Interaction between Residues Glu269 (Helix VIII) and His322 (Helix X) of the Lactose Permease of *Escherichia coli* Is Essential for Substrate Binding

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ABSTRACT: Site-directed and Cys-scanning mutagenesis of the lactose permease of *Escherichia coli* reveals that as few as four residues—Glu269 (helix VIII), Arg302 (helix IV), His322 (helix X), and Glu325 (helix X)—are irreplaceable for coupling substrate and H^+ translocation. Interestingly, the four residues are in close physical proximity, Glu269 interacting with His322 and Arg302 with Glu325. In addition, the substrate translocation pathway is located close to the four residues at the interface between helices V and VIII. To investigate the importance of the four residues and their interactions for substrate binding, mutation Glu269→Asp, Glu269→Gln, Arg302→Ala, Arg302→Lys, His322→Ala, His322→Phe, Glu325→Asp, or Glu325→Gln was introduced into single-Cys148 permease, where the reactivity of Cys with 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS) is blocked by binding of substrate. The double mutants were purified, and the rates of MIANS labeling were measured in the absence or presence of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG), lactose, or galactose at various concentrations. Remarkably, substrate binding by the Glu269 or His322 mutants is abolished or decreased dramatically, while binding by the Arg302 or Glu325 mutants is not altered. The observations are consistent with the notion that the interaction between Glu269 and His322 stabilizes the interface between helices V and VIII and thereby leads to binding of substrate.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic membrane transport protein encoded by the *lacY* gene. The permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for the coupled stoichiometric translocation of β-galactosides and H^+ as a monomer (Kaback et al., 1994; Kaback, 1996). All available evidence indicates that the permease is composed of 12 α-helical rods that traverse the membrane with both N and C termini in the cytosolic side (Figure 1). Moreover, extensive site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) reveal (Kaback, 1996) that as few as 4 out of over 400 residues are irreplaceable with respect to the coupling between lactose and H^+ translocation—Glu269 (helix VIII), Arg302 (helix IV), His322 (helix X), and Glu325 (helix X).

Site-directed excimer fluorescence, site-directed mutagenesis, and second-site suppressor studies have led to a model describing helix packing in which Glu269 (helix VIII) interacts with His322 (helix X) and Arg302 (helix IV) with Glu325 (helix X, Figure 2; Kaback, 1996a). In addition, there are two pairs of interacting Asp and Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] that are not essential for activity. The interactions have been confirmed and extended by engineering divalent metal-binding sites (bis- or tris-His residues) between many of the interacting pairs (Jung et al., 1995; He et al., 1995a,b). Furthermore, site-directed chemical cleavage (Wu et al., 1995), thiol cross-linking experiments, and site-directed spin-labeling studies (Wu et al., 1996) have further extended the model by placing helix V near helices VII and VIII with Cys148 (helix V) directed toward the interface of helices VII and VIII. Finally, helices I−IV, VI, and XII have also been localized by site-directed thiol cross-linking (Wu & Kaback, 1996, 1997; Sun & Kaback, 1997).

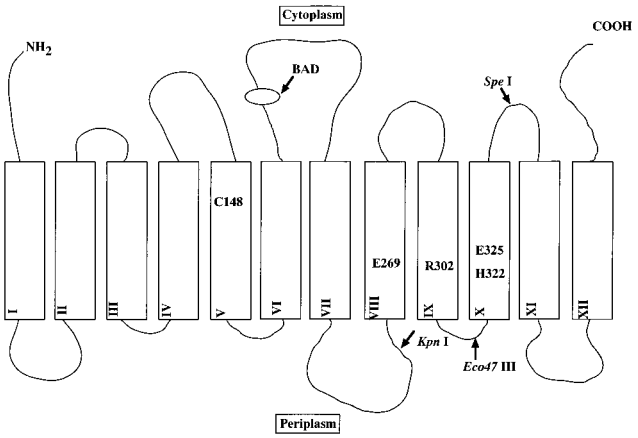

FIGURE 1: Secondary structural model of lac permease. Putative transmembrane helices are shown in boxes. The positions of four essential residues (Glu269, Arg302, His322, and Glu325) and Cys148 are indicated. Also shown are the restriction endonuclease sites used for construction of the mutants and the biotin acceptor domain (BAD) used for purification of the lac permease by the avidin affinity column.

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid; DM, dodecyl β-D-maltoside.

