J. Membrane Biol. 211, 101–113 (2006)
DOI: 10.1007/s00232-005-7020-x

The Journal of
Membrane
Biology

A Suppressor Analysis of Residues Involved in Cation Transport in the Lactose Permease: Identification of a Coupling Sensor

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Received: 2 December 2005/Revised: 5 May 2006

Abstract. Four amino acids critical for lactose permease function were altered using site-directed mutagenesis. The resulting Quad mutant (E269Q/R302L/H322Q/E325Q) was expressed at 60% of wild-type levels but found to have negligible transport activity. The Quad mutant was used as a parental strain to isolate suppressors that regained the ability to ferment the α-galactoside melibiose. Six different suppressors were identified involving five discrete amino acid changes and one amino acid deletion (Q60L, V229G, Y236D, S306L, K319N and ΔI298). All of the suppressors transported α-galactosides at substantial rates. In addition, the Q60L, ΔI298 and K319N suppressors regained a small but detectable amount of lactose transport. Assays of sugar-driven cation transport showed that both the Q60L and K319N suppressors couple the influx of melibiose with cations (H^+ or H_3O^+). Taken together, the data show that the cation-binding domain in the lactose permease is not a fixed structure as proposed in previous models. Rather, the data are consistent with a model in which several ionizable residues form a dynamic coupling sensor that also may interact directly with the cation and lactose.

Key words: Suppressor analysis — Cation Transport — Lactose permease — Coupling sensor

Introduction

Bacterial secondary active transporters capture free energy from the movement of cations down their electrochemical gradient to drive the transport of solutes such as sugars, amino acids, Krebs cycle intermediates, antibiotics and inorganic ions against a gradient [41, 46, 51]. The most extensively studied example of this type of protein is the lactose permease (LacY) of *Escherichia coli*. Having been the subject of intensive study for the better part of three decades, many aspects of the relationship between structure and function have been well characterized for the lactose permease. Cloning and sequencing of *lacY*, the gene encoding the lactose permease, predicts a protein consisting of 417 amino acids [7, 55]. Topological studies predicted a secondary structural model in which the permease contains 12 α-helical transmembrane domains, with the amino and carboxyl terminal portions of the protein residing in the cytoplasm [9, 10]. The protein has been purified to homogeneity, restored into functional proteoliposomes and found to be functional as a monomer [44, 45, 59].

LacY belongs to a large superfamily of transport proteins (the major facilitator superfamily, MFS) that includes uniporters, symporters and antiporters [20, 25, 40, 46]. MFS proteins are predicted to exist in a wide range of cells including bacteria, eukaryotes and archaea [54]. Phylogenetic analysis predicts a similar topological structure for most family members that includes 12 α-helical transmembrane segments (TMS) suggestive of an evolutionary relatedness [16, 54]. A general homology found between the two halves of MFS proteins also indicates that a gene duplication/fusion event took place from a gene encoding a six-transmembrane domain protein early in the evolution of this family [40]. Analysis of the MFS for hydrophobicity, amphipathicity and loop length combined with potential salt-bridges between transmembrane domains in the lactose permease led our lab to propose a tertiary structural model [16]. A critical aspect of this model was that the two halves of the protein form distinct domains that are situated in a rotational symmetric manner [16]. The MFS contains a highly conserved decapeptide motif, G-X-

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X-X-D/E-R/K-X-G-R/K-R/K, located in the loop connecting TMS-2 to TMS-3 and repeated in the loop that connects TMS-8 to TMS-9 [20, 25, 46]. With this information as a starting point, site-directed mutagenesis coupled with suppressor analysis was used to identify functionally important amino acids within these motifs and the neighboring helices of TMS-2 and TMS-8 [8, 17–19, 26–28, 47–49]. Information obtained from these studies led us to propose that residues of TMS-2 and TMS-8 are located at the interface between the two halves of the protein and that these domains are the location of global conformational changes that take place during the transport cycle, possibly involving a scissoring motion of transmembrane domains relative to one another. The recently reported crystal structure of the lactose permease supports such a model [2, 60, 61].

Extensive mutagenesis studies have subjected every residue of LacY to substitution and some form of functional characterization [*for reviews*, *see* 15, 35, 36, 57]. These studies have led to the surprising conclusion that only a small number of amino acids are critical for protein function. These include eight ionizable amino acids: Glu-126, Arg-144, Asp-240, Glu-269, Arg-302, Lys-319, His-322 and Glu-325 [4, 13, 14, 37–39, 42, 50, 53, 56, 58, 63, 64]. While Glu-126 and Arg-144 are believed to form a salt-bridge important for sugar recognition, the remaining six residues are important for many aspects of coupled transport between sugar and cations. Biochemical and physiological evidence also suggests that these residues are located on transmembrane segments and involved in a complex salt-bridge and/or hydrogen bonding network [21–24, 33, 38, 39]. The recent crystallization of an inwardly facing, sugar-bound permease confirmed the proximity and relative location of many of these residues [2].

A critical aspect of the study of the lactose permease is elucidation of the cation (H^+ or H_3O^+) transport pathway during lactose cotransport across the membrane. The importance of six ionizable residues (Asp-240, Glu-269, Arg-302, Lys-319, His-322 and Glu-325) for coupled transport in the lactose permease has led to the proposal of several models for cation translocation. A model from another laboratory proposes that an H^+ ion is initially shared between Glu-269 and His-322. The proton is transferred to Glu-325 through a hydrogen bond to His-322 during a complex conformational change that alters the protein from an outward-facing to an inward-facing structure. This change releases Glu-325 from a salt-bridge with Arg-302 and facilitates the formation of a salt-bridge between Arg-144 and Glu-269 [2]. The proton is then released from Glu-325 into the cytoplasm. A model from our laboratory also proposed that Glu-325 is responsible for cation binding and transport. In our model, cation binding to Glu-325 disrupts a salt-bridge between Glu-325 and Lys-319, facilitating the formation of a salt-bridge between Glu-269 and Lys-319. Sugar binding then causes a conformational change in LacY from an outward-facing to an inward-facing structure. This conformational change disrupts the salt-bridge between Lys-319 and Glu-269, and formation of a new interaction between Lys-319 and Asp-240 results. Sugar and cations are released into the cytoplasm following this conformational change [30]. In this model, Lys-319 controls coupling between cation and sugar transport and Glu-325 is responsible for cation binding.

To address the issue of cation binding and coupling, we have previously used a combinatorial approach in which multiple mutants are made within a single permease protein [29]. In the present study, we combined a combinatorial approach with a suppressor analysis. We began with a quadruple mutant (E269Q/R302L/H322Q/E325Q) that has four ionic residues neutralized. Most importantly, both Glu-269 and Glu-325, which are critical components for cation binding and coupling in previous models, have been changed to nonionizable residues. The results show that previous models from our laboratory and another laboratory that involve obligatory binding of H^+ to Glu-269 and/or Glu-325 to achieve H^+/sugar cotransport are not correct. Rather, the results are consistent with a mechanism in which several ionizable residues function as a dynamic coupling sensor.

