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Roles of Charged Residues in the Conserved Motif, G-X-X-X-D/E-R/K-X-G-[X]-R/K-R/K, of the Lactose Permease of *Escherichia coli*

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Abstract. The lactose permease is a polytopic membrane protein that has a duplicated conserved motif, GXXX(D/E)(R/K)XG[X](R/K)(R/K), located in cytoplasmic loops 2/3 and 8/9. In the current study, the roles of the basic residues and the acidic residue were investigated in greater detail. Neutral substitutions of two positive charges in loop 2/3 were tolerated, while a triple mutant resulted in a complete loss of expression. Neutral substitutions of a basic residue in loop 8/9 (i.e., K289I) also diminished protein stability. By comparison, neutral substitutions affecting the negative charge in loop 2/3 had normal levels of expression, but were defective in transport. A double mutant (D68T/N284D), in which the aspartate of loop 2/3 was moved to loop 8/9, did not have appreciable activity, indicating that the negative charge in the conserved motif could not be placed in loop 8/9 to recover lactose transport activity. An analysis of site-directed mutants in loop 7/8 and loop 8/9 indicated that an alteration in the charge distribution across transmembrane segment 8 was not sufficient to alleviate a defect caused by the loss of a negative charge in loop 2/3. To further explore this phenomenon, the double mutant, D68T/N284D, was used as a parental strain to isolate suppressor mutations which restored function. One mutant was obtained in which an acidic residue in loop 11/12 was changed to a basic residue (i.e., Glu-374 → Lys). Overall, the results of this study suggest that the basic residues in the conserved motif play a role in protein insertion and/or stability, and that the negative charge plays a role in conformational changes.

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Introduction

The lactose permease, located in the *Escherichia coli* inner membrane, is a model protein for the study of sugar transport processes. The protein cotransports a lactose molecule and a proton into the bacterial cytoplasm with a stoichiometry of 1:1 [34, 35]. The gene for the lactose permease has been cloned and sequenced, and it encodes a polypeptide of 417 amino acids with a predicted molecular weight of 46,504 daltons [2, 30]. Studies using purified lactose permease indicate that it functions as a monomer [16].

The lactose permease is a member of the Major Facilitator Superfamily (MFS) (*see reference* 23 for a recent review). Most members of the MFS are predicted to contain twelve membrane-spanning segments by hydropathicity analysis [6, 8]. In the case of the lactose permease, this model has been confirmed by gene fusions with alkaline phosphatase and β-galactosidase [3, 4]. The N- and C-terminal segments are cytoplasmic as shown by antibody binding studies [5, 12, 29]. The general homology of the two halves of the proteins are evidence for an early evolutionary gene duplication which led to the current superfamily of proteins [20]. Analysis of the MFS for hydrophobicity, amphipathicity, loop length, and potential salt-bridges between helices of the lactose permease has led to a proposed tertiary structure model [8]. This model shows the two halves as distinct domains which may move relative to one another to transport lactose [8, 14].

Although the level of homology varies among members of the MFS, a consistent feature is the presence of a duplicated motif, GXXX(D/E)(R/K)XG[X](R/K)(R/K),

in cytoplasmic loops 2/3 and 8/9 [9, 10, 11]. The positive charges, particularly the last two, are usually conserved within members of the superfamily, and an aspartate or glutamate is highly conserved in at least one of the two loops. The conserved motif of the lactose permease and the tetracycline antiporter have been investigated by site-directed mutagenesis of conserved residues [13, 17, 25, 36, 37]. Loop 2/3 of the lactose permease and the tetracycline antiporter both contain an aspartate residue that is critical for transport. Instead of an aspartate, loop 8/9 of the lactose permease has an asparagine at the fifth position. In the tetracycline antiporter, the aspartate is also missing from loop 8/9, but a glutamate residue is present in the ninth position. Even though the residue is functionally equivalent, this glutamate is not critical for tetracycline efflux [38].

Other studies also suggest that loop 2/3 and loop 8/9 perform analogous roles in the lactose permease. Single site-directed mutations in loop 2/3 and loop 8/9, which greatly inhibited the V_{max} values for lactose uptake, were used as parental strains for the isolation of spontaneous suppressor mutations that restored transport velocity [14, 15, 24]. Both loop 2/3 and loop 8/9 suppressor mutations colocalized in three regions of the permease (*see* reference 24, for a discussion), and a kinetic analysis showed that the primary effect of the suppressor mutations was to partially or completely restore the maximal velocity for lactose transport. The locations and kinetic effects of the suppressor mutations are consistent with a rotationally symmetrical structure for the lactose permease in which the loop 2/3 and loop 8/9 motif plays a role in maintaining the topology at the interface between the two halves of the protein in order to facilitate conformational changes [24].

