Properties of Interacting Aspartic Acid and Lysine Residues in the Lactose Permease of *Escherichia coli*

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ABSTRACT: The side chains of the interacting pair Asp237(helix VII)–Lys358(helix XI) or Asp240(helix VII)–Lys319(helix X) in the lactose permease of *Escherichia coli* were extended by replacement with Glu and/or Arg or by site-specific derivatization of single-Cys replacement mutants. Iodoacetic acid was used to carboxymethylate Cys, or methanethiosulfonate derivatives [Akabas, M. H., Stauffer, D. A., Xu, M., & Karlin, A. (1992) *Science 258*, 307] were used to attach negatively charged ethylsulfonate or positively charged ethylammonium groups. Replacement of Asp237 with Glu, carboxymethyl-Cys, or sulfonylethylthio-Cys yields active permease with Lys or Arg at position 358. Similarly, the permease tolerates replacement of Lys358 with Arg or ammonioethylthio-Cys with Asp or Glu at position 237. Remarkably, moreover, permease with Lys, Arg, or ammonioethylthio-Cys in place of Asp237 is highly active when Lys358 is replaced with Asp or Glu, in agreement with the conclusion that the polarity of the charge interaction can be reversed without loss of activity [Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547]. In contrast, replacement of Asp240 with Glu abolishes lactose transport, and permease with carboxymethyl-Cys, at position 240 is inactive when paired with Lys319, but it exhibits significant activity with Arg319. Interestingly, sulfonylethylthio-Cys substitution for Asp240 also results in significant transport activity. Permease with Arg or ammonioethylthio-Cys in place of Lys319 exhibits high activity with Asp240 as the negative counterion, but no lactose transport is observed when either of these modifications is paired with Glu240. Lactose permease mutants in the charge pair Asp237–Lys358 are inserted into the membrane at wild-type levels if the charge pair is maintained with either polarity, while disruption of the interacting pair often causes a marked decrease in the amount of protein inserted into the membrane; mutations in Asp240–Lys319 do not affect insertion of the permease. The results demonstrate that neither Asp237, Lys358, nor the interaction between these residues is important for permease activity and that this putative salt bridge probably plays a role in membrane insertion. In contrast, the interaction between Asp240 and Lys319 exhibits much more stringent properties, and the polarity of the interaction appears to be important for activity.

Lactose (lac)[1] 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, 1992) and Kaback et al. (1990) for reviews]. On the basis of circular dichroism 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 hydrophobic domains in an α-helical configuration that traverse the membrane in a zig-zag fashion connected by hydrophilic loops, and a 17-residue hydrophilic C-terminal tail (Figure 1). Evidence favoring 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; Page & Rosenbusch, 1988), immunological studies with monoclonal (Carrasco et al., 1982, 1984a; Herzlinger et al., 1984, 1985) and site-directed polyclonal antibodies (Seckler et al., 1983, 1984, 1986; Carrasco et al., 1984b), and chemical modification (Page & Rosenbusch, 1988). Unequivocal support for the 12-helix motif has been obtained from the analyses of an extensive series of lac permease–alkaline phosphatase (*lacY–phoA*) fashions (Calamia & Manoil, 1990).

An interaction between Asp237 and Lys358 (Figure 1) was proposed initially by King et al. (1991) on the basis of second-site suppressor analyses. Permease mutants with Thr in place of Lys358 or Asn in place of Asp237 are defective in lactose transport, and second-site suppressor mutations of K358T[2] yield neutral amino acid substitutions for Asp237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys358. It was proposed that Asp237 and Lys358 neutralize each other via a salt bridge, that replacement of either charged residue with a neutral residue creates an unpaired charge that causes a functional defect while simultaneous neutral substitutions preserve activity, and that Asp237 is located in the middle of helix VII rather than in the loop between helices VII and VIII.

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[1] Abbreviations: lac, lactose; IPTG, isopropyl 1-thio-β,D-galactopyranoside; KP_i, potassium phosphate; PMS, phenazine methosulfate; $NaDodSO_4$, sodium dodecyl sulfate; MTSES, methanethiosulfonate ethylsulfonate; MTSEA, methanethiosulfonate ethylammonium.

[2] Site-directed mutants are designated as follows: the one-letter amino acid code is used followed by a number indicating the position of the residue in wild-type lac permease. The sequence is followed by a second letter denoting the amino acid replacement at this position.

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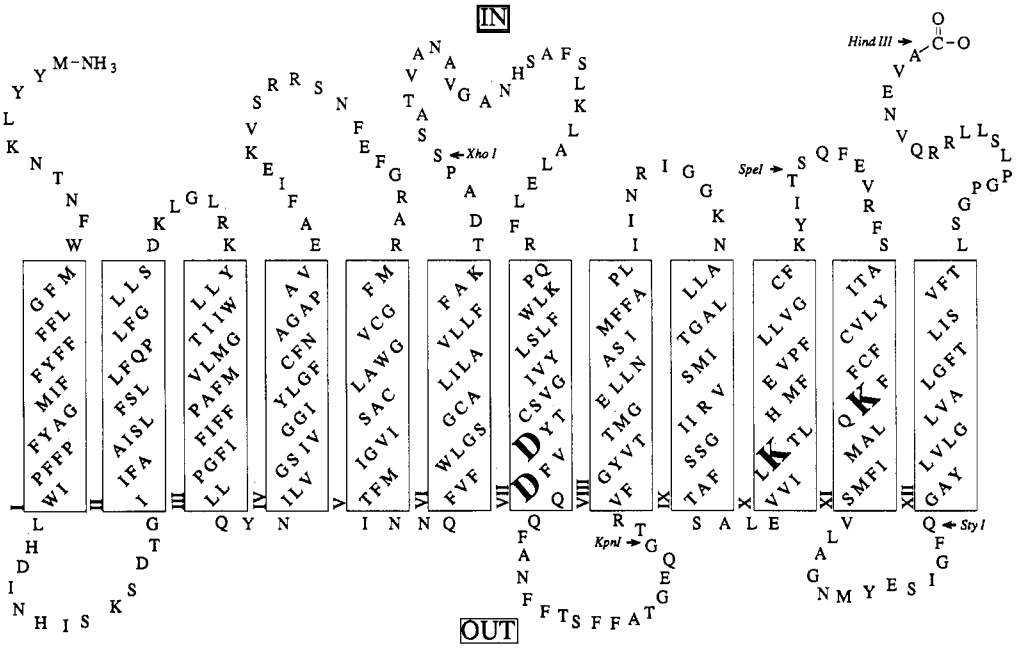

FIGURE 1: Secondary structure model of lac permease based on hydropathy analysis (Foster et al., 1983). The single-letter amino acid code is used, and Asp237, Asp240, Lys319, and Lys358 are highlighted. Hydrophobic transmembrane helices are shown in boxes, and the topology of helix VII was modified according to results obtained from a series of *lacY–phoA* fusions in this region (M. L. Ujwal and H. R. Kaback, unpublished observations). Also indicated are the restriction endonuclease sites used for construction of the mutants.

