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Control of H^+/Lactose Coupling by Ionic Interactions in the Lactose Permease of *Escherichia coli*

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Abstract. A combinatorial approach was used to study putative interactions among six ionizable residues (Asp-240, Glu-269, Arg-302, Lys-319, His-322, and Glu-325) in the lactose permease. Neutral mutations were made involving five ion pairs that had not been previously studied. Double mutants, R302L/E325Q and D240N/H322Q, had moderate levels of downhill [^{14}C]-lactose transport. Mutants in which only one of these six residues was left unchanged (pentuple mutants) were also made. A Pent269$^-$ mutant (in which only Glu-269 remains) catalyzed a moderate level of downhill lactose transport. Pent240$^-$ and Pent 322$^+$ also showed low levels of downhill lactose transport. Additionally, a Pent240$^-$ mutant exhibited proton transport upon addition of melibiose, but not lactose. This striking result demonstrates that neutralization of up to five residues of the lactose permease does not abolish proton transport. A mutant with neutral replacements at six ionic residues (hextuple mutant) had low levels of downhill lactose transport, but no uphill accumulation or proton transport. Since none of the mutants in this study catalyzes active accumulation of lactose, this is consistent with other reports that have shown that each residue is essential for proper coupling. Nevertheless, none of the six ionizable residues is individually required for substrate-induced proton cotransport. These results suggest that the H^+ binding domain may be elsewhere in the permease or that cation binding may involve a flexible network of charged residues.

Key words: Lactose permease — *Escherichia coli* — Symport — Proton transport — Coupling — Mutagenesis

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Introduction

Integral membrane proteins known as cation-solute transporters or symporters catalyze the uptake of sugars, amino acids, and Krebs cycle intermediates. The lactose permease of *Escherichia coli* transports protons and galactosides in a 1:1 stoichiometry [42, 43]. The inwardly directed proton gradient generated across the plasma membrane by components of the respiratory chain pathway can thus be used to accumulate sugars against a concentration gradient [10]. Lactose permease is a well understood symporter, having been the subject of mutagenesis for many years. Cloning and sequencing of *lacY*, the gene encoding lactose permease, gives a structure of 417 amino acids with a molecular weight of 46,504 Da [6, 40]. Secondary structural models are consistent with the permease forming 12 transmembrane segments with N and C termini facing the cytoplasm [7]. The lactose permease belongs to a larger group of transport proteins known as the major facilitator superfamily (MFS) [32, 37]. Studies of MFS members indicate that they are evolutionarily related, and most are predicted to contain 12 transmembrane segments [9, 15].

All 417 residues of the lactose permease have been subjected to site-directed mutagenesis, and these studies have given insight as to the mechanism of protein function. Of particular interest is the role of six charged residues that are predicted to lie within the transmembrane region of the protein. These are: Asp-240, Glu-269, Arg-302, Lys-319, His-322, and Glu-325. Hydropathy analysis predicts that these residues lie on successive helices from TMS-7 through TMS-10, and a variety of experiments have demonstrated that these residues are close to each other in three-dimensional space. For example, fluorescent labeling of cysteine mutants [23] as well as site-directed spin-labeling [16, 19] indicated that TMS-10 is

close to TMS-8 and TMS-9. Binding of manganese in double-His mutant permeases showed that TMS-7 and TMS-10 are close together [17], and also that TMS-8 and TMS-10 [16], TMS-9 and TMS-10 [18, 19] and TMS-8 and TMS-9 [19] are adjacent to each other. Very recently, the proximity of these residues was confirmed by crystallization of the inward-facing conformation of the permease [1].

There is debate over the exact nature of the species of the transported cation in symporters such as the lactose permease. In the *E. coli* melibiose permease, sugar transport is coupled to protons (or possibly H_3O^+) as well as the larger ions Na^+ and Li^+ [2, 34], and changes in cation specificity can be conferred by mutations as small as a single residue [14]. Because of the flexibility in cation recognition in the wild-type permease and the observation that single substitutions can result in a major change in cation specificity, it has been suggested that transport may take place by coupling with hydronium (H_3O^+) ions, not proton translocation [3]. Likewise, it is possible that the region of the lactose permease comprising TMS-7 through TMS-10 might form such a coordination site for H_3O^+ ions, although proton translocation cannot be ruled out.

With regard to Asp-240, Glu-269, Arg-302, Lys-319, His-322, and Glu-325 in the lactose permease, neutral substitutions at these sites have a detrimental effect on transport, disrupt active accumulation, change kinetics of net lactose transport, and alter cation/lactose coupling [4, 11, 27, 28, 29, 33, 35, 41]. Because of their proximity, it is also inferred that these residues form transmembrane salt-bridge interactions important to the overall mechanism of lactose permease [22, 36]. The interactions that take place among these charged side chains during lactose transport may be important in controlling proton/lactose coupling, which is necessary for active lactose accumulation. In previous studies, mutations involving double replacements of a putative charge pair have been useful in determining whether an interaction is taking place. For example, a double mutant K319N/E325Q relieves a defect in downhill transport caused by single neutralization of Glu-325, in part because an unpaired charge within the membrane is disruptive. Given that there are three positive and three negative charges among the six ionic residues, there are a total of nine positive/negative combinations that could take place. Mutations involving four of these pairs (Asp-240/Lys-319, Glu-269/Lys-319, Lys-319/Glu-325, and His-322/Glu-325) have been studied extensively [22, 26, 27, 28, 35]. There remain five pairs without detailed transport data, and these charge pairs might also be important to the overall function of the lactose permease.

An important problem to address with regard to cation/lactose coupling is exactly how cations (i.e., H^+ or H_3O^+) are taken across the membrane. Early models of proton transport included a "charge-relay" system, transferring a proton sequentially among many ionic side chains [24], or binding and release at a single site in the protein, most likely a carboxyl side chain [20, 41]. Aspects of the charge-relay model were later shown to be incorrect, but one residue, Glu-325, still seemed important [12, 20, 25]. Neutral replacements of Glu-325 catalyzed equilibrium exchange of lactose, but the efflux phase did not vary with pH [12]. Further, it appeared that E325Q single mutants were unable to cotransport protons with lactose or other galactosides. However, this simple model for proton transport was challenged with experiments on a K319N/E325Q mutant, which catalyzed proton transport with both lactose and TDG [20], and a triple mutant K319N/H322Q/E325Q that transported protons with TDG [22]. Because of these striking results, it became clear that proton or H_3O^+ recognition is a very complicated process, and it was of interest to determine which of the six essential residues, if any, are required for substrate-induced proton cotransport, and which residues may be interacting with a partner during the transport cycle to control coupling. With these goals in mind, this paper describes the construction and analysis of five neutral mutant pairs, as well as pentuple and hextuple mutants of the lactose permease.