The goal of the current study is to gain insight as to how the charged residues within the conserved motifs affect the structure of the lactose permease in a way that facilitates conformational changes associated with lactose transport. The beginning of the motif may promote a turn; the rest of the motif is highly charged, containing one negative and three positive charges. Within members of the MFS, the first position is predicted to be near the cytoplasmic edge of transmembrane segment-2 (TMS-2) and TMS-8. The negative charge at the fifth position is predicted to initiate hydrophilic loop 2/3 and loop 8/9. The last positive charge in the motif precedes the entry of TMS-3 and TMS-9 into the membrane. Overall, we hypothesize that the role of the charged residues is to sharply demarcate the aqueous/lipid boundary of TMS-2/TMS-3 and TMS-8/TMS-9. If each half of the permease is a tightly folded domain, the conserved motif may play a principal role in positioning the entire protein within the plane of the lipid bilayer. In the current study, we have investigated the role of charged residues within this motif in greater detail.

Materials and Methods

Reagents

Lactose (*O*-β-D-galactopyranosyl-[1,4]-α-D-glucopyranose) was purchased from Sigma Chemical, St. Louis, MO. [^{14}C]-lactose and Sequenase, V2.0, were purchased from Amersham, Cleveland, OH. Restriction enzymes and ligase were purchased from New England Biolabs, Beverly, MA. The remaining reagents were analytical grade.

Bacterial Strains and Methods

The relevant genotypes of the bacterial strains and plasmids are described in Table 1. Plasmid DNA was purified using PERFECT-prep Plasmid DNA kit obtained from 5 Prime → 3 Prime, Boulder, CO. Bacterial transformations were done using the $CaCl_2$ procedure of Mandel and Higa [21]. Restriction digests and ligations were performed according to manufacturers' recommendations. Cell cultures were grown in YT media [22] supplemented with tetracycline (0.01 mg/ml).

In vitro Galactoside Transport

Cells were grown at 37°C with shaking to mid-log phase in YT media supplemented with 5 μg/ml of tetracycline and 0.25 mM isopropylthiogalactoside (IPTG). The cells were pelleted by centrifugation at 5,000 × *g* for 5 min. The pellet was washed in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4, then resuspended in the same buffer at a concentration of about 0.5 mg of protein/ml. The cells were equilibrated at 30°C for 5–10 min before [^{14}C]-lactose (2.5 μCi/ml) was added to a final concentration of 0.1 mM. Aliquots of 200 μl were removed at the appropriate time points, and the cells were captured on 0.45 μm Metricel membranes (Gelman Sciences, Ann Arbor, MI). The cells were then washed with 5–10 ml of ice-cold phosphate buffer by rapid filtration. The filter with the cells was then placed in liquid scintillation fluid and counted using a Beckman LS1801 liquid scintillation counter. Uphill and downhill transport assays were similar except that a *lacZ* minus strain was used in the uphill assays.

Calculations

The K_m and V_{max} for lactose transport were determined by plotting *1/V vs.* 1/[S] in a Lineweaver-Burke double reciprocal plot [28].

Membrane Isolation and Western Blot Analysis

Ten ml of mid-log cells grown as for transport assays were harvested by centrifugation (5,000 × *g*, 5 min). The pellet was quickly frozen in liquid nitrogen and resuspended in 400 μl of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4). The suspension was quickly frozen two more times in liquid nitrogen. The cell suspension was then sonicated three times for 20 sec each. Triton-X 100 was added to a final concentration of 1%, and the membrane fraction was harvested by centrifugation. The pellet was resuspended in 50 μl of MTPBS, and subjected to a Modified Lowry Protein Assay (Sigma Chemical, St. Louis, MO). A sample of one hundred μg protein was subjected to SDS-PAGE using a 12% acrylamide gel. The proteins were electroblotted to nitrocellulose and Western Blot analysis was performed according to Sambrook et al. [27]. The primary polyclonal antibody rec-

ognizes the lactose permease C-terminal 10 amino acids. The secondary antibody, goat-anti-rabbit, conjugated to alkaline phosphatase, was purchased from Sigma Chemical, St. Louis, MO. The Western blot was developed using the NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) colorimetric reaction. The Western blot was then scanned using a Molecular Dynamics laser densitometer and analyzed by comparison to wild-type values for the same preparation and Western blot. As shown in Table 1, values are reported as a percentage of wild-type for three separate preparations.

Site-Directed Mutagenesis

The plasmid, pTE18, was digested with *Eco*RI to yield a 2384 base pair fragment containing the entire *lacY* gene. The fragment was ligated into the vector M13mp18 [39] so that the antisense strand of the *lacY* gene was colinear with the plus-strand of the viral DNA. Site-directed mutagenesis was performed according to Zoller and Smith [40], as modified by Kunkel et al. [19]. The oligonucleotide primers introducing 1–3 base pair changes were obtained from Biosource International, Camarillo, CA. Clones with appropriate amino acid substitution were identified by sequencing. The double-stranded replicative form of M13 was digested with *Eco*RI, coprecipitated with linearized pACYC184, ligated, and transformed into T184. The resultant colonies were screened for inserts by plating on YT containing tetracycline and YT containing tetracycline and chloramphenicol. The insert disrupts the chloramphenicol gene preventing the appropriate colonies from growing on the YT with tetracycline and chloramphenicol. The clones containing the insert were screened for the correct orientation by digestion with *Ava*I. The mutant plasmids were confirmed by sequencing, and two independent clones were kept for further study.