As part of a Cys-scanning mutagnesis study (Sahin-Tóth & Kaback, 1993) of a functional permease mutant devoid of Cys residues (C-less permease; van Iwaarden et al., 1991), putative intramembrane charged residues were systematically replaced with Cys (Sahin-Tóth et al., 1992). Individual replacements for Asp237, Asp240, Glu269, Arg302, Lys319, His 322, Glu325, and Lys358 abolish active lactose transport. By using the single-Cys mutants, 14 double mutants were constructed in which all possible interhelical combinations of negative and positively charged residues were replaced pairwise with Cys ("charge-pair neutralization"). Out of all the combinations, only D237C/K358C and D240C/K319C exhibit significant lactose transport activity, but striking differences are observed between the two sets of residues. (i) While the double mutant D237C/K358C accumulates lactose to essentially the same steady state as C-less permease, only 25–30% of the C-less steady state is observed when Asp240 and Lys319 are neutralized simultaneously with Cys or Ala. (ii) The interchange of Asp237 and Lys358 yields active permease, but reversal of Asp240 and Lys319 abolishes lactose transport, indicating that the polarity of Asp240–Lys319 is critical for activity. (iii) Although D237C/K358C permease exhibits high activity, markedly decreased levels of permease are observed in the membrane; conversely, D240C/K319C permease is inserted normally. These findings confirm the proposed interaction between Asp237 and Lys358 and suggest a more stringent interaction between Asp240 and Lys319 [see Lee et al. (1992) in addition]. In any case, the observations indicate that transmembrane helix VII neighbors helices X and XI in the tertiary folded structure of mature lac permease.

The low levels of insertion observed for many of the mutants lacking the putative salt bridge between Asp237 and Lys358 suggest a role for the salt bridge in either permease folding or stability and raise the possibility that the salt bridge may exist in a folding intermediate but not in the mature protein (Dunten et al., 1993). However, a mutant with Cys in place of Asp237 is restored to full activity by carboxymethylation, which recreates a negative charge at position 237, and 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 that of C-less. Therefore, the interaction between Asp237 and Lys358 is likely to be important for permease folding, but it is maintained in the mature protein. In addition, the restoration of activity by carboxymethylation of the D237C mutant suggests that the interaction between Asp237 and Lys358 may be relatively tolerant to changes in the lengths of the side chains.

In this study, the side chains at positions 237, 240, 319, or 358 in lac permease were modified by introducing Glu and/or Arg residues or by derivatizing single-Cys replacements at these positions with charged thiol reagents. The results support the following conclusions: (i) The interaction between Asp237 and Lys358 is remarkably tolerant with respect to the length of the side chains, so long as a negative and positive charge interaction is maintained. (ii) The Asp240–Lys319 pair can be altered to a relatively small extent without complete loss of activity, and this pair exhibits much more stringent requirements, particularly with respect to the polarity of the interaction. The results are consistent with the idea that the Asp240–Lys319 interaction plays a role in the mechanism of action of the permease.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose and [^{35}S]methionine were purchased from Amersham (Arlington Heights, IL). Oligonucleotides 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., 1984b) was prepared by BabCo (Richmond, CA). 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*,λ^-/F$^-$] (Boyer & Roulland-Dussoix, 1969) was used as carrier for the plasmids described and for detection of lac permease activity on MacConkey plates (Difco Laboratories) containing 25 mM lactose. *E. coli* T184-

Table I: DNA Sequence Analysis of Glu and Arg Replacements in Asp237–Lys358 or Asp240–Lys319 in lac Permease

mutant		mutagenic oligonucleotide(s)[a]	codon change
D237E	sense	TCCACCTACGAAGTTTTTGACCAA	GAT → GAA
	antisense	TTGGTCAAAAACTTCGTAGGTGGAG	
D237R	sense	TCCACCTACCGTGTTTTTGACCAACAG	GAT → CGT
	antisense	TTGGTCAAAAACACGGTAGGTGGAGGA	
D240E	sense	GATGTTTTTGAACAACAGTTTGCT	GAC → GAA
	antisense	AGCAAACTGTTGTTCAAAAACATCGTA	
K319R	sense	GTTATTCTGAGAACGCTGCATATG	AAA → AGA
	antisense	ATGCAGCGTTCTCAGAATAACCAC	
K358E	sense	TTCAGCTTCTTTGAGCAACTGGCGATG	AAG → GAG
	antisense	CGCCAGTTGCTCAAAGAAGCTGAAACT	
K358R	sense	AGCTTCTTTAGGCAACTGGCG	AAG → AGG
	antisense	CGCCAGTTGCCTAAAGAAGCTG	

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

[*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*),*rpsL*, *met*$^-$,*thr*$^-$,*recA*,*hsdM*,*hsdR*/*F*′,-*lacq*^{q}*O*$^+$*Z*^{D118}(*Y*$^+$*A*$^+$)] (Teather et al., 1980), harboring plasmid pT7-5/*lacY* with given mutations in the *lacY* gene, was used for expression from the *lac* promoter by induction with isopropyl 1-thio-β,D-galactopyranoside (IPTG). A cassette *lacY* gene (EMBL-X56095) devoid of Cys codons (van Iwaarden et al., 1991) containing the *lac* promoter/operator was used for all *lacY* gene manipulations.

Oligonucleotide-Directed Site-Specific Mutagenesis. The cassette *lacY* gene encoding C-less permease was inserted into plasmid pT7-5 and used as the template for mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers. Arg and Glu replacements were constructed by a two-stage PCR method (overlap extension; Ho et al., 1989) with two complementary mutagenic primers, the sequences of which are given in Table I. The PCR product was digested with *Kpn*I and *Xho*I for the Asp237 and Asp240 replacements, with *Kpn*I and *Spe*I for the Lys319 mutations, and with *Spe*I and *Sty*I for the Lys358 substitutions and then ligated into pT7-5/*lacY* encoding C-less permease that had been digested with the same enzymes (see Figure 1). After propagation in *E. coli* HB101, selected clones were sequenced. In addition to the site of mutation, the entire sequence between the restriction sites used for subcloning was verified by DNA sequencing. Double mutants between positions 237–358 and 240–319 were constructed by a "cut and paste" method using *Kpn*I and *Hind*III restriction endonuclease sites (see Figure 1) from appropriate single mutants, and the mutations were verified by DNA sequencing.

DNA Sequencing. Double-stranded plasmid DNA prepared by Magic Minipreps (Promega) was sequenced by using the dideoxynucleotide termination method (Sanger et al., 1977; Sanger & Coulson, 1978) and synthetic sequencing primers after alkaline denaturation (Hattori & Sakaki, 1986).

