Thiol Cross-Linking of Transmembrane Domains IV and V in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Glu126 (helix IV) and Arg144 (helix V) in the lactose permease of *Escherichia coli* are critical for substrate binding and transport, and the two residues are in close proximity and charge-paired. By using a functional permease construct with two tandem factor Xa protease sites in the cytoplasmic loop between helices IV and V, it is shown here that Cys residues in place of Glu126 and Arg144, as well as Ala122 and Val149, spontaneously form disulfide bonds in situ, indicating that this region of transmembrane domains IV and V is in the α-helical conformation. To determine if the local structure or environment is perturbed by the presence of an unpaired charge, either Glu126 or Arg144 or both were replaced with Ala, and cross-linking between the Cys pair Ala122→Cys/Val149→Cys was studied. Ala replacement for Arg144 causes a marked decrease in cross-linking, while Ala replacement for Glu126 alone or for both Glu126 and Arg144 has little effect. The data provide strong support for the argument that Glu126 and Arg144 are within close proximity and suggest that an unpaired carboxylate at position 126 causes a structural change at the interface between helices IV and V.

The lactose permease (lac permease)[1] of *Escherichia coli* is representative of 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*−*4*). This polytopic membrane protein catalyzes the coupled stoichiometric symport of galactosides and H^+. The *lac Y* gene which encodes the permease has been cloned and sequenced, and the gene product has been solubilized, purified, reconstituted into proteoliposomes, and shown to be solely responsible for *β*-galactoside transport (reviewed in *5*) as a monomer (see *6*). The permease consists of 12 hydrophobic, transmembrane domains which are in α-helical conformation and that transverse the membrane in zigzag fashion connected by relatively hydrophilic loops with the N and C termini on the cytoplasmic face of the membrane (reviewed in *7, 8*).

In a functional mutant devoid of native Cys residues (Cys-less permease), each residue has been replaced with Cys (reviewed in *9*). Analysis of the mutant library utilizing a variety of site-directed biophysical and biochemical techniques has led to the following developments (reviewed in *9, 10*): (1) The great majority of the mutants are expressed normally in the membrane and exhibit significant transport activity, and only six side chains are clearly irreplaceable with respect to active transport—Glu126 (helix IV) and Arg144 (helix V), which are essential for ligand binding, and Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X), which are critical for H^+ translocation and coupling. (2) Helix packing, tilts, and ligand-induced conformational changes have been determined. (3) Positions that are accessible to solvent have been revealed. (4) Positions where the reactivity of the Cys replacement is increased or decreased by ligand binding have been identified. (5) The permease has been shown to be a highly flexible molecule. (6) A working model describing a mechanism for lactose and H^+ symport has been formulated.

Glu126 (helix IV) and Arg144 (helix V) are irreplaceable with respect to all translocation reactions catalyzed by the permease (*11, 12*). By studying site-directed *N*-ethylmaleimide (NEM) labeling of single-Cys148 permease or site-directed fluorescence of single-Cys V331C permease (*13*), as well as ligand binding by flow dialysis (*12*), it was shown that a carboxylate side chain at position 126 and a guanidino side chain at position 144 are obligatory for substrate binding. Moreover, the reactivity of Cys148 with NEM is dramatically decreased when either Glu126 or Arg144 is replaced with Ala, but interchanging the residues, double-Ala replacement, or replacement of Arg144 with Lys or His does not alter reactivity, thereby indicating that Glu126 and Arg144 are charge-paired (*13*).

Based on the observations, the following model for the substrate binding site was postulated (*13*): (i) One of the guanidino NH_2 groups of Arg144 forms an H-bond with the OH group at the C4 and/or C3 position(s) of the galactosyl moiety of the substrate, an interaction that plays a key role

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[1] Abbreviations: lac permease, lactose permease; Cys-less permease, functional permease devoid of native Cys residues; PCR, polymerase chain reaction; KP_I, potassium phosphate; EDTA, ethylenediaminetetraacetate; NEM, *N*-ethylmaleimide; *o*-PDM, *N*,*N*′-*o*-phenylenedimaleimide; DTT, dithiothreitol; $NaDodSO_4$/PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; N_4/C_8, permease expressed as nonoverlapping fragments with a discontinuity in the cytoplasmic loop between helices IV and V.

