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Characterization of Site-Directed Mutants in the lac Permease of Escherichia coli.

1. Glutamate-325 Replacements

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ABSTRACT: lac permease with Ala in place of Glu325 was solubilized from the membrane, purified, and reconstituted into proteoliposomes. The reconstituted molecule is completely unable to catalyze lactose/H+ symport but catalyzes exchange and counterflow at least as well as wild-type permease. In addition, Ala325 permease catalyzes downhill lactose influx without concomitant H+ translocation and binds p-nitrophenyl a-p-galactopyranoside with a KD only slightly higher than that of wild-type permease. Studies with right-side-out membrane vesicles demonstrate that replacement of Glu325 with Glu, Phe, Asp, His, Trp, or Tyr results in behavior similar to that observed with Ala in place of Glu325. On the other hand, permease with Glu325 catalyzes lactose/H+ symport about 20% as well as wild-type permease. The results indicate that an acidic residue at position 325 is essential for lactose/H+ symport and that hydrogen bonding at this position is insufficient. Taken together with previous results and those presented in the following paper [Lee, J. A., Püttner, I. B., & Kaback, H. R. (1989) Biochemistry (third paper of three in this issue)], the findings are consistent with the idea that Arg302, His322, and Glu325 may be components of a H+ relay system that plays an important role in the coupled translocation of lactose and H+.

The preceding paper (Püttner et al., 1989) confirms and extends earlier observations (Padan et al., 1979, 1985; Patel et al., 1982; Garcia et al., 1982; Püttner et al., 1986) focusing on the importance of His322 in lactose/H+ symport by the lac permease. Thus, evidence was presented indicating that His322 may be directly involved in lactose-coupled H+ translocation. Although a high-resolution structure for lac permease is not available, recent studies (Costello et al., 1984, 1987; Li & Tooth, 1987) suggest the presence of a notch or groove within the molecule. Therefore, the number of amino acid residues directly involved in substrate and H+ translocation may be fewer than the number of residues required to span the full thickness of the membrane.

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On the basis of various algorithms for secondary structure, lac permease is proposed to consist of 12-14 transmembrane α-helical domains (Foster et al., 1983; Bieseler et al., 1985; Wright et al., 1985; Vogel et al., 1985) with His322 and Glu325 located in putative transmembrane helix X (Foster et al., 1983). General aspects of the models are supported by biochemical and biophysical studies [cf. Kaback (1983, 1986)]. Bieseler et al. (1985) have also provided evidence that His322 and Glu325 are located in an α-helical portion of the polypeptide.

When putative helix X (Foster et al., 1983) is subjected to molecular modeling (Kaback, 1987), Glu325 is on the same face of the helix as His322, and the carboxylic is about 1.5 Å from the imidazole. On these grounds, it seems likely that the two residues are ion-paired, and for this reason, Carrasco et al. (1986) replaced Glu325 with Ala by using site-directed mutagenesis. Permease altered in this fashion does not catalyze lactose/H⁺ symport, but the protein catalyzes exchange and counterflow as well as wild-type permease. On the basis of these observations, it was suggested that His322 and Glu325 may be components of a charge-relay system that is important in lactose-coupled H⁺ translocation. Subsequently, it was shown that Arg302 (putative helix IX), which is also important for lactose/H⁺ symport, may be sufficiently close to His322 to form a hydrogen bond, thereby forming the third component in a so-called catalytic triad [Menick et al., 1987; cf. Kaback (1987, 1988) for reviews].

In view of the critical importance of Glu325 in lactose/H⁺ symport, permease with Ala at position 325 was solubilized from the membrane, purified, and reconstituted into proteoliposomes. As shown here, the purified, reconstituted mutant permease behaves in a fashion identical with that reported for intact cells and right-side-out (RSO)¹ membrane vesicles. In addition, other amino acid replacements were made for Glu325, and the properties of the altered permeases were studied in RSO membrane vesicles. Although permease with Asp325 is still able to catalyze lactose/H⁺ symport, permeases with Gln, His, Val, Cys, or Trp at position 325, like permease with Ala325, do not catalyze lactose/H⁺ symport but exhibit normal exchange and counterflow activity. In the last paper of the series (Lee et al., 1989), further evidence supporting the notion that His322 and Glu325 are ion-paired is presented.

