Cysteine-Scanning Mutagenesis of Helix VI and the Flanking Hydrophilic Domains in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Using a functional lactose permease mutant devoid of Cys residues (C-less permease), each amino acid residue in putative transmembrane helix VI and the flanking hydrophilic loops (residues 164−211) was replaced individually with Cys. Of the 48 mutants, 43 accumulate lactose at highly significant rates to >80% of the steady state observed with C-less permease. Three mutants (Phe185 → Cys, Ala187 → Cys, and Phe208 → Cys) exhibit lower but significant levels of accumulation (30−60% of C-less). Cys replacement for Ala177 or Leu184 results in low transport activity (ca. 20%) in the C-less background but much higher activity (60−70%) in the wild type. Immunoblot analysis reveals that all of the mutants are inserted into the membrane at concentrations comparable to that of C-less permease. The transport activity of the great majority of the mutants is unaffected by treatment with *N*-ethylmaleimide (NEM). Relatively modest but significant inactivation (ca. 50%) is observed with mutants Phe170 → Cys, Gly173 → Cys, and Ala187 → Cys, and these positions cluster on the same face of helix VI. Moreover, the two positions where single Cys replacements result in low activity (Ala177 and Leu184) are on the same face of helix VI. The results demonstrate the following. (i) Permease function is not disrupted by replacement of most residues with Cys, but function is disrupted when some residues are further altered by addition of the NEM moiety. (ii) The latter residues lie on a stripe down one face of an α-helix, and within the same stripe are residues where Cys substitution itself leads to inhibition of function.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the coupled stoichiometric translocation of β-galactosides and H^+. Encoded by the *lacY* gene, the permease 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)] as a monomer [see Sahin-Tóth et al. (1994b)]. On the basis of circular dichroism and hydropathy analysis (Foster et al., 1983), a secondary structure was proposed in which the permease has 12 putative transmembrane α-helices connected by hydrophilic loops (Figure 1). Support for the general features of the model was subsequently obtained from other spectroscopic techniques, limited proteolysis, immunological studies, and chemical modification [see Kaback (1983, 1989, 1992)], and exclusive support for the 12-helix motif was provided by an extensive series of lac permease−alkaline phosphatase (*lacY*−*phoA*) fusions (Calamia & Manoil, 1990). Furthermore, use of second-site suppressor analysis, site-directed mutagenesis, and site-directed excimer fluorescence has led to a model describing the packing of helices VII−XI in the C-terminal half of the permease [Jung et al., 1993; see Kaback et al. (1993, 1994)]. The model has been confirmed and extended recently by engineering divalent metal-binding sites (bis-His residues) within the transmembrane domains of the permease (Jung et al., 1995b; He et al., 1995a,b) and use of site-directed chemical cleavage [Wu et al., 1995b; reviewed in Kaback (1996)].

Many observations indicate that the C-terminal half of the permease plays a direct role in the transport mechanism. Significant downhill transport activity is retained in mutants deleted of helices II−V (N_1C_6; Bibi et al., 1991), and the C-terminal half of the permease (C_6) containing a single Cys residue can be expressed independently, inserted into the membrane [see Wu et al. (1996)], and shown to retain the ability to bind ligand by site-directed fluorescence labeling [see Wu and Kaback (1994)]. Extensive mutagenesis [reviewed in Kaback et al. (1994) and Kaback (1996)] shows that the four charged residues found to be mandatory for activity thus far are located in transmembrane domains in the C-terminal half of the permease. Furthermore, the epitope for a monoclonal antibody that uncouples lactose from H^+ translocation has been localized recently (Sun et al., 1996) to the periplasmic loop between helices VII and VIII. On the other hand, the N-terminal 22 amino acid residues can be deleted from the permease without abolishment of activity (Bibi et al., 1992), and no essential residues have been found in the N-terminal half of the permease, as judged by site-directed and Cys-scanning mutagenesis [see Kaback et al. (1994) and Kaback (1996)].

Although remarkably few amino acid residues appear to be critically involved in the transport mechanism, the activity of various active Cys replacement mutants is altered by

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[1] Abbreviations: lac, lactose; C-less permease, functional lactose permease devoid of Cys residues; $\Delta\bar{\mu}_{H^+}$ the H^+ electrochemical gradient across the membrane; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside.