Materials and Methods

Reagents

Lactose (O-β-D-galactopyranosyl-[1,4]-α-D-glucopyranose), melibiose (O-α-D-galactopyranosyl-[1,6]-α-D-glucopyranose), maltose (O-α-D-glucopyranosyl-[1,4]-α-D-glucopyranose), cellobiose (O-β-D-glucopyranosyl-[1,4]-α-D-glucopyranose), and TDG (thiodigalactoside) were purchased from Sigma. [^{14}C]-lactose was purchased from Pharmacia. Restriction enzymes were purchased from New England Biolabs. The remaining reagents were analytical grade.

Bacterial Strains

For downhill lactose transport, *E. coli* strain HS4006/F′$I^QZ^+Y^-$ was used. It is *lacZ* positive but *lacY* negative [5]. For uphill lactose transport, *E. coli* strain T184 was used, which lacks functional *lacZ* and *lacY* genes [39].

Stock cultures of cells were grown in YT media [30] supplemented with tetracycline (0.01 mg/ml). For transport assays, cells were grown to midlog phase in YT media containing tetracycline (0.005 mg/ml), and 0.25 mM isopropylthiogalactoside (IPTG) to induce the synthesis of the lactose permease.

Plasmid DNA was isolated using the PERFECTPREP plasmid isolation kit (Eppendorf, Westbury, NY) and introduced into the appropriate bacterial strain by the RbCl method [31].

Construction of Mutant Permeases

A permease completely devoid of the six charged residues (Hextuple mutant) was created in stages. First, a mutant D240N/K319N/H322Q/E325Q was made with the Transformer mutagen-

esis kit from Clonetech. The plasmid pK319N/H322Q/E325Q (*see* [22]) was used as a template along with a primer changing Asp-240 to Asn. The pentuple mutants D240N/E269Q/K319N/H322Q/E325Q (Pent302$^+$) and D240N/R302L/K319N/H322Q/E325Q (Pent269$^-$), were made using the Quikchange mutagenesis kit from Stratagene. The D240N/K319N/H322Q/E325Q plasmid was used as a template with primers separately changing Glu-269 to Gln or Arg-302 to Leu. Next, the *Eco*RI fragments of these pentuple mutants were subcloned into pLac 184-*Sty*I. This plasmid is identical to pLAC184 [12], but has a small deletion in a nonessential region of pACYC184 (between the *Sty*I and *Nco*I sites) created by digestion and re-ligation of the complementary ends. This creates a unique *Bcl*I restriction site between codons 269 and 302, and the hextuple mutant D240N/E269Q/R302L/K319N/H322Q/E325Q was created by combining restriction fragments from Pent269$^-$ and Pent302$^+$. The other pentuple mutants (Pent240$^-$, Pent319$^+$, Pent322$^+$, and Pent325$^-$) were made through combinations with existing mutants, or by PCR mutagenesis.

Paired-neutral combinations (D240N/R302L, E269Q/R302L, D240N/H322Q, D240N/H322Q, and R302L/E325Q) were made either with PCR mutagenesis or by combining restriction fragments of existing single mutants. Verification of all mutants was done by sequencing of plasmid DNA through the entire *lacY* gene.

Sugar Transport

Downhill lactose transport experiments were performed by analyzing [^{14}C]-lactose uptake with rapid filtration as previously described [21], at an external lactose concentration of 1.0 mM, pH 7.0, at 37°C. Uphill transport was carried out as described [21] at 0.1mM, pH 7.0 at 30°C.

Proton Transport

Proton transport experiments in anaerobic T184 cells were carried out as previously described [21], using anaerobic melibiose and lactose at a final concentration of 10 mM.

Membrane Isolation and Immunoblot Analysis

T184 strains containing the appropriate plasmid were grown as described above for the sugar transport assays and subjected to membrane isolation by freeze-thaw/sonication and immunoblot analysis as described previously [22]. Normalized to wild type at 100%, the levels are: Hextuple, 73% ± 12, Pent240$^-$, 48% ± 10, Pent269$^-$, 73% ± 24, Pent302$^+$, 42% ± 18, Pent 319$^+$, 49% ± 12, Pent322$^+$, 84% ± 2, Pent 325$^-$, 91% ± 8, D240N/R302L, 90% ± 6, D240N/H322Q, 111% ± 21, E269Q/R302L, 99% ± 18, E269Q/H322Q, no expression detected, and R302L/E325Q, 139% ± 32.

Results

Characterization of Mutants by MacConkey Phenotype

As discussed in the Introduction, a single mutation at an ionizable residue in the lactose permease has a detrimental effect if this leaves an essential salt bridge unpaired within the membrane. If this effect is suppressed by a complementary neutralization of a second ionic residue, such a result suggests that the two residues form an ionic interaction. To determine if such ionic interactions take place, double mutants of the lactose permease comprising five pairs that had not been previously studied in detail were made as stated in Materials and Methods.

To initially evaluate the effects of the double mutations, the five strains were streaked onto MacConkey indicator plates. A red color on MacConkey indicator agar is one qualitative method of assaying lactose permease function. When plated on this medium, enteric bacteria that transport and ferment sugars will grow as red colonies, while those that are defective are white. The phenotype of the double mutants on MacConkey plates containing either lactose or melibiose is shown in Table 1. Compared to wild type, which was a deep red after overnight incubation, D240N/H322Q and R302L/E325Q are the most active, with a phenotype nearly indistinguishable from wild type. The mutants D240N/R302L and E269Q/R302L are less active, and only grew red on 1% sugar plates. Therefore, mutants missing an arginine at position 302 were rescued by neutralizing a complementary negative charge in a non-equivalent manner, namely Glu-325 > Asp-240 > Glu-269.

