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Functional interactions between putative intramembrane charged residues in the lactose permease of *Escherichia coli*

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ABSTRACT **Using a lactose permease mutant devoid of Cys residues ("C-less permease"), we systematically replaced putative intramembrane charged residues with Cys. Individual replacements for Asp-237, Asp-240, Glu-269, Arg-302, Lys-319, His-322, Glu-325, or Lys-358 abolish active lactose transport. When Asp-237 and Lys-358 are simultaneously replaced with Cys and/or Ala, however, high activity is observed. Therefore, when either Asp-237 or Lys-358 is replaced with a neutral residue, leaving an unpaired charge, the permease is inactivated, but neutral replacement of both residues yields active permease [King, S. C., Hansen, C. L. & Wilson, T. H. (1991) *Biochim. Biophys. Acta* 1062, 177–186]. Remarkably, moreover, when Asp-237 is interchanged with Lys-358, high activity is observed. The observations provide a strong indication that Asp-237 and Lys-358 interact to form a salt bridge. In addition, the data demonstrate that neither residue nor the salt bridge plays an important role in the transport mechanism. Thirteen additional double mutants were constructed in which a negative and a positively charged residue were replaced with Cys. Only Asp-240 → Cys/Lys-319 → Cys exhibits significant activity, accumulating lactose to 25–30% of the steady state observed with C-less permease. Replacing either Asp-240 or Lys-319 individually with Ala also inactivates the permease, but double mutants with neutral substitutions (Cys and/or Ala) at both positions exhibit essentially the same activity as Asp-240 → Cys/Lys-319 → Cys. In marked contrast to Asp-237 and Lys-358, interchanging Asp-240 and Lys-319 abolishes active lactose transport. The results demonstrate that Asp-240 and Lys-319, like Asp-237 and Lys-358, interact functionally and may form a salt bridge. However, the interaction between Asp-240 and Lys-319 is clearly more complex than the interaction between Asp-237 and Lys-358. In any event, the findings suggest that putative transmembrane helix VII lies next to helices X and XI in the tertiary structure of lactose permease.**

Lactose 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 refs. 1–3 for reviews). On the basis of circular dichroic studies and hydropathy analysis of the primary amino acid sequence (4), a secondary structure was proposed in which the permease has a short hydrophilic N terminus, 12 hydrophobic domains in α-helical configuration that traverse the membrane in 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 (5), limited proteolysis (6, 7), immunological studies (8–15), and chemical modification (16). Exclusive support for the 12-helix motif has been obtained from analyses of an extensive series of *lac* permease–alkaline phosphatase (*lacY–phoA*) fusions (17).

Recently, King *et al.* (18) found that *lac* permease mutants with Thr in place of Lys-358 or Asn in place of Asp-237 are defective with respect to active transport. Second-site suppressor mutations of K358T† yield neutral amino acid substitutions for Asp-237 (Asn, Gly, or Tyr), while suppressors of D237N exhibit Gln in place of Lys-358. On the basis of these findings, it was proposed that Asp-237 and Lys-358 interact via a salt bridge, thereby neutralizing each other. Replacement of either charged residue with a neutral residue creates 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 Asp-237 and Lys-358 in the low dielectric environment of the membrane by placing residues Phe-247 to Thr-235 in transmembrane helix VII rather than in the hydrophilic domain between helices VII and VIII (Fig. 1).

As part of an extensive site-directed mutagenesis study with a *lac* permease mutant devoid of Cys residues ("C-less permease"; ref. 19), putative intramembrane residues Asp-237, Asp-240, Glu-269, Arg-302, Lys-319, His-322, Glu-325, and Lys-358 were systematically replaced with Cys, and individual replacement of any of the residues essentially abolishes active lactose transport. Using the single Cys mutants D237C and K358C, we constructed a double mutant containing both Cys substitutions in the same molecule. D237C/K358C transports lactose with close to C-less activity. Moreover, replacement of Asp-237 and Lys-358, respectively, with Ala and Cys or Cys and Ala or even interchanging the residues causes little change in permease activity. The observations confirm and extend the observations of King *et al.* (18) and provide the groundwork for direct biochemical and biophysical studies on the proximity and environment of the residues.

