Cysteine Scanning Mutagenesis of Helix V in the Lactose Permease of *Escherichia coli*

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ABSTRACT: Using a functional lactose permease mutant devoid of Cys (C-less permease), each amino acid residue in putative transmembrane helix V was replaced individually with Cys (from Met145 to Thr163). Of the 19 mutants, 13 are highly functional (60−125% of C-less permease activity), and 4 exhibit lower but significant lactose accumulation (15−45% of C-less permease). Cys replacement of Gly147 or Trp151 essentially inactivates the permease (<10% of C-less); however, previous studies [Menezes, M. E., Roepe, P. D., & Kaback, H. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 1638; Jung, K., Jung, H., et al. (1995) *Biochemistry 34,* 1030] demonstrate that neither of these residues is important for activity. Immunoblots reveal that all of the mutant proteins are present in the membrane in amounts comparable to C-less permease with the exception of Trp151→Cys and single Cys154 permeases which are present in reduced amounts. Finally, only three of the single-Cys mutants are inactivated significantly by *N*-ethylmaleimide (Met145→Cys, native Cys148, and Gly159→Cys), and the positions of the three mutants fall on the same face of helix V.

Lactose (lac) permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+ (i.e., H^+/substrate symport or cotransport). The *lacY* gene which encodes the permease has been cloned and sequenced, and the protein has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport [reviewed in Kaback (1983, 1989, 1992) and Poolman and Konings (1993)] as a monomer [see Sahin-Tóth et al. (1994)]. On the basis of circular dichroic studies and hydropathy analysis of the primary amino acid sequence (Foster et al., 1983), a secondary structure was proposed in which the permease is composed of a short hydrophilic N terminus, 12 α-helical hydrophobic domains that traverse the membrane in zig-zag fashion connected by hydrophilic loops, and a 17 residue hydrophilic C-terminal tail (Figure 1). Evidence supporting the general features of the model and demonstrating that both the N and C termini, as well as the loops between helices IV and V and VI and VII, are on the cytoplasmic face of the membrane was obtained from laser Raman spectroscopy (Vogel et al., 1985), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1984, 1986; Herzlinger et al., 1984, 1985; Danho et al., 1985), and chemical modification (Page & Rosenbusch, 1988). Exclusive support for the 12-helix motif was obtained from analyses of an extensive series of lac permease−alkaline phosphatase (*lacY−phoA*) fusions (Calamia & Manoil, 1990). Recently, use of second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence has led to a model describing helix packing in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993, 1994).

Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) reveal that less than a half-dozen out of over 320 residues mutated thus far are mandatory for activity [reviewed in Kaback et al. (1993, 1994) and Kaback (1995)]. Moreover, the functionally essential residues which are charged and in transmembrane domains are located predominantly in the C-terminal half of the molecule. Mutagenesis of Glu269 in putative helix VIII, Arg302 in helix IX, and His322 or Glu325 in helix X indicates that these residues play key roles in lactose/H^+ symport and/or substrate recognition. Although very few amino acid residues appear to be critically involved in the transport mechanism, the activity of various active Cys-replacement mutants is altered by alkylation, and these mutants appear in clusters, suggesting that surface contours within the permease may be important (Sahin-Tóth & Kaback, 1993; Dunten et al., 1993; Sahin-Tóth et al., 1994a; Frillingos et al., 1994). Furthermore, by using site-directed fluorescence labeling, it has been shown that the reactivity of individually placed Cys residues in a number of transmembrane domains is altered as a result of ligand binding or imposition of a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] (Sahin-Tóth & Kaback, 1993; Jung et al., 1994a,b; Wu & Kaback, 1994; Wu et al., 1994, 1995). In brief, therefore, it appears likely that permease turnover involves relatively simple chemistry coupled to widespread conformational changes.