Materials and Methods

Reagents

Lactose (*O*-β-D-galactopyranosyl-[1, 4]- α-D-glucopyranose), melibiose (*O*-α-D-galactopyranosyl-[1, 6]- α-D-glucopyranose and 4-nitrophenyl-α-D-galactopyranoside (αNPG) were purchased from Sigma (St. Louis, MO). [^{14}C]-Lactose was purchased from Pharmacia (Gaithersburg, MD). The lactose permease antibody and [^{3}H]-melibiose were generous gifts from Dr. Thomas H. Wilson (Harvard Medical School, Boston, MA). Anti-rabbit immunoglobulin G (IgG, alkaline phosphatase conjugate) and alkaline phosphatase substrate tablets (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) were purchased from Sigma. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The remaining reagents were analytical grade.

Bacterial Strains and Plasmids

Mutant plasmids used in this study are listed in Table 1. Wild-type lactose permease was expressed from the pACYC184 plasmid as described previously [14]. Plasmid DNA was isolated using the Perfectprep Plasmid Mini kit (Eppendorf, Hamburg, Germany) and introduced into the appropriate bacterial strains by RbCl transformation. HS4006/F'$I^qZ^+Y^-$ cells were used for plating on lactose MacConkey media and downhill lactose transport experiments [6]. This strain is able to metabolize lactose (*lacZ*$^+$) but does not encode a functional lactose permease (*lacY*$^-$). DW2

Table 1. Genotypes of Quad mutant and Quad suppressors

Suppressor genotype[a]	Short name	Number of isolates	Suppressor location
E269Q/R302L/H322Q/E325Q	Quad	Parent	—
E269Q/R302L/H322Q/E325Q/Y236D	QS-A	2	TMS-7
E269Q/R302L/H322Q/E325Q/S306L	QS-B	3	TMS-9
E269Q/R302L/H322Q/E325Q/ΔI298	QS-C	1	TMS-9
E269Q/R302L/H322Q/E325Q/Q60L	QS-D	8	TMS-2
E269Q/R302L/H322Q/E325Q/K319N	QS-H[b]	6	TMS-10
E269Q/R302L/H322Q/E325Q/V229G	QM-2	1	TMS-7

[a]Mutant residues are designated using the single-letter amino acid code. Amino acids present in the wild-type lactose permease are listed first, followed by the number designating the amino acid position within the protein and single-letter designation of the mutant. For example, *E269Q* designates that a glutamic acid residue at position 269 is changed to glutamine. Multiple mutations are separated by a backslash.
[b]This suppressor carries the same set of mutations as the previously characterized Pent240$^-$ mutant.

(*lacI*$^+$Δ*ZY melA*$^+$Δ*B*) [3] was used for plating on melibiose MacConkey media, downhill α-NPG transport, downhill melibiose transport, cation transport experiments and Western blots. This strain is deleted for both the lactose (Δ*lacY*) and melibiose (Δ*melB*) permeases but is still able to metabolize melibiose (*melA*$^+$). Stock cultures were grown in YT medium supplemented with tetracycline (0.01 mg/ml). DW1 (*lacI*$^+$Δ*ZY mel*Δ*AB)* [62] was used for uphill lactose and melibiose transport experiments.

Construction of the Quad Mutant Lactose Permease

The Pent240$^-$ (E269Q/R302L/K319N/H322Q/E325Q) and Pent319$^+$ (D240N/E269Q/R302L/H322Q/E325Q) plasmids [29] were digested with *Bam*HI and *Kpn*I. *Bam*HI cuts once within the plasmid, while *Kpn*I cuts between amino acids 269 and 302 within the lactose permease coding region. The unique *Kpn*I site was introduced during construction of the Pent240$^-$ and Pent319$^+$ mutants. A DNA fragment containing the E269Q mutation was isolated from the Pent240$^-$ digest, and a fragment containing the R302L/H322Q/E325Q mutations was isolated from Pent319$^+$. These two DNA fragments were ligated to produce a lactose permease with the following mutations: E269Q/R302L/H322Q/E325Q. This mutant was designated as the Quad mutant.

Isolation of Suppressors

DW2 cells were transformed with a plasmid expressing the *lacY* Quad mutant (E269Q/R302L/H322Q/E325Q). Cells were plated on MacConkey agar containing 0.4% melibiose (~12 mM) and incubated at 37°C. Initially, colonies displayed a white phenotype on this medium. Following 5–7 days of incubation at 37°C, small red spots were seen within the primary cell streak. These red isolates were picked and restreaked on the same media to purify the red cells. Plasmid DNA was isolated from the purified red colonies and used to transform DW2 cells to verify that the red phenotype was the result of changes within the plasmid carrying the *lacY* gene. Red clones were picked and saved for further analysis. Base substitutions within the *lacY* gene were identified by sequencing the entire gene using several primers that anneal at roughly 350-bp intervals.

DNA Sequencing

Plasmid DNA sequencing was done by the Advanced Genetic Analysis Center at the University of Minnesota using an ABI (Foster City, CA) model 377 DNA sequencer.

Sugar Transport Assays

For downhill lactose transport assays, HS4006/F'I^{q}Z$^+$Y$^-$ cells carrying plasmid DNA expressing normal or mutant *lacY* were grown to mid-log phase in YT medium supplemented with tetracycline (0.005 mg/ml) and isopropylthiogalactoside (IPTG, 0.25 mM). Cells were collected by centrifugation (5,000 × *g*, 4°C, 10 min) and the resulting cell pellets washed once in potassium phosphate buffer (100 mM K_2HPO_4, 100 mM KH_2PO_4, pH 6.0) followed by resuspension in the same buffer to a concentration of approximately 0.5 mg total protein/ml. Aliquots of cells were equilibrated at 37°C for 10 min before addition of [^{14}C]-lactose (final concentration 0.3 mM) to initiate transport assays. At appropriate time points, cells were withdrawn (0.2 ml aliquots) and captured on 0.45 μm nitrocellulose membrane filters (Millipore, Bedford, MA) by rapid filtration. Filters were immediately washed with 5–10 ml of ice-cold potassium phosphate buffer prior to determination of radioactivity via scintillation counting. As a control, the *lacY*$^-$ strain HS4006/F'I^{q}Z$^+$Y$^-$/pACYC184 was also assayed for [^{14}C]-lactose uptake to obtain an accurate value for nonspecific lactose transport. Sugar transport values obtained for this control strain were then subtracted from those for the experimental strains to determine permease-mediated lactose uptake. Lactose transport rates were calculated based on time points taken in the linear range of transport for mutant and suppressor strains (within 30 min) and for wild-type (within 1 min). Reported lactose transport rates are the average of triplicate measurements taken from three independent experiments (n = 9).

