Role of the Charge Pair Aspartic Acid-237–Lysine-358 in the Lactose Permease of *Escherichia coli*

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Received October 19, 1992

ABSTRACT: Using a lactose permease mutant devoid of Cys residues (C-less permease), Asp237 and Lys358 were replaced with Cys or other amino acids to pursue the proposal that the two residues form a charge pair [King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177–186]. Individual replacement of Asp237 with Cys, Ala, or Lys or replacement of Lys358 with Cys, Ala, or Asp virtually abolishes active lactose transport. However, simultaneous replacement of both residues with Cys and/or Ala yields permease with high activity. Therefore, neutral amino acid substitutions at either position are detrimental only because they leave the opposing charge unpaired. Strikingly, moreover, when Asp237 is interchanged with Lys358, high activity is observed. The results indicate strongly that Asp237 and Lys358 interact to form a salt bridge and that neither residue nor the salt bridge per se is important for activity. Immunoblots reveal low membrane levels of the active mutants lacking the putative salt bridge, suggesting a role for the salt bridge in either permease folding or stability and raising the possibility that the salt bridge may exist in a folding intermediate but not in the mature protein. Remarkably, however, a mutant with Cys in place of Asp237 is restored to full activity by carboxymethylation which recreates a negative charge at position 237. Pulse–chase analysis and heat-inactivation studies indicate that the stability of the double mutant with Cys at positions 237 and 358 is comparable to C-less. Therefore, the interaction between Asp237 and Lys358 is likely to be important for permease folding and is maintained in the mature protein. Finally, permease molecules with Cys at position 237 and/or 358 are inactivated more readily by lipophilic than lipophobic sulfhydryl reagents, although both types of compounds inactivate to the same extent. Thus, the charge pair may be located within the membrane bilayer close to the membrane–water interface rather than in the middle of the membrane.

Lactose (*lac*) permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of β-galactosides and H^+ with a 1:1 stoichiometry (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 [see Kaback (1989, 1990, 1992) for reviews]. On the basis of circular dichroic studies and hydropathy analysis of the 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 a 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; Danho et al., 1985), and chemical modification (Page & Rosenbusch, 1988). Exclusive 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).

Recently, King et al. (1991) found that *lac* permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective with respect to active transport. Second-site suppressor mutations of K358T[1] yield neutral amino acid substitutions for Asp237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys358. On the basis of these findings, it was proposed that Asp237 and Lys358 interact via a salt bridge, thereby neutralizing each other. Replacement of either charged residue with a neutral residue presumably leaves an unpaired charge which causes a functional defect, while neutral substitutions for both residues do not cause inactivation. Consequently, the secondary structure model of the permease was altered to accommodate a putative salt bridge between Asp237 and Lys358 within the low dielectric of the membrane by shifting residues Phe247–Thr235 from the hydrophilic domain between helices VII and VIII into transmembrane helix VII (Figure 1). Subsequent studies (Sahin-Tóth et al., 1992; Lee et al., 1992) also reveal a functional interaction between Asp240 and Lys319, leading to the hypothesis that putative helix VII which contains Asp237 and Asp240 neighbors helices X (Lys319) and XI (Lys358) in the tertiary structure of the permease.

In this study, Asp237 and Lys358 were replaced with Cys or other amino acids by site-directed mutagenesis of a functional *lac* permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991). The results confirm the observations of King et al. (1991) indicating that Asp237 and Lys358 form a charge pair. In addition, data are presented supporting the following conclusions: (i) neither residue nor

