Structural Topology of Transmembrane Helix 10 in the Lactose Permease of Escherichia coli*

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In the lactose permease of Escherichia coli, transmembrane helix 10 has been shown to be functionally important. The structure of this helix has been examined in greater detail in this study. A total of 46 substitution and 8 insertional mutants were constructed and analyzed along the entire length of transmembrane helix 10. The results identified amino acids that are tolerant of substitutions by a variety of amino acids. Since a number of these amino acids (Thr-320, Val-331, Phe-325, and Ile-317) are clustered in one region in a helical wheel projection of transmembrane helix 10, it seems likely that this face of helix 10 is interacting with the membrane. The channel lining domain is thought to consist of the helical face containing Glu-325, Leu-318, Leu-329, His-322, Val-315, Cys-333, Val-326, and Lys-319 based on the results here and from earlier findings. Deleterious mutations along this face tended to greatly increase the $K_m$ value for lactose transport with only minor effects on the $V_{max}$. Analysis of insertional mutants revealed that perturbation of the spatial relationship between amino acids at the periplasmic edge is less deleterious than perturbation in the center of the helix or the cytoplasmic edge. Using all of the above information, a detailed structural topology of transmembrane helix 10 is proposed.

Transport of solutes across biological membranes is a fundamental biochemical process in bacteria (1), fungi (3, 4), plant (5, 6), and animal cells (7, 8). In these organisms, the transport of substrates such as sugars, amino acids, antibiotics, etc. is often coupled to the transport of a cation such as H\(^+\), Na\(^+\), or Li\(^+\) and this cotransport is mediated by membrane embedded proteins. Energetically, cotransport is accomplished by an inwardly directed cation electrochemical gradient that acts as the driving force for the transport of the substrate so that secondary active transport can be achieved with regard to the solute (9, 10). The cotransport of substrate/cation has been studied extensively, most notably in the lactose permease of Escherichia coli (11, 12). Extensive biochemical and genetic analysis have been conducted on the lactose permease. The cloned sequence indicates a polypeptide of 417 amino acids with molecular weight of 46,504 (13, 14). Hydropathicity and genetic analysis have suggested the presence of 12 transmembrane domains that traverse the membrane in an \(\alpha\)-helical manner (15–17).

Sequence analyses have shown that a number of transporters, including the lactose permease of E. coli, are evolutionarily related (18–20). Mechanistically, these transporters function as uniporters, symporters, or antiporters. Functionally, they transport a wide variety of substrates including sugars, amino acids, antibiotics, and Kreb's cycle intermediates. Most of the transporters are thought to contain 12 transmembrane \(\alpha\)-helices traversing the membrane (21). More recently, we have conducted a structural comparison of 65 members of the superfamily (22). Based on a number of parameters, including hydropathicity, amphipathicity, interhelical loop length, and putative helical interactions in the lactose permease, we proposed a general, three-dimensional arrangement for all 12 transmembrane helices in the superfamily.

In the lactose permease of E. coli, transmembrane helix 10 has been implicated to contain a number of functionally important amino acids (23–28). For example, Glu-325 has been identified as a critical residue for cation recognition and transport. Lys-319 and His-322 have been shown to affect proton leakiness and coupling. In addition, changes in sugar specificity of the lactose permease can be attributed to mutations in transmembrane helix 10. Therefore, we have decided to examine the structure of transmembrane helix 10 in greater detail. Based on our model concerning the three-dimensional arrangement of transmembrane segment, helix 10 contains a putative channel lining face and a lipid bilayer interacting face. The model also predicts two sections of the helix that potentially interact with other helices (22).

Previously, Hinkle et al. (29) examined transmembrane helix 8 of the lactose permease of E. coli for mutability. Analysis of functional single and multiple mutations in transmembrane helix 8 suggested a "mutable stripe" that was thought to be in contact with the membrane phospholipids. We used a similar approach in this study. A number of high and low activity single site substitution mutants were isolated along the entire length of transmembrane helix 10. In addition, we examined the importance of the spatial relationship between the amino acids in transmembrane helix 10. A number of insertional mutants were constructed in which a hydrophobic amino acid was inserted at various positions along the length of transmembrane helix 10. Based on the analyses of all these mutants, we propose a more detailed model of transmembrane helix 10.