DW2 (*lacI*$^+$Δ*ZY melA*$^+$Δ*B*) cells were used for downhill melibiose transport assays. Cells were grown to mid-log phase in YT medium supplemented with tetracycline (0.005 mg/ml), IPTG (0.25 mM) and α-methyl galactoside (1 mM) to induce expression of the chromosomal α-galactosidase. Cells were prepared and the assay carried out as described for lactose transport with [^{3}H]-melibiose (3 mM) used as the transport substrate. As a control, the *lacY*$^-$ strain DW2/pACYC184 was also assayed for [^{3}H]-melibiose uptake to obtain an accurate value for nonspecific melibiose transport. Sugar transport values obtained for this control strain were then subtracted from those for the experimental strains to determine permease-mediated melibiose uptake. Melibiose transport rates were calculated based on time points taken in the linear range of transport for wild-type, mutant and suppressor strains (within 1 min). Reported melibiose transport rates are the average of duplicate measurements taken from three independent experiments (n = 6).

Downhill α-NPG transport assays were also conducted using the DW2 strain. Growth medium was supplemented with

tetracycline (0.005 mg/ml), IPTG (0.25 mM) and α-methylgalactoside (1 mM); and cells were prepared as described above. Aliquots of cells were equilibrated at 37°C for 10 min before addition of α-NPG (final concentration 10 mM) to initiate transport assays. At appropriate time points, cells were withdrawn (0.2 ml aliquots) and added to 0.6 ml of Stop Solution (1 M sodium carbonate, 0.01% sodium dodecyl sulfate [SDS]). Samples were then stored at −80°C until the assay was completed. Samples were thawed and centrifuged at 13,000 rpm for 10 min. An aliquot (0.7 ml) was then transferred to a cuvette (10 × 4 × 45 mm; Sarstedt, Newton, NC), and absorbance was read at a wavelength of 420 nm. An extinction coefficient of 4,600 L mol^{-1} cm^{-1} was used for α-NPG. As a control, the *lacY*$^-$ strain DW2/pACYC184 was also assayed for α-NPG uptake to obtain an accurate value for nonspecific α-NPG transport. Sugar transport values obtained for this control strain were then subtracted from those for the experimental strains to determine permease-mediated αNPG uptake. Transport remained linear for this sugar for an extended period (60 min), and rates were calculated based on time points taken within this period. Reported NPG transport rates are the average of triplicate measurements taken from three independent experiments ($n = 9$).

A two-tailed *t*-test was applied to assess whether the transport rate for the Quad mutant differed significantly from each suppressor strain for each sugar tested for downhill transport ($n = 6$ for melibiose transport, $n = 9$ for NPG transport).

DW1(*lacI*$^+$Δ*ZY melΔAB)* cells were used for lactose and melibiose accumulation assays. Cells were grown to mid-log phase in YT medium supplemented with tetracycline (0.005 mg/ml) and IPTG (0.25 mM). Cells were collected, washed and resuspended as described above for downhill lactose transport. Transport assays were initiated by addition of [^{14}C]-lactose (final concentration 0.1 mM) or [^{3}H]-melibiose (0.1 mM). At appropriate time points, cells were withdrawn (0.2 ml aliquots) and captured on 0.45 μm nitrocellulose membrane filters by rapid filtration. Filters were immediately washed with 5–10 ml of ice-cold potassium phosphate buffer prior to determination of radioactivity via scintillation counting. A value of 0.6 μl/6×10^8 cells was used for the volume of intracellular water for calculations of sugar concentration. Accumulation values are reported as ratios of intracellular to extracellular sugar concentration ([IN]/[OUT]). Reported values represent transport after 1 min. Longer time points did not yield greater accumulation values.

During the time course of the experiment, it is assumed that there is negligible change in the extracellular sugar concentration. Reported accumulation values are the average of triplicate measurements taken from three independent experiments ($n = 9$) for both lactose and melibiose.

Cation Transport

DW2 cells were grown to mid-log phase in YT medium supplemented with tetracycline (0.005 mg/ml) and IPTG (0.25 mM). Cells were washed twice in unbuffered KCl (120 mM) and resuspended in the same buffer to a concentration of approximately 3.5 mg total protein/ml; 2.5 ml of cells supplemented with potassium thiocynate (30 mM) 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 a gas-impermeable Hamilton syringe. Cells were made anaerobic under a continuous stream of argon for approximately 30 min. To initiate sugar-induced cation transport, an anaerobic solution of melibiose was added via the syringe to a final concentration of 20 mM. Changes in extracellular pH were measured with a Radiometer pH meter and electrode and continuously monitored with a Radiometer chart recorder (Copenhagen, Denmark).

Membrane Preparation

For Western blot analysis, DW2 cells containing the appropriate plasmid were grown to late log phase in YT medium (10 ml) supplemented with tetracycline (50 μg/ml) and IPTG (0.25 mM). Cells were collected by low-speed centrifugation (5,000 × *g*, 4°C, 10 min), washed once with H_2O (10 ml) and resuspended in 0.5 ml lysis buffer (50 mM TES [pH 8.0], 100 mM NaCl) supplemented with the protease inhibitors TPCK (0.1 mg/ml) and phenylmethyl sulfonyl fluoride (PMSF, 25 mg/ml). Cells were subjected to three freeze-thaw cycles, using liquid nitrogen to flash freeze and cold H_2O to thaw. Each sample was then sonicated three times for 20 s, with cooling on ice between each round of sonication. Membranes were collected by centrifugation (185,000 × *g*, 4°C, 45 min) and resuspended in 0.2 ml of HAS buffer (50 mM TES [pH 8.0], 100 mM NaCl, 1 mM lactose, 20% glycerol) supplemented with TPCK and PMSF. Protein concentration was measured using the Bradford method (Bio-Rad Protein Assay Kit; Bio-Rad, Richmond, CA).

Western Blots

For Western blots, 25 μg of total membrane protein from each strain was subjected to SDS-polyacrylamide gel electrophoresis (PAGE, 12% gel). Proteins were then transferred to nitrocellulose using a TransBlot® semidry transfer cell (Bio-Rad). Blots were washed once in 1× PBS and incubated overnight in blocking buffer (1× PBS, 0.1% Tween-20, 3% bovine serum albumin) at room temperature. Blots were placed in a sealed bag containing blocking buffer (10 ml) and anti-LacY antibody (1/10,000 dilution) and incubated for 2 h. Anti-LacY is a polyclonal antibody developed in rabbits that recognizes the carboxyl-terminal 10 amino acids of the lactose permease. Following three washes in 1× PBS (10 min/wash), blots were placed in a sealed bag with blocking buffer (10 ml) and goat anti-rabbit IgG alkaline phosphatase conjugate (1/20,000 dilution, Sigma) and incubated for 1 h. Blots were then washed three times with 1× PBS. The Sigma-Fast™ alkaline phosphatase substrate was used to develop blots. Blots were scanned using the GS-700 Imaging Densitometer and analyzed with Molecular Analyst software (both from Bio-Rad). Mutant and suppressor bands were compared with the wild-type band for the same blot. Values are reported as a percentage of wild-type averaged over three separate blots.

