Cysteine-Scanning Mutagenesis of Helix IV and the Adjoining Loops in the Lactose Permease of *Escherichia coli:* Glu126 and Arg144 Are Essential

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ABSTRACT: Cys-scanning mutagenesis has been applied to the remaining 45 residues in lactose permease that have not been mutagenized previously (from Gln100 to Arg144 which comprise helix IV and adjoining loops). Of the 45 single-Cys mutants, 26 accumulate lactose to >75% of the steady state observed with Cys-less permease, and 14 mutants exhibit lower but significant levels of accumulation (35−65% of Cys-less permease). Permease with Phe140→Cys or Lys131→Cys exhibits low activity (15−20% of Cys-less permease),while mutants Gly115→Cys, Glu126→Cys and Arg144→Cys are completely unable to accumulate the dissacharide. However, Cys-less permease with Ala or Pro in place of Gly115 is highly active, and replacement of Lys131 or Phe140 with Cys in wild-type permease has a less deleterious effect on activity. In contrast, mutant Glu126→Cys or Arg144→Cys is inactive with respect to both uphill and downhill transport in either Cys-less or wild-type permease. Furthermore, mutants Glu126→Ala or Gln and Arg144→Ala or Gln are also inactive in both backgrounds, and activity is not rescued by double neutral replacements or inversion of the charged residues at these positions. Finally, a mutant with Lys in place of Arg144 accumulates lactose to about 25% of the steady state of wild-type, but at a slow rate. Replacement of Glu126 with Asp, in contrast, has relatively little effect on activity. None of the effects can be attributed to decreased expression of the mutants, as judged by immunoblot analysis. Although the activity of most of the single-Cys mutants is unaffected by *N*-ethylmaleimide, Cys replacement at three positions (Ala127, Val132, or Phe138) renders the permease highly sensitive to alkylation. The results indicate that the cytoplasmic loop between helices IV and V, where insertional mutagenesis has little effect on activity [McKenna, E., et al. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954−11958], contains residues that play an important role in permease activity and that a carboxyl group at position 126 and a positive charge at position 144 are absolutely required.

The lactose permease (lac permease)[1] of *Escherichia coli* is a paradigm for secondary active transport proteins that transduce free energy stored in electrochemical ion gradients into work in the form of a concentration gradient (reviewed in 1 and 2). This hydrophobic, polytopic, cytoplasmic membrane protein catalyzes the coupled, stoichiometric translocation of β-galactosides and H^+, and it has been solubilized, purified, reconstituted into artificial phospholipid vesicles, and shown to be solely responsible for β-galactoside transport (reviewed in 3) as a monomer (see 4). The *lacY* gene which encodes the permease has been cloned and sequenced, and all available evidence indicates that the protein has 12 transmembrane domains in α-helical configuration that traverse the membrane in zigzag fashion connected by hydrophilic loops with the N and C termini on the cytoplasmic face of the membrane (Figure 1; reviewed in 5 and 6−8). Extensive use of site-directed and Cys-scanning mutagenesis indicates that few residues are directly involved in the mechanism, but the permease undergoes widespread conformational changes during turnover (*9*−*19*). On the basis of a variety of site-directed approaches which include second-site suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance, thiol cross-linking, and identification of discontinuous mAb epitopes, a helix packing model has been formulated (reviewed in 20).

A mechanism for the coupled translocation of substrate and H^+ by the lac permease of *E. coli* has been proposed recently (*21*). In brief, the residues that are irreplaceable with respect to coupling between lactose and H^+ translocation are paired in the tertiary structure—Arg302 (helix IX) with Glu325 (helix X) and His322 (helix X) with Glu269 (helix VIII). In an adjacent region of the molecule at the interface between helices VIII and V is the substrate translocation pathway, a conclusion based on indications that Cys148 and Met145 in helix V (*12*, *22*) and Val264, Gly268, and Asn272 in helix VIII (*17*, *23*) are involved in substrate binding and are in close proximity. Because of this arrangement, interfacial changes between helices VIII and V are transmitted to the interface between helices IX and X and vice versa. Upon substrate binding, a structural change at the interface between helices V and VIII disrupts the interaction between Arg302 and Glu325, resulting in protonation of Glu325, and

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[1] Abbreviations: lac, lactose; C-less permease, functional lac permease devoid of Cys residues; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside.

