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

Stathis Frillingos, Jianzhong Sun, Alberto Gonzalez, and H. Ronald Kaback*

Howard Hughes Medical Institute, Departments of Physiology and of Microbiology & Molecular Genetics, Molecular Biology Institute, University of California, Los Angeles, Los Angeles, California 90024-1570

Received July 26, 1996; Revised Manuscript Received October 8, 1996[⊗]

ABSTRACT: Using a functional lactose permease mutant devoid of Cys residues (C-less permease), each amino acid residue in putative transmembrane helix II and flanking hydrophilic loops (from Leu34 to Lys74) was replaced individually with Cys. Of the 41 single-Cys mutants, 28 accumulate lactose to >70% of the steady state observed with C-less permease, and an additional 10 mutants exhibit lower but significant levels of accumulation (25−60% of C-less). His35→Cys permease exhibits very low activity (ca. 20% of C-less), while Gly64→Cys or Asp68→Cys permease is unable to accumulate lactose. However, His35 can be replaced with Arg without effect on transport activity [Padan, E., Sarkar, H. K., et al. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765−6768]. In addition, even though mutant Gly64→Cys or Glu68→Cys is inactive both in the C-less background and in the wild-type, neither Gly64 [Jung, K., Jung, H., et al. (1995) *Biochemistry 34*, 1030−1039] nor Glu68 [Jessen-Marshall, A. E., & Brooker, R. J. (1996) *J. Biol. Chem. 271*, 1400−1404] is essential for active lactose transport. Immunoloblot analysis reveals that all of the mutants except His35→Cys permease are inserted into the membrane at concentrations comparable to that of C-less permease. The transport activity of the single-Cys mutants is altered by *N*-ethylmaleimide (NEM) treatment in a highly specific manner. Most of the mutants are insensitive, but Cys replacements render the permease sensitive to NEM inactivation at positions that cluster in a manner indicating that they are on one face of an α-helix (Thr45→Cys, Gly46→Cys, Phe49→Cys, Ser53→Cys, Ser56→Cys, Gln60→Cys, and Ser67→Cys). Interestingly, the same face contains positions where Cys substitution itself leads to low transport activity (Ile52→Cys, Leu57→Cys, Gln60→Cys, and Gly64→Cys). The results demonstrate that although no residue *per se* in this region of the permease is irreplaceable, the surface of one face of helix II is important for active lactose transport.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic membrane transport protein encoded by the *lacY* gene. The permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for the coupled stoichiometric translocation of β-galactosides and H^+ as a monomer [reviewed in Kaback et al. (1994) and Kaback (1996)]. A variety of experimental approaches indicate that the permease is composed of 12 α-helical rods that traverse the membrane, with both N and C termini in the cytosolic side, and unequivocal support for the 12-helix motif has been obtained from analysis of fusions between lac permease and alkaline phosphatase (*lacY*−*phoA*) (Calamia & Manoil, 1990). Moreover, site-directed excimer fluorescence, site-directed mutagenesis, and second-site suppressor studies have led to a model describing the packing of helices VII−XI [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) within the permease (Jung et al., 1995b; He et al., 1995a,b), site-directed chemical cleavage (Wu et al., 1995a), thiol cross-linking experiments, and site-directed spin-labeling (Wu et al., 1996a).

Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) reveal that as few as four residues in the permease are irreplaceable with respect to active lactose transport [reviewed in Kaback et al. (1994) and Kaback (1996)]. However, the activity of certain active Cys-replacement mutants is inhibited by alkylation, and these mutants appear in clusters on faces of putative transmembrane helices, suggesting that surfaces within the permease may be important for turnover. In addition, site-directed fluorescence spectroscopy with purified permease (Jung, H., et al., 1994; Jung, K., et al., 1994; Wu & Kaback, 1994; Wu et al., 1994, 1995b) and site-directed sulfhydryl modification *in situ* (Frillingos & Kaback, 1996a,c) show that the reactivity of individually placed Cys residues in a number of transmembrane domains is altered as a result of β-galactoside binding or imposition of a H^+ electrochemical gradient. Therefore, it appears likely that permease turnover involves widespread conformational changes and few essential side chain−substrate (H^+ and sugar) or side chain−side chain interactions.

