Cysteine Scanning Mutagenesis of Putative Helix XI 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 in putative transmembrane helix XI was individually replaced with Cys (from Ala347 to Ser366). Fifteen of the 20 mutants are highly functional and accumulate lactose to >60% of the level achieved by C-less permease, and an additional three mutants, all located at the cytoplasmic end of the helix, exhibit lower but significant lactose accumulation. Cys replacements for Thr348 or Lys358 result in virtually inactive permease. Lys358, however, is not essential for active lactose transport but plays a role in permease folding or membrane insertion by interacting with Asp237. Immunoblots reveal that all mutant proteins are present in the membrane in amounts comparable to C-less with the exception of Lys358→Cys which is hardly detectable, as expected. The results highlight Thr348 as a potentially important residue for further analysis. Finally, all active mutants were assayed after treatment with the sulfhydryl reagent *N*-ethylmaleimide, and results range from nearly complete inhibition to almost 2-fold stimulation. Remarkably, all of the strongly inhibited positions lie on one face of helix XI. The implications of the findings for packing of transmembrane helices in the C-terminal half of the permease are discussed.

The lactose (lac)[1] permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled, stoichiometric translocation of β-galactosides and H^+ (i.e., symport or cotransport). 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, 1989, 1992). On the basis of circular dichroic studies and hydropathy analysis of primary amino acid sequence (Foster et al., 1983), a secondary structure was proposed in which the permease has 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. Evidence supporting the general features of the model and demonstrating that both the N and C termini are on the cytoplasmic face of the membrane has been 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), and chemical modification (Page & Rosenbusch, 1988). Unequivocal support for the 12-helix motif has been obtained from analyses of an extensive series of lac permease-alkaline phosphatase (*lacY-phoA*) fusions (Calamia & Manoil, 1990).

Although the mechanism by which lac permease accomplishes coupled lactose/H^+ symport remains obscure, oligonucleotide-directed, site-specific mutagenesis has allowed the identification of certain functionally important residues, in particular Arg302 (helix IX), His322 (helix X), and Glu325 (helix X), as well as other charged residues in putative transmembrane domains (reviewed in Kaback et al. (1993)). Once such a region of interest has been identified, additional side chains that are important for activity can be located by systematic "scanning" mutagenesis, wherein each amino acid is individually replaced with a "scanner" residue, usually Ala, which eliminates the side chain beyond the β carbon (Cunningham & Wells, 1989). With hydrophobic membrane proteins, scanning with Cys has advantages over Ala because the Cys side chain is of intermediate bulk and is relatively hydrophobic (Kyte & Doolittle, 1982). Furthermore, by using a Cys-free protein, the unique reactivity of Cys residues introduced into specified positions in the protein can be exploited to gain structural information (Falke & Koshland, 1987, Altenbach et al., 1990; Pakula & Simon, 1992; Jung et al., 1993). In a functional lac permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991), helices IX and X were recently subjected to systematic Cys scanning mutagenesis (Sahin-Tóth and Kaback, 1993a), and only the previously identified residues Arg302, His322, and Glu 325 were found to be important for active lactose transport. In addition, it was demonstrated that the *N*-ethylmaleimide (NEM) reactivity of permease with a single Cys at position 315 is markedly enhanced in the presence of β-D-galactopyranosyl 1-thio-β-D-galactopyranoside (TDG) or an H^+ electrochemical gradient ($\Delta\bar{\mu}_H^+$).

Residues outside of helices IX and X also play a role in transport (see Kaback (1992)) but a three-dimensional structure of lac permease is not available. Recently, however, interactions between helices VII to XI in the C-terminal half of the permease have been identified (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b; Jung et al., 1993). Since helix VII interacts with both helices X and XI, the latter may be adjacent to one another, and in addition, helix XI may also contain residues that participate in the transport mechanism. Con-

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[1] Abbreviations: lac, lactose; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside; $\Delta\bar{\mu}_H^+$, the H^+ electrochemical gradient across the membrane; $NaDodSO_4$, sodium dodecyl sulfate; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; Tris, tris(hydroxymethyl)aminomethane; TBST, 10 mM Tris–HCl (pH 7.4)/0.9% NaCl/0.2% Triton X-100.