Construction of Multiple Mutants

Single mutant strains made via site-directed mutagenesis were used as starting material to construct double and triple mutants. The plasmid DNAs were digested with a restriction enzyme that had a unique site between the relevant codons, and separated by gel electrophoresis. The appropriate fragments were then isolated from agarose slices using QIAquick Extraction Kit (Qiagen, Chatsworth, CA). The purified fragments were ligated, transformed into strain T184 (Table 1), and confirmed by sequencing over the mutations and the junction within the *lacY* gene.

DNA Sequencing

Mutations were confirmed by sequencing the appropriate regions of the lactose permease. Sequencing was performed on double stranded plasmid according to Kraft et al. [18].

Results

Role of Basic Residues in Loop 2/3

In our previous studies of the lactose permease, each of the three basic residues in loop 2/3 (i.e., Lys-69, Arg-73, and Lys-74) was substituted individually with a neutral residue [13]. The resulting single mutants had normal levels of protein expression, and exhibited lactose transport that was similar to the wild-type strain. A subsequent study investigated the basic residues in loop 8/9, which only contains two positively charged residues (i.e., Arg-285 and Lys-289 [25]. In whole cell transport assays, R285S, K289I, K289T, and R285L/K289T strains had normal levels of transport. However, Western analysis revealed that neutral mutations at position 289 resulted in a permease that was very susceptible to proteolytic degradation during membrane vesicle preparation [25]. Taken together, this later study suggested that the positive charges in loop 8/9 may play a role in maintaining protein structure.

In light of the results obtained with neutral substitutions in loop 8/9, the basic residues in loop 2/3 were investigated by making double and triple neutral substitutions. With regard to expression levels, the double mutant combinations had substantial levels of permease in the membrane, although the R73C/K74C strain appeared moderately defective (*see* Table 1). The triple mutant, however, had negligible levels of permease in the membrane. As shown in Fig. 1, the defect in expression of the triple mutant also correlated with a loss of lactose transport activity. As shown here, the triple mutant was completely defective in lactose transport activity while the double mutant strains behaved normally. These results indicate that a loss of two positive charges in loop 2/3 can be tolerated, while a loss of all the basic residues results in a permease that is either not inserted into the membrane, or is highly unstable and proteolytically degraded.

Interchange of Acidic Residues in Loop 2/3 and Loop 8/9

A striking feature of the conserved motif within the MFS is that a negative charge is highly conserved at the fifth position within one of the two conserved motifs, but not necessarily both. In previous work concerning the lactose permease and tetracycline antiporter, a negative charge in loop 2/3 was found to be essential, but unnecessary in loop 8/9 [13, 17, 25, 36, 37, 38]. In the wild-type lactose permease, the analogous (fifth) position of the conserved negative charge is an asparagine in loop 8/9, not an aspartic acid. To examine the requirement of a negative charge in one of the two conserved motifs, we constructed single substitutions for Asp-68 (i.e., the negative charge in loop 2/3), Asn-284 (i.e., the analogous residue in loop 8/9), and a double mutant strain. Position 68 was changed to asparagine, and position 284 was changed to aspartic acid or isoleucine. The Asn-68 mutant had normal protein expression levels, whereas the single substitutions at position 284 had lower levels of permease (Table 1). As discussed below, these strains actually had downhill transport levels that were higher than wild-type. Therefore, the lower levels of protein described in Table 1 are probably due to an increased rate of protein degradation that occurs during membrane iso-

Table 1. Bacterial strains and plasmids

Strain	Relevant genotype (chromosome/F′/plasmid)	Reference
T184	*lacI*⁺ *lacO*⁺ *lacZ*⁻ *lacY*⁻/ *lacI*^{Q} *lacO*⁺ *lacZ*^{U118a} (*lacY*⁺)/–	31
HS4006/F′I^{Q}Z⁺Y⁻	Δ(*lac-pro*)Δ*malB101*/*lacI*^{Q} *lacO*⁺ *lacZ*⁺ *lacY*⁻/–	1
pTE18 (plasmid)	–/–/Δ(*lacI*) *lacO*⁺ Δ(*lacZ*) *lacY*⁺ Δ(*lacA*) Amp^R Tet^R	31
pLac184[b]	–/–/Δ(*lacI*) *lacO*⁺ Δ(*lacZ*) *lacY*⁺ Δ(*lacA*) Tet^R	7

Plasmid[c]	Level of protein expression (%WT ± SE)
pLac184	100
pD68N	88 ± 4
pK69C/R73C	78 ± 6
pK69C/K74C	106 ± 10
pR73C/K74C	54 ± 9
pK69C/R73C/K74C	3 ± 2
pN284D	17 ± 4
pN284I	38 ± 7
pD68N/N284D	113 ± 40
pD68N/T45R	92 ± 5
pR259L	22 ± 3
pR259Q	162 ± 6
pR259V	122 ± 17
pR259L/N284D	77 ± 18
pR259Q/N284D	87 ± 15
pD68N/R259L	168 ± 20
pD68N/R259Q	133 ± 29
pD68N/R259V	172 ± 21
pD68N/R259L/N284D	104 ± 18
pD68N/R259Q/N284D	110 ± 22

