Cysteine-Scanning Mutagenesis of Transmembrane Domain XII and the Flanking Periplasmic Loop 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 transmembrane domain XII and the periplasmic loop between putative helices XI and XII (loop XI/XII) was replaced individually with Cys. Out of 34 mutants, 31 exhibit 60−100% or more of C-less activity, mutants Gly377 → Cys and Leu385 → Cys exhibit lower rates of transport but accumulate lactose about 60−70% as well as C-less, and mutant Leu400 → Cys exhibits <20% of C-less activity. Immunoblots reveal that all of the mutant proteins are present in the membrane in amounts comparable to that of C-less with the exception of mutants Gly377 → Cys and Leu385 → Cys which are expressed about 40% as well as C-less and mutant Leu400 → Cys which is hardly detectable. When transferred to the wild-type background, however, mutant Leu400 → Cys is expressed normally and exhibits highly significant transport activity. Finally, each active Cys-replacement mutant was assayed for sensitivity to *N*-ethylmaleimide, and with three exceptions, the mutants are essentially unaffected by the alkylating agent. Mutants Val367 → Cys, Gly370 → Cys, and Tyr373 → Cys which are predicted to be immediately distal to helix XI in loop XI/XII are significantly inactivated. The periodicity observed suggests that the periplasmic end of transmembrane domain XI may extend to position 373. In the following paper [Voss, J., He, M. M., Hubbell, W. L., & Kaback, H. R. (1996) *Biochemistry 35*, 12915−12918], site-directed spin labeling of single-Cys mutants at positions 387−402 is used to demonstrate that transmembrane domain XII is in an α-helical conformation.

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^+. The permease is encoded by the *lacY* gene which has been cloned and sequenced, and the *lacY* gene product has been solubilized from the membrane, purified to homogeneity, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport as a monomer [reviewed in Kaback et al. (1994) and Kaback (1996)]. On the basis of circular dichroism and hydropathy analysis of the primary amino-acid sequence, a secondary structure was proposed in which the permease is composed of 12 hydrophobic α-helices connected by hydrophilic loops (Figure 1). Support for general features of the model was subsequently obtained from various experimental approaches, 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). More recently, 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 [see Kaback et al. (1994) and Kaback (1996)]. The model has been confirmed and extended by engineering divalent metal-binding sites (bis- or tris-His residues) between the transmembrane domains of the permease (He et al., 1995a,b; Jung et al., 1995), site-directed chemical cleavage (Wu et al., 1995), and site-directed spin labeling (Wu et al., 1996).

Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) have been used extensively to reveal functionally important residues. Almost all of the 417 residues in the permease have been mutagenized, and the great majority of the mutants are both expressed and active [see Kaback et al. (1994) and Kaback (1996)]. Specifically, only Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) are irreplaceable with respect to active lactose transport. However, the activity of certain active Cys-replacement mutants is inhibited by alkylation, and these mutants appear in clusters, suggesting that helical surfaces within the permease may be important for substrate binding and/or the conformational changes that occur during turnover.

Although the 17-amino acid carboxyl-terminal tail of the permease (residues 401−417) is not involved in insertion of the protein into the membrane, its stability or its ability to catalyze transport, a 5-amino acid segment near the carboxyl terminus of putative helix XII (residues 396−400) is important for stability and hence activity once the protein is inserted into the membrane (Roepe et al., 1989). Permease sequentially truncated at positions 396−401 exhibits a progressive increase in transport activity and life time after insertion into the membrane (McKenna et al., 1991).

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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; $NaDodSO_4$, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $\Delta\bar{\mu}_{H^+}$, the H^+ electrochemical gradient across the membrane.