Membrane isolation and Western blotting of the double mutants was done to check for proper expression in the plasma membrane. While nearly all of the double mutants had over 90% expression compared to wild type (*see* Materials and Methods), the E269Q/H322Q mutant showed no lactose permease expression, which explains its white color on MacConkey agar. It seems that this particular charge pair is extremely important to the overall structure of the lactose permease, since neutralization leads to a protein that is degraded or otherwise does not properly insert into the membrane.

Single neutral mutants of the lactose permease are disruptive in function if they leave an unpaired charge within the hydrophobic membrane. Another method to assess the effect of such a disruption is to neutralize all residues except one. To this end, six pentuple mutants were created, in which only one of the six essential residues remains charged. The changes made to the wild-type permease in each mutant are listed in Table 2. For simplicity in discussing these results, each mutant has been given a short name that derives from the only residue of the six that remains unchanged. When the pentuple mutants were plated on MacConkey agar (Table 3), a large variation in phenotype across the six mutants was seen. While Pent240$^-$, Pent269$^-$, and Pent325$^-$ were red to dark pink, Pent302$^+$, Pent319$^+$, and Pent322$^+$ were lighter pink or white. Remarkably, a difference in sugar specificity was noticed as well. Pent240$^-$ and Pent269$^-$ were red on lactose plates, but pink on melibiose. Pent 302$^+$ was red on melibiose, but pink on lactose. Pent322$^+$ and Pent325$^-$

Table 1. Phenotype of lactose permease mutants on MacConkey agar

Strain	Melibiose		Lactose	
	0.4%	1%	0.4%	1%
pLAC184	red	red	red	red
D240N/R302L	white	pink	white	red center
D240N/H322Q	red	red	red	red
E269Q/R302L	white	pink	white	white
E269Q/H322Q	white	white	white	white
R302L/E325Q	red	red	red	red

Cells containing the indicated plasmid in strain HS4006/F′ were plated on MacConkey agar with the designated sugar, and color was analyzed after overnight growth at 37°C.

were slightly darker pink on lactose, and Pent319$^+$ was white on both. However, unlike the E269Q/H322Q double mutant, the inactivity of Pent319$^+$ does not stem from a defect in membrane expression. Western data showed that the Pent319$^+$ mutant is expressed at 48% of wild-type, similar to the expression levels of Pent302$^+$ and Pent240$^-$.

Previous studies have given examples of mutations in the lactose permease that lead to a change in sugar specificity. Specifically, an H322Q mutant led to a decrease in recognition of β-glucosides and β-galactosides, and an increased recognition of α-glucosuch as maltose [8, 13]. To examine if a change in specificity was taking place with the pentuple mutants, they were grown on MacConkey agar containing maltose, and also plates containing cellobiose (a β-glucoside). Wild-type lactose permease does not transport either of these sugars, yielding white colonies. Among the pentuple mutants, no difference in phenotype was seen, even after an extended incubation period of 40 hours (Table 3). Therefore, no significant change in sugar specificity has occurred.

With the striking result that a triple mutant K319N/H322Q/E325Q catalyzed proton transport with TDG [22], as well as the MacConkey plating results from Table 3, it became of interest to determine if any of the six ionizable residues are required for H^+/lactose cotransport. As stated in the Materials and Methods section, the hextuple mutant D240/N/E269Q/R302L/K319N/H322Q/E325Q was created through successive rounds of mutagenesis. Lactose transport capabilities of the hextuple mutant were assayed by plating on MacConkey Agar. The phenotype of the hextuple mutant is a very light pink on MacConkey plates containing either 1% lactose or 1% melibiose (Table 3), and white at 0.4% of each sugar, indicating a level of transport only slightly above colonies that contain the pACYC vector, but definitively higher than some of the pentuple mutants. As with the pentuple mutants, there was no increased uptake of maltose or cellobiose compared to wild type (Table 3).

Table 2. Genotypes of permease hextuple and pentuple mutants

Mutation	Short name
E269Q/R302L/K319N/H322Q/E325Q	Pent240$^-$
D240N/R302L/K319N/H322Q/E325Q	Pent269$^-$
D240N/E269Q/K319N/H322Q/E325Q	Pent302$^+$
D240N/E269Q/R302L/H322Q/E325Q	Pent319$^+$
D240N/E269Q/R302L/K319N/E325Q	Pent322$^+$
D240N/E269Q/R302L/K319N/H322Q	Pent325$^-$
D240N/E269Q/R302L/K319N/H322Q/E325Q	Hextuple

Uptake of [^{14}C]-Lactose by Mutants

Further quantitative analysis of lactose transport was done by measuring the uptake of ^{14}C-lactose. In cells that express β-galactosidase (i.e., the strain HS4006/F′), lactose transport is always "downhill" with respect to the outside concentration, because the sugar is metabolized as soon as it enters the cell. Downhill transport of [^{14}C]-lactose was carried out with the double mutants, and the results are shown in Fig. 1. Both the R302L/E325Q and D240N/H322Q catalyzed moderate levels of transport, around 20–25% of wild type at the steady state. The D240N/R302L was substantially less active, at only 1%, and there was no transport above background for both mutants containing substitutions at Glu-269.

Downhill lactose transport was conducted on the pentuple mutants (Fig. 2). An interesting result was that those mutants containing a single negative charge had a consistently higher level of net lactose transport than those containing a single positive charge. Pent269$^-$ had the highest level of downhill transport, which implies that lactose permease works best with a carboxyl group at position 269. From there, the mutants decreased in activity: Pent240$^-$ > Pent 322$^+$ > Pent 325$^-$ > Pent302$^+$ > Pent 319$^+$. Explaining these differences in transport may be done by examining the local environment of the lactose binding site in each mutant permease. Pent322$^+$ leaves an unpaired imidazole side chain, which has a pK_a close to neutrality, and transports downhill at an intermediate level compared to wild type. The guanido group left unpaired in the Pent302$^+$ mutant has a pK_a of 12, and the amino end of Pent319$^+$ has a pK_a of 11, which make these highly positive side chains at neutral pH. The unpaired carboxyl groups of Pent240$^-$, Pent269$^-$, and Pent325$^-$ were consistently better at transporting lactose. With a pKa of 4, these unpaired negative charges may provide a better cation binding site than either positive or neutral charges. This is consistent with the role these residues may play in cation recognition during lactose transport. However, it should be noted the Pent302$^+$ mutant did have a red phenotype on melibiose MacConkey agar. It is possible that while the local cation binding environment of the Pent302$^+$ mutant

