J. Membrane Biol. 181, 215–224 (2001)
DOI: 10.1007/s00232-001-0024-2

The Journal of
Membrane
Biology

A Triple Mutant, K319N/H322Q/E325Q, of the Lactose Permease Cotransports H^+ with Thiodigalactoside

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Received: 6 November 2000/Revised: 23 March 2001

Abstract. In a previous study, we characterized a lactose permease mutant (K319N/E325Q) that can transport H^+ ions with sugar. This result was surprising because other studies had suggested that Glu-325 plays an essential role in H^+ binding. To determine if the lactose permease contains one or more auxiliary H^+ binding sites, we began with the K319N/E325Q strain, which catalyzes a sugar-dependent H^+ leak, and isolated third site suppressor mutations that blocked the H^+ leak. Three types of suppressors were obtained: H322Y, H322R, and M299I. These mutations blocked the H^+ leak and elevated the apparent K_m value for lactose. The M299I and H322Y suppressors could still transport H^+ with β-D-thiodigalactoside (TDG), but the H322R strain appeared uncoupled for H^+/sugar cotransport.

Four mutant strains containing a nonionizable substitution at codon 322 (H322Q) were analyzed. None of these were able to catalyze uphill accumulation of lactose, however, all showed some level of substrate-induced proton accumulation. The level seemed to vary based on the substrate being analyzed (lactose or TDG). Most interestingly, a triple mutant, K319N/H322Q/E325Q, catalyzed robust H^+ transport with TDG. These novel results suggest an alternative mechanism of lactose permease cation binding and transport, possibly involving hydronium ion (H_3O^+).

Key words: Lactose permease—Symporter—Sugar transporter—Cotransporter

Introduction

Integral membrane proteins, known as cation/substrate cotransporters or symporters, facilitate the uptake of many solutes necessary for cellular function [12, 37]. Transported solutes include amino acids, metabolic intermediates, inorganic ions, and a variety of sugars. The lactose permease of *Escherichia coli* is an extensively studied symporter that has provided a model system for the study of structure and function in transport proteins [51]. The lactose permease is a cytoplasmic integral membrane protein that couples the transport of H^+ and lactose into the bacterial cytoplasm with a stoichiometry of 1:1 [54, 55]. The gene for the permease has been cloned and sequenced, and it encodes a polypeptide of 417 amino acids with a predicted molecular weight of 46,504 Daltons [6, 48]. Studies using purified lactose permease indicate that it functions as a monomer [29].

The lactose permease is a member of the Major Facilitator Superfamily (MFS) (*see* reference 38 for a recent review). Most members of the MFS are predicted to contain twelve membrane-spanning segments by hydropathicity analysis [15, 21]. In the case of the lactose permease, this model has been confirmed by gene fusions with alkaline phosphatase and β-galactosidase [7, 8]. The *N*- and *C*-terminal segments are cytoplasmic as shown by antibody binding studies [10, 24, 46]. The general homology of the two halves of the family members suggests an early evolutionary gene duplication, which led to the current superfamily of proteins [35]. Analysis of the MFS for hydrophobicity, amphipathicity, loop length, and potential salt-bridges between helices of the lactose permease has led our laboratory to propose a tertiary structure model [21]. This model shows the two halves as distinct domains which may move relative to one another to transport lactose [21, 25]. Other tertiary models have been proposed based on the proximity of amino acids suggested from biophysical and crosslinking studies [52, 53].

All 417 amino acid residues within the lactose permease have been altered by site-directed mutagenesis, and this approach has made it possible to identify residues that may play an important role in the mechanism of

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H^+/lactose symport and active lactose transport (*see* ref. 21 for a review). Six ionizable residues within the lactose permease have been implicated to play a central role, or an auxiliary role, in the mechanism of H^+/lactose coupling [4, 9, 11, 16, 23, 27, 31, 32, 42, 49]. These residues are Asp-240, Glu-269, Arg-302, Lys-319, His-322, and Glu-325. In addition, Glu-126 and Arg-144 may form an essential salt bridge [19, 44].

With regard to the actual cation recognition site in the lactose permease, nonionizable substitutions at codons 240, 269, 302, 319, and 322 have been shown previously to catalyze H^+/lactose symport, indicating that they are not absolutely required for H^+ binding or transport [4, 9, 16, 23, 27, 31, 32, 42, 49]. Single, nonionizable substitutions at codon-325 can catalyze a normal exchange of lactose, but are almost completely defective in unidirectional transport [9, 16]. Due to the very low rate of net transport, these previous studies have not definitively determined if codon-325 substitutions can catalyze net H^+ transport. However, the efflux reaction appears pH insensitive, suggesting that codon-325 mutations are unable to recognize H^+ ions [16]. In a very recent study, we obtained the surprising result that an acidic residue at position 325 is not obligatorily required for H^+ transport via the lactose permease [26]. When a nonionizable substitution at codon-325 was coupled with a nonionizable substitution at codon-319, the velocity for unidirectional net transport was substantially restored, and the double K319N/E325Q mutant was able to catalyze H^+/sugar symport to near wild-type levels. In light of these results, we speculated that one or more other residues might act as auxiliary H^+ binding sites when position 325 contains a nonionizable amino acid. The goal of the current study was to identify such a putative auxiliary H^+ binding site and/or to understand how the K319N/E325Q strain is able to cotransport H^+ with lactose.