EXPERIMENTAL PROCEDURES

Materials

All materials utilized were of reagent grade and obtained from commercial sources as described (Sarkar et al., 1986).

Methods

Bacterial Strains. The bacterial strains used have been described (Sarkar et al., 1985; Püttner et al., 1986, 1989; Menick et al., 1987).

Oligonucleotide Synthesis. Deoxyoligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer and purified by polyacrylamide gel electrophoresis as described in the Applied Biosystems manual.

Site-Directed Mutagenesis. Oligonucleotide-directed, site-specific mutagenesis (Zoller & Smith, 1983) was performed essentially as described (Sarkar et al., 1986) with one of the following modifications to improve the frequency of mutant recovery: (i) For E325A² (Carrasco et al., 1986) and E325D, template single-stranded (ss) DNA was isolated from phage grown in Escherichia coli BW313 (duv* ung*) as described by Kunkel (1985). Closed-circular heteroduplex DNA with the desired mutations was synthesized in vitro as described (Sarkar et al., 1986) and transfected into E. coli JM109 (ung*). Mutant phage were screened as described below. (ii) For each of the other mutants described, mismatch repair was minimized by transfecting the heteroduplex into the mutator strain E. coli BMM711-18 mutL (Kramer et al., 1984). Phage harboring a given mutation were identified initially by colony blot hybridization with the appropriate 32P-labeled mutagenic primer (Carter et al., 1984). Phage from positive colonies were plaque purified, and the mutations were verified by dideoxyoligonucleotide sequencing (Sanger et al., 1977; Sanger & Coulson, 1978) utilizing a synthetic primer complementary to a region of lac Y 50-150 bases downstream from codon 325. In addition, the entire lac Y gene encoding the E325A and E325D mutations was sequenced by using six synthetic primers complementary to appropriate regions of lac Y.

Double-stranded lac Y DNA from each mutant was restricted from M13mp19 replicative form (RF) DNA and ligated into the EcoRI site of pACYC184 DNA. The resulting plasmids (Table I) were used to transform E. coli T184 (Z⁺ Y⁺) or HB101 (Z⁺ Y⁺), as indicated.

Measurement of Lactose-Induced H⁺ Translocation. Measurements of intravesicular pH were measured in proteoliposomes containing entrapped 8-hydroxy-1,3,6-pyrenesulfonate (pyranine) as described by Matsushita et al. (1984). Proteoliposome reconstituted with wild-type or E325A permease in 50 mM potassium phosphate (pH 7.5)/1 mM dithiothreitol were diluted 5-fold, and pyranine was added to a final concentration of 2.5 mM. The preparations were then sonicated briefly in order to equilibrate the intravesicular and extravesicular compartments. Pyranine-loaded proteoliposomes were diluted 100-fold with 10 mM potassium phosphate (pH 7.5), harvested by centrifugation for 1 h at 17500g, and resuspended in 10 mM potassium phosphate (pH 7.5) to a final concentration of 42 µg of protein/mL and 31.3 mg of phospholipid/mL. Valinomycin was then added to a final concentration of 10 µM. In order to monitor changes in intravesicular pH, proteoliposomes loaded with pyranine were diluted to 0.14 µg of protein/mL in 10 mM potassium phosphate (pH 7.5), and the fluorescence of the entrapped pyranine was measured in a Perkin-Elmer MPF-66 fluorescence spectrophotometer (excitation, 460 nm; emission, 520 nm).

Other methods are described in the preceding paper (Püttner et al., 1989).

