Evidence That Transmembrane Segment 2 of the Lactose Permease Is Part of a Conformationally Sensitive Interface between the Two Halves of the Protein

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A conserved motif, GXX(D/E)(R/K)XG(R/K)(R/K), is found in a large group of evolutionarily related membrane proteins involved in the transport of small molecules across the membrane. This motif is located within the cytoplasmic side of transmembrane domain 2 (TM-2) and extends through the hydrophilic loop that connects transmembrane domains 2 and 3. The motif is repeated again in the second half of the protein. In a previous study concerning the loop 2/3 motif (Jessen-Marshall, A. E., Paul, N. J., and Brooker, R. J. (1995) J. Biol. Chem. 270, 16251–16257), it was shown that the conserved aspartate at the fifth position in the motif is critical for transport activity since a variety of site-directed mutations were found to greatly diminish the rate of transport. In the current study, two of these mutations, in which the conserved aspartate was changed to threonine or serine, were used as parental strains to isolate second site suppressor mutations that restore transport function. A total of 10 different second site mutations were identified among a screen of 19 independent mutants. One of the suppressors was found within loop 1/2 in which Thr-45 was changed to arginine. Since the conserved aspartate and position 45 are at opposite ends of TM-2, these results suggest that the role of the conserved aspartic acid residue in loop 2/3 is to influence the topology of TM-2. Surprisingly, the majority of suppressor mutations were found in the second half of the protein. All of these are expected to alter helix topology in either of two ways. Some of the mutations involved residues within transmembrane segments 7 and 11 that form a scaffolding function. The model also suggests that the effects of these suppressor mutations are to alter the helical topologies in the second half of the protein to facilitate a better interaction with the first half. Overall, these results are consistent with a transport model in which TM-2 acts as an important interface between the two halves of the lactose permease. According to our tertiary model, this interaction occurs between TM-2 and TM-11.

The uptake of a variety of solutes, including sugars, amino acids, and inorganic ions, is mediated by integral membrane proteins known as cation/substrate cotransporters or symporters. The lactose permease of Escherichia coli has provided a model system in which to investigate the molecular mechanism of symport (1, 2). This protein is found in the E. coli cytoplasmic membrane and couples the transport of H+ and lactose. From the cloning and nucleotide sequencing of the lacY gene, the lactose permease contains 417 amino acids with a molecular weight of 46,504 (Refs. 3 and 4). Hydrophathy plots as well as genetic studies are consistent with a secondary structural model in which the protein contains 12 hydrophobic segments that traverse the membrane in an α-helical manner (5–7).

Evolutionary analyses have indicated that the lactose permease is a member of a large group of solute transporters that are homologous to each other (8–10). This superfamily includes proteins that transport substrates such as sugars, K+ or other ions, and antibiotics. Interestingly, the superfamily includes not only symporters but also uniporters and antiporters. It has been referred to as the uniporter-symporter-antiporter (USA) superfamily and as major facilitator superfamily (10, 11). Secondary structural models for members of the USA superfamily are commonly found to predict a membrane protein with 12 transmembrane segments traversing the membrane in an α-helical manner. Furthermore, it has been proposed that this arrangement of 12 segments has arisen by a gene duplication/fusion event of an ancestral gene encoding a protein with six transmembrane segments (12). Based on the structural characteristics of the members of the USA superfamily, we have recently proposed a three-dimensional model that describes the orientation of the 12 transmembrane segments (11). In this model, eight of the transmembrane segments act as channel-lining domains while the other four perform a scaffolding function. The model also suggests that the two halves of the protein are folded in a similar fashion and that they associate with each other in a rotationally symmetric manner.

