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The role of helix VIII in the lactose permease of *Escherichia coli*: II. Site-directed sulfhydryl modification

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Abstract

Cys-scanning mutagenesis of putative transmembrane helix VIII in the lactose permease of *Escherichia coli* (Frillingos S, Ujwal ML, Sun J, Kaback HR, 1997, *Protein Sci* 6:431–437) indicates that, although helix VIII contains only one irreplaceable residue (Glu 269), one face is important for active lactose transport. In this study, the rate of inactivation of each N-ethylmaleimide (NEM)-sensitive mutant is examined in the absence or presence of β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG). Remarkably, the analogue affords protection against inactivation with mutants Val 264 → Cys, Gly 268 → Cys, and Asn 272 → Cys, and alkylation of these single-Cys mutants in right-side-out membrane vesicles with [^{14}C]NEM is attenuated by TDG. In contrast, alkylation of Thr 265 → Cys, which borders the three residues that are protected by TDG, is enhanced markedly by the analogue. Furthermore, NEM-labeling in the presence of the impermeant thiol reagent methanethiosulfonate ethylsulfonate demonstrates that ligand enhances the accessibility of position 265 to solvent. Finally, no significant alteration in NEM reactivity is observed for mutant Gly 262 → Cys, Glu 269 → Cys, Ala 273 → Cys, Met 276 → Cys, Phe 277 → Cys, or Ala 279 → Cys. The findings indicate that a portion of one face of helix VIII (Val 264, Gly 268, and Asn 272), which is in close proximity to Cys 148 (helix V), interacts with substrate, whereas another position bordering these residues (Thr 265) is altered by a ligand-induced conformational change.

Keywords: active transport; bioenergetics; Cys modification; Cys replacement; ligand binding

In the companion paper (Frillingos et al., 1997), Cys-scanning mutagenesis was employed to examine the role of putative transmembrane helix VIII in the lactose permease of *Escherichia coli*. Residues were found on one face of putative helix VIII where Cys-replacement followed by NEM treatment inactivates the permease. The face also contains Glu 269, a residue that cannot be replaced without abolishing active lactose transport (Ujwal et al., 1994; Franco & Brooker, 1994). According to the recently-formulated packing model of helices V and VII–XI (Wu et al., 1995b, 1996), part of this face of helix VIII is close to residues Arg 302, His 322, and Glu 325, which are essential for lactose/H^+ symport (see Kaback, 1987, 1990), and another part is close to Cys 148 and Met 145, which interact directly with substrate (Jung et al., 1994b; Wu & Kaback, 1994) (see Fig. 5). The observations imply that some of the NEM-sensitive positions in helix VIII may interact directly with substrate (sugar or H^+) and/or be involved in structural changes associated with permease turnover. In order to investigate these possibilities, the effect of ligand binding on reactivity of the single-Cys permease mutants was studied by using both transport assays and in situ site-directed sulfhydryl modification with [^{14}C]NEM (Frillingos & Kaback, 1996b). The results indicate that Val 264, Gly 268, and Asn 272 in helix VIII are in contact with substrate, whereas a nearby residue, Thr 265, is effected by a conformational alteration elicited by substrate binding.

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Abbreviations: lac, lactose; NEM, N-ethylmaleimide; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside; MTSES, methanethiosulfonate ethylsulfonate; IPTG, isopropyl 1-thio-β,D-galactopyranoside; C-less permease, functional lactose permease devoid of Cys residues; DTT, dithiothreitol; PMS, phenazine methosulfate; RSO, right-side-out; KP_i, potassium phosphate; DM, *n*-dodecyl-β,D-maltopyranoside.

Results

Effect of TDG on inactivation of NEM-sensitive mutants

As shown in Figure 7 of the companion paper (Frillingos et al., 1997), the NEM-sensitive Cys-replacement mutants lie on a face of helix VIII that lines part of an interface between helices V, VII, VIII, IX, X, and XI. In order to understand the role of these residues more thoroughly, the rate of NEM-inactivation of transport by each Cys-replacement mutant in the absence or presence of the high-affinity substrate TDG was examined (Fig. 1). At a final concentration of 2 mM NEM, T265C or G268C permease is inactivated at a rate comparable to that of wild-type permease

