Role of Glutamate-126 and Arginine-144 in the Lactose Permease of *Escherichia coli*

Jerry L. Johnson and Robert J. Brooker*

Department of Genetics, Cell Biology and Development and Biotechnology Institute, University of Minnesota, St. Paul, Minnesota 55108

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ABSTRACT: Several previous studies have suggested that glutamate-126 and arginine-144 in the lactose permease of *Escherichia coli* form an ion pair that is essential for sugar binding. To further investigate the role of these residues, E126Q, R144Q, and R144S mutants were made. The R144Q and R144S strains, which had negligible levels of transport, were used as parental strains to isolate suppressor mutations that partially restored sugar transport. The R144Q parent only yielded first-site revertants, but the R144S strain produced three types of second-site replacements: E126Q, V229A, and L330R. In downhill transport assays, the E126Q strain was able to transport lactose at low levels, with an apparent K_m 3-fold higher than the wild-type strain but a severely depressed apparent V_{max}. A triple mutant, E126Q/R144S/V229A, showed a relatively robust V_{max} value for downhill transport and could actively accumulate lactose against a concentration gradient. Taken together, these results indicate that Glu-126 and Arg-144 are not essential for sugar binding. An alternative explanation for their role in maintaining secondary structure is discussed.

Integral membrane proteins known as secondary active transporters or symporters facilitate the coupled transport of solutes across a membrane (*1*, *2*). Many of these proteins are grouped into a large superfamily called the major facilitator superfamily (MFS),[1] members of which transport such diverse substrates as antibiotics, sugars, amino acids, and metabolic intermediates (*3*). Sequence analysis has shown MFS members are phylogenetically related and might share similar protein structures (*4*, *5*).

The best-characterized symporter is the lactose permease of *Escherichia coli*, which transports protons and galactosides in a 1:1 stoichiometry (*6*, *7*). By use of the proton electrochemical gradient generated across the inner membrane, uptake of sugars can be accomplished to many times the external concentration. The permease consists of 417 amino acids with a molecular mass of 46 504 Da and functions as a monomer (*8*−*10*). A battery of studies including hydropathy plots, *phoA* fusions, and *lacZ* gene fusions are consistent with a model arranging the secondary structure into 12 transmembrane α-helical domains (*11*−*13*). Analysis of bioinformatic data from the MFS was combined with data involving salt-bridge interactions to create a tertiary model for the lactose permease (*5*, *14*). Central to this model is the assertion that the first six and last six transmembrane regions form a rotationally symmetrical structure and that the two halves move relative to one another during transport (*14*). Other models have been proposed on the basis of biophysical and cross-linking studies (*15*).

The role of charged residues on putative transmembrane segments in the lactose permease has been the subject of many studies (*16*−*27*). All 417 amino acids in the lactose permease have been changed by site-directed mutagenesis, and six ionic residues within the transmembrane region (Asp-240, Glu-269, Lys-319, Arg-302, His-322, and Glu-325) have been proposed to interact with each other in controlling proton/lactose coupling (*16*−*19*). For example, neutralizations at Glu-325 completely abolish downhill transport and are the only single mutations that cannot catalyze substrate-induced proton cotransport (*20*). However, double mutants K319N/E325Q and H322Q/E325Q have restored activity, consistent with the characteristics of a charge pair (*21*, *22*).

Two other charged residues, Glu-126 and Arg-144, have also been extensively investigated. Several studies have led to the novel suggestion that Glu-126 and Arg-144 may play multiple roles, forming a charge pair that is also essential in sugar recognition (*23*−*27*). For example, cysteine-scanning mutagenesis revealed an overall lack of transport in E126C and R144C mutants (*23*). Similarly, other mutations involving nonconserved changes at these positions were found to inactivate the lactose permease (*23*). Conservative substitutions, however, had measurable activities. Both E126D and R144K mutants showed measurable activity, as did a double mutant E126D/R144K (*23*). Electron paramagnetic resonance (EPR) spectroscopy showed Mn(II) binding in E126H/R144H mutants, consistent with Glu-126 and Arg-144 lying close to each other in the tertiary structure (*25*). NEM binding and NPG flow-dialysis measurements revealed a lack of thiodigalactoside binding in E126A, R144A, R144K, and R144H replacements (*24*). Double mutants E126R/R144E

* To whom correspondence should be addressed: GCD Department, 6-160 Jackson Hall, 321 Church St. SE, Minneapolis, MN 55455. E-mail robert-b@biosci.cbs.umn.edu.