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Table I: DNA Sequence Analysis of Cysteine Replacement Mutants in the C-less Cassette *lacY* Gene

mutant		mutagenic oligonucleotides[a]	codon change
A347C	sense	CGTTTTTCATGTACGATTTATCTG	GCG→TGT
	antisense	ATAAATCGTACATGAAAAACGCAC	
T348C	sense	TTTTCAGCGTGTATTTATCTGGTC	ACG→TGT
	antisense	CAGATAAATACACGCTGAAAAACG	
I349C	sense	TCAGCGACGTGTTATCTGGTC	ATT→TGT
	antisense	GACCAGATAACACGTCGCTGA	
Y350C	sense	GCGACGATTTGTCTGGTCAGT	TAT→TGT
	antisense	ACTGACCAGACAAATCGTCGC	
L351C	sense	ACGATTTATTGTGTCAGTTTCAGC	CTG→TGT
	antisense	GAAACTGACACAATAAATCGTCGC	
V352C	sense	ATTTATCTGTGCAGTTTCAGC	GTC→TGC
	antisense	GCTGAAACTGCACAGATAAATCGT	
S353C	sense	TATCTGGTCTGTTTCAGCTTC	AGT→TGT
	antisense	GAAGCTGAAACAGACCAGATA	
F354C	sense	CTGGTCAGTTGCAGCTTCTTT	TTC→TGC
	antisense	AAAGAAGCTGCAACTGACCAG	
S355C	sense	GTCAGTTTCTGCTTCTTTAAG	AGC→TGC
	antisense	CTTAAAGAAGCAGAAACTGAC	
F356C	sense	AGTTTCAGCTGCTTTAAGCAA	TTC→TGC
	antisense	TTGCTTAAAGCAGCTGAAACT	
F357C	sense	TTCAGCTTCTGTAAGCAACTG	TTT→TGT
	antisense	CAGTTGCTTACAGAAGCTGAA	
K358C	sense	AGCTTCTTTTGTCAACTGGCGATG	AAG→TGT
	antisense	CGCCAGTTGACAAAAGAAGCTGAA	
Q359C	sense	TTCTTTAAGTGTCTGGCGATGATT	CAA→TGT
	antisense	CATCGCCAGACACTTAAAGAAGCT	
L360C	sense	TTTAAGCAATGTGCGATGATTTTT	GTG→TGT
	antisense	AATCATCGCACATTGCTTAAAGAA	
A361C	sense	AAGCAACTGTGTATGATTTTTATG	GCG→TGT
	antisense	AAAAATCATACACAGTTGCTTAAA	
M362C	sense	CAACTGGCGTGTATTTTTATGTCT	ATG→TGT
	antisense	CATAAAAATACACGCCAGTTGCTT	
I363C	sense	CTGGCGATGTGTTTTATGTCTGTA	ATT→TGT
	antisense	AGACATAAAACACATCGCCAG	
F364C	sense	GCGATGATTTGTATGTCTGTA	TTT→TGT
	antisense	TACAGACATACAAATCATCGC	
M365C	sense	ATGATTTTTTGTTCTGTACTGGCG	ATG→TGT
	antisense	CAGTACAGAACAAAAAATCATCGC	
S366C	sense	ATTTTTATGTGTGTACTGGCG	TCT→TGT
	antisense	CGCCAGTACACACATAAAAAT	

[a] Sequences of mutagenic primers are presented in the 5′→3′ order with altered codons in boldface type.

sequently, as described here, Cys-scanning mutagenesis was employed to study helix XI (residues Ala347 to Ser366). The effects of individual Cys replacements on permease activity and expression were determined, and each mutant was tested for sensitivity to the sulfhydryl reagent NEM. In addition to Lys358 which interacts with Asp237, Thr348 does not tolerate Cys substitution and may be an important residue. Strikingly, the NEM studies show that the strongly inhibited residues reside on the same face of helix XI, a finding that is consistent with a recently formulated model delineating the arrangement of the helices in the C-terminal half of the permease (Jung et al., 1993; Kaback et al., 1993).

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., 1984a) was prepared by BabCo, Richmond, CA. All restriction endonucleases, T4 DNA ligase, and Vent$_R$ DNA polymerase were from New England Biolabs, Beverly, MA. Sequenase was from United States Biochemical, Cleveland, OH. All other materials were reagent grade and obtained from commercial sources.

Methods

Bacterial Strains. E. coli HB101 [*hsdS20*(r^-_B,m^-_B), *recA13*, *ara14*,*proA2*,*lacY1*,*galK2*,*rpsL20*(*Sm^r*), *xyl-5*,*mtl-1*,*SupE44*,λ^-/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. Cys-replacement 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[2]; van Iwaarden et al., 1991). The sequences of the two mutagenic primers used to create each mutation are presented in Table I. The polymerase chain reaction (PCR) overlap extension method described by Ho et al. (1989) was employed with the substitution of Vent$_R$ DNA polymerase for *Taq* DNA polymerase in the two stages of PCR. First-stage PCR products were purified in agarose gels and gene-cleaned (Bio 101) prior to the second stage of PCR. The second-stage PCR products, of approximately 400 base pairs, were extracted with chloroform, ethanol precipitated, and digested with *Sty*I, *Spe*I, and *Eco*47III restriction