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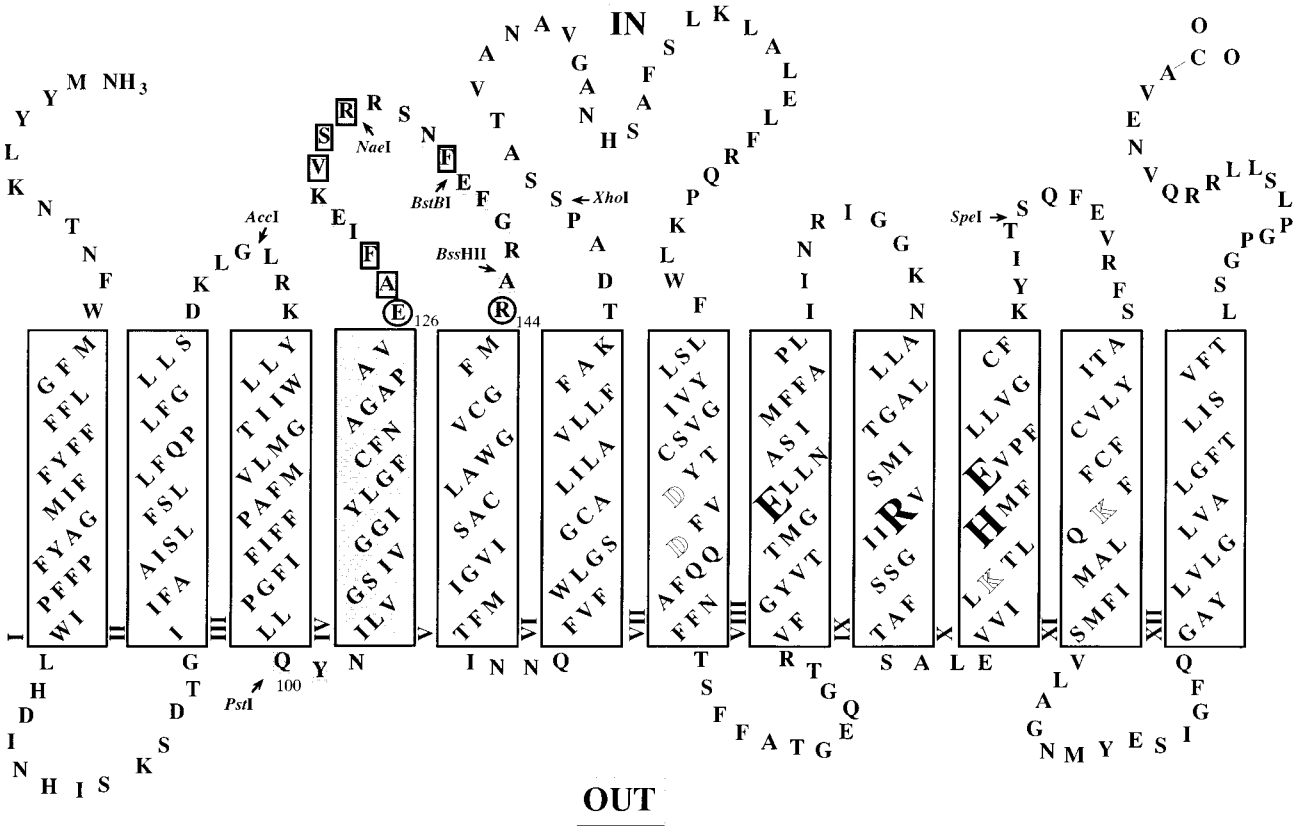

FIGURE 1: Secondary structure model of *E. coli* lac permease. The one-letter amino acid code is used, and transmembrane helices are shown in boxes. The shaded area highlights the region of the permease subjected to Cys-scanning mutagenesis. Residues E126 and R144 are encircled, and the positions of the NEM-sensitive mutants are boxed. Also indicated are the restriction endonuclease sites used for construction of the mutants.

simultaneously the carboxylic acid becomes inaccessible to water which drastically increases its pK_a. Upon loss of substrate, the interaction between Arg302−Glu325 is re-established with loss of H^+ from Glu325. The H^+ is released into a water-filled crevice between helices IX and X which becomes transiently accessible to both sides of the membrane due to a change in helix tilt, where it is acted upon equally by either the membrane potential or the pH gradient across the membrane.