Results

Mutant Construction and Suppressor Isolation

In an effort to identify residues important for the coupling of cation/substrate cotransport, a lactose permease mutant was made that contained the following four substitutions: E269Q/R302L/H322Q/E325Q. Each residue substituted in this Quad mutant has been shown to be important for the coupled transport of sugar and cations in the wild-type protein. These four residues have also been predicted to provide the initial cation binding site (Glu-269, His-322), accept cations during transport (Glu-325), influence cation release into the cytoplasm (Arg-302) and control the interaction of cation and sugar binding sites (Glu-269) [2]. Thus, it would be expected that the Quad mutant is particularly defective for coupled transport.

The Quad mutant was characterized initially by plating on MacConkey indicator plates with either lactose or melibiose as the sole fermentable carbon source. Cells that can appreciably transport and subsequently metabolize sugar will form red colonies, whereas a defect in this process results in colonies with a white phenotype. In general, a lactose permease mutant must possess a significant transport defect (< 5%) compared to wild-type to prevent a red colony phenotype. In the presence of a high concentration (1%) of lactose or melibiose, the Quad mutant exhibited a white colony phenotype.

The white phenotype on MacConkey agar allows for a convenient screen of transport-competent suppressors. Incubation of lactose permease mutants on melibiose-containing MacConkey agar for a prolonged period can produce spontaneous suppressors that grow as red areas within a background of white cells. We used DW2 as a host strain to select for mutations that would suppress the transport defect of the Quad mutant. Cells were plated on 0.4% melibiose-MacConkey agar and incubated at 37°C. After 5–7 days, small red areas appeared within the white colonies. These red cells were purified by restreaking on the same medium until a uniform red colony phenotype was observed. To ensure that the mutation responsible for the red phenotype was carried on the *lacY*-bearing plasmid, DNA was isolated from red colonies and used to transform DW2 cells. Only those samples that retained a red phenotype after transformation were saved for further study. Plasmid DNA was isolated from red colonies, and the entire *lacY* gene was sequenced to verify the presence of the original Quad mutations, as well as any new changes within the coding sequence. Twenty-one independent suppressors were isolated that included five distinct single-amino acid substitutions (Q60L, V229G, Y236D, S306L and K319N) and deletion of a single codon at Ile-298 (*see* Table 1). All of these suppressors retained the four original mutations present in the Quad parental strain.

Colony Phenotype and Protein Expression Levels

The function of lactose permease mutants can be evaluated qualitatively by plating on MacConkey agar with a fermentable carbon source that is transported by the protein. The phenotype of isolated Quad suppressors was tested on lactose and melibiose plates (0.4% and 1%, respectively). The Quad mutant gave a white phenotype indistinguishable from the pACYC184 vector control. As expected, all of the suppressor strains are red on melibiose MacConkey plates, suggesting they regained the ability to transport this sugar effectively (Table 2). Interestingly, some of the suppressors also seem to have partially restored lactose transport. The Q60L, ΔI298 and K319N suppressors are red on 1% lactose (~29 mM) but white on 0.4% (~12 mM). Thus, transport of lactose in these three suppressor strains is compromised but demonstrable.

Table 2. Phenotype of Quad mutant and suppressors on MacConkey plates

	Lactose		Melibiose	
Strain	0.4%	1%	0.4%	1%
pACYC184	White	White	White	White
pLac184	Red	Red	Red	Red
Quad	White	White	White	White
QM-A (Y236D)	White	White	Red	Red
QM-B (S306L)	White	White	Red	Red
QM-C (ΔI298)	White	Red	Red	Red
QM-D (Q60L)	White	Red	Red	Red
QM-H (K319N)	White	Red	Light red	Red
QM-2 (V229G)	White	White	Red	Red

Cells containing the indicated plasmid in strain HS4006/F'$I^qZ^+Y^-$ were plated on lactose MacConkey agar, and color was analyzed after overnight growth at 37°C. Cells containing the indicated plasmid in strain DW2 were plated on MacConkey agar with melibiose, and color was analyzed after overnight growth at 37°C. Suppressor mutations are listed in parentheses.

To determine the relative amount of lactose permease present in mutant and suppressor strains, we performed immunoblots on isolated membranes using a polyclonal antibody directed against the carboxyl-terminal 10 amino acids of the protein (Fig. 1). The amount of lactose permease expressed in each strain was calculated as a percentage of the value obtained for wild-type (Table 3). The Quad mutant had relatively good expression at 59% of wild-type. Likewise, the suppressor mutants showed expression levels that were similar to the parental strain.

IN VITRO Sugar Transport Assays

DW2 cells were transformed with plasmid DNA expressing wild-type, mutant or suppressor lactose permease proteins. This strain is deleted for the lactose permease (Δ*lacY*) and melibiose permease (Δ*melB*) but is still able to metabolize melibiose (*melA*$^+$). Upon entry into the cell, melibiose is rapidly cleaved by α-galactosidase, allowing the extracellular concentration to remain higher than the intracellular concentration such that melibiose transport moves "downhill," or with its concentration gradient, throughout the assay. Measurement of initial melibiose transport rates (within 1 min) showed that the Quad parental strain has negligible transport and that all of the suppressors, with the exception of ΔI298, are able to transport melibiose with improved rates (Fig. 2). While the V229G, S306L and K319N suppressors have transport rates more than twofold above that of the Quad parent (Fig. 2), the Q60L and the Y236D suppressors

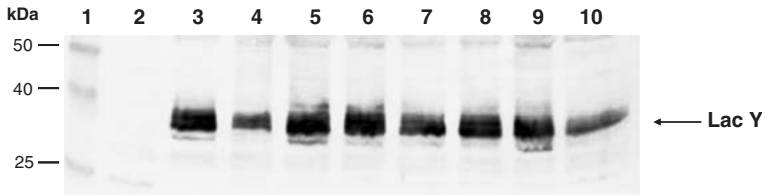

Fig. 1. A representative Western blot is shown for the comparison of protein expression levels for wild-type lactose permease, the Quad mutant and Quad suppressor strains. DW2 cells containing the appropriate plasmid were used for expression of *lacY*. Total membrane protein from each strain (25 μg) was subjected to SDS-PAGE (12% gel), followed by transfer to nitrocellulose membrane. An anti-LacY polyclonal antibody was used to detect lactose permease protein, as described under Materials and Methods. Lane 1, Protein molecular weight standard; 2, pACYC184 (no *lacY* insert); 3, pLac184 (wild-type); 4, Quad mutant; 5, QM-A; 6, QM-B; 7, QM-C; 8, QM-D; 9, QM-H; 10, QM-2.