MATERIALS AND METHODS

Reagents—Lactose (\(\beta-D\)-galactopyranosyl-[1,4]-\(\alpha\)-D-glucopyranose) was purchased from Sigma. [\(^{14}C\)]Lactose was purchased from American Corp. All other chemicals were analytical grade.

Bacteria and Plasmids—Bacterial strains and plasmids used in this work are listed in Table 1.

Plasmid DNA was isolated by using the plasmid isolation kit purchased from 5 Prime – 3 Prime, Inc. DNA fragments were isolated from 1% agarose gels using the gel extraction kit from Qiagen. Plasmid DNA
was introduced into the appropriate bacterial strain by the CaCl₂ transfection procedure of Mandel and Higa (30). Stock cultures of cells were grown in YT media (31) supplemented with tetracycline (0.01 mg/ml) or ampicillin (0.1 mg/ml). For transport assays, cells were grown to midlog phase in YT media containing tetracycline (0.005 mg/ml) or ampicillin (0.05 mg/ml) and 0.25 mM isopropylthiogalactoside to induce the synthesis of the lactose permease.

Site-directed Mutagenesis—The plasmid, pTE18 (32), was digested with EcoRI to yield a 2300-base pair fragment containing the lacY gene. This fragment was ligated into the EcoRI site of pBR322 to produce the 2300-base pair fragment containing the lacY gene. This fragment was ligated into the EcoRI site of either pBR322 or pACYC184. Positive hybrid clones into pBR322 were identified on 1% lactose MacConkey plates containing ampicillin (0.1 mg/ml) and tetracycline (0.01 mg/ml). The hybrid clones were restriction mapped to verify the orientation of the lacY gene in the plasmid. Only those clones that contained the lacY gene and the ampicillin resistance gene in the same transcriptional direction were used. (Note: this is the same orientation as parent plasmid, pTE18.) Positive clones into pACYC184 were identified by selecting for tetracycline resistance followed by screening for the loss of chloramphenical resistance. Only those clones that contained the lacY gene and the tetracycline resistance gene in the opposite transcriptional direction were used. (Note: this is the same orientation as parent plasmid, pLac184.)

Finally, the mutant plasmids were sequenced throughout the entire lacY coding sequence to verify the presence of the mutation and to be certain that no other secondary mutations had occurred. At least two independent clones for each mutant type were saved for further study.

DNA Sequencing—Single-stranded viral DNA was sequenced by the Sanger dideoxy method (36) using oligonucleotide primers that anneal within the lacY gene. Double-stranded plasmid DNA was isolated and sequenced as described by Kraff et al. (37).

Transport Assays—Midlog cells were washed in phosphate buffer, pH 7.0, containing 60 mM K₂HPO₄ and 40 mM KH₂PO₄ and resuspended in the same buffer to a density of approximately 0.5 mg of protein/ml. Cells were then equilibrated at 30 °C and radioactive sugar (final concentration = 0.1 μM) was added. At appropriate time intervals, 0.2-ml aliquots were withdrawn and filtered over a membrane filter (pore size, 0.45 μm). The external medium was then washed away with 5–10 ml of the wash buffer during uphill transport experiments to rapidly inhibit the lactose permease and thereby minimize sugar efflux during removal of the extracellular medium. At 1-min time points, the activity of mutants was compared to the activity of the wild-type. Activity of the mutants that were cloned into pACYC184 was compared to the activity of the wild-type lactose permease cloned into pACYC184 (plac184). Similarly, activity of the mutants that were cloned into pBR322 was compared to the activity of the wild-type lactose permease cloned into pBR322 (pTE18).