In this communication, the results of Cys-scanning mutagenesis of the remaining 45 residues in the permease that have not yet been mutagenized which comprise transmembrane helix IV (*24*) and flanking loops are presented. Helix IV is one of the least-conserved transmembrane sequence regions in the five homologous oligosaccharide/H^+ symporters from enteric bacteria encompassing cluster 5 in the major facilitator superfamily (MFS) (*8*, *25*). Although permease activity is hardly affected by insertional mutagenesis of either periplasmic loop III/IV or cytoplasmic loop IV/V (*26*), the results reported here demonstrate surprisingly that loop IV/V is highly important, and more specifically that Glu126 and Arg144 are irreplaceable residues. Furthermore, loop IV/V is proximal in sequence to Cys148 and Met145 (helix V), residues that interact sterically with substrate (see 12 and 22), which is consistent with the conclusion that this region of the permease is an important part of the substrate translocation pathway.

EXPERIMENTAL PROCEDURES

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 (*27*) 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 Corporation (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^-] (*28*) 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'*, $lacI^qO^+Z^{U118}$] (*29*) 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 (*30*) 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 (*31*) or by one-step PCR using pT7-5/cassette *lacY* encoding Cys-less permease (or wild-type permease, where indicated) as template. The PCR products were digested using the restriction sites *Acc*I, *Pst*I, *Bst*BI or *Nae*I, and *Xho*I (Figure 1) and were ligated to similarly treated pT7-5/cassette *lacY* encoding Cys-less or wild-type permease.

Mutants E126A,[2] E126Q, E126R, E126D, R144A, R144Q, R144E and R144K were also constructed by two-stage PCR, as described. The double-substitution mutants E126C/R144C, E126A/R144A, E126R/R144E and E126D/R144K were constructed from the corresponding single-replacement mutants by using *Bst*BI−*Spe*I restriction fragment replacement.

Double-stranded plasmid DNA was sequenced using dideoxynucleotide termination (*32*) after alkaline denaturation (*33*). 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 native 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). Fully grown 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. After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

Active Transport. T184 cells were assayed for active transport of [1-^{14}C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) by rapid filtration (*34*).

Downhill Transport. Assays were performed with *E. coli* HB101 (Z^+Y^-) using 1 mM [1-^{14}C]lactose (10 mCi/mmol) (*35, 36*).

Membrane Preparation. Crude membrane fractions from T184 were prepared by osmotic lysis and sonication (*37*).

Immunological Analyses. Membrane fractions were subjected to sodium dodecyl sulfate/12% polyacrylamide gel electrophoresis (*38*). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-PVDF; Millipore) and probed with site-directed polyclonal antibody against the C terminus of the permease (*27*). Quantitation was performed with a model 425F PhosphorImager (Molecular Dynamics) as described (*39*).

RESULTS

Colony Morphology. 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 metabolism of the sugar causes acidification and appearance of red colonies. Cells expressing inactive mutants form white colonies, while 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 or not the cells catalyze accumulation. Of the 45 Cys-replacement mutants described, 40 grow as red colonies indistinguishable from cells expressing Cys-less permease, K131C and F140C grow as red colonies with a white halo, and G115C, E126C, and R144C yield white colonies. White colonies are also produced by all other mutants with replacements for Glu126 or Arg144, except E126D (Table 1).