RESULTS

Verification of Mutations by DNA Sequencing

The lac Y gene in each plasmid used was cloned initially from pGM21 into M13mp19 RF DNA, and ss phage DNA was isolated and used as a template for site-directed mutagenesis. Subsequently, ss phage DNA containing mutated lac Y was isolated and sequenced (Sanger et al., 1977; Sanger & Coulson, 1978) with appropriate primers complementary to regions of lac Y 50-150 bases downstream from codon 325. The sequence analyses summarized in Table I demonstrate

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¹ Abbreviations: RSO, right side out; ss, single stranded; RF, replicative form; EMB, eosin-methylene blue; PMS, phenazine methosulfate; DnX-Gal, 6-(N-dansylamino)hexyl β-D-thiogalactopyranoside; NPG, p-nitrophenyl α-D-galactopyranoside.

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 of this position (e.g., E325A designates that Glu325 is replaced with Ala).
that the mutated lac Y genes contain changes in codon 325 such that Glu is replaced with Ala, Asp, Gln, His, Cys, Val, or Trp as indicated. In addition, the entire nucleotide sequence of lac Y encoding the E325A and E325D mutations was determined by using six synthetic sequencing primers. With the exception of the alterations given in Table I, the remainder of the lac Y sequence was identical with that reported by Büchel et al. (1980).

**E325A Permease**

Preliminary studies by Carrasco et al. (1986) with intact cells and RSO membrane vesicles suggest that Glu325 plays a critical role in the release of H+ from the permease during turnover. Thus, permease with E325A is defective in all translocation reactions that involve net H+ movement but catalyzes equilibrium exchange and counterflow normally. In view of the unique properties of the E325A permease, the molecule was solubilized from the membrane, purified, and reconstituted into proteoliposomes by previously described techniques [cf. Viitanen et al. (1986)].

**Active Transport.** As shown previously (Newman et al., 1981; Foster et al., 1982) and in Figure 1, proteoliposomes reconstituted with purified wild-type permease catalyze lactose accumulation in the presence of a potassium diffusion gradient ([K+]in > [K+]out) and the ionophore valinomycin. In marked contrast, proteoliposomes reconstituted with purified E325A permease exhibit essentially no lactose transport under identical conditions.

**Downhill Lactose Influx and Lactose-Induced H+ Translocation.** Like the phenotypes conferred by plasmids pH322R (Padan et al., 1985), pH322N, pH322Q, pH322K (Püttner et al., 1989), and pR302L (Menick et al., 1987), when the cryptic strain HB101 is transformed with pE325A and grown at high lactose concentrations, the cells appear as red colonies on eosin–methylene blue (EMB) indicator plates (Carrasco et al., 1986). Therefore, although E325A permease is unable to catalyze lactose/H+ symport, the polypeptide is apparently able to facilitate downhill lactose translocation at high concentrations of the disaccharide presumably without concomitant H+ translocation.

This contention receives strong support from direct measurements of lactose-facilitated diffusion and lactose-induced H+ translocation (Figure 2). When 10 mM lactose is added to a suspension of proteoliposomes reconstituted with purified wild-type permease or purified E325A permease in the presence of valinomycin and nigericin, the internal lactose concentration equilibrates with the external medium within 10–15 min, and the rate of equilibration is dramatically inhibited by addition of p-(chloromercuri)benzenesulfonate (pCMBS) (Figure 2A). Although not shown, equilibration occurs within 3 h in the presence of pCMBS with both preparations.

In the corollary experiment, lactose-induced internal pH changes were monitored in the same proteoliposomes containing entrapped pyranine, an impermeant pH-sensitive fluorophore (Clement & Gould, 1981; Matsushita et al., 1984)

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**Table I: DNA Sequence Analyses of E325 Mutants in lac Y***