Table 3. Phenotype of lactose permease mutants on MacConkey agar

Strain	Melibiose		Lactose		Maltose		Cellobiose	
	0.4%	1%	0.4%	1%	0.4%	1%	0.4%	1%
pLAC184	red	red	red	red	white	white	white	white
Pent240$^-$	pink	dark pink	red	red	white	white	white	white
Pent269$^-$	pink	pink	red	red	white	white	white	white
Pent302$^+$	red	red	white	pink	white	white	white	white
Pent319$^+$	white	white	white	white	white	white	white	white
Pent322$^+$	white	white	white	pink	white	white	white	white
Pent325$^-$	pink	pink	white	dark pink	white	white	white	white
Hextuple	white	light pink	white	light pink	white	white	white	white

Cells containing the indicated plasmid in strain HS4006/F′ were plated on MacConkey agar with the designated sugar, and color was analyzed after overnight growth at 37°C.

in the presence of lactose is perturbed, this may not be the case when other sugars are transported, such as melibiose. The hextuple mutant transported at approximately 2% of wild-type activity, between that of Pent325$^-$ and Pent 302$^+$ (Fig. 2). This confirmed the result of the MacConkey plating experiment, namely that the hextuple mutant carries out at least a low level of net transport.

In cells of the strain T184, which do not have functional β-galactosidase, lactose transport may be done "uphill" only if the transport of lactose is properly coupled to the internally directed proton gradient. While cells expressing the wild-type lactose permease quickly accumulate lactose to high levels, none of the double mutants studied could transport lactose above a 1:1 equilibrium (Fig. 3). This was the case even for those mutants with moderate downhill transport, such as R302L/E325Q. This result is similar to past studies on double mutants where a moderate to high level of net lactose transport did not correlate to a similar level of uphill transport [11, 20, 22]. It is evident that both members of a charge pair are often required for correct H^+/lactose coupling, even if certain substitutions allow for net transport. The pentuple mutants were also not able to carry out uphill lactose accumulation (Fig. 4). This was not an unexpected result, given that most double mutants and the triple mutant K319/N/H322Q/E325Q were previously shown to not have uphill activity [22]. Essentially, the Pent240$^-$, Pent269$^-$, and Pent302$^+$ mutants combine this triple mutant with another double combination, and it is very unlikely that the resultant mutant would carry out active accumulation. The hextuple mutant was similarly defective in uphill transport (Fig. 4).

Proton Transport Analysis

Direct proton transport studies were done by measuring changes in extracellular pH in response to sugar addition, as in Materials and Methods. In cells expressing an active lactose permease, protons are transported in as sugars are taken up, leading to an alkalinization of the external media. This alkalinization does not occur in cells without lactose permease. With the hextuple mutant, there was no alkalinization relative to background upon addition of either 10 mM lactose or 10 mM melibiose (Fig. 5). However, this negative result could be due simply to the extremely low levels of net sugar transport, and not a defect in proton transporter per se.

Based on [^{14}C]-lactose transport data from Fig. 3, proton transport measurements were done with the Pent269$^-$ and Pent240$^-$ mutants. Because these had higher levels of net lactose transport than the hextuple mutant, it was less likely that they would yield the unambiguous results of the hextuple mutant experiment. No proton transport was seen in the Pent269$^-$ mutant upon addition of either 10 mM lactose or 10 mM melibiose (Fig. 5). This is a particularly striking result, given the fact that downhill transport studies on Pent269$^-$ (Fig. 3) would seem to suggest that its level of net lactose transport should have been sufficient to detect proton transport, if the mutant was properly coupled. Remarkably, though, substrate-induced proton transport did occur in the Pent240$^-$ mutant upon addition of 10 mM melibiose, but not with 10 mM lactose. Given the MacConkey-plating results of Table 3, which implied Pent240$^-$ had a better recognition of lactose than melibiose, this is interesting, indeed. One explanation for this result is that the coupling site is still active in Pent240$^-$, but the five mutations have changed the site such that it couples melibiose transport, while lactose transport remains uncoupled.

Discussion

Studies of lactose permease mutants have shown that six ionic residues are critical for function [4, 11, 12, 20, 22, 24–29, 33, 35, 36, 41]. The characteristics of neutral mutant combinations in the lactose permease are summarized in Table 4. It is notable