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in substrate specificity. (ii) The other guanidino NH_2 of Arg144 forms a salt-bridge with Glu126, and the interaction holds Arg144, as well as Cys148, in an orientation that allows specific interaction with the galactosyl moiety of the substrate. One of the oxygen atoms of the carboxylate at position 126 could also act as an H-bond acceptor from the C6-OH of the galactosyl moiety. (iii) Cys148 interacts weakly and hydrophobically with the galactosyl end of lactose and other galactosides. (iv) Met145 interacts even more weakly with the nongalactosyl end of certain substrates.

One important aspect of the proposed model is that Glu126 and Arg144 are charge-paired and thus in close physical proximity. Support for this contention is provided by the demonstration that purified E126H/R144H[2] permease binds Mn(II) with high affinity in a pH-dependent manner. In addition, pyrene- or spin-labeled E126C/R144C permease reconstituted into proteoliposomes shows eximer fluorescence or spin−spin interactions, respectively (*14*).

In contrast to the experiments of Zhao et al. (*14*) which utilized purified permease, in this study, in situ site-directed thiol cross-linking is used to establish the proximity of positions 126 and 144, as well as neighboring amino acyl side chains. E126C/R144C permease forms a disulfide bond spontaneously, and A122C on the same face of helix IV as Glu126 also forms a disulfide bond spontaneously with V149C on the same face of helix V as Arg144. Finally, replacement of Arg144 with Ala so as to leave an unpaired carboxylate causes decreased cross-linking between Cys pair A122C/V149C, while Ala replacement of Glu126 or both Glu126 and Arg144 has little or no effect.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lactose and [^{125}I]protein A were obtained from Amersham (Sunnyvale, CA). Restriction endonucleases, T4 DNA ligase, factor Xa protease, and appropriate reaction buffers were from New England Biolabs (Beverly, MA). Sequenase (modified T7 polymerase) and Sequence reaction kits were from United States Biochemicals (Cleveland, OH). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of permease was prepared as described (*15*). *N,N′-o*-Phenylenedimaleimide (*o*-PDM) and NEM were obtained from Sigma Chemical Co. (St. Louis, MO). Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. All other materials were reagent grade obtained from commercial sources.

Bacterial Strains and Plasmids. Subcloning grade *E. coli* XL-1blue (Stratagene Cloning Systems, Inc., La Jolla, CA) [F′:: Tn10 *proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)*M15/ *recA*1 *endA*1 *gyr*A96 (Nal^r) *thi hsd*R17 ($r_k^-m_k^+$) *sup* E44 *rel* A1 *lac*] was used as host for transformation of the products of all subcloning procedures. *E. coli* HB101 (Promega, Inc., Madison, WI) [F− Δ(*gpt-proA*) *leuB*6 *sup*E44 *ara*-14 *gal*k2 *lacY*1 Δ(*mcr*C-*mmr*) *rps*L20 *xyl*-5 *mtl*-1 *recA*13] was used for qualitative assessment of permease activity by growth on MacConkey indicator plates containing lactose. *E. coli* T184 [F′ *lacI*^{q}*O*$^+$*Z*^{U118} (*lacY*$^+$*lacA*$^+$) /*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*$^+$), *rps*L *met thr recA hsd*MR] was used for quantitative transport assays and for cross-linking experiments. All Cys-replacement mutants were made in plasmid pT7-5/cassette *lacY* (EMBL X56095) encoding Cys-less permease (*16*).