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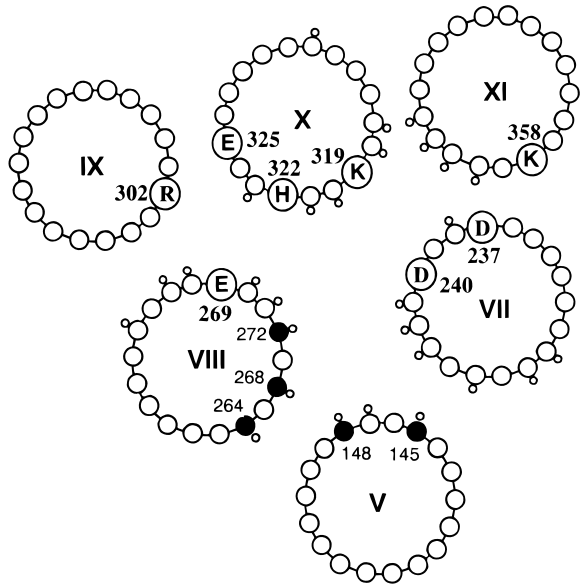

FIGURE 2: Helical wheel model of putative helices V and VII−XI in lac permease viewed from the periplasmic surface. Four essential residues and the Asp-Lys charge pairs are highlighted and indicated by larger circles. The smallest circles represent the single-Cys mutants whose activities are inhibited by *N*-ethylmaleimide (NEM). The mid-sized filled circles represent residues Val264, Gly268, Asn272, Cys148, and Met145 where the NEM reactivity of Cys-replacement mutants is blocked by substrate.

Site-directed mutagenesis and site-directed fluorescence studies demonstrate that Cys148 and Met145 are located in the substrate translocation pathway (Jung et al., 1994; Wu & Kaback, 1994). Cys148 was replaced with a variety of amino acid residues, and the size and polarity of the side chain at this position modify transport activity and substrate specificity. Moreover, permease with a single Cys at position 148 reacts rapidly with 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid (MIANS), a fluorophore whose quantum yield increases dramatically upon reaction with a thiol. Various ligands of the permease block the reaction, and the concentration dependence is commensurate with the affinity of each ligand [i.e., β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) $\ll$ lactose $<$ galactose]. Furthermore, *in situ* sulfhydryl modification of single-Cys148 permease is blocked by addition of β-galactosides, and this residue is accessible from both sides of the membrane (Frillingos & Kaback, 1996). Recent Cys-scanning mutagenesis studies on helix VIII show that the reactivity of single-Cys mutants V264C, G268C, and N272C with *N*-ethylmaleimide (NEM) is decreased dramatically in the presence of TDG, indicating that the face of helix VIII where the three residues are located is also probably part of the substrate translocation pathway (Frillingos & Kaback, 1997; Frillingos et al., 1997). Since this face of helix VIII is adjacent to Cys148 and Met145, it was proposed that at least a portion of the substrate translocation pathway lies at the interface between helices V and VIII.

According to the proposed helix packing model (Figure 2), it is clear that substrate-induced structural changes at the interface between helices V and VIII will be transmitted through the network of interacting residues to the interface between helices VIII, IX, and X, where the four irreplaceable residues reside. Conversely, changes in the interactions between the four essential residues will be transmitted to the interface between helices V and VIII, altering substrate interaction and therefore active transport. To test the possibility that the interaction between Glu269 and His322 or between Arg302 and Glu325 is important for substrate binding, mutation E269D, E269Q, R302A, R302K, H322A, H322F, E325D, or E325Q was introduced into single-Cys148 permease. The double mutants were then purified, and MIANS reactivity was measured in the absence or presence of various ligands. The results demonstrate that mutant E269D, E269Q, or H322A no longer binds substrate, and H322F binds TDG with 30-fold lower affinity. On the other hand, mutant R302A, R302K, E325D, or E325Q retains the ability to bind substrate normally. The observations are consistent with the notion that the interaction between Glu269 and His322 stabilizes the interface between helices V and VIII and thereby leads to binding of substrate.

MATERIALS AND METHODS

Materials. Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. MIANS was from Molecular Probes Inc. All restriction endonucleases, T4 DNA ligase, and Vent^{r}™ DNA polymerase were from New England Biolabs, Beverly, MA. Sequenase was from United States Biochemical, Cleveland, OH. All other materials were reagent grade and obtained from commercial sources.