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

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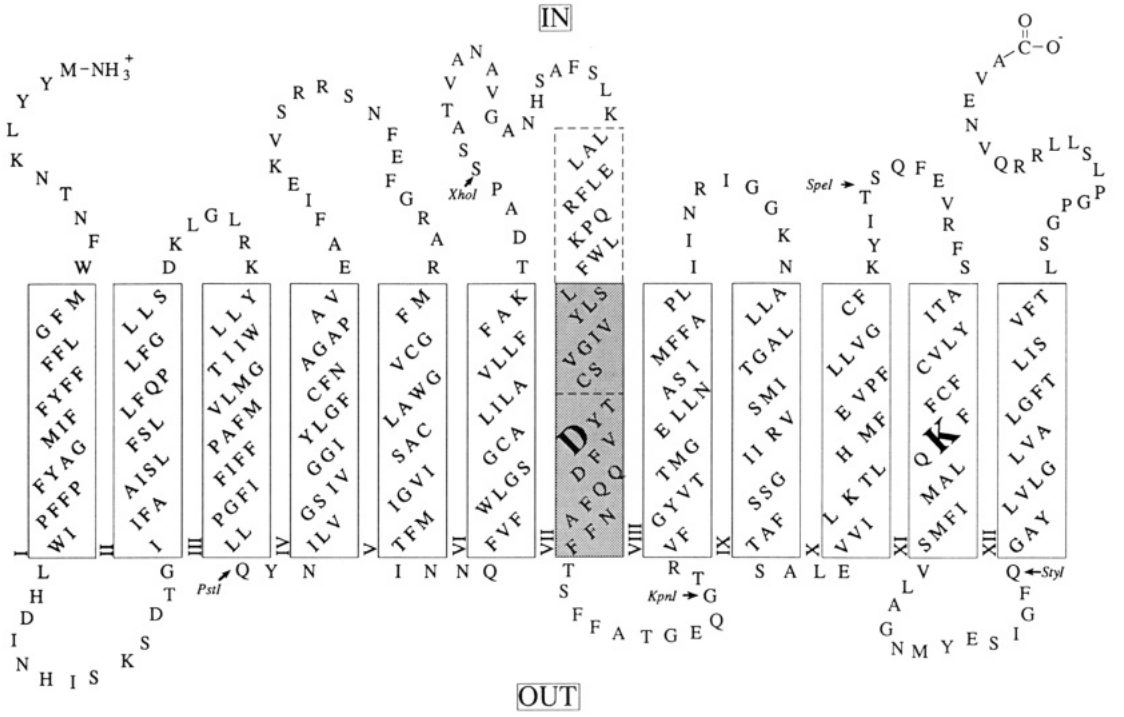

FIGURE 1: Secondary structure model of *E. coli lac* permease based on hydropathy analysis (Foster et al., 1983). The single-letter amino acid code is used, and Asp237 and Lys358 are highlighted. Hydrophobic transmembrane helices are shown in solid boxes except for putative helix VII, which is enclosed by a dashed line. The shaded box shows the modification of helix VII proposed by King et al. (1991). The locations of relevant restriction sites in the corresponding DNA sequence are also indicated.

the salt bridge is important for permease activity; (ii) the interaction between Asp237 and Lys358 is probably important for folding of the permease; (iii) the interaction is maintained in the mature molecule; and (iv) the two residues may be within the bilayer close to the membrane–water interface.

MATERIALS AND METHODS

Materials

[1-^{14}C]Lactose, [α-^{35}S]dATP, and L-[^{35}S]methionine 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. Methanethiosulfonate ethylsulfonate (MTSES)[2] was the generous gift of Arthur Karlin, Columbia University, New York, NY. All other materials were reagent grade and obtained from commercial sources.

Methods

Bacterial Strains. E. coli HB101 [*hsdS20*(r^-_B,m^-_B),-*recA13*,*ara-14*,*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.

Mutagenesis. The single mutants (D237C, D237A, D237K, K358C, K358A, and K358D) were prepared by oligonucleotide-directed, site-specific mutagenesis of a cassette version of the *lacY* gene (EMBL-X56095) encoding C-less permease in the plasmid pT7-5 (pC$_7$S/C154V; van Iwaarden et al., 1991). The sequences of the two mutagenic primers used to create each mutation are presented in Table I. The two-stage polymerase chain reaction (PCR) overlap extension method of Ho et al. (1989) was employed with Vent$_R$ DNA polymerase. 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 were extracted with chloroform, ethanol-precipitated, and digested with appropriate restriction endonucleases (see Figure 1 for location of sites). The *Xho*I–*Kpn*I fragment (Asp237 mutants) and the *Spe*I–*Sty*I fragment (Lys358 mutants) were isolated from low melting point agarose gels and ligated to similarly treated pC$_7$S/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).