Putative helix V of lac permease contains a number of residues that exhibit interesting properties when mutated, although none are obligatory for function. Cys148 is

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; TBST, 10 mM Tris-HCl/0.9% NaCl/0.2% Triton X-100; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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required for substrate protection against alkylation (Fox & Kennedy, 1965; Beyreuther et al., 1981; Trumble et al., 1984; Viitanen et al., 1985; Neuhaus et al., 1985; Sarkar et al., 1986), but the permease tolerates various replacements at this position with little or no loss of activity (Jung et al., 1994). By using site-directed mutagenesis (Jung et al., 1994) and site-directed fluorescence labeling (Wu & Kaback, 1994), however, it has been shown that Cys148 is located in a sugar binding site, interacting hydrophobically with the galactosyl moiety of the substrate, and that Met145 is in close proximity. C154G[2] permease is inactive with respect to transport, while C154S or C154V permease transports lactose at 10% or 30%, respectively, the rate of wild-type permease to a comparable steady-state level of accumulation (Menick et al., 1985). It is also noteworthy that C154G and C154V permeases bind *p*-nitrophenyl α-D-galactopyranoside with higher affinities than wild-type permease (van Iwaarden et al., 1993). G159C permease is a partially uncoupled mutant (Wong et al., 1970; Matos & Wilson, 1994; Jung et al., 1995) that exhibits normal exchange and counterflow and may be altered in deprotonation (Herzlinger et al., 1985). Finally, G147C permease exhibits enhanced reactivity in the presence of ligand (Wu & Kaback, 1994). Consequently, Cys-scanning mutagenesis was employed in order to systematically examine the remaining residues in this transmembrane domain. The effect of individual Cys replacement for Met145 to Thr163 on both permease activity and expression was determined, and the active mutants were tested for sensitivity to alkylation by *N*-ethylmaleimide (NEM). The results demonstrate that none of the residues play an essential role in transport, but the three mutants that are inactivated significantly by NEM fall on the same face of the helix.

MATERIALS AND METHODS

Materials

[1-^{14}C]Lactose was purchased from Amersham, Arlington Heights, IL. Deoxyoligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of lac permease (Carrasco et al., 1984a) was prepared by BabCo, Richmond, CA. All restriction endonucleases, T_4 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.

Methods

Bacterial Strains. E. Coli HB101 [*hsdS20(r^-_B,m^-_B),recA13,ara-14,proA2,lacY1,galK2,rpsL20(Sm^r),xyl-5,mtl-1, SupE44,l$^-$/F$^-$*] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described. *E. coli* T184 [*lacI$^+$O$^+$Z$^-$Y$^-$(A),rpsL,met$^-$,thr$^-$,recA,hsdM,hsdR/F′, lacI^{q}O$^+$Z^{D118}(Y$^+$A$^+$)*] (Teather et al., 1980) was used for expression of lac permease and lactose transport.

Mutagenesis. The single-Cys mutants were prepared by oligonucleotide-directed, site-specific mutagenesis of the C-less version of the cassette *lacY* gene (EMBL-X56095) in the plasmid pT7-5 pC$_7$S/C154V; van Iwaarden et al., 1991). The polymerase chain reaction (PCR) overlap extension method of Ho et al. (1989) was employed with the substitution of Vent$_R$ DNA polymerase for *Taq* DNA polymerase in the two stages of PCR. PCR products were isolated from low melting point agarose gels and purified with a Magic PCR Preps DNA purification system (Promega, Madison, WI). The purified second-stage PCR products, of approximately 400 base pairs, were digested with *Bss*HII and *Xho*I restriction endonucleases (see Figure 1 for location of sites). The digested fragments were isolated from low melting point agarose gels as described above and ligated to similarly treated pC$_7$S/C154V vector. The resulting plasmids were transformed into *E. coli* HB101 (*Z$^+$Y$^-$*), and transformants were selected on MacConkey (Difco) indicator plates containing 25 mM lactose. Plasmid DNA was prepared from single colonies with Magic Minipreps (Promega). Mutations were verified by sequencing the length of the PCR-generated segment through the ligation junctions in double-stranded DNA using the dideoxynucleotide termination method and synthetic sequencing primers (Sanger et al., 1977; Sanger & Coulsen, 1978) after alkaline denaturation (Hattori & Sakaki, 1986).

Growth of Bacteria. For lactose transport assays and membrane preparation, *E. coli* T184 transformed with each plasmid described were grown aerobically at 37 °C in Luria broth with streptomycin (10 μm/mL) and ampicillin (100 μg/mL). Dense cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM IPTG. After further growth for 2 h at 37 °C, cells were harvested by centrifugation.