<table>
<thead>
<tr>
<th>plasmid</th>
<th>mutagenic primer</th>
<th>DNA sequence of codon 325</th>
<th>amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGM21</td>
<td>5'-GTAATGCAAACTATGC-3'</td>
<td>GAA</td>
<td>Glu</td>
</tr>
<tr>
<td>pE325A</td>
<td>5'-GAACTGACAAACATATG-3'</td>
<td>GCA</td>
<td>Ala</td>
</tr>
<tr>
<td>pE325C</td>
<td>5'-GAATGCTACAAACATATGC-3'</td>
<td>TGT</td>
<td>Cys</td>
</tr>
<tr>
<td>pE325D</td>
<td>5'-GATACCTACAAACATATGC-3'</td>
<td>GAC</td>
<td>Asp</td>
</tr>
<tr>
<td>pE325H</td>
<td>5'-GAACTGCATGAAACATATGC-3'</td>
<td>CAT</td>
<td>His</td>
</tr>
<tr>
<td>pE325Q</td>
<td>5'-GAACTGACTAACACATATGC-3'</td>
<td>CAA</td>
<td>Gin</td>
</tr>
<tr>
<td>pE325V</td>
<td>5'-GAACTGACTAACACATATGC-3'</td>
<td>GTA</td>
<td>Val</td>
</tr>
<tr>
<td>pE325W</td>
<td>5'-GAGAACCTGATCAGAACATATGC-3'</td>
<td>TGG</td>
<td>Trp</td>
</tr>
</tbody>
</table>

*Italics indicates nucleotide or amino acid replaced in mutants.

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**FIGURE 1:** Membrane potential (ΔΨ) driven lactose transport in proteoliposomes reconstituted with purified wild-type (A, A) or E325A permease (B, B). lac permease from E. coli T206 or E325A was solubilized, purified, reconstituted into proteoliposomes, and assayed for ΔΨ-driven lactose transport as described [cf. Viitanen et al. (1986)]. The suspension contained 50 μg of protein/mL and 37.5 mg of E. coli phospholipid/mL in 50 mM potassium phosphate (pH 7.5)/1 mM EDTA/0.5 mM dithiothreitol. Valinomycin was added to 20 μM final concentration, and aliquots (1 μL) were diluted 200-fold into 50 mM sodium phosphate (pH 7.5; closed symbols) or 50 mM potassium phosphate (pH 7.5; open symbols) containing 0.3 mM [1-14C]lactose (19.4 mCi/mmole). At given times, reactions were terminated, and the samples were assayed by rapid filtration and liquid scintillation spectrometry.

(Figure 2B). When added to proteoliposomes containing wild-type permease, lactose causes a transient decrease in fluorescence, indicating that the internal space becomes acidic as the result of lactose/H+ symport. The decrease in fluorescence reaches a minimum in about 5–6 min and is rapidly and completely reversed upon addition of nigericin. In contradistinction, proteoliposomes containing E325A permease do not exhibit any change in fluorescence upon addition of lactose. Thus, E325A permease catalyzes downhill influx of lactose, but the process does not occur in symport with H+. Although not shown, similar results were obtained when changes in external pH were measured directly with a pH electrode [cf. Püttner et al. (1989)].