A conserved motif, GXX(D/E)(R/K)XG(R/K)(R/K), is found in all members of the USA superfamily (8–10). This motif is located within the cytoplasmic side of transmembrane segment 2 and extends through the hydrophilic loop that connects TM-21 and TM-3. It is repeated again in loop 8/9. In our previous study with the lactose permease and also in studies with the tetracycline antiporter, it has been found that the negative charge in the fifth position of the motif is critical with regard to transport function (13, 14). In the lactose permease, substitutions of alanine, serine, threonine, tyrosine, asparagine, histidine, and even glutamate exhibited transport activities that
were less than 5% the wild-type rate. In the current study, the serine and threonine mutants were used as parental strains to identify second site suppressor mutations that restore transport function.

**MATERIALS AND METHODS**

Reagents—Lactose (O-β-D-galactopyranosyl-1,4)-α-D-glucopyranose) and melibiose (O-α-D-galactopyranosyl-1,6)-α-D-glucopyranose) were purchased from Sigma. [14C]Lactose was purchased from Amersham Corp. The remaining reagents were analytical grade.

Bacterial Strains and Methods—The bacterial strains and plasmids are described in Table I. Plasmid DNA was isolated using PERFECT-prep Plasmid DNA kit obtained from 5 Prime Corp. The remaining reagents were introduced into the appropriate bacterial strain by the CaCl2 transformation procedure of Mandel and Higa (15).

Stock cultures of cells were grown in YT media (16) supplemented with tetracycline (0.01 mg/ml). For transport assays, cells were grown to midlog phase in YT media containing tetracycline (0.005 mg/ml) and 0.25 mM isopropyl-1-thio-β-D-galactopyranoside to induce the synthesis of the lactose permease.

**RESULTS**

Isolation of Second Site Suppressor Mutants—In our previous work, it was shown that the aspartate residue at position 68 in the lactose permease is important for transport activity (13). As shown in Fig. 1, this aspartate is located in the hydrophilic loop region connecting TM-2 and TM-3. Since this residue is conserved in a variety of permeases that transport different substrates such as sugars, antibiotics, and Kreb’s cycle intermediates, it seems unlikely that it is directly involved with substrate recognition. Additionally, it may be important in maintaining protein conformation and/or facilitating conformational changes. Along these lines, it is possible that the negative charge interacts with other residues within cytoplasmic hydrophilic loops to maintain protein conformation. A second possibility is that the negative charge is important in position-
Suppressor Mutants in the GXXX(D/E)(R/K)XG(R/K)(R/K) Motif

...ing TM-2 and/or TM-3 within the tertiary structure of the protein or within the phospholipid bilayer.

To explore the role of the conserved aspartate in the loop 2/3 motif, the Ser-68 and Thr-68 mutants were used as parental strains to isolate second site suppressor mutations that restore transport activity. These two parental strains were chosen because the serine and threonine codons require a two-base change back to the original aspartate codon. Therefore, it seemed likely that the use of these strains would favor our ability to identify single base substitutions at a “second site” within the gene that restores transport function.

The Ser-68 and Thr-68 strains were streaked on 1% melibiose MacConkey plates as a method to identify suppressor mutants that restore activity. It should be noted that the wild-type lactose permease is able to transport melibiose (an α-galactoside). Therefore, when streaked onto 1% melibiose MacConkey plates, a strain containing the wild-type permease forms red colonies since it can transport (and ferment) the sugar. In contrast, the Ser-68 and Thr-68 strains were observed to form white or pink colonies (see Table II). After several days, “red flecks” became apparent within the primary streak. These red flecks were restreaked to isolate individual red colonies. From 19 independent red colonies, the plasmid DNA containing the lacY gene was then isolated and transformed into a lacZ− strain. In all cases, the transformants exhibited a red phenotype on both 1% lactose and 1% melibiose MacConkey plates (see Table II).

DNA Sequencing of the Suppressors—To identify the mutations that suppressed the defect in lactose permease function, the mutants were sequenced within the entire lacY gene. They retained the original mutation (Ser-68 or Thr-68) in the loop 2/3 motif. In addition, they all had second site mutations within the lacY coding sequence. These mutations are summarized in Table III. It was striking that the mutations fell into three categories. First, one of the mutants involved a nonconservative substitution in loop 1/2 (i.e. Thr-45 → Arg). Since TM-2 lies between loop 1/2 and loop 2/3, a reasonable explanation for this result is that the mutation in loop 1/2 restores a correct topology in TM-2.