Construction of Mutants. Cys-less cassette *lacY* was digested with *Ngo*MI and *Bst*BI, and linkers encoding tandem factor Xa sites (Ile-Glu-Gly-Arg)$_2$ (*17*) or a "split" (*18*) were ligated into the DNA encoding cytoplasmic loop IV/V between codons 136 and 137 (Figure 1, inset). Single- and double-Cys mutants E126C and/or R144C were constructed by cloning either or both the *Pst*I/*Ngo*MI fragment which contains the E126C mutation or the *Bst*I/*Xho*I fragment which contains the R144C mutation from pT7-5 single-Cys mutants (*11*) into pT7-5 encoding Cys-less permease with tandem factor Xa sites. The single-Cys mutant at position 149 in helix V was constructed by ligating the *Bst*BI/*Xho*I fragment from pT7-5 encoding V149C permease into similarly digested pT7-5 encoding Cys-less permease with the tandem Xa sites. Double-Cys mutants were made by ligating the *Pst*I/*Ngo*MI fragment from pT7-5 encoding single-Cys mutants A120C, G121C, A122C, P123C, or A124C in helix IV (*11*) into the V149C Cys-less background with tandem Xa sites.

A122C/V149C mutants containing E126A and R144A were constructed by using the polymerase chain reaction (PCR). For introducing the R144A mutation into the appropriate backgrounds, DNA encoding single-Cys V149C was used as template with the following primers: 135 Sense (R144A/V149C), 5′-CGCAGTAATTTCGAATTTGGTCG-CGCGGCGATGTTTGGCAGTTGTGGCTGGGCGCTG-GTT; 219 Antisense, 5′-CTGTCTGAACAGTTCCAGT-GCC. A product of 257 bp was synthesized, restricted with *Bst*BI/*Xho*I, and ligated into the background desired. For introduction of mutation E126A into the appropriate background, DNA encoding single-Cys A122C was used as a template with the following primers: 96 Sense, 5′-GGGC-CACTGCTGCAGTACAACA; 139 Antisense (A122C/E126A), 5′CGAAATTACTGCGCCGGCTGACTTTCTCAA-TAAATGCCGCTACTACTGCTGGACAACCGGC. A product of the 132 bp fragment was synthesized, restricted with *Pst*I/*Ngo*MI, and ligated into the appropriate background. Sequences of the restricted fragments and ligation junctions were confirmed by double-stranded DNA sequencing after alkaline denaturation (*19*) using the dideoxynucleotide chain-termination reaction (*20*).

Colony Morphology. For qualitative assessment of permease activity, *E. coli* HB101 (Y^-Z^+) was transformed with plasmid encoding a given mutant, and the cells were plated on MacConkey indicator agar containing 25 mM lactose. The phenotypes were scored after a 24 h incubation at 37 °C as red (high activity), haloed (low activity), or white (no activity).

Growth of Cells. E. coli T184 (Z^-Y^-) transformed with plasmid encoding a given mutation was grown aerobically at 37 °C in Luria−Bertani broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Overnight cultures of cells were diluted 10-fold and grown for 2 h before permease expression was induced by addition of 0.5 mM isopropyl-1-thio-β,D-galactopyranoside. After further growth for 2 h, cells were harvested by centrifugation.

Active Transport. Cells were washed with 100 mM potassium phosphate (KP_I, pH 7.5)/10 mM $MgSO_4$ and adjusted to an OD_{420} of 10 in the same buffer (approximately

[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, and followed by a second letter indicating the amino acid replacement.