Table 3. Lactose permease expression for mutant and suppressor strains

Strain	% Expression ± SE
pLac184 (wild-type)	100
Quad	59 ± 6
QM-A (Y236D)	118 ± 10
QM-B (S306L)	95 ± 9
QM-C (ΔI298)	77 ± 11
QM-D (Q60L)	73 ± 7
QM-H (K319N)	67 ± 5
QM-2 (V229G)	41 ± 8

Expression levels of lactose permease of each strain are reported as a percentage of the value found for the wild-type protein. Values are averages of three separate experiments ± standard error (SE).

transport melibiose dramatically better than the Quad parental strain.

We also tested the transport of a related α-galactoside, NPG. NPG is also cleaved by α-galactosidase, allowing measurement of downhill transport for this sugar. Cleavage of NPG produces a yellow color that can be monitored with a spectrophotometer. Transport of NPG remained linear over a long time course (60 min) for this sugar substrate in the wild-type, mutant and suppressor strains. Thus, transport rates were calculated using time points within 1 h of transport. As can be seen in Figure 3, the parental Quad mutant has a low rate of NPG transport. In contrast, all of the suppressor strains, with the exception of K319N, transport NPG at a rate that is clearly better than that of the Quad mutant. Interestingly, three distinct types of suppressors gave the best transport of NPG. These included the introduction of a negative charge (Y236D), substitution of a polar residue with a neutral residue (Q60L) and deletion of a neutral residue (ΔI298). This suggests that reconstituting a sugar binding site within the Quad mutant can be accomplished in a variety of ways.

The red phenotype of Q60L, K319N and ΔI298 suppressor strains on 1% lactose MacConkey medium suggested that they can achieve lactose transport to some degree. Lactose transport rates were measured in the β-galactosidase-positive strain HS4006/F'$I^{Q}Z^{+}Y^{-}$. After entering the cell, lactose is quickly metabolized such that the external concentration remains higher than the intracellular concentration. Thus, lactose transport in this strain is considered "downhill." Transport rates for the Quad parent and suppressor strains were much lower than for the wild-type lactose permease and had to be measured over a longer time course (30 min compared to 1 min). However, in agreement with the plating results, downhill [^{14}C]-lactose transport rates for the Q60L, K319N and ΔI298 suppressors were significantly higher than for the Quad parent and the remaining suppressor strains that exhibited a white phenotype on lactose MacConkey plates (Fig. 4).

An important aspect of sugar transport via the lactose permease is the ability of this protein to move sugar substrates against an intracellular concentration gradient. To accomplish this "uphill" transport, the protein must capture free energy from the proton electrochemical gradient and utilize it for sugar uptake. Uphill transport can be accomplished only when sugar and cation transport are coupled to one another. Thus, sugar accumulation assays are one way to probe the ability of mutant and suppressor strains to couple cation/sugar cotransport. We tested the ability of the Quad parent and suppressor strains to catalyze uphill transport for both lactose and melibiose in the DW1 strain. DW1 carries deletions of both the lactose permease (Δ*lacY*) and the melibiose carrier (Δ*melB*) as well as deletions for α-galactosidase (Δ*melA*) and β-galactosidase (Δ*lacZ*) so that it is unable to transport or metabolize either sugar. The data in Figure 5 show that the wild-type permease is able to accumulate both lactose and melibiose. However, the Quad parent strain and isolated suppressors are not able to accumulate these sugars significantly above the background levels seen for the negative control strain DW1/pACYC184.

When considering the sugar transport results, it is interesting to note that while some of the suppressors restore transport in a general way, others appear to be sugar-specific. For example, Q60L and

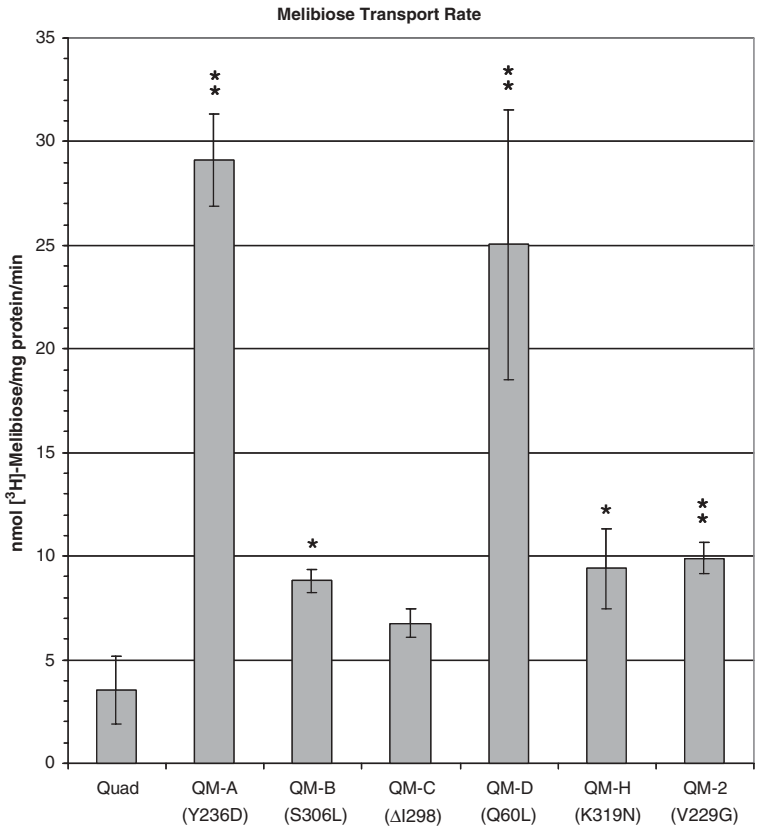

Fig. 2. Melibiose transport was measured in the DW2 strain containing the designated mutant or suppressor plasmids. Cells were incubated at 37°C for 15 min at an external pH of 6.0 prior to addition of [^{3}H]-melibiose (final concentration 3 mM). Error bars represent the standard error (SE). A two-tailed *t*-test was applied to assess whether the transport rate for the Quad mutant differed significantly from that of each suppressor strain. Significant difference from the Quad mutant, $*P < 0.05$ and $**P < 0.01$. The rate for melibiose transport in the wild-type permease was 98.58 ± 8.36 nmol [^{3}H]-melibiose/mg protein/min.