Plasmid Construction. Mutant permease R302A/C148, R302K/C148, H322A/C148, H322F/C148, E325A/C148, or E325D/C148 was constructed by individually introducing mutation R302A, R302K, H322A, H322F, E325A, or E325D into plasmid pT7-5/Cys148-L6XB (encoding single-Cys148 permease with a *Klebsiella pneumoniae* biotin acceptor domain in loop VI/VII) by oligonucleotide-directed, site-specific mutagenesis using a two-stage polymerase chain reaction (PCR). The PCR products were digested with *Kpn*I and *Spe*I (Figure 1) and ligated into similarly treated pT7-5/Cys148-L6XB. Mutant E269D/C148 or E269Q/C148 was constructed by restriction fragment replacement of the DNA fragment encoding E269D or E269Q (Ujwal et al., 1994) into pT7-5/Cys148-L6XB using the *Kpn*I and *Eco*47III restriction sites. Mutations were verified by sequencing the length of the PCR-generated or replacement segment through the ligation junctions in double-stranded DNA using the dideoxynucleotide termination method (Sanger et al., 1977) and synthetic sequencing primers, after alkaline denaturation (Hattori & Sakaki, 1986).

Purification of Mutant Lac Permeases. E. coli T184 (*lacZ*$^{-}$*Y*$^{-}$; Teather et al., 1980) was transformed with plasmid encoding a given mutant. Cultures (6 L) were grown at 37 °C in LB broth with streptomycin (10 μg/mL) and ampicillin (100 μg/mL) and induced with 0.5 mM IPTG for 3 h when the OD_{600} reached 0.8. Cells were harvested and disrupted by passage through a French pressure cell. A membrane fraction was isolated by centrifugation and extracted with 2% dodecyl β-D-maltoside (DM), and permease was purified by affinity chromatography on immobilized monomeric avidin as described (Wu & Kaback, 1994). The purity of each preparation was assessed by electrophoresis on sodium dodecyl sulfate−12% polyacrylamide gels (Laemmli, 1970), followed by silver staining.

Protein Determination. Protein was assayed by using a Micro BCA protein determination kit (Pierce Inc., Rockford, IL).

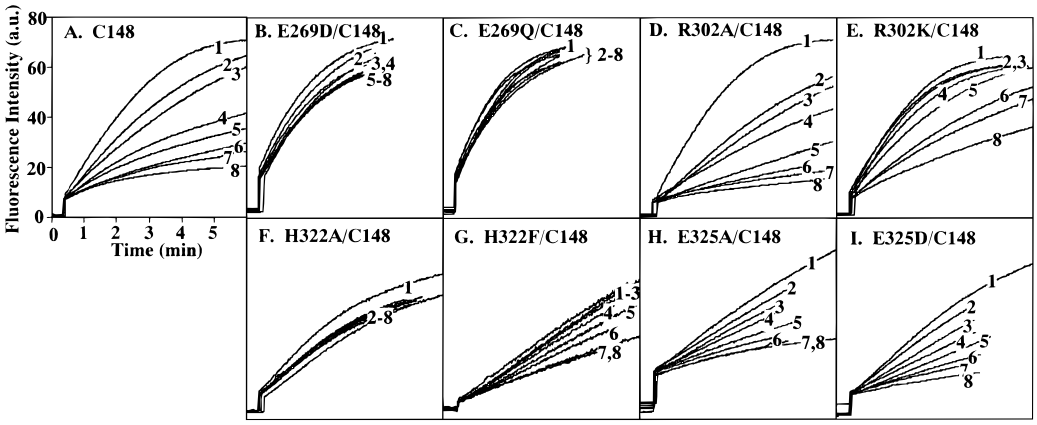

FIGURE 3: Time course of MIANS labeling of mutant permeases in the presence of TDG. Affinity-purified mutant lac permease at a concentration of 15−20 μg/mL was preincubated with a given concentration of TDG in reaction mixtures containing 0.5 mL of 50 mM KP_i (pH 7.5)/100 mM NaCl/0.01% DM. MIANS labeling was initiated by adding MIANS to a final concentration of 4 μM, and fluorescence was recorded continuously at 420 nm as a function of time (excitation, 330 nm) as described under Materials and Methods. Addition of ligands was as follows: (curve 1) no addition; (curves 2−8, respectively) 0.1, 0.2, 0.5, 1, 2.5, 5, or 10 mM TDG was added prior to addition of MIANS.