Colony Morphology. For preliminary qualitative assessment of permease activity, *E. coli* HB101 (*Z*$^+$*Y*$^-$) was transformed with pT7-5/*lacY* plasmids carrying given mutations, and the cells were plated on MacConkey indicator plates containing 25 mM lactose.

Active Transport. Active lactose transport was measured in *E. coli* T184 (*Z*$^-$*Y*$^-$) transformed with a given plasmid. Fully grown overnight cultures of cells were diluted 10-fold and grown aerobically for 2 h at 37 °C in the presence of 10 μg/mL streptomycin and 100 μg/mL ampicillin. Expression of lac permease was then induced by the addition of 0.5 mM IPTG (final concentration), and the cultures were grown for an additional 2 h. Cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$, and assayed by rapid filtration (Consler et al., 1991).

Modification of Cys Residues. Cells grown and washed as described above were treated with given thiol reagents immediately before the transport assays. Aliquots (500 μL) of cells were incubated at room temperature with 1 mM (final concentration) freshly prepared thiol reagent. After a 30-min incubation, cells were assayed for lactose uptake in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (PMS; Konings et al., 1971).

Preparation of Membranes. Crude membrane fractions from *E. coli* T184 harboring plasmids with given mutations were prepared as follows: Cells were resuspended in ice-cold osmotic shock buffer (25 mM Tris-HCl (pH 8.0)/45% sucrose/1 mM ethylenediaminetetraacetate), kept on ice for 20 min, harvested by centrifugation, resuspended in cold distilled water, and allowed to stand for 10 min on ice before the addition of 0.1 mg/mL lysozyme. After incubation for 20 min, the suspensions were briefly sonicated. Unlysed cells were removed by low-speed centrifugation, and membranes were harvested by ultracentrifugation in a Beckman Optima TL ultracentrifuge.

Immunological Analyses. Membrane fractions were subjected to sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis (Newman et al., 1981). Proteins were electroblotted, and immunoblots were probed with site-directed polyclonal antibody against the C-terminus of lac permease (Carrasco et al., 1984b).

Protein Determinations. Protein was assayed in the presence of $NaDodSO_4$ by a modified Lowry procedure (Peterson, 1977).

RESULTS

Construction of Mutants with Extended Side Chains. The amino acid residues comprising the interacting pairs Asp237–Lys358 and Asp240–Lys319 (Figure 1) were altered to create side chains of increasing length while preserving the negative–positive charge interactions. To create negatively charged side chains of varying lengths, Asp237 or Asp240 was replaced with Glu or Cys, and the latter was modified with iodoacetic acid or methanethiosulfonate ethylsulfonate (MTSES). Elongation of positively charged side chains was carried out by replacing Lys319 or Lys358 with Arg or Cys and modifying the latter with methanethiosulfonate ethylammonium (MTSEA). Modification of Cys with iodoacetic acid results in the formation of a negatively charged carboxymethyl-Cys, while the methanethiosulfonate derivatives attach a negative ethylsulfonate or a positive ethylammonium group through mixed disulfide linkages to Cys (Figure 2). Single Glu or Arg substitutions were carried out by oligonucleotide-directed site-specific mutagenesis using PCR, and except for the base changes summarized in Table I, the sequences were identical

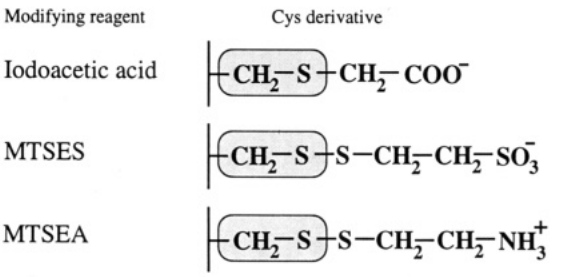

FIGURE 2: Structure of charged side chains created by modification of Cys residues with iodoacetic acid, MTSES, and MTSEA. The protein backbone is represented by the perpendicular line, and the Cys moiety of the Cys derivatives is shaded.

Table II: Single and Double Mutants Used for Cys Modification

mutant	reference
D237C	Sahin-Tóth et al., 1992; Dunten et al., 1993
D240C	Sahin-Tóth et al., 1992
K319C	Sahin-Tóth et al., 1992; Sahin-Tóth & Kaback, 1993
K358C	Sahin-Tóth et al., 1992; Dunten et al., 1993
D237C/K358D	this work
D237C/K358E	this work
D237C/K358R	this work
D237E/K358C	this work
D237K/K358C	this work
D237R/K358C	this work
D240C/K319R	this work
D240E/K319C	this work

to that reported for *lacY* encoding C-less permease. Single-Glu and -Arg mutants were then combined by cut and paste to create the double mutants D237E/K358R and D240E/K319R. Double mutants at positions 237 and 358 with reversed polarity (i.e., D237K/K358E, D237R/K358D, and D237R/K358E) were also constructed by combining the appropriate single mutants by cut and paste using the *Kpn*I and *Hind*III restriction sites. Single-Cys mutants D237C and K358C (Sahin-Tóth et al., 1992) were paired with mutants K358D, K358E, and K358R and D237E, D237K, and D237R, respectively, to yield the double mutants used for Cys modification. Similarly, D240C/K319R and D240E/K319C were constructed from the appropriate single mutants. The single- and double-Cys mutants are listed in Table II.

Colony Morphology. *E. coli* HB101 (*lacZ*$^+$*Y*$^-$) is a "cryptic" strain that produces active β-galactosidase but carries a defective *lacY* gene. Cells expressing functional lac permease allow access of external lactose to the cytosolic β-galactosidase, and subsequent metabolism of the monosaccharides causes acidification and the appearance of red colonies on MacConkey indicator plates containing high concentrations of lactose. Cells impermeable to lactose appear as white colonies, and permease mutants with low activity grow as red colonies with a white halo. It is important that indicator plates report only "downhill" lactose translocation and give no indication as to whether or not a given mutant catalyzes lactose accumulation. *E. coli* HB101 expressing mutants in Asp237 or Lys358 where the positive–negative charge interaction is preserved (D237E, K358R, D237E/K358R, D237K/K358D, D237K/K358E, D237R/K358D, and D237R/K358E) or mutants K319R, D240E, and D240E/K319R grow as red colonies indistinguishable from cells expressing C-less permease. Mutants with single-Cys replacements (D237C, D237C/K358D, D237C/K358E, D237C/K358R, D237E/K358C, D237K/K358C, D237R/K358C, D240C, D240C/K319R, D240E/K319C, K319C, and K358C) grow as red colonies with white halos of varying size. Therefore, all of the mutants retain at least some ability to transport lactose.