To test "charge-pair neutralization" as an approach to identify other interactions between charged residues, 13 additional double-Cys mutants were constructed, each harboring two Cys substitutions, one replacing a negative and the other a positively charged residue. Recovery of transport activity in mutant D240C/K319C exclusively (and in other mutants at these positions containing double neutral replace-

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Abbreviations: IPTG, isopropyl 1-thio-β-D-galactopyranoside; NEM, *N*-ethylmaleimide; *p*CMB, *p*-chloromercuribenzoate; *p*CMBS, *p*-chloromercuribenzenesulfonate.

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†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 number is followed by a second letter, denoting the amino acid replacement at this position.

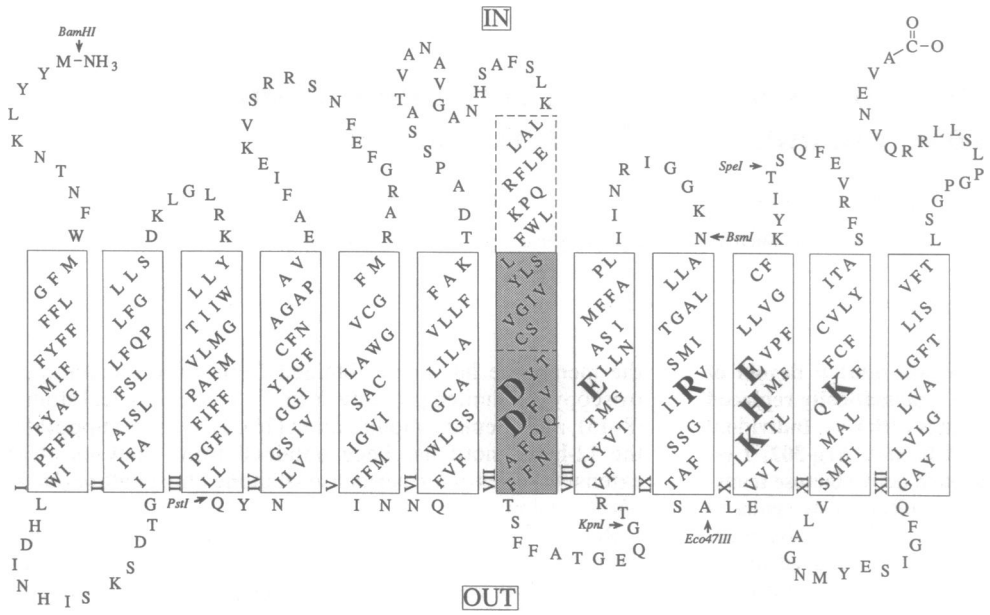

FIG. 1. Secondary structure model of *E. coli lac* permease based on hydropathy analysis (4), showing modification suggested by King *et al.* (18). Putative intramembrane charged residues (Asp-237, Asp-240, Glu-269, Arg-302, Lys-319, His-322, Glu-325, and Lys-358) are in boldface. The single-letter amino acid code is used, and hydrophobic transmembrane helices are shown in boxes. The shaded box contains transmembrane helix VII as proposed by King *et al.* (18), and the box enclosed by the broken line defines putative helix VII as described in ref. 4. Also indicated are the restriction endonuclease sites used for construction of the double mutants.