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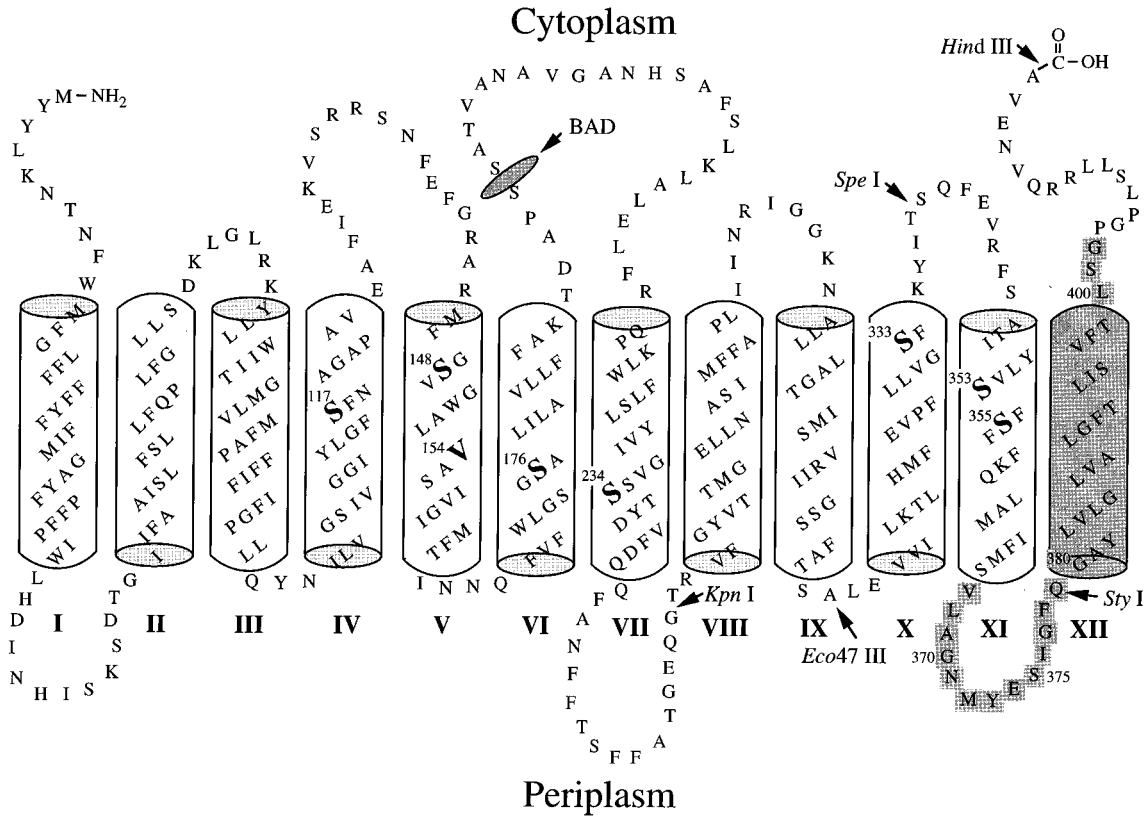

FIGURE 1: Secondary structure model of lac permease. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. Positions at which native Cys residues were replaced with given residues in C-less permease are highlighted and numbered. The shaded area highlights the region of the permease subjected to Cys-scanning mutagenesis. Also indicated are the restriction endonuclease sites used for construction of the mutants and the biotin acceptor domain (BAD).

Moreover, permease with four Leu residues at positions 397−400 is fully functional and completely stable, while permease with Gly-Pro-Gly-Pro at the same positions is inactive and unstable (McKenna et al., 1992). The results suggest that the last turn of putative helix XII may interact with another portion of the permease and that this interaction is important for folding of the permease into an active conformation which is resistant to proteolysis.

Cys-scanning mutagenesis has now been employed systematically to examine the residues in putative helix XII and the flanking periplasmic loop. In this study, the effects of individual Cys replacements for Val367−Gly402 on permease activity and expression are reported, as well as the sensitivity of the mutants to *N*-ethylmaleimide (NEM). The results demonstrate that, although none of the residues plays an important role in the transport mechanism, three single-Cys mutants (V367C,[2] G370C, and Y373C) are significantly inactivated by NEM, indicating that the periplasmic end of helix XI may extend as far as Tyr373. In the following paper (Voss et al., 1996) site-directed spin labeling of single-Cys mutants is used to demonstrate that transmembrane domain XII is in an α-helical conformation.

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, 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.

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 a carrier for the plasmids described and for detection of lac permease activity on MacConkey-agar indicator plates (Difco Laboratories) containing 25 mM lactose. *E. coli* T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$ (*A*), *rspL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR/F′*, *lacI*^{q}*O*$^+$*Z*^{D118} (*Y*$^+$*A*$^+$)] (Teather et al., 1980) harboring plasmid pT7-5 or pKR35/*lacY* with given mutations was used for expression of lac permease from the *lac* promoter/operator.