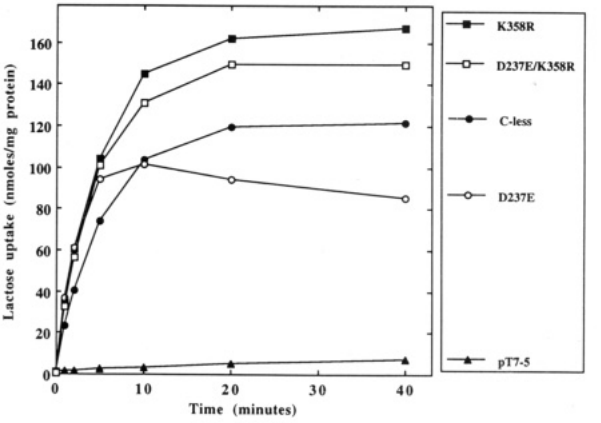

FIGURE 3: Active transport of lactose by *E. coli* T184 harboring plasmids encoding C-less permease or Glu or Arg replacements in Asp237 or Lys358. Cells were grown at 37 °C, as described in Materials and Methods. Aliquots of cells (50 μL) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (5 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at the times given by addition of 3.0 mL of 100 mM KPi (pH 5.5)/100 mM LiCl and rapid filtration through Whatman GF/F filters: ●, C-less; ○, D237E; ■, K358R; □, D237E/K358R; ▲, pT7-5 with no *lacY* insert.

Active Lactose Transport. [1-^{14}C]Lactose transport was assayed in *E. coli* T184 expressing each mutant permease, and as expected, single-Cys mutants in either of the interacting pairs of Asp–Lys residues cause loss of activity by creating an unpaired charge (King et al., 1991; Sahin-Tóth et al., 1992). Prior to treatment with thiol reagents, mutants D237C and D237C/K358E accumulate lactose to about 10–15% of the steady-state level exhibited by C-less permease (data not shown), while the transport activity of the remainder of the Cys mutants (D237C/K358D, D237C/K358R, D237E/K358C, D237K/K358C, D237R/K358C, D240C, D240C/K319R, D240E/K319C, K319C, and K358C) is similar to that of *E. coli* T184 transformed with pT7-5 containing no *lacY* gene. Modification of the single-Cys mutants with methyl methanethiosulfonate, which is uncharged, or iodoacetamide causes no significant change in transport activity. Since thiol reagents inhibit the generation of the H^+ electrochemical gradient with physiological electron donors (Kaback & Patel, 1978), all transport measurements with thiol-modified mutants were carried out in the presence of ascorbate/PMS (Konings et al., 1971). Although data are not shown, the restoration of transport by reduced PMS varies depending on the thiol reagent used. Thus, higher levels of lactose accumulation are observed when *E. coli* T184 expressing C-less permease is treated with iodoacetic acid or MTSES (70-80% of untreated cells), and slightly lower levels are obtained after treatment with MTSEA (50–60% of untreated cells).

Increasing the Lengths of the Side Chains at Positions 237 and 358. Replacement of Asp237 with Glu or Lys358 with Arg results in fully active permease (Figure 3). Moreover, the double mutant containing both alterations in the same molecule (D237E/K358R) also exhibits high activity. Interestingly, replacement of Lys358 with Arg significantly increases the steady-state level of accumulation relative to that of the C-less permease. When permease with Cys in place of Asp237 is carboxymethylated to recreate the negative charge, the altered permease transport lactose essentially as well as C-less permease, with either Lys or Arg at position 358 (Figure 4A). Similarly, modification of D237C permease with MTSES results in highly active permease with Lys358 as the counterion (Figure 4B). Remarkably, with Arg as the

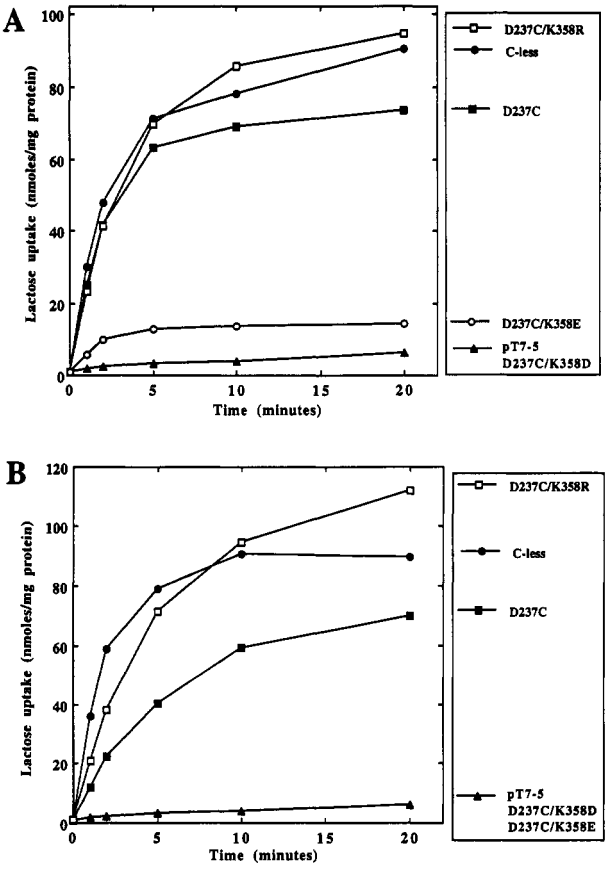

FIGURE 4: Effect of iodoacetic acid (A) and MTSES (B) on the time course of lactose accumulation by *E. coli* T184 expressing C-less permease (●), no permease (▲), or mutants D237C (■), D237C/K358R (□), D237C/K358D (▲), and D237C/K358E (○ in A and ▲ in B). Cells were grown and washed as described in Materials and Methods. Aliquots (500 μL) were treated with 1 mM iodoacetic acid or MTSES for 30 min at room temperature, and cells were assayed directly in the presence of 20 mM potassium ascorbate and 0.2 mM PMS (Konings et al., 1971) as described in the legend to Figure 3.

counterion to the MTSES derivative of Cys237, the activity is essentially the same as that observed with C-less. The latter combination represents an overall increase of 5 bond lengths between the original Asp and Lys residues at positions 237 and 358, respectively. It is also clear that the presence of a positive charge at position 358 is required for activity if position 237 carries a negatively charged side chain (Figure 4A,B). Thus, mutants D237C/K358D and D237C/K358E exhibit no increase in activity when Cys237 is modified with either iodoacetic acid or MTSES. Although the data are not shown, treatment of Cys237 with *p*-(chloromercuri)benzoic acid or *p*-(chloromercuri)benzenesulfonate fails to restore transport activity in mutants D237C or D237C/K358R, indicating that, although the introduction of a negative charge is necessary for reactivation, other constraints are also important.