Double mutants (D237C/K358C, D237C/K358A, D237A/K358C, and D237K/K358D) were constructed from single mutants using restriction endonucleases *Pst*I and *Spe*I (see Figure 1). Both the 716 base pair *Pst*I–*Spe*I "inserts" from

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid: IPTG, isopropyl 1-thio-β-D-galactopyranoside; KP$_i$, potassium phosphate; MMTS, methyl methanethiosulfonate; MTSES, methanethiosulfonate ethylsulfonate; NEM, *N*-ethylmaleimide; NaDodSO$_4$, sodium dodecyl sulfate; *p*CMB, *p*-(chloromercuri)benzoic acid; *p*CMBS, *p*-(chloromercuri)-benzenesulfonate; PMS, phenazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; RSO, right-side-out; Tris, tris(hydroxymethyl)aminomethane; TBST, 10 mM Tris-HCl/0.9% NaCl/0.2% Triton X-100.

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

mutant		mutagenic oligonucleotides[a]	codon change
D237C	sense	TCCACCTACTGTGTTTTTGACCAA	GAT→TGT
	antisense	GTCAAAAACACAGTAGGTGGA	
D237A	sense	TCCACCTACGCTGTTTTTGAC	GAT→GCT
	antisense	GTCAAAAACAGCGTAGGTGGA	
D237K	sense	TCCACCTACAAGGTTTTTGACCAA	GAT→AAG
	antisense	GTCAAAAACCTTGTAGGTGGAGGA	
K358C	sense	AGCTTCTTTTGTCAACTGGCGATG	AAG→TGT
	antisense	CGCCAGTTGACAAAAGAAGCTGAA	
K358A	sense	AGCTTCTTTGCGCAACTGGCGATG	AAG→GCG
	antisense	CGCCAGTTGCGCAAAGAAGCTGAA	
K358D	sense	AGCTTCTTTGACCAACTGGCGATG	AAG→GAC
	antisense	CGCCAGTTGGTCAAAGAAGCTGAA	

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

the plasmids encoding the Asp237 mutations and the approximately 3200 base pair "vectors" lacking the *Pst*I–*Spe*I fragment from the Lys358 mutations were isolated from low melting point agarose gels and ligated to produce the appropriate double mutants. Plasmids were isolated and sequenced as described above to confirm the presence of the mutations.

Growth of Bacteria. For lactose transport assays and membrane preparations, *E. coli* T184, transformed with each plasmid described, was grown aerobically at 37 °C in LB 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 additional growth for 2 h at 37 °C, cells were harvested by centrifugation.

Alternatively, for overexpression via the T7 RNA polymerase system (Tabor & Richardson, 1985), *E. coli* T184 harboring plasmids pGP1-2 and either pT7-5 or one of the derivatives described was grown at 30 °C in LB broth supplemented with streptomycin (10 μg/mL), ampicillin (100 μg/mL), and kanamycin (50 μg/mL) to an OD_{600} between 0.85 and 1.10. Cells were induced by heat shock at 42 °C for either 12 min (10-mL cultures) or 1 h (3-L cultures) and allowed to recover for 1 h at 30 °C before being harvested by centrifugation.

Preparation of Membranes. Right-side-out (RSO) membrane vesicles were prepared by lysozyme–EDTA treatment and osmotic lysis as described (Kaback, 1971; Short et al., 1975) from 3-L cultures of *E. coli* T184 harboring pGP1-2 and given plasmids. Cells were grown and induced as described above.

Alternatively, crude membrane fractions were prepared as follows: 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 250000*g* for 1 h at 4 °C in a Beckman Optima TL ultracentrifuge.

Active Lactose Transport. Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 Ci = 37 CBq) at a final concentration of 0.4 mM was assayed in intact cells as described by Consler et al. (1991). Transport in RSO membrane vesicles was performed similarly except that vesicles were assayed under oxygen and in the presence of ascorbate and phenazine methosulfate (PMS; Konings et al., 1971; Kaback, 1974).

Immunoblotting. Membrane fractions prepared as described above were resuspended in $NaDodSO_4$ gel loading buffer and heated at 37 °C for 20 min prior to electrophoresis performed as described (Newman et al., 1981). Proteins were electroblotted onto poly(vinylidene 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 TBST (10 mM Tris-HCl/0.9% NaCl/0.2% Triton X-100). After 1 h, rabbit polyclonal antiserum directed against the C-terminal dodecapeptide of *lac* permease was added at a final dilution of 1:2000. Blots were incubated a further 2 h, washed 3 times with TBST for 20 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 four 15-min washes with TBST, blots were developed with fluorescent substrate (Amersham) according to the manufacturer's instructions.