ments) clearly identifies Asp-240 and Lys-319 as functionally interacting residues. The same conclusion has been derived independently by Lee *et al.* (ref. 20; T. H. Wilson, personal communication). Interestingly, as opposed to Asp-237/Lys-358, the interaction between Asp-240 and Lys-319 appears to be relatively important, although not essential, for active transport.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose was purchased from Amersham. Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of *lac* permease (13) 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*, $1^-/F^-$] (21) was used as carrier for the plasmids described and for detection of *lac* permease activity on MacConkey plates (Difco) containing 25 mM lactose. *E. coli* T184 [$lacI^+O^+Z^-Y^-$(A), *rpsL, met*$^-$, *thr*$^-$, *recA, hsdM, hsdR*/F′, $lacI^qO^+Z^{D118}(Y^+A^+)$] (22) harboring plasmid pT7-5 with given mutations in *lacY* 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 (19) 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 (19) was inserted into the replicative form of phage M13 or into plasmid pT7-5 and used as the template for mutagenesis. All site-specific mutations were directed by synthetic mutagenic oligonucleotide primers. Mutations at Arg-302, Lys-319, His-322, and Glu-325 were made by the method of Kunkel (23) with M13mp18 and a single antisense mutagenic primer as described (24). Mutant E269C was created by polymerase chain reaction (PCR) mutagenesis using a single sense mutagenic primer (25), while mutants D237C, D237K, D240C, D240A, D240K, K319A, K319D, K358C, and K358D were constructed by a two-stage PCR method (overlap-extension) with two largely complementary mutagenic primers (26).

Construction of Double Mutants. Mutants containing two amino acid replacements in the same molecule were constructed by "cut and paste," making use of the restriction endonuclease sites shown in Fig. 1. Briefly, for any given pair of mutations, the plasmid carrying the single mutation closer to the 3′ end of the gene was used as recipient (vector) for an insert obtained from the plasmid with the mutation closer to the 5′ end. Thus, double mutants of the K358C series were made by digestion of the individual plasmids with *Bam*HI or *Pst* I and *Spe* I and ligation of the isolated inserts from mutants D237C, D240C, E269C, or E325C into the vector carrying the K358C mutation also digested with *Bam*HI or *Pst* I and *Spe* I. Restriction sites for *Bam*HI and *Kpn* I were used for construction of double mutants harboring mutations at position 237 or 240. Mutants E269C/R302C, E269C/K319C, and E269C/H322C were made by using *Bam*HI and *Bsm* I restriction sites. Finally, mutant R302C/E325C was created by using *Bam*HI and *Eco*47III.

DNA Sequencing. Double-stranded plasmid DNA was sequenced by using the dideoxynucleotide termination method and synthetic sequencing primers (27, 28) after alkaline denaturation (29).

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

Active Transport. Active transport was measured in *E. coli* T184 (Z^-Y^-) transformed with each plasmid described. Fully grown suspensions of cells were diluted 200-fold, grown overnight (12–14 hr) in the presence of 1 mM IPTG at 30°C, and harvested by centrifugation. Transport of [1-^{14}C]lactose [10 mCi/mmol (1 mCi = 37 MBq); final concentration 0.4 mM] was assayed by rapid filtration (24). When the effect of sulfhydryl reagents was tested, transport measurements were carried out in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (30).

Immunological Analyses. Sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis (3) and Western blotting (31) were carried out with given membrane preparations. Immunoblots were probed with site-directed polyclonal antibody against the C terminus of *lac* permease.

Protein Determinations. Protein was assayed in the presence of $NaDodSO_4$ by a modified Lowry method (32).

RESULTS

Construction and Verification of Mutants. Mutant permeases containing individual single-Cys replacements in puta-

tive intramembrane charged residues (Fig. 1) were cloned in plasmid pT7-5. Using the 8 single-Cys mutants, we constructed the following 14 double mutants carrying two Cys substitutions in the same molecule, one replacing a negatively and the other a positively charged residue as described in *Materials and Methods*: D237C/R302C, D237C/K319C, D237C/H322C, D237C/K358C, D240C/R302C, D240C/K319C, D240C/H322C, D240C/K358C, E269C/R302C, E269C/K319C, E269C/H322C, E269C/K358C, E325C/R302C, and E325C/K358C. To test the effect of length and charge of the amino acid side chain on the double mutants at positions 240 and 319, other replacements were also made. Mutants with single-Cys (D240C and K319C) or single-Ala replacements (D240A and K319A) were used to construct double-Cys (D240C/K319C) and double-Ala (D240A/K319A) mutants, as well as mixed Cys/Ala double mutants (D240C/K319A and D240A/K319C). Double mutants in which the positions of the charged residues were interchanged (D237K/K358D and D240K/K319D) were constructed from the appropriate single mutants.