Several findings indicate that the C-terminal half of the permease plays a direct role in the transport mechanism. In addition to Cys-scanning mutagenesis studies showing that helices VII−XI contain the four irreplaceable residues

* Address correspondence to this author at HHMI/UCLA 6−720 MacDonald Research Labs, Box 951662, Los Angeles, CA 90095-1662. Telephone: (310) 206-5053. Telefax: (310) 206-8623. E-mail: RonaldK@HHMI. UCLA.edu.

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.

[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; NEM, *N*-ethylmaleimide; PMS, phenazine methosulfate; TDG, β-D-galactopyranosyl 1-thio-β-D-galactopyranoside.

S0006-2960(96)01862-4 CCC: $14.00

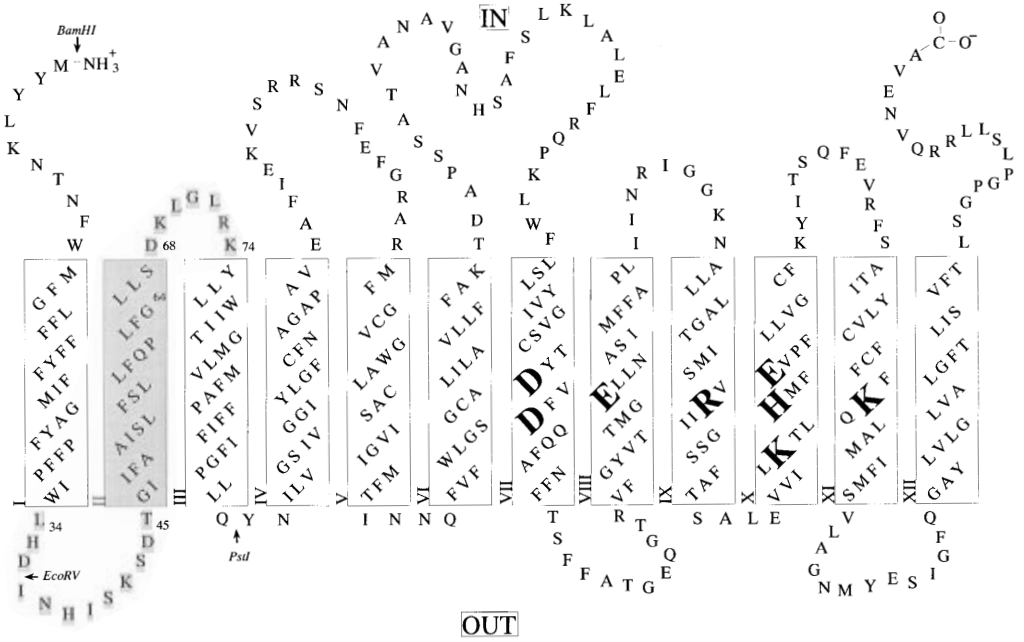

FIGURE 1: Secondary structure model of *E. coli* lac permease. The one-letter amino acid code is used, putative transmembrane helices are shown in boxes, and the irreplaceable residues [Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X)] as well as the interacting pairs Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X) are enlarged and emboldened. 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.

[Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X)], as well as the majority of the *N*-ethylmaleimide (NEM)-sensitive mutants, downhill transport activity and/or ligand binding is retained in mutants deleted of the N-terminal half of the permease (Bibi et al., 1991; Wu et al., 1996b). Furthermore, the epitope for monoclonal antibody (mAb) 4B1 which uncouples lactose from H^+ translocation has been localized (Sun et al., 1996) to the periplasmic loop between helices VII and VIII, and conformational changes elicited by 4B1 binding have been detected primarily in the C-terminal half of the permease (Frillingos et al., 1996; Frillingos & Kaback, 1996b). On the other hand, the N-terminal 22 amino acid residues can be deleted from the permease without abolishing transport (Bibi et al., 1992), and no irreplaceable residues have been found in helices I, III, V, or VI.

In this paper, we present a systematic Cys-scanning mutagenesis study of putative transmembrane helix II and the flanking loops in the N-terminal half of the permease. Study of helix II is particularly interesting since recent findings (Jessen-Marshall & Brooker, 1996; Sahin-Tóth et al., 1996; Wu & Kaback, 1996) indicate that part of this helix might interact with the C-terminal half of the protein. Moreover, the cytoplasmic end of helix II and the loop between helices II and III (loop II/III) contain the conserved sequence motif ^{64}G-X-X-X-D-(R/K)-X-G-X-(R/K)-(R/K)^{74} (Henderson, 1990), within which insertional mutagenesis (McKenna et al., 1992) or site-directed mutagenesis of Gly64 (Jung et al., 1995a) or Asp68 (Jessen-Marshall et al., 1995) inactivates the permease. In the present report, each residue in loop I/II, helix II, and loop II/III was replaced individually with Cys (from Leu34 to Lys74) and tested for function. The results demonstrate that none of the residues in this region is obligatory for active lactose transport, and residues where the Cys substitution itself or Cys substitution followed by NEM treatment results in low permease activity are on one face of helix II.