[a] *LacZ*^{U118} is a polar nonsense mutation which results in a *LacZ*⁻-*LacY*⁻ phenotype [31].
[b] pLac184 was constructed by cloning the 2300 bp EcoRI fragment from pTE18 which carries the wild-type *lacY* gene into the EcoRI site of pACYC184. The *lacY* gene and the tetracycline resistance gene are in the opposite transcriptional direction.
[c] The following plasmids are identical with pLac184 except for the noted substitutions within the *lacY* gene.

lation for Western analysis. A similar phenomenon was observed previously for a mutation at position 289 [38].

As shown in Fig. 2, the single D68N is very defective in downhill and uphill lactose transport. The N284D strain, which has aspartic acids in both loops, had an improved level of downhill transport, and even the N284I strain showed influx rates that were similar to wild-type. However, both of these strains showed uphill levels of accumulation that were defective. The impaired uphill transport rates could be due to a faster rate of efflux compared to the wild-type strain, or due to a defect in H⁺/lactose coupling, which is required for the active accumulation of lactose. The double mutant,

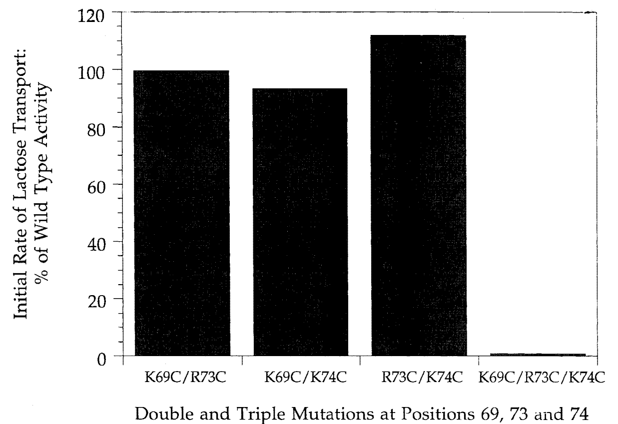

Fig. 1. Downhill lactose transport in the wild-type strain and in mutant strains replacing two or three basic residues in loop 2/3. [^{14}C]-Lactose uptake was measured as described in the Materials and Methods in strain HS4006/F′I^{Q}Z⁺Y⁻ carrying the wild-type or designated mutant plasmids. Note: in downhill lactose transport assays, the wild-type or mutant plasmids were transformed into an *E. coli* strain which is *lacZ*⁺ (i.e., β-galactosidase positive). Upon entry into the cell, lactose is rapidly metabolized so that the extracellular lactose concentration remains higher than the intracellular concentration [26].

D68N/N284D, which switches the essential aspartic acid residue in loop 2/3 to the analogous position in loop 8/9, was also defective in both modes of transport. These results indicate that a defect in transport caused by a loss of the negative charge in loop 2/3 cannot be alleviated by the gain of a negative charge at the corresponding position in loop 8/9.

Charge Distribution Across TMS-2 and TMS-8

In a previous study, neutral substitutions of Asp-68 (i.e., D68T and D68S), which were severely defective in lactose transport, were used as parental strains to isolate second-site suppressors that restored activity [14]. One suppressor involved a change of Thr-45 in loop 1/2 to an arginine. This observation suggested that the charge distribution across TMS-2 may greatly influence the activity of the lactose permease. Interestingly, when we examined the amino acid sequence in the second half of the wild-type lactose permease, it was noticed that a basic residue is already found in loop 7/8 (i.e., Arg-259), while the acidic residue in loop 8/9 is replaced with a neutral residue (i.e., Asn-284). In this regard, the locations of charges across TMS-2 in the double mutant (i.e., T45R/D68S) resembled the charge distribution across TMS-8 in the wild-type permease (i.e., Arg-259/Asn-284). With these observations in mind, we wanted to determine if the basic residue in loop 7/8 was offsetting the requirement for a negative charge in loop 8/9. Figure 3 illustrates the relative locations of these residues in the wild-type lactose permease.