All single mutants were subjected to double-stranded DNA sequencing, and the entire portion of cassette *lacY* between the two restriction sites used for subcloning was sequenced by dideoxynucleotide termination (27, 28). With the exception of the base changes made in the mutated codon, the sequences were identical to those of cassette *lacY* encoding C-less permease (19). The double mutants were also verified by sequencing through the sites of the mutations.

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 an indicator plate. 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 "downhill" translocation of lactose and give no indication as to whether or not the cells catalyze lactose accumulation. Cys or Ala replacement for each of the charged residues highlighted in Fig. 1 yields either red colonies indistinguishable from *E. coli* HB101 expressing C-less permease or red colonies with a white halo. A similar range of phenotypes is observed for the double mutants. Therefore, judging from indicator plates, all of the mutants appear to retain at least some ability to translocate lactose "downhill."

Active Transport. With the exception of D237C which accumulates lactose up to 10% as well as C-less permease, all other mutants with single-Cys replacements for putative intramembrane charged residues do not accumulate lactose as judged by comparison to *E. coli* T184 (*Z*$^-$*Y*$^-$) transformed with pT7-5 devoid of a *lacY* insert (data not shown). The observations are consistent with earlier findings that amino acid substitutions other than Cys for Asp-237 (18), Asp-240 (20), Glu-269 (ref. 33 and M. L. Ujwal, B. Persson, M.S.-T., and H.R.K., unpublished observations), Arg-302 (34), His-322 (35), Glu-325 (36), Lys-319 (37), or Lys-358 (18) cause virtually complete loss of active lactose transport. Time courses of lactose transport in *E. coli* T184 transformed with pT7-5 encoding C-less permease, no permease (i.e., pT7-5 without a *lacY* insert) or given double-Cys mutants are shown in Fig. 2. Only two of the double mutants, D237C/K358C and D240C/K319C, catalyze significant lactose accumulation at rates of about 60% and 45–50% of C-less permease, respectively. Moreover, the steady-state level of accumulation in D237C/K358C approximates that of C-less permease, while D240C/K319C accumulates lactose to a steady state of about 25–30% of the control. Since the internal volume of *E. coli* is about 5.8 μl/mg of protein (38), it can be calculated that these levels of accumulation correspond to concentration gradients

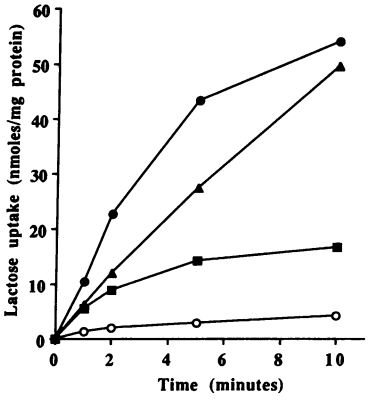

FIG. 2. Active transport of lactose by *E. coli* T184 harboring plasmids encoding C-less permease or double-Cys mutants. Cells were grown overnight at 30°C. Aliquots of cells (50 μl) in 100 mM KP_i, pH 7.5/10 mM $MgSO_4$ were assayed at room temperature. Transport was initiated by the addition of [1-^{14}C]lactose (10 mCi/mmol) to a final concentration of 0.4 mM. Reactions were quenched at given times by addition of 3.0 ml of 100 mM KP_i, pH 5.5/100 mM LiCl and rapidly filtered through Whatman GF/F filters. ●, C-less; ▲, D237C/K358C; ■, D240C/K319C; ○, pT7-5 with no *lacY* insert or double mutants D237C/R302C, D237C/K319C, D237C/H322C, D240C/R302C, D240C/H322C, D240C/K358C, E269C/R302C, E269C/K319C, E269C/H322C, E269C/K358C, E325C/R302C, E325C/K358C, where no significant differences were observed. Although not shown, D237C/K358C achieves the same steady-state level of accumulation as C-less in about 15–20 min, which is then maintained for at least 30 min.

of at least 30-fold and 10-fold, respectively. The other double-Cys mutants exhibit transport activities similar to the activity observed with pT7-5 devoid of a *lacY* gene.