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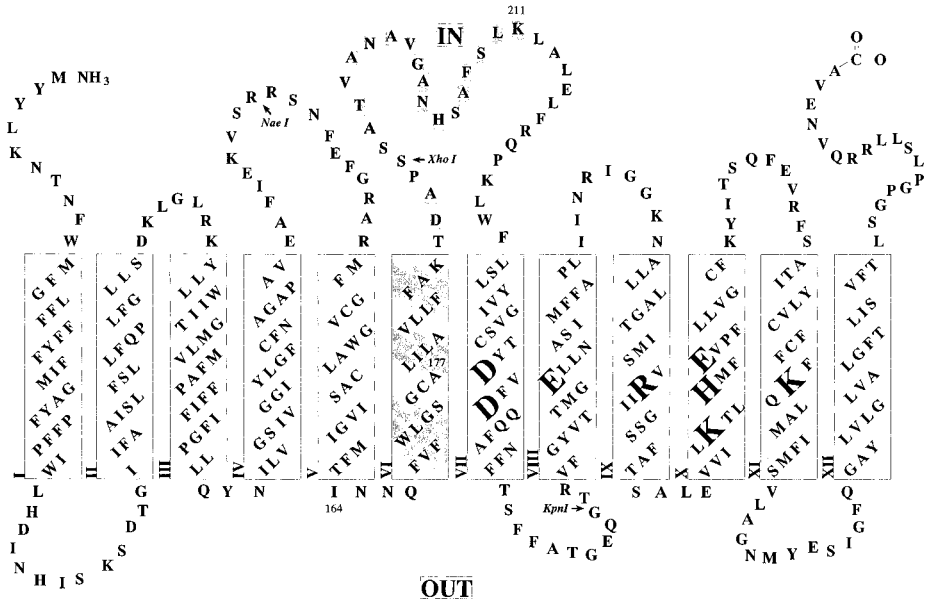

FIGURE 1: Secondary structure model of *E. coli* lac permease. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. The shaded area highlights the region of the permease subjected to Cys-scanning mutagenesis in this study. Also indicated are the restriction endonuclease sites used for construction of the mutants.

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; Weitzman & Kaback, 1995). Moreover, site-directed fluorescence with purified permease containing single Cys residues (Jung, H., et al., 1994; Jung, K., et al., 1994; Wu & Kaback, 1994; Wu et al., 1994, 1995a; Weitzman et al., 1995), as well as site-directed sulfhydryl modification *in situ* (Frillingos & Kaback, 1996; Wu et al., 1995a), shows that the reactivity of Cys residues in a number of transmembrane domains is altered as a result of ligand binding or imposition of a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$). Therefore, it appears likely that permease turnover involves widespread multidetermined conformational changes and very few essential side chain substrate (H^+ and sugar) and side chain interactions.

Putative helix VI and the flanking hydrophilic domains are among the least-conserved sequence regions in the five homologous oligosaccharide/H^+ symporters from enteric bacteria encompassing cluster 5 in the major facilitator superfamily [MFS; Marger & Saier, 1993; see Bockman et al. (1992) and Lee et al. (1994)]. However, these regions in *E. coli* lac permease contain residues that exhibit interesting properties when mutated. An Ala or Gly residue is conserved at position 177 of helix VI in all members of cluster 5. Lac permease mutants with bulkier residues (e.g. Val) in place of Ala177 alter sugar specificity (Brooker & Wilson, 1985; King & Wilson, 1990a; Goswitz & Brooker, 1993), as well as coupling between lactose and H^+ translocation (King & Wilson, 1990b; Brooker, 1991). In contrast, insertional mutagenesis of loop VI/VII of the permease shows that increasing the length of the loop by two to over 200 residues does not affect activity (McKenna et al., 1992; Consler et al., 1993; Privé et al., 1994); similarly, permease with two or six contiguous His residues inserted into loop V/VI retains significant activity (McKenna et al., 1992). Conversely, in-frame deletion of 17 residues from the loop VI/VII abolishes activity, thereby suggesting that a minimal length may be important for function (McKenna et al., 1992). In addition, evidence has been presented that loop VI/VII is involved in regulation by enzyme II glucose (IIA^{glc}) of the phosphoenolpyruvate/sugar phosphotransferase system (PTS; Saier et al., 1978; Wilson et al., 1990).