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 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 25 mM lactose. *E. coli* T184 [$lacI^+O^+Z^-Y^-$(*A*), *rspL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR/F′*, $lacI^qO^+Z^{D118}$ (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 site-directed mutagenesis.

Oligonucleotide-Directed Site-Specific Mutagenesis. Cys-replacement mutants were constructed either by a two-stage PCR method (PCR overlap−extension; Ho et al., 1989) or by one-step PCR using pT7−5/*lacY*/cassette encoding C-less permease as template. The PCR products were digested with *Bam*HI and *Pst*I (L34C to I37C) or *Eco*RV and *Pst*I (all other mutants) and ligated to similarly treated pT7−5/*lacY*/C-less vector (see Figure 1 for location of sites). For construction of mutant p(wt)D68C (D68C in the wild-type background), the *Eco*RV−*Pst*I restriction fragment of the single-Cys D68C

was isolated and ligated to similarly treated pT7−5/cassette wild-type *lacY*. Mutant p(wt)G64C (G64C in the wild-type background) was constructed previously (Jung et al., 1995a).

DNA Sequencing. Double-stranded plasmid DNA was sequenced using the dideoxynucleotide termination method of Sanger et al. (1977) 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 or wild-type cassette *lacY*.

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 medium 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 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 and used for transport assays or preparation of membranes.

Active 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; final concentration 0.4 mM) was assayed by rapid filtration as described (Consler et al., 1991).

Membrane Preparation. Crude membrane fractions from T184 were prepared by osmotic lysis and sonication as described (Frillingos et al., 1994).

Immunological Analyses. Membrane fractions were subjected to 12% sodium dodecyl sulfate−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

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 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, while mutants with low activity grow as red colonies with a white halo. It is important that indicator plates report "downhill" translocation of lactose and give no indication as to whether or not the cells catalyze lactose accumulation. Of the 41 Cys-replacement mutants described, 38 grow as red colonies indistinguishable from cells expressing C-less permease, H35C and D68C grow as red colonies with a white halo, and G64C yields white colonies. Therefore, judging from indicator plates, all of the single-Cys mutants except G64C retain at least some ability to translocate lactose downhill.

Active Transport. *E. coli* T184 ($lacZ^-Y^-$) was used to test the ability of the mutants to catalyze active lactose transport. The majority of the 41 mutants transport lactose at very significant rates (Figure 2A). Twenty-eight mutants