For the kinetic experiments, the same procedure for downhill transport was followed but several different final sugar concentrations were used to evaluate the Kₘ and Vₘₐₓ values. The Kₘ and Vₘₐₓ values for lactose transport were determined by plotting 1/V versus 1/[S] in a Lineweaver-Burke double-reciprocal plot (38). Membrane Isolation and Immunoblot Analysis—For Western blot analysis, MS109 cells containing the appropriate plasmid were grown as described for the sugar transport assays. 10 ml of late log cells were pelleted by low speed centrifugation, quick frozen in liquid N₂, and thawed at room temperature. Cells were resuspended in 800 μl of MTPBS (150 mM NaCl, 16 mM Na₂HPO₄, 4 mM NaH₂PO₄). Cells were quick frozen in liquid N₂ and thawed twice. Cells were then sonicated three times for 20 s each. The membranes were harvested by centrifugation and resuspended in 100 μl of MTPBS. 10 μg of total membrane protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose and then probed using an antibody that recognizes the carboxyl terminus of the lactose permease (graciously provided by Dr. Tom Wilson of Harvard University). The amount of lactose permease was then determined by laser densitometry.

Construction of pASTM10—pASTM10-kan—The plasmid, pASTM10, used for cassette mutagenesis was derived from plac184. Plasmid plac184 is a low copy plasmid derived from pACYC184 with a 2300-base pair fragment containing the entire lacY gene cloned into the EcoRI site of the vector. Site-directed mutagenesis was performed as described above to construct two unique restriction sites flanking the transmembrane helix 10 sequence of the lacY gene. An AattI restriction site was constructed at the 5’ end of the sequence encoding transmembrane helix 10 and an SpeI site at the 3’ end. In order to construct the AattI site, the mutagenic primer, 5’-ctt cca gcg cgt cag cag atg agc gc-3’, was used. This primer has two base mismatches at Ala-309 and Thr-310. The two mismatches create a AattI site but also conserve the wild-type amino acid sequence. Similarly, the SpeI mutagenic primer, 5’-ctt caa gac tag taa tat att taa gcg cc-3’, has two base mismatches at Thr-338 and Ser-339. In this case the mismatches create an SpeI site but also conserve the wild-type amino acid sequence. The two restriction sites were generated independently of each other and the plasmids referred to as pASTM10 and pSpe339.

In order to construct pASTM10, plasmids pAat310 and pSpe339 were digested with BamHI and Ndel. There is a unique Ndel restriction site in the middle of the transmembrane helix 10. The resulting 3542-base pair fragment from pAat310 was ligated with the 3087-base pair fragment from pSpe339 to generate pASTM10.

Another plasmid, pASTM10-kan, was constructed by cloning a 2.7-kilobase pair kanamycin cartridge into the Ndel site present in the middle of TM10. It was advantageous to use pASTM10-kan in the cassette mutagenesis protocol because any incompletely digested vector (9.3 kilobase pairs) could easily be separated from the completely digested plasmid (6.6 kilobase pairs). By removing the incompletely digested vector, the number of background red colonies derived from incompletely digested vector was minimized. Thus, most of the colonies on the transformation plate contained TM10 that originated from the synthetic oligonucleotide. Plasmid pASTM10-kan, was constructed by digesting pACYC177 with Bsal and pASTM10 with Ndel and ligating the two mixtures and selecting for kanamycin and tetracycline resistance on YT plates. The resulting plasmid, pASTM10-kan has the kanamycin cartridge cloned into the Ndel site located in the middle of TM10.

Cassette Mutagenesis—A 96-mer oligonucleotide encoding transmembrane helix 10 and the flanking regions was synthesized and gel purified (Keystone Laboratories) with 0.33% contamination at each of the bases between codon 315 and codon 335. A complementary primer

\[ \text{TABLE I} \]

| Strains | Relevant genotype (chromosome/F plasmid) | Ref. |
|---------|-----------------------------------------|-----|
| HS4006/F' I" Z' Y" | Δ(Lac-Pro)malB101/lac I" lac O" lac Z' lac Y'/-- | 23 |
| T184 | Lad lacO' lacZ' lacY' lacO" lacZ" lacO" lacZ' lacY'/-- | 32 |
| MS1019 | Δ(lacY) thr-leu-arg, B1, SmR'/-- | 40 |