Active Lactose Transport. Cells were washed with 100 mM KP$_i$ (pH 7.5)/10 mM $MgSO_4$ and adjusted with the same buffer to an OD_{420} of 10. Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 Ci = 37 CBq) at a final concentration of 0.4 mM was assayed by rapid filtration (Consler et al., 1991), except that the assays were conducted under oxygen in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) (Konings et al., 1971; Kaback, 1974).

Membrane Preparation. Cells were washed with 50 mM Tris-HCl (pH 8.0)/100 mM NaCl/1 mM ethylenediaminetetraacetate (EDTA) and resuspended in ice-cold osmotic shock buffer (25 mM Tris-HCl, pH 8.0/45% sucrose/1 mM EDTA/0.1 mg of lysozyme per milliliter). After 20 min on ice, spheroplasts were harvested and resuspended in 0.75 mL of cold water. After incubation for 30 min at 4 °C, cell suspensions were briefly sonicated. Unlysed cells were removed by low-speed centrifugation, and membranes were harvested by ultracentrifugation at 250000g_{max} for 1 h at 4 °C in a Beckman Optima TL ultracentrifuge.

Protein Determinations. Protein was assayed as described (Peterson, 1977) with bovine serum albumin as standard.

Immunoblotting. Membranes prepared as indicated were resuspended in sample buffer and subjected to sodium dodecyl sulfate (NaSDS)−polyacrylamide gel electrophoresis (Newman et al., 1981). Proteins were electroblotted to poly(vinylidene difluoride) membranes (Immobilon-PVDF, Millipore) for 3 h at 0.5 A in transfer buffer (25 mM Tris-HCl/200 mM glycine/20% methanol/pH 8.3) at 4 °C. Blots were then blocked in 5% bovine serum albumin in TBST (10 mM Tris-HCl/0.9% NaCl/0.2% Triton X-100). After 1

[2] Site-directed mutants are designated by the single-letter amino acid abbreviations for the targeted residue, followed by the sequence position of the residue in the wild-type lac permease, followed by a second letter indicating the amino acid replacement.