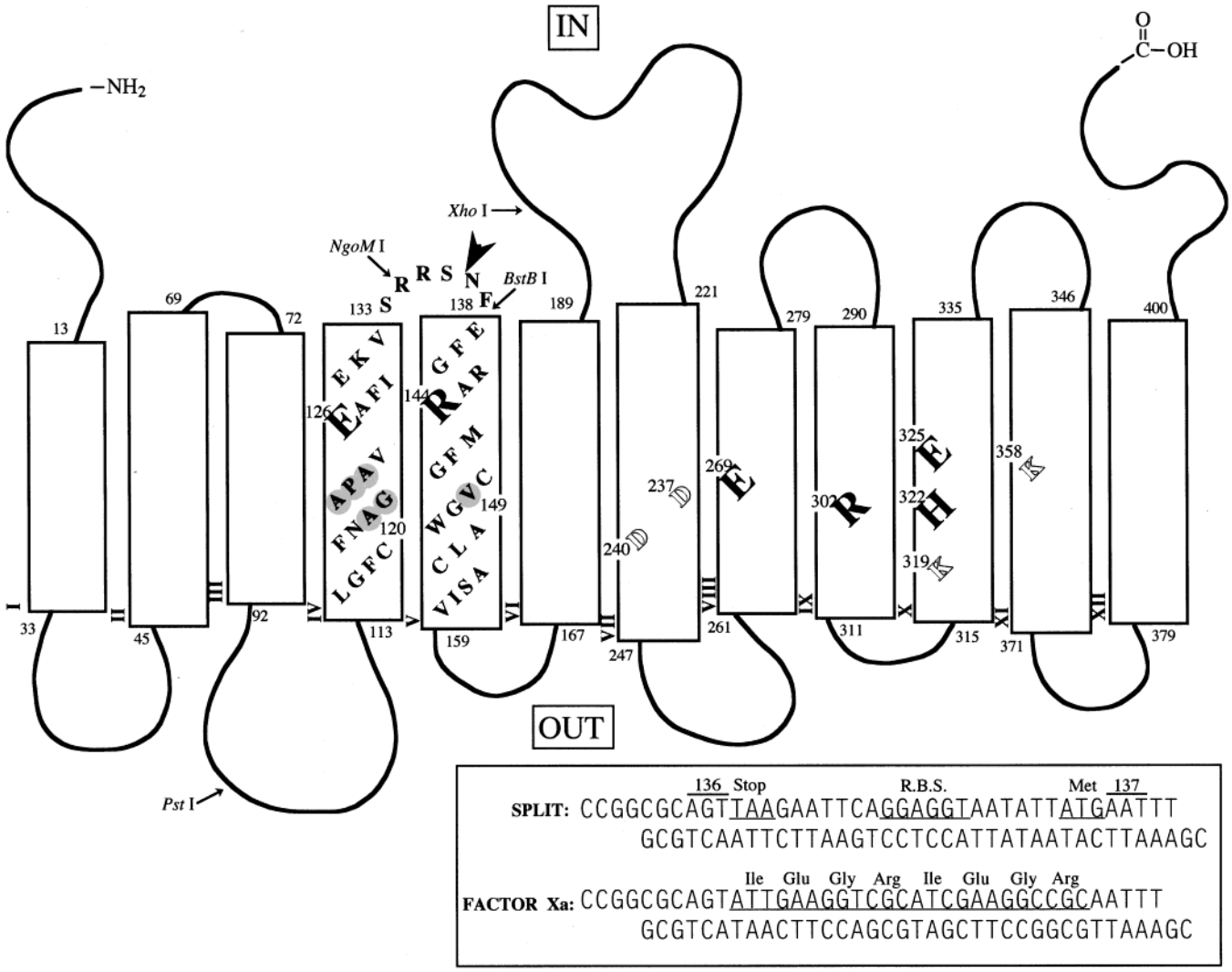

FIGURE 1: Secondary structure of lac permease. The single-letter amino acid code is used, and transmembrane domains are shown in boxes. The residues that are irreplaceable with respect to active transport are shown in large boldfaced type, and charge pairs Asp237/Lys358 and Asp240/Lys319 are shown in open-face type. The residues in helices IV and V utilized in these studies are shaded. The large boldfaced arrow indicates the site of insertion of the split or the tandem factor Xa sequences. The approximate locations of the *Pst*I, *NgoM*I, *BstB*I, and *Xho*I restriction endonuclease sites utilized for construction of given mutants are indicated as they would appear in the protein sequence. Inset: The sequence of the *NgoM*I/*BstB*I linkers synthesized for the construction of the split permease or tandem factor Xa sites between Ser136 and Asn137 in loop IV/V. The locations of the ocher stop codon, ribosomal binding site (R.B.S.), and start codon (Met) in the split linker are indicated. The amino acids encoded by the tandem factor Xa linker are also indicated.