**Efflux, Exchange, and Counterflow.** When proteoliposomes reconstituted with purified wild-type permease are equilibrated with 10 mM [1-14C]lactose, treated with valinomycin, and diluted into medium devoid of lactose (Figure 3A) or into medium containing 10 mM lactose (Figure 3B), efflux and equilibrium exchange occur at rates approximating those described previously [i.e., t1/2 ~ 1 min and 10 s, respectively; cf. García et al. (1983), Viitanen et al. (1983), and Püttner et al. (1989)]. In contrast, proteoliposomes reconstituted with E325A permease catalyze efflux at a rate that is only marginally greater than that observed in the presence of pCMBS, and the phenomenon is totally unaffected by increasing ambient pH to 9.5 (Figure 3A). As shown previously (Viitanen
concentrated to approximately 250 pg/mL in 50 mM potassium
GSTF filters (0.2 pm) and washed twice with the same volume of
cold buffer. The data were corrected for zero-time control values
by rapid dilution with 4 mL of ice-cold 50 mM potassium phosphate
potassium phosphate (pH
concentration of lactose as the samples incubated in the absence of
was assayed as described [cf. Viitanen et al. (1986)]. Valinomycin
and nigericin were added to final concentrations of 20 and 2 µM,
respectively, and aliquots (1 µL) were diluted 100-fold into 50 mM
potassium phosphate (pH 7.5) containing 10 mM [1-14C]lactose (7
mCi/mmol) at 25 ౾. At indicated times, reactions were terminated
by rapid dilution with 4 mL of ice-cold 50 mM potassium phosphate
(pH 7.5), and the samples were filtered immediately through Millipore
GSTF filters (0.2 µm) and washed twice with the same volume of
cold buffer. The data were corrected for zero-time control values
obtained by adding an aliquot (1 µL) of proteoliposomes to reaction
mixtures that had already been diluted with 4 mL of cold buffer,
followed by filtration and washing. (A and A) Proteoliposomes
reconstituted with wild-type and A325 permease, respectively, after
removal of dithiothreitol and incubation with 2.5 mM pCMBS.
Although not shown, after 3 h, the samples achieved the same internal
concentration of lactose as the samples incubated in the absence of
pCMBS. (B) Lactose-induced H+ translocation. Proteoliposomes
reconstituted with wild-type (a) or E325A permease (b) were loaded
with pyrane as described under Experimental Procedures, and
changes in intravesicular pH were measured by monitoring the
fluorescence of entrapped pyrane (excitation, 460 nm; emission, 520
nm). At indicated times, lactose or nigericin was added to the cuvette
to final concentrations of 10 mM or 8.3 nM, respectively. The
nigericin-sensitive fluorescence change observed with proteoliposomes
containing wild-type permease (trace a) was 15% of the initial pyranine
fluorescence.

![Graph](image1.png)

**Figure 2:** (A) Facilitated diffusion of lactose in proteoliposomes
reconstituted with purified permease. Proteoliposomes reconstituted
with purified lac permease from E. coli T206 (a) or A325 (O) were
concentrated to approximately 250 pg/mL in 50 mM potassium
phosphate (pH 7.5)/1 mM dithiothreitol, and facilitated diffusion
was assayed as described [cf. Viitanen et al. (1986)]. Valinomycin
and nigericin were added to final concentrations of 20 and 2 µM,
respectively, and aliquots (1 µL) were diluted 100-fold into 50 mM
potassium phosphate (pH 7.5) containing 10 mM [1-14C]lactose (7
mCi/mmol) at 25 ౾. At indicated times, reactions were terminated
by rapid dilution with 4 mL of ice-cold 50 mM potassium phosphate
(pH 7.5), and the samples were filtered immediately through Millipore
GSTF filters (0.2 µm) and washed twice with the same volume of
cold buffer. The data were corrected for zero-time control values
obtained by adding an aliquot (1 µL) of proteoliposomes to reaction
mixtures that had already been diluted with 4 mL of cold buffer,
followed by filtration and washing. (A and A) Proteoliposomes
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removal of dithiothreitol and incubation with 2.5 mM pCMBS.
Although not shown, after 3 h, the samples achieved the same internal
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to final concentrations of 10 mM or 8.3 nM, respectively. The
nigericin-sensitive fluorescence change observed with proteoliposomes
containing wild-type permease (trace a) was 15% of the initial pyranine
fluorescence.

![Graph](image2.png)

**Figure 3:** Lactose efflux (A) and equilibrium exchange (B). (A)
Proteoliposomes containing purified lac permease from E. coli T206
(a) or E325A (O) were resuspended to 50 µg of protein/mL in 50
mM potassium phosphate/1 mM dithiothreitol at given pH values.
A small aliquot of [1-14C]lactose (10 mCi/mmol) was added to each
suspension to a final concentration of 10 mM, and valinomycin was
added to a final concentration of 20 µM. After equilibration at room
temperature for 1 h, aliquots (1 µL) were rapidly diluted into 200
µL of 50 mM potassium phosphate at the appropriate pH at 25 ౾.
At indicated times, samples were diluted and filtered as described
[cf. Viitanen et al. (1986)]. The experiments were conducted at pH
7.5 (a), 8.5 (A), or 9.5 (C). Data are presented as the concentration
of lactose (mM) retained within the proteoliposomes as a function
of time. (*) Identical experiments were performed with proteoliposomes
incubated with pCMBS at a final concentration of 2.5 mM, as
described in Figure 2. (B) Experiments were performed as described
in (A), except that 10 mM unlabeled lactose was included in the
medium into which the proteoliposomes were diluted.