The properties of permeases with other mutations at Asp-237 and Lys-358 will be described in detail elsewhere (see ref. 18 in addition); here, we note that it is particularly striking that interchanging the positions of these residues (i.e., D237K/K358D) does not dramatically alter transport activity (Fig. 3). On the other hand, interchanging Asp-240 and Lys-319 in D240K/K319D abolishes lactose accumulation. As expected, replacement of Asp-237, Asp-240, Lys-319, or Lys-358 with their counterpart charged residue (i.e., D237K, D240K, K319D, and K358D), which yields either positive or negative charges at both positions of the respective charge pairs, inactivates.

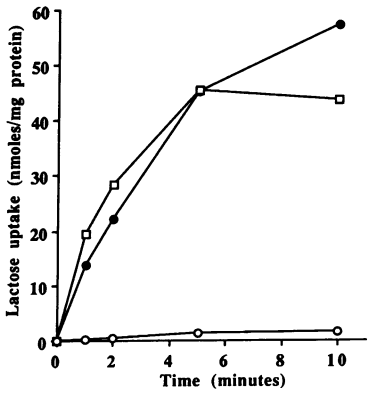

FIG. 3. Active transport of lactose in *E. coli* T184 transformed with plasmids encoding C-less permease (●), no permease (pT7-5; ○), or permease mutants D237K/K358D (□), D240K/K319D (○), D237K (○), D240K (○), K319D (○), and K358D (○). Cells were grown overnight at 30°C and assayed as described for Fig. 2. There were no significant differences between pT7-5 with no *lacY* insert and D240D/K319D, D237K, D240K, K319D, or K358D.

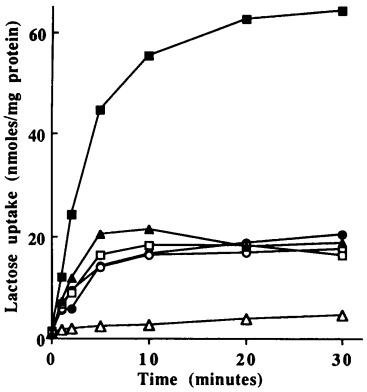

FIG. 4. Active transport of lactose in *E. coli* T184 transformed with plasmids encoding C-less permease or double mutants at positions 240 and 319. Cells were grown overnight at 30°C and assayed as described for Fig. 2. ■, C-less; △, pT7-5 with no *lacY* insert, D240A, or K319A; ●, D240C/K319C; □, D240C/K319A; ○, D240A/K319C; ▲, D240A/K319A.

Individual replacement of Asp-240 or Lys-319 with Ala in C-less permease also abolishes active lactose transport (Fig. 4). Double mutants constructed from single-Ala and -Cys mutants (D240A/K319C and D240C/K319A) and the double-Ala mutant (D240A/K319A), in contrast, exhibit activities similar to the activity observed with D240C/K319C. The mutants transport at rates that are 45–50% of C-less and accumulate lactose to steady states that are about 25–30% of control.

Immunological Analyses of Asp-240 and Lys-319 Mutants. Western blot analysis was carried out on membrane fractions with anti-C-terminal antibody (Fig. 5). The concentration of the single or double mutants at positions 240 and 319 is comparable to that of C-less, demonstrating that the differences observed in permease activity are due to the mutations *per se* and not to variations in expression, insertion, or stability of the mutant permeases.