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

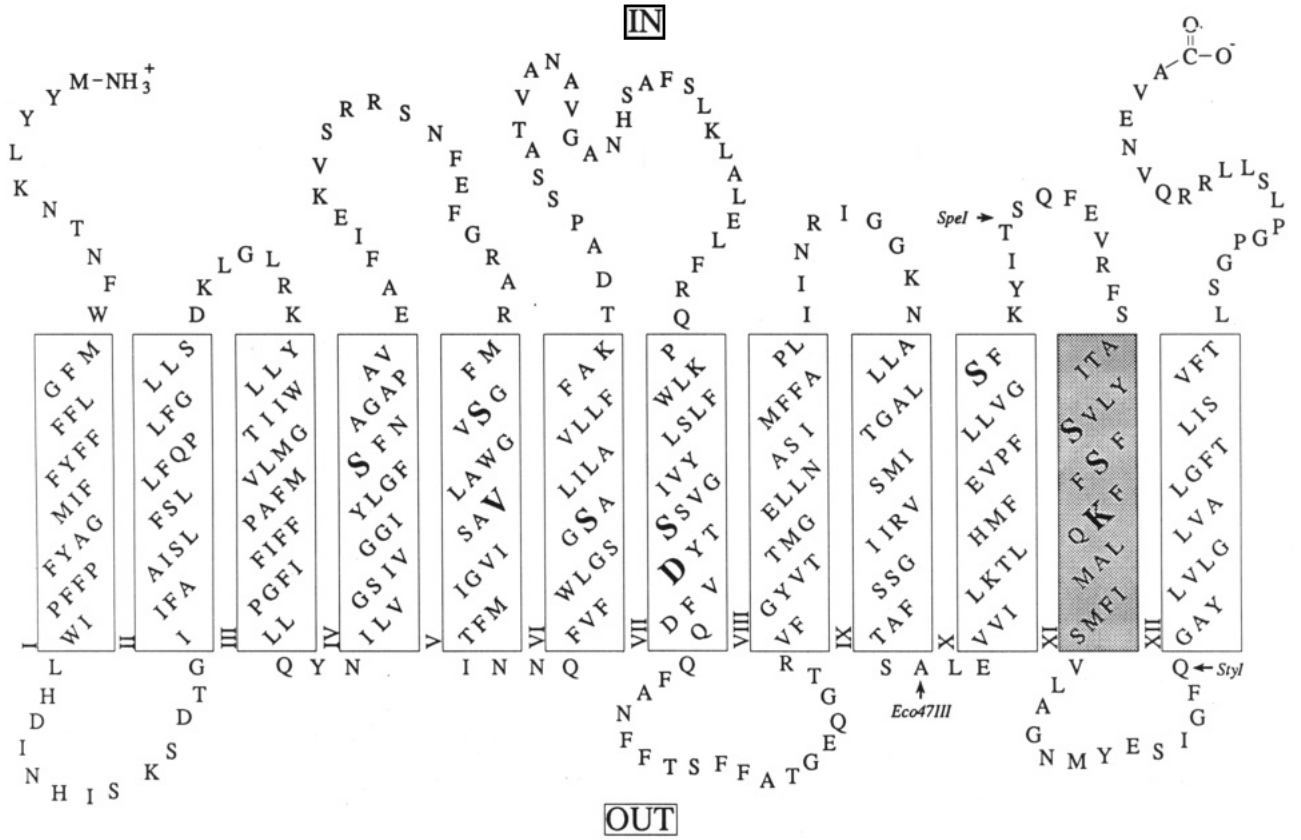

FIGURE 1: Secondary-structure model of *E. coli* lac permease based on hydropathy analysis (Foster et al., 1983). The single letter amino acid code is used, and the C-less version is shown with the residues replacing the eight native cysteines highlighted. Asp237 and Lys358, which form a putative charge pair, are also highlighted. Hydrophobic transmembrane helices are enclosed in boxes, and the shaded box shows helix XI, which was subjected to Cys scanning mutagenesis. The region enclosed as helix VII differs from the original model based on the results of a series of *lacY-phoA* fusions (M. L. Ujwal and H. R. Kaback, unpublished observations). The locations of relevant restriction endonuclease sites in the corresponding DNA sequence are also indicated.

endonucleases (see Figure 1 for location of sites). The *Spe*I–*Sty*I fragments were isolated from low melting point agarose gels and ligated to similarly treated $pC_7S/C154V$ 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 by 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, was grown aerobically at 37 °C in Luria broth with streptomycin (10 μg/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 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 KP_i (pH 7.5)/10 mM $MgSO_4$ and then adjusted with the same buffer to an optical density of 10 at 420 nm. 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 the rapid filtration method as described by Consler et al. (1991). Transport after NEM treatment was performed similarly except that the assays were conducted in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS) according to Konings et al. (1971) and Kaback (1974).