Materials and Methods

Reagents

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

Bacterial Strains and Methods

For downhill lactose transport, *Escherichia 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 [47]. The plasmids used in this study are derivatives of the *lacY*-carrying plasmid pLac184 (*see* ref. 16).

Plasmid DNA was isolated by the NaOH-sodium dodecyl sulfate method [2] and introduced into the appropriate bacterial strain by the $CaCl_2$ transformation procedure of Mandel and Higa [34].

Stock cultures of cells were grown in YT media [36] 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 to induce the synthesis of the lactose permease.

Sugar Transport Assays

Midlog cells were washed in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4 and resuspended in the same buffer to a density of approximately 0.5 mg protein per ml. Cells were then equilibrated at 30°C and radioactive sugar was added at the designated concentrations. At appropriate time intervals, 0.2 ml aliquots were withdrawn and filtered over a membrane filter (pore size = 0.45 μm). The external medium was then washed away with 5–10 ml of ice-cold phosphate buffer, pH 7.0, by rapid filtration. As a control, the *lacY* strain, HS4006/$F'I^QZ^+Y^-$/pACYC184, was also assayed for radiolabeled sugar uptake to obtain an accurate value for nonspecific sugar uptake.

Kinetic Calculations

Apparent K_m and V_{max} values for lactose transport were determined by observing initial rates of downhill transport at four lactose concentrations. Since the pH is constant, the transport velocity follows Michaelis-Menten kinetics with respect to lactose concentration [45]. The apparent K_m has also been previously shown to vary with a change in sugar specificity, i.e., a change in the affinity of the permease for lactose [50]. Results were plotted by the method of Hanes-Woolf, graphing [S]/v versus [S] [45]. Data from three independent trials were averaged to give a final value for apparent K_m and V_{max}, plus or minus SEM.

H^+ Transport

For the H^+ transport experiments of Figures 1 and 3, cells were grown to mid-log phase and washed twice with 120 mM KCl. The cells were then suspended in 120 mM KCl and 30 mM potassium thiocyanate to a density of approximately 5 mg protein per ml. 2.5 ml of cells were placed in a closed vessel with a lid containing tight-fitting openings for the insertion of a pH electrode, the introduction of argon, and the insertion of (gas-impermeable) Hamilton syringes. Cells were made anaerobic under a continuous stream of argon for at least 30 min. To initiate sugar-induced H^+ transport, an anaerobic solution containing lactose or TDG was added to a final concentration of 2 mM. The change in external pH was measured with a Radiometer pH meter (PHM82) and electrode (GK2401C). Changes in pH were continuously recorded on a Radiometer chart recorder which had been modified to expand the scale of pH changes to a range where a 0.1 unit pH change caused a 10 cm deflection in the chart recording.

Construction of Plasmids Carrying Mutant Permeases

In a previous study, we have described the construction of mutant *lacY* strains carrying single mutations at codon 319 (K319N), codon 325

The abbreviations used are: TDG, thiodigalactoside, and TMG, methyl-β-D-thiogalactopyranoside.

(E325Q), and the double mutant strain, K319N/E325Q [26]. Using mutagenic PCR primers, we generated a mutation at codon 322, which changed histidine to glutamine. The mutagenic primers also eliminated a unique *Nde*I site and produced a unique *Pst*I site. To make this single H322Q strain, a mutagenic primer running in the clockwise direction was used with a second nonmutagenic primer that anneals across a unique *Sac*II site within the plasmid. The template DNA was the plasmid, pLac184, carrying the wild-type *lacY* gene. This PCR reaction produces a 3.8 kbp fragment carrying codon 319. A mutagenic primer across codon 322 running in the counterclockwise direction was also used with a nonmutagenic primer across the *Sac*II site to produce a 2.8 kbp fragment carrying codon 325. Ligation of the 3.8 kbp and 2.8 kbp fragments to each other produces a pLac184 derivative that has codon 322 changed from histidine to glutamine and also has the *Nde*I site changed to a *Pst*I site.

The same mutagenic PCR primers used to make this single H322Q mutation were also used to make double mutants K319N/H322Q and H322Q/E325Q except that the single K319N or E325Q mutant DNA was used as the template DNA. The triple mutant K319N/H322Q/E325Q was made by digestion of the corresponding double mutants with *Sac*II and *Pst*I and the ligation of the 3.8 kbp fragment from the K319N/H322Q strain to the 2.8 kbp fragment from the H322Q/E325Q strain.