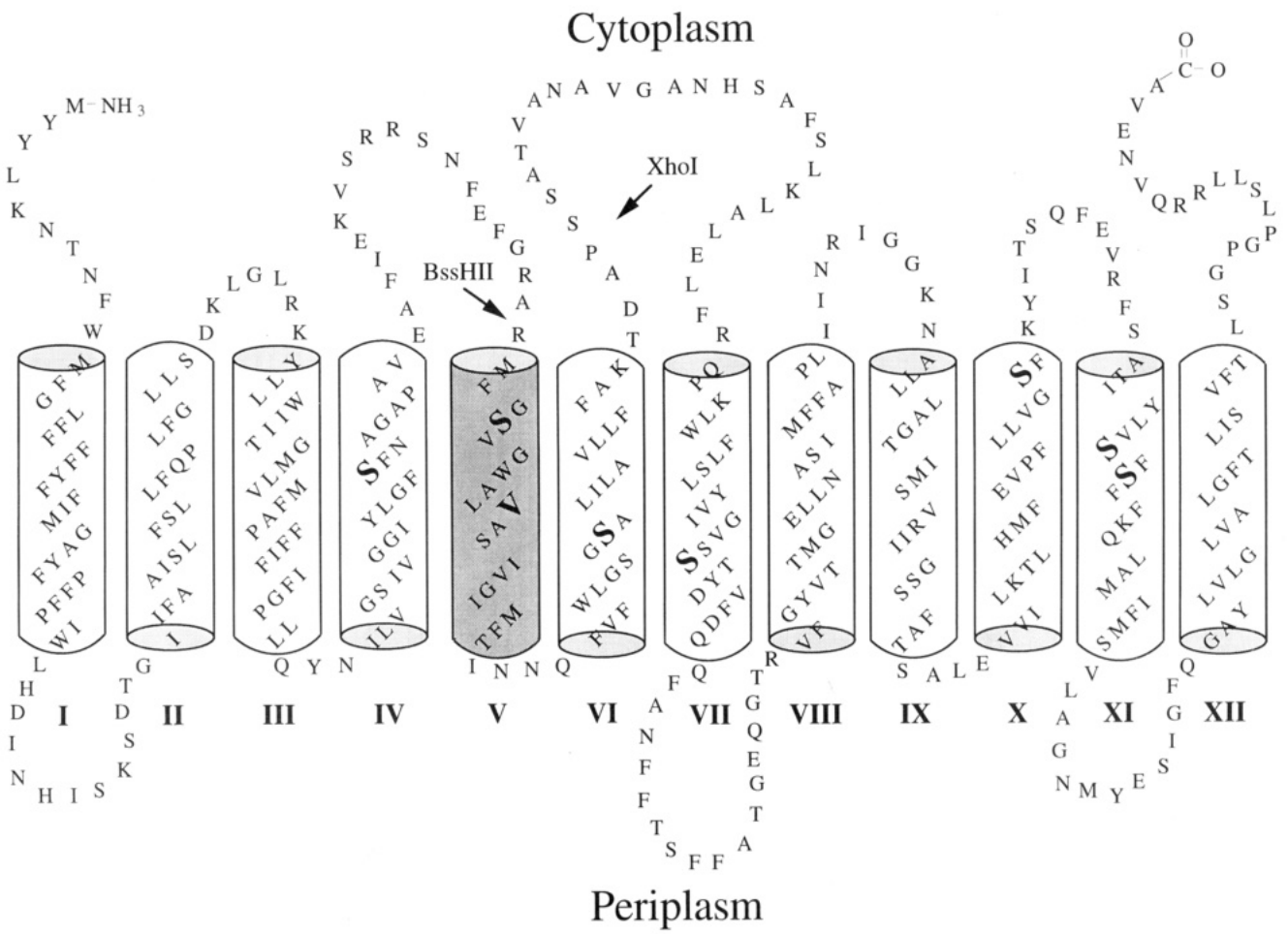

FIGURE 1: Secondary structure model of *E. coli* lac permease. The single-letter amino acid code is used, and C-less lac permease is shown with the residues replacing the eight native Cys residues highlighted. Hydrophobic transmembrane helices are enclosed in cylinders, and helix V which was subjected to Cys-scanning mutagenesis is shaded. The locations of relevant restriction endonuclease sites in the corresponding DNA sequence are also indicated.

h, rabbit polyclonal antiserum directed against the C-terminal dodecapeptide of lac permease was added at a final dilution of 1:2500. Blots were incubated a further 2 h, washed 3 times with TBST for 20 min each, and incubated again in TBST containing 5% bovine serum albumin for 30 min prior to addition of horseradish peroxidase linked-protein A (Amersham) at a final dilution of 1:40 000. After a 1 h incubation and three 20-min washes with TBST, blots were developed with fluorescent substrate (Amersham) according to the instructions of the manufacturer.

RESULTS

Construction and Verification of Mutants. Each amino acid in putative helix V, residues M145 through T163 (Figure 1), was individually replaced with Cys in the C-less permease (van Iwaarden et al., 1991) as described under Methods. Mutants S148C and V154C restore single native Cys residues to C-less permease and are referred to as Cys148 and Cys154 permease. All mutations were verified by double-stranded DNA sequencing of the *Bss*HII−*Xho*I segment including the restriction site junctions (Figure 1), and except for the desired base changes, the sequences were identical to those of cassette *lacY* encoding C-less permease.

Colony Morphology. *E. coli* HB101 (*lacZ*$^+$*Y*$^-$) is a "cryptic" strain that expresses active β-galactosidase but carries a defective *lacY* gene. The ability of lac permease mutants to translocate lactose "downhill" was assessed qualitatively by transforming *E. coli* HB101 with plasmid encoding each mutant and growing the transformants on MacConkey indicator plates containing 25 mM lactose. Cells expressing functional lac permease hydrolyze imported lactose, and metabolism of the monosaccharide causes acidification which makes the colonies appear red. Cells impermeable to lactose appear as white colonies, and mutants with low activity grow as red colonies with a white halo. With the exception of G147C and W151C which grow as white colonies with a small central red dot, all of the other mutants yield red colonies indistinguishable from cells expressing C-less permease.