Membrane Preparation. Cells were washed twice with 50 mM Tris–HCl (pH 8.0)/100 mM NaCl/1 mM EDTA/1 mM PMSF and resuspended in ice-cold osmotic shock buffer (25 mM Tris–HCl (pH 8.0)/45% sucrose/1 mM EDTA/1 mM PMSF). After 20 min on ice, cells were pelleted, resuspended in 0.75 mL of cold water with 1 mM PMSF, and allowed to stand 10 min on ice before adding 0.1 mg of lysozyme. 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 250 000*g* for 1 h at 4 °C in a Beckman Optima TL ultracentrifuge.

Protein Determinations. Protein was assayed by a modified Lowry method (Peterson, 1977) with bovine serum albumin as standard.

Immunoblotting. Membranes prepared as described were resuspended in sodium dodecylsulfate ($NaDodSO_4$) gel-loading buffer prior to electrophoresis performed as described (Newman et al., 1981). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-PVDF, Millipore) for 2 h at 0.5 A in transfer buffer (25 mM Tris–HCl/192 mM glycine/20% methanol/pH 8.3) at 4 °C. Blots were then blocked in 5% bovine serum albumin in 10 mM Tris–HCl (pH 7.4)/0.9% NaCl/0.2% Triton X-100 (TBST). After 1 h, rabbit polyclonal antisera 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 four times with TBST for 5 min each, and incubated again in TBST containing 5% bovine serum albumin for 10 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 15-min washes with TBST, blots were developed with luminescent substrate (Amersham) according to the manufacturer's instructions.

RESULTS

Construction and Verification of Mutants. Each amino acid of putative helix XI, residues A347–S366 (Figure 1), was replaced individually with Cys in C-less permease (van Iwaarden et al., 1991). As described in the Methods, each mutant was constructed using synthetic oligonucleotide primers and the PCR overlap extension method (Ho et al., 1989). The PCR products were cloned into C-less permease using the *Sty*I and *Spe*I restriction endonuclease sites (see Figure 1 for location of sites). Note that mutants S353C and S355C each restore a single native Cys to C-less permease. All mutations were verified by double-stranded DNA sequencing, and except for the desired base changes summarized in Table I, 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" can be 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 the imported lactose, and metabolism of the monosaccharides released causes acidification which makes the colonies appear red. Cells impermeable to lactose appear as white colonies, whereas mutants with low activity grow as red colonies with a white halo. The four mutants at the N terminus of helix XI (A347C, T348C, I349C, and Y350C) and mutant K358C yield red colonies with a white halo. The remainder grow as red colonies indistinguishable from cells expressing C-less permease. Therefore, all the mutants retain some ability to translocate lactose downhill.

Active Lactose Transport. The ability of mutant permeases to transport lactose "uphill" against a concentration gradient was assayed in *E. coli* T184 (*Z*$^-$*Y*$^-$) which lacks β-galactosidase and cannot metabolize lactose. One-h time courses of [1-^{14}C]-lactose uptake for each mutant, the negative control (pT7–5 with no insert), and the C-less positive control were determined, and both the rates of lactose uptake measured at 1 min and the steady-state levels of lactose accumulation are presented as a percentage of the C-less control in Figure 2. Three categories of mutants emerge from the analysis. The first, representing the majority of the mutants, catalyze lactose accumulation at significant rates and to steady states that approximate C-less permease. The second group, four mutants at the N terminus of helix XI (A347C, T348C, I349C, and Y350C) and K358C, exhibits poor activity (<35% of the initial rate and steady state of C-less). F354C has a low initial rate (ca. 35% of C-less), but the steady-state level of accumulation reaches 73% of C-less, indicating that the ability to concentrate lactose is not greatly compromised by this mutation. The third group, S353C, S355C, M362C, and M365C, are mutants with significantly enhanced activity (>150% the initial rate and >125% the steady state of C-less). F364C also achieves a high steady state (179% of C-less), but the initial rate is not as enhanced (128% of C-less).

Time courses of lactose transport for the mutants with lowest and highest activities are shown in Figure 3. The only Cys replacement in helix XI that completely inactivates is at position 358, where the positive charge of the native lysine