Mutagenesis. Single-Cys mutants from V367C to Q379C were constructed by oligonucleotide-directed, site-specific mutagenesis of the C-less version of the cassette *lacY* gene (EMBL-X56095) in the plasmid pT7-5 (van Iwaarden et al., 1991) by either one- or two-stage polymerase chain reaction (PCR) (overlap−extension; Ho et al., 1989). The PCR products were digested with *Spe*I and *Hin*dIII (for two-stage PCR) or *Eco47*III and *Sty*I (for one-stage PCR) and ligated into similarly treated pT7-5/cassette *lacY* encoding C-less

[2] Site-directed mutants are designated as follows. The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type lac permease and then a second letter denoting the amino acid replacement at this position.

permease (see Figure 1 for locations of sites). Single-Cys mutants from G381C to G402C were prepared by two-stage PCR mutagenesis of cassette *lacY* encoding C-less permease in pKR35 with a biotin acceptor domain in the middle cytoplasmic loop (pKR35/L6XB) (Consler et al., 1993) to facilitate purification (Voss et al., 1996). All Gly → Cys mutants were prepared as descibed, but G380C and G386C pemeases were omitted from the study, as these mutants are poorly expressed as single-Cys proteins in the C-less background (Jung et al., 1995a). For consistency and for purposes of purification, pT7-5/cassette *lacY* encoding G391C or G402C permease was transferred into pKR35/L6XB by using restriction fragment replacement with the *Kpn*I and *Hin*dIII restriction sites. Cys replacement for Leu400 in the wild-type permease was made by restriction fragment replacement of the DNA fragment encoding the mutation into pT7-5/cassette *lacY* using the *Sty*I and *Hin*dIII restriction sites in the cassette gene. All plasmids were transformed into *E. coli* HB101 (Z^+Y^-), and transformants were selected on MacConkey (Difco) indicator plates containing 25 mM lactose. 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 (Sanger et al., 1977) and synthetic sequencing primers, after alkaline denaturation (Hattori & Sakaki, 1986).

Growth of Bacteria. For lactose transport assays and membrane preparation, *E. coli* T184 (Z^-Y^-) transformed with each of the plasmids described was grown aerobically at 37 °C in LB medium containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Dense cultures were diluted 10-fold and allowed to grow 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 by centrifugation.

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 the rapid filtration method (Consler et al., 1991).

Membrane Preparation. Crude membrane fractions from *E. coli* T184 were prepared essentially as described (Sahin-Tóth & Kaback, 1993). Briefly, cells were suspended in ice-cold osmotic shock buffer (25 mM Tris-HCl (pH 8.0)/45% sucrose/1.0 mM ethylenediaminetetraacetic acid), centrifuged, resuspended in ice-cold water, allowed to stand for 10 min on ice, and then incubated with lysozyme (0.13 mg/mL) for 30 min. Suspensions were sonified, and after removal of unlysed cells, membranes were harvested by centrifugation at 250000g_{max} for 1 h at 4 °C in a Beckman Optima TL™ ultracentrifuge.

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

Immunological Analyses. Membrane fractions were subjected to 12% $NaDodSO_4$−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).

RESULTS

Verification of Mutants. All mutations described in Materials and Methods were verified by sequencing the length of subcloned double-stranded DNA through the ligation junctions. Except for the desired base changes, the sequences were identical to that of C-less cassette *lacY*.

Colony Morphology. As a preliminary qualitative assay of transport activity, each mutant was transformed into *E. coli* HB101 ($lacZ^+Y^-$), and colonies were grown on MacConkey indicator plates containing 25 mM lactose. This strain expresses active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of the external lactose 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. Indicator plates report "downhill" translocation of lactose and give no indication as to whether the cells catalyze accumulation. With the exception of L400C which yields red colonies with a white halo in the C-less background and dark red colonies in the wild type, the other Cys-replacement mutants grow as red colonies indistinguishable from cells expressing C-less permease. Therefore, judging from indicator plates, all of the mutants appear to retain at least some ability to translocate lactose downhill.