Treatment of K358C permease with MTSEA yields active permease when a negative charge (Asp or Glu) is present at position 237 (Figure 5). MTSEA derivatives of K358C or D237E/K358C permease exhibit only about 30–35% of the rate of C-less permease with essentially normal steady-state levels of accumulation, because expression of these mutants is significantly diminished relative to C-less (see Figure 11B). Finally, no reactivation of transport by MTSEA is observersed when Cys358 is modified in mutants D237K/K358C or D237R/K358C, since this results in the presence of positive charges at both positions 237 and 358.

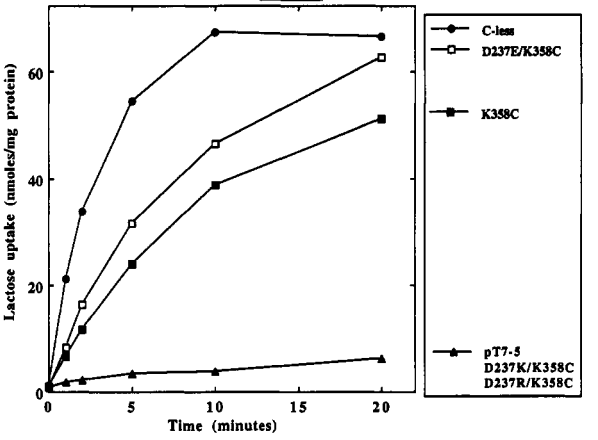

FIGURE 5: Effect of MTSEA on the active transport of lactose in *E. coli* T184 transformed with plasmids encoding C-less (●), pT7-5 (vector with no *lacY* gene, ▲), or mutants K358C (■), D237E/K358C (□), D237K/K358C (▲), and D237R/K358C (▲). Cells were grown and washed as described in Materials and Methods. Aliquots (500 μL) were treated with 1 mM MTSEA for 30 min at room temperature, and cells were assayed directly in the presence of potassium ascorbate and PMS (Konings et al., 1971) as given in the legend to Figure 3.

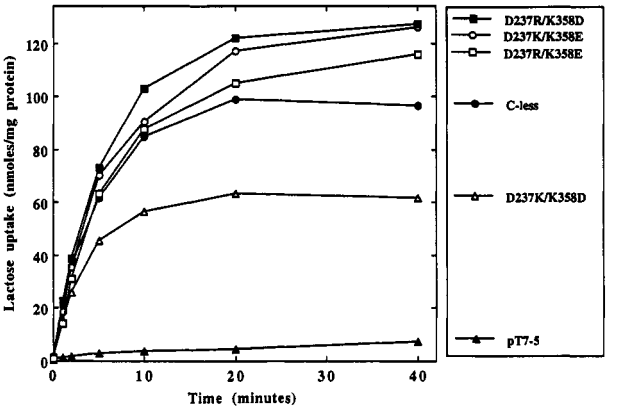

FIGURE 6: Active transport of lactose in *E. coli* T184 harboring plasmids encoding C-less permease, no permease (pT7-5 vector without the *lacY* gene), or mutants with reversed polarity of Asp237–Lys358. Cells were grown and assayed exactly as described in the legend to Figure 3: ●, C-less; ▲, pT7-5; △ D237K/K358D; ○, D237K/K358E; ■, D237R/K358D; □, D237R/K358E.

Reversal of Charge Polarity at Positions 237 and 358. Mutants D237K/K358E, D237R/K358D, and D237R/K358E accumulate lactose at approximately the same rate as C-less, to approximately 15–25% higher steady-state levels of accumulation (Figure 6). In contrast, D237K/K358D accumulates lactose to about 60% of the steady state exhibited by C-less permease (Dunten et al., 1993). The effect of polarity was also tested by modifying Cys237 with MTSEA in mutants D237C/K358D and D237C/K358E (Figure 7). As shown, these mutants exhibit very significant lactose accumulation (approximately 60–70% of the C-less steady state) after treatment with the positively charged thiol reagent. The low activity observed with D237C permease after MTSEA treatment (Figure 7) is unexplained, since this mutant contains positive charges at both positions 237 and 358. In any event, D237C/K358R permease is unaffected by MTSEA, demonstrating that charge neutralization is obligatory for high transport activity. Although the data are not shown, iodoacetic acid or MTSES fails to restore transport activity when K358C is modified in mutants with a positive charge in place of Asp237 (D237K/K358C and D237R/K358C) for reasons that are not apparent.

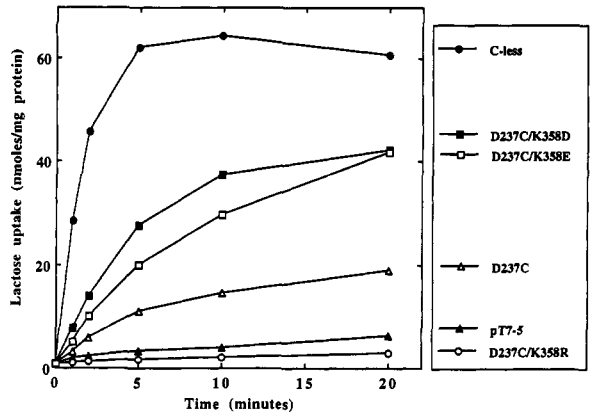

FIGURE 7: Effect of MTSEA on lactose transport in *E. coli* T184 transformed with plasmids encoding C-less (●), pT7-5 (vector with *no lacY* gene, ▲), D237C (△), D237C/K358D (■), D237C/K358E (□), and D237C/K358R (○). Cells were grown and washed as described in Materials and Methods. Aliquots (500 μL) were treated with 1 mM MTSEA for 30 min at room temperature, and cells were assayed directly in the presence of potassium ascorbate and PMS (Konings et al., 1971) as given in legend to Figure 3.

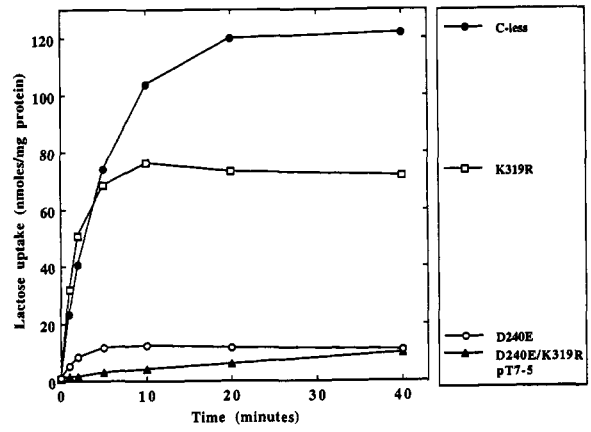

FIGURE 8: Effects of Glu and Arg replacements on Asp240–Lys319. Transport of lactose in *E. coli* T184, expressing C-less permease (●), no permease (pT7-5 with no *lacY* gene, ▲), or mutants D240E (○), K319R (□), and D240E/K319R (△), was assayed as described in the legend to Figure 3.