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[1] Abbreviations: MFS, major facilitator superfamily; NEM, *N*-ethylmaleimide; TDG, thiodigalactoside; NPG, nitrophenylgalactoside; TMS, transmembrane segment; IPTG, isopropyl thiogalactoside.

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and E126A/R144A behaved similarly. However, E126D substitutions showed some NPG displacement activity (*26*). These results suggest that TDG has negligible recognition in the R144K, R144A, and E126A strains but has some recognition by the E126D strain. Noticeably, there was very little binding of lactose in E126D mutant as evidenced by ligand protection of Cys-148 in NEM labeling studies (*26*). Therefore, the E126D strain is also defective in the recognition of certain sugars (i.e., lactose) but not completely defective in TDG recognition. Taken together, these studies led to the assertion that a carboxyl group at position 126 and a guanidino group at position 144 are absolutely required for substrate binding. Furthermore, two models suggested specific interactions between Glu-126, Arg-144, and the galactosyl ring of lactose (*26*, *27*). In particular, the first model proposed a hydrogen-bonding network between these residues and the sugar. According to this model, the presence of sugar breaks the ionic interaction, but the two residues remain hydrogen-bonded together as they also hydrogen-bond to the sugar. A very recent study involving the binding of NPG analogues indicated that Arg-144 may form a hydrogen bond with the C-4 hydroxyl of galactose (*27*). This compelling model provides a way to envision how a sugar molecule could bind to a transporter, thereby breaking an ionic interaction between these two residues. Presumably, this event would facilitate conformational changes associated with transport.

In the current study, the goal was to reexamine the role of Glu-126 and Arg-144 in the function of the lactose permease. The implication has been made that both residues are essential for sugar binding and interact directly with the sugar via hydrogen bonding once the ionic interaction is broken. While this model is original and unique, it should be emphasized that the experimental evidence to date is primarily negative. All neutral residues at positions 126 and 144 that have been studied thus far have negligible or nearly negligible transport activity (*23*, *26*). As an alternative explanation to the previously mentioned data, it is possible that mutations at positions 126 and 144 may disrupt secondary structure in a way that severely inhibits activity even though Glu-126 and Arg-144 may not play a key role in sugar recognition. To examine this possibility, the current study examined neutral substitutions at positions 126 and 144 and included a suppressor analysis in which R144Q and R144S strains were used as parental strains to isolate suppressor mutations. The results contradict the original assertion that Glu-126 and Arg-144 play an essential role in sugar recognition.

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. [^{14}C]-Lactose was from Pharmacia (Piscataway, NJ). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). The remaining reagents were analytical-grade.

Bacterial Strains and Methods. For downhill lactose transport, *E. coli* strain HS4006/F′I^{Q}Z^{+}Y^{-} was used. It is *lacZ* positive but *lacY* negative (*28*). For uphill lactose transport, *E. coli* strain T184 was used, which lacks functional *lacZ* and *lacY* genes (*29*). Strain DW2, Δ*lacYZ*, (*30*) was used for the isolation of suppressor mutants. The plasmids in this study are derivatives of the *lacY*-carrying plasmid pLAC184 (*21*).

Plasmid DNA was isolated with the Perfectprep plasmid isolation kit (Eppendorf Corp., Westbury, NY) and introduced into the appropriate bacterial strain by the RbCl method (*31*).

Stock cultures of cells were grown in YT medium (*32*) supplemented with tetracycline (0.01 mg/mL). For transport assays, cells were grown to midlog phase in YT medium containing tetracycline (0.005 mg/mL), and 0.25 mM isopropyl thiogalactoside (IPTG) to induce the synthesis of the lactose permease.