Labeling of Purified Mutant Lac Permease with MIANS and Fluorescence Measurements. MIANS (Molecular Probes Inc., Eugene, OR) was dissolved in methanol, and the concentration was determined by measuring the absorbance at 322 nm and using an extinction coefficient of 17 000 M^{-1} cm^{-1} (Haugland, 1994). Fluorescence was measured at 30 °C with an SLM 8000C spectrofluorometer (SLM−Amico Instruments Inc., Urbana, IL).

To determine the rate of MIANS reaction with Cys residues in purified mutant permeases, purified permease (15−20 μg/mL) was preincubated with given ligands in an assay buffer containing 50 mM KP_i (pH 7.5)/100 mM NaCl/0.01% DM for 5 min at 30 °C. The reaction was initiated by addition of MIANS to a final concentration of 4 μM from a 2 mM stock solution, and fluorescence was monitored continuously at an emission wavelength at 420 nm (excitation, 330 nm) with 4-nm slits for both excitation and emission.

RESULTS

Purification of Biotinated Mutant Lac Permeases. Biotinated mutant lac permease E269D, E269Q, R302A, R302K, H322A, H322F, E325D, or E325Q in the background of single-Cys148 was purified by monovalent avidin affinity chromatography as described by Wu and Kaback (1994). The proteins were homogeneous as judged by silver-stained sodium dodecyl sulfate−polyacrylamide gels (data not shown).

Effect of TDG on MIANS Labeling of Mutant Permeases. To test the effect of mutation E269D, E269Q, R302A, R302K, H322A, E325D, or E325Q on TDG binding, each mutation was put into single-Cys148 permease, and the rate of MIANS labeling of Cys148 was measured in the presence of various concentrations of TDG. As shown previously (Wu & Kaback, 1994), Cys148 is readily accessible to MIANS (Figure 3, panel A, curve 1). Addition of MIANS to a reaction mixture containing purified single-Cys148 permease in DM results in a rapid and linear increase in fluorescence emission intensity for about 3 min, and the reaction terminates after about 6 min. Preincubation of Cys148 permease with increasing concentrations of TDG (panel A) sequentially decreases the rate of reaction until little, if any, increase in fluorescence is observed at 10 mM TDG.

Strikingly, TDG has essentially no effect on the rate of MIANS labeling of mutant E269D/C148 or E269Q/C148 at any concentration tested, and the rate of the reaction is comparable to that observed for single-Cys148 permease in the absence of ligand (Figure 3, compare panels A, B, and C). Similarly, TDG does not alter the fluorescence increase observed with mutant H322A/C148 (Figure 3, panel F). However, mutant H322F/C148 exhibits decreased MIANS labeling rates in the presence of higher concentrations of TDG (Figure 3, panel G). Remarkably, none of the mutations at positions 302 and 325 alters TDG protection against MIANS labeling of Cys148 (Figure 3, panels D, E, H, and I). In every set of experiments, 100 mM sucrose, which is not a substrate of lac permease, was added as a control for viscosity. The results show that 100 mM sucrose decreases the MIANS labeling rate by about 10−15%.

To quantitate the TDG binding properties of these mutants, the concentration of TDG required to yield half-maximal protection of MIANS labeling for each mutant was determined. Individual MIANS labeling rates were calculated from the linear portions of the fluorescence curves and corrected for nonspecific background labeling by subtracting the labeling rate of Cys148 in the presence of 10 mM TDG. The rates were further normalized for protein concentration. Figure 4 shows the percentage of the MIANS labeling rate of each mutant as a function of TDG concentration relative to the rate of labeling without TDG. The results demonstrate clearly that the mutants are divided into three groups. Mutants R302A/C148, R302K/C148, E325D/C148, or E325A/C148 have an affinity for TDG that is similar to that of single-Cys148 (Table 1; Wu & Kaback, 1994). The second class of mutants includes E269D/C148, E269Q/C148, and H322A/C148, where the MIANS labeling rates do not decrease significantly in the presence of TDG, thereby indicating that the apparent affinity of the mutants for TDG is extremely low. The third type of mutant is H322F/C148, where a TDG concentration of about 10 mM is required to yield half-maximum protection (Table 1), suggesting that mutation H322F decreases the apparent affinity for TDG by about 30-fold. Taken together, the results demonstrate that mutations at positions 269 and 322 abolish or dramatically decrease the affinity for TDG, while mutations at positions 302 and 325 have little or no effect.