Table 1: Properties of Lac Permeases Mutated at Glu126 and/or Arg144

permease mutant	phenotype[a]	active transport[b] (steady state)	membrane expression[c]
A. In the C-Less Permease Background			
C-less	red	100%	100%
E126C, A, Q, or R	white	bkgr	+++
R144C, A, Q, or E	white	bkgr	+++
E126C/R144C	white	bkgr	+++
E126A/R144A	white	bkgr	nd
E126R/R144E	white	bkgr	nd
E126D/R144K	white	bkgr	nd
B. In the Wild-Type Permease Background			
wild-type	red	100%	100%
p(wt)E126C, A, Q, or R	white	bkgr	+++
p(wt)E126D	red center	100%	nd
p(wt)R144C	white	bkgr	++
p(wt)R144A, Q, or E	white	bkgr	+++
p(wt)R144K	white	25%	nd
p(wt)E126A/R144A	white	bkgr	nd
p(wt)E126R/R144E	white	bkgr	nd
p(wt)E126D/R144K	white	25%	nd

[a] *E. coli* HB101 ($lacZ^+Y^-$) expressing a given mutant was plated on MacConkey agar containing 25 mM lactose and incubated at 37 °C for 15 h. Downhill lactose transport was also assayed quantitatively with mutants E126C, A or Q, R144C, A or Q, p(wt)E126C, A or Q, and p(wt)R144C, A or Q. In each case, activity similar to that of cells transformed with plasmid pT7-5 with no *lacY* insert was observed (Figure 5). [b] *E. coli* T184 ($lacZ^-Y^-$) expressing each mutant was incubated with 0.4 mM [1-^{14}C]lactose at pH 7.5 and assayed by rapid filtration. Levels of accumulation achieved at 1h are given as percentages of the Cys-less or wild-type control. "bkgr" (background) indicates the same level as that of T184 transformed with pT7-5 with no *lacY* insert (see also Figures 2−4). [c] Membranes prepared from *E. coli* T184 expressing each mutant were subjected to immunoblot analysis as described in the Experimental Procedures. "+++," more than 75% of Cys-less (A) or wild-type (B); "++," 20−50% of Cys-less; nd, not determined.

Active Transport. *E. coli* T184 ($lacZ^-Y^-$) was used to test the mutants for active transport. The majority of the 45 mutants transport lactose at very significant rates (Figure 2A). Twenty-six mutants exhibit rates that are between 75% and 100% or more of Cys-less permease, and eight mutants (N102C, I103C, S107C, I108C, G111C, A120C, S133C, R134C) transport at low but significant rates (30−50% of Cys-less). However, seven mutants (G121C, P123C, E130C, S136C, E139C, F140C, G141C) exhibit very low rates (15−25% of Cys-less), and rates which approximate cells transformed with vector containing no *lacY* insert are observed for mutants G115C, E126C, K131C, and R144C. Steady state levels of lactose accumulation for the great majority of mutants also approximate Cys-less permease (Figure 2B); steady states of 75−100% or more of Cys-less are achieved by 26 mutants and intermediate levels (35−65% of Cys-less) are observed with 14 mutants. Mutant F140C or K131C accumulates lactose to a low level (13−20% of Cys-less), while G115C, E126C, and R144C are unable to accumulate the sugar to any significant extent.

Gly115 can be replaced with Ala or Pro with retention of high transport activity (*40*). Furthermore, when mutations K131C and F140C are transferred to the wild-type background, significantly greater accumulation is observed (ca.

[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 the wild-type lac permease, followed by a second letter indicating the amino acid replacement.