DNA Sequencing

The presence of the mutations within the plasmids was confirmed by the sequencing of double-stranded plasmid DNA isolated using the PERFECTPREP plasmid isolation kit (Eppendorf, Westbury, NY) and sequenced according to Kraft et al. [30].

Membrane Isolation and Immunoblot Analysis

For Western blot analysis, T184 cells containing the appropriate plasmid were grown as described above for the sugar transport assays. Ten ml of late log phase cells were pelleted by low speed centrifugation, and resuspended in 800 μl MTPBS buffer (in mM: 150 NaCl, 16 Na_2HPO_4, 4 NaH_2PO_4) with protease inhibitors PMSF (25 mg/ml) and TPCK (0.1 mg/ml). Cells were freeze-thawed twice in liquid nitrogen and subjected to three rounds of sonication. The membrane fraction was collected by centrifugation (10 min, 11,000 × g) and resuspended in 100 μl MTPBS plus protease inhibitors. Triton X-100 was added to a concentration of 1%. Fifty μg total membrane protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were electroblotted to nitrocellulose and then probed using an antibody that recognizes the carboxyl-terminus of the lactose permease. The amount of lactose permease was then determined by laser densitometry. All of the mutant strains constructed for this study had levels of permease that were very similar to wild-type. Normalized to wild-type (at 100%), they were as follows: H322Q, 99.5%, K319N/H322Q, 100.7%, H322Q/E325Q, 104.3%, and K319N/H322Q/E325Q, 115.5%.

Results

Suppressor Analysis

As mentioned in the Introduction, previous studies have shown that the K319N/E325Q mutant strain catalyzes H^+/sugar symport. These results indicate that H^+ ions are bound and efficiently transported via this strain. In addition, the K319N/E325Q strain catalyzes a sugar-dependent H^+ leak. The leak occurs by the cotransport of H^+ and sugar into the cell in conjunction with the uncoupled export of sugar (*see* ref. 13). The sugar-dependent H^+ leak also indicates that the K319N/E325Q strain catalyzes robust H^+/sugar cotransport. When streaked on YT plates containing the nonmetabolizable sugar, TDG, the growth of the K319N/E325Q strain is severely inhibited due to the rapid import of H^+ ions. This collapses the H^+ electrochemical gradient. Furthermore, this phenotype provides a method to identify suppressor mutations that do not catalyze the sugar-dependent H^+ leak, as described below.

Starting with the K319N/E325Q strain, there are several ways that an additional (third) mutation in the *lacY* gene could prevent an H^+ leak. One possibility is that a mutation could prevent H^+ binding and/or transport. Such a mutation may identify residues that play a role in H^+ binding or transport. This is the category of mutations that were sought in the current study. Secondly, however, a mutation could block the sugar-dependent H^+ leak by preventing sugar binding or transport. Finally, a mutation could prevent lactose permease expression and thereby stop the H^+ leak. To distinguish among these various possibilities, MacConkey plates can be used. MacConkey plates contain a pH indicator dye, a sugar (as the sole fermentable substrate), and nonfermentable energy sources. All *E. coli* strains can grow on these plates, but only strains that take up and ferment the sugar will form red colonies, because they excrete acidic waste products and thereby lower the pH. Those strains that are unable to transport the sugar form white colonies. Therefore, mutations in the lactose permease that prevent sugar binding or transport or prevent lactose permease expression would be expected to form white colonies on lactose MacConkey plates. By comparison, a mutation that blocks H^+ binding or transport without inhibiting sugar binding and transport would form red colonies.

With these ideas in mind, we developed a plating strategy to identify mutations in the K319N/E325Q strain that prevented the H^+ leak but did not block sugar transport or prevent lactose permease expression. The K319N/E325Q strain was streaked on 1% melibiose MacConkey plates containing 2.0 mM TDG. (*Note:* melibiose is an α-galactoside that is also transported by the lactose permease. We used melibiose instead of lactose because our parent strain is deleted for both the genes that encode the melibiose permease and β-galactosidase). When streaked on these plates, the parent strain grows extremely slowly. After several days, fast-growing colonies are seen. While most of these colonies were white, we did obtain several fast growers that were very red. These were saved for further study.

Six independent red mutants (Table 1) were sub-

Table 1. Third site suppressor mutations isolated from the K319N/E325Q parent strain*

Third Site Mutation	Number of Isolates
H322R	3
H322Y	2
M299I	1

* In addition to the third site mutation shown here, these strains also retained the K319N and E325Q mutations.

jected to DNA sequencing as in Materials and Methods. All of these mutants had a third site mutation, in addition to the mutations at codons 319 and 325. Five of the six mutations occurred at codon 322, changing His-322 to arginine or tyrosine. The other mutation changed Met-299 to an isoleucine.