Table 1: Properties of Double-Cys Mutants with a Given Cys Replacement in Helix IV and V149C in Helix V[a]

helix IV residue	rate of transport [% Cys-less(Xa)$_2$]	steady state [% Cys-less(Xa)$_2$]	phenotype
A120C	10	23	halo
G121C	0	0	white
A122C	48	64	red
P123C	0	0	white
A124C	35	53	red

[a] See Experimental Procedures for experimental details.

0.7 mg of protein/mL). Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 mCi = 37 Mbq) at a final concentration of 0.4 mM was assayed by rapid filtration at 25 °C (*21*). Rates of transport were determined at 2 min and steady-state levels of accumulation at 60 min. Values given in Table 1 are expressed as a percentage of activity relative to Cys-less permease with tandem Xa sites in loop IV/V.

Membrane Preparation. Cells from 100 mL of culture were resuspended in 12 mL of 20 mM Tris-HCl (pH 7.5)/5.0 mM ethylenediaminetetraacetate (EDTA, Na^+ salt). Cells were disrupted by sonification, and the preparations were centrifuged at low speed to remove cellular debris. The supernatant was then centrifuged at 300000g_{max} for 10 min, and the membrane pellet was resuspended in 1.0 mL of 20 mM Tris-HCl (pH 7.5)/5.0 mM EDTA and stored at 4 °C overnight. Prior to use, the samples were sonified and subjected to low-speed centrifugation again to remove additional debris.

Cross-Linking. Cross-linking was carried out on aliquots of membrane suspensions (100 μg of membrane protein in a volume of 100 μL). Mutants that cross-link spontaneously were incubated with 10 mM *N*-ethylmaleimide (NEM) after preparation of membranes and then treated as described below. Iodine-catalyzed cross-linking was initiated by the addition of iodine to a final concentration of 50 μM (1.0 μL of 5.0 mM iodine dissolved in ethanol). After 5 min at 25 °C, NEM was added to a final concentration of 10 mM to stop the reaction. Chemical cross-linking was initiated by

addition of *N*,*N*′-*o*-phenylenedimaleimide (*o*-PDM) to a final concentration of 0.5 mM (2.5 μL of a 20 mM stock solution). After 30 min at 25 °C, 10 mM dithiothreitol (DTT) was added to quench unreacted *o*-PDM. Samples were washed twice with 1.0 mL of 20 mM Tris-HCl (pH 7.5)/100 mM NaCl/2.0 mM $CaCl_2$, and the membranes were harvested by centifugation at 300000g_{max} for 10 min. The pellet was resuspended in 100 μL of 20 mM Tris-HCl (pH 7.5)/100 mM NaCl/2.0 mM $CaCl_2$ containing 1% dodecyl maltoside. The samples were then incubated overnight at 4 °C after addition of 3 μg of factor Xa protease to each sample (*17*).

An aliquot containing 25 μg of membrane protein from each sample was electrophoresed in sodium dodecyl sulfate/12% polyacrylamide gels ($NaDodSO_4$/PAGE) (*22*). Proteins were electroblotted onto polyvinylidene fluoride membranes (Immobilon-PVDF; Millipore) and probed with site-directed polyclonal antibody against the C terminus of lac permease (*15*). The blots were then analyzed by chemilumenesence and/or autoradiography utilizing [^{125}I]protein A and quantitated with a Model 425F Phosphorimager (Molecular Dynamics) (*23*).