Active Lactose Transport. E. coli T184 ($lacZ^-Y^-$) transformed with plasmids encoding given permease mutants was used to test the ability of the mutants to catalyze active lactose transport. The majority of the 34 mutants transport the disaccharide at highly significant rates (Figure 2A). Thirty-one mutants exhibit rates that are between 60 and 130% of that of C-less permease, while G377C and L385C transport at about 40% of the rate of C-less. Only L400C exhibits a markedly diminished rate of transport (<20% of that of C-less). Steady state levels of lactose accumulation for 33 of the mutants range between 60 and 140% of that of C-less permease (Figure 2B), while L400C accumulates only about 20% as well as C-less. In order to investigate the properties of L400C permease further, the mutation was transferred to the wild-type background (Figure 3). In the presence of the eight native Cys residues, mutant L400C transport exhibits 60−70% of wild-type activity. Thus, Leu400 is not essential for active transport.

Expression of Permease Mutants. Western blot analyses of membrane fractions prepared from *E. coli* T184 expressing the Cys-replacement mutants demonstrates that the majority of the mutants are present in the membrane at levels comparable to that of C-less permease (Figure 4). On the other hand, mutants G377C and L385C exhibit reduced expression (ca. 40% of that of C-less) which is consistent with relatively low rates of transport and essentially normal steady state levels of accumulation (Figure 2). Expression of mutant L400C is markedly reduced in the C-less background; however, when the mutation transferred to the wild type, expression at about 70% of the wild-type level is observed (data not shown).

Effect of NEM. The effect of NEM, a membrane-permeable thiol reagent, on the initial rate of lactose transport by each mutant, with the exception of L400C which has very low activity, is shown in Figure 5. A clear periodicity is

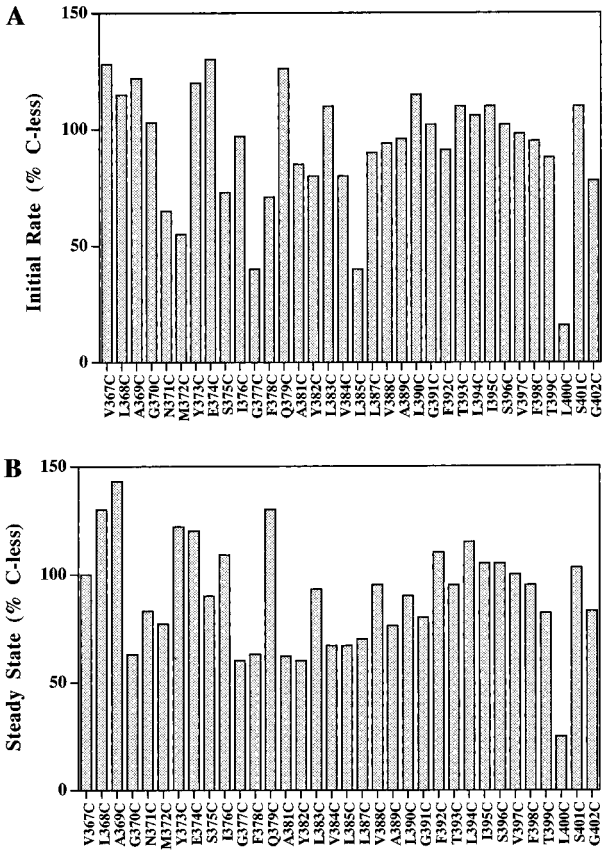

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants or C-less permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described in Materials and Methods. (A) Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 50 nmol min^{-1} (mg of $protein^{-1}$); 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) averaged 2 nmol min^{-1} (mg of $protein^{-1}$) (i.e. 4% of that of C-less). (B) Steady state levels of lactose accumulation. Results are expressed as a percentage of C-less permease which averaged 170 nmol of lactose/(mg of protein). Although not shown (see Figure 3), T184 cells harboring pT7-5 accumulated 10 nmol of lactose/(mg of protein) in 1 h (i.e. 5.9% of that of C-less).