These novel suppressor strains were analyzed for their ability to transport lactose and their ability to transport H^+ ions upon the addition of sugar. Table 2 shows the results of a kinetic analysis in which the downhill transport of lactose was measured in the wild-type strain, the K319N/E325Q parent strain, and the suppressor strains. As seen here, the wild-type strain transports lactose with an apparent K_m of 0.6 mM and an apparent V_{max} of 169.6 nmol lactose $\cdot$ min^{-1} $\cdot$ mg protein^{-1}. The suppressors had elevated K_m values ranging from 1.0 mM to 3.0 mM, and V_{max} values that were less than 10% of the wild-type level.

The suppressors were also analyzed for the ability to transport H^+ in conjunction with sugar. External pH measurements of cell suspensions upon substrate addition were measured as in Materials and Methods, with the results in Fig. 1. The wild-type permease rapidly transported H^+ upon the addition of TDG. Two of the three mutant suppressor strains (M299I and H322Y) also showed substantial levels of H^+ transport upon the addition of TDG. In contrast, the H322R strain showed negligible alkalinization when TDG was added.

Construction of Strains Containing a Glutamine at Position 322

Thus far, the results indicate that mutations at positions 299 and 322 substantially affect the kinetics of lactose transport, and also prevent the sugar-dependent H^+ leak. Two of the three suppressors were able to cotransport H^+ with sugar, while the H322R was uncoupled for sugar transport. Overall, the results indicate that the suppressor mutations have significant effects on H^+ transport and H^+/sugar coupling. Since two of the suppressors were located at position 322, one explanation for these results is that His-322 plays a role in H^+ transport and/or H^+/sugar coupling. In this regard, His-322 lies between Lys-319 and Glu-325 on the same face of transmembrane segment 10. The M299I mutation could also mediate its effect through His-322; according to tertiary models, Met-299 and His-322 are found on faces of adjacent helices and positions 299 and 322 are predicted to project toward each other [20].

To further explore the role of His-322, it was important to establish if an ionizable side chain at position 322 is needed to facilitate H^+/sugar cotransport in the K319N/E325Q background. The three suppressor strains still contain an ionizable side chain at position 322, although the H322Y and H322R strains have side chains that deprotonate at a relatively high pH. Even so, the ability of the M299I and H322Y strains to cotransport H^+ with TDG could involve the binding and release of H^+ ions via an ionization reaction at position 322. To determine if an ionizable side chain at position 322 is required for H^+ transport, we changed His-322 to a glutamine by site-directed mutagenesis as described in Materials and Methods. We then analyzed the single H322Q strain, the double mutant strains K319N/H322Q and H322Q/E325Q, and the triple mutant strain K319N/H322Q/E325Q with regard to sugar and H^+ transport.

Phenotype on MacConkey plates was used as an initial, qualitative, measure of transport activity of the wild type and mutant strains. We also compared these strains with single mutations at codons 319 and 325. As shown in Table 3, all of the strains, except the single E325Q strain, could effectively transport lactose (a β-galactoside) or melibiose (an α-galactoside), as evidenced by their red or pink phenotypes. When the E325Q mutation was coupled with either the K319N mutation, the H322Q mutation, or both mutations, the defect in sugar transport was substantially alleviated compared to the single E325Q strain.

Table 3 also shows the growth of these strains on YT plates with and without TDG. Lactose permease mutants that catalyze a sugar-dependent H^+ leak are unable to grow when these rich plates are supplemented with TDG (*see* also ref. 13). As seen here, the K319N single mutant and the K319N/E325Q double mutant strains cannot grow on YT + TDG plates because they catalyze a robust sugar-dependent H^+ leak. The single H322Q mutation does not promote an H^+ leak by itself. Furthermore, when coupled with the K319N mutation or both the K319N and E325Q mutations, the presence of the H322Q mutation appears to block the sugar-dependent H^+ leak as evidenced by the growth of the K319N/H322Q and K319N/H322Q/E325Q strains on the YT + TDG plates. These results are consistent with our suppressor analysis which showed that other mutations at position 322 (i.e., H322R and H322Y) prevent H^+ leakage in the K319N/E325Q background.

To obtain a quantitative description of the transport process, in vitro transport experiments were conducted. Table 4 shows the results of a kinetic analysis of these strains, and the results are compared with a previous kinetic analysis of the K319N, E325Q, and K319N/

Table 2. Kinetic analysis of wild-type, parent, and suppressor strains

Strain	apparent K_m* ± SEM (mM)	apparent V_{max}* ± SEM (nmol · mg^{-1} · min^{-1})	Reference
pLac184 (wild-type)	0.6 ± 0.2	169.6 ± 13.1	This study
pK319N/E325Q (parent)	0.4 ± 0.1	18.5 ± 3.6	26
pK319N/E325Q/H322R	2.7 ± 1.3	9.2 ± 2.8	This study
pK319N/E325Q/H322Y	3.0 ± 0.9	7.1 ± 1.3	This study
pK319N/E325Q/M299I	1.0 ± 0.2	2.1 ± 0.5	This study

* The K_m and V_{max} values were determined at 30°C as described in Materials and Methods.