Increasing the Lengths of the Side Chains at Positions 240 and 319. In contrast to Asp237, when Asp240 is replaced with Glu, lactose transport is diminished drastically (Figure 8). However, replacement of Lys319 with Arg results in a highly active permease that catalyzes lactose transport at essentially the same rate as C-less permease, to approximately 70% of the control steady state. Furthermore, combination of both mutations in the same molecule leads to complete inactivation (D240E/K319R). When Lys319 is retained as the counterion, iodoacetic acid is unable to restore transport activity to D240C permease (Figure 9A), while treatment of the same mutant with MTSES restores significant levels of accumulation (Figure 9B). Interestingly, replacement of Lys319 with Arg alters the effect of both iodoacetic acid with MTSES or D240C permease. When paired with Arg319, carboxymethyl-Cys at position 240 yields permease with about 25% of the C-less activity (Figure 9A), and MTSES-treated D240C transports about 35–40% as well as C-less permease (Figure 9B). MTSEA treatment of K319C yields a permease that transports lactose with a rate approximating that of C-less permease and a steady-state level of accumulation that is about 65–70% of control. However, no activity is observed when K319C is combined with D240E and modified with MTSEA.

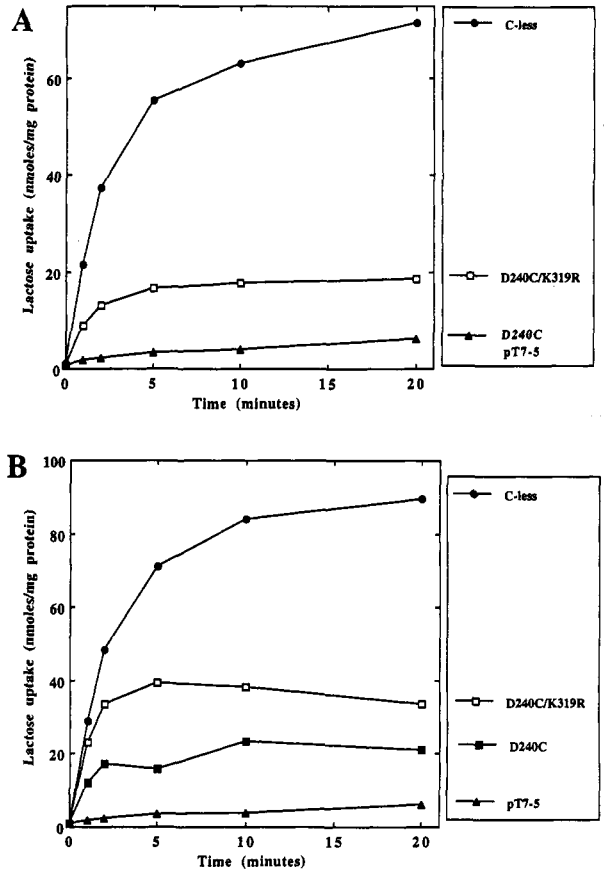

FIGURE 9: Effect of iodoacetic acid (A) or MTSES (B) on the time course of lactose accumulation by *E. coli* T184 expressing C-less permease (●), *no* permease (pT7-5 without the *lacY* insert, ▲), or mutants D240C (▲ in A and ■ in B), and D240C/K319R (□). Cells were grown and washed as described in Materials and Methods. Aliquots (500 μL) were treated with 1 mM iodoacetic acid or MTSES for 30 min at room temperature, and cells were assayed directly in the presence of potassium ascorbate and PMS (Konings et al., 1971) as described in the legend to Figure 3.

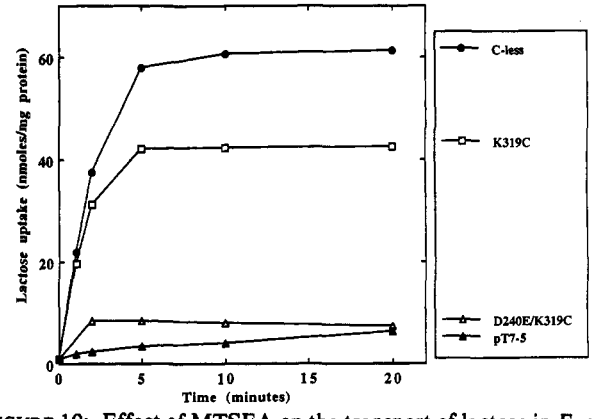

FIGURE 10: Effect of MTSEA on the transport of lactose in *E. coli* T184 transformed with plasmids encoding C-less (●), pT7-5 (vector with no *lacY* gene, ▲), or mutants K319C (□) and D240E/K319C (△). Cells were grown and washed as described in Materials and Methods. Aliquots (500 μL) were treated with 1 mM MTSEA for 30 min at room temperature, and cells were assayed directly in the presence of potassium ascorbate and PMS (Konings et al., 1971) as given in the legend to Figure 3.

Expression of Mutant Permeases. In order to test whether mutations in Asp237–Lys358 or Asp240–Lys319 alter the permease concentration in the cell membrane, immunoblots were carried out on membrane preparations from T184 cells expressing given mutants (Figure 11A–C). Glu and/or Arg replacements in Asp237–Lys358 do not affect expression so