In this study, Cys-scanning mutagenesis was employed systematically to investigate the importance of the residues in loop V/VI, helix VI, and loop VI/VII. The results reveal that, although none of the 48 residues is essential for activity, residues on one face of helix VI may be important. Thus, the three single-Cys mutants that are significantly inactivated by *N*-ethylmaleimide (NEM) cluster on the same face of helix VI as Ala177 and Leu184, both of which exhibit low activity when replaced with Cys.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose and [α-^{35}S]dATP were 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., 1984) was prepared by BabCo, Richmond, CA. Restriction endonucleases and T4 DNA ligase were from New England Biolabs, Beverly, MA. Taq DNA polymerase was from Promega Corp., Madison, WI. Sequenase was from United States Biochemical, Cleveland, OH. All other materials were reagent grade and were obtained from commercial sources.

Bacterial Strains and Plasmids. 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 and for detection of lac permease activity on MacConkey-agar indicator plates (Difco Laboratories) containing 25mM lac. *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 pT7-5/*lacY* with given mutations was used for expression of lac permease from the *lacZ* promoter/operator. A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (C-less permease; van Iwaarden et al., 1991) containing the *lacZ* promoter/operator was used for all *lacY* gene manipulations.

Oligonucleotide-Directed Site-Specific Mutagenesis. Each amino acid residue from Ile164 to Lys211, a region contain-

ing the short periplasmic loop between helices V and VI (loop V/VI; residues 164−167), transmembrane helix VI (residues 168−188), and the cytoplasmic loop between helices VI and VII (loop VI/VII; residues 189−211), was replaced with Cys in C-less permease (Figure 1). Cys replacement mutants were constructed by two-stage polymerase chain reaction (PCR) (overlap−extension; Ho et al., 1989) using pT7-5/cassette *lacY* encoding C-less permease (van Iwaarden et al., 1991) as template. The PCR products were digested with *Nae*I and *Xho*I (I164C to P192C) or *Xho*I and *Kpn*I (A195C to K211C) and ligated to similarly treated pT7-5/*lacY*/C-less vector (see Figure 1 for location of sites). For construction of mutants S193C and S194C, the PCR products were restricted with *Nae*I and *Kpn*I, as the *Xho*I site is destroyed by the mutations.

For construction of mutants p(wt)A177C and p(wt)L184C (A177C and L184C in the wild-type background), mutagenic primers were used that included the native Cys176 codon and pT7-5/cassete *lacY* template encoding wild-type permease. The PCR products were digested with *Nae*I and *Xho*I and ligated to similarly treated pT7-5/*lacY*/wild-type vector.

DNA Sequencing. Double-stranded plasmid DNA was sequenced using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulsen, 1978) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Mutations were verified by sequencing the length of subcloned double-stranded DNA through the ligation junctions. Except for the base changes introduced, the sequences were identical to that of C-less cassette *lacY*. In the case of p(wt)A177C or p(wt)L184C, sequencing of the subcloned fragments verified the presence of mutations GCA(Ala) → TGC(Cys) at position 177 or CTC(Leu) → TGC(Cys) at position 184, as well as the presence of the native Cys codons (TGT) at positions 148, 154, and 176.

Growth of Bacteria. E. coli HB101 (Z^+Y^-) or T184 (Z^-Y^-) transformed with each plasmid described was grown aerobically at 37 °C in Luria-Bertani broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). HB101 cultures were used for preparation of plasmid DNA. Fully grown cultures of T184 were diluted 10-fold and grown for another 2 h before induction with 0.5 mM isopropyl 1-thio-β,D-galactopyranoside (IPTG). After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

Active Lactose Transport. Cells were washed with 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10.0 at 420 nm (approximately 0.7 mg of protein/mL). Transport of [1-^{14}C]-lactose (2.5 mCi/mmol; 1 mCi = 37 MBq) at a final concentration of 0.4 mM was assayed by rapid filtration, as described (Consler et al., 1991).

Membrane Preparation. Crude membrane fractions from T184 were prepared as described (Frillingos et al., 1994).

Immunological Analyses. Membrane fractions were subjected to 12% sodium dodecyl sulfate ($NaDodSO_4$)−polyacrylamide gel electrophoresis, as described (Newman et al., 1981). Proteins were electroblotted to poly(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore) and probed with a site-directed polyclonal antibody against the C-terminus of lac permease (Carrasco et al., 1984; Herzlinger et al., 1985).