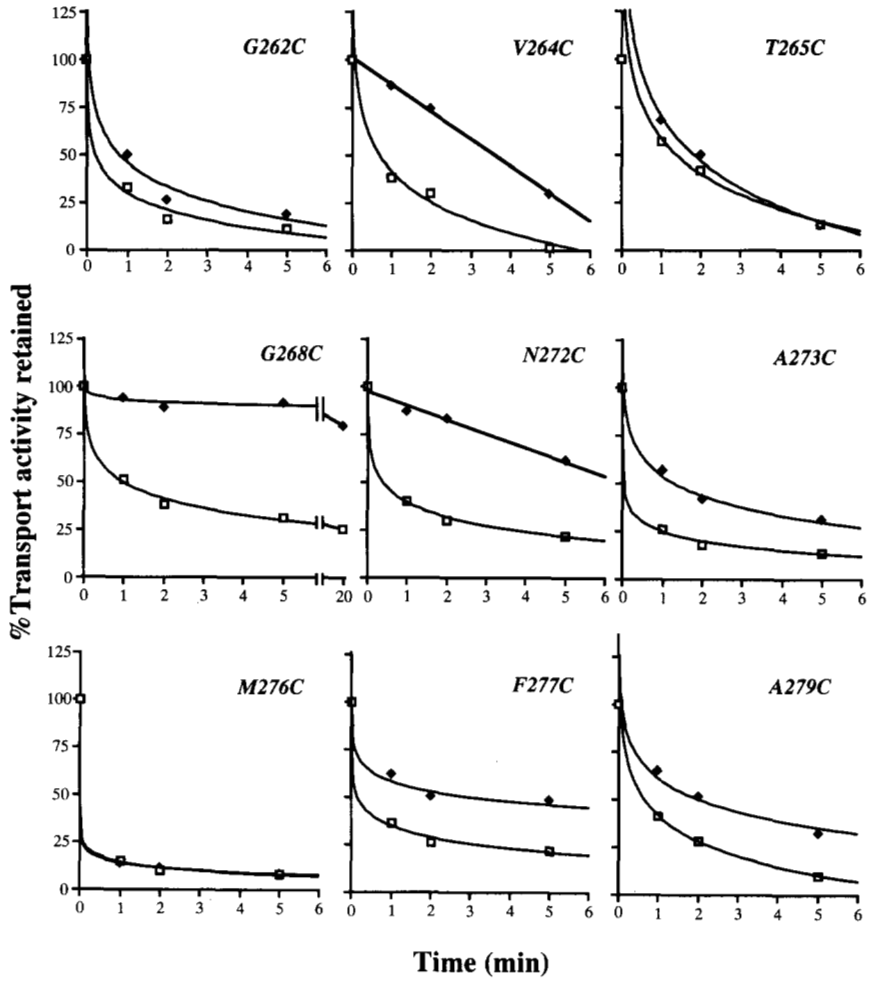

Fig. 1. Effect of TDG on the rate of NEM inactivation of lactose transport by single Cys-replacement mutants in helix VIII. *E. coli* T184 harboring pT7-5 encoding given single-Cys mutants were incubated with 2 mM NEM for the times indicated in the absence (□) or presence (♦) of 10 mM TDG as described (Frillingos et al., 1997). Reactions were terminated by addition of 20 mM DTT (final concentration), and the cells were washed extensively with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and resuspended at a final concentration of 0.7 mg protein/mL in the same buffer. Samples were then assayed for initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971).

($t_{1/2}$ ca. 1 min; Viitanen et al., 1986). The other seven mutants exhibit higher rates of inactivation, with M276C permease exhibiting the greatest sensitivity. In the presence of TDG (10 mM), NEM inactivation is markedly attenuated with mutants V264C, G268C, and N272C, which exhibit $t_{1/2}$ values of approximately 4 min, >20 min, and 6 min, respectively. Less pronounced protection is observed with mutants A273C, F277C, and A279C, and no significant protection is observed with mutants G262C, T265C, and M276C.

In situ sulfhydryl modification

In order to examine alkylation of the single-Cys mutants by NEM and the effect of TDG directly, given single-Cys mutations were transferred into pKR35/*lacY*-CXB, thereby placing a biotin acceptor domain at the C terminus of the mutant proteins. The mutants were then expressed in *E. coli* T184 (*lacZ*$^-$*Y*$^-$), RSO membrane vesicles were prepared, and site-directed sulfhydryl modification was studied in situ with [1-^{14}C]NEM (Frillingos & Kaback, 1996b). All ten mutants examined are labeled, and TDG blocks reactivity with mutant V264C, G268C, or N272C (Fig. 2). The results are generally consistent with the transport data presented in Figure 1. No significant TDG effect is observed with mutant G262C, E269C, A273C, M276C, F277C, or A279C. However, the reactivity of T265C permease is stimulated markedly in the presence of ligand, indicating that ligand binding induces a conformational change that enhances the reactivity of the Cys residue at position 265. Similar ligand-induced increases in reactivity have been described previously for single-Cys replacements at positions 315 (helix X; Sahin-Tóth & Kaback, 1993; Jung et al., 1994a; Frillingos & Kaback, 1996b), 28, and 31 (helix I; Wu et al., 1995a).