![Graph](image3.png)

**Figure 4:** Lactose counterflow at saturating (A) and subsaturating
(B) external lactose concentrations. Proteoliposomes reconstituted
with purified lac permease from E. coli T206 (a) or E325A (O) were
equilibrated with 10 mM lactose in the presence of 20 µM valinomycin.
Counterflow was then assayed at given times at 25 ౾ by diluting
an aliquot (1 µL) into 200 µL of 50 mM potassium phosphate (pH
7.5) containing 1.6 mM [1-14C]lactose (10 mCi/mmol) as described
by Viitanen et al. (1986). (B) Experiments were conducted as
described in (A), except that the dilution medium contained 0.25 mM
[1-14C]lactose (40 mCi/mmol).

with deuterium in place of protium (Viitanen et al., 1983),
in the presence of monoclonal antibody 4B1 (Carrasco et al.,
1984), or in RSO vesicles from the uncoupled mutant E. coli
ML 308-22 (Herzlinger et al., 1985) and is consistent with
the notion that E325A permease is unable to release H+ from
the periplasmic side of the membrane (Viitanen et al., 1983), the rate of efflux in proteoliposomes reconstituted
with wild-type permpase is increased approximately 5-fold over
this pH range. Remarkably, however, the rate of exchange in proteoliposomes with E325A permease is identical with that
of proteoliposomes with wild-type permease within experimental error (Figure 3B). Furthermore, proteoliposomes with
E325A permease catalyze counterflow at essentially the same
rate and to the same extent as proteoliposomes with wild-type
permease, but the internal concentration of [1-14C]lactose in
E325A proteoliposomes is maintained at high levels for a prolonged period relative to proteoliposomes with wild-type
permease (Figure 4A).

Interestingly, when the external [1-14C]lactose concentration is
decreased to about half the Km for entrance counterflow,
the maximum level achieved during the overshoot in E325A
proteoliposomes is about 3-fold greater than that in proteo-
liposomes containing wild-type permease (Figure 4B). The
effect is significantly more marked than observed previously

\[ \text{p-Nitrophenyl \alpha-D-Galactopyranoside (NPG) Binding.} \]

As shown previously, wild-type lac permease binds NPG stoichiometrically with a Kd of about 20 µM (Rudnick et al.,
1976; Herzlinger et al., 1985), while permeases with R302L (D. R.
Site-Directed Mutants of lac Permease

Menick, L. Patel, and H. R. Kaback, unpublished information) or H322R (Pütter et al., 1989) appear to bind ligand with markedly decreased affinity. Although not shown, Scatchard analyses of NPG binding data obtained with RSO membrane vesicles containing wild-type permease yield a $K_D$ value of 35 μM. Interestingly, the $K_D$ observed with E325A vesicles is about 70 μM, a value very significantly lower than that observed with either the R302L or H322R permease. Thus, the affinity of the permease for NPG appears to be relatively unaffected by replacement of Glu325 with Ala.

Other Amino Acid Replacements for Glu325

In order to more fully elucidate the role of Glu325 in lactose/H+ symport, codon 325 of lac Y was modified by site-directed mutagenesis so that Glu325 in the permease was replaced with Asp, Gln, His, Cys, Val, or Trp (Table I). Subsequently, the effects of the replacements on permease activity were studied qualitatively by plating E. coli HB101 transformed with the appropriate plasmid on EMB/lactose and more quantitatively by assaying various translocation reactions in RSO membrane vesicles.

** Colony Morphology on EMB/Lactose.** E. coli HB101 (Z+Y-) transformed with pE325D grows as dark red colonies on EMB containing 25 mM lactose. That is, the cells exhibit a phenotype indistinguishable from that of cells that express wild-type permease. HB101 transformed with each of the other plasmids also appear as dark red colonies, indicating that they too are able to metabolize lactose effectively at high concentrations.