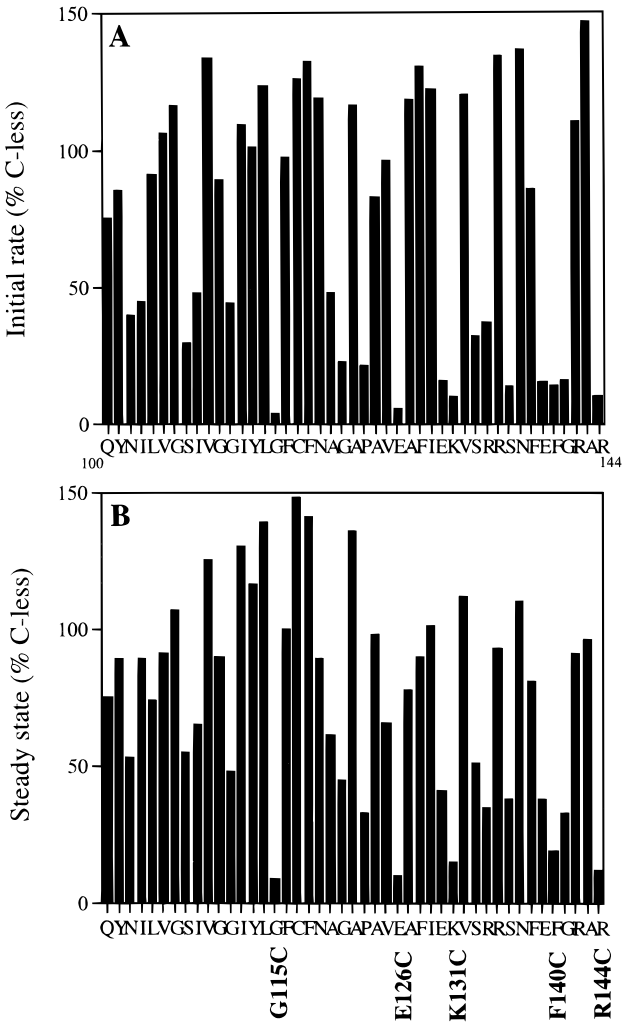

FIGURE 2: Active lactose transport by *E. coli* T184 expressing individual Cys-replacement mutants or C-less permease. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed as described in Experimental Procedures. The single-letter amino-acid code along the horizontal axis denotes the original residues replaced with Cys in increasing order from Gln100 to Arg144. (A) Rates of lactose transport measured at 1 min. The rate for Cys-less permease averaged 45 nmol/min/mg of protein. Results are expressed as a percentage of this value. Although not shown (see Figure 3), T184 harboring pT7-5 with no *lacY* gene transported at a rate of 2 nmol/min/mg of protein (i.e. 4.5% of Cys-less). (B) Steady state levels of lactose accumulation. Results are expressed as a percentage of Cys-less which averaged 118 nmol of lactose/mg of protein. Although not shown (see Figure 3), T184 harboring pT7-5 with no *lacY* insert accumulated 8 nmol of lactose/mg of protein in 1 h (i.e. 6.8% of C-less).

45% of wild-type steady state; Figure 3). In contrast, when mutation E126C or R144C is transferred to the wild-type background, the mutants remain totally inactive.

Positions Glu126 and Arg144 were examined further, and mutants E126A, E126Q, E126R, R144A, R144Q, and R144E are inactive in either Cys-less or wild-type permease (Table 1). Double neutral replacement (E126C/R144C or E126A/R144A), as well as inversion of the charged residues (E126R/R144E), also leads to inactive permease. However, mutant E126D catalyzes lactose accumulation at about one fourth the rate of wild-type to a comparable steady state, while R144K permease transports lactose at a very slow rate to about 25% of wild-type steady state (Figure 4).

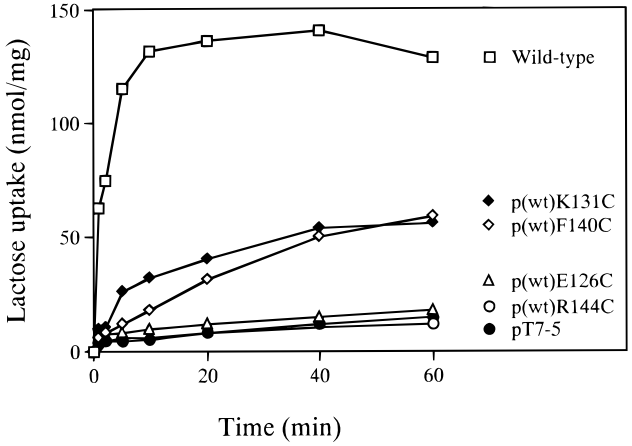

FIGURE 3: Time courses of lactose transport by mutants p(wt)-E126C, p(wt)K131C, p(wt)F140C, and p(wt)R144C (mutants E126C, K131C, F140C, and R144C in the wild-type background). *E. coli* T184 transformed with plasmid pT7-5 with no *lacY* insert, pT7-5 encoding wild-type permease or pT7-5 encoding a given mutant was grown and assayed as described in the legend to Figure 2 and in Experimental Procedures.