RESULTS

Activity and Expression of Permease with Tandem Factor Xa Protease Sites or a Split in Loop IV/V. A particularly powerful approach for studying proximity relationships that is carried out in situ (*24*−*31*) involves expression of functional lac permease in two contiguous, nonoverlapping fragments (split lac permease), each with a single-Cys residue. Proximity of paired Cys residues is then assayed on Western blots by disulfide or chemical cross-linking of the fragments. Alternatively, lac permease with an engineered factor Xa protease site(s) can be used for the same purpose by carrying out proteolysis after cross-linking (*17*, *32*−*34*).

Cells expressing Cys-less permease containing two factor Xa protease sites in tandem in cytoplasmic loop IV/V catalyze lactose transport at a rate and to a steady-state level of accumulation comparable to Cys-less permease, while the split construct (N_4/C_8) exhibits a significantly decreased initial rate but a comparable steady-state (Figure 2). With regard to expression (Figure 2, inset), Cys-less permease with tandem Xa sites is expressed at levels similar to Cys-less permease, exhibiting a single band at approximately 35 kDa (compare lanes a and b). Incubation of Cys-less permease containing two in-tandem Xa sites with factor Xa protease overnight at 4 °C yields complete conversion to an anti-C-terminal reactive fragment with a molecular mass of about 25 kDa (Figure 2, inset, lane c). Although data are not presented, permease with a single Xa site between residues 136 and 137 is not digested at a significant rate (see *17*). In contrast to Cys-less permease with tandem Xa sites, N_4/C_8 permease with a discontinuity between positions 136 and 137 exhibits a single band at about 25 kDa, but the steady-state level of expression is only about 5% of intact Cys-less permease (Figure 2, inset, lane d). Therefore, although N_4/C_8 permease exhibits activity, it is poorly expressed. For this reason, Cys-less permease with tandem Xa protease sites was used for the cross-linking studies to be presented.

Cross-Linking Cys Pairs at Positions 126 and 144. To study the proximity of positions 126 and 144 in situ, mutants with Cys at either or both positions were constructed in Cys-less permease with tandem Xa protease sites in cytoplasmic loop IV/V. Remarkably, approximately 32% of the double-Cys mutant migrates with a molecular mass of 35 kDa under nonreducing conditions after overnight digestion with factor Xa protease, indicating that a disulfide bond forms spontaneously between the two thiols, and neither of the single-Cys mutants exhibits a band at 35 kDa (Figure 3A). Furthermore, the band observed at 35 kDa with the double-Cys mutant disappears upon incubation of the sample with DTT prior to electrophoresis (Figure 3B). When membranes from cells expressing E126C/R144C are first treated with DTT and washed, the residues cross-link spontaneously at room temperature at a rate of 6%/h and at almost 10 times the rate in the presence of 50 μM iodine (data not shown). These results obtained in situ support and extend previous findings (*14*) with purified permease solubilized in dodecyl maltoside or reconstituted into proteoliposomes, indicating that residues 126 and 144 are within close proximity.

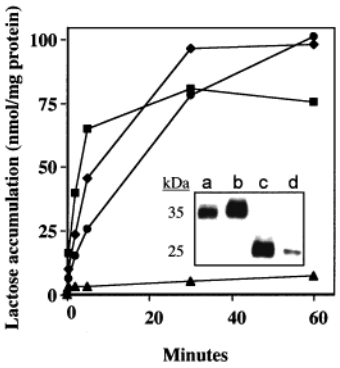

FIGURE 2: Lactose transport and permease expression in *E. coli* T184 harboring plasmid pT7-5 encoding Cys-less permease with given mutations. Lactose transport was carried out as described under Experimental Procedures. (■) C-less permease; (◆) C-less permease containing tandem factor Xa protease sites; (●) Cys-less split permease; (▲) pT7-5 with no *lacY* insert. (Inset) Permease expression in the membrane. Western blots with anti-C-terminal antibody were performed as described under Experimental Procedures. Lane a, Cys-less permease; lane b, Cys-less permease with tandem factor Xa sites; lane c, Cys-less permease with tandem factor Xa sites after overnight incubation with factor Xa protease; lane d, split permease.