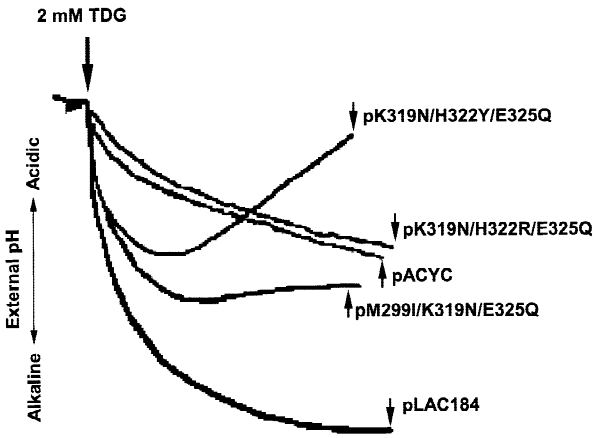

Fig. 1. Sugar-induced H^+ transport in the parent and suppressor strains. H^+ transport was measured in the strain HS4006/F′I^{Q}Z$^+$Y$^-$ containing the designated plasmids as described in Materials and Methods. The pACYC plasmid is a negative control that does not carry the *lacY* gene. The pLAC184 plasmid is a derivative of pACYC184 that carries the wild-type *lacY* gene. The other plasmids carry a *lacY* gene with the designated mutations.

E325Q strains. For these experiments, plasmids containing the wild-type or mutant *lacY* genes were transformed into a *lacZ*$^+$ *E. coli* strain (HS4006/F′Z$^+$Y$^-$), which is β-galactosidase positive. When lactose enters the cell, it is rapidly metabolized so that the external lactose concentration is always higher than the internal concentration [41]. Therefore, this in vitro assay measures lactose transport as it moves from a higher to lower concentration or "downhill."

As shown in Table 4, the wild-type permease catalyzed downhill transport with an apparent K_m of 0.6 mM and an apparent V_{max} value of 169.6 nmol lactose · min^{-1} · mg protein^{-1}. The K319N and H322Q single mutants both had elevated K_m values and moderately defective V_{max} values. The single E325Q mutant had a slightly lower K_m, but a V_{max} value that was less than 1% of the value of the wild-type strain. The coupling of these single mutations in the double mutant strains had some interesting effects. When K319N was coupled with H322Q, the apparent K_m was about the same, at 1.6 mM, but the apparent V_{max} decreased substantially to 7.1 nmol lactose · min^{-1} · mg protein^{-1}. Compared to the single mutations at codons 319 and 322, the defect in sugar binding is similar in the K319N/H322Q strain (as evidenced by K_m), but the defect in transport is greater (as shown by lower V_{max}).

When the K319N or H322Q mutations were coupled with the E325Q mutation, quite different results were observed. As shown in Table 4, when the K319N mutation was coupled with the E325Q mutation, the apparent K_m was similar to wild-type, meaning that the double mutant has a near wild-type affinity for lactose. By comparison, this effect is not seen in the H322Q/E325Q strain, which has a relatively high apparent K_m value of 2.5 mM. The apparent V_{max} values in the K319N/E325Q and H322Q/E325Q strains are intermediate compared with the corresponding single mutant strains. These double mutants have V_{max} values that are much higher than the single E325Q strain, but significantly lower than the single K319N or H322Q strains. Finally, when all three mutations are coupled in the triple mutant strain, K319N/H322Q/E325Q, the K_m value is relatively high, and the V_{max} value is slightly higher than any of the corresponding double mutant strains. These results indicate interestingly that having three mutations instead of two does not have an additional detrimental effect on V_{max}, and that the effect on K_m is no worse than the effect seen in the K319N/H322Q strain.

A second way to evaluate lactose transport in wild-type and mutant strains is via an "uphill" transport assay. The wild-type lactose permease obligatorily couples the transport of H^+ and lactose with a 1:1 stoichiometry. This coupling enables the bacterium to actively accumulate lactose against a concentration gradient. Therefore, this uphill accumulation of lactose provides a way to examine the coupling between H^+ and lactose transport. To conduct an uphill transport assay, plasmid DNA was transformed into a bacterial strain that is β-galactosidase negative. The assays were done at a low external lactose concentration (0.1 mM) and the intracellular accumulation of [^{14}C]-lactose was measured at various time points by rapid filtration. The results of an uphill transport assay are shown in Fig. 2. As seen here, the wild-type permease catalyzed uphill transport to accumulation lev-