Transport Assays. HS4006/F′I^{Q}Z^{+}Y^{-} cells containing plasmids carrying the wild-type or mutant permease were grown at 37 °C with shaking to midlog phase in YT medium supplemented with 0.005 mg/mL tetracycline and 0.25 mM IPTG. The cells were collected by centrifugation at 5000*g* for 5 min. The cell pellet was washed in phosphate buffer, pH 7.0, containing 60 mM K_2HPO_4 and 40 mM KH_2PO_4, and then resuspended in the same buffer at a concentration of 0.5 mg of protein/mL. In a standard assay, the cells were equilibrated at 30 °C for 5−10 min before [^{14}C]lactose (1.0 μCi/mL) was added to a final concentration of 0.1 mM. For kinetic analyses, the external lactose concentration was varied between 0.1 and 10.0 mM. Aliquots of 200 μL were removed at the appropriate time points and the cells were captured on 0.45 μm Metricel membranes (Gelman Sciences, Inc., Ann Arbor, MI). The cells were then washed with 5−10 mL of ice-cold phosphate buffer by rapid filtration. The filter with the cells was then placed in liquid scintillation fluid and counted in a Beckman LS1801 liquid scintillation counter. The HS4006/F′I^{Q}Z^{+}Y^{-} strain carrying the pACYC184 vector with no *lacY* insert was used to determine the background level of lactose transport. This background value was subtracted from the experimental values to determine the nanomoles of [^{14}C]lactose taken up per milligram of total cellular protein. Uphill and downhill transport assays were similar except that a *lacZ* minus strain was used in the uphill assays.

Site-Directed Mutagenesis. The mutant R144Q was made by PCR mutagenesis as described previously in ref *21*. Briefly, pLAC184 plasmid DNA was used as a template for amplification with a primer that simultaneously changed codon 144 to gln and created a *Sac*I restriction site. This was combined with a nonmutagenic primer over a unique *Sac*II site in the vector region of the plasmid. Two fragments corresponding to each half of the plasmid were thus amplified. This DNA was digested, ligated, and transformed to create pR144Q. Mutations were confirmed by DNA sequencing of the entire *lacY* gene.

Construction of pE126Q and pR144S mutations was carried out with the Transformer mutagenesis kit from Invitrogen (Carlsbad, CA). Plasmid pLAC184 provided the template, and a selection primer changed a native *Sca*I site to *Stu*I. This primer was used in tandem with a mutagenic primer to change the desired codon in the *lacY* gene. Screening of potential mutants was carried out with digestion by *Sca*I and confirmed by DNA sequencing. All mutants were sequenced throughout the entire *lacY* gene.

Kinetic Calculations. For all mutant strains tests, apparent K_m and V_{max} values for lactose transport were determined

by observing initial rates of downhill transport at eight external lactose concentrations. A minimum of six transport measurements at each of the eight concentrations were made for all the mutant strains. Since the pH is constant, the transport velocity follows Michaelis−Menten kinetics with respect to lactose concentration (*33*). 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 (*34*). Results were calculated by nonlinear regression analysis of a plot of v vs [S] with the graphic analysis program KaleidaGraph (Synergy Software, Reading, PA). The Michaelis−Menten equation was programmed into the curve-fitting function of the program, which was used to determine the apparent K_m and V_{max} values. Data from three independent runs were averaged to give a final value for apparent K_m and V_{max}, plus or minus SEM.

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 and immunoblot analysis as described previously (*22*). For strains harboring an R144S mutation, the sonication step in this procedure appeared to cause protein instability. For these mutants, membranes were isolated by a French pressure procedure. Briefly, a 1-L culture of cells was pelleted and suspended to a final wet-weight concentration of 0.2 g/mL in 50 mM TES/NaOH, pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol, 0.1 mg/mL TPCK, 0.7 μg/mL pepstatin, and 25 μg/mL PMSF. The cell suspension was pressed at approximately 20 000 psi, making sure that the sample was maintained at 4 °C. After pressing, the sample was transferred to a centrifuge tube and spun at 20000*g* for 10 min to remove cell debris. This step was repeated until there was not a significant pellet. The supernatant was then transferred to an ultracentrifuge tube and spun at 180000*g* for 45 min. The membrane pellet was resuspended in the French press buffer. The remaining steps in the Western analysis were identical to those described previously (*22*). Normalized to wild type, the expression levels of the mutants were as follows: E126Q, 100%; R144Q, 94%; R144S, 25%; R144S/V229A, 79%; R144S/L330R, 32%; R144S/E126Q, 93%, E126Q/R144S/V229A, 40%; E126Q/ R144S/L330R, not determined; V229A, 92%. By this method, the average standard error was approximately 10%.