Surprisingly, all of the other mutations were in the second half of the protein. These mutants fell into two other categories. Several of the mutants were found within transmembrane domains (C234, C234F, Q241L, F247V, and S566F). All of these mutations involved a dramatic change in side chain volume either by gaining or losing a large, hydrophobic side chain. Alternatively, other mutants were found at the edges of transmembrane domains or within hydrophilic loop regions. In all of these cases, the mutations are expected to be highly disruptive with regard to protein structure (G257D, A369P, V367E, and a 5-codon insertion). The most unusual example was a 5-codon insertion in loop 11/12 that arose as a small duplication of codons 372 through 376 and inserted between codons 376 and 377. Overall, both categories of mutations in the second half of the permease are expected to alter transmembrane topology. Assuming that the first site mutation disrupts TM-2, the suppressor mutations in the second half of the protein may act to restore the proper topology of helix 2 or create a more favorable interface between helix 2 and the second half of the protein. Interestingly, according to our tertiary model, TM-2 is expected to interact with TM-11. Most of the second site suppressors were found within or near TM-11 or were side chain volume changes within neighboring TM-7.

In Vitro Galactoside Transport—The experiments of Table II suggest that the suppressor mutations result in a substantial restoration of transport activity. In the experiments of Figs. 2 and 3, in vitro transport assays were carried out to provide a more quantitative description of the uptake process. In the 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. Therefore, under these conditions, lactose transport is “downhill” or with its concentration gradient (18). In the uphill assays of Fig. 3, the plasmids were transformed into a β-galactosidase negative strain. As expected from the results of Table II, the suppressor mutations substantially increased the level of lactose transport compared with the Ser-68 and Thr-68 parental strains. In downhill transport assays, the parents had transport values that were less than 4% of that of the wild type, while the suppressor mutants ranged from 

| Strain | Number of isolates | Second site codon change | Amino acid substitution | Location in secondary model | Level of expression |
|--------|-------------------|--------------------------|-------------------------|-----------------------------|-------------------|
| pD68S/T45R | 1 | ACG → AGG | Thr-45 → Arg | Loop 1/2, near TM-2 | 92 ± 5 |
| pD68X/C234W | 4 | TGC → TGG | Cys-234 → Trp | TM-7, central region | 51 ± 3 |
| pD68X/C234F | 3 | TGC → TTC | Cys-234 → Phe | TM-7, central region | 63 ± 19 |
| pD68T/Q241L | 1 | CAA | Gin-241 → Leu | TM-7, central region | 104 ± 10 |
| pD68T/G257D | 2 | GTG → CAT | Phe-247 → Val | TM-7, near periplasm | 102 ± 2 |
| pD68S/S366F | 2 | TAT → TTG | Ser-366 → Phe | Loop 7/8, near TM-8 | 86 ± 10 |
| pD68T/V367E | 1 | GTA → GAA | Val-367 → Glu | Loop 11/12, very near TM-11 | 99 ± 6 |
| pD68T/A369P | 2 | GCC → CCG | Ala-369 → Pro | Loop 11/12, near TM-11 | 57 ± 17 |
| pD68T/ins5 | 1 | GGC → CGC | Val-367Ins5 | Loop 11/12 | 43 ± 16 |

a The suppressor strains were identified as having a red phenotype of 1% melibiose MacConkey plates.

b The amount of permease protein expressed as the percentage of wild type ± S.E.

c X indicates that the same second site mutation was obtained from both the Ser-68 and Thr-68 parental strains.

d The duplicated codons are Met-Tyr-Glu-Ser-Ile.
from 15 to 75%. Likewise, in the uphill transport assays, the suppressor mutants displayed steady state accumulation values that were at least 50% of the wild-type level. Certain mutants, such as the T45R and Q241L strains, even had uphill accumulation values that were significantly higher than wild type. Taken together, the results of Figs. 2 and 3 indicate that the suppressor mutations restore a substantial amount of transport activity.