Table 3. Phenotypes on Plates

Plasmid[a]	Plate Type						Reference
	Lactose MacConkey[b]		Melibiose MacConkey[b]		YT Plates[c]		
	0.4%	1.0%	0.4%	1.0%	No Sugar	+2 mM TDG	
pLac184	red	red	red	red	+	+	This study
pK319N	red	red	red	red	+	−	26
pH322Q	red	red	red	red	+	+	This study
pE325Q	white	white	white	white	+	+	26
pK319N/H322Q	pink	red	pink	pink	+	+	This study
pK319N/E325Q	pink	red	white	pink	+	−	This study
pH322Q/E325Q	pink	red	pink	red	+	+	This study
pK319N/H322Q/E325Q	pink	red	red	red	+	+	This study

[a] The plasmids were introduced into strain HS4006/F′$I^QZ^+Y^-$.
[b] A red phenotype indicates the ability to ferment the added sugar whereas a white phenotype indicates a lack of significant fermentation. Pink reflects an intermediate level of fermentation.
[c] A + designation indicates normal growth. A − designation indicates no growth.

Table 4. Kinetic analysis of wild-type and mutant strains

Strain	Apparent K_m* ± SEM (mM)	Apparent V_{max}* ± SEM (nmol · mg^{-1} · min^{-1})	Reference
pLac184	0.6 ± 0.2	169.6 ± 13.1	This study, from Table 2
pK319N	1.3 ± 0.3	48.3 ± 11.2	26
pH322Q	1.7 ± 0.4	28.7 ± 6.1	This study
pE325Q	0.3 ± 0.1	1.3 ± 0.3	26
pK319N/H322Q	1.6 ± 0.2	7.1 ± 1.3	This study
pK319N/E325Q	0.4 ± 0.1	18.5 ± 3.6	26
pH322Q/E325Q	2.5 ± 1.0	9.6 ± 2.8	This study
pK319N/H322Q/E325Q	3.5 ± 2.5	21.2 ± 11.8	This study

* The K_m and V_{max} values were determined at 30°C as described in Materials and Methods.

els that were over 30-fold higher than the external lactose concentration. However, the single, double, and triple mutants were completely defective in their ability to actively transport lactose. Except for the E325Q strain, which has a very low rate of downhill transport, all of these strains have significant rates of downhill transport (refer back to Table 4). Therefore, their inability to actively transport lactose against a concentration gradient is due to an anomaly in H^+/lactose coupling, not due to an inability to transport lactose across the membrane. This could be because of an inability to recognize and transport protons, or it could be the result of the previously discussed proton leak transporting protons or lactose alone.

Proton transport in response to sugar addition was directly measured as before with a pH electrode (Fig. 3). As expected, when lactose was added, the wild-type strain exhibited a rapid alkalinization of the medium due to the cotransport of H^+ and sugar into the bacterial cytoplasm (*see* Fig. 3*A*). After a short period of time, this alkalinization was followed by an acidification due to the metabolism of lactose and the excretion of acidic products. The addition of the nonmetabolizable sugar TDG also promoted a rapid alkalinization (*see* Fig. 3*B*).

In the current study, we examined the ability of H322Q mutant strains to cotransport H^+ with these two sugars. Note that a previous study showed that the K319N and K319N/E325Q strains could cotransport H^+ with lactose or TDG [26]. In the case of lactose, significant H^+ sugar cotransport was not observed with any of the strains carrying an H322Q mutation (*see* Fig. 3*A*). With the H322Q strains, there was not a significant alkalinization with lactose, although the sugar was getting into the cells as evidenced by the later acidification phase. With TDG, however, very different results were obtained. All of the strains carrying an H322Q mutation could transport H^+ with TDG, though to varying degrees. Particularly striking was the result that the triple mutant with nonionizable side chains at all three positions, K319N/H322Q/E325Q, showed robust H^+/TDG cotrans-