RESULTS

Suppressor Analysis. Previous studies concerning the lactose permease and other transporters have identified ionic interactions between residues via a suppressor analysis (*13*, *16*−*18*, *22*, *35*). In this approach, one residue is altered by site-directed mutagenesis to a neutral residue, resulting in a permease with very low activity. Suppressors that restore activity are then obtained. This strategy can identify ionic interactions that are functionally important. For example, suppressor analyses have indicated interactions between Lys-319 and Glu-269 and between His-322 and Glu-325 (*18*, *22*). These paired interactions appear to be essential for the proper coupling between H^+ and lactose cotransport since the neutralization of both charged residues results in the elimination of uphill lactose accumulation. Alternatively, suppressor analyses can identify ionic interactions that are structurally important but not functionally essential. For example, suppressor analyses have shown convincingly that Asp-237 and Lys-358 form a salt bridge (*13*, *35*). When one of the two residues is left unpaired, the effect is highly inhibitory. However, neutralization of charges at both sites results in a functional permease that effectively accumulates lactose against a gradient. These results indicate that Asp-237 and Lys-358 are not essential in the mechanism of H^+/lactose cotransport.

Table 1: Suppressor Mutations of Position 144 Parent Strains

parent strain	mutation	no. of isolates	codon change	MacConkey phenotype (melibiose)	MacConkey phenotype (lactose)
R144Q	Q144R	3	CAG → CGG	red	red
R144S	L330R	4	CTG → CGG	red	red
R144S	V229A	3	GTT → GCT	red	red center
R144S	E126Q	2	GAG → CAG	red	red

E. coli strain HS4006/F′$I^QZ^+Y^-$ harboring the designated plasmid was streaked on 1% melibiose or 1% lactose MacConkey plates, and the color of individual colonies was assessed after 20 h.

To explore the importance of the putative Glu-126/Arg-144 interaction, neutral substitutions were made at either site. Glu-126 was changed to glutamine, or Arg-144 was changed to glutamine or serine. Strains harboring the single E126Q mutation formed red colonies on MacConkey agar with either lactose (a β-galactoside) or melibiose (an α-galactoside) as the sugar source, indicating an appreciable level of transport and fermentation. It should be noted that this plating result is different than seen in previous studies (*23*). One explanation for this is that the plates were incubated for a different length of time than in the previous study. Another is the fact that a different *lacZ*$^+$ strain was used in the current study (HS4006/F′ vs HB101). Due to the red phenotype, it was not possible to use this strain for the isolation of spontaneous suppressor mutations. In contrast, the R144Q and R144S strains formed white colonies, indicating a negligible level of transport.

To isolate suppressor mutations, cells containing the pR144Q or pR144S plasmid in the strain DW2 (Δ*lacZ* Δ*lacY*) were plated on MacConkey agar containing either 1.0% or 0.4% melibiose. This sugar was used because the DW2 cells cannot metabolize lactose. While the colonies were white initially, after about 8 days of incubation, mutant red flecks appeared in the primary streak. These areas were picked and restreaked to isolate individual red colonies. Plasmid DNA from these colonies was isolated and transformed into DW2 competent cells to ensure that the restoring mutation was located on the plasmid. The plasmids from red retransformants were then isolated and sequenced.

The results of this suppressor analysis are shown in Table 1. From the R144Q strain, all suppressors of the white parental phenotype were first-site revertants in which codon 144 was changed back to an arginine codon. This reversion can occur by a single base change in the glutamine codon, and these revertants were not studied further. However, in the R144S strain, the serine codon was chosen in such a way as to prevent the isolation of such revertants. In the pR144S construct, serine is a two-base change (TCG) that cannot revert back to arginine with a single substitution. In this way, the appearance of second-site suppressors was favored. As shown in Table 1, only second-site suppressors were obtained from this strain. These can be divided into three classes, R144S/V229A, R144S/L330R, and R144S/E126Q. Of these, all were red on melibiose plates in the