MTSES accessibility

The membrane-impermeant sulfhydryl reagent MTSES (Akabas et al., 1992; Stauffer & Karlin, 1994) was used to investigate the accessibility of the single-Cys mutants to solvent. Thus, RSO membrane vesicles were pretreated with MTSES, washed free of excess

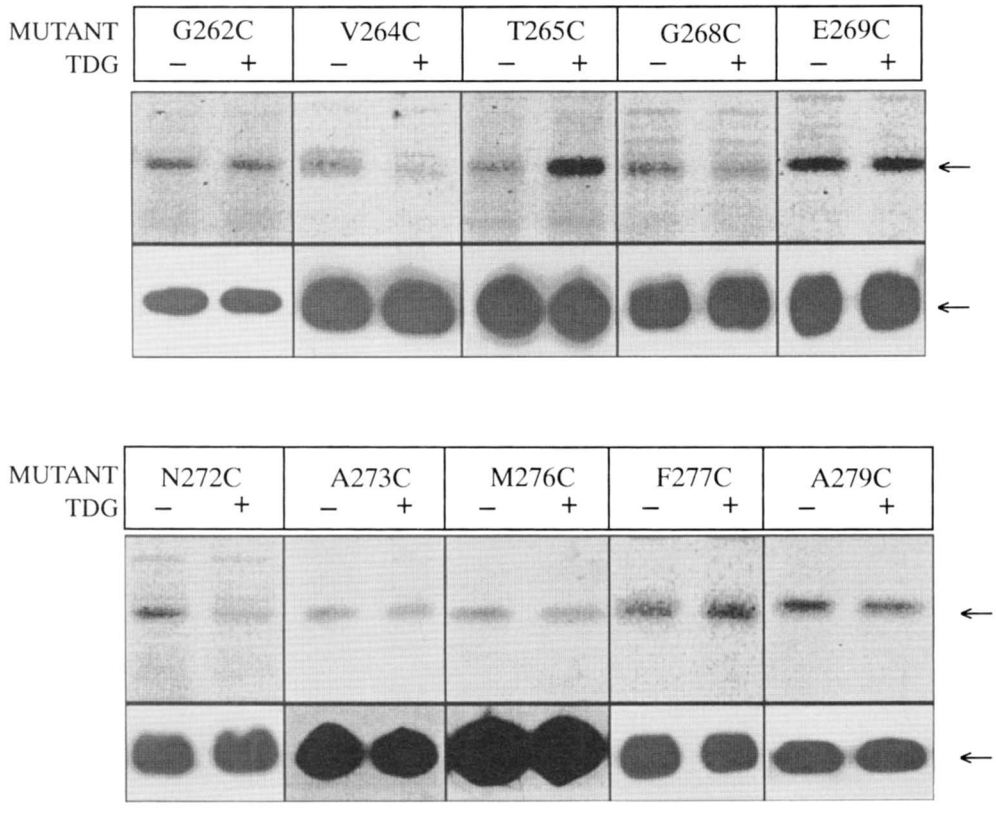

Fig. 2. Effect of TDG on the reactivity of G262C, V264C, T265C, G268C, E269C, N272C, A273C, M276C, F277C, and A279C permeases with [1-^{14}C]NEM in RSO membrane vesicles. RSO membrane vesicles (0.3 mg of protein in 50 μL) prepared from *E. coli* T184 transformed with pKR35/*lacY* CXB encoding the indicated single-Cys mutants were labeled with 0.4 mM [1-^{14}C]NEM in the absence or presence of 10 mM TDG. Reactions were terminated with DTT at 10 min (30 min in the case of G268C), and biotinylated permease was solubilized and purified as described in Materials and methods. Aliquots containing 5 μg of protein were separated by 12% SDS-PAGE, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography (upper panels). A fraction of the protein eluted from the avidin beads (0.5 μg of protein) was analyzed by Western blotting in order to ascertain the amount of permease in the samples incubated without and with TDG (lower panels). Arrows on the right indicate the migration of LacY-CXB permease. In the case of E269C, identical results were also obtained when E269C-L6XB (containing the biotin-acceptor domain in the middle cytoplasmic loop; Jung et al., 1994a) was used in place of E269C-CXB (not shown).

reagent, and incubated with [1-^{14}C]NEM. MTSES reactivity is evaluated from the decrease in the amount of NEM-labeled permease relative to the untreated control (Frillingos & Kaback, 1996b). As shown in Figure 3, NEM labeling of G262C, E269C, or F277C permease is clearly decreased by MTSES. In addition, V264C permease exhibits a significant but less marked decrease in NEM labeling. In contrast, NEM labeling of T265C, G268C, N272C, A273C, M276C, or A279C permease remains essentially unaffected after preincubation with MTSES. Interestingly, NEM labeling of T265C permease is not blocked by MTSES; however, when incubation is performed in the presence of TDG, MTSES reactivity is clearly enhanced (i.e., NEM labeling is attenuated to the level observed without TDG) (Fig. 4). Thus, the accessibility of position 265 to solvent is increased in the presence of ligand.