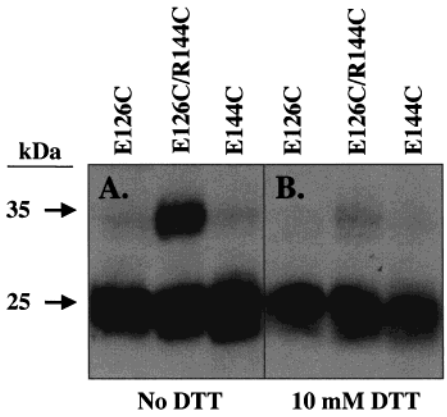

FIGURE 3: Spontaneous cross-linking of E126C/R144C permease and reversal by DTT. Western blots of membrane aliquots (25 μg of total protein) containing permease mutants E126C, R144C, or E126C/R144C with tandem factor Xa sites after overnight digestion with factor Xa protease were performed with anti-C-terminal antibody as described under Experimental Procedures. (A) Nonreducing conditions; (B) membranes from the same digestion incubated with 10 mM DTT prior to electrophoresis.

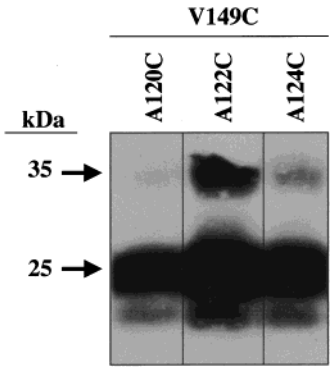

FIGURE 4: Cross-linking of A122C/V149C permease. Membrane aliquots (25 μg of total protein) containing permease mutants A120C/V149C, A122C/V149C, or A124C/V149C with tandem factor Xa sites were incubated with 50 μM iodine for 5 min followed by overnight digestion with factor Xa protease and subjected to Western blotting with anti-C-terminal antibody as described under Experimental Procedures.

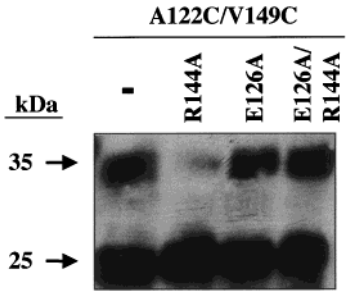

FIGURE 5: Effect of an unpaired charge at position 126 or 144 on spontaneous cross-linking of A122C/V149C permease. Western blots of membrane samples (25 μg of total protein) containing the double-Cys pair A122C/V149C and tandem factor Xa sites, and, in addition, mutation E126A, R144A, or E126A/R144A after overnight digestion with factor Xa protease were performed with anti-C-terminal antibody as described under Experimental Procedures.

Cross-Linking between a Cys Residue at Position 149 in Helix V and Cys Residues at Positions in Helix IV. To study the relationship between helices IV and V in more detail, a series of double-Cys mutants were constructed with the active single-Cys mutants A120C, G121C, A122C, P123C, or A124C in helix IV (*35*) and V149C in helix V which is presumably one turn of a helix removed from position 144 and exhibits good activity (*36*). Mutants A120C/V149C, A122C/V149C, and A124C/V149C show significant transport activity, while mutants G121C/V149C and P123C/V149C are devoid of activity even when treated with DTT and grow as white colonies on indicator plates (Table 1). In addition, cross-linking is observed with mutants G121C/V149C and P123C/V149C, although these mutants are inactive (data not shown).