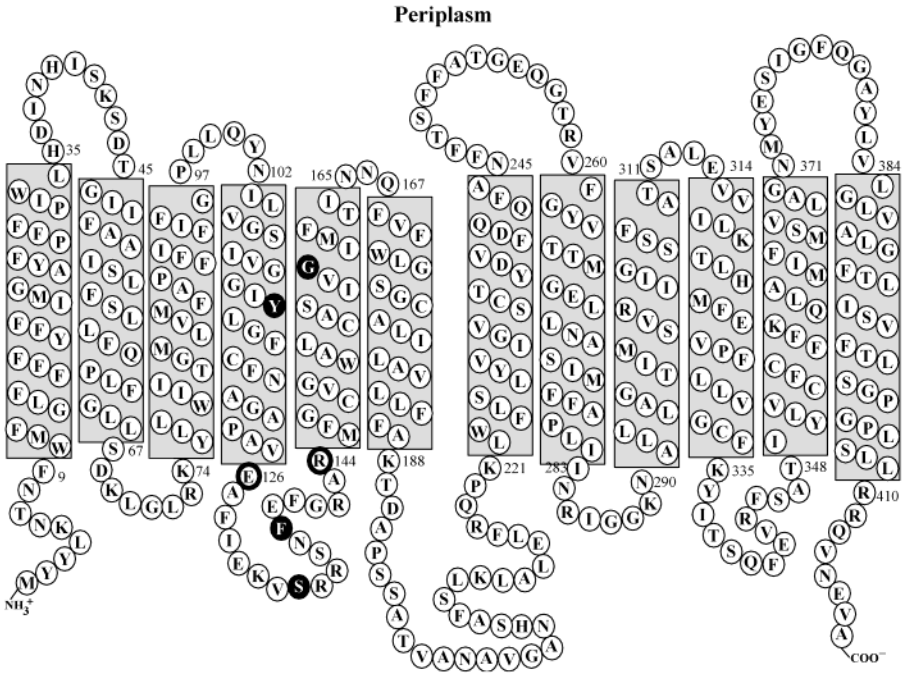

FIGURE 1: Secondary topology model of the lactose permease (see ref *39*). The modified borders of TMS-4 and TMS-5 proposed in ref *40* are indicated by darkened circles. In this alternative model, TMS-4 includes Tyr-113 to Ser-133 and TMS-5 includes Phe-138 to Gly-159. Therefore, this alternative model suggests that Glu-126 is near the center of TMS-4 and Arg-144 is approximately six amino acids from the cytoplasmic edge.

Table 2: Transport Characteristics of Lactose Permease Mutants

strain	apparent K_m[a] ± SEM (mM)	apparent V_{max}[a] ± SEM (nmol ·mg^{-1}·min^{-1}) [adjusted V_{max}]	uphill transport[b] [in]/ [out] 0.1 mM	1.0 mM
pLAC184 (wild type)	0.6 ± 0.1	323.9 ± 39.4	17.8 ± 1.8	6.0 ± 1.1
pE126Q	2.0 ± 0.8	7.7 ± 2.1 [7.7]	1.4 ± 0.04	1.2 ± 0.2
pR144Q	*c, d*	*c, d*	≪1	c
pR144S	*c, d*	*c, d*	≪1	c
pR144S/V229A	>5	>15	≪1	c
pR144S/L330R	>5	>15	≪1	c
pR144S/E126Q	3.3 ± 0.4	15.1 ± 2.9 [16.2]	2.5 ± 0.4	1.9 ± 0.5
pE126Q/R144S/V229A	2.1 ± 0.6	56.9 ± 1.1 [142]	4.2 ± 0.1	2.9 ± 0.6
pE126Q/R144S/L330R	*c, d*	*c, d*	≪1	c
pV229A	0.4 ± 0.06	198.6 ± 4.2 [215]	c	c

[a] K_m and V_{max} values were determined at 30 °C as described under Materials and Methods. [b] Steady-state level at 15 min, determined at 37 °C as described under Materials and Methods. [c] Not determined. [d] Transport levels were too low for accurate kinetic measurement. Velocity was less than 1% that of the wild-type strain.

DW2 strain. After transformation into a strain that was *lacZ*$^+$, they were also red on lactose MacConkey plates, though the V229A suppressor was less so. Val-229 and Leu-330 are predicted in our secondary model to lie within the transmembrane region of helices 7 and 10, respectively (see Figure 1).

Sugar Transport Analysis. Kinetic analyses of the wild-type and mutant strains were carried out by transforming plasmids into *E. coli* strain HS4006/F′Z$^+$Y$^-$, which produces β-galactosidase. In this genetic background, lactose transported into the cells is quickly broken down into glucose and galactose. Therefore transport of lactose is always "downhill" with respect to its external concentration. As shown in Table 2, the wild-type permease effectively transports lactose, exhibiting an apparent K_m for lactose of 0.6 mM, and a V_{max} value of 323.9 nmol of lactose min^{-1} (mg of protein)$^{-1}$.