Under identical conditions, MTSES treatment abolishes NEM labeling of S41C or E255C permeases, which contain a single-Cys replacement in periplasmic loop I/II or VII/VIII, respectively (data not shown), whereas labeling of single-Cys 148 permease, which contains a single Cys disposed toward the cytoplasmic face of helix V, is low (i.e., MTSES treatment inhibits NEM labeling by only ca. 30%; see Fig. 5B in Frillingos & Kaback, 1996b). Therefore, it is likely that MTSES reactivity reflects accessibility to solvent from the periplasmic surface of RSO vesicles.

Discussion

The results presented here and in the companion paper (Frillingos et al., 1997) demonstrate that transmembrane helix VIII of lac permease plays an important role in the mechanism. Specifically, Cys-scanning mutagenesis of helix VIII identified a face that contains Glu 269, one of four irreplaceable residues in the permease, as well as nine other positions where Cys-replacement followed by alkylation with NEM inactivates transport. Each of the ten single-Cys mutants was examined here for reactivity with a membrane-permeant (NEM) and a membrane-impermeant (MTSES) sulfhydryl reagent in RSO vesicles in the absence or presence of TDG, a high-affinity ligand. As schematized in Figure 5, this helical face contains three positions (Val 264, Gly 268, and Asn 272) where TDG protection against NEM labeling of single-Cys replacements is observed, and Cys residues at these positions exhibit low MTSES reactivity. Another portion of the same face contains positions (Gly 262, Glu 269, and Phe 277) where reactivity with [^{14}C]NEM