\[ ^{a} \text{Δ, before a bracketed symbol indicates a deletion.} \]

\[ ^{b} \text{LacZ" is a polar nonsense mutation which results in a Lac Z' Lac Y" phenotype.} \]
was made to the 96-mer between positions 77 and 96. 200 pmol of each of the mutagenic and complementary primer were annealed by slow cooling (about 1 h) from 70 °C to 40 °C. The cassette was synthesized by extending the complementary primer with Klenow enzyme. After synthesis, the mixture was desalted using Qiagen Gel extraction kit followed by digestion with AatII and SphI. The digest was desalted again prior to ligation.

The vector was prepared by digesting approximately 2 μg of pASTM10-kan with AatII and SphI and run on a 1% agarose gel. The resulting 6.5-kilobase pair band was cut and eluted from the agarose. Complete digestion by AatII and SphI was verified by the presence of the DNA band lacking the kanamycin cartridge. The cassette synthesized above was ligated to this vector, transformed into MS1019, and plated on 1% lactose MacConkey plates. Plasmid DNA from a number of red, pink, and white colonies was isolated and the DNA sequenced.

RESULTS

Isolation of Single Site Mutants—A variety of studies have shown an important role of transmembrane helix 10 within the lactose permease (23–28). According to our model, transmembrane helix 10 is a putative channel lining segment that interacts with several other transmembrane helices (22). To gain insight into the topology of transmembrane helix 10 within the lactose permease, the technique of cassette mutagenesis was used to generate random single site mutations within transmembrane helix. On 1% lactose MacConkey plates, the transformed colonies were predominantly red but some pink and white colonies were also apparent. Plasmid DNA from a total of 400 red, pink, and white colonies was isolated and sequenced in the transmembrane 10 region of the lacY gene. Although most were wild-type or had multiple mutations, 46 unique amino acid substitutions were identified and saved for further analysis.

Phenotype on MacConkey Plates—The phenotypes of bacterial colonies on lactose MacConkey plates provides qualitative information about the level of activity of the lactose permease. Colonies containing active lactose permease have a red phenotype on 0.4% lactose MacConkey plates. Mutants retaining the red phenotype indicating the presence of a functional lactose permease. Other mutations had a significant effect on the function of the lactose permease. For example, G332D, M323K, etc., had a red center phenotype. One of the mutations, E325K, resulted in a lactose permease that was dramatically defective as indicated by a white phenotype. Overall, although most of the single mutations resulted in functional lactose permease as seen by the red phenotype, a few substitutions caused significant decreases in lactose permease activity.

In Vitro Galactoside Transport—In vitro galactoside transport was performed to obtain more quantitative information about the level of activity of the lactose permease. In the results shown in Table I, mutant strains were analyzed for their ability to transport [14C]lactose “downhill” relative to the wild-type. In downhill transport assays, plasmids containing the wild-type lacY gene or mutant genes were introduced into an E. coli strain that 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 (39). Therefore, under these conditions, lactose transport is downhill or with its concentration gradient.

As expected from the phenotypes on MacConkey plates, most of the mutants with the red phenotype showed high levels of lactose permease activity, whereas the mutants with red center phenotypes exhibited a significant decrease in lactose transport. Generally, the mutants with red center phenotypes had less than 15% of the activity compared to the wild-type. The E325K mutant’s white phenotype on MacConkey plates is consistent with the extremely low level of lactose transport. In fact, this mutant had the lowest level of activity. Four of the mutants with red phenotypes on 0.4% MacConkey plates contain only 4, 9, and 14% of lactose transport relative to the wild-type. Although it is not surprising that occasionally a mutant with the red phenotype has only about 10–15%, it is rare that a mutant with a red phenotype, i.e. G332V, has only 4% activity. These results from in vitro galactoside transport were generally consistent with the phenotypes seen on the MacConkey plates. Mutants E325K, L329P, H322Y, L330P, M323K, F334S, P327L, T320P, and G332D had low activities as expected from the plating results. Furthermore, mutants V326G, M323L, G332V, and G332C also exhibited low activity even though they were red on MacConkey plates.