** Active Transport.** In the absence of exogenous electron donors, RSO membrane vesicles containing wild-type permease or each of the mutated permeases described transport lactose at negligible initial rates and to minimal steady-state levels of accumulation (Figure 5). On addition of ascorbate and phenazine methosulfate (PMS), the initial rate of transport in vesicles containing wild-type permease increases dramatically, and the vesicles achieve a steady-state level of accumulation by about 5 min. In contrast, vesicles containing

![Figure 5: Time course of lactose transport in RSO vesicles from E. coli T206 (O), E325A (O), E325D (O), E325C (O), E325H (O), E325Q (O), E325V (O), or E325W (O) or for T184 (Δ). RSO membrane vesicles were prepared and assayed for lactose transport in the presence and absence of PMS as described (Kaback, 1971, 1974). Transport in the presence or absence of electron donors was considered as steady-state and the vesicles achieve a steady-state level of accumulation by about 5 min.](image)

![Figure 6: Lactose efflux (A) and exchange (B) in RSO membrane vesicles from E. coli T206 (O), E325C (O), E325H (O), E325Q (O), E325V (O), and E325W (Δ). Membrane vesicles were equilibrated with 10 mM [1-14C]lactose (6 mCi/mmol), and aliquots were rapidly diluted into media devoid of lactose (A) or media containing equimolar concentrations of unlabelled lactose (B). At the times indicated, the reactions were terminated with 3 mL of 0.1 M potassium phosphate (pH 5.5)/0.1 M lithium chloride/20 mM HgCl2, and the samples were immediately filtered as described (Kaczorowski & Kaback, 1979). Similar experiments were performed in the presence of 1 mM pCMBS (Δ). Although not shown, similar results were obtained for equilibrium exchange with E325C, E325H, and E325V vesicles.](image)

E325D permease transport lactose at about 20% of the rate of wild-type vesicles, and the steady-state level of accumulation is also reduced to a similar extent. Furthermore, no effect is observed after addition of PMS/ascorbate to vesicles containing permease with Ala, Gln, His, Cys, Val, or Trp in place of Glu325. Significantly, each vesicle preparation exhibits an amount of permease comparable to that observed in vesicles containing wild-type permease, as judged by immunoblot analyses (cf. Herzlinger et al. (1985)), and each preparation catalyzes respiration-driven proline transport normally (data not shown). Therefore, it is apparent that lac permease with Asp in place of Glu325 retains partial activity, while permease with each of the other amino acid replacements at position 325 is inactive.

** Efflux, Exchange, and Counterflow.** As shown with E325A vesicles (Carrasco et al., 1986) and with proteoliposomes containing purified E325A permease (Figure 3), RSO vesicles containing permease with Gln, His, Cys, Val, or Trp in place of Glu325 catalyze efflux at markedly defective rates (Figure 6A). In addition, it is noteworthy that the rate of efflux in vesicles containing E325C permease is not increased significantly when ambient pH is increased to pH 9.5 (data not shown). In contrast, the rate of equilibrium exchange catalyzed by each lac permease mutant is indistinguishable from the rate catalyzed by wild-type permease (Figure 6B). Moreover, E325A, E325Q, E325H, E325C, E325V, and E325W vesicles catalyze counterflow at similar rates and to similar extents as observed in T206 vesicles, but the increase in Dns6-Gal fluorescence is maintained for longer periods of time (Figure 7). Finally, as observed with purified, reconstituted E325A permease (Figure 4B), the mutant lac permeases catalyze lactose/Dns6-Gal counterflow more effectively than the wild type when the external concentration of Dns6-Gal is decreased to below the apparent $K_m$.