[^{35}S]*Methionine Labeling.* Specific [^{35}S]methionine labeling of *lac* permease mutants was performed using the T7 RNA polymerase system (McKenna et al., 1991). Membrane fractions were prepared and electrophoresed as described above, and the gel was exposed to film for 40 min.

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

RESULTS

Construction and Verification of Mutants. In order to test the hypothesis that Asp237 and Lys358 in *lac* permease form a charge pair in an environment that cannot tolerate an unpaired charge (King et al., 1991), site-directed mutagenesis was used to replace the two residues with Ala and/or Cys (D237A, D237C, K358A, and K358C and D237C/K358C, D237A/K358C, and D237C/K358A). All amino acid replacements were made in C-less permease (van Iwaarden et al., 1991) so that specific modification of engineered Cys residues can be exploited in further studies. Additionally, the charged residues at positions 237 and 358 were reversed in the double mutant D237K/K358D which was constructued from D237K and K358D. All mutations were verified by double-stranded DNA sequencing as described under Methods. Except for the base changes summarized in Table I, the sequences were identical to those of cassette *lacY* encoding C-less permease.

Colony Morphology. The ability of mutants to translocate lactose "downhill" can be assessed qualitatively by transforming *E. coli* HB101 (*lacZ*$^+$*Y*$^-$) with plasmid encoding each mutant and growing the transformants on MacConkey

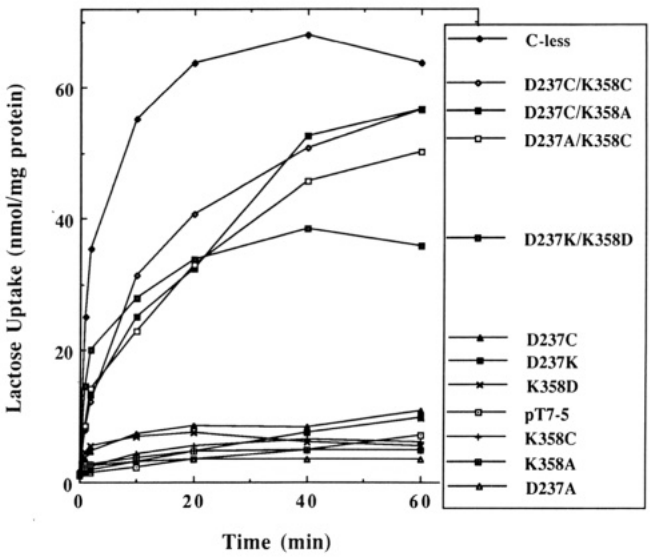

FIGURE 2: Active transport of lactose by *E. coli* T184 harboring plasmid encoding C-less permease or given mutants at Asp237 and/or Lys358. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature by rapid filtration as described under Methods.

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, while mutants with low activity grow as red colonies with a white halo. All the double mutants, including the charge-inversion mutant D237K/K358D, appear as red colonies indistinguishable from cells expressing C-less permease. With the exception of D237A which yields red colonies, the single mutants grow as red colonies with a white halo.

Active Lactose Transport. Time courses of [1-^{14}C]lactose transport for each mutant expressed in *E. coli* T184 (Z^-Y^-), the negative control (pT7-5 with no insert), and the positive control (pT7-5 encoding C-less permease) are shown in Figure 2. All three mutants in which Asp237 and Lys358 were simultaneously replaced with neutral residues catalyze lactose accumulation to 75–90% of the steady-state level of C-less permease at approximately 30% of the rate. Furthermore, permease with Lys in place of Asp237 and Asp in place of Lys358 (i.e., the charge-inversion mutant D237K/K358D) accumulates lactose to approximately 50% of the steady-state level of C-less at nearly 60% of the rate. In contrast, none of the single mutants reach a steady-state above that of the negative control, and only D237C and K358D exhibit measurable rates (<10% of C-less).