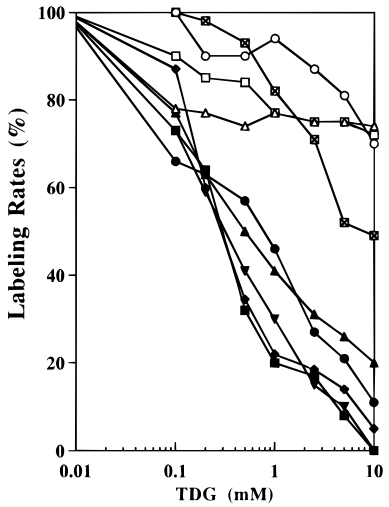

FIGURE 4: Effect of TDG on the rates of MIANS labeling of mutant permeases: ■, single-Cys148; ▲, R302A/C148; ●, R302K/C148; ◆, E325D/C148; ▼, E325A/C148; □, E269D/C148; ○, E269Q/C148; △, H322A/C148; and × in open square, H322F/C148. The labeling rates were obtained from the initial fluorescence increase after addition of MIANS and plotted as a function of TDG concentration (see Materials and Methods). Although not shown, sucrose which is not a substrate of the permease decreases the MIANS labeling rate by 10−15% depending on different mutants at a concentration of 100 mM.

Table 1: Half-Maximal Protection of MIANS Labeling by Substrates[a]

mutants	[TDG] (mM)	[lactose] (mM)
Cys148	0.3	10
148C/302A	0.5	25
148C/302K	0.7	28
148C/325A	0.37	20
148C/325D	0.3	25
148C/269D	>10	>100
148C/269Q	>10	>100
148C/322A	>10	>100
148C/322F	10	>100

[a] The galactose concentrations that are required for half-maximal protection of MIANS labeling are not listed because the highest concentration tested (100 mM) is not enough to yield half-maximal protection except for single-Cys148 where 60 mM is needed (see Figure 6).

Effect of Lactose or Galactose. MIANS labeling rates of each mutant in the presence of various concentrations of lactose or galactose were also determined. The data in Figure 5 and Table 1 show the ability of lactose to block MIANS labeling. As shown for TDG, mutations at 302 or 325 have little effect on lactose "binding", while the mutations at 269 or 322 abolish lactose binding ability. Interestingly, mutant H322F/C148 which binds TDG with low affinity does not bind lactose up to a concentration of 50 mM. The result is consistent with the finding that mutant H322F/C148 has 30-fold lower affinity for substrate relative to single-Cys148 permease (Table 1). On the other hand, lactose decreases the MIANS labeling rate for mutant E269D/C148, E269Q/C148, or H322A/C148 to an extent similar to TDG. The decrease is due largely to a nonspecific increase in viscosity, as shown by addition of sucrose. Finally, the data presented in Figure 6 demonstrate the effect of galactose on MIANS labeling of the mutants. Although the pattern is less distinctive due to the low affinity of the permease for galactose (Wu & Kaback, 1994), it is clear that mutants R302A/C148, R302K/C148, E325A/C148, and E325D/C148 have higher affinity for galactose than mutants E269Q/C148, E269D/C148, H322F/C148, and H322A/C148.

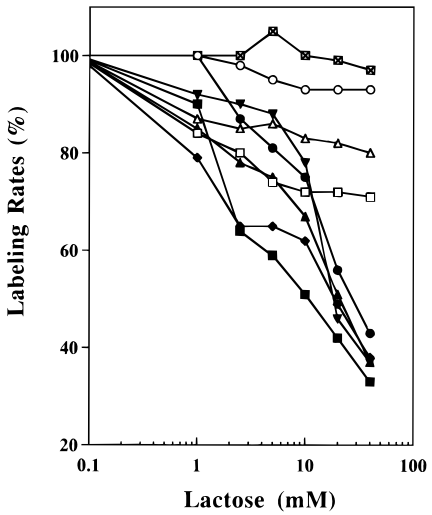

FIGURE 5: Effect of lactose on the rates of MIANS labeling of mutant permeases: ■, single-Cys148; ▲, R302A/C148; ●, R302K/C148; ◆, E325D/C148; ▼, E325A/C148; □, E269D/C148; ○, E269Q/C148; △, H322A/C148; and × in open square, H322F/C148. The rates were calculated as described in the legend for Figure 4.