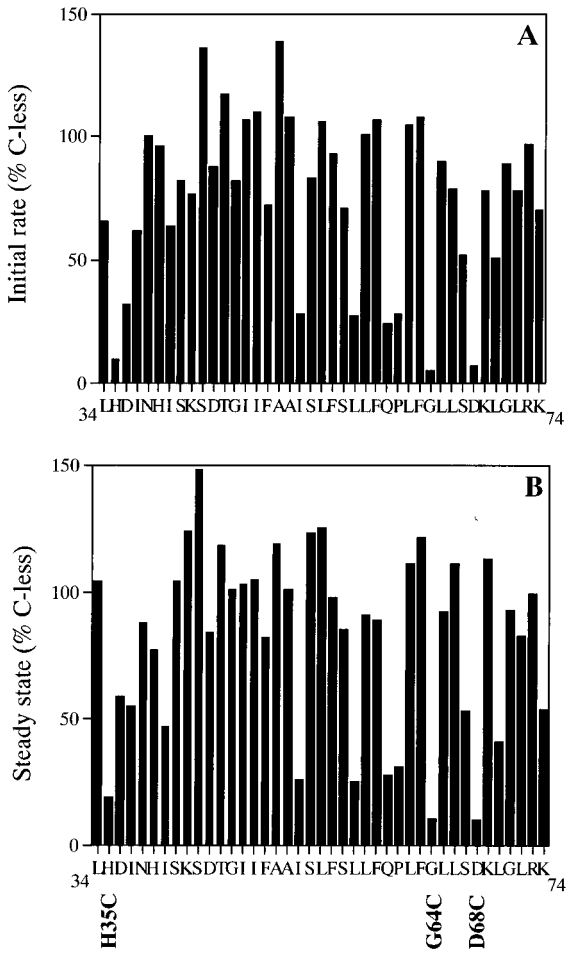

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants or C-less permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described under Materials and Methods. The single-letter amino acid code along the horizontal axis denotes the original residues replaced with Cys in increasing order from Leu34 to Lys74. (A) Rates of lactose transport measured at 1 min. The rate for C-less permease averaged 59 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) transported at a rate of 2.4 nmol min^{-1} (mg of protein)$^{-1}$ (i.e. 4.3% of C-less). (B) Steady state levels of lactose accumulation. Results are expressed as a percentage of the C-less value, which averaged 150 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., 6.7% of C-less).

exhibit rates that are between 70% and 100% or more of C-less permease, 5 mutants exhibit intermediate rates (50−65% of C-less), and an additional 5 mutants (D36C, I52C, L57C, Q60C, P61C) transport at low but significant rates (25−30% of C-less). However, negligible transport rates (5−10% of C-less) are observed for mutants H35C, G64C, and D68C. Steady-state levels of lactose accumulation for the great majority of mutants also approximate those of C-less permease (Figure 2B); steady states of 70−100% or more of C-less are achieved by 28 mutants, and intermediate levels (45−60% of C-less) are achieved by 6 mutants. Mutants I52C, L57C, Q60C, and P61C accumulate lactose to levels of 25−30% of C-less, while H35C accumulates to ca. 20%. G64C and D68C are unable to accumulate the sugar to any significant extent. Although data are not presented, when mutations G64C and D68C are transferred to the background of wild-type lac permease, the resulting

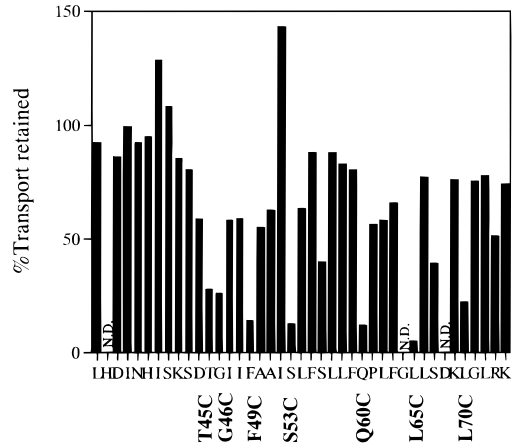

FIGURE 3: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 1 mM NEM (final concentration) at room temperature for 30 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and cells were assayed for initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (Konings et al., 1971). Rates are presented as percentages of the rate measured in the absence of NEM. Inhibition of mutants H35C, G64C, and D68C which display low initial rates of transport (≤20% of C-less) could not be assessed with accuracy (N.D., not determined).

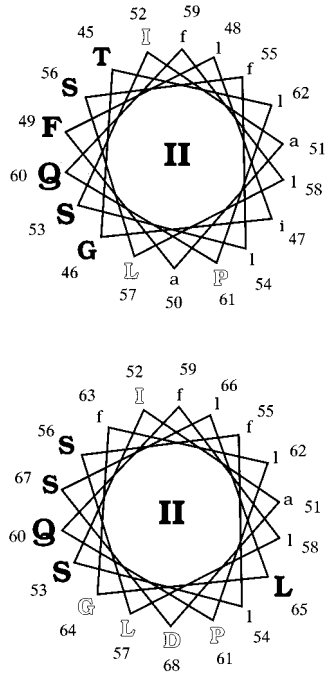

FIGURE 4: Helical wheel plots of residues in putative helix II viewed from the periplasmic surface of the membrane. Residues from Thr45 to Leu62 are plotted in the upper panel, and residues from Ala51 to Asp68 are plotted in the lower panel. Positions sensitive to NEM treatment (>60% inhibition of initial rate; see Figure 3) are shown as enlarged boldface letters. Positions where a single-Cys replacement by itself results in low activity (≤25−30% of C-less) are outlined.

mutants are still unable to catalyze lactose accumulation. However, previous studies demonstrate that His35 can be replaced with Arg (Padan et al., 1985) and Gly64 can be replaced with Ala (Jung et al., 1995a) with retention of highly significant activity, while second-site suppressor mutations for D68S or D68T in various regions of the permease are able to restore transport to high levels (Jessen-Marshall & Brooker, 1996). Therefore, none of the residues in this region plays a direct role in the transport mechanism.

