by the addition of 50 \( \mu \)M \([14C]lactose\). Efflux and exchange were measured in de-energized cells that were preloaded with 2.75 mM \([14C]lactose\). The exit of \([14C]lactose\) from E. coli cells expressing LacS(C320A) (closed symbols) and LacS(K138C) (open symbols) under conditions of \([14C]lactose\)/lactose exchange (squares) and efflux of \([14C]lactose\) down the concentration gradient (triangles).]

![Table II. Second-site suppressors of LacS D71C and LacS R64C](https://example.com/table2.png)

| Mutations | Location of suppressor mutation | Activity |
|-----------|--------------------------------|---------|
| R64C suppressor mutations | | |
| S61F | 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 |
| R303C | Loop 6–7 | 60 |
| R303C, 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 |

* 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).

* 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%.

![Helix Packing in the Lactose Transport Protein](https://example.com/helix_packing.png)
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.

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 Km of the low affinity site) (6), protects Cys-67 and Cys-133 from alkylation. The control experiment shows that downhill uptake of LacS[C320A] is not affected by the incubation with NEM or TMG (Fig. 5A).

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 (Kd 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 Kd of the low affinity site). After labeling, LacS was purified by Ni2+-affinity chromatography, and the

![Fig. 4. Downhill lactose uptake of site-directed double mutants of LacS.](image-url)

![Fig. 5. Substrate protection of NEM inactivation of Cys-67 in helix II, and Cys-133 in helix IV.](image-url)
amount of biotinylated LacS was determined with streptavidin-
aline phosphatase. The substrate protection of biotin-
aleimide labeling of LacS(R64C) and LacS(D71C) de-
strates 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 pos-
sibility 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 in LacS, 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 pres-
ence 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 pro-
tein 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 up-
take), but not essential for translocation per se. Mutants car-
rying a single cysteine substitution at these positions catalyze
dowhill 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 aspar-
ate 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 in-
crease in the efflux rate. How can this gain of efflux activity be
explained? In a transport protein catalyzing the coupled trans-
port 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 (up-
take) 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 (Eout → Ein). The increased efflux rate together with
wild type exchange and facilitated influx rates can be explained
when the reorientation EHout → EHin 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

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**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 immu-
noblotting 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 lac-
tose 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 (be-
tween helix II and loop 6–7), D71C-R577H (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 sup-
pressor analysis in MelB, i.e. R528-D19Q (between helices II and I),
R528-W116R and R528-G117D (between helices II and IV), R528S-
247R, R528-N244S, and R528-N248K (between helices II and VII),
R528-T338R (between helices II and X), R22S-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
grey (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).
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, 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. 1352V 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).

Acknowledgments—We thank Dirk Jan Slotboom for his advice on the labeling studies and Erik Hamminga for DNA sequencing.

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