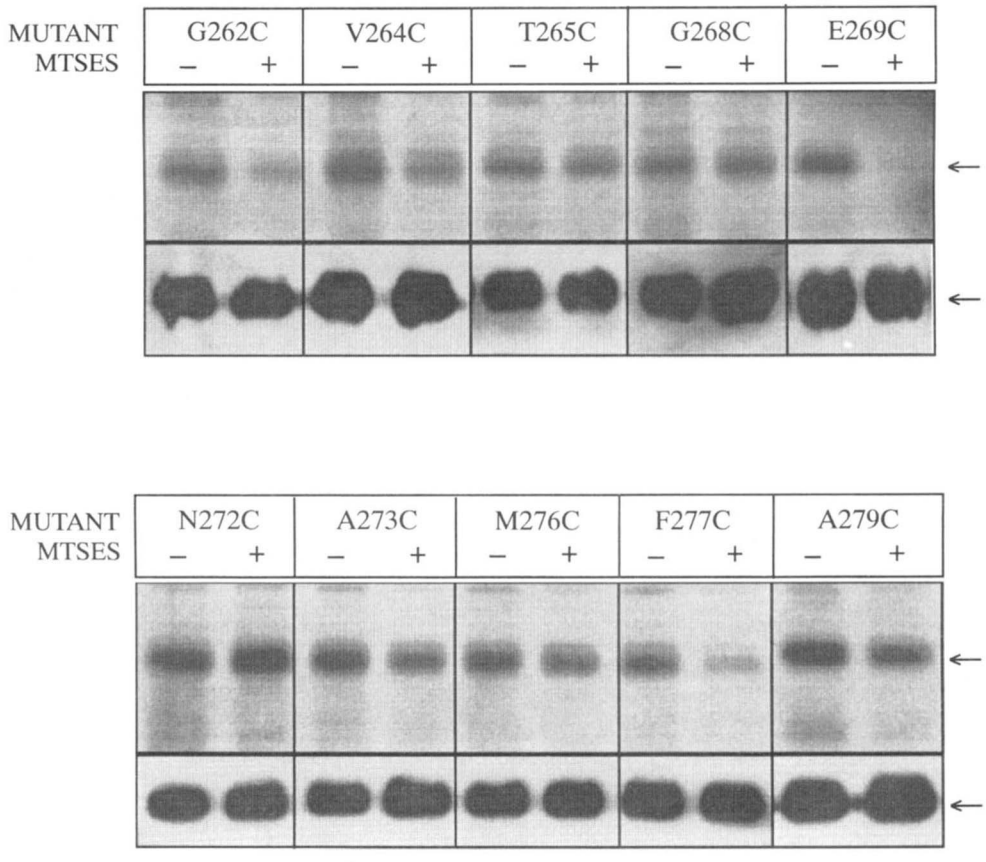

Fig. 3. Reactivity of RSO vesicles containing G262C, V264C, T265C, G268C, E269C, N272C, A273C, M276C, F277C, or A279C permease with MTSES. RSO membrane vesicles (0.3 mg of protein in 50 μL) prepared from *E. coli* T184 transformed with pKR35/*lacY* CXB encoding the indicated single-Cys mutants were incubated with or without 200 μM MTSES at pH 7.5 for 3 min, washed once, resuspended in 50 μL of the same buffer and labeled with 0.4 mM [1-^{14}C]NEM for 30 min. Reactions were quenched with DTT and biotinylated permease was solubilized and purified as described in the Materials and methods. Aliquots containing 5 μg of protein were separated by 12% SDS-PAGE, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography (upper panels). A fraction of the protein eluted from the avidin beads (0.5 μg of protein) was analyzed by Western blotting in order to ascertain the amount of permease in the samples incubated without and with TDG (lower panels). Arrows on the right indicate the migration of LacY-CXB permease. Although not shown, results identical to those seen for E269C-CXB (i.e., abolishment of [1-^{14}C]NEM labeling by MTSES treatment) were obtained with permeases S41C-CXB or E255C-L6XB containing single-Cys replacements in the periplasmic loops I/II or VII/VIII, respectively.

does not change significantly in the presence of TDG, and these positions are readily accessible to MTSES. Finally, position 265 is identified as a position that is sensitive to a conformation change induced by TDG. Thus, NEM labeling of T265C permease is markedly increased in the presence of TDG, and the increase in labeling induced by ligand is blocked by MTSES (Fig. 4).

The observations are particularly important for at least three reasons:

First, the finding that positions 264, 268, and 272 in helix VIII, as well as positions 148 and 145 in helix V (Jung et al., 1994b; Wu & Kaback, 1994), interact with substrate focuses on the interface between helices V and VIII as an important part of the substrate translocation pathway. In this respect, it is noteworthy that these positions are on one face of helix VIII and distributed over two turns of the helix, which is approximately twice the length of a disaccharide. Moreover, the permease seems to behave in a similar manner with galactose as substrate (Frillingos et al., 1997). Therefore, the findings are more consistent with a "pathway" than a fixed binding site.

Second, the observation that a Cys residue at position 265 exhibits a marked increase in reactivity with NEM in the presence of ligand, which is blocked by MTSES, indicates that position 265 has minimal accessibility to solvent in the absence of ligand (no effect of MTSES), but becomes accessible when TDG is bound (increased reactivity with NEM and inhibition by MTSES). The findings are consistent with the idea that ligand binding induces a conformational change in helix VIII, which may initiate turnover. Position 265 lies between Glu 269 and the substrate protectable residues on one face of helix VIII (Fig. 5). Glu 269 is one of four irreplaceable residues in the permease and interacts with His 322 (helix X) (Jung et al., 1993, 1995), another irreplaceable residue, presumably by H-bonding. Therefore, it is tempting to speculate that the ligand-induced conformation change in helix VIII disrupts the interaction between Glu 269 and His 322, which results ultimately in protonation or deprotonation of Glu 325, a residue that is probably involved directly in H^+ translocation (Carrasco et al., 1986, 1989; Frillingos & Kaback, 1996a). It is also noteworthy that, although TDG markedly enhances the NEM reactivity of

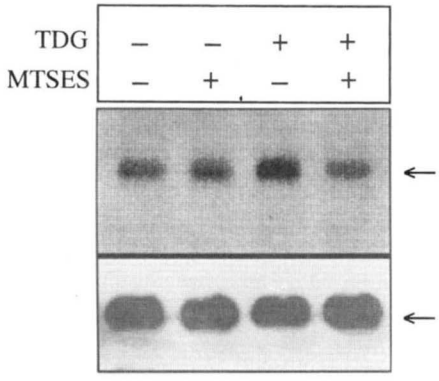

Fig. 4. Effect of TDG on the accessibility of T265C permease to MTSES. RSO membrane vesicles (0.3 mg of protein in 50 μL) prepared from *E. coli* T184 transformed with pKR35/*lacY* CXB encoding single-Cys mutant T265C permease were incubated with or without MTSES (200 μM) for 3 min in the absence or presence of TDG (10 mM) as indicated. Excess TDG and unreacted reagent were removed by extensive washing, and the vesicles were incubated with [1-^{14}C]NEM (0.4 mM) for 30 min. Reactions were quenched with DTT and biotinylated permease was solubilized and purified as described in Materials and methods. Aliquots containing 5 μg of protein were separated by 12% SDS-PAGE, and the [^{14}C]NEM-labeled proteins were visualized by autoradiography (upper panel). A fraction of the protein eluted from the avidin beads (0.5 μg of protein) was analyzed by Western blotting in order to ascertain the amount of permease in the samples incubated without and with TDG (lower panel). Arrows on the right indicate the migration of LacY-CXB permease.