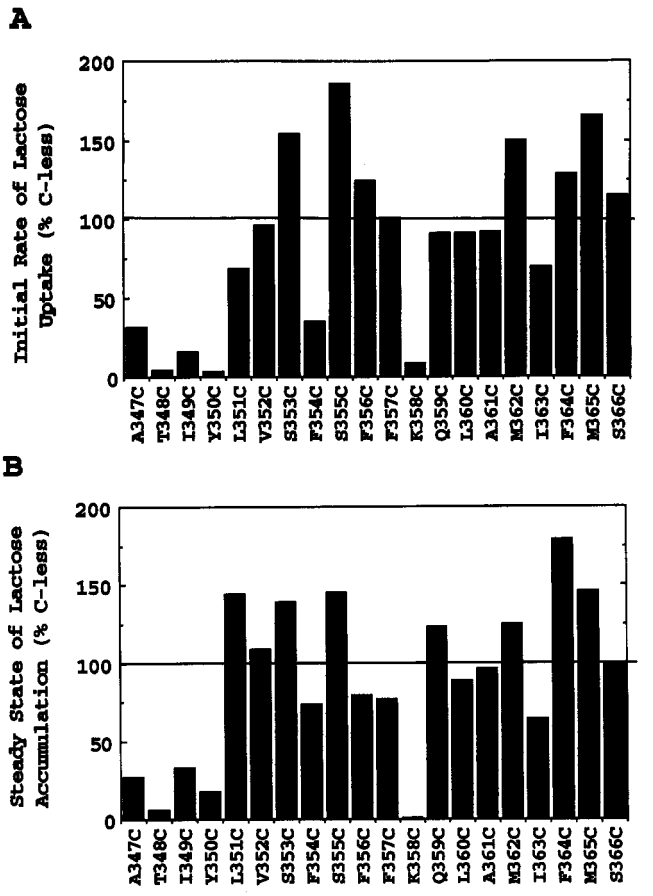

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants of C-less lac permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL, containing 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed for [1-^{14}C]lactose uptake at room temperature as described in the Methods. A. Rates of lactose transport at 1 min. The rate of C-less permease averaged 19 nmol lactose/min/mg protein. Results are expressed as a percentage of C-less activity after correcting for non-specific transport by T184 cells harboring pT7–5 (vector with no *lacY* gene) which typically transported at a rate of 0.6 nmol lactose/min/mg protein (i.e., 3% C-less). B. Steady-state levels of lactose accumulation. Results are expressed as a percentage of the C-less value (80 nmol lactose/mg protein) after correcting for nonspecific accumulation by T184 harboring pT7–5, which typically accumulated 8 nmol of lactose/mg protein in 1 h (i.e., 10% of C-less).

residue has been shown to be essential for neutralization of Asp237 in helix VII (King et al., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b). Both T348C and Y350C exhibit very low rates (<5% of C-less), although Y350C climbs to 18% of the steady state of C-less in 1 h. A347C and I349C both reach approximately 30% of the steady state of C-less, but A347C has twice the rate of I349C (32% and 16% of C-less, respectively). The two positions where a native Cys is restored, 353 and 355, achieve steady states of approximately 140% of C-less at rates of 154% and 186% of C-less, respectively. Interestingly, similar rate enhancements occur when either of the Met residues are replaced with a less bulky Cys residue.

Expression of Mutant Permeases. The relative concentration of each mutant in *E. coli* T184 membranes was assessed by Western blot analysis with anti-C-terminal antibody (Figure 4). All the mutants with normal or enhanced activity are present in amounts comparable to C-less permease (left lane in each panel). Among the low activity mutants, the completely inactive mutant, K358C, is virtually absent due to disruption of the Asp237-Lys358 charge pair which probably

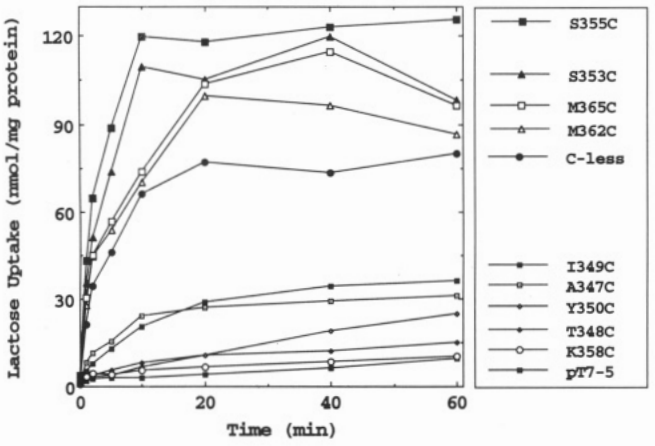

FIGURE 3: Time courses of lactose transport by individual Cys-replacement mutants in helix XI. *E. coli* T184 harboring plasmid pT7–5 (vector with no *lacY* gene), pT7–5 encoding C-less permease, or pT7–5 encoding given Cys-replacement mutants were grown at 37 °C and assayed as described in the Methods.