All of the mutants which showed less that 30% downhill transport were analyzed with regard to the amount of lactose permease protein (see Table II). There was significant variation in protein levels among the mutant strains. In most mutants with less than 30% transport activity, the low rate of lactose transport did not quantitatively correlate with the amount of permease protein. For example, M323V had only 27% transport activity while its value for protein level was 75% of the wild-type level. In these types of cases, the mutation in the permease appears to be directly affecting the function of the protein. In two mutants with very low transport activity, however, a lower level of protein did correlate with lower transport activity (e.g. M323K and G332V). In these two cases, the low rate of lactose transport can be explained by a defect in protein levels.

Several of the mutants with low transport activity were also analyzed with regard to the kinetic parameters of lactose transport (i.e. K_m and V_max; see Table II). As expected, the mutants tended to raise the K_m and/or lower the V_max for lactose transport. Most mutants with low transport exhibited both a higher K_m and a lower V_max. As discussed later under “Discussion,” the mutants in Table II are arranged according to our model of the topology of TM-10 (see Fig. 3 under “Discussion”). Mutations in the putative channel lining side of TM-10 tended to have higher K_m values compared to mutations in other regions. In two cases (L329P and C333Y), mutations along the channel lining face had little or no effect on the velocity of lactose transport.

Insertional Mutants—To further explore the importance of transmembrane helix 10 topology, hydrophobic insertional mutants were systematically made along helix 10. As shown in Fig. 1, a hydrophobic amino acid of either valine or leucine was inserted every 2 or 3 amino acids along the entire transmembrane helix 10. In order to qualitatively assess the level of lactose permease activity, each of the insertional mutants was streaked on 1 and 0.2% lactose MacConkey plates. Results from in vitro galactoside transport further confirmed that insertional mutants Leu-315 and Val-318 had a red phenotype on both 1 and 0.2% lactose MacConkey plates, whereas Leu-320 and Val-323 were still red on 1% but white on 0.2% MacConkey plates (Table II). In contrast, Leu-326, Val-329, Leu-332, and Leu-335 were white on both 1 and 0.2% lactose MacConkey plates. Results from in vitro galactoside transport further confirmed that insertional mutants Leu-315 and Val-318 transport lactose uphill and downhill relatively well (see Fig. 2). However, the other insertional mutants showed dramatically diminished abilities to transport lactose. Phenotypes on 1 and 0.2% lactose MacConkey and [14C]lactose transport assays are consistent with the notion that the insertions near the periplasmic edge are less deleterious than the insertions in the middle or the cytoplasmic edge.
In order to examine the topology of transmembrane helix 10 in greater detail, a number of substitution and insertional mutants were constructed and analyzed in this study. The results have identified amino acids in transmembrane helix 10 that are tolerant of substitution by various other amino acids and those that are less tolerant. In particular, amino acids at positions 320, 331, 324, and 317 were shown to allow many substitutions. Interestingly, in a helical wheel projection of transmembrane helix 10, most of the amino acids that permit substitutions by a variety of amino acids are clustered together in one region (see bilayer face of helix 10 in Fig. 3). Mutants that significantly reduce the lactose permease activity are scattered throughout the remaining regions of the helical wheel projection.

Based on our three-dimensional model (Fig. 3), transmembrane helix 10 should contain a channel lining face, a lipid bilayer interacting face, and two or three putative helical interacting regions. Analyses of the data from amino acid substitutions suggest that the helical face containing Thr-320, Val-315, Cys-323, Val-326, Lys-319, Leu-330, Met-323, and Phe-328 is crucial for lactose permease activity. The amino acids at these positions may play a critical role in the binding and transport of lactose.