Active Lactose Transport. The ability of the mutant permeases to catalyze active lactose transport was assayed in *E. coli* T184 (*Z*$^-$*Y*$^-$) which lacks β-galactosidase and cannot metabolize lactose. The majority of the Cys-replacement mutants transport lactose at high rates (Figure 2A) to steady-state levels of accumulation comparable to C-less permease (Figure 2B). When initial rates of transport are measured over the first minute, 16 of 19 mutants exhibit rates that are 50−100% or more of C-less, and 3 mutants (G147C, G150C, and W151C) transport at 10% or less of C-less (Figure 2A). Steady-state levels of lactose accumulation at 30 min by the great majority of the mutants also approximate or exceed that of C-less permease (Figure 2B).

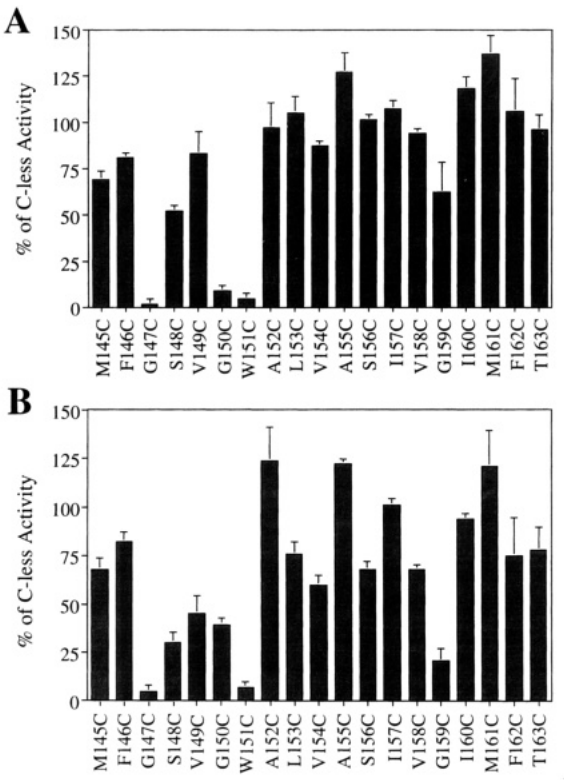

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants in C-less lac permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed for [1-^{14}C]lactose uptake at 25 °C as described under Methods. (A) Rates of lactose transport calculated from time points up to 1 min. The rate of C-less permease averaged 21 nmol of lactose min^{-1} (mg of protein)$^{-1}$. Results are expressed as a percentage of C-less activity after correcting for nonspecific uptake by T184 harboring pT7-5 (vector with no *lacY* gene) which typically transported at a rate of 0.8 nmol of lactose min^{-1} (mg of protein)$^{-1}$ (i.e., 4% C-less). (B) Steady-state levels of lactose accumulation. Results are expressed as a percentage of C-less activity (97 mnol of lactose/mg of protein) after correcting for nonspecific accumulation by T184 harboring pT7-5 which typically accumulated 5 nmol of lactose/mg of protein in 30 min (i.e., 5% of C-less).

Thirteen mutants accumulate to levels of 60−100% or more of C-less, and 3 mutants (Cys148, V149C, and G150C) achieve steady-states that are 30−50% of C-less. Only G147C, W151C, and G159C permeases accumulate the disaccharide to steady-states that are less than 25% of C-less. Thus, three broad categories of mutants are apparent: (i) M145C, F146C, A152C, L153C, V154C, A155C, S156C, I157C, V158C, I160C, M161C, F162C, and T163C which are comparable in activity to C-less; (ii) S148C, V149C, G150C, and G159C which exhibit 15−45% of C-less activity. In this category, two phenotypes are observed: S148C, V149C, and G159C which display relatively higher initial rates than steady-state levels of accumulation, due probably to partial uncoupling of lactose from H^+ translocation; and G150C, which exhibits a low initial rate relative to the steady-state level of accumulation, an effect consistent with a decreased turnover rate. (iii) The third category is G147C and W151C which are virtually inactive. However, it must be emphasized that permease with Ala in place of Gly147 (Jung et al., 1995) or Phe in place of Trp151 (Menezes et al., 1990) exhibits activity comparable to C-less or wild-type permease, respectively.