Iodine-catalyzed thiol cross-linking was studied with the active mutants A120C/V149C, A122C/V149C, and A124C/149C (Figure 4). Only mutant A122C/V149C exhibits efficient cross-linking. Thus, an intense band is observed at 35 kDa with mutant A122C/V149V, while mutants A120C/V149C and A124C/V149C exhibit only faint bands at the position of the full-length protein. Importantly, mutant A122C/A149C also exhibits spontaneous cross-linking (Figure 5). In contrast, at 25 °C, all three double-Cys mutants cross-link to approximately the same extent in the presence of *o*-PDM, a rigid homobifunctional cross-linking agent in which the bis-maleimides are separated by 6 Å (data not shown). The results support the interpretation that this region of the permease is in the α-helical conformation (*37*) with positions 126 and 144, as well as positions 122 and 149, in close proximity. In addition, the cross-linking results with iodine relative to *o*-PDM are consistent with the notion that the region is conformationally flexible (*38*), since periodicity is observed only at lower temperature with iodine (i.e., under oxidizing conditions with minimal protein thermal movement).

Effect of an Unpaired Charge at Position 126 or 144 on Cross-Linking between Positions 122 and 149. Replacement of Glu126 or Arg144 with Ala so as to leave an unpaired charge results in decreased reactivity of Cys148 (helix V) with NEM (*13*). In contrast, double-Ala replacement for Glu126 and Arg144, reversal of Glu126 and Arg144, or replacement of Arg144 with Lys or His does not significantly alter the reactivity of Cys148. If the effect of an unpaired charge on Cys148 reactivity is due to a structural perturbation, cross-linking efficiency between Cys pair A122C/V149C might be altered. As shown in Figure 5 (lane 3), replacement of Arg144 with Ala so as to leave Glu126 unpaired results in a marked decrease in spontaneous cross-linking between Cys pairs A122C/V149C. In contrast, replacement of Glu126 with Ala so as to leave Arg144 unpaired (lane 2) or replacement of both residues with Ala (lane 4) has little or no effect on cross-linking. Similar results were obtained with iodine-catalyzed disulfide formation or with *o*-PDM as a cross-linking agent (data not shown).

DISCUSSION

In this study, the proximity of Cys residues at positions 126 and 144 in helices IV and V, respectively, in lac permease is documented in situ, and the results confirm and extend previous studies with purified permease solubilized in detergent or reconstituted into proteoliposomes (*14*). Thus, by using a construct with tandem factor Xa protease sites in cytoplasmic loop IV/V in order to assess cross-linking, permease with Cys replacements for Glu126 and Arg144, two residues that are critical for substrate binding, spontaneously forms a disulfide bond that is reversed by reducing agent, and no cross-linking is observed with either of the single-Cys mutants. In addition, spontaneous cross-linking is observed with double-Cys replacements at positions 122 and 149 which lie on the same faces of helices IV and V, but one turn removed from positions 126 and 144, respectively, thereby providing further evidence that this region of the permease is in the α-helical conformation (see *14, 36*).

The NEM reactivity of Cys148 in the Glu126 and Arg144 mutants (*13*) is highly analogous to the transport properties of mutants in Asp237 (helix VII) and Lys358 (helix XI) which are charge-paired (reviewed in *39*). Neutral replacement of Asp237 or Lys358 abolishes active lactose transport by leaving an unpaired charge, while simultaneous neutral replacement or reversal of the charged residues yields active permease. Furthermore, D237H/K358H permease binds Mn(II) (*40*), providing more direct evidence that the two positions are in close proximity. Similarly, normal reactivity of Cys148 is observed in the charge-reversal mutant E126R/R144E/C148 and the double-neutral mutant E126A/R144A/C148, in marked contrast to the low reactivity in the single mutants E126A/C148 and R144A/C148. The findings pro-

vide strong support for charge-pairing between Glu126 and Arg144. Based on the assumption that an unpaired charge reduces the activity of Cys148 by causing a structural change at the interface between helices IV and V, the effect of Ala replacement for Glu126 or Arg144 or both on cross-linking between Cys pairs A122C/V149C was examined. Surprisingly, although cross-linking is markedly decreased with Ala in place of Arg144, no significant effect is observed with Ala in place of Glu126 or both Glu126 and Arg144. Thus, while the presence of an unpaired negative charge at position 126 appears to alter the conformation at the interface between helices IV and V, the presence of an unpaired positive charge does not, although both perturbations decrease the reactivity of Cys148.

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