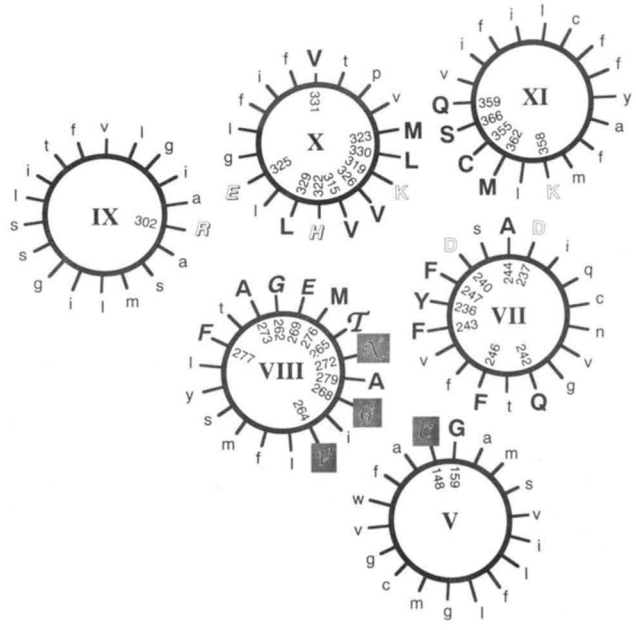

Fig. 5. Helical wheel model of helices V and VII–XI in lac permease highlighting the positions of Cys-replacement mutants examined in this study (see Frillingos et al., 1997 in addition). Bold capital letters represent positions where a Cys-replacement mutant is inactivated by treatment with NEM. Val 264, Gly 268, and Asn 272 (as well as Cys 148 in helix V) are boxed where protection against NEM labeling is observed in the presence of TDG (Figs. 1, 2). Thr 265 is an italicized arabic letter where TDG enhances the reactivity of the Cys-replacement mutant (Figs. 2, 4), and Gly 262, Glu 269, and Phe 277 are italicized block letters where Cys-replacements are accessible to MTSES (Fig. 3).

T265C permease (Fig. 2), NEM inactivation of transport is not accelerated by the analogue (Fig. 1). A possible explanation for the apparent discrepancy is that the transport studies were conducted in the presence of an H^+ electrochemical gradient, whereas the labeling studies were conducted in RSO vesicles in the absence of electron donors.

Third, the observation that single-Cys residues at positions 262, 269, and 277 are accessible to MTSES confirms and extends previous findings (Jung et al., 1995), indicating that Glu 269 is accessible to solvent, although it is within the membrane at about the middle of helix VIII. Thus, replacement of Glu 269 with a His residue leads to formation of a divalent metal binding site due to proximity to His 322, and the apparent pK_a for metal binding approximates that of an unperturbed imidazole. The findings presented here indicate that positions 262 and 277, in addition to position 269, are also accessible to solvent from the surface of the membrane.

Materials and methods

Bacterial strains and plasmids: *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*), *rspL*, *Met*$^-$, *Thr*$^-$, *recA*, *hsdM*, *hsdR/F'*, *lacI*^{q}*O*$^+$*Z*^{D118} (*Y*$^+$*A*$^+$)] (Teather at al., 1980) harboring plasmid pKR35/*lacY*-CXB (Consler et al., 1993), encoding given single-Cys permease mutants containing a biotin acceptor domain at the C terminus was used for expression from the *lacZ* promoter/operator by induction with IPTG.

N-[*ethyl*-1-^{14}C]maleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, Massachusetts). [1-^{14}C]Lactose and [^{125}I] protein A were from Amersham (Arlington Heights, Illinois). Immobilized monomeric avidin was from Pierce (Rockford, Illinois). MTSES was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984) was prepared by BabCo (Richmond, California). All other materials were reagent grade and obtained from commercial sources.

DNA manipulation

For construction of the plasmid pKR35/C-less *lacY*-CXB, a DNA fragment encoding the biotin acceptor domain from a *Klebsiella pneumoniae* oxaloacetate decarboxylase was inserted into the *Esp* I site at the 3'-end of the cassette *lacY* gene (EMBL-X56095) encoding C-less permease (van Iwaarden et al., 1991) cloned into pKR35 as described (Consler et al., 1993). Single-Cys mutants (Frillingos et al., 1997) were transferred into plasmid pKR35/C-less *lacY*-CXB by restriction fragment replacement. Mutations were verified by sequencing the length of the inserted DNA fragment through the ligation junctions using the dideoxynucleotide termination method (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986).