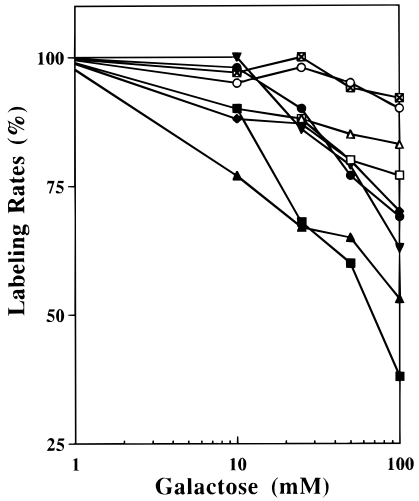

FIGURE 6: Effect of galactose on the rates of MIANS labeling of mutant permeases: ■, Single-Cys148; ▲, R302A/C148; ●, R302K/C148; ◆, E325D/C148; ▼, E325A/C148; □, E269D/C148; ○, E269Q/C148; △, H322A/C148; and × in open square, H322F/C148. The rates were calculated as described in the legend for Figure 4.

DISCUSSION

Recently (Kaback, 1997), a mechanism explaining the coupled translocation of substrate and H^+ by the lac permease of *Escherichia coli* was proposed based on helix packing and a variety of experimental observations. Cys-scanning and site-directed mutagenesis of almost every residue in the permease reveal that as few as four residues are irreplaceable with respect to the coupling between substrate and H^+ translocation—Glu269, Arg302, His322, and Glu325—and the residues are in close proximity, Glu269 interacting with His322 and Arg302 with Glu325. In an adjacent region of the molecule at the interface between helices VIII and V are residues in the substrate translocation pathway—Cys148, Met145, Val264, Gly268, and Asn272. The mechanism of coupling proposed is such that upon ligand binding a structural change at the interface between helices V and VIII

disrupts the interaction between His322 and Glu269, Glu269 displaces Glu325 from Arg302, and Glu325 is protonated. Simultaneously, helix X moves so as to bring protonated Glu325 into contact with the hydrophobic phase of the membrane which markedly increases the pK_a of the carboxylic acid. In order to return to ground state after release of substrate, the Arg302−Glu325 interaction must be reestablished which necessitates loss of H^+ from Glu325.

According to the model, neither His322 nor Glu269 is directly involved in H^+ translocation, but both residues lie close to the interface between helices V and VIII where substrate binding is postulated to occur, and their interaction may act to stabilize this interface. By studying substrate protection of single-Cys148 permease against MIANS labeling, this paper demonstrates that mutants in Arg302 or Glu325 bind TDG, lactose, and galactose essentially normally, while mutants in Glu269 or His322 are markedly defective. The findings suggest that the interaction between Glu269 and His322 may play an important role in maintaining the stability of the substrate translocation pathway and are consistent with the proposed model.

Although mutant permease H322A is defective in substrate binding, mutant H322F maintains the ability to bind substrate TDG with about 30-fold lower affinity than single-Cys148. This result is consistent with the previous finding indicating that various mutants at position 322 are uncoupled, although His322 is probably not directly involved in H^+ translocation (Padan et al., 1985; Püttner et al., 1986; King & Wilson, 1989a,b, 1990; Kaback, 1997). For instance, mutant H322N is defective in all transport modes except downhill influx of sugar without H^+. On the other hand, mutant H322F catalyzes sugar-dependent H^+ transport with low efficiency, and melibiose efflux remains coupled to H^+ translocation. In addition, a double mutant with Val in place of Ala177 and Asn in place of His322 catalyzes lactose-dependent H^+ influx with a stoichiometry close to unity (Brooker, 1990). Taken together, although His322 is irreplaceable with respect to active transport, it does not appear to play a direct role in H^+ translocation. Rather, His322 is important for substrate recognition, acting in concert with Glu269 to stabilize the interface between helices V and VIII, and for coupling conformational changes at the interface between helices V and VIII to the interface between helices IX and X.

Although mutants with Asp, Gln, or Cys in place of Glu269 do not catalyze active transport of lactose, equilibrium exchange, counterflow, and facilitated influx or efflux down a concentration gradient (Ujwal et al., 1994), E269D permease accumulates TDG in a partially uncoupled fashion with an increase in H^+/TDG stoichiometry. Superficially, the result seems contradictory with the observation presented here that mutant E269D is defective in TDG binding. However, the discrepancy may be due to the fact that the previous experiments were carried out with whole cells expressing mutant E269D in the wild-type background, while the binding studies described here were performed with mutant E269D/Cys148 in the Cys-less background purified in detergent DM. In any event, the studies, together with other evidence (Franco & Brooker, 1994; Ujwal et al., 1994), indicate that Glu269 plays an essential role in conformational coupling and stabilization of the interface between helices V and VIII, but is not directly involved in H^+ translocation.

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