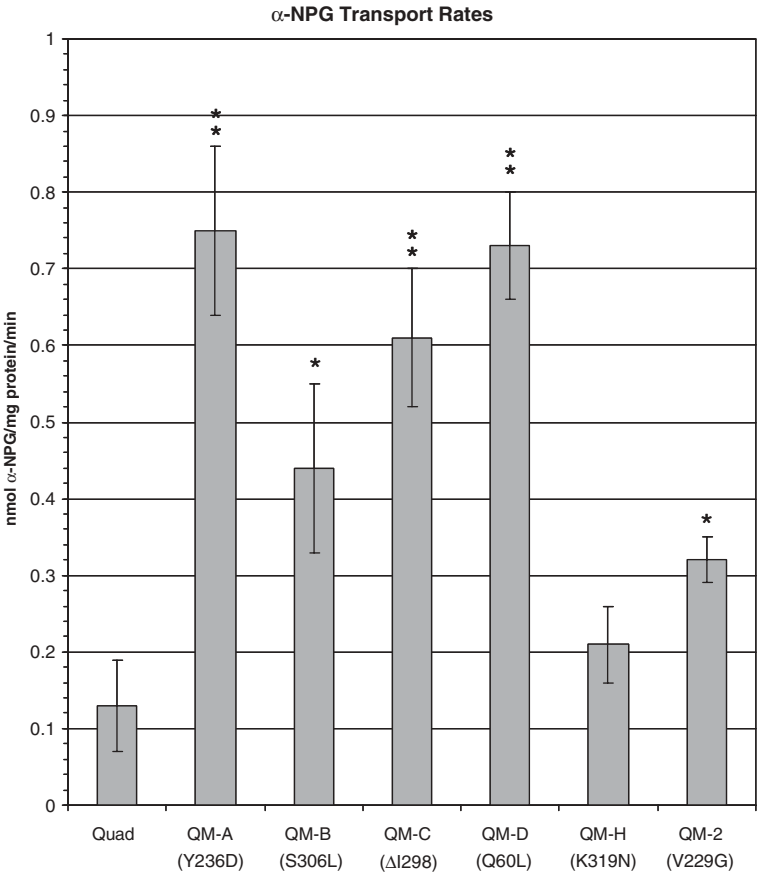

Fig. 3. NPG transport was measured in DW2 containing the designated mutant or suppressor plasmids. Cells were incubated at 37°C for 15 min at an external pH of 6.0 prior to addition of NPG (final concentration 10 mM). Error bars represent standard error (SE). Significant difference from the Quad mutant, $*P < 0.05$ and $**P < 0.01$. The rate for NPG transport in the wild-type lactose permease was 10.3 ± 1.0 nmol NPG/mg protein/min.

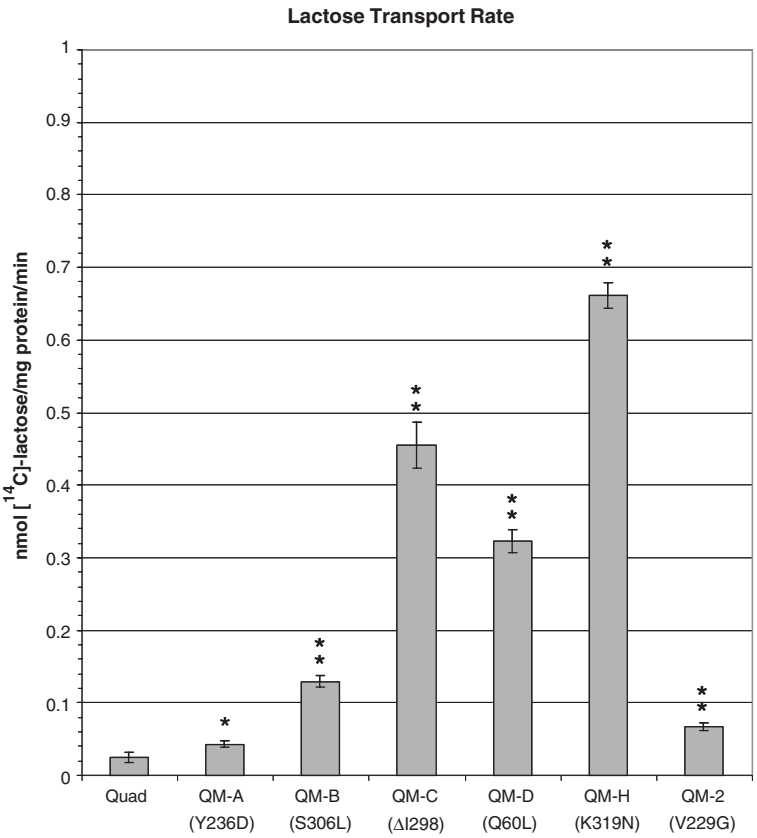

Fig. 4. Lactose transport was measured in the HS4006/F'$I^qZ^+Y^-$ strain. Cells were incubated at 37°C for 15 min at an external pH of 6.0 prior to addition of [^{14}C]-lactose (final concentration 0.3 mM). Error bars represent standard error (SE). Significant difference from the Quad mutant, $*P < 0.05$ and $**P < 0.01$.The rate for lactose transport in the wild-type permease was 121.8 ± 9.6 nmol [^{14}C]-lactose/mg protein/min.

ΔI298 were able to restore transport of both α- and β-galactosides. In contrast, Y236D and S306L were better at transporting α-galactosides. It is possible that the more general effect on sugar transport seen in Q60L and ΔI298 has to do with restoration of a protein structure that is competent to make the conformational changes from an outward- to an inward-facing structure. It has been shown in previous studies from our lab that Gln-60 is positioned at the interface of the N- and C-terminal halves of the protein and influences global conformational changes associated with sugar transport [17–19]. Perhaps the ΔI298 suppressor plays a similar role in repositioning TMS-9 for the Quad mutant to allow recovery of sugar transport. Both Tyr-236 and Ser-306 are close to Arg-302, as revealed by the crystal structure [2]. Thus, they may influence sugar selectivity by interaction with, or repositioning of, Arg-302. While the V229G suppressor showed low transport recovery overall, it is a residue that has been shown to influence residues in TMS-2, TMS-8 and TMS-11, all of which are at the interface of the two halves of the protein [17–19].

Measurement of Cation Transport

Wild-type lactose permease couples the movement of cations and sugar into the cells during the transport cycle. This obligatory coupling allows direct measurement of sugar-induced cation transport by monitoring extracellular pH. In such an experiment, cells are made anaerobic under a continuous argon stream. An anaerobic sugar solution is then added, and any change in the pH of extracellular medium is monitored with an electrode and pH meter. As sugar and cations move into the cells, alkalinization of the external medium occurs, which can be visualized with a chart recorder. The Quad mutant and suppressor strains were tested to determine whether melibiose could induce cation uptake. For the wild-type strain, addition of melibiose (20 mM) caused rapid alkalinization indicated by a downward deflection of the chart recording (Fig. 6). In contrast, cells not expressing the lactose permease showed no alkalinization. Among the suppressors, the K319N strain gave a modest response. In addition, the Q60L strain had a robust alkalinization that was very similar to wild-type. The Quad mutant and remaining suppressors gave curves similar to the vector control. The efficient cation transport observed for the Q60L suppressor has important implications for identification of the cation transport pathway. It strongly suggests that none of the residues altered in the Quad parent – namely, Glu-269, Arg-302, His-322 and Glu-325 – is essential for sugar-driven cation transport.