With regard to the parental mutations at positions 126 or 144, the effects of the E126Q mutation were quite surprising in light of previously reported results (*23*−*27*). The K_m value was moderately elevated, approximately 3-fold, compared with the wild-type strain. In contrast, the V_{max} value was severely depressed (i.e., 2.4% of wild type). Thus, the E126Q mutation has an effect on both K_m and V_{max}. Quantitatively, a larger effect is on the velocity for transport, while sugar binding is only moderately affected. In the case of the R144Q and R144S strains, the level of lactose transport was negligible, even over long time periods (e.g., 10 min). Therefore, it was not possible to measure the kinetic parameter of lactose transport in these strains.

Kinetic analyses were also conducted on the second-site suppressors. The suppressors had relatively high values for apparent K_m and apparent V_{max} values in the range of 5% or higher. Kinetic values for the V229A and L330R suppressors could not be accurately determined because their transport did not saturate even at an external lactose concentration of 5−10 mM. It can be estimated that their K_m values are substantially above 5 mM.

Since second-site suppressor mutations were obtained in the first half (E126Q) and second half (V229A and L330R) of the permease, it was of interest to determine the effects of multiple suppressors when coupled to the R144S mutation. Two different triple mutants were constructed: E126Q/R144S/V229A and E126Q/R144S/L330R. Kinetic analysis of these triple mutants yielded striking results. For the triple mutant containing L330R, the transport levels were too low for an accurate kinetic measurement. In this case, the values were less than 1% that of the wild-type strain. In sharp contrast, the E126Q/R144S/V229A triple mutant had relatively robust activity. Its K_m value was elevated 3−4-fold, and its V_{max} value was approximately 17% of the wild-type value. The single V229A mutant was also analyzed and it exhibited kinetics that were similar to those of the wild-type strain.

A second way to analyze permease function is the ability to transport galactosides into a cell against a concentration gradient. This requires effective coupling between H^+ and lactose transport. To conduct such an assay, plasmids are introduced into an *E. coli* strain that cannot metabolize lactose. The ability to accumulate lactose is then monitored over time, and the results are expressed as the ratio of lactose inside the cell compared to the external concentration. The results for two lactose concentrations are shown in Table 2. The wild-type permease transports to a steady-state level of about 18 times the external concentration of 0.1 mM and about 6 times an equilibrium of 1.0 mM. The R144S, R144Q, R144S/L330R, and E126Q/R144S/L330R strains have negligible transport, while the E126Q strain transports lactose to a 1:1 equilibrium value. The R144S/E126Q suppressor shows significant accumulation to levels that are approximately 2 times the external concentration. The triple mutant E126Q/R144S/V229A shows even higher levels of transport, 4 times the equilibrium value at 0.1 mM and approximately 3 times the level at 1.0 mM. These results clearly indicate that an ionic interaction between the putative E126/R144 charge pair is not required for coupled H^+/lactose transport or the uphill accumulation of galactosides. An interaction, possibly through hydrogen bonds, may still be required for optimal activity. This may be important in holding the protein together at the secondary structure level.

DISCUSSION

A series of publications gave compelling evidence that Glu-126 and Arg-144 may be required for substrate binding in the lactose permease (*23*−*27*). The assertion was made that these residues are clearly essential, and that a carboxylate group at position 126 and a guanidino group at position 144 are absolutely required for sugar recognition. This was an attractive model which also suggested that the breakage of this essential ion pair is a consequence of sugar binding. However, two lines of data in the current work dispute this model. First, a kinetic analysis of the single E126Q mutation shows that this strain has a fairly good affinity for lactose, though the V_{max} is relatively poor (see Table 2). The second and most convincing argument against the functional importance of these residues lies with the E126Q/R144S/V229A triple mutant. In this strain, both residues are neutralized, and the arginine, in particular, is changed to a serine residue; serine is a much smaller residue and would not be expected to have hydrogen-bonding properties resembling those of arginine. Nevertheless, the triple mutant recognizes lactose with a reasonable affinity, has a moderate transport rate, and can catalyze the uphill accumulation of lactose. Taken together, these data argue that Glu-126 and Arg-144 are not essential for sugar recognition, H^+/sugar coupling, or the uphill accumulation of sugar. However, they could play a minor role in sugar recognition and/or play a role in maintaining secondary structure. Furthermore, the data are not inconsistent with the possibility that Glu-126 and Arg-144 form a charge pair in the wild-type protein. Indeed, the data of the current study suggest that this is a likely possibility. Nevertheless, such a putative pairing between Glu-126 and Arg-144 is not essential for function, as is particularly evident in the E126Q/R144S/V229A strain.