Expression of Mutant Permeases. The relative concentration of each mutant in *E. coli* T184 membranes was estimated by Western blot analysis with site-directed polyclonal antibody against a dodecapaptide corresponding to the C terminus of the permease (Carrasco et al., 1984). All of the mutants are expressed at levels that are roughly comparable to C-less permease with the exceptions of W151C and V154C permease which are clearly expressed at reduced levels (Figure 3).

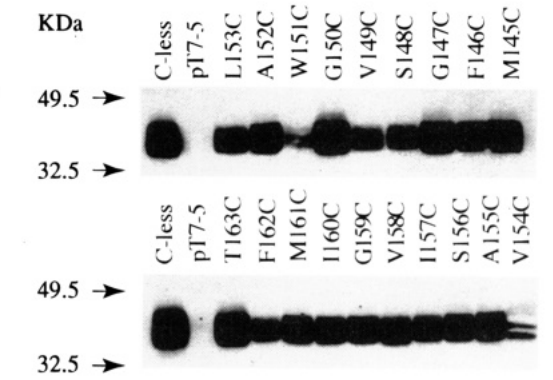

FIGURE 3: Western blot of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring each plasmid as described under Methods, and 50 μg of membrane protein was subjected to 12% SDS−polyacrylamide gel electrophoresis and electroblotting. The blot was incubated with antibody directed against the C-terminal dodecapeptide of lac permease, followed by horseradish peroxidase-linked protein A, and, finally, fluorescent substrate before exposure to film.

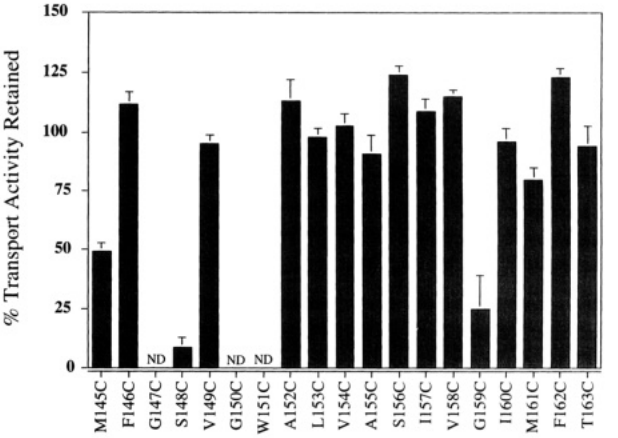

FIGURE 4: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding Cys-replacement mutants. Cells were incubated with 1 mM NEM at room temperature for 10 min, and 5 mM dithiothreitol was added before assaying initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM PMS under oxygen (Konings et al., 1971). The rates are presented as a percentage of the rate measured in cells incubated without NEM treatment. Mutants displaying initial rates below 10% of C-less permease were not studied (ND, not determined).

Effect of NEM on Lactose Transport Activity. The effect of the membrane-permeable sulfhydryl reagent NEM on the initial rate of lactose transport of each mutant, with the exception of G146C, G150C, and W151C permeases which have very low activities, is shown in Figure 4. Clearly, only three of the mutants exhibit significant sensitivity to alkylation by the alkylating agent. Mutants M145C, Cys148, and G159C are inhibited by about 50%, 90%, and 75%, respectively, relative to control samples assayed in the absence of NEM. When viewed on a helical wheel plot (Figure 5), the sensitive positions cluster on the same face of helix V.

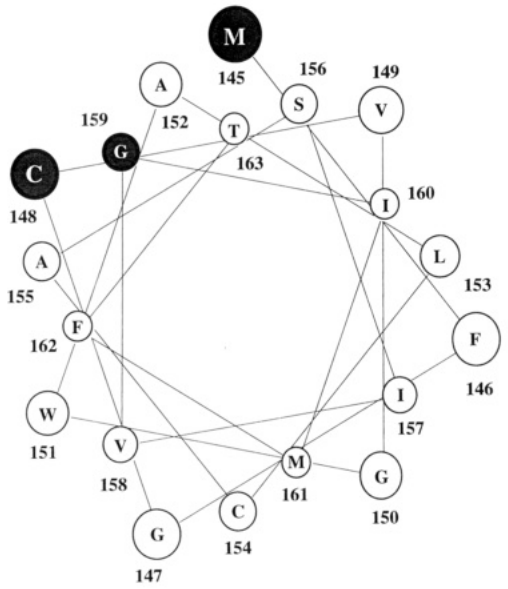

FIGURE 5: Helical wheel plot of residues in putative helix V. Positions sensitive to NEM treatment (i.e., >50% inhibition of initial rate as shown in Figure 4) are highlighted against a dark background. For consistency with the model presented in Jung et al. (1993), the helix is drawn with the C-terminus at the top.