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 3). The amount of permease detected differs greatly among the mutants, and none is as abundant as the C-less control (lane 11). Among the four double mutants that are highly active, only D237K/K358D (lane 10) is present in relatively high amounts, approximately 50% of the C-less control. All three double mutants with neutral amino acid substitutions [i.e., D237C/K358C (lane 3), D237A/K358C (lane 6), and D237C/K358A (lane 7)] are present in low amounts, at most 10–15% of C-less. The inactive single mutants exhibit a range of levels from as low as a few percent [e.g., K358A (lane 5)] to as high as 20–40% of C-less [e.g., K358D (lane 9) and D237A (lane 4)].

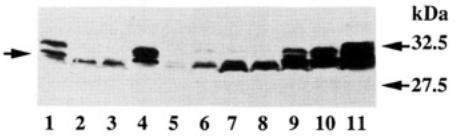

FIGURE 3: Western blot of membranes containing C-less *lac* permease or various single or double mutants. Membranes were prepared from IPTG-induced cultures of *E. coli* T184 harboring given plasmids as described under Methods, and 200 μg of membrane protein was subjected to 12% $NaDodSO_4$–polyacrylamide gel electrophoresis and electroblotting. The blot was incubated with anti-C-terminal antibody, folowed by horseradish peroxide linked protein A, and finally with fluorescent substrate before a 3-min exposure to film. The arrow on the left indicates the position of *lac* permease. Lane 1, D237C; lane 2, K358C; lane 3, D237C/K358C; lane 4, D237A; lane 5, K358A; lane 6, D237A/K358C; lane 7, D237C/K358A; lane 8, D237K; lane 9, K358D; lane 10, D237K/K358D; lane 11, C-less. Although not shown, membranes prepared from cells harboring pT7-5 with no *lacY* insert exhibit no immunoreactive material between the marker proteins shown on the right: soybean trypsin inhibitor (27.5 kDa) and carbonic anhydrase (32.5 kDa).

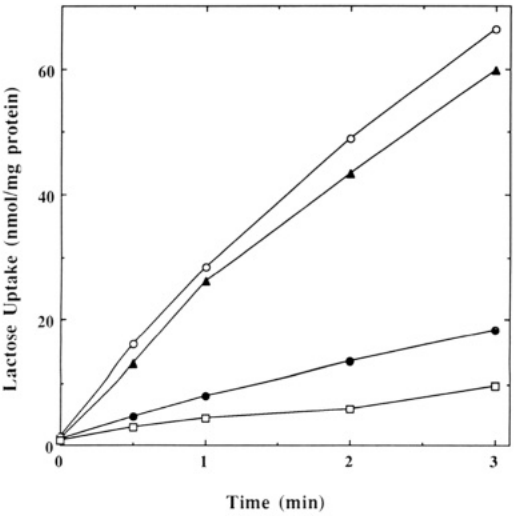

FIGURE 4: Effect of iodoacetate and iodoacetamide on active lactose uptake in cells expressing C-less or D237C mutant permease. IPTG-induced cultures of *E. coli* T184 harboring plasmid encoding C-less or D237C permease were washed with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$, preincubated for 30 min at room temperature with given sulfhydryl reagents, and immediately assayed for transport in the presence of ascorbate and PMS as described under Methods. Preincubations contained C-less permease alone (▲), D237C alone (●), D237C with 1 mM iodoacetate (○), and D237C with 1 mM iodoacetamide (□).

Effect of Carboxymethylation on D237C Permease Activity. Decreased expression of permease mutants in which the interaction between Asp237 and Lys358 is disturbed raises the possibility that the charge pair may exist in a folding intermediate, but not in the mature protein. To approach this problem, we tested the effect of iodoacetic acid or iodoacetamide on mutant D237C which is presumably inactive because the permease contains an uncompensated positive charge (i.e., Lys) at position 358. Remarkably, treatment of the mutant with iodoacetic acid completely restores the activity of D237 permease, while iodoacetamide which is uncharged has no significant effect (Figure 4). Although data are not shown, the activities of C-less permease or D237A permease are unaffected by either reagent. The results provide a strong indication that formation of the negatively charged adduct (carboxymethyl)cysteine at position 237 is responsible for restoration of activity and are consistent with the conclusion that the interaction between Asp237 and Lys358 is present in the mature permease.