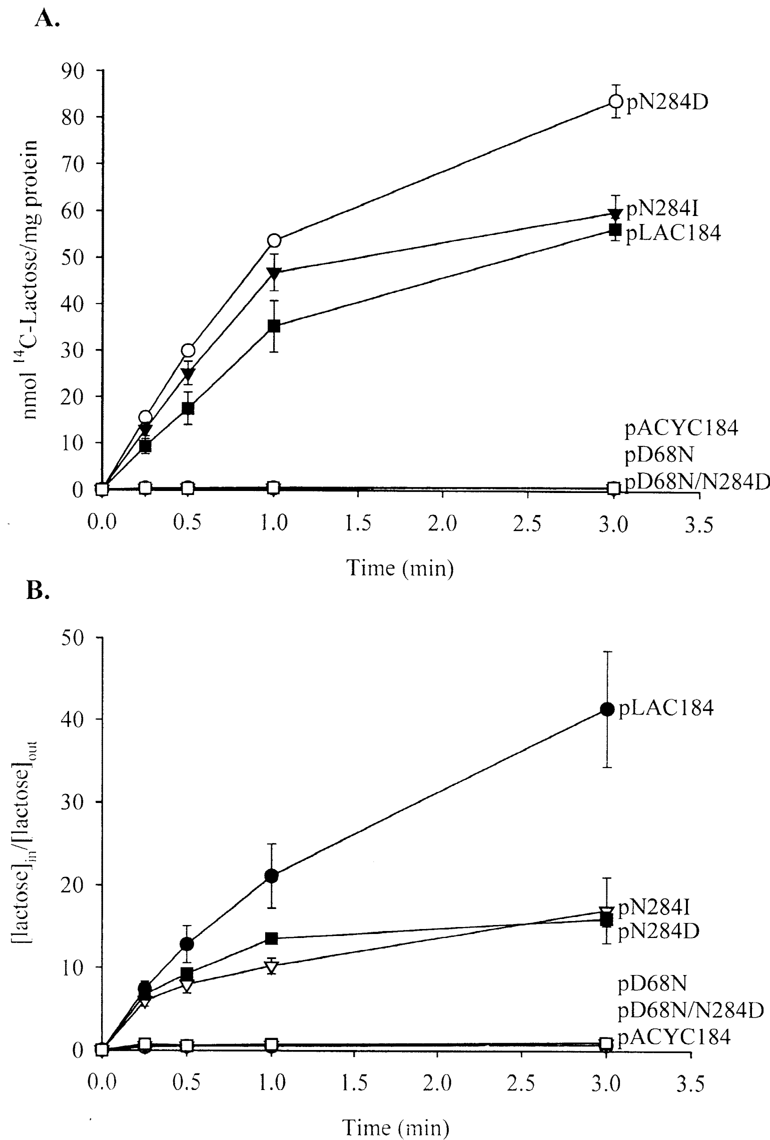

Fig. 2. Lactose transport in the wild-type strain and in mutant strains replacing the acidic residue in loop 2/3 and/or the analogous asparagine residue (Asn-284) in loop 8/9. (*A*) Downhill [^{14}C]-lactose uptake was measured as described in the Materials and Methods in strain HS4006/F′I^{Q}Z^{+}Y^{-} carrying the wild-type or designated mutant plasmids. (*B*) Uphill [^{14}C]-lactose accumulation was measured in strain T184 carrying the wild-type or designated mutant plasmids.

In the experiment of Fig. 4, we tested if the positive charge in loop 7/8 was important for lactose permease activity, and whether its functional effects were influenced by a neutral or negative charge in loop 8/9 (i.e., Asn-284 *vs.* Asp-284). Although some minor differences in transport were observed, Leu-259, Gln-259, and Val-259 mutant strains exhibited normal levels of downhill transport (Fig. 4, part *A*) and uphill transport (Fig. 4, part *B*). In downhill transport, the coupling of an Asp-284 mutation with position 259 mutations only had minor effects. In uphill transport assays, the coupling of neutral mutations at postion 259 with Asp-284 resulted in higher levels of lactose accumulation. These results indicate that the charge distribution across TMS-8 has some effects on conformational changes associated with lactose transport (e.g., the relative rates of influx *vs.* efflux). However, mutations affecting a single charge across TMS-8 do not dramatically inhibit lactose transport.

Overall, the results of Fig. 4 suggest that the charge distribution across TMS-8 is not a critical feature for lactose transport. Even so, if the lactose permease is composed of two rigid domains which interact at the interface between the two halves of the protein, the translational topology of the entire protein may be governed by the charge distribution in only one half of the protein. In this regard, the charge distribution across TMS-2 may play a dominant role in maintaining the proper translational topology of the permease, and may mask the changes in charge distribution across TMS-8. To explore this possibility, Fig. 5 shows the results of downhill (part *A*) and uphill (part *B*) transport assays comparing the wild-type permease with mutants having a D68N mutation in loop 2/3 plus additional mutations in loop