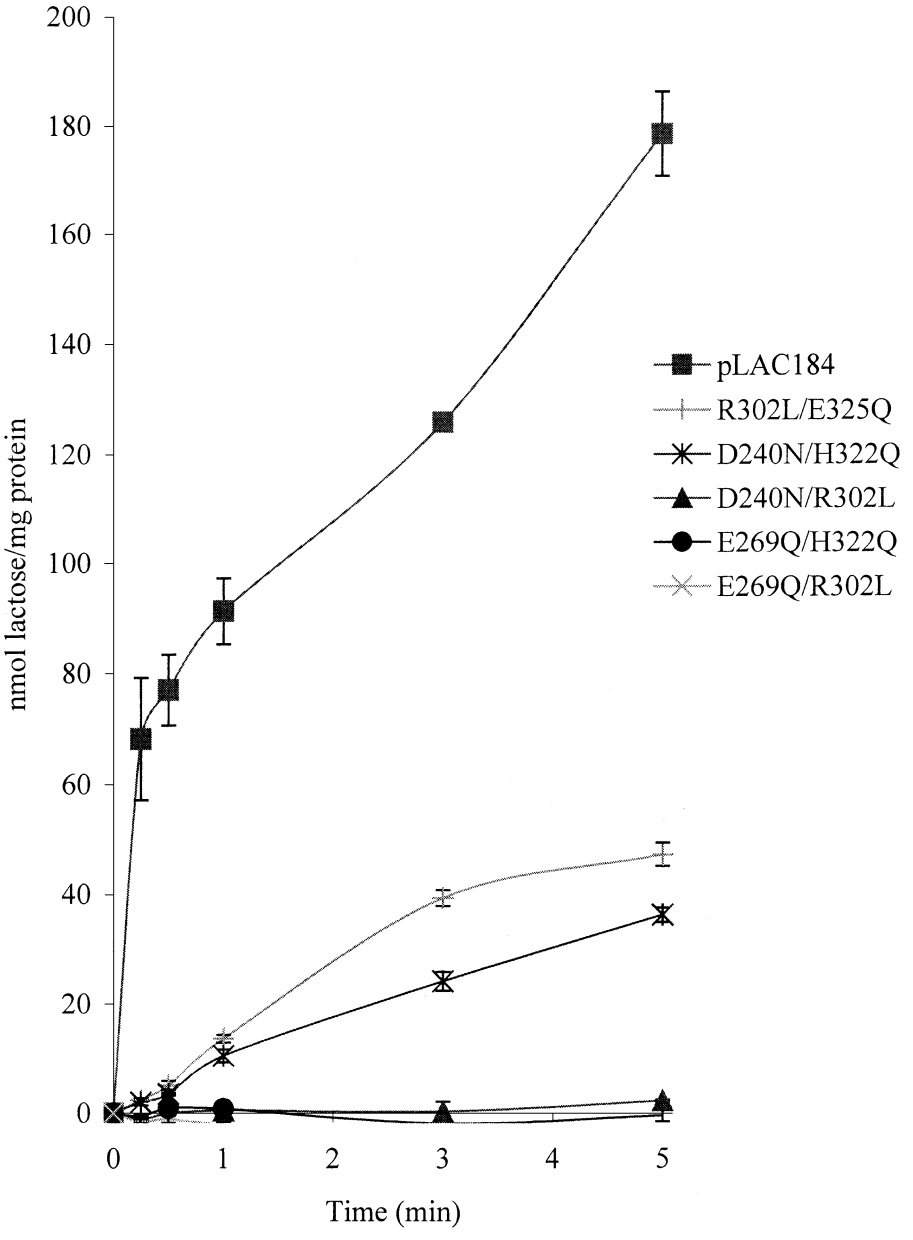

Fig. 1. Downhill transport of lactose permease double neutral mutants. Cells of the strain HS4006/F′ containing the indicated plasmid were prepared and uptake of ^{14}C lactose was measured as described in Materials and Methods.

that while some single mutants have moderate to high levels of downhill transport, all except H322Q at pH 6 are deficient in uphill accumulation. Double mutants (with the exception of D240/K319 mutants), as well as the pentuple mutants and hextuple mutant are similarly defective. The coupling of cation and lactose transport is likely a complicated system, with many factors at work. A lactose permease mutant may have normal levels of downhill transport, as well as substrate-induced cation transport, but still be unable to accumulate lactose against a concentration gradient, as is the case with the K319N mutation. This potential paradox can be explained by understanding how the binding of cations might control the coupling mechanism.

Two models for H^+/lactose coupling in the lactose permease have previously been published [20, 36]. Our laboratory proposed a simple mechanism for coupling, with the most important interactions taking place between Lys-319 and Glu-325, Lys-319 and Glu-269, and Lys-319 and Asp-240 [20]. While none of the data presented in this study refutes this mechanism, our concept of a simple, directly connected proton and lactose binding site requires modification. A second mechanism from another laboratory proposes that the most important interactions take place among His-322 and Glu-269 and Arg-302 and Glu-325 [36]. In the ground state of lactose permease, Arg-302 and Glu-325 interact, and a proton is shared between His-322 and Glu-269. Sugar binding transfers this proton to Glu-325,

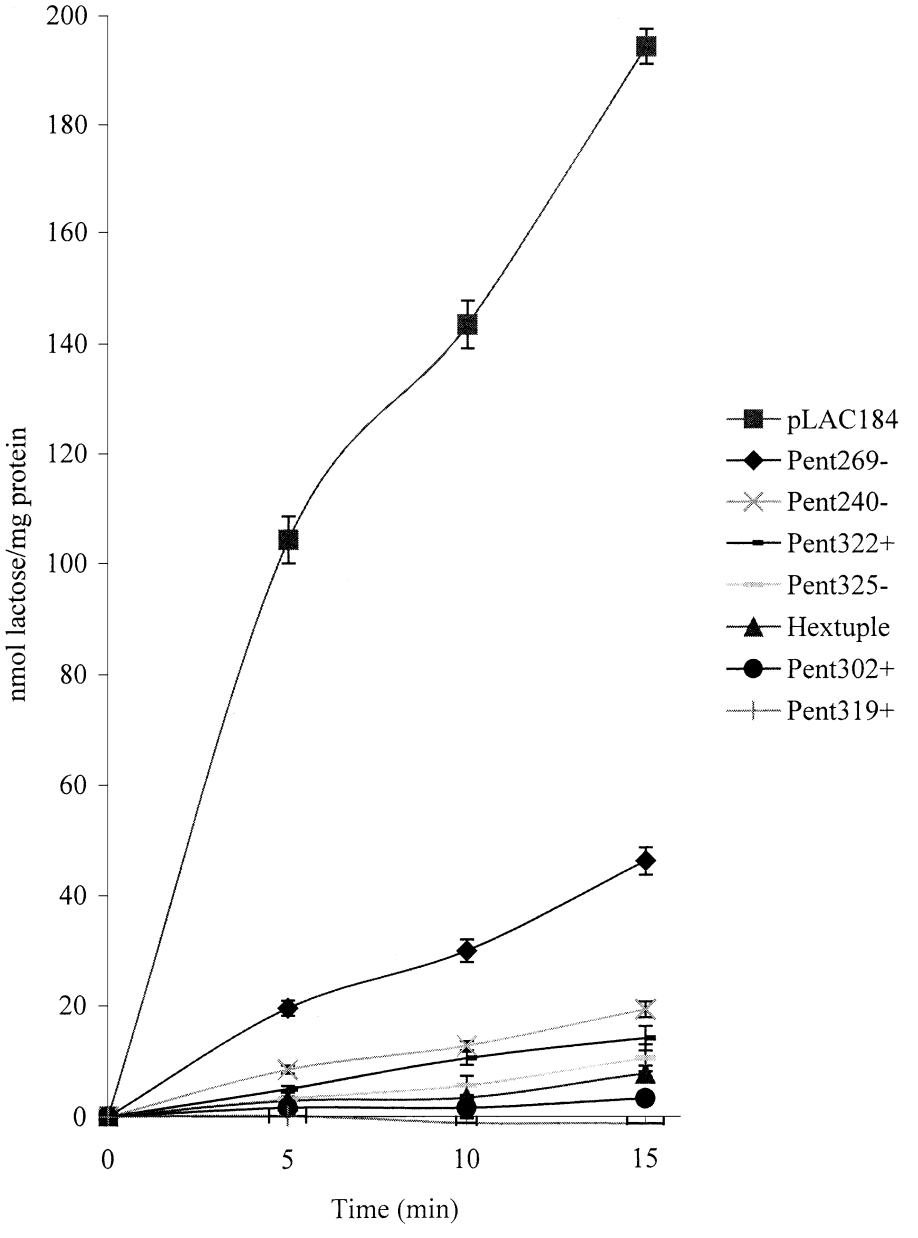

Fig. 2. Downhill transport of lactose permease hextuple and pentuple mutants. Cells of the strain HS4006 containing the indicated plasmid were prepared and uptake of ^{14}C lactose was measured as described in Materials and Methods.