Expression of Permease Mutants. Western blot analysis of membrane fractions prepared from *E. coli* T184 expressing individual Cys-replacement mutants demonstrates that all of the mutants are present in the membrane at levels comparable to C-less permease with the exception of H35C (data not shown). H35C is expressed at reduced levels, which is consistent with the finding that this mutant exhibits a very low rate of transport and a low but significant level of accumulation in 1 h (Figure 2).

NEM Inactivation. The effect of NEM, a membrane-permeable sulfhydryl reagent, on the initial rate of lactose transport for each mutant is shown in Figure 3. Although the activity of the majority of the Cys-replacement mutants is not altered significantly, seven mutants (T45C, G46C, F49C, S53C, Q60C, L65C, and L70C) are inhibited by the alkylating reagent by more than 70%, and an additional two mutants (S56C, S67C) are inhibited by 60%. With the exception of L65C, the NEM-sensitive mutants cluster on one face of helix II (Figure 4).

DISCUSSION

The results presented in this paper extend an almost completed series of site-directed and Cys-scanning mutagenesis studies of lac permease [see Kaback et al. (1994) and Kaback (1996)] that lead to two major conclusions. First, only four residues are irreplaceable with respect to the mechanism of lactose/H^+ symport (Glu269, Arg302, His322, and Glu325). Second, the activity of a specfic set of active Cys-replacement mutants is compromised by alkylation, and these mutants appear in clusters, suggesting that helical surfaces within the permease are important for the conformational changes that occur during turnover. Out of 41 residues studied here (from Leu34 to Lys74), none appears to be directly involved in the mechanism, while the activity of 9 Cys-replacement mutants is inhibited strongly after treatment with NEM. With one exception, the NEM-sensitive positions cluster on one face of helix II, and the same face contains residues where Cys substitution itself leads to low transport activity (Figure 4). No NEM-sensitive mutants (except L65C) or mutants of low activity are located on the opposite, Leu-rich face of the helix (Figure 4).

Even though none of the residues in the sequence Leu34−Lys74 of lac permease is irreplaceable with respect to activity, Cys replacement at seven positions leads to low activity. In periplasmic loop I/II, replacement of His35 with Cys results in strikingly low activity, paralleled by reduced expression in the membrane. However, replacement of His35 with Arg in mutant H35R/H39R (Padan et al., 1985) results in wild-type activity, demonstrating that His35 is not important. Within helix II, low activity (25−30% of C-less) is observed with Cys replacements for Ile52, Leu57, Gln60, and Pro61. Interestingly, the transport activity of mutant I52C is enhanced by alkylation (Figure 3), indicating that a minimal side-chain volume at this position might be required for optimal function. Conversely, NEM treatment inactivates Q60C completely (Figure 3), indicating that more specific interactions may occur at this position. On the other hand, replacement of Pro61 with other amino acid residues (Ala, Leu, or Gly) does not affect activity significantly (Consler et al., 1991), and P61C exhibits a low but significant rate of lactose transport even after alkylation (Figure 3). Finally, the cytoplasmic end of the helix contains Gly64 and Asp68 that cannot be replaced with Cys in either the C-less or the wild-type permease background. Even though replacement

of Gly64 with Pro, Val, or Ser or replacement of Asp68 with Ala, Ser, Thr, Tyr, Asn, His, or Glu essentially abolishes activity (Jung et al., 1995a; Jessen-Marshall et al., 1995), G64A in the wild-type background (Jung et al., 1995a) and several second-side revertants of D68S or D68T (Jessen-Marshall & Brooker, 1996) exhibit high activity. Interestingly, in 7 of 18 revertants, the inactive phenotype of the Asp68 mutant is reversed by a mutation in the cytoplasmic half of helix VII in which Cys234 is replaced with a bulkier residue (Phe or Trp). In addition, G64A is completely inactive in the C-less background, but highly active in the wild-type background (Jung et al., 1995a). Therefore, neither Gly64 nor Asp68 plays a direct role in the transport mechanism.