### Table II

| Wild-type residue | Location in model | Mutation | Phenotype on 1% MacConkey | Transport activity | Protein level | Transport kinetics
|------------------|-------------------|----------|--------------------------|-------------------|---------------|---------------------|
|                  |                   |          |                          |                   |               | Km/Vmax
| Glu-325          | Channel lining    | K        | W                        | 100               | 100           | 0.3/126.4          |
|                  |                   | D        | R                        | 42                | ND            | ND                 |
| Leu-318          | Channel lining    | M        | R.C.                     | 147               | 45            | 4.3972             |
| Leu-329          | Channel lining    | P        | R.C.                     | 6                 | 148           | ND                 |
| His-322          | Channel lining    | Q        | R                        | 19                | 137           | 3.5/54.4           |
| Val-315          | Channel lining    | None     | R                        | 21                | 125           | 2.0/192.5          |
| Cys-333          | Channel lining    | Y        | R                        | 9                 | 167           | 1.9/35.6           |
| Val-326          | Channel lining    | G        | R                        | 137               | ND            | ND                 |
|                  |                   | I        | R                        | 18                | 189           | ND                 |
| Lys-319          | Channel lining    | E        | R                        | 16                | 184           | 1.21/63.5          |
| Leu-330          | Interhelical      | P        | R.C.                     | 8                 | 39            | 1.8/18.4           |
| Met-323          | Interhelical      | V        | R                        | 27                | 75            | ND                 |
|                  |                   | K        | R.C.                     | 7                 | 4             | ND                 |
|                  |                   | L        | R                        | 14                | 44            | 1.8/74.4           |
| Phe-328          | Interhelical      | C        | R                        | 120               | ND            | ND                 |
| Leu-321          | Interhelical      | M        | R                        | 134               | ND            | ND                 |
|                  |                   | V        | R                        | 93                | ND            | ND                 |
|                  |                   | Q        | R                        | 92                | ND            | ND                 |
| Gly-332          | Interhelical      | D        | R.C.                     | 9                 | 39            | ND                 |
|                  |                   | S        | R                        | 22                | 33            | ND                 |
|                  |                   | V        | R                        | 4                 | 7             | ND                 |
|                  |                   | C        | R                        | 14                | 107           | 0.8/35.2           |
| Val-316          | Bilayer           | G        | R                        | 31                | ND            | ND                 |
|                  |                   | I        | R                        | 139               | ND            | ND                 |
|                  |                   | F        | R                        | 175               | ND            | ND                 |
| Phe-334          | Bilayer           | L        | R                        | 62                | ND            | ND                 |
| Pre-327          | Bilayer           | A        | R                        | 70                | ND            | ND                 |
|                  |                   | S        | R                        | 69                | ND            | ND                 |
|                  |                   | L        | R.C.                     | 6                 | 109           | 2.8/31.5           |
| Thr-320          | Bilayer           | S        | R                        | 143               | ND            | ND                 |
|                  |                   | P        | R.C.                     | 15                | 154           | 0.5/4.2            |
| Val-331          | Bilayer           | M        | R                        | 150               | ND            | ND                 |
|                  |                   | L        | R                        | 111               | ND            | ND                 |
|                  |                   | M        | R                        | 86                | ND            | ND                 |
| Phe-324          | Bilayer           | L        | R                        | 117               | ND            | ND                 |
|                  |                   | W        | R                        | 92                | ND            | ND                 |
|                  |                   | C        | R                        | 105               | ND            | ND                 |
| Ile-317          | Bilayer           | M        | R                        | 122               | ND            | ND                 |
|                  |                   | V        | R                        | 108               | ND            | ND                 |

**a** The residues in TM-10 are arranged according to our putative model that is described under “Discussion.”

**b** R, red phenotype which indicates very good lactose fermentation; R.C., red center which indicates low but significant fermentation; W, white which indicates poor fermentation.

**c** Downhill lactose transport was carried out as described under “Materials and Methods.” The results are expressed as the percentage of the initial rate of wild-type activity.

**d** The amount of lactose permease proteins was determined by Western blotting analysis as described under “Materials and Methods.” The values are expressed as the percentage of mutant permease compared with the wild-type protein.

**e** Km and Vmax values were determined as described under “Materials and Methods.” The wild-type Km was 0.3 mM and the Vmax was 126.4 nmol of lactose/min/mg protein.