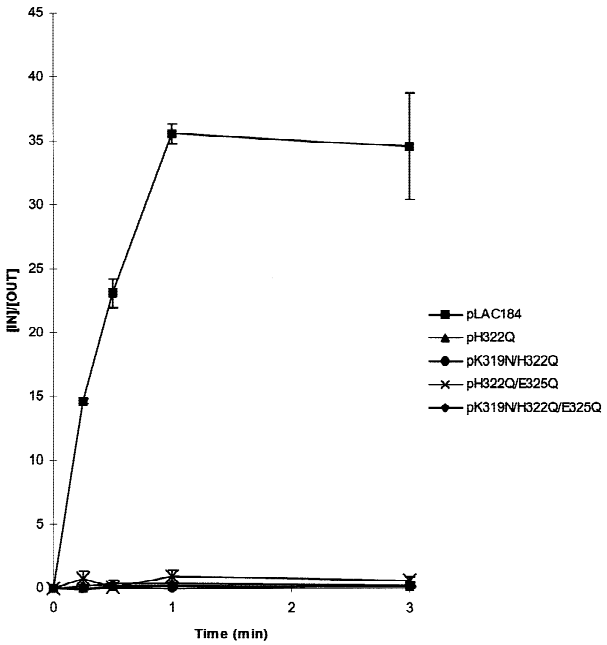

Fig. 2. Uphill lactose transport by wild-type and mutant strains. The uptake of [^{14}C]-lactose was measured as described in Materials and Methods. Lactose uptake was performed at an external lactose concentration of 0.1 mM in strain T184 containing the wild-type *lacY* plasmid or mutant plasmids as designated to the right of the figure.

port. These results indicate that no ionizable side chain at any of these positions is essential for H^+/sugar cotransport.

Discussion

The goal of the current study was to gain a greater understanding of how the lactose permease recognizes and transports H^+ ions. The binding and transport of cations has been studied in a variety of transporters. In the case of ion pumps, it has been found that acidic residues often play a central role in cation recognition. For example, in bacteriorhodopsin, an aspartic acid residue at position 96 acts as an H^+ acceptor on the cytoplasmic side of the membrane, and an aspartic acid at position 85 releases the H^+ to the external medium [33]. Other residues, such as Arg-82, Glu-194, Glu-204, and Asp-212 also play auxiliary roles in H^+ binding and transport. In subunit *c* in the F_0 portion of the F_0F_1-ATPase of *E. coli*, an aspartic acid at position 61 is essential for H^+ binding [14]. In addition, residues at positions 28 and 62 play a role in the binding of Li^+ or Na^+ via the F_0F_1-ATPase. For P-type ATPases, such as the Ca^{+2}-ATPase of the sarcoplasmic reticulum, multiple residues on TMS-4, TMS-5, TMS-6, and TMS-8 are thought to coordinate the binding of two Ca^{2+} ions [1]. These residues include Glu-309, Glu-771, Asn-796, Thr-799, Asp-800, and Glu-908.

For cation/solute symporters, less information is available regarding the cation binding site. Cation binding has been extensively investigated in the melibiose permease, which cotransports H^+, Li^+, and/or Na^+ with α- and β-galactosides (*see* ref. 39 for a review). In the melibiose permease, melibiose is transported with H^+, Na^+, and Li^+, while methyl-β-D-thiogalactopyranoside (TMG) is transported with Na^+ or Li^+, but not with H^+. These results are consistent with the idea that the cation and sugar binding sites are overlapping so that only certain combinations of cation and sugar can be bound and transported. The cation binding site of the melibiose permease in *E. coli* has been studied through site-directed mutagenesis, suppressor analysis, and chimera analysis with the melibiose permease from *Klebsiella pneumoniae* [17, 18, 22, 40, 56]. Substitutions of aspartic acid residues at positions 19, 56, 59, and 124 lead to a loss of Na^+-coupled transport without greatly affecting TMG recognition. Other residues, such as Asn-58 may also play a role in cation recognition.

The cation binding site in the lactose permease still remains an unresolved question. The cation is generally described as H^+ ion, though it remains a possibility that hydronium ion (H_3O^+) is the transported species. If hydronium ions are transported, the cation binding site might involve the coordination of a hydronium ion by several residues [3]. Since backbone carbonyl groups may be involved with coordination, it may be difficult to identify such a coordination site via site-directed mutagenesis studies. With regard to H^+ as the putative transported species, there are various possibilities to explain the mechanism of H^+ binding and its symport with lactose. The simplest mechanism involves a single ionizable residue within a transmembrane segment that functions as an H^+ binding site. Following H^+ and sugar binding, the lactose permease would then undergo a conformational change to release H^+ and lactose on the opposite side of the membrane. Although such a scenario remains possible, evidence is mounting that the H^+ binding site is not as simple as a single amino acid residue.

One result of the current study that may seem paradoxical is that while these mutants can catalyze downhill transport of lactose, as well as substrate-induced proton accumulation, they are unable to actively accumulate lactose. An explanation for this is that the overall transport cycle is the sum of many individual reactions. While those reactions regulating the protonmotive force that drives uphill accumulation of sugars may be inhibited in certain mutants, this does not mean that uphill accumulation of protons (driven by lactose) is necessarily abolished.