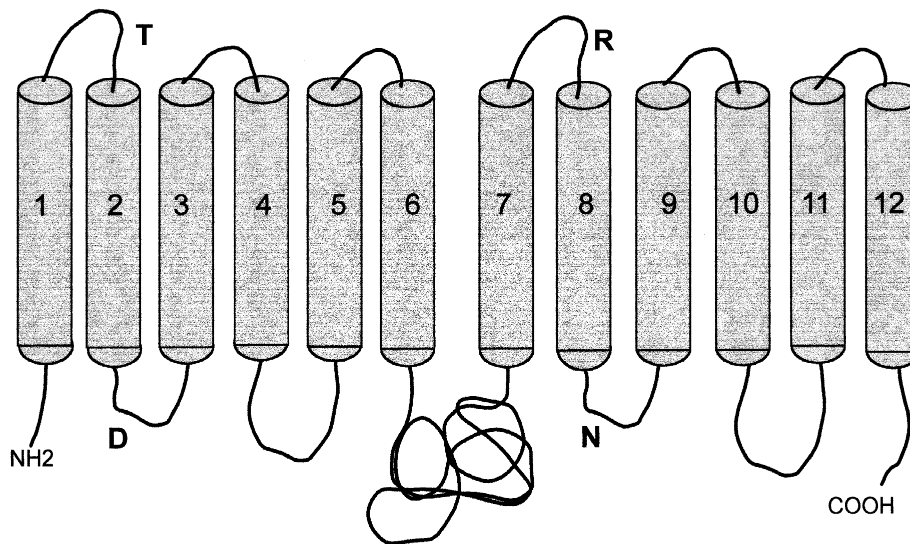

Fig. 3. Secondary structure topology of the lactose permease mutants showing the locations of critical residues that are altered in the experiments of Figs. 4 and 5. Wild-type distribution of residues are Thr-45 in loop 1-2, Asp-68 in loop 2-3, Arg-259 in loop 7-8, and Asn-284 in loop 8-9.

7/8 and/or loop 8/9. As shown here, a loss of lactose transport activity, caused by a neutral substitution in loop 2/3, could not be rescued by changes in charge distribution across loop 8/9. These results indicate that changes in charge distribution across loop 8/9 could not compensate for a loss of a negative charge in loop 2/3.

Suppressor Analysis

To further explore why a negative charge is essential in loop 2/3 but not in loop 8/9, the double mutant, D68T/N284D, which has a neutral substitution in loop 2/3 and a negative charge in loop 8/9, was used as a parent strain for the isolation of suppressor mutations. When streaked on MacConkey plates, the D68T/N284D strain has a white phenotype, due to its low level of transport. After much effort, and the restreaking of many plates, we eventually were able to identify one suppressor as a red fleck in the primary streak; this suppressor partially restored sugar transport as judged by its red phenotype. When subjected to DNA sequencing, the suppressor was found to have a single base change, in addition to retaining the two parent mutations which were already present. The third mutation in the suppressor changed a glutamic acid codon at position 374 to a lysine codon. This mutation, which changes a negative to a positive charge, is predicted to lie within the periplasmic loop that connects TMS-11 and TMS-12.

After we had identified the spontaneous E374K mutation, molecular genetic techniques were used to separate this mutation from the two parent mutations, and also to combine the E374K mutation with just one of the two parent mutations (*see* Materials and Methods). We then examined the transport properties of the wild-type, D68T, N284D, E374K, D68T/N284D, D68T/E374K, N284D/E374K, and D68T/N284D/E374K strains.

Colony phenotypes of sugar MacConkey plates provide a qualitative measure of transport ability. If a strain can transport and ferment a sugar, it will form red colonies on these plates due to the presence of a pH indicator dye. On MacConkey plates containing 1% lactose (a β-galactoside) or 1% melibiose (an α-galactoside), the wild-type strain has a red phenotype because the lactose permease is able to transport either of these sugars, which are subsequently fermented. The *lacY* minus strain (vector only) has a white phenotype. Similarly the double mutant D68T/N284D, which was used as a parent strain in the isolation of the suppressor mutant, was also white on both types of plates. When comparing the D68T/N284D strain (which is white on MacConkey plates) with the corresponding single mutants, D68T or N284D, it was found that the D68T strain is also white, while the N284D strain is red. These results indicate that the defect seen in the double mutant is explained by the effects of the D68T mutation. As a single mutant, the E374K mutation is also red on MacConkey plates, as are the D68T/E374K and D68T/N284D/E374K strains.

To gain greater insight into the effects of these mutations, a kinetic analysis was performed (*see* Table 2). Unfortunately, the D68T/N284D parent strain had negligible levels of transport which prevented the measurement of kinetic parameters in this strain. The D68T single mutant showed a slightly lower K_m but a dramatically lower V_{max} for lactose transport, suggesting that the primary effect of this mutation is to inhibit conformational changes associated with lactose transport. The