breaking its interaction with Arg-302. The proton is then released from Glu-325, solute is released, and the permease recycles. It should be noted that in this mechanism, Lys-319 forms a stable salt bridge with Asp-240 throughout transport, which does not explain how mutations at Lys-319 would affect coupling.

The results presented in this work are inconsistent with the latter mechanism of proton/lactose coupling, because removal of one of the salt bridges (Arg-302 and Glu-325) does not abolish downhill transport. Further, because E269Q/H322Q seems completely defective in expression, it is inconclusive whether another pair could substitute for this interaction. One way to definitively explore this model would be to combine these neutral pairs into a single permease mutant (i.e., E269Q/R302L/H322Q/E325Q) and assess its activity. Along these lines, the Pent240$^-$ mutant does have all of these neutralizations in place (along with K319N) and retains a low level of downhill transport (Fig. 1), as well as substrate-induced cotransport (Fig. 3).

One model that can explain the effects of these mutants is that interactions among the six charged residues in the lactose permease may form a "coupling sensor". Interactions among the six residues are altered when zero, one or two substrates (meaning cations or lactose) are bound to the permease. As stated before, crystallography has shown these residues to be in close proximity to each other [1], and small rearrangements would transmit conformational changes that would alter the affinity for substrates

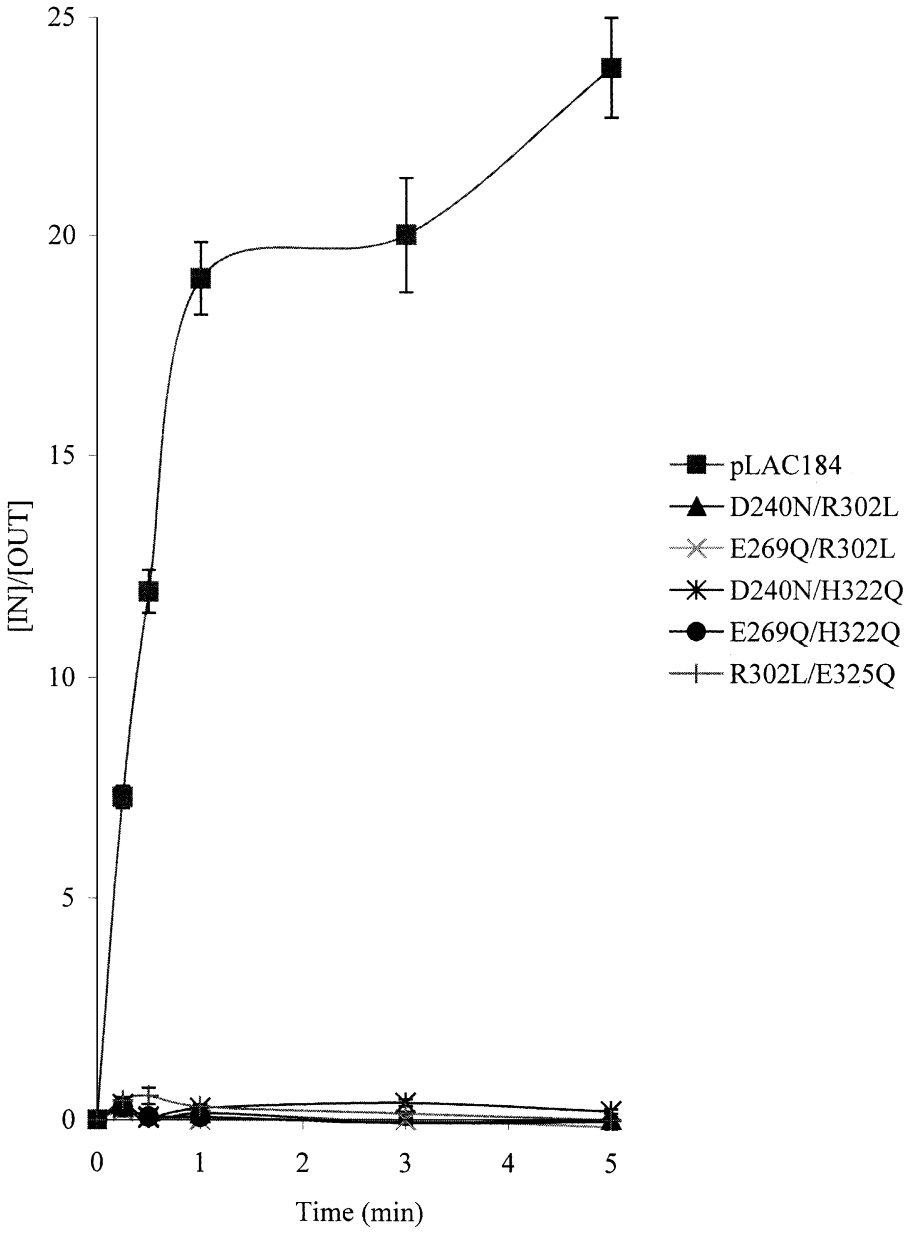

Fig. 3. Uphill transport of double neutral mutants. Cells of the strain T184 containing the indicated plasmid were prepared and uptake of ^{14}C lactose was measured as described in Materials and Methods.

and/or affect the velocity of the C1 → C2 interconversion. Thus, the sensor would form an ionic network for communicating this information between the sites for cation recognition and lactose binding, and areas that are important in controlling the C1 → C2 conformational change. Mutations removing one member (or both members) of an interacting pair might therefore simultaneously affect cation recognition, substrate affinity, and transport kinetics.