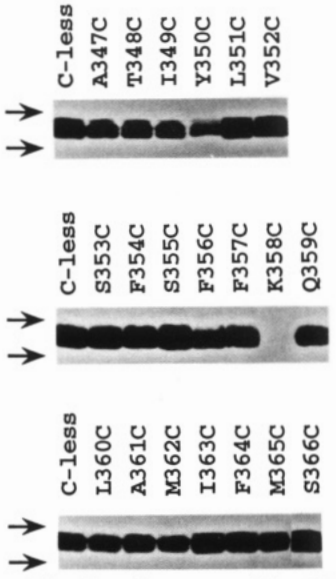

FIGURE 4: Western blot of membranes containing C-less lac permease or Cys-replacement mutants. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids as described in the Methods, and 100 μg of membrane protein was subjected to 12% $NaDodSO_4$-polyacrylamide gel 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, luminescent substrate before exposure to film. The arrows on the left indicate the positions of marker proteins: upper arrow, carbonic anhydrase (32.5 kDa), lower arrow, soybean trypsin inhibitor (27.5 kDa).

plays a role in permease folding and/or insertion (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b). Three of the four N-terminal mutants with reduced activities (A347C, T348C, and I349C) are present in relatively high amounts, at least 50% of the level of C-less. Therefore, the low activities of these mutants cannot be attributed to a defect in protein folding or insertion into the membrane. In contrast, the low rate of Y350C may be the result of a partial defect in insertion or stability because Y350C is found in lower amounts (approximately 20% of C-less), and although the rate is <5% of C-less, lactose accumulation is still rising after 1 h (see Figure 3). In any case, Y350F permease exhibits wild-type activity (Roepe & Kaback, 1989), indicating that Tyr350 is not an essential residue.

Effect of NEM on Lactose Transport Activity. The effect of the permeant sulfhydryl reagent NEM on the rate of lactose

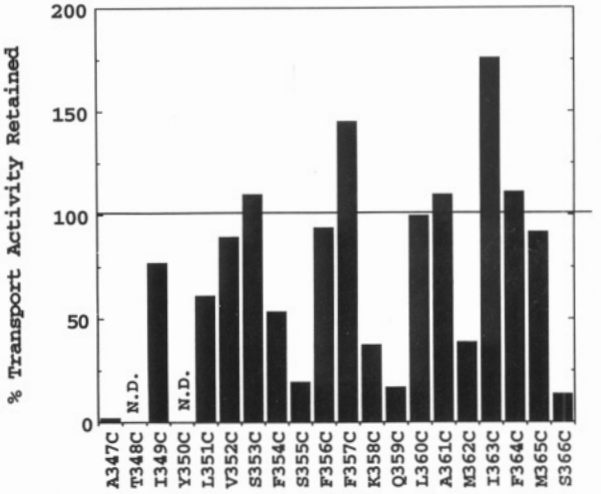

FIGURE 5: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single Cys-replacement mutants. Cells were incubated with 1 mM NEM at room temperature for 30 min and then quenched by addition of 10 mM dithiothreitol before assaying initial rates of lactose uptake in 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. Mutants with initial rates below 10% of C-less were not determined (N.D.) except for K358C which was determined using the highly active double mutant D237A/K358C (Dunten et al., 1993).

transport of each mutant is shown in Figure 5. The mutants exhibit a range of responses to NEM from virtually complete inhibition (A347C) to no effect or even stimulation (F357C and I363C). Because NEM treatment results in addition of a hydrophobic 5-membered ring to the sulfhydryl group of the introduced Cys residue, stimulation at positions that were originally occupied by bulky hydrophobic residues like Phe or Ile is not surprising. Of interest, however, is the striking periodicity observed for positions found to be inhibited by NEM modification. After the N-terminal portion containing the less active mutants, where sensitivity to NEM cannot be determined accurately, every third or fourth mutant (S355C, Q359C, M362C, and S366C) is inhibited by >60%, while mutants at the intervening positions are relatively unaffected. When viewed on a helical wheel plot (Figure 6), the sensitive positions cluster on the face of helix XI that contains a higher percentage of relatively polar residues.

DISCUSSION

In this study, Cys-scanning mutagenesis was employed to determine the functional importance of the amino acid residues in putative transmembrane helix XI of lac permease. As observed with transmembrane helices IX and X (Sahin-Tóth & Kaback, 1993a), the majority of the single-Cys replacement mutants in C-less permease are both highly active and expressed normally. Such residues can therefore be excluded from playing a role in the mechanism of lactose transport, while residues that do not tolerate substitution with Cys may or may not be essential to the mechanism. Among the 20 Cys mutants in helix XI, only K358C is completely defective. Also in contrast to the other mutants, K358C permease is hardly detectable in the membrane. Detailed studies on this mutant and other replacements at this position (King et al., 1991; Sahin-Tóth et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b) reveal that Lys358 itself is not essential for transport and that a positive charge at this position is required to interact with a negative charge at position 237 in helix VII, thereby providing an interaction that is important for folding