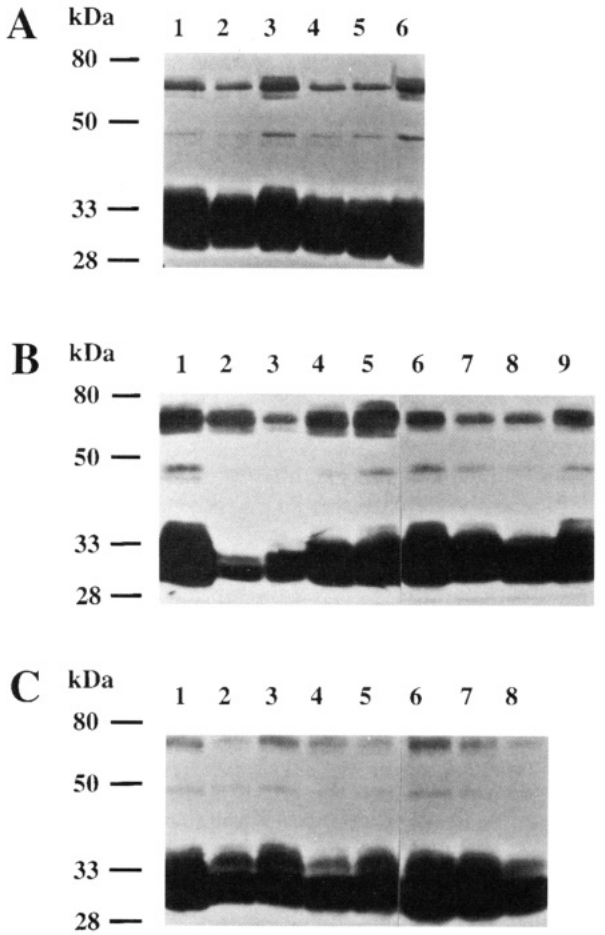

FIGURE 11: Western blots of membranes containing C-less lac permease or mutants in Asp237–Lys358 or Asp240–Lys319. *E. coli* T184 transformed with pT7-5/C-less *lacY* encoding given mutations was induced with IPTG. Membranes were prepared, and approximately 100 μg of membrane protein was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and electroblotted. The nitrocellulose paper was incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase-conjugated protein A, followed by a short incubation with a fluorescent substrate (Amersham), the nitrocellulose paper was exposed to film for 1 min. The immunoreactive band between the 50- and 80-kDa markers corresponds to a dimeric aggregate form of lac permease. Although not shown, membranes prepared from T184 expressing no lac permease exhibit no immunoreactive material [see Meckenna et al. (1992)]. (A) Mutants in Asp237–Lys358; lane 1, C-less; lane 2, K358R; lane 3, D237E; lane 4, D237E/K358R; lane 5, D237K/K358D; lane 6, D237R/K358E. (B) Single-Cys mutants in Asp237–Lys358; lane 1, C-less; lane 2, K358C; lane 3, D237E/K358C; lane 4, D237K/K358C; lane 5, D237R/K358C; lane 6, D237C; lane 7, D237C/K358R; lane 8, D237C/K358D; lane 9, D237C/K358E. (C) Mutants in Asp240–Lys319; lane 1, C-less; lane 2, D240C; lane 3, D240C/K319R; lane 4, K319C; lane 5, D240E/K319C; lane 6, D240E; lane 7, K319R; lane 8, D240E/K319R.

long as the negative–positive charge interaction is preserved, regardless of polarity. Thus, mutants K358R, D237E, D237E/K358R, D237K/K358D, and D237R/K358E are present in the membrane in amounts comparable to that of C-less permease (Figure 11A). Although not shown, mutants D237R/K358D and D237K/K358E also exhibit levels of permease in the membrane that are identical to the C-less level. Mutants K358C [see Dunten et al. (1993) in addition] and D237E/K358C exhibit marked reductions in the membrane levels of lac permease, but expression of mutants D237K/K358C and D237R/K358C is only slightly decreased (Figure 11B). Also, mutants containing Cys in place of Asp237 [D237C (Dunten et al., 1993), D237C/K358R, D237C/K358D, and D237C/K358E] exhibit close to normal levels of lac permease in the membrane. Finally, mutations in Asp240–Lys319 have no significant effect on the expression of lac permease [see Sahin-Tóth et al. (1992) in addition]. Thus, mutants D240C, D240C/K319R, K319C, D240E/K319C, D240E, K319R, and D240E/K319R exhbit concentrations of permease in the membrane that are comparable to that of C-less (Figure 11C).

DISCUSSION

Site-directed mutagenesis and site-specific Cys modification have been employed to investigate the tolerance of the interacting pairs Asp237–Lys358 and Asp240–Lys319 to alterations in the length of the side chains. In addition to replacement with natural amino acids, the specific reactivity of Cys was utilized to create novel side chains by modifying Cys with charged thiol reagents (Figure 2). In preliminary studies (Dunten et al., 1993), carboxymethylation of D237C lac permease with iodoacetic acid was shown to restore the transport activity of the mutant to control levels. Similarly, modification of Cys by iodoacetic acid has been used to investigate the flexibility of Asp85 in bacteriorhodopsin (Greenhalgh et al., 1992), and the methanesulfonate derivatives, MTSES and MTSEA, have been used to probe the channel structure of the nicotinic acetylcholine receptor (Akabas et al., 1992). In the present study, the use of these reagents demonstrates the remarkable flexiblity of the residues comprising the putative intramembrane salt bridges between Asp237 and Lys358. Extension of the native side chains in this interacting pair by up to 5 bond lengths yields fully functional permease. Even more surprising is the finding that reversal of the polarity of the interaction has no significant effect on transport activity [see Sahin-Tóth et al. (1992) and Dunten et al. (1993) in addition], although the flexibility seems to be compromised to some extent. Thus, reverse polarity mutants with Glu and Arg replacements exhibit high activity, but mutants created by Cys modification show either moderately decreased activity [e.g., MTSEA derivatives of D237C/K358D and D237C/K358E (Figure 7)] or are completely inactive [e.g., iodoacetic acid or MTSES derivatives of D237K/K358C and D237R/K358C]. However, it should be noted that, unlike the Glu and Arg replacement mutants which are synthesized *in vivo*, Cys modification is carried out with the mature, folded protein in the membrane. Therefore, it is possible that the introduction of positive charge into helix VII in mutants D237K/K358C and D237R/K358C alters local conformation such that residues 237 and 358 cannot form a salt bridge. On the other hand, the restoration of functional interactions in most of the Cys mutants argues that the conformation of the mutant proteins in the membrane is probably close to normal.

The high tolerance for a variety of side chains at positions 237 and 358 in lac permease is consistent with the argument that neither Asp237 nor Lys358 nor the salt bridge itself plays a role in the transport mechanism. On the other hand, a role in folding and membrane insertion was proposed for Asp237–Lys358 (Dunten et al., 1993) on the basis of the observation that disruption of the charge interaction by Cys or Ala substitution leads to diminished amounts of protein in the membrane secondary to a defect in insertion. Consistent with this proposal, the immunoblots presented here reveal that mutants in the putative salt bridge exhibit normal amounts of permease in the membrane, so long as a negative–positive interaction is maintained regardless of polarity. Given these observations, it is intriguing to speculate that insertion of helix VII into the membrane is unfavored until the negative charge

in Asp237 is neutralized by Lys358 in helix XI, thereby implying that the C-terminal half of the permease may insert into the membrane as a unit. The presence of an unpaired intramembrane charged residue may significantly reduce the insertion of a transmembrane helix, as demonstrated for Arg302 in helix IX of lac permease (Calamia & Manoil, 1992) or for intramembrane Asp residues in the pBR322-encoded tetracycline transporter (Allard & Bertrand, 1992). By neutralizing uncompensated charged residues which are unfavored in the low dielectric of the membrane, charge-pairing may play an important role in the insertion of transmembrane domains containing charged residues. Disruption of such interactions might lead to defective folding of a membrane protein and to decreased insertion into the membrane. The observations with Cys substitutions for Asp237 or Lys358 are not completely clear-cut in this respect, however. While neutralization of Lys358 in mutants K358C and D237E/K358C causes a marked decrease in permease expression, mutants D237K/K358C and D237R/K358C exhibit only a slight reduction in expression (see Figure 11B). Moreover, mutants with Cys substitution for Asp237 (D237C, D237C/K358R, D237C/K358D, and D237C/K358E; Figure 11B) are expressed to levels comparable to that of C-less. Possibly, alternative folding pathways are used in some of the mutants which may compensate for the loss of the salt bridge. In this respect, the finding that these mutants are inactive, even though Asp237 and Lys358 are unimportant for transport activity, may indicate that they are inserted in a "misfolded" state in a manner that can be corrected when the appropriate charge is introduced by Cys modification with restoration of the salt bridge. In any event, whatever the ultimate explanation for the behavior of the single Cys mutants in Asp237–Lys358, the results are generally consistent with the proposed role for the salt bridge in folding and/or membrane insertion.