Active lactose transport

E. coli T184 (Z^-Y^-) transformed with each plasmid was grown, induced, and harvested as described previously (Frillingos et al., 1997). Cells were pretreated with NEM (2 mM) at 25 °C in the absence or presence of TDG (10 mM) for a given time, the reactions were stopped by addition of 20 mM DTT, and cells were washed extensively with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$

to remove TDG and excess reagent. Transport of [1-^{14}C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) was assayed in the presence of 20 mM potassium ascorbate and 0.2 mM PMS as described (Konings et al., 1971; Sahin-Tóth & Kaback, 1993).

Membrane preparation

RSO membrane vesicles were prepared from *E. coli* T184 by lysozyme-ethylenediaminotetraacetic acid treatment and osmotic lysis (Kaback, 1971; Short et al., 1975).

[^{14}C]NEM labeling

Modification with [1-^{14}C]NEM was performed essentially as described (Frillingos & Kaback, 1996b). Briefly, RSO membrane vesicles (0.3 mg protein in 50 μL) were incubated in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ containing 0.4 mM [^{14}C]NEM (40 mCi/mmol), in the absence or presence of 10 mM TDG at 25 °C. Labeling was terminated by addition of 5 mM DTT and membranes were treated with 2.0% (w/v) DM for 5 min. The DM extract was then incubated with immobilized monomeric avidin. Biotinylated permease was eluted from the resin with 5 mM *d*-biotin, electrophoresed, and the extent of [^{14}C]NEM labeling was analyzed by autoradiography. Quantitation of the radioactive signal was performed with a model 425F PhosphorImager (Molecular Dynamics).

Western blot analysis

Fractions containing biotinylated lac permease were analyzed electrophoretically on a SDS-polyacrylamide (12%) gel (Newman et al., 1981). Protein was electroblotted to polyvinylidene difluoride membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984). The PVDF membrane was subsequently incubated with horseradish peroxidase-conjugated protein A (Amersham) and finally developed with fluorescent substrate (Renaissance; DuPont NEN) before exposure to film. Alternatively, after treatment with the anti-C-terminal antibody, the blot was incubated with [^{125}I]protein A (30 mCi/mg, 100 μCi/mL), autoradiographed, and the amount of permease was quantitated with a model 425F PhosphorImager as described (Frillingos & Kaback, 1996b; Sun et al., 1996).

References

Akabas MH, Stauffer DA, Xu M, Karlin A. 1992. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Science 258*:307–310.

Carrasco N, Antes LM, Poonian MS, Kaback HR. 1986. *Lac* permease of *Escherichia coli*: Histidine-322 and glutamic acid-325 may be components of a charge-relay system. *Biochemistry 25*:4486–4488.

Carrasco N, Herzlinger D, Mitchell R, DeChiara S, Danho W, Gabriel TF, Kaback HR. 1984. Intramolecular dislocation of the COOH terminus of the *lac* carrier protein in reconstituted proteoliposomes. *Proc Natl Acad Sci USA 81*:4672–4676.

Carrasco N, Puttner IB, Antes LM, Lee JA, Larigan JD, Lolkema JS, Roepe PD, et al. 1989. Characterization of site-directed mutants in the *lac* permease of *Escherichia coli*. 2. Glutamate-325 replacements. *Biochemistry 28*:2533–2539.

Consler TG, Persson BL, Jung H, Zen KH, Jung K, Prive GG, Verner GE, et al. 1993. Properties and purification of an active biotinylated lactose permease from *Escherichia coli*. *Proc Natl Acad Sci USA 90*:6934–6938.

Franco PJ, Brooker RJ. 1994. Functional roles of Glu-269 and Glu-325 within the lactose permease of *Escherichia coli*. *J Biol Chem 269*:7379–7386.

Frillingos S, Kaback HR. 1996a. Monoclonal antibody 4B1 alters the pK_a of a carboxylic acid at position 325 (helix X) of the lactose permease of *Escherichia coli*. *Biochemistry 35*:10166–10171.

Frillingos S, Kaback HR. 1996b. Probing the conformation of the lactose permease of *Escherichia coli* by in situ site-directed sulfhydryl modification. *Biochemistry 35*:3950–3956.

Frillingos S, Ujwal ML, Sun J, Kaback HR. 1997. The role of helix VIII in the lactose permease of *Escherichia coli*: I. Cys-scanning mutagenesis. *Protein Sci 6*:431–437.