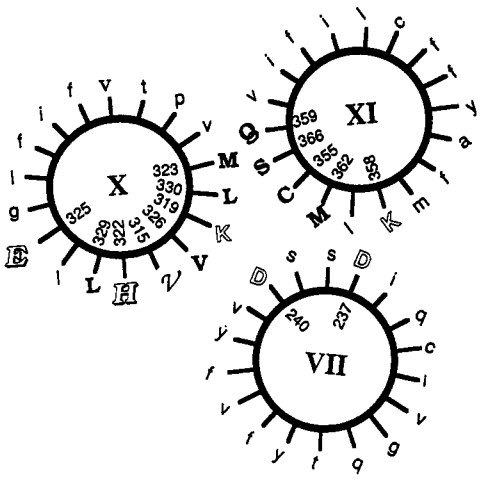

FIGURE 6: Relationship between helices VII, X, and XI in lac permease. Eighteen residues of each putative helix are shown on helical wheels viewed from the periplasmic surface of the membrane. Residues shown as filled capital letters in helices X (Sahin-Tóth & Kaback, 1993a) and XI represent positions where a Cys replacement mutant is significantly inactivated by treatment with NEM (i.e., >60% inhibition of initial rate; cf. Figure 5). Also shown (open capital letters) are the interactions between Lys358 and Asp237 and between Lys319 and Asp240 (King et al., 1991; Sahin-Tóth et al., 1992; Lee et al., 1992; Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b). Positions 325 (Glu) and 322 (His) (shadowed open capital letters) have been shown to be in close proximity by excimer fluorescence (Jung et al., 1993), and permease with Cys in place of Val315 (*v*) exhibits markedly enhanced rates of NEM inactivation in the presence of TDG or $\Delta\bar{\mu}_H^+$ (Sahin-Tóth & Kaback, 1993a).

and/or insertion. Note, however, that these two residues remain in close proximity in the tertiary structure of the permease (Dunten et al., 1993; Sahin-Tóth & Kaback, 1993b).

The other Cys-replacement mutants in helix XI with substantially impaired activity are at the four positions closest to the cytoplasmic face of the membrane (Ala347, Thr348, Ile349, and Tyr350). Among these mutants, A347C and I349C are more active, reaching 30% of the steady-state level of lactose accumulation achieved by C-less permease, and both mutants are present in high amounts in the membrane. Since neither of these residues contains functional groups likely to be involved in proton translocation or sugar binding, the defects may result from structural perturbations caused by introducing Cys at these positions. Y350C has very low activity relative to C-less and is expressed at significantly lower levels (ca. 20%) in the membrane. Although the Tyr hydroxyl could act as a H^+ donor or acceptor, Roepe and Kaback (1989) demonstrated that substitution of Phe for Tyr350 in wild-type *lacY* results in fully functional permease. Therefore, the defect in Y350C is likely due to aberrant permease folding or insertion. In contrast, mutant T348C is virtually inactive, but present in the membrane at near C-less levels. In addition, a fortuitous C-less mutant with Met in place of Thr348 also exhibits low activity (18% the rate and 14% the steady state accumulation of C-less permease) and is present in amounts comparable to C-less (data not shown).

Comparison of the primary amino acid sequence of *E. coli* lac permease with the lac permease of *Klebsiella pneumoniae* (McMorrow et al., 1988), the raffinose permease of *E. coli* (Aslandis et al., 1989), and an *E. coli* sucrose permease (Bockmann et al., 1992; Lengeler et al., 1992) reveals that Thr348 is one of only five residues in helix XI that are conserved in all four proteins. The other four conserved residues (Leu351, Phe354, Leu360, and Ser366) are all relatively unaffected by Cys substitution. In light of these observations and because Thr contains a hydroxyl group that might participate in hydrogen bonding, Thr348 is of interest as a target for further mutagenic analysis.

In a few instances, Cys substitutions cause significant enhancement in transport activity. Reversion of either Ser353 or Ser355 to the native Cys residue stimulates the rate of transport to 154% or 186% the rate of C-less, respectively. This result is consistent with the observation of Menick et al. (1987) that wild-type permease mutated to Ser at both Cys353 and Cys355 loses 50% of its activity. Surprisingly, rate enhancements also result when either Met362 or Met365 are replaced with Cys. These Met residues represent the bulkiest side chains on the face of helix XI that presumably interacts with helix VII via Lys358 and Asp237. It has also been postulated that helix VII interacts with helix X via Asp240 and Lys319 (Sahin-Tóth et al., 1992, Lee et al., 1992; Sahin-Tóth & Kaback, 1993b), thereby bringing helices X and XI into close proximity (see Jung et al. (1993) and Kaback et al. (1993) for models describing the packing of helices VII to XI). Replacement of either Met with the smaller Cys residue may facilitate conformational changes occurring in the helix X region during lactose transport. Evidence for such a conformational change was recently obtained (Sahin-Tóth & Kaback, 1993a) using C-less permease with a single Cys replacement for Val315 which is presumably the N-terminal residue in helix X (Figure 1). In the presence of TDG or $\Delta\bar{\mu}_H^+$, V315C permease becomes 10-fold more sensitive to NEM inhibition and thus appears to report a conformational change induced by ligand binding or $\Delta\bar{\mu}_H^+$.