Protein Determinations. Protein was assayed in the presence of $NaDodSO_4$ as described (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Colony Morphology. As a preliminary, qualitative assay of transport activity, each mutant was transformed into *E. coli* HB101, and colonies were grown on MacConkey indicator plates containing 25 mM lactose. HB101 ($lacZ^+Y^-$) expresses active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of the external lac to cytosolic β-galactosidase, and subsequent metabolism of the sugar leads to acidification and the appearance of red colonies. Cells expressing inactive mutants form white colonies, and mutants with low activity grow as red colonies with a white halo. It is important that indicator plates report "downhill" translocation only and give no indication as to whether the cells catalyze lactose accumulation. Of the 48 Cys replacement mutants described, 46 grow as dark red colonies indistinguishable from cells expressing C-less permease, while A177C and L184C yield red colonies with a white halo in the C-less background and dark red colonies when introduced into wild-type permease. Therefore, judging from the indicator plates, all of the mutants retain the ability to translocate lac downhill.

Active Lactose Transport. The ability of the mutants to catalyze active lactose transport was tested in *E. coli* T184 ($lacZ^-Y^-$) transformed with an appropriate plasmid. The majority of the 48 mutants transport lactose at highly significant rates (Figure 2A). Forty mutants exhibit rates that are between 70 and 100% or more of that of C-less permease, five mutants (G173C, I179C, F185C, A187C, and L210C) transport at 40−60% of the rate of C-less, and three mutants (A177C, L184C, and F208C) transport at 20−25% of the rate of C-less. Steady state levels of lactose accumulation for the great majority of mutants also approximate that of C-less permease (Figure 2B). Steady states of 80−100% or more of C-less are achieved by 43 mutants, F185C or A187C exhibits intermediate levels of accumulation (40−60% of C-less), and A177C, L184C, or F208C catalyzes accumulation about 20−30% as well as C-less permease.

Time courses of lactose transport by the mutants with low or intermediate activity are presented in Figure 3. Mutants F185C, A187C, and F208C transport the disaccharide to approximately 60, 40, and 30%, respectively, of the level achieved by C-less permease. It is also apparent that A177C or L184C permease retains some ability to accumulate lac against a concentration gradient but reaches only about 20% of the control steady state. Since the steady state level of accumulation is clearly reduced with each of these mutants, it is likely that the stoichiometry between H^+ and lactose is altered (i.e. the permease is partially uncoupled). In order to investigate the properties of A177C and L184C further, the mutations were transferred to the wild-type background (Figure 4). As shown, in the presence of the eight native Cys residues, both mutants transport lactose at about 50% of the wild-type rate to a steady state level of accumulation that is 60−65% of that of wild type. The results demonstrate that neither Ala177 nor Leu184 is essential for active transport and that relatively low activity in the C-less background is due to an unspecified interaction(s) between