**f** ND, not determined.
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| Plasmid | Phenotype of insertional mutants |
|---------|---------------------------------|
|         | 1% Lactose | 0.2% Lactose |
| Wild-type | Red | Red |
| Leu-315' | Red | Red |
| Val-318' | Red | Red |
| Leu-320' | Red | White |
| Val-323' | Red | White |
| Leu-326' | White | White |
| Val-329' | White | White |
| Leu-332' | White | White |
| Leu-335' | White | White |

*The phenotype was determined on 1% and 0.2% lactose MacConkey plates. A red phenotype indicates the ability to ferment lactose whereas white indicates a lack of significant fermentation.

**Activity of Insertional Mutants**

![Activity of insertional mutants](Image)

**FIG. 2. Activity of insertional mutants.** The uptake of [14C]lactose was measured as described under "Materials and Methods." Downhill lactose uptake was carried out in strain HS4006F' (23) containing the lacY plasmids described within the figure. Uphill accumulation was carried out in strain T184, that is β-galactosidase negative. The percentage of downhill and uphill lactose transport activity of each of the insertional mutants is compared to the wild-type at the 1-min time point.

Our results suggest that the helical face containing Glu-325, Leu-318, Leu-329, His-322, Val-315, Cys-333, Val-326, and Lys-319 is likely to be the channel lining domain for a number of reasons. First, this face of the helix contains three amino acids with ionizable side chains. Second, many previous studies have indicated that amino acids on this face may be involved in binding and/or H+ recognition (23–28). This observation is consistent with the current study since detrimental mutations along this face tended to have a substantially higher Km for lactose transport. Even so, it is not surprising that mutations along this putative channel lining face had variable effects. The substitution of bulky amino acids at some positions may inhibit lactose transport by sterically blocking the channel or by affecting residues that are directly involved in substrate recognition and transport. However, substitution by bulky amino acids at other positions may not inhibit lactose transport because there may be sufficient space in the lumen of the channel to accommodate the increase in side chain volume without affecting the normal substrate recognition and transport. With regard to the putative channel lining face in Fig. 3, it is interesting to comment on the location of Glu-325 that has been implicated to function as a H+ binding site. Our data suggests that Glu-325 is not projecting into the center of the putative channel pathway but rather is displaced toward the edge that interacts with transmembrane helices 8 and 9. This result is consistent with spectroscopic studies that have shown that His-322 (on TM-10) is in close proximity with Glu-269 (on TM-8) and Arg-302 (on TM-9) is close to His-322 and Glu-325 (Refs. 41 and 42).

According to our model, there are several residues in helix 10 (Leu-330, Met-323, Phe-328, Leu-321, and Gly-332) that are potentially interacting with other transmembrane segments. In cases where the wild-type residue is already bulky (Phe-328, Leu-321, and Leu-330), amino acid substitutions were tolerated in these regions. An exception is the L330P mutation which is expected to alter helix topology. By comparison, the Met-323 and Gly-332 positions were relatively intolerant of amino acid substitutions. For example, all four of the mutations at position 332 significantly reduced the downhill lactose transport activity. Since glycine is the smallest amino acid, it would seem reasonable to suggest that substitution with another amino acid which has a larger volume may be deleterious if the glycine is involved in helical interactions. It is also noteworthy that the Western results described in Table I indicated that substitutions in these interhelical regions are...
more likely to lower the protein level of the lactose permease compared with substitutions in the putative channel lining or bilayer face of helix 10 (see Table II).

Insertion of amino acids at the periplasmic edge of transmembrane helix 10 were less deleterious than insertions in the center or near the cytoplasmic edge of the helix. Analysis of the insertional mutants indicates the importance of the spatial relationship between the amino acids. For example, pLeu-326, which is adjacent to a functionally critical amino acid, glutamate 325, has a white phenotype on 1% lactose MacConkey plates and is totally inactive, whereas Leu-315, has a white phenotype on 1% lactose MacConkey which is adjacent to a functionally critical amino acid, glutamate. Compared with substitutions in the putative channel lining or more likely to lower the protein level of the lactose permease.

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