While many studies have focused on the effects of mutations at Asp-240, Glu-269, Arg-302, Lys-319, His-322, and Glu-325, the elimination of ionizable side chains at any of these positions does not prevent H^+/

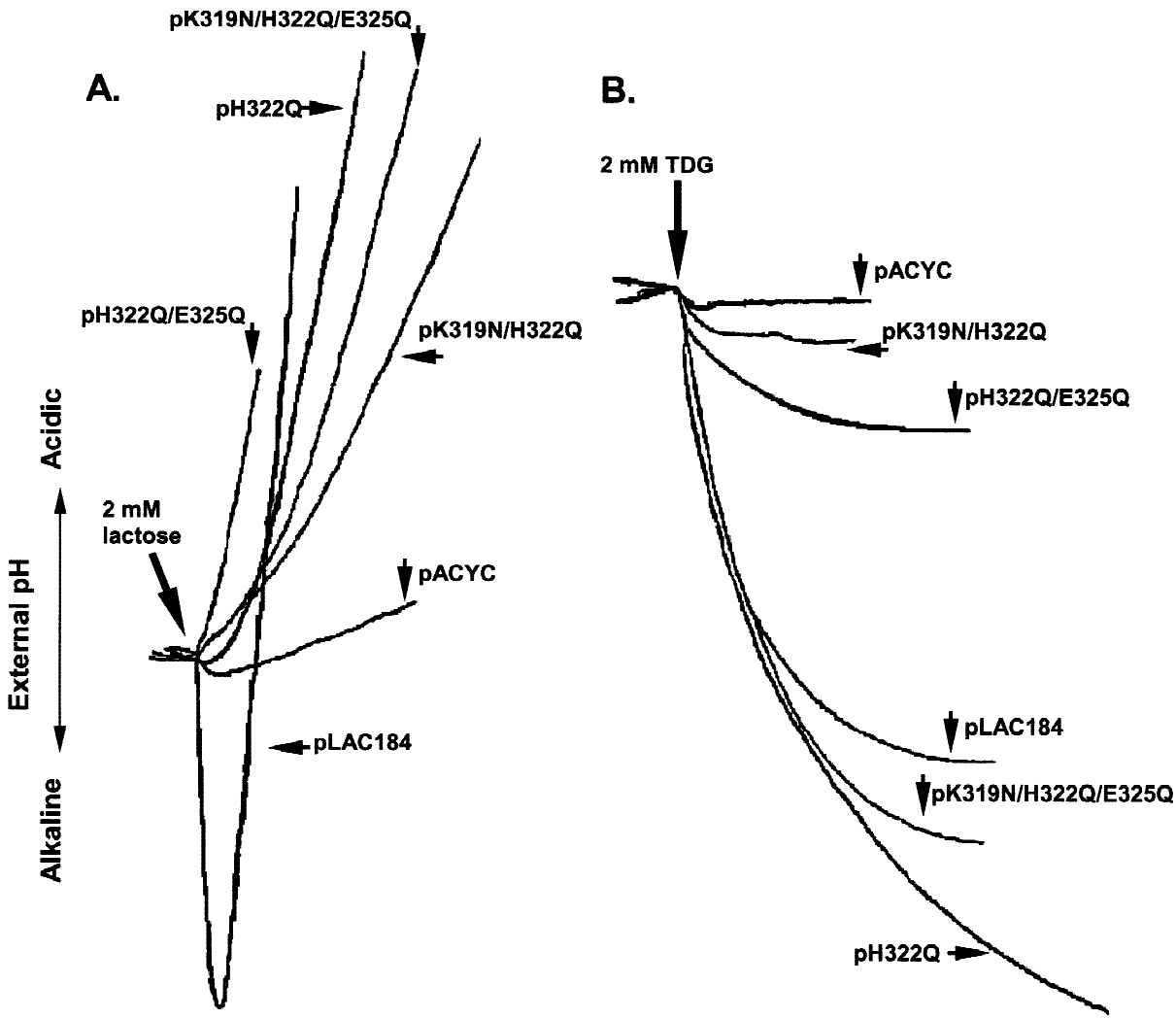

Fig. 3. Sugar-induced H^+ transport. H^+ transport was measured in the strain HS4006/F′$I^QZ^+Y^-$ containing the designated plasmids as described in Materials and Methods. Part *A,* Addition of lactose. Part *B,* Addition of TDG.

sugar cotransport [4, 9, 11, 16, 23, 26, 27, 31, 32, 42, 49]. The results of the current study would seem to contradict a recently published mechanism for lactose transport [43]. In that model, His-322 shares the proton-binding site with Glu-269. Substrate binding induces a conformational change, transferring the proton to Glu-325, which then de-protonates to start the cycle over again. However, as shown here, a triple mutant containing non-ionizable side chains at positions 319, 322, and 325 is still able to catalyze H^+/TDG cotransport to near wild-type levels. It is possible these residues function only in the coupling of H^+/ sugar cotransport without playing a direct role in H^+ binding. In such a scenario, the cation (H^+ or H_3O^+) may bind elsewhere, and the binding of cation and/or lactose may lead to local conformational changes that affect the ionic interactions among these residues. Although the nature of these interactions remains highly speculative, previous models from our laboratory and another laboratory have suggested that many of these residues play a key role in the coupling mechanism [26, 28].

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