**DISCUSSION**

The conservation of a motif within a large superfamily is indicative of an important functional and/or structural role. The results of the current study provide evidence that the aspartate within the GXXX(D/E)(R/K)XG(R/K)(R/K) motif is important in maintaining the conformation of the permease. In particular, it appears to be very important for the positioning of TM-2 within the tertiary structure of the protein. At the current time, it is not clear how the Ser-68 and Thr-68 substitutions exert an incorrect positioning of TM-2. One possibility is that the absence of an aspartate in loop 2/3 may allow a small perturbation in helix 2 toward the periplasm. If this is the case, the suppressor mutations may circumvent this perturbation in different ways. The T45R mutation introduces a positive charge in loop 1/2 that may counteract the movement of helix 2 toward the periplasm. In contrast, second site suppressor mutations in the second half of the protein are expected to exert their effects in a different way. For example, they may promote a stronger interaction between helix 11 and helix 2 that would prevent the repositioning of helix 2 toward the periplasm. This idea is consistent with the observation that most of the suppressors were found within or near TM-11 or were side chain volume changes within neighboring TM-7.

The transport of H+ and lactose via the lactose permease requires conformational changes that promote an interconversion between outwardly accessible and inwardly accessible solute binding sites. Within the entire superfamily, the conservation of the loop 2/3 and 8/9 motifs may be related to their abilities to maintain transporters in a conformationally competent state. In our tertiary model, it is interesting that TM-2 and TM-8 lie at the interface between the two halves of the

**Fig. 2.** Downhill lactose transport by wild-type (W.T.) and mutant strains. The uptake of [14C]lactose was measured as described under "Materials and Methods." Downhill lactose uptake was carried out in strain HS4006/F12Z/2Y containing the lacY plasmids described within the figure.

**Fig. 3.** Uphill lactose accumulation by wild-type (W.T.) and mutant strains. The uptake of [14C]lactose was measured as described under "Materials and Methods." Uphill lactose uptake was carried out in strain T184 containing the lacY plasmids described within the figure.

**Fig. 4.** The three-dimensional arrangement of transmembrane domains was proposed by Goswitz and Brooker (11). The C1 conformation has its solute binding site accessible from the outside, while the same site in the C2 conformation is accessible from the inside. The model suggests that two halves of the protein act as fairly rigid domains that move along the TM-2/TM-11 and TM-8/TM-5 interfaces to convert between the C1 and C2 conformation.
protein. Most of the suppressor mutations are located near the TM-2/TM-11 interface, although one suppressor (i.e., G257D) is expected to be near the TM-8/TM-5 interface. Overall, it is interesting to speculate that these interfaces form two conformationally sensitive sites that are involved in the interconversion between the C1 and C2 conformations (see Fig. 4). According to this model, the interhelical arrangements of TM-2/TM-11 and TM-8/TM-5 form a critical juncture for protein motion. The two halves of the permease are considered to be relatively rigid domains that rotate at these two interfaces.

The motion of proteins is usually facilitated by hinge motions or shearing motions (19). The model of Fig. 4 can be explained by small shear motions at the TM-2/TM-11 and TM-8/TM-5 interfaces. In other proteins that have been crystallized in both opened and closed conformations, shear motions are commonly involved in conformational changes that alter accessibility to the solute binding site (19). The motion does not involve the repacking of interdigitating side chains at the interface (i.e. between α-helices). Instead, the side chains can alter their torsional angles so that they move among conformational states of nearly the same energy without crossing large energy barriers. However, it is important to point out that the amount of rotation that is accomplished by this type of shear motion is relatively small (i.e. typically a maximum of 15°). Without further structural information, it is difficult to assess whether a 15° rotation is sufficient to account for the types of conformational changes described in Fig. 4.

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