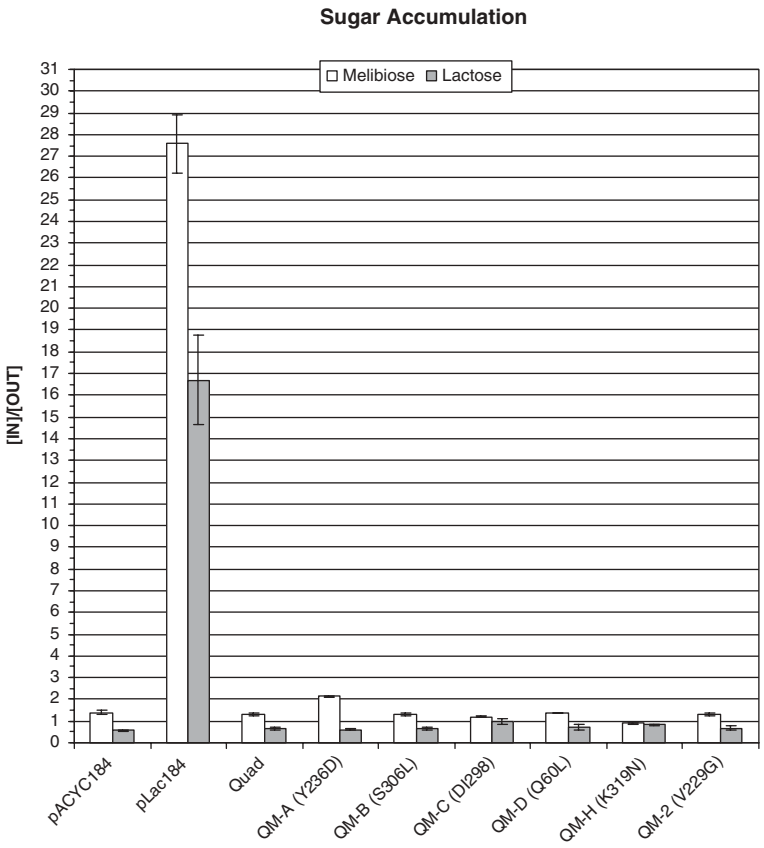

Fig. 5. Sugar accumulation was measured in the DW1 strain containing the designated mutant or suppressor plasmids. Cells were incubated at 37°C for 15 min at an external pH of 6.0 prior to addition of 0.1 mM [^{3}H]-melibiose (*white bars*) or 0.1 mM [^{14}C]-lactose (*gray bars*). Error bars represent standard error (SE).

Discussion

Exhaustive genetic, biochemical, physiological and molecular research on the structure and function of the lactose permease has shown that relatively few amino acids are critical for coupled transport [4, 13–15, 35–39, 42, 50, 53, 56–58, 63, 64]. Among these are six ionizable residues on various transmembrane α-helices. These include Asp-240 (TMS-7), Glu-269 (TMS-8), Arg-302 (TMS-9), Lys-319 (TMS-10), His-322 (TMS-10) and Glu-325 (TMS-10). It was long believed that these residues, and others, formed a complex network of charge-charge and hydrogen bond interactions. The recent publication of an inwardly facing, substrate-bound crystal structure has confirmed the proximity of these residues [2].

Based on mutagenesis, biophysical and physiological studies, molecular mechanisms for the coupled transport of cations and lactose have been proposed [1, 2]. In our model, Glu-325 played a key role in H^+ recognition, with Lys-319 and Glu-269 communicating conformational changes that control the coupling of sugar and cation transport. In a model from another laboratory, cations are accepted to the unloaded permease in the outwardly facing conformation by His-322 and Glu-269, which are linked by a hydrogen bond. The binding of H^+ to this site is postulated to allow sugar to bind, which induces a transition to the inward-facing conformation. The model predicts that this conformational change shifts the cation to Glu-325, breaks the hydrogen bond between Glu-269 and His-322 and allows Glu-269 to form a salt-bridge with Arg-144 (Arg-144 is part of the sugar binding site). Sugar substrate is then released, followed by cation release. Cation release is thought to be driven by an interaction between Glu-325 and Arg-302 in this conformation. Cation release causes transition to the outward-facing conformation and reformation of the hydrogen bond between His-322 and Glu-269.

While the details of the two models have parallels and differences, both models make two predictions that are not consistent with the data of the current work. First, both models propose that Glu-269 and/or Glu-325 are critical residues for H^+ recognition and transport. The data in Figure 6 refute this idea. Second, both models propose that interplay between specific ionizable residues is required for the permease to undergo conformational changes associated with sugar transport. The data in Figures 1–3 demonstrate that this is not the case. As an alternative possibility, several ionizable residues within the lactose permease, including Asp-240, Glu-269, Arg-302, Lys-319, His-322 and/or Glu-325, may constitute a "coupling sensor." The coupling sensor would be required to facilitate uphill accumulation of sugar but is not required for binding of either substrate or for conformational changes associated with downhill

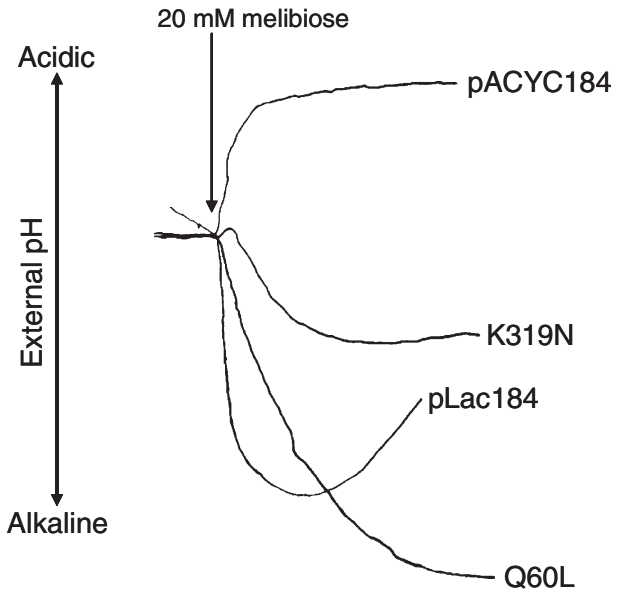

Fig. 6. Sugar-induced H^+ transport was measured in strain DW2 containing the designated plasmids, as described in Materials and Methods. *Arrow* indicates the point of injection of anaerobic melibiose to a final concentration of 20 mM. A deflection of the chart recording in a downward direction indicates alkalinization of the external medium caused by sugar-driven cation transport into cells. A representative experiment is shown. Each strain was tested on three separate days, with the magnitude of the difference between wild-type and suppressors being similar on each day.

transport. Along these lines, the coupling sensor may preclude uncoupled transport by preventing conformational changes when only one substrate (i.e., cation or sugar) is bound to the protein. In this regard, one might speculate that the coupling sensor would be close to the cation and sugar recognition sites and perhaps even contribute partially to the binding of these substrates. However, the data argue strongly that Glu-269, Arg-302, Lys-319 and Glu-325, the residues changed in the Quad parental strain, do not solely constitute either the cation or the sugar recognition sites in the lactose permease.