In contrast to Asp237–Lys358, the interaction between Asp240 and Lys319 exhibits considerable sensitivity toward changes in the nature of the side chains. Even short extension of Asp240 by replacement with Glu or carboxymethyl-Cys inactivates the protein. Surprisingly, however, further extension of the side chain at position 240 by derivatization with MTSES (Figure 9B) and simultaneous replacement of Lys319 with Arg yield permease with significant transport activity. Although no obvious explanation is apparent, this type of behavior may be consistent with the notion that the interaction participates in the transport mechanism. If the interaction between Asp240 and Lys319 is dynamic and undergoes association–dissociation during turnover of the permease, the strength of the interaction would be important for optimal activity. Thus, alterations in the lengths of the side chains at these positions may favor or unfavor interaction, depending upon steric constraints. Consistent with the notion that these two residues play a role in the mechanism is the observation that the polarity of the charge interaction between residues at positions 240 and 319 is critical for activity. In this context, it is noteworthy that the interchange of Asp240 and Lys319 in a background where both Asp237 and Lys358 are neutralized with Ala and Cys (D237A/D240K/K319D/K358C) also inactivates transport (data not shown). Thus, loss of activity in D240K/K319D cannot be due to a spurious interaction between Asp237 and Lys240. Finally, in agreement with previous observations (Sahin-Tóth et al., 1992), mutations in Asp240–Lys319 have no significant effect on the expression and membrane insertion of lac permease.

As a result of these observations, the secondary structure model of lac permease has been modified to accommodate the interactions between Asp237–Lys358 and Asp240–Lys319. Although King et al. (1991) placed Asp237 and Asp240 in the middle of helix VII, site-directed spin and fluorescent labeling experiments indicate that these residues are located in an amphipathic environment, probably at the membrane–water interphase (i.e., close to the N-terminus of helix VII).[3] Moreover, data obtained from a series of lactose permease–alkaline phosphatase chimeras in helix VII indicate that the midpoint of this helix is between Tyr228 and Ile230 (M. L. Ujwal and H. R. Kaback, unpublished observations), which places residues Asp237 and Asp240 at the periplasmic border of this transmembrane domain. On the basis of these observations, it reasonable to suggest that the interacting pairs Asp237–Lys358 and Asp240–Lys319 are indeed within transmembrane domain VII, but are located at or near the periplasmic surface of the membrane rather than in the middle of the domain.

There are increasing indications that charge-pairing may be a general feature for the intramembrane charged residues in lac permease. Purified, reconstituted double-Cys mutants R302C/E325C, E325C/H322C, and E269C/H322C labeled with pyrene exhibit excimer fluorescence, indicating that these pairs of residues are in close proximity (K. Jung, H. Jung, J.-W. Wu, G. G. Privé, & H. R. Kaback, manuscript in preparation). Conversely, despite the functional studies supporting the existence of interactions between Asp237–Lys358 and Asp240–Lys319, direct physical evidence showing that these two sets of residues are in close proximity is lacking. In this respect, efforts are underway to demonstrate chemical cross-linking between double-Cys replacements in these interacting pairs of residues.

[3] Jung, H., Lopez, R., Altenbach, C., Hubbell, W. L., & Kaback, H. R. (1993) *Biophys. J. 64*, A14 (Abstr. MPMH6).

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REFERENCES

Akabas, M. H.; Stauffer, D. A., Xu, M., & Karlin, A. (1992) *Science 258*, 307–310.

Allard, J. D., & Bertrand, K. P. (1992) *J. Biol. Chem. 267*, 17809–17819.

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

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

Calamia, J., & Manoil, C. (1992) *J. Mol. Biol. 224*, 539–543.

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

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

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

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

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

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

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

Greenhalgh, D. A., Subramaniam, S., Alexiev, U., Otto, H., Heyn, M. P., & Khorana, H. G. (1992) *J. Biol. Chem. 36*, 25734–25738.

Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 232–238.
Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688–3693.
Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221–229.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene (Amsterdam) 77*, 51–59.
Kaback, H. R. (1989) *Harvey Lectures 83*, 77–105.
Kaback, H. R. (1992) *International Review of Cytology* (Jeon, K. W., Friedlander, M., Eds.) Vol. 137A, pp 97–125, Academic Press, Inc., San Diego.
Kaback, H. R., & Patel, L. (1978) *Biochemistry 17*, 1640–1646.
Kaback, H. R., Bibi, E., & Roepe, P. D. (1990) *Trends Biochem. Sci. 15*, 309–314.
King, S. C., Hansen, C. L., & Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177–186.
Konings, W. N., Barnes, E. M., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5857–5861.
Lee, J.-L., Hwang, P. P., Hansen, C., & Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758–20764.
McKenna, E., Hardy, D., & Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954–11958.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804–11808.
Page, M. G. P., & Rosenbusch, J. P. (1988) *J. Biol. Chem. 263*, 15906–15914.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Sahin-Tóth, M., & Kaback, H. R. (1993) *Protein Sci 2*, 1024–1033.
Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., & Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547–10551.
Sanger, F., & Coulson, A. R. (1978) *FEBS Lett. 87*, 107–110.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5468.
Seckler, R., & Wright, J. K. (1984) *Eur. J. Biochem. 142*, 268–279.
Seckler, R., Wright, J. K., & Overath, P. (1983) *J. Biol. Chem. 258*, 10817–10820.
Seckler, R., Möröy, T., Wright, J. K., & Overath, P. (1986) *Biochemistry 25*, 2403–2409.
Stochaj, V., Bieseler, B., & Ehring, R. (1986) *Eur. J. Biochem. 158*, 423–248.
Teather, R. M., Bramhall, J., Riede, I., Weight, J. K., Furst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223–231.
van Iwaarden, P. R., Pastore, J. C., Konings, W. N., & Kaback, H. R. (1991) *Biochemistry 30*, 9595–9600.
Vogel, H., Wright, J. K., & Jähnig, F. (1985) *EMBO J. 4*, 3625–3631.