Inhibition by Sulfhydryl Reagents. The effect of *N*-ethylmaleimide (NEM) on lactose transport by *E. coli* T184 expressing D240C/K319C, D240C/K319A, or D240A/K319C was tested initially (Fig. 6). The double mutant D240C/K319C is inactivated by 1 mM NEM with a $t_{1/2}$ of about 15 min. Surprisingly, when the Cys replacements at position 240 or 319 are tested individually with D240C/K319A and D240A/K319C, it is clear that the Cys-319 is specifically responsible for inactivation. Thus, D240A/K319C exhibits a $t_{1/2}$ of about 5 min, while the transport

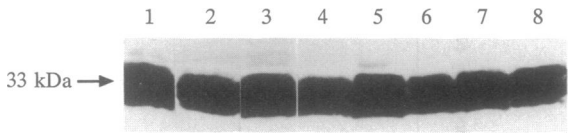

FIG. 5. Western blots of membranes containing C-less *lac* permease or mutants with single or double replacements. *E. coli* T184 transformed with pT7-5 encoding C-less permease or a given mutation were induced with IPTG. Membranes were prepared and approximately 200 μg of membrane protein from C-less (lane 1) or mutants D240C (lane 2), K319C (lane 3), D240C/K319C (lane 4), D240C/K319A (lane 5), D240A/K319C (lane 6), D240A/K319A (lane 7), and D240K/K319D (lane 8) were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis and electroblotted, and the nitrocellulose paper was incubated with anti-C-terminal antibody. After incubation with horseradish peroxidase-conjugated staphylococcal protein A, followed by a short incubation with fluorescent substrate (Amersham), the nitrocellulose paper was exposed to film for 60 min. Although not shown, membranes prepared from cells harboring pT7-5 with no *lacY* insert exhibit no immunoreactive material of 33 kDa.

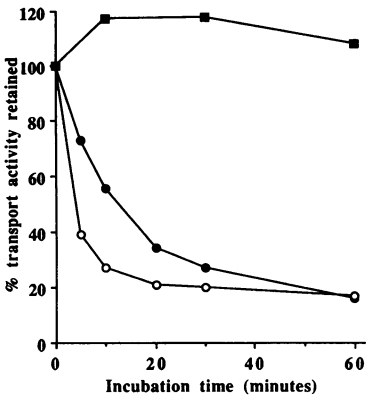

FIG. 6. Effect of NEM on active lactose transport in *E. coli* T184 harboring plasmids encoding the double mutants D240C/K319C (●), D240C/K319A (■), or D240A/K319C (○). Cells were incubated with 1 mM NEM at room temperature, and reactions were stopped at given times by addition of 10 mM dithiothreitol. Experimental conditions for transport are given in the legend of Fig. 2, except that transport was assayed in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (30). The measurements were made during the initial phase of transport, when the rates were linear.

activity of D240C/K319A is unaffected or even slightly stimulated by the alkylating agent. In an effort to estimate whether the effects of NEM are due to differences in accessibility of the Cys residues in the mutants, *p*-chloromercuribenzoate (*p*CMB) or *p*-chloromercuribenzenesulfonate (*p*CMBS), respectively, were used as lipid- or water-soluble sulfhydryl reagents. Both reagents inhibit each double mutant to about the same extent over a concentration range from 10 to 100 μM (data not shown). It is also important that treatment of D240C/K319A with NEM blocks inactivation by *p*CMB or *p*CMBS, demonstrating that NEM does indeed alkylate the Cys residue at position 240 in this mutant.