** DISCUSSION**

Use of site-directed mutagenesis has focused attention on specific amino acid residues that clearly play an important role in the mechanism of lactose/H+ symport [cf. Kaback (1987a,b, 1988) for reviews]. Thus, Arg302 (putative helix IX), His322
A hydrogen-bond network that is directly involved in H+ Arg302, His322, and Glu325 may be sufficiently close to form steps that require net H+ translocation but catalyzes exchange efflux down a concentration gradient consists of a minimum of Glu325 may mediate loss of Glu325 with Ala results in permease that is defective in all from T206, E32SW, E325Q, and counterflow normally. For this reason, it was suggested that protonation or deprotonation are blocked in the R302L scheme presented in Figure 8. By the mechanism shown, for lactose/H+ symport, while certain other residues play no apparent role in the transport mechanism. Furthermore, evidence has been presented that is consistent with the idea that Arg302, His322, and Glu325 may be sufficiently close to form a hydrogen-bond network that is directly involved in H+ translocation.

The behavior of permease mutants specifically altered in Arg302, His322, or Glu325 is easily rationalized by the kinetic model presented in Figure 8. By the mechanism shown, efflux down a concentration gradient consists of a minimum of five steps: (1) binding of substrate and H+ to the permease on the outer surface (order unspecified); (2) translocation of the ternary complex to the outer surface; (3) release of substrate; (4) release of H+; (5) return of the unloaded permease to the outer surface. Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1–3 only (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983; Carrasco et al., 1984, 1986; Herzlinger et al., 1985). Since all steps in the mechanism that involve protonation or deprotonation are blocked in the R302L and H322R mutants and the primary effect of the H+ electrochemical gradient on the kinetics of lactose transport (i.e., a decrease in the apparent K₈ₐ for lactose; Kaczorowski et al., 1979; Viitanen et al., 1983) is not observed in these mutants, it seems likely that protonation of His322 is involved in step 1 in a manner that involves Arg302. In contrast, replacement of Glu325 with Ala results in permease that is defective in all steps that require net H+ translocation but catalyzes exchange and counterflow for this reason, it was suggested (Carrasco et al., 1986) that Glu325 may be involved in step 4 of the mechanism (i.e., Glu325 may mediate loss of H+ from the permease).

The results presented here provide strong support for previous observations with E325A permease (Carrasco et al., 1986) and extend the conclusions significantly. Thus, it has been demonstrated that although purified, reconstituted permease with Ala in place of Glu325 is unable to catalyze
relative to that of the wild type when the external substrate concentration is below the apparent $K_m$.

The observations have important implications on different levels: (1) Given the nature of the replacements and the ability of the modified permeases to catalyze exchange and counterflow, it seems unlikely that either the bulk or hydropathy of the side chain at position 325 has a significant effect on substrate binding or translocation of the loaded permease. (2) Since permease with Asp325 retains partial ability to catalyze lactose/H+ symport, while permease with Gln325 catalyzes exchange and counterflow but is totally defective in all reactions involving net H+ translocation, it is likely that an acidic residue at position 325 is required for lactose-coupled H+ translocation and that hydrogen bonding is insufficient. (3) As discussed previously (Menick et al., 1987; Kaback, 1987), Arg302, His322, and Glu325 might function as components of a charge relay, in which H+ would be expected to move physically from one residue to the other. Alternatively, however, His322 might be the only residue involved in H+ translocation, and its $pK_a$ may be modulated by the proximity of a positive charge in Arg302 and a negative charge in Glu325. In this regard, the behavior of the E325C mutant may be significant. As indicated, this mutant is unable to catalyze reactions involving net H+ translocation even when the ambient pH is increased to pH 9.5, where Cys would be expected to exist as a thiolate anion. Although the observation tends to favor the charge-relay notion, it should be emphasized that the local pH within the protein may not be in equilibrium with the bulk phase and/or that the $pK_a$ of a Cys within the permease may be perturbed (i.e., at an ambient pH of 9.5, Cys325 may not be negatively charged).

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Registry No. Glu, 56-86-0; Ala, 56-41-7; Gln, 56-85-9; His, 71-00-1; Val, 72-18-4; Cys, 52-90-4; Trp, 73-22-3; Asp, 56-84-8; NPG, 7493-95-0; H+, 12408-02-5; lactose, 63-42-3; lactose permease, 9086-45-5.

REFERENCES