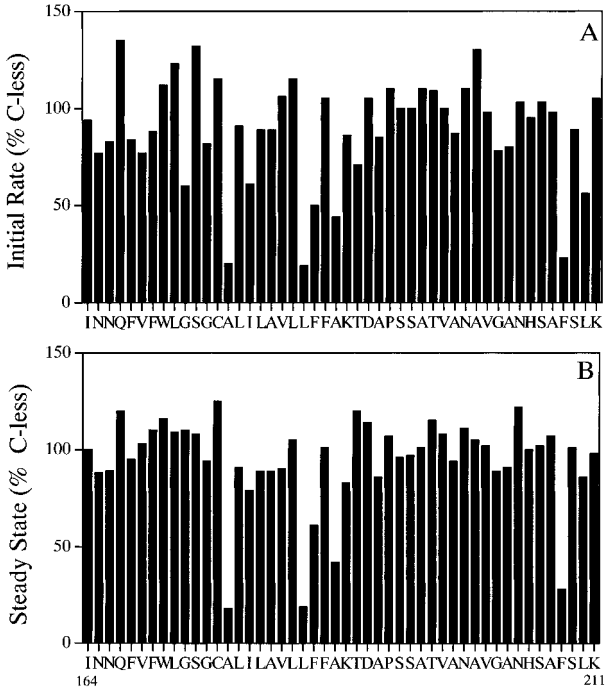

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys replacement mutants or C-less permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described in Materials and Methods. The one-letter amino acid code along the horizontal axis denotes the original residues replaced with Cys in increasing order from Ile164 to Lys211. (A) Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 55 nmol min^{-1} (mg protein)$^{-1}$. Results are expressed as a percentage of this value. Although not shown (see Figure 3), T184 cells harboring pT7-5 (vector with no *lacY* gene) transported at a rate of 2.5 nmol min^{-1} (mg protein)$^{-1}$ (i.e. 4.5% of C-less). (B) Steady state levels of lac accumulation. Results are expressed as a percentage of the steady state observed with C-less permease which averaged 160 nmol of lactose/(mg of protein). Although not shown (see Figure 3), T184 cells harboring pT7-5 accumulated 11 nmol of lactose/(mg of protein) in 1 h (i.e. 6.8% of C-less).

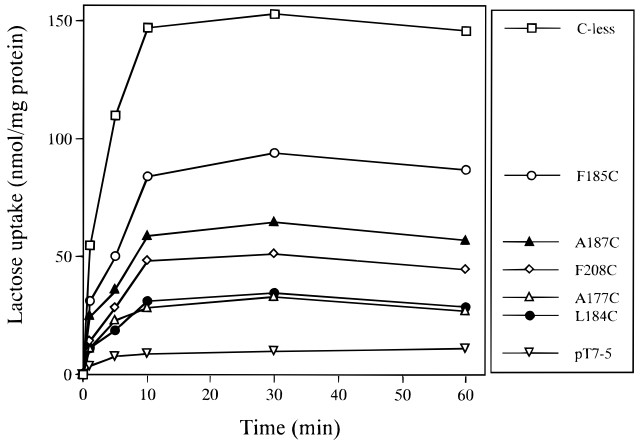

FIGURE 3: Time courses of lactose transport by single Cys mutants with low or intermediate activity. *E. coli* T184 transformed with plasmid pT7-5 (vector with no *lacY* gene), pT7-5 encoding C-less permease, or pT7-5 encoding given Cys replacement mutants were grown and assayed as described in the legend to Figure 2 and in Materials and Methods.

these mutations and one or more of the eight other mutations in C-less permease.

Expression of Permease Mutants. Western blot analysis of membrane fractions prepared from *E. coli* T184 expressing the Cys replacement mutants demonstrates that all of the

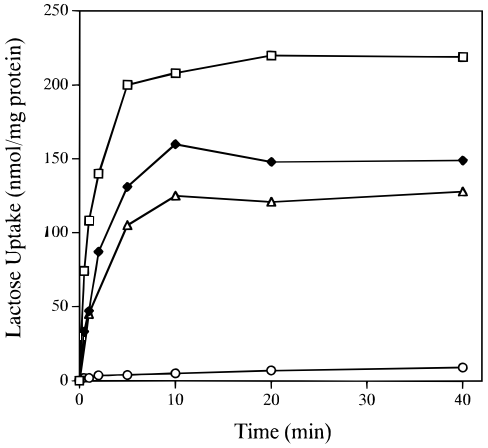

FIGURE 4: Time course of lactose transport by mutants p(wt)A177C and p(wt)L184C (mutants A177C and L184C in the wild-type background). *E. coli* T184 transformed with plasmid pT7-5 alone (○), pT7-5 encoding wild-type permease (□), p(wt)A177C permease (◆), or p(wt)L184C permease (△) was grown and assayed as described in the legend to Figure 2 and in Materials and Methods.