Another question regarding Glu-126 and Arg-144 concerns their location within secondary topological models of the lactose permease. On the basis of a variety of studies (i.e., refs *4* and *11*−*13*), our laboratory proposed a model in which Glu-126 and Arg-144 are predicted to lie at the cytoplasmic interface (see Figure 1). Alternatively, the lipid/aqueous boundaries have also been investigated by placing single amino acid deletions within the lactose permease (*40*). The assumption of this approach is that deletions should be tolerated in loop regions but not in transmembranous regions. Because deletions at positions 113 and 114 inactivated the permease, as did 132 and 133 deletions, it was surmised that the area between those residues is transmembranous. Similar results were seen with positions 138 and 139 and with positions 158 and 159 to define that region as transmembranous. However, it should be pointed out that the study did not include point deletions between positions 139 and 158. Nevertheless, as a result of this deletion approach, a secondary model was proposed, suggesting that Glu-126 and Arg-144 are located within transmembrane segments 4 and 5, respectively (also see Figure 1).

Given the results obtained with the triple mutant, it is important to ask why single, neutral substitutions at positions 126 and 144 have such detrimental effects. One possibility may be related to their locations within the secondary structure of the lactose permease. According to our model, these residues lie at the interface between the lipid/aqueous boundaries of TMS-4 and TMS-5 (see Figure 1). Neutral substitutions may cause significant perturbations in the proper topology of TMS-4 and TMS-5 within the plane of the membrane. Along these lines, sequence analyses of MFS members may provide insight into this question.

Table 3 shows the results of MFS alignments in the TMS-4/TMS-5 region. For family 5, which includes the lactose permease, an arginine at the cytoplasmic lipid/aqueous boundary of TMS-5 is completely conserved. At the cytoplasmic lipid/aqueous boundary of TMS-4, a glutamic acid residue tolerates aspartic acid replacement. In the consensus sequences of 14 families studied, seven of them show a negative− positive border approximating the predicted region that contains the connecting loop between TMS-4 and TMS-5. Twelve of the 14 families showed a conserved basic residue within three amino acids of the predicted loop/TMS-5 boundary. Overall, the results of this analysis suggest that charges at the cytoplasmic ends of TMS-4 and TMS-5 are frequently found in a majority of family members. This conservation of charges may indicate a structural role for these residues in maintaining the proper topology of TMS-4

Table 3: Predicted Loop 4/5 Region among Families of the MFS[a]

family[b]	loop 4/5 region (shown underlined)
1	YlsEmA-envRGkmiSm
3	vvaDit-ker--r-fg
4	pcgrtmthWfskkeRGtw
5	gaieayiErvsR-s-FEyGkaRmfG
6	vGgEygg-avym-Eva--grkgFaySfq-
7	Pyvtvlg---tat-rlnl
8	nisfffPkkkqG-alGlngg-
9	atI-SEyankktRGafia
10	Nsisy--l-qaglDivtdFPpIRvfG
11	iamn-avkWfpdkrGLa
12	yavEsWPk-lr-KAsa
13	migkyfykkrplAn
15	llsey-p-r-rgtlvg
17	PslIIKrkfp-kailig

[a] For each family, one member was chosen as a query sequence to search for homologous members with the database program FASTA (36). High-scoring matches were saved, and subsequently aligned with CLUSTALW (37) to create a consensus sequence. Predicted transmembrane segments of sequences were individually analyzed with the program TMHMM (38), and these were aligned with the consensus sequence to obtain an overall TMS-4/TMS-5 structure for each family. Underlined residues are predicted to lie in the cytoplasmic loop connecting TMS-4 and TMS-5. Uppercase residues are completely conserved among the family members studied, lowercase letters are >50% conserved among similar residues, and a dash indicates no conservation. Due to substantial variation among family members, a consensus sequence for the loop 4/5 region could not be established for families 2, 14, and 16. [b] Family numbers are described in ref *3*.

and TMS-5. Therefore, mutants that neutralize charged residues at these boundaries may greatly perturb the locations of TMS-4 and/or TMS-5 in the tertiary structure of the permease.