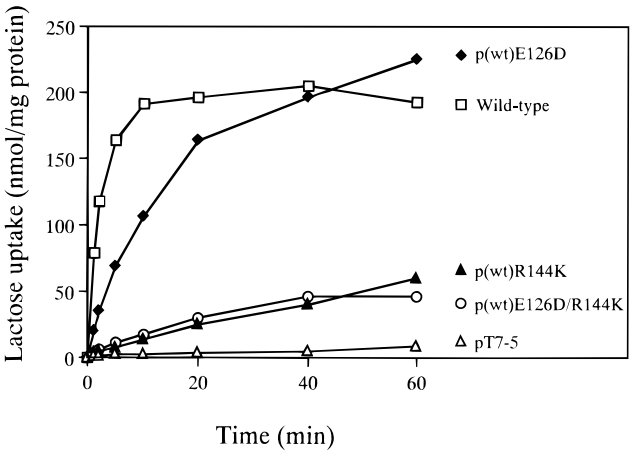

FIGURE 4: Time courses of lactose transport by mutants p(wt)-E126D, p(wt)R144K and p(wt)E126D/R144K. Conditions of cell growth and assay were as described in Figure 3.

Downhill Transport. Translocation of lactose down a concentration gradient was also measured in *E. coli* HB101 (Z^+Y^-) (Figure 5). Since β-galactosidase is expressed in this strain, any [1-^{14}C]lactose that enters the cell is rapidly cleaved, thereby providing a "sink" that drives accumulation of radioactivity, and a steady state is not achieved. As shown, HB101 expressing Cys-less permease takes up large amounts of radioactivity, while cells expressing permease with Cys, Ala, or Gln in place of Glu126 or Arg144 in either the Cys-less or wild-type background exhibit the same activity within experimental error as cells transformed with plasmid pT7-5 containing no *lacY* insert.

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 Cys-less permease with the exceptions of G115C and p(wt)R144C (data not shown). G115C which exhibits negligible transport activity (Figure 2) is present at very low levels; however, mutants G115V, G115P, and G115A are expressed to levels comparable to that of Cys-less permease (*40*). Although the R144C mutant exhibits somewhat

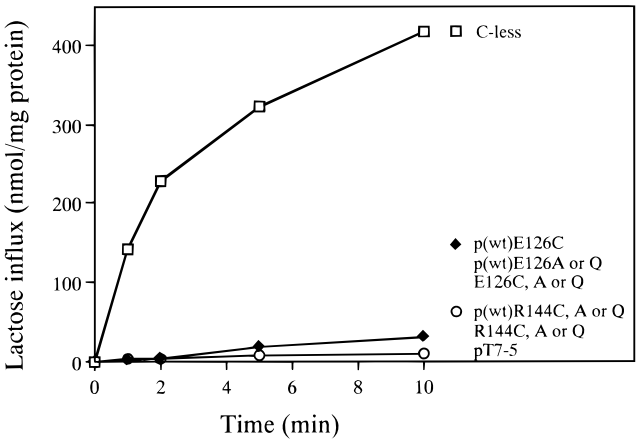

FIGURE 5: Downhill lactose transport by *E. coli* HB101 harboring plasmid pT7-5 with no *lacY* insert (negative control), pT7-5/Cys-less cassette *lacY* (positive control) or pT7-5 encoding a given amino acid replacement for either Glu126 or Arg144 in either the wild-type (wt) or Cys-less background. Experiments were carried out with [1-^{14}C]lactose at a final concentration of 1.0 mM as described in Experimental Procedures.