Many other studies also have provided evidence that Asp-240, Glu-269, Arg-302, Lys-319, His-322 and Glu-325 are not required for cation transport or for conformational changes associated with downhill transport. Analysis of single mutants and double mutants has shown that none of these residues is required for cation transport [5, 13, 14, 30, 32, 37–39, 42]. Though single mutations at any of these sites tend to have very low transport activity, multiple mutations sometimes have restored levels of downhill transport [13, 14, 21, 29, 30, 32, 36–39, 42, 43, 53, 56]. For example, a K319N/E325Q mutant had a normal K_m and showed a downhill transport rate that was about 20% of the wild-type rate. This same mutant showed cation transport with lactose and with thiodigalactoside (TDG). In fact, its TDG-induced cation transport was faster and more robust than that of wild-type (Fig. 4*B*) [30].

Although accumulating evidence indicates that Asp-240, Glu-269, Arg-302, Lys-319, His-322 and Glu-325 are not essential for cation transport, a large amount of data has shown that they are critical for the uphill accumulation of sugar. Single mutations at these sites usually show negligible uphill transport [11, 14, 22, 34, 36–14, 22, 34, 36–39, 42, 50, 53, 56, 57], and all multiple mutants at two or more of these sites are completely defective in the uphill accumulation of sugar [29, 30, 32, 37–39, 53]. In some cases, mutants have shown substantial downhill transport of sugar and cation but were unable to accumulate that same sugar against a gradient [30, 32]. These striking results are consistent with the notion that these residues are not necessary for cation and sugar recognition but are required to control conformational changes that promote coupled transport. Indeed, certain multiple mutants have been shown to exhibit a proton-leaky phenotype [30].

If the hypothesis is correct that Asp-240, Glu-269, Arg-302, Lys-319, His-322 and/or Glu-325 constitute a coupling sensor, it is important to consider how the Quad mutant might disrupt transport. In the wild-type permease (normal coupling sensor), net sugar transport is accomplished by a transition of the loaded permease from the outward-facing conformation to an inward-facing conformation (C1 to C2 conversion). This must be followed by release of substrates and a second transition of the empty permease from inward- to outward-facing (C2 to C1 conversion). Net transport is also dependent on the ability of the protein to restrict forbidden transitions from one side of the membrane to the other. These forbidden transitions include those times when only one substrate is bound (sugar or cation). The combination of mutations in the Quad mutant may lock the protein in a conformation that prevents the C1 to C2 conversion. Individually, each residue substituted in the Quad mutant has been shown to influence transport in an important way. Nonionizable substitutions for Glu-269 are particularly defective in sugar recognition but can still drive sugar-induced cation transport [14]. Arg-302 substitutions allow a cation leak where cations are transported without sugar [42]. Neutral substitutions for His-322 or Glu-325 disrupt coupled transport but retain low levels of downhill sugar transport [14, 52]. In addition, individual substitutions for Glu-325 are completely defective in cation transport and unidirectional transport, but can still catalyze sugar exchange and counterflow [14]. In combination, the changes in the Quad mutant would be expected to create a nonfunctional permease protein that should not be able to interconvert between the C1 and C2 conformation.

With these ideas in mind, it is interesting to speculate regarding the effects of the suppressors

obtained in the current study. K319N alters a residue within the putative coupling sensor itself. Perhaps a realignment of sensor residues in this way could allow C1 to C2 conversion of the loaded permease. The Y236D suppressor introduces a negative charge at a residue that lies in close proximity to another sensor residue (Asp-240) and that forms hydrogen bonds with two others (H322 and R302) in the crystal structure [2]. Perhaps the effect of this suppressor is twofold. The newly introduced negative charge might be in a better position to stabilize the H322Q residue during the transport cycle via a hydrogen bond. In addition, the added charge density in the vicinity of Asp-240 might provide for a weak cation binding site. The deletion of I298 is intriguing. In the crystal structure, E325 is in close proximity to Met-299 [2]. Given its proximity to Met-299, it is plausible that the hydrophobic environment around Met-299 influences the structure of the coupling sensor. The importance of Met-299 in relation to Glu-325 was also revealed when a suppressor mutation was isolated (M299I) that blocked a cation leak in the K319N/E325Q double mutant [30]. The deletion of Ile-298 might push sensor residues into a conformation more competent for transport by repositioning the uncharged E325Q in relation to Met-299. Perhaps the most interesting suppressor obtained in this work is the Q60L mutation, which is very far from the residues of the putative coupling sensor in the tertiary structure of the protein. It is, however, positioned directly at the interface of the two halves of the permease and appears to be critical for conformational changes, as determined from our studies of residues in TMS-2 [17, 19]. Single mutations at this site block transport. One interpretation of this suppressor is that the Quad mutations cause a misalignment between the two halves of the permease at the interface and that the Q60L suppressor compensates for this misalignment. It is not clear how the V229G suppressor would affect residues of the coupling sensor. However, a second-site suppressor (V229A) that restored lactose transport to a R144S mutant was isolated in a previous study, suggesting this residue may affect sugar binding [31]. The effect of V229G might act independently of the coupling sensor by increasing the affinity for sugar and/or by realigning the interaction between transmembrane domains important for transport. Val-229 was also the location of suppressor mutations that restore transport to mutants of residues in both TMS-8 and TMS-2 [17–19]. The S306L suppressor is close to a coupling sensor residue (R302). Substitution of the relatively small serine residue with a large bulky amino acid such as leucine could influence the position of the R302L residue in the Quad mutant, repositioning the coupling sensor to a more transport-competent form.

In summary, the cation binding site and translocation pathway remain enigmatic. We have proposed, and provided evidence for, the idea that coupled transport may be accomplished via a coupling sensor composed primarily of six critical ionizable residues. This sensor has two characteristics critical to permease function: (1) the six residues that make up the sensor must be intact for energy coupling to occur and (2) residues of the sensor monitor the binding of substrates such that the permease is allowed to undergo global conformational changes only under the proper conditions. In addition, these two characteristics are separable. This would help to explain the many instances that have been described where a mutant cannot couple energy from the cation electrochemical gradient to sugar accumulation but can still transport sugar and/or cations downhill. The idea of a coupling sensor allows for another important possibility when considering the results of this study. If a coupling sensor were indeed monitoring substrate binding (cations and sugar), it may not have to directly participate in that binding to control conformational changes associated with transport. This would be particularly important in mutant permease proteins with multiple substitutions within the sensor itself (e.g., the Quad mutant). This would allow for the possibility of compensatory changes (suppressor mutations) that reset or realign interactions among transmembrane domains important for the transport reaction under control of the sensor. Thus, cation and/or sugar transport might be accomplished in an uncoupled manner even when some of the six critical ionizable residues have been replaced with neutral amino acids.

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