In helix XI, each Cys mutant was also tested for sensitivity to NEM, and a remarkable periodicity of inhibition at every third or fourth residue is observed. Thus, the sensitive positions all reside on the same relatively polar face of helix XI (Figure 6) which contains the salt-bridged Lys358 and therefore interacts directly with helix VII and is probably in close proximity to helix X as well (Jung et al., 1993; Kaback et al., 1993). In fact, four positions that are relatively strongly inhibited, Gln359, Ser366, Cys355, and Met362, are thought to directly adjoin helix X. Addition of a bulky maleimide might hinder conformational flexibility in this region of the protein, or alternatively these residues might provide part of an interface between helices in the C-terminal half of the permease that comprises the pathway for substrate.

NEM sensitivity may simply reflect the greater accessibility of residues positioned at these particular helical interfaces. However, stimulation of I363C and particularly F357C, which is located on the opposite side of the helix, suggests that these residues are also accessible to NEM and that modification of Cys residues at these positions may improve activity perhaps by restoring some of the bulk of the original residues. Therefore, although it has not been demonstrated directly that NEM reacts with each of the Cys-replacement mutants described, it seems reasonable to assume that most of the Cys mutants react with NEM, but in given instances, modification fails to affect activity. In any case, the pattern of NEM inhibition is consistent with the notion that the face of helix XI containing the strongly inhibited positions forms part of a crevice between helices VII, X and XI and provides additional support for the model describing the interactions between helices VII through XI in the C terminus of lac permease (Jung et al., 1993; Kaback et al., 1993).

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REFERENCES

Altenbach, C., Martin, T., Khorana, H. G., & Hubbell, W. L. (1990) *Science 248*, 1088–2010.

Aslandis, C., Schmid, K., & Schmitt, R. (1989) *J. Bacteriol. 171*, 6753–6763.

Bockmann, J., Heuel, H., & Lengeler, J. W. (1992) *Mol. Gen. Genet. 235*, 22–32.

Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459–472.

Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937–4941.

Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894–6898.

Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984a) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672–4676.

Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984b) *Biochemistry 23*, 3681–3687.

Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291–1298.

Cunningham, B. C., & Wells, J. A. (1989) *Science 244*, 1081–1085.

Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 3139–3145.

Falke, J. J., & Koshland, D. E. (1987) *Science 237*, 1596–1600.

Foster, D. L.; Boublik, M., & Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31–34.

Goldkorn, T., Rimon, G., & Kaback, H. R. (1983) *Proc. Acad. Natl. Sci. U.S.A. 80*, 3322–3326.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232–238.

Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688–3693.

Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221–229.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene (Amst.) 77*, 51–59.

Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry* (in press).

Kaback, H. R. (1974) *Methods Enzymol. 31*, 698–709.

Kaback, H. R. (1989) *Harvey Lectures 83*, 77–105.

Kaback, H. R. (1992) in *International Review of Cytology 137A* (Jeon, K. W., & Friedlander, M. Eds.) pp 97–125, Academic Press, Inc., New York.

Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., & Zen, K. (1993) *J. Bioenerg. Biomembr.* (in press).

King, S. C., Hansen, C. L.; & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177–186.

Konings, W. N., Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5857–5861.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Lee, J.-I., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758–20764.

Lengeler, J. W., Bockmann, J., Heuel, H., & Titgemeyer, F. (1992) in *Molecular Mechanisms of Transport* (Quagliariello, E., & Palmieri, Y. F., Eds.) pp 77–85, Elsevier, New York.

McMorrow, I., Chin, D. T., Fiebig, K., Pierce, J. L., Wilson, D. M., Reeve, F. C. R., & Wilson, T. H. (1988) *Biochim. Biophys. Acta 945*, 315–323.

Menick, D. R., Lee, J. A., Brooker, R. J., Wilson, T. H., & Kaback, H. R. (1987) *Biochemistry 26*, 1132–1136.

Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804–11808.

Page, M. G. P., & Rosenbusch, J. P. (1988) *J. Biol. Chem. 263*, 15906–15914.

Pakula, A. A., & Simon, M. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4144–4148.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 6127–6132.

Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547–10551.

Sahin-Tóth, M., & Kaback, H. R. (1993a) *Protein Sci. 2*, 1024–1033.

Sahin-Tóth, M., & Kaback, H. R. (1993b) *Biochemistry 32*, 10027–10035.

Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107–110.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. U.S.A. 74*, 5463–5468.

Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817–10820.

Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 268–279.

Seckler, R., Möröy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403–2409.

Stochaj, V., Bieseler, B., & Ehring, R. (1986) *Eur. J. Biochem. 158*, 423–428.

Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223–231.

van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595–9600.

Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625–3631.