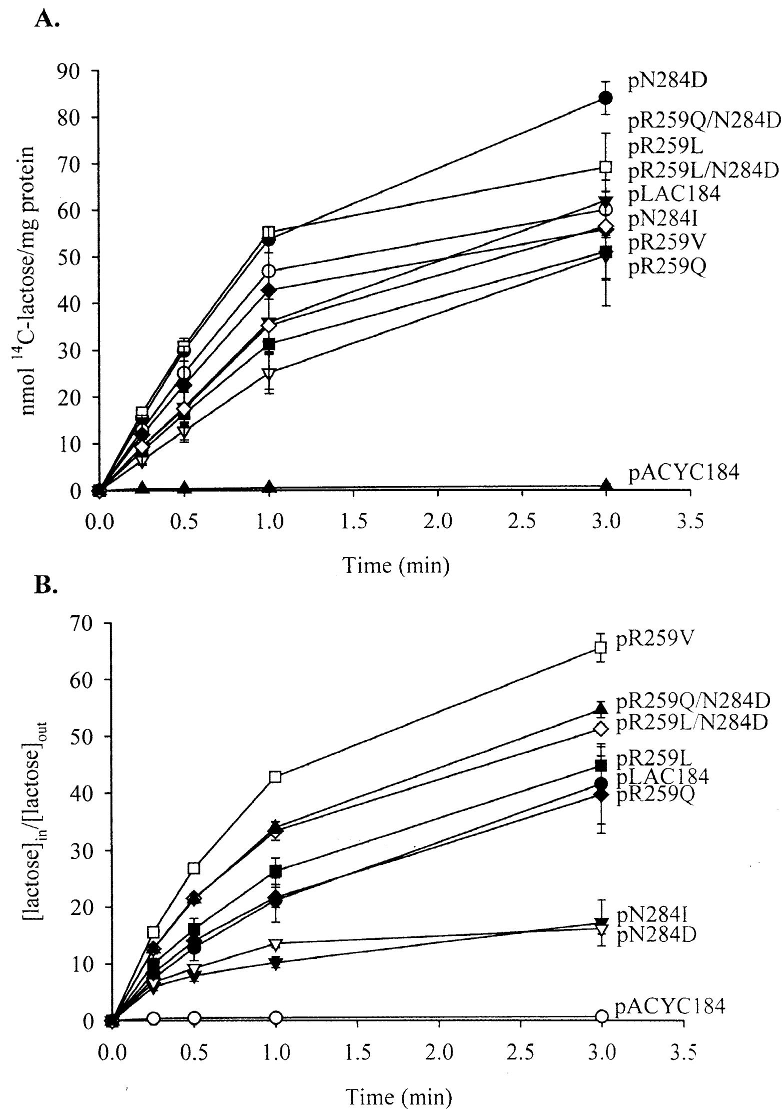

Fig. 4. Lactose transport in the wild-type strain and in mutant strains which alter the charge distribution across TMS-8. (*A*) Downhill [^{14}C]-lactose uptake was measured as described in the Materials and Methods in strain HS4006/F′I^{Q}Z^{+}Y^{-} carrying the wild-type or designated mutant plasmids. (*B*) Uphill [^{14}C]-lactose accumulation was measured in strain T184 carrying the wild-type or designated mutant plasmids.

single N284D and E374K mutations had higher K_m values, and V_{max} values that were slightly higher than the wild type strain. In the triple mutant, the kinetic parameters became fairly similar to the wild-type strain. In the double mutant, D68T/E374K, the E374K mutation substantially alleviated the defect in V_{max} seen in the single D68T strain, but its V_{max} value of 103 nmoles of lactose/min.mg protein was still much lower than the triple mutant strain.

Discussion

The requirement for charged residues within the conserved loop 2/3 and loop 8/9 motif of the lactose permease was further examined in this study, and compared with results obtained in previous studies [13, 15, 17, 24, 25, 36, 37, 38]. The basic residues primarily appear to have an effect on protein insertion and/or stability. When all three basic residues were neutralized in loop 2/3, a negligible level of permease was observed in the membrane. Similarly, in a previous study, neutral mutations at a basic residue in loop 8/9 (i.e., Lys-289) resulted in a permease that was very susceptible to proteolytic degradation during membrane vesicle preparation [25]. Taken together, these observations are consistent with the positive inside rule which states that cytoplasmic loops are generally enriched in arginyl and lysyl residues, and is a major determinant of transmembrane topology in membrane proteins [32, 33].

In contrast, the conserved negative charge in loop 2/3 is not consistent with the positive inside rule. Therefore, one would speculate that the conservation of such a negative charge in the cytoplasmic loop of a membrane transport protein may be related to an important functional role during transport. It is unlikely that this resi-