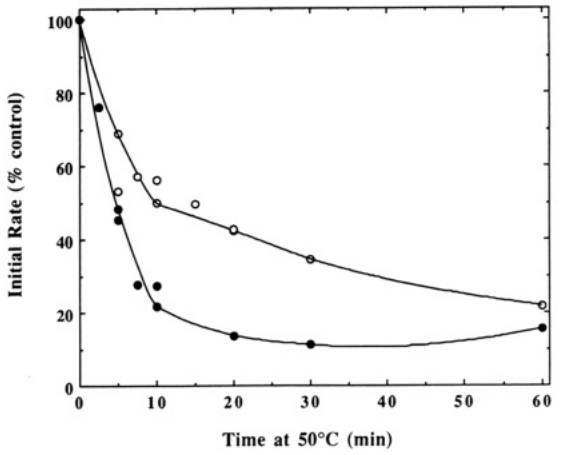

FIGURE 5: Heat inactivation of the initial rate of lactose transport in C-less and D237C/K358C RSO membrane vesicles. C-less (open symbols) or D237C/K358C (filled symbols) vesicles were incubated for the given times at 50 °C and then equilibrated at room temperature prior to assay of ascorbate/PMS-driven lactose uptake as described under Methods. Activities, expressed as a percentage of the initial rate observed without heat treatment, were corrected for transport in the absence of ascorbate and PMS.

Stability of D237C/K358C Permease. In order to determine whether decreased expression observed for the mutants described is due to instability after insertion into the membrane, heat inactivation of transport activity and [^{35}S]methionine pulse–chase experiments were performed. When the time course of heat inactivation of lactose transport in RSO vesicles containing D237C/K358C permease is compared to that of RSO vesicles containing C-less permease, 50% inactivation is achieved at about 5 min for D237C/K358C and at about 10 min for C-less (Figure 5). Similarly, pulse–chase studies with [^{35}S]methionine during expression from the T7 promoter in the presence of rifampicin show that both proteins are stable for at least 20 h, although the double Cys mutant is expressed at a lower level (Figure 6A,B). The results indicate that the decreased level of D237C/K358C permease in the membrane cannot be attributed to instability after the protein is inserted.

Accessibility of Cys Residues at Position 237 or 358. Neither Asp237 nor Lys358 is important for permease activity, but a variety of sulfhydryl reagents inactivate transport to varying degrees when either or both residues are replaced with Cys (data not shown). Therefore, in order to estimate the accessibility of reactive Cys residues at positions 237 or 358, single Cys mutants with Ala at the alternate position were tested for inactivation by lipophilic or lipophobic sulfhydryl reagents. Methyl methanethiosulfonate (MMTS), which is relatively lipophilic, inhibits the rate of lactose transport by D237C/K358A (Figure 7A) or D237A/K358C permease (Figure 7B) by about a factor of 2, and the concentration that produces half-maximal inhibition approximates 25 μM with both mutants. In contrast, methanethiosulfonate ethylsulfonate (MTSES), which is 50 000 times as soluble in water as in 1-octanol (A. Karlin, personal communication), inhibits to approximately the same extent as MMTS, but the concentration that produces half-maximal inhibition is about 150 or 400 μM for D237C/K358A or D237A/K358C permease, respectively.

Although data are not presented, three additional points should be emphasized: (i) MTSES is intrinsically 2.5–4.0-fold more reactive than MMTS (A. Karlin, personal communication), suggesting that the difference between the two compounds may be underestimated. (ii) Incomplete inactivation with MMTS is not due to incomplete reaction with