REFERENCES

1. Crane, R. K. (1977) *Rev. Physiol. Biochem. Pharmacol. 78*, 99−159.
2. Mitchell, P. (1963) *Biochem. Soc. Symp. 22*, 142−168.
3. Pao, S. S., Paulsen, I. T., and Saier, M. J., Jr. (1998) *Microbiol. Mol. Biol. Rev. 62*, 1−34.
4. Foster, D. L., Boublik, M., and Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31−34.
5. Goswitz, V. C., and Brooker, R. J. (1995) *Protein Sci. 4*, 543−537.
6. West, I. C. (1970) *Biochem. Biophys. Res. Commun. 41*, 655−661.
7. West, I. C., and Mitchell, P. (1973) *Biochem. J. 132*, 587−592.
8. Büchel, D. E., Gronenborn, B., and Müller-Hill, B. (1980) *Nature 283*, 541−545.
9. Teather, R. M., Müller-Hill, B., Abrutsch, U., Aichele, G., and Overath, P. (1978) *Mol. Gen. Genet. 159*, 239−248.
10. Kaback, H. R., Jung, K., Jung, H., Wu, J., Privé, G. G., and Zen, K. H. (1993) *J. Bioenerg. Biomembr. 25*, 627−635.
11. Calamia, J., and Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937−4941.
12. Calamia, J., and Manoil, C. (1992) *J. Mol. Biol. 224*, 539−543.
13. King, S. C., Hansen, C. L., and Wilson, T. H. (1991) *Biochim. Biophys. Acta 1062*, 177−186.
14. Green, A. L., Anderson, E. A., and Brooker, R. J. (2000) *J. Biol. Chem. 275*, 23240−23246.
15. Wang, Q., and Kaback, H. R. (1999) *Biochemistry 38*, 16777−16782.
16. Lee, J. I., Hwang, P. P., Hansen, C., and Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−20764.
17. Lee, J. I., Hwang, P. P., and Wilson, T. H. (1993) *J. Biol. Chem. 268*, 20007−20015.
18. Lee, J. I., Varela, M. F., and Wilson, T. H. (1996) *Biochim. Biophys. Acta 1278*, 111−118.
19. Jung, K., Jung, H., Wu, J., Privé, G. G., and Kaback, H. R. (1993) *Biochemistry 32*, 12273−12278.
20. Franco, P. J., and Brooker, R. J. (1994) *J. Biol. Chem. 269*, 7379−7386.
21. Johnson, J. L., and Brooker, R. J. (1999) *J. Biol. Chem. 274*, 4074−4081.
22. Johnson, J. L., Lockheart, M. S. K., and Brooker, R. J. (2001) *J. Membr. Biol. 181*, 215−224.
23. Frillingos, S., Gonzalez, A., and Kaback, H. R. (1997) *Biochemistry 36*, 14284−14290.
24. Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802−9807.
25. Zhao, M., Zen, K. C., Hubbell, W. L., and Kaback, H. R. (1999) *Biochemistry 38*, 7407−7412.
26. Sahin-Toth, M., le Coutre, J., Kharabi, D., le Maire, G., Lee, J. C., and Kaback, H. R. (1999) *Biochemistry 38*, 813−819.
27. Sahin-Toth, M., Lawrence, M. C., Nishio, T., and Kaback, H. R. (2001) *Biochemistry 43*, 13015−13019.
28. Brooker, R. J., and Wilson, T. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3959−3963.
29. Teather, R. M., Bramhall, H., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, U., and Overath, P. (1980) *Eur. J. Biochem. 108*, 223−231.
30. Wilson, D. M., and Wilson, T. H. (1987) *Biochim. Biophys. Acta 904*, 191−200.
31. New England Biolabs (1994) *New England Biolabs Transcript*, Vol. 6, p 7, New England Biolabs, Beverly, MA.
32. Miller, J. (1972) in *Experiments in Molecular Genetics*, p 433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. Segel, I. (1975) *Enzyme Kinetics*, Wiley and Interscience, New York.
34. Varela, M. F., Brooker, R. J., and Wilson, T. H. (1997) *J. Bacteriol. 179*, 5570−5573.
35. Dunten, R. L., Sahin-Toth, M., and Kaback, H. R. (1993) *Biochemistry 32*, 12644−12650.
36. Pearson, W. R., and Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444−2448.
37. Thompson, J. D., Higgins D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
38. Krogh, A., Larsson, B., von Heijne G., and Sonnhammer, E. L. (2001) *J. Mol. Biol. 305*, 567−580.
39. Green, A. L., and Brooker, R. J. (2001) *Biochemistry 40*, 12220−12229.
40. Wolin, C., and Kaback, H. R. (1999) *Biochemistry 38*, 8590−8597.

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