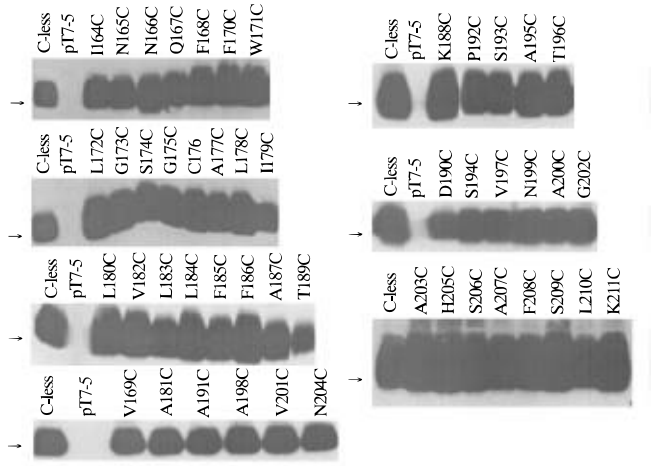

FIGURE 5: Western blots of membranes containing C-less lac permease or Cys replacement mutants. Membranes were prepared from IPTG-induced cultures of T184 harboring given plasmids, as described in Materials and Methods. Samples containing approximately 100 μg of membrane protein were subjected to $NaDodSO_4$−polyacrylamide gel electrophoresis and electroblotted, and the blots were incubated with anti-C-terminal lac permease antibody, followed by incubation with horseradish peroxidase-conjugated protein A (Amersham). The blots were developed with chemiluminescent substrate (Renaissance; New England Nuclear) and exposed to film for 1 min. Membranes prepared from cells harboring pT7-5 with no *lacY* gene exhibited no immunoreactive material. The arrows at the left indicate the position of the marker protein carbonic anhydrase (32.5 kDa).

mutants are present in the membrane at levels comparable to that of C-less permease (Figure 5).

Effect of NEM on the Activity of Single Cys Mutants. The effect of NEM, a membrane-permeable sulfhydryl reagent, on the initial rate of lactose transport with each mutant is shown in Figure 6. The activity of the great majority of the Cys replacement mutants is not altered significantly by treatment with the alkylating agent. Only mutants F170C, G173C, and A187C are inactivated significantly and reproducibly by the alkylating agent, albeit by only about 50%. When viewed on a helical wheel plot (Figure 7), the sensitive positions cluster on one face of helix VI.

DISCUSSION

The results presented here extend a series of ongoing observations that have led to the conclusion that remarkably

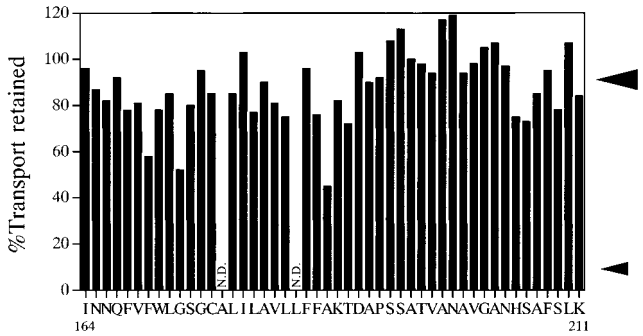

FIGURE 6: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single Cys mutants. Cells were incubated with 1 mM NEM (final concentration) at room temperature for 30 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971). Rates are presented as percentages of the rate measured in the absence of NEM. The arrows at the right indicate the corresponding rates measured in parallel assays for C-less permease [90%; see Sahin-Tóth et al. (1994b) in addition] and single-Cys148 permease [10%; see Weitzman and Kaback (1995) in addition]. NEM inhibition of mutants A177C and L184C which display low rates of transport (ca. 20% of C-less) could not be assayed with accuracy (N.D., not determined).

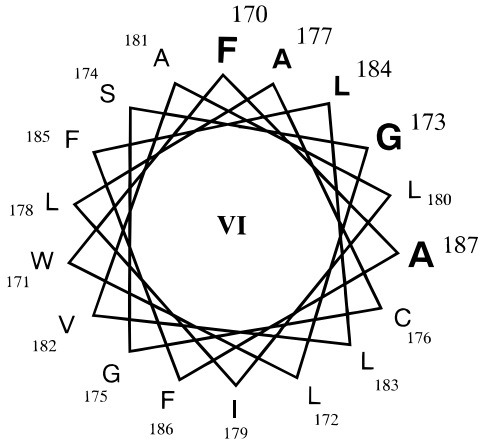

FIGURE 7: Helical wheel plot of residues in putative helix VI (from Phe170 to Ala187) viewed from the periplasmic surface of the membrane. Positions sensitive to NEM treatment (>40% inhibition of initial rate; see Figure 6) are shown as enlarged bold letters. Also shown (bold letters of regular size) are the two positions where Cys replacement results in low activity in the C-less background (Ala177 and Leu184).