DISCUSSION

The functional relationship between Asp-237 and Lys-358 in *lac* permease led King *et al.* (18) to propose that the two residues are in close proximity in the tertiary structure of the molecule, possibly forming a salt bridge. As a result, the secondary-structure model of the permease (4) was altered (Fig. 1). The modification not only places Asp-237 and Asp-240 within putative transmembrane helix VII but balances the distribution of putative intramembrane charged residues, leaving four negative and four positively charged amino acid residues in the transmembrane domains. The equal distribution of negative and positive charges and the functional interaction between Asp-237 and Lys-358 form the basis of an appealing notion that intramembrane charged residues in the permease are not only balanced in number but may exist as charge pairs, neutralizing each other via salt bridges. Disruption of the salt bridges would lead to the presence of an unpaired charge in the membrane and cause a functional defect. The proposed alteration in the secondary-structure model and the idea that certain putative intramembrane charged residues may form salt bridges are consistent with some of the findings presented here and with additional observations of R.L.D., M.S.-T., and H.R.K. Individual replacement of each of the eight putative intramembrane charged residues with Cys or in some cases Ala inactivates the permease. In addition, Cys replacement of Lys-221, Arg-218, or Glu-215, originally assigned to putative helix VII (Fig. 1), yields mutants with significant transport activity (B. Persson, M.S.-T., and H.R.K., unpublished information), implying that these residues may not be within the membrane. Most convincingly, as demonstrated with Asp-237 and Lys-358 which provide a particularly clearcut example, re-

placement of either of these residues with neutral residues so as to leave the other charge uncompensated leads to inactivation, while double replacement with Cys (Fig. 2) or interchanging Asp-237 and Lys-358 (Fig. 3) has little effect on activity. In addition, the results demonstrate that neither residue nor the salt bridge plays an important role in the transport mechanism.

To test the possibility that the other charged residues in putative transmembrane helices are neutralized by charge pairing, we constructed 13 additional double mutants in which all possible interhelical combinations of negative and positively charged residues were replaced pairwise with Cys. Out of the combinations of double-Cys mutants, only D240C/K319C exhibits significant activity, the remaining double mutants exhibiting essentially no ability to concentrate lactose. However, the functional interaction between Asp-240 and Lys-319 is phenomenologically quite different from that between Asp-237 and Lys-358. Active transport in D240C/K319C is clearly diminished relative to C-less permease, and although significant activity is observed with the double-Ala mutant or with the two possible Ala–Cys combinations, interchanging Asp-240 and Lys-319 abolishes active transport. Therefore, although neither Asp-240 nor Lys-319 *per se* is mandatory for active lactose transport, the polarity of the interaction is apparently important for full activity.

The results presented here suggest that charge pairing between intramembrane charged residues is probably not a general feature of *lac* permease and may be exclusive to D237/K358 and D240/K319. However, it is important to emphasize that the charge-pair neutralization approach is dependent upon permease activity and will not reveal charge-paired residues if they are essential for activity. In this regard, it is noteworthy that double-Cys mutants involving residues suggested to be hydrogen-bonded and directly involved in lactose-coupled H^+ translocation and/or substrate recognition [i.e., Arg-302, His-322, and Glu-325 (34–36)], as well as Glu-269, which has been shown to be essential (ref. 33 and M. L. Ujwal, B. Persson, M.S.-T., and H.R.K., unpublished results), are inactive with respect to active lactose transport.

As discussed above, the modification of the secondary-structure model proposed by King *et al.* (18) is based on the functional interaction between Asp-237 and Lys-358 and on the idea that the intramembrane charged residues must be balanced. Despite the indication that Asp-240 and Lys-319 may also participate in a salt bridge, 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 240 and 319, the sensitivity of given mutants to NEM was studied (Fig. 6). Clearly, D240A/K319C is readily inactivated by alkylation, while D240C/K319A is not. However, as indicated from studies with *p*CMB and *p*CMBS, a clear pattern is not apparent. It is anticipated that the use of spectroscopic techniques with purified double-Cys and Cys–Ala mutants will provide a means to resolve this issue. Similarly, we have made preliminary efforts to demonstrate disulfide bond formation directly by oxidation of appropriate double-Cys mutants and to examine this aspect of the problem by site-directed spin-labeling (39) as well. In any event, on the basis of the evidence currently available, it is clear that Asp-237 and Lys-358 and Asp-240 and Lys-319 interact functionally, and it is reasonable to postulate that both pairs of residues may be salt-bridged in the tertiary structure of the permease. It follows that putative helix VII (Asp-237 and Asp-240) may be in close proximity to helices X (Lys-319) and XI (Lys-358) in the tertiary structure of *lac* permease (see ref. 20 in addition).

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