DISCUSSION

In this study, Cys-scanning mutagenesis was employed to assess the functional importance of the amino acid residues that comprise putative transmembrane helix V of lac permease. As shown previously [reviewed in Kaback et al. (1993, 1994) and Kaback (1995)], site-directed mutagenesis and Cys-scanning mutagenesis of over 320 residues in the premease have revealed that the great majority of the single-Cys replacement mutants are both expressed and active. More specifically, only Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) have been shown to be obligatory for active lactose transport at this time. While other residues, such as Cys148 and Met145 (Jung et al., 1994; Wu & Kaback, 1994), may play a role in the mechanism, any residue that can be replaced with Cys in either the C-less or the wild-type background (Sahin-Tóth et al., 1994; Jung et al., 1994) without leading to complete loss of active transport is unlikely to be essential for the mechanism. Among the 19 Cys-replacement mutants in helix V, only G147C and W151C appear to be completely defective, as both mutants exhibit a negative morphology on indicator plates and display little if any ability to accumulate lactose. Importantly, however, neither of these residues is essential for transport, as Ala replacement for Gly 147 is well tolerated (Jung et al., 1995) as is Phe replacement for Trp151 (Menezes et al., 1990). The low activity displayed by G147C is not due to a defective insertion into the membrane, since Western blot analysis shows high expression of the mutant. In contrast, the low activity displayed by W151C permease may result in part from an insertion defect, since this mutant is expressed in the membrane in significantly reduced quantity. The results presented for G159C permease are consistent with previous results (Wong et al., 1970; Matos & Wilson, 1994; Herzlinger et al., 1985; Jung et al., 1995) demonstrating that this mutant is partially uncoupled. In any case, the results as a whole indicate that none of the residues in putative helix V play an essential role in the mechanism.

The observation that G147C permease exhibits little or no activity is interesting in the context of the finding (Wu & Kaback, 1994) that this mutant exhibits properties which reflect ligand-induced conformational changes in the permease. The high-affinity ligand TDG accelerates the rate of labeling of G147C permease with 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS) like other mutants with single-Cys residues at positions 315 (Sahin-Tóth & Kaback, 1993; Jung et al., 1994b), 322, and 269 (Jung et al., 1994a). Moreover, like V331C permease (Wu et al., 1994), in addition to increasing reactivity with MIANS, TDG also quenches the fluorescence observed with MIANS-labeled Cys147 permease and causes a blue-shift in the fluorescence emission spectrum. Thus, although Gly147 is not obligatory for activity, as the Ala-replacement mutant exhibits high activity (Jung et al., 1995), the presence of a Cys residue at position 147 may interfere with an important step in the overall turnover cycle of the permease.

Each Cys-replacement mutant with transport activity was also tested for sensitivity to NEM, and only M145C, Cys148, and G159C permeases are significantly inactivated. The most straightforward explanation for the observations is that the other Cys residues are inaccessible to NEM and do not react. Although this is a possibility, it is unlikely as NEM is relatively permeant, and Cys-replacement mutants with single-Cys residues at positions 145, 146, 147, and 148 which are presumably near the cytoplasmic terminus of helix V react with MIANS (Wu & Kaback, 1994). Furthermore, Cys154 permease is inactivated by NEM over longer incubation periods (data not shown), and a number of Cys mutants located at various positions in helices I, III, VII, X, and XI are readily inactivated by the alkylating agent (Sahin-Tóth & Kaback, 1993; Dunten et al., 1993; Sahin-Tóth et al., 1994; Frillingos et al., 1994; Jung et al., 1995). Finally, as shown for many of the other helices that have been subjected to Cys-scanning mutagenesis [see Kaback et al. (1993, 1994) and Kaback (1995)], the three mutants described here that are inactivated significantly by NEM cluster on the same face of helix V.

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