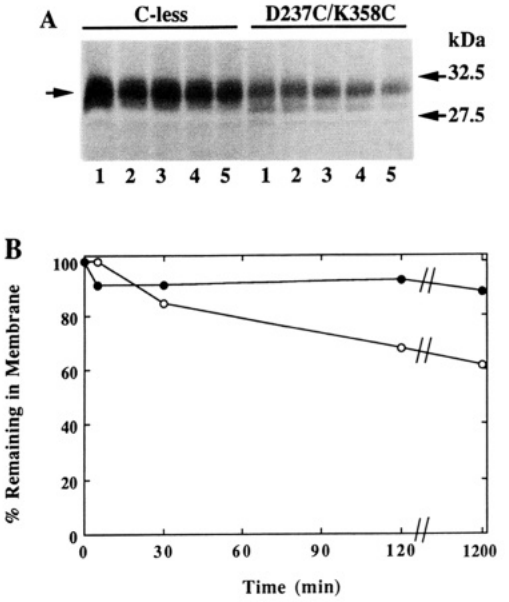

FIGURE 6: Stability of C-less permease and D237C/K358C mutant permease assessed by [^{35}S]methionine pulse–chase analysis. (A) As described under Methods, cells were incubated with [^{35}S]methionine for 10 min before removal of the zero-time aliquot (lanes 1) and addition of a 200-fold excess of unlabeled methionine. Subsequently, aliquots were removed at 5 min (lanes 2), 30 min (lanes 3), 2 h (lanes 4), and 20 h (lanes 5). Membranes were prepared from each time point as described under Methods and subjected to 12% NaDodSO$_4$–polyacrylamide gel electrophoresis. The arrow on the left indicates the position of *lac* permease, and marker proteins shown on the right are given in Figure 3. (B) The amount of either C-less (○) or D237C/K358C (●) permease labeled with [^{35}S]methionine as shown in (A) was quantitated with a Model 400E PhosphorImager (Molecular Dynamics). Permease remaining at each time point is expressed as a percentage of the counts measured in the zero-time lane.

either D237C/K358A or D237A/K358C permease. Although *p*-(chloromercuri)benzoate (*p*CMB) inactivates almost completely, only 50% inactivation is observed (data not shown) after pretreatment with 0.5 mM MMTS, indicating that the Cys residues react completely with MMTS. (iii) Similar but less marked differences are observed with *p*CMB versus *p*-(chloromercuri)benzenesulfonate (*p*CMBS). Thus, 100 μM *p*CMB or *p*CMBS inactivates lactose transport almost completely with both mutants, and the concentration that causes 50% inactivation is about 50 or 70 μM, respectively.

DISCUSSION

In this paper, site-directed mutagenesis of Asp237 and Lys358 in a functional *lac* permease devoid of Cys residues (van Iwaarden et al., 1991) was employed to test the hypothesis that these residues form a charge pair, thereby placing putative transmembrane helix VII in close proximity to transmembrane helix XI (King et al., 1991; Sahin-Tóth et al., 1992). Individual replacement of Asp237 with Cys, Ala, or Lys or replacement of Lys358 with Cys, Ala, or Asp leads to inactivation, whereas simultaneous replacement of both residues with Cys and/or Ala or interchanging Asp237 and Lys358 has little effect on active lactose transport. Thus, single neutral substitutions for either residue leave an uncompensated charge which causes a functional defect, while simultaneous replacement of both residues with Cys and/or Ala or interchanging the residues either removes charge altogether or reverses the polarity of the interaction, neither of which seriously compromises activity. The results provide strong support for the idea that Asp237 and Lys358 interact in a salt bridge and, in addition, demonstrate that neither Asp237, Lys358, nor the salt bridge per se plays an important role in the transport mechanism.

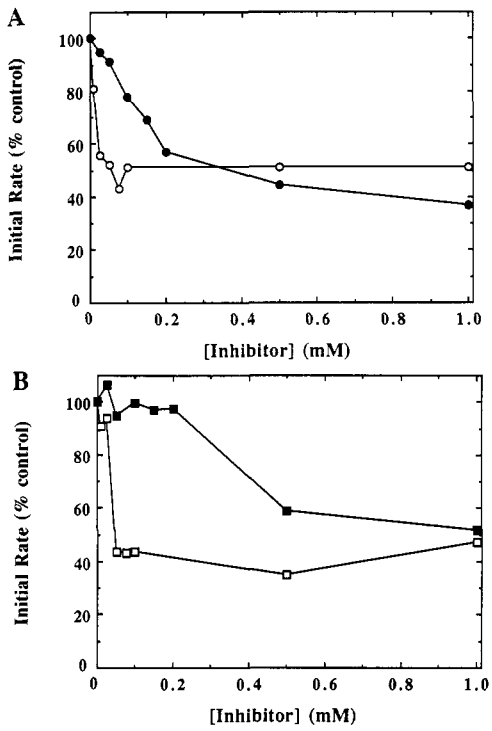

FIGURE 7: Inactivation of active lactose uptake in D237C/K358A (A) or D237A/K358C (B) RSO vesicles by MMTS or MTSES. Vesicles (3 mg of protein/mL) in 50 mM KP_i (pH 7.2)/10 mM $MgSO_4$ were incubated with the given concentrations of MMTS (open symbols) or MTSES (filled symbols) for 30 min at room temperature and assayed immediately for ascorbate/PMS-driven lactose accumulation. Activities are expressed as a percentage of the initial rate observed in the absence of inhibitor and were corrected for lactose transport in the absence of ascorbate/PMS. Virtually no inhibition was observed with C-less permease with either inhibitor over the concentration range shown.