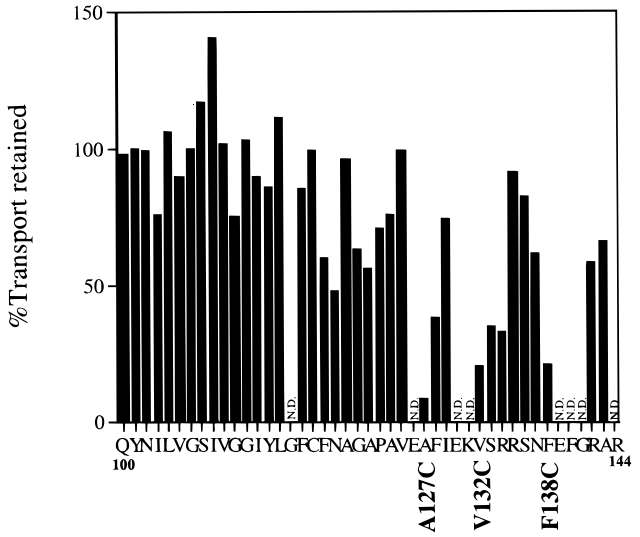

FIGURE 6: Effect of NEM on active lactose transport by *E. coli* T184 harboring plasmids encoding single-Cys mutants. Cells were incubated with 1.0 mM NEM (final concentration) at room temperature for 30 min, the reaction was quenched by addition of 10 mM dithiothreitol (final concentration), and the cells were assayed for initial rates of lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (*58*). Rates are presented as percentages of the rate measured in the absence of NEM. The effect of NEM on mutants displaying low initial rates of transport (≤20% of C-less) cannot be assessed with accuracy (N.D., not determined).

reduced protein levels, permease with other replacements at position 144 is expressed normally (Table 1).

Effect of NEM on Transport Activity. Although the activity of the majority of the Cys-replacement mutants is not altered significantly by membrane-permeant *N*-ethylmaleimide (NEM), three mutants (A127C, V132C, and F138C) are inhibited by the alkylating reagent by more than 80% and an additional three mutants (F128C, S133C, and R134C) are inhibited by ca. 65% (Figure 6). All of the NEM-sensitive mutants are located in loop IV/V, and none of the mutants in loop III/IV and transmembrane helix IV is inhibited by more than 50%, under the same conditions.

DISCUSSION

The results presented in this paper conclude the systematic Cys-scanning mutagenesis of lac permease. Unexpectedly, cytoplasmic loop IV/V is identified as a region containing several interesting and important residues. As judged by Cys-scanning and site-directed mutagenesis *per se* (*16*, *23*, *37*, *41*−*47*), Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix X) are the only residues that are irreplaceable with respect to coupling between lactose and H^+ translocation (*21*). Furthermore, none of the residues in the loops between helices appears to play a direct role in the transport mechanism. However, the activity of about 40 active Cys-replacement mutants is compromised by alkylation, and these mutants appear to be involved either in interactions with substrate or with conformationally active helical interfaces (see 20). Like most of the other transmembrane helices and intervening loops, none of the residues in loop III/IV or transmembrane helix IV is irreplaceable with respect to active transport. Moreover, none of the Cys-replacement mutants in loop III/IV or helix IV are particularly sensitive to NEM. In striking contrast, Glu126 and Arg144, which are presumed to be at the membrane−water interface at the cytoplasmic ends of helices IV and V, respectively, are clearly essential. In addition, a number of other Cys-replacement mutants with low transport activity or sensitivity to inactivation by NEM are found in loop IV/V. The findings provide a strong indication that loop IV/V plays a critical role in the transport mechanism.

With the exception of E126D and R144K permease, which exhibit high and significant but low activity, respectively, permease with other amino-acid replacements for Glu126 or Arg144 are not only completely defective with respect to $\Delta\bar{\mu}_{H^+}$-driven active transport, but are also unable to catalyze lactose influx down a concentration gradient. These properties contrast dramatically with those of mutations in the four residues essential for coupling (*21*). Moreover, NEM-labeling experiments *in situ* show that the reactivity of Cys148 is decreased and unaffected by TDG in the double mutants E126A/single-Cys148 or R144A/single-Cys148 (P. Venkatesan, S. Frillingos, and H. R. Kaback, manuscript in preparation). Thus, it appears that Glu126 and Arg144 play an important role in substrate binding either by direct interaction or by stabilizing the conformation of a binding site.