There are different ways that cation recognition (i.e., H^+, or H_3O^+) by the lactose permease could affect uphill transport of lactose. One explanation is that binding of a cation to the unloaded carrier may increase the affinity of lactose permease for its substrate. Upon cation release into the cytoplasm, the permease would lower its affinity and release the substrate as well. The carrier would then recycle for another round of transport. A mutation might alter the cation binding site, or some other region such that binding of cations no longer has the effect of changing the affinity for lactose. A second explanation is that cation binding has an effect on the velocity of the C1 → C2 conformational change that takes place during transport. In the fully loaded carrier, the C1 → C2 interconversion takes place at a fast rate in the wild-type transport cycle. If neither cations nor lactose are bound, the C1 → C2 change takes place at a slow rate, and if the carrier is singly loaded, the C1 → C2 change is essentially blocked. The higher concentration of cations outside the plasma membrane relative to inside favors the fully

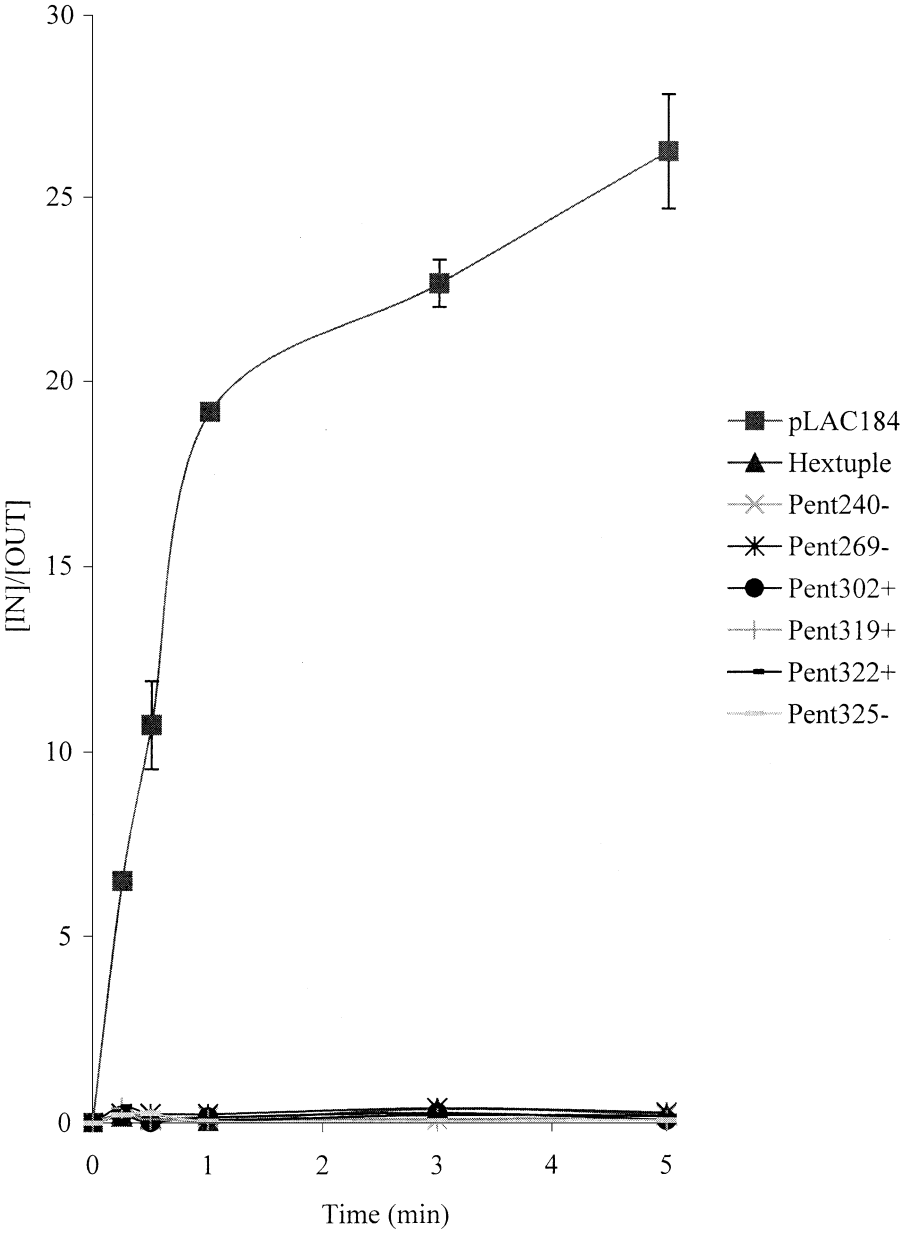

Fig. 4. Uphill transport of hextuple and pentuple mutants. Cells of the strain T184 containing the indicated plasmid were prepared and uptake of ^{14}C lactose was measured as described in Materials and Methods.

loaded carrier state, while recycling to the unloaded carrier ensures the overall cycle will transport lactose against its concentration gradient. However, if mutations in the carrier prevent it from discriminating among loaded, partially loaded, and unloaded carrier states, the effects on the C1 → C2 interconversion may be disrupted, and uphill transport will not occur. In the case of the K319N mutant, for example, cations are recognized and transported, but not exclusively with lactose—this results in a permease with a sugar-dependent proton leak [4, 20]. It should be noted that these two explanations presented above (i.e., effects on sugar binding and effects on the C1 → C2 interconversion) are not mutually exclusive, either or both may be fundamental to the coupling mechanism.

Along these lines, it is interesting to consider the effects of the mutations summarized in Table 4. Previous research has shown that K_m effects are seen in the single mutants R302L, K319N, and H322Q, all increasing the apparent K_m for lactose [20, 22, 29]. These K_m effects act in an additive manner; a K319N/H322Q double mutant has a higher K_m for lactose than either single mutant [22]. Studies also support V_{max} effects on coupling in lactose permease mutants. The proton leak of the K319N mutant suggests that it allows a rapid C1 → C2 change in all carrier states, meaning it acts as though two substrates are always bound. However, the E269Q mutant seems to never allow the C1 → C2 change to occur, suggesting that the mutant results in a permease that always acts as though only one substrate is bound. Since double