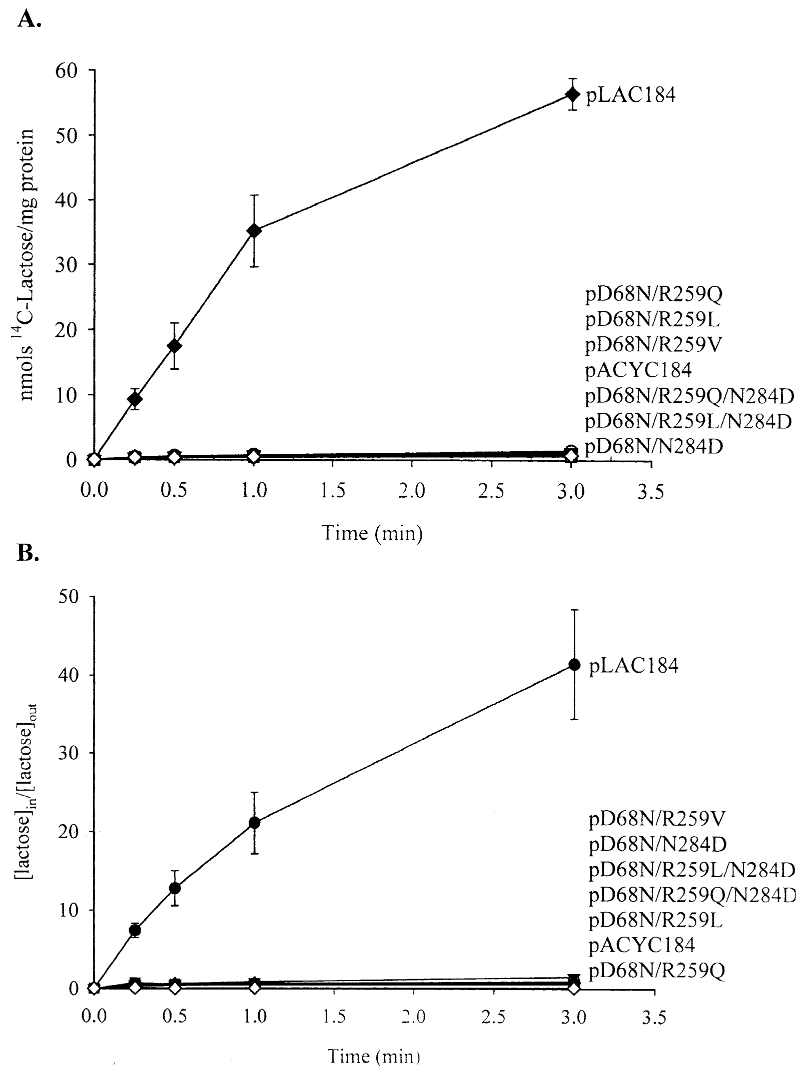

Fig. 5. Lactose transport in the wild-type strain and in mutant strains which carry a D68N mutation in loop 2/3 and also alter the charge distribution across TMS-8. (*A*) Downhill [^{14}C]-lactose uptake was measured as described in the Materials and Methods in strain HS4006/F′I^{Q}Z^{+}Y^{-} carrying the wild-type or designated mutant plasmids. (*B*) Uphill [^{14}C]-lactose accumulation was measured in strain T184 carrying the wild-type or designated mutant plasmids.

due would play a role in substrate recognition because it is conserved among transporters which recognize widely different substrates. Likewise, it is not likely to play a role in cation recognition, because it is conserved in transporters, such as mammalian glucose transporters, which are uniporters. Instead, the results from this study, as well as previous studies, suggest that it plays a role in facilitating conformational changes associated with lactose transport. Neutralization of the acidic residue in loop 2/3 results in a dramatic decrease in V_{max} (*see* Table 2), and the main effect of suppressor mutations is to restore the maximal velocity for lactose transport [15, 24].

Compared with loop 8/9, the results described in the current study also indicate that the negative charge in loop 2/3 plays a dominant role in maintaining the lactose permease in a functional state. As shown in Fig. 2 and Table 2, the neutralization of the negative charge in loop 2/3 results in a dramatic loss of transport activity that is

Table 2. Table of apparent K_m and V_{max} values*

Strain	K_m ± SE (mM)	V_{max} ± SE (nmol lactose/ min mg protein)
pLAC184 (wild type)	0.55 ± 0.1	247 ± 60
pD68T	0.18 ± 0.1	2 ± 0.4
pN284D	0.84 ± 0.3	377 ± 144
pE374K	1.60 ± 0.7	291 ± 99
pD68T/N284D (parent)	too low to measure	
pD68T/E374K	0.48 ± 0.1	103 ± 24
pN284D/E374K	1.18 ± 0.1	364 ± 45
pD68T/N284D/E374K		
(suppressor)	0.24 ± 0.1	291 ± 99

* K_m and V_{max} values were determined in downhill lactose transport assays as described under Materials and Methods.

not recovered in mutant constructs that contain variations in charge distribution across TMS-8 (i.e., D68N/N284D, D68N/R259L, D68N/R259Q, D68N/R259L/N284D, and D68N/R259Q/N284D). However, a negative charge in loop 8/9 is not completely without effect. The E374K suppressor mutation was better at restoring maximal transport velocity when a negative charge was located at position 284 (compare D68N/E374K with D68N/N284D/E374K, *see* Table 2). Taken together, these results indicate that the negative charge in loop 2/3 plays an important role in maintaining the permease in functionally competent state, but mutations in other parts of the protein can compensate for a loss of negative charge at this site.

Overall, the charges throughout the lactose permease are expected to contribute to the positioning of the protein within the plane of the lipid bilayer. Since the twelve transmembrane segments in the lactose permease are the evolutionary result of a gene duplication/fusion event, the two halves of the protein (TMS-1 to TMS-6 and TMS-7 to TMS-12) may behave as independently folded domains that interact at an interface. The locations of charges in the conserved loop 2/3 and 8/9 motif may be important in the positioning of the two halves of the permease relative to each other.

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