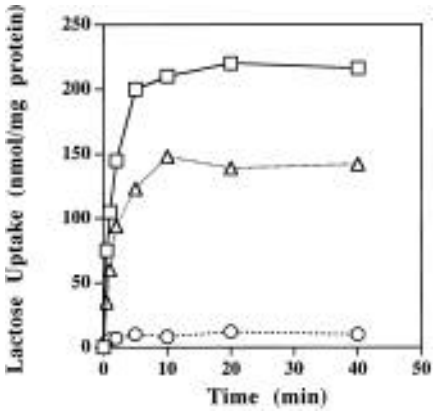

FIGURE 3: Time course of lactose transport by mutant p(wt)L400C (mutant L400C in the wild-type background). *E. coli* T184 cells transformed with plasmid pT7-5 with no *lacY* insert (○), pT7-5 encoding wild-type permease (□), or p(wt)L400C permease (△) were grown and assayed as described in Figure 2 and in Materials and Methods.

observed for positions 367−373 such that mutants V367C, G370C, and Y373C are inactivated by 85, 75, and 70%,

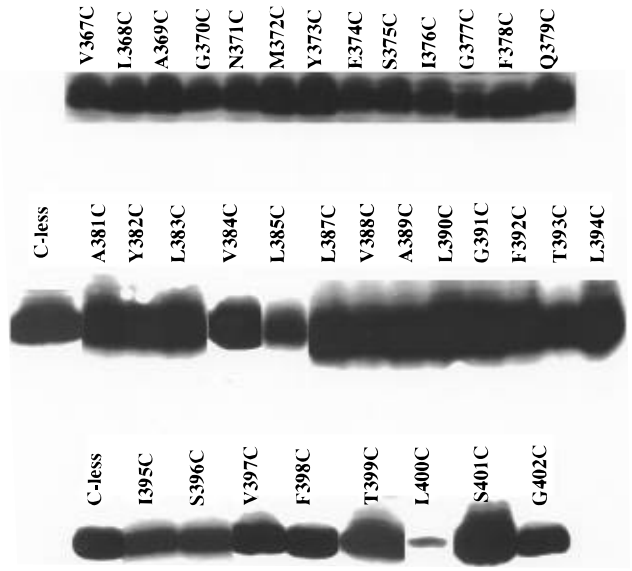

FIGURE 4: Western blots of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 cells harboring given plasmids, as described in Materials and Methods. Samples containing approximately 200 μg of membrane protein were subjected to $NaDodSO_4$−polyacrylamide gel electrophoresis and electroblotted, and the blots were incubated with anti-C-terminal 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.

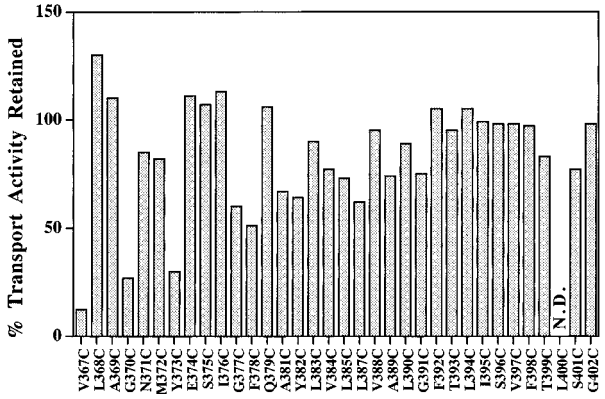

FIGURE 5: Effect of NEM on active lactose transport by *E. coli* T184 cells harboring plasmids encoding single-Cys mutants. Cells were incubated with 1 mM NEM (final concentration) at room temperature for 30 min, and the reaction was quenched by addition of 10 mM dithiothreitol (final concentration) and 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 a percentage of the rate measured in the absence of NEM. NEM inhibition of mutant L400C which displays low rates of transport (ca. 20% of that of C-less) was not determined (N.D.).

respectively, while single-Cys mutants at the intervening positions are relatively unaffected. These positions cluster on the same face of helix XI as the NEM-sensitive Cys-replacement mutants in helix XI described by Dunten et al. (1993) (Figure 6). On the other hand, the Cys-replacement mutants in the remainder of loop XI/XII and transmembrane domain XII are relatively insensitive to NEM and exhibit no obvious periodicity.