The remarkable ability of *lac* permease to tolerate paired neutral replacements in Asp237 and Lys358 or reversal of the charge pair distinguishes this interaction from that observed for Asp240 and Lys319 (Sahin-Tóth et al., 1992; Lee et al., 1992). It has been shown (Sahin-Tóth et al., 1992) that simultaneous replacement of Asp240 and Lys319 with Cys and/or Ala results in significant but diminished active lactose transport and that interchanging the residues leads to complete inactivation. Therefore, although neither Asp240 nor Lys319 is mandatory for activity, the polarity of the interaction appears to be important. Parenthetically, it is also noteworthy that mutant permeases in which the interaction between Asp240 and Lys319 has been disrupted are present in normal concentrations in the membrane.

Surprisingly, despite the high activity observed for the mutants carrying double neutral replacements for Asp237 and Lys358, low concentrations of these mutant permeases and most of the single mutants as well are found in the membrane. In addition to standing in contrast to the Asp240–Lys319 pair (Sahin-Tóth et al., 1992), the findings raise the possibility that the charge pair may exist in a folding intermediate but not in the mature permease. Strikingly, however, when the single mutant D237C is carboxymethylated with iodoacetic acid, full activity is restored. This result clearly demonstrates that restoration of the negative charge at position 237 corrects the defect in activity and implies that the charge pair exists in the mature permease. Nonetheless, the low concentration of the mutants, particularly those exhibiting high activity, indicates that the salt bridge plays a role in folding or stability.

Since it is difficult, if not impossible, to study folding of polytopic membrane proteins during insertion into the membrane, the stability of the active double mutant D237C/K358C after insertion into the membrane was investigated by testing either the heat inactivation of permease activity or the lifetime in the membrane by means of [^{35}S]methionine pulse–chase experiments. The mutant is inactivated only twice as fast as C-less permease at 50 °C, and its lifetime is indistinguishable from that the of the control. Therefore, the charge pair is likely to play a role in permease folding at a stage prior to complete insertion into the membrane.

The modification of the secondary structure model proposed by King et al. (1991) is based on the functional interaction between Asp237 and Lys358 and on the idea that the intramembrane charged residues must be balanced. Despite the indication that Asp240 and Lys319 may also participate in a salt bridge (Sahin-Tóth et al., 1992; Lee et al., 1992), the evidence is indirect, and other approaches are required to determine the location of the residues relative to the plane of the membrane and to demonstrate directly that both sets of residues are in close proximity. In both respects, C-less permease mutants containing double Cys or paired Cys-Ala replacements should be useful. In a preliminary effort to estimate the accessibility of Cys at positions 237 and 358, the sensitivity of given mutants to lipophilic and lipophobic sulfhydryl reagents was studied. Both D237C/K358A and D237A/K358C are inactivated to the same extent by MMTS or MTSES, but in both instances, the relatively lipophilic MMTS is significantly more effective. Similar observations were made with *p*CMB and *p*CMBS, although the difference between the compounds is much less marked. The results suggest that Asp237 and Lys358 are located within the membrane near the membrane–water interface, rather than in the middle of the membrane as suggested by King et al. (1991). Initial experiments using site-directed spin-labeling (Altenbach et al., 1990) and fluorescent labeling are consistent with this suggestion. Thus, the nature of the spectra and the quenching behavior of purified, reconstituted mutant permeases labeled with appropriate electron paramagnetic resonance or fluorescence probes at either position 237 or position 358 are consistent with the interpretation that both residues are in an amphipathic environment (H. Jung, R. Lopez, C. Altenbach, W. L. Hubbell, and H. R. Kaback, unpublished results). On the basis of the observations, we suggest that the salt bridge between Asp237 and Lys358 is located near the C-termini of putative transmembrane helices VII and XI, respectively, and not in the middle of the helices (see Figure 1).

ACKNOWLEDGMENT

We are indebted to Arthur Karlin for the generous gift of MTSES.

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