In an initial screen for accessibility or reactivity of the Cys residues at each position from Leu34 to Lys74, the effect of NEM on lactose transport was studied in each active mutant. While most of the 41 mutants are insensitive, 9 mutants are inactivated by at least 60%. One possible explanation is that the other Cys residues do not react with the alkylating agent. Another possibility is that each position is accessible to NEM and that the sensitive mutants reflect positions that are either close to a binding site or important for conformational flexibility in this region of the protein. The second explanation seems more likely in general since NEM is membrane-permeant and a large number of single-Cys replacement mutants within transmembrane domains or disposed toward the inner surface of the membrane are readily inhibited (Sahin-Tóth & Kaback, 1993; Dunten et al., 1993; Frillingos et al., 1994; Weitzman & Kaback, 1995) and react with [1-^{14}C]NEM *in situ* (Frillingos & Kaback, 1996a; Frillingos et al., 1996). In this respect, with the sole exception of L65C, the NEM-sensitive positions of helix II lie on one face, and the low activity single-Cys mutants lie on or close to the same face (Figure 4). Recent thiol cross-linking experiments (Wu & Kaback, 1996) indicate that this face of helix II is close to helix VII. The opposite face of the helix which is more hydrophobic (Figure 4) contains positions that are insensitive to either Cys-replacement or Cys-replacement and NEM treatment, implying that this face may be in contact with either the hydrophobic face of another helix or the lipid phase of the membrane.

ACKNOWLEDGMENT

We thank Kerstin Stempel for synthesizing deoxyoligonucleotide primers.

REFERENCES

Bibi, E., Verner, G., Chang, C.-Y., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7271−7275.
Bibi, E., Stearns, S. M., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3180−3184.
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., Herzlinger, D., Mitchell, R., DeChiara, S., Dahno, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672−4676.
Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291−1298.
Dunten, R. L., Sahin-Tóth, M., & Kaback, H. R. (1993) *Biochemistry 32*, 12644−12650.
Frillingos, S., & Kaback, H. R. (1996a) *Biochemistry 35*, 3950−3956.
Frillingos, S., & Kaback, H. R. (1996b) *Biochemistry 35*, 10166−10171.
Frillingos, S. & Kaback, H. R. (1996c) *Biochemistry* (in press).
Frillingos, S., Sahin-Tóth, M., Persson, B., & Kaback, H. R. (1994) *Biochemistry 33*, 8074−8081.
Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232−238.
He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Biochemistry 34*, 15667−15670.
He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 15661−15666.
Henderson, P. J. F. (1990) *J. Bioenerg. Biomembr. 22*, 525−569.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene (Amsterdam) 77*, 51−59.
Jessen-Marshall, A. E., & Brooker, R. J. (1996) *J. Biol. Chem. 271*, 1400−1404.
Jessen-Marshall, A. E., Paul, N. J., & Brooker, R. J. (1995) *J. Biol. Chem. 270*, 16251−16257.
Jung, H., Jung, K., & Kaback, H. R. (1994) *Protein Sci. 3*, 1052−1057.
Jung, K., Jung, H., & Kaback, H. R. (1994) *Biochemistry 33*, 3980−3985.
Jung, K., Jung, H., Colacurcio, P., & Kaback, H. R. (1995a) *Biochemistry 34*, 1030−1039.
Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 6272−6277.
Kaback, H. R. (1996) in *Handbook of Biological Physics II: Transport in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., & Lolkema, J. S., Eds.) pp 203−227, Elsevier, Amsterdam.
Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Zen, K. (1994) *J. Exp. Biol. 196*, 183−195.
Konings, W. N., Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5857−5861.
McKenna, E., Hardy, D., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954−11958.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804−11808.
Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765−6768.
Sahin-Tóth, M., & Kaback, H. R. (1993) *Protein Sci. 2*, 1024−1033.
Sahin-Tóth, M., Kaback, H. R., & Friedlander, M. (1996) *Biochemistry 35*, 2016−2021.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5468.
Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−998.
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.
Weitzman, C., & Kaback, H. R. (1995) *Biochemistry 34*, 9374−9379.
Wu, J., & Kaback, H. R. (1994) *Biochemistry 33*, 12156−12171.
Wu, J., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Wu, J., Frillingos, S., Voss, J., & Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.
Wu, J., Perrin, D., Sigman, D., & Kaback, H. (1995a) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−9190.
Wu, J., Frillingos, S., & Kaback, H. R. (1995b) *Biochemistry 34*, 8257−8263.
Wu, J., Voss, J., Hubbell, W. L., & Kaback, H. R. (1996a) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10123−10127.
Wu, J., Sun, J., & Kaback, H. R. (1996b) *Biochemistry 35*, 5213−5219.

BI9618629