Since the cytoplasmic ends of helices IV and V may be in close proximity (*48*), it is possible that Glu126 and Arg144 interact by charge pairing. However, unlike charge pairs Asp237 (helix VII)−Lys358 (helix XI) and Asp240 (helix VII)−Lys319 (helix X), which are not required for active transport (*49*−*54*), Glu126 and Arg144 are essential residues. Therefore, if Glu126 and Arg144 are charge paired, it cannot be demonstrated by functional assays, and it is not surprising that double neutral replacement or inversion of the charged residues between positions 126 and 144 inactivates the permease. On the other hand, the only replacements for these residues that do not completely inactivate are Lys in place of Arg144, which yields permease with low activity, and Asp in place of Glu126, which yields permease with high activity.

Extensive mutagenesis and site-directed sulfhydryl modification studies on single-Cys148 permease demonstrate that all of the specificity of the permease is directed toward the

asymmetry of the hydroxyl group at the fourth position of the galactosyl moiety of the substrate (*12*, *22*). Moreover, Cys148 interacts hydrophobically with the galactosyl moiety of the substrate and Met145 which is on the same face as Cys148 at the cytoplasmic end of helix V also interacts with substrate. Helix V is in close proximity to helix VIII in the tertiary structure of the permease (*55*, *56*), and on the adjoining face of helix VIII are three positions (Val264, Gly268, and Asn272) where the reactivity of single-Cys replacements with NEM is blocked by ligand (*17*, *23*). Thus, part of the substrate translocation pathway lies between helices V and VIII. However, it is noteworthy that none of these residues is irreplaceable with respect to activity. Since Arg144 is only one peptide bond removed from Met145 in helix V and the cytoplasmic boundary of this domain has not been defined, Arg144 may be in close proximity to Cys148. This notion is appealing, as it implies that Arg144, a residue that plays an essential role in stabilizing the conformation of a substrate binding site, may also interact directly with substrate (P. Venkatesan, S. Frillingos, and H. R. Kaback, manuscript in preparation). In this context, it is also interesting to speculate about a possible role for Glu126. As discussed above, Glu126 may be charge paired with Arg144, and its role may be to stabilize local conformation in the vicinity of a binding site. In addition, the carboxyl group at this position could also be an H^+ acceptor from Glu325 (helix X) which is thought to play a primary role in H^+ translocation in the permease (*21*). By this means, Glu126 might play a dual role in substrate binding and H^+ translocation. Thus, H^+ transfer from Glu325 might destabilize the binding site by causing dissociation of the putative Glu126-Arg144 charge pair, thereby leading to release of sugar on the cytoplasmic face of the membrane.

In an initial test for accessibility or reactivity of the Cys replacement mutants, the effect of NEM on lactose transport was studied in each active mutant. While most of the 45 mutants are insensitive, three mutants are highly sensitive to NEM and inactivated by more than 80%, and an additional three mutants are inactivated by 65%. A possible explanation is that Cys replacements at the other positions do not react with NEM. However, on the basis of previous observations (*15*, *17*, *18*, *23*), it seems more likely that most of the positions in hydrophilic loop IV/V react with the alkylating agent to some extent at least. Therefore, the NEM-sensitive Cys-replacement mutants may reflect positions that are important for conformational flexibility. In this respect, it is surprising that insertion of two or six contiguous His residues between Arg134 and Arg135 in loop IV/V has little effect on activity (*26*). On the other hand, a later study (*57*) demonstrates that the position of the insertion is critical. In any case, the loop clearly plays an essential role in permease turnover. The findings distinguish loop IV/V from the other loops in the permease and add credence to the notion that the cytoplasmic end of helix V, as well as loop IV/V, comprises an important part of the substrate translocation pathway in the permease.

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