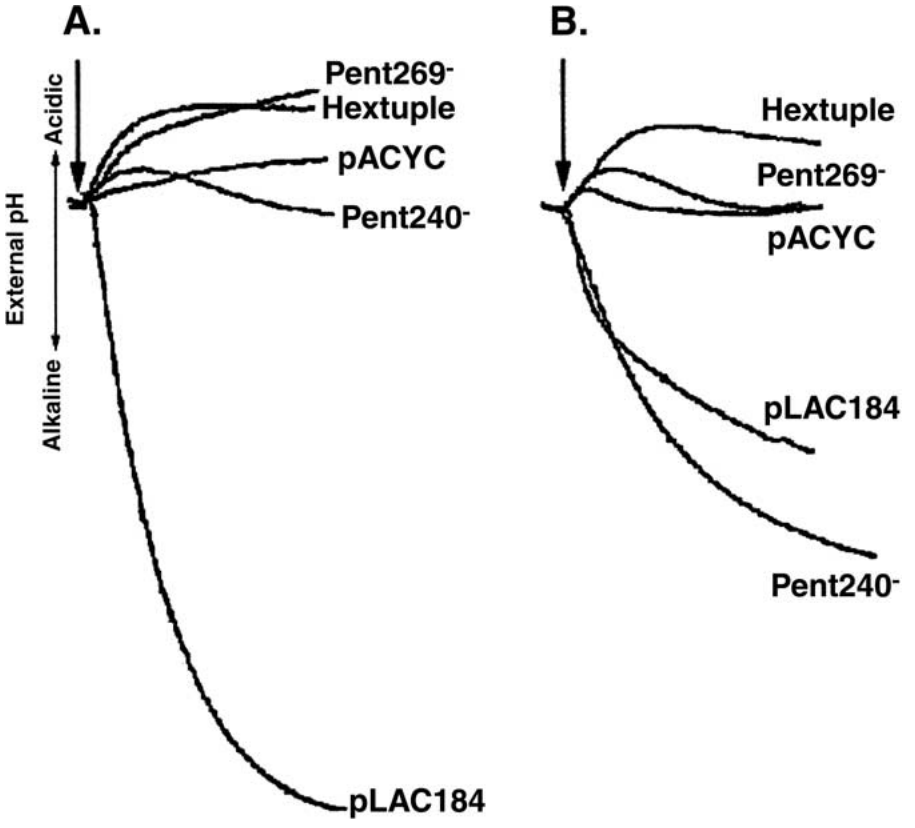

Fig. 5. Proton transport in lactose permease mutants. Cells containing the indicated plasmid were made anaerobic as described in Materials and Methods. At the time indicated by the large arrow, anaerobic sugar was added to a final concentration of 10 mM. Part *A*, lactose; Part *B*, melibiose.

Table 4. Characteristics of mutants at six key residues in the lactose permease

Mutant	Lactose transport[a]		Proton transport	Source
	Downhill	Uphill		
D240A	Mod.	None	Yes	[26]
E269Q	None	None	Yes	[12, 27]
R302L, R302S	Low	None	Yes	[29]
K319/N	High	None	Yes	[27]
H322Q	Low	Low, pH 6	Yes	[11]
E325Q	Low	None	No	[12, 20]
D240A/K319Q, D240A/K319A	High	Mod.	Yes	[26, 35]
E269Q/K319N	None	None	No	[27]
K319N/E325Q	High	None	Yes	[20]
H322Q/E325Q	Low	None	Yes	[22]
D240N/R302L	None	None	N. D.[b]	This study
D240N/H322Q	Mod.	None	N. D.	This study
E269Q/R302L	None	None	N. D.	This study
E269Q/H322Q	None	None	N. D.	This study
R302L/E325Q	Mod.	None	N. D.	This study
Pent240$^-$	Low	None	Yes, 10 mM Mel	This study
Pent269$^-$	Mod.	None	No	This study
Pent302$^+$	None	None	N. D.	This study
Pent319$^+$	None	None	N. D.	This study
Pent322$^+$	Low	None	N. D.	This study
Pent325$^-$	Low	None	N.D.	This study
Hextuple	Low	None	No	This study

[a] None = < 1%, Low = between 1% and 10%, Mod. = between 10% and 50%, High = greater than 50% of wild-type transport at steady-state levels.
[b] N. D. = not determined.

mutants containing E269Q are not rescued by other paired neutralizations (Table 4), Glu-269 appears to be extremely important to overall function, as has been seen in this and other studies [12, 27]. This is in contrast to Glu-325 mutants. While E325Q is severely defective in downhill transport, this defect can be relieved, for example, by pairing the mutation with R302L or K319N (Table 4). In the latter case, though, the proton leak still exists and uphill transport does not take place. E325Q single mutants catalyze efflux and exchange of lactose [12], so this defect seems to be in the proper recycling of the unloaded carrier—that is, the E325Q permease does not properly sense when zero substrates are bound to allow the C1 $\rightarrow$ C2 interconversion.

The question of the location of the actual cation (H^+ or H_30^+) binding site in the lactose permease remains unclear. Most importantly, the results of this study along with previous work [12, 22, 26, 29] indicate that none of the six ionic residues are (individually) necessary for cation/sugar transport. If the six residues are indeed directly involved with cation recognition, there must be a mechanistically flexible H^+ or H_30^+ binding site that does not require any particular residue. Recent work on the mechanism of proton uptake in the bacterial photosynthetic reaction center of *Rhodobacter capsulatus* suggests that this takes place through an extended hydrogen bond network [38]. The photosynthesis complex is formed of three proteins, termed L, M, and H. Electrons are shuttled to two quinone acceptors, termed Q_A and Q_B which then take up protons. Crystallization of the protein has shown that the Q_B quinone is surrounded by acidic side chains, including Glu-212 and Asp-213 from the L protein. The corresponding residues near Q_A are two alanines, Ala^{M246} and Ala^{M247}. Proton transfer through the Q_B quinone is normally high over a pH range from 5 to 11. However, the double mutant $Glu^{L212} \rightarrow Ala/Asp^{L213} \rightarrow Ala$ disrupts proton uptake by Q_B at above pH 8. Mutations near Q_A can restore wild-type transport at Q_B. A triple mutant $Glu^{L212} \rightarrow Ala/Asp^{L213} \rightarrow Ala/Ala^{M247} \rightarrow Tyr$ had a wild-type proton-uptake profile, and this happens even though the spacing between the two quinones is over 18 Å. This suggests a far-reaching hydrogen bond interaction between the two sites, and indicates that acidic residues may not be absolutely required for a functional proton translocation site. It is possible that a similar network is present in the lactose permease.

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