Hattori M, Sakaki Y. 1986. Dideoxy sequencing method using denatured plasmid templates. *Anal Biochem 152*:1291–1297.

Jung H, Jung K, Kaback HR. 1994a. A conformational change in the lactose permease of *Escherichia coli* is induced by ligand binding or membrane potential. *Protein Sci 3*:1052–1057.

Jung H, Jung K, Kaback HR. 1994b. Cysteine 148 in the lactose permease of *Escherichia coli* is a component of a substrate binding site. I. Site-directed mutagenesis studies. *Biochemistry 33*:12160–12165.

Jung K, Jung H, Wu J, Privé GG, Kaback HR. 1993. Use of site-directed fluorescence labeling to study proximity relationships in the lactose permease of *Escherichia coli*. *Biochemistry 32*:12273–12278.

Jung K, Voss J, He M, Hubbell WL, Kaback HR. 1995. Engineering a metal binding site within a polytopic membrane protein, the lactose permease of *Escherichia coli*. *Biochemistry 34*:6272–6277.

Kaback HR. 1971. Bacterial membranes. *Methods Enzymol 22*:99–120.

Kaback HR. 1987. Use of site-directed mutagenesis to study the mechanism of a membrane transport protein. *Biochemistry 26*:2071–2076.

Kaback HR. 1990. *Lac* permease of *Escherichia coli*: On the path of the proton. *Phil Trans R Soc (Lond) 326*:425–436.

Konings WN, Barnes E Jr, Kaback HR. 1971. Mechanisms of active transport in isolated membrane vesicles. 2. The coupling of reduced phenazine methosulfate to the concentrative uptake of β-galactosides and amino acids. *J Biol Chem 246*:5857–5861.

Newman MJ, Foster DL, Wilson TH, Kaback HR. 1981. Purification and reconstitution of functional lactose carrier from *Escherichia coli*. *J Biol Chem 256*:11804–11808.

Sahin-Tóth M, Kaback HR. 1993. Cysteine scanning mutagenesis of putative transmembrane helices IX and X in the lactose permease of *Escherichia coli*. *Protein Sci 2*:1024–1033.

Sanger F, Nicklen S, Coulsen AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Short SA, Kaback HR, Kohn LD. 1975. Localization of D-lactate dehydrogenase in native and reconstituted *Escherichia coli* membrane vesicles. *J Biol Chem 250*:4291–4296.

Stauffer DA, Karlin A. 1994. Acetylcholine receptor channel structure probed in cysteine-substitution mutants. *Biochemistry 33*:6840–6849.

Sun J, Wu J, Carrasco N, Kaback HR. 1996. Identification of the epitope for monoclonal antibody 4B1 which uncouples lactose and proton translocation in the lactose permease of *Escherichia coli*. *Biochemistry 35*:990–998.

Teather RM, Bramhall J, Riede I, Wright JK, Furst M, Aichele G, Wilhelm V, et al. 1980. Lactose carrier protein of *Escherichia coli*. Structure and expression of plasmids carrying the Y-gene of the *lac* operon. *Eur J Biochem 108*:223–231.

Ujwal ML, Sahin-Toth M, Persson B, Kaback HR. 1994. Role of glutamate-269 in the lactose permease of *Escherichia coli*. *Mol Membr Biol 1*:9–16.

van Iwaarden PR, Pastore JC, Konings WN, Kaback HR. 1991. Construction of a functional lactose permease devoid of cysteine residues. *Biochemistry 30*:9595–9600.

Viitanen P, Newman MJ, Foster DL, Wilson TH, Kaback HR. 1986. Purification, reconstitution and characterization of the lac permease of *Escherichia coli*. *Methods Enzymol 125*:129–152.

Wu J, Frillingos S, Kaback HR. 1995a. Dynamics of lactose permease of *Escherichia coli* determined by site-directed chemical labeling and fluorescence spectroscopy. *Biochemistry 34*:8257–63.

Wu J, Kaback HR. 1994. Cysteine 148 in the lactose permease of *Escherichia coli* is a component of a substrate binding site. 2. Site-directed fluorescence studies. *Biochemistry 33*:12166–12171.

Wu J, Perrin D, Sigman D, Kaback H. 1995b. Helix packing of lactose permease in *Escherichia coli* studied by site-directed chemical cleavage. *Proc Natl Acad Sci USA 92*:9186–9190.

Wu J, Voss J, Hubbell WL, Kaback HR. 1996. Site-directed spin labeling and chemical crosslinking demonstrate that helix V is close to helices VII and VIII in the lactose permease of *Escherichia coli*. *Proc Natl Acad Sci USA 93*:10123–10127.