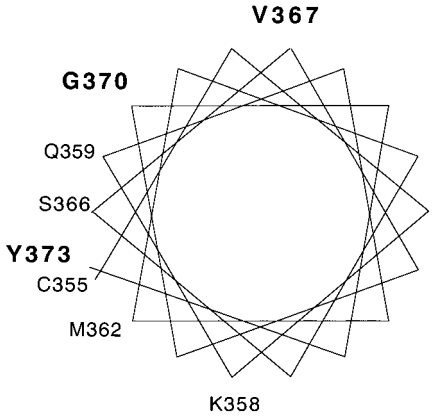

FIGURE 6: Helical wheel plot of modified helix XI, including positions 367−373. Positions in helix XI that are sensitive to NEM treatment (>60% inhibition of initial rate) are shown as normal capital letters [from Dunten et al. (1993)]. Bold capital letters show the positions previously thought to be in loop XI/XII that are sensitive to NEM inhibition when replaced with Cys. The helix is viewed from the cytoplasmic surface of the membrane.

DISCUSSION

The results presented here extend a series of ongoing observations that have led to the conclusion that remarkably 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 reveal that the great majority of mutants are both expressed and active, and only Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X) are irreplaceable with respect to active transport [see Kaback et al. (1994) and Kaback (1996)]. It is also apparent from these studies that the active Cys-replacement mutants which are sensitive to inactivation by NEM appear in clusters, suggesting that helical surfaces within the permease may be important for the conformational changes that occur during turnover. Both properties are investigated here for putative helix XII and flanking region loop XI/XII. Clearly, none of the individual residues is irreplaceable with respect to active lactose transport. However, a periodicity for NEM inhibition is observed within putative loop XI/XII, indicating that residues 367−373 may comprise the periplasmic end of helix XI.

Among the 36 Cys-replacement mutants in the region studied here, mutants G380C and G386C exhibit significantly decreased rates of transport, and mutant L400C exhibits both a diminished rate and steady state level of lactose accumulation. As shown previously (Jung et al., 1995a), G380C and G386C are expressed at significantly low levels from *lac* promoter/operator. However, the mutants are inserted into the membrane in a stable form when expressed at a high rate from the T7 promoter, indicating that both mutant proteins may be proteolyzed at a point between translation and insertion into the membrane. In addition, both mutants accumulate lactose to significant steady state levels of accumulation. Taken together, the results indicate that neither of the mutants is uncoupled and that they probably retain close to normal specific activities. Similarly, mutant L400C exhibits an even lower level of expression from the *lac* promoter/operator in C-less background. However, when the mutation is transferred to wild-type permease, expression and activity are restored to near control levels. Therefore, Leu400 is not important for activity, and the expression defect with L400C is observed in the C-less but not in the wild-type background for reasons that are not apparent.

In an initial test for accessibility or reactivity of Cys residues at the positions mutated, the effect of NEM on lactose transport was studied, and it is clear that none of the mutants in the putative transmembrane helix XII is significantly inactivated. Although it is possible that the Cys-replacement mutants are unreactive, this seems unlikely because (i) NEM is relatively permeant, (ii) a number of single-Cys mutants located presumably in the middle of the membrane or disposed toward the inner surface in other helices are reactive [see Kaback et al. (1994), Kaback (1996), and Frillingos and Kaback (1996)], and (iii) most of the residues in helix XII react with the methane thiosulfonate spin-label (Voss et al., 1996). Therefore, it is concluded that most of the Cys-replacement mutants in helix XII are probably accessible to NEM.

In contrast, three Cys-replacement mutants at positions presumed to be in loop XI/XII (Val367, Gly370, and Tyr373) are relatively strongly inactivated by NEM. Previous Cys-scanning mutagenesis of putative helix XI shows that the activities of single-Cys permeases Cys355, Q359C, M362C, and S366C are strongly inhibited by NEM, and these positions are located on the same face of helix XI, probably lining part of a crevice between helices in the C-terminal half of permease (Dunten et al., 1993). The three positions that exhibit NEM inhibition here (i.e. 367, 370, and 373) display helical periodicity, and moreover, they reside on the same face of helix XI as positions 355, 359, 362, and 366 when modeled on a helical wheel plot (Figure 6). The results suggest that the periplasmic end of helix XI may include an additional two turns containing residues 367−373.

In the following paper (Voss et al., 1996), Cys-replacement mutants L387C−G402C are used to confirm the α-helical conformation of putative helix XII by site-directed spin labeling.

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