few residues in lac permease are directly involved in the mechanism of lactose/H^+ symport. Thus, site-directed mutagenesis and Cys-scanning mutagenesis of almost all of the 417 residues in the permease have revealed that the great majority of mutants are both expressed and active [reviewed in Kaback et al. (1993, 1994) and Kaback (1996); see also Sun et al. (1996) and S. Frillingos, J. Sun, M. He, A. Gonzalez, and H. R. Kaback, unpublished results]. More specifically, only Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) have been shown to be mandatory for active lactose transport. It has also become apparent from these studies that the active Cys replacement mutants that are sensitive to inactivation by alkylation appear in clusters, suggesting that the surfaces of certain helices within the permease may be important for the conformational changes that occur during turnover. Both properties are reported here for putative transmembrane helix VI and the flanking hydrophilic loops. None of the individual residues is essential for active lactose transport, but the active mutants that are sensitive to inactivation by NEM cluster on one face of helix VI. Moreover, the data confirm previous findings showing that Trp171 (Menezes et al., 1990), Cys176 (Menick et al., 1987), Pro192 (Consler et al., 1991), His205 (Puttner et al., 1986, 1989), Gly173, Gly175, Gly202 (Jung et al., 1995a), and Ala177 (King & Wilson, 1990a; Brooker, 1991) do not play a direct role in the transport mechanism.

As observed previously with mutants L76C (helix III; Sahin-Tóth et al., 1994c), G64A (helix II; Jung et al., 1995a), and T348C (helix XI; S. Frillingos, R. Dunten, and H. R. Kaback, unpublished results), the single Cys mutants A177C and L184C exhibit low activity in the C-less background but highly significant activity when introduced into wild-type permease. In addition to demonstrating that these residues are not essential for lac transport, the data suggest that the mutations interact with one or more of the other eight mutations in C-less permease. For example, it is conceivable that Cys replacement for Ala177 in helix VI interacts with the bulky Val residue which replaces Cys154 in helix V of C-less permease (van Iwaarden et al., 1991). The two residues are at approximately the same depth in the membrane, and they are likely to be in close proximity, since the connecting periplasmic loop is very short (Figure 1). In this context, it is particularly interesting that Ala177 and Leu184 cluster on the same face of helix VI as the three NEM-sensitive positions (Figure 7), implying that this helical face may be important for lactose transport due to an interaction with another helix, possibly helix V.

On the basis of the observation that the mutation A177V alters the sugar specificity of the permease, it has been suggested that Ala177 is involved in substrate recognition (Brooker & Wilson, 1985; King & Wilson, 1990a; Goswitz & Brooker, 1993). As discussed by King and Wilson (1990a,b), a direct role seems unlikely, since neither Ala nor Val can H bond to sugars or bind H^+, while replacement of Ala177 with Val has pleiotropic effects, altering specificity for many sugars *and* modifying the coupling between lactose and H^+ translocation. In agreement, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) does not change the reactivity of A177C permease with [^{14}C]NEM or 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (S. Frillingos, J. Sun, and H. R. Kaback, unpublished results), indicating that Ala177 is not in contact with substrate.

In an initial test for accessibility or reactivity of the Cys residues at the positions mutated in this study, the effect of NEM on lactose transport was studied in each active mutant. The great majority of the mutants are insensitive; only F170C, G173C, and A187C are inactivated significantly (ca. 50%) by the alkylating agent. Although it is possible that most of the Cys replacement mutants are unreactive, this seems unlikely because NEM is relatively permeant, and in addition, a number of single Cys mutants in helices V, VII, X, and XI which are located presumably in the middle of the membrane or disposed toward the inner surface are readily inhibited (Sahin-Tóth et al., 1992; Sahin-Tóth & Kaback, 1993; Dunten et al., 1993; Frillingos et al., 1994; Weitzman & Kaback, 1995). Most importantly, alkylation with [^{14}C]NEM has been demonstrated *in situ* for a number of Cys replacement mutants at various positions within transmembrane helices of the permease (Frillingos & Kaback, 1996). In this respect, it is important that the three NEM-sensitive mutants described here cluster on one face of helix

VI (Figure 7), the same face on which Ala177 and Leu184 are located. It is also interesting that replacement of Gly173 or Ala187 with a bulkier Cys residue partially compromises activity (Figure 2A), and NEM treatment causes additional loss of activity (Figure 6), indicating further that one face of helix VI is relatively important (Figure 7). Similarly, replacement of Ala177 with bulkier amino acid residues (Brooker, 1991; Gram & Brooker, 1992; Goswitz & Brooker, 1993) alters sugar specificity and leads to a partial defect in β-galactoside transport, while replacement with Gly (Goswitz & Brooker, 1993) has no effect on sugar specificity.

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