

# The proton electrochemical gradient induces a kinetic asymmetry in the symport cycle of LacY

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LacY catalyzes accumulation of galactosides against a concentration gradient by coupling galactoside and H<sup>+</sup> transport (i.e., symport). While alternating access of sugar- and H<sup>+</sup>-binding sites to either side of the membrane is driven by binding and dissociation of sugar, the electrochemical H<sup>+</sup> gradient ( $\Delta\tilde{\mu}_{H^+}$ ) functions kinetically by decreasing the K<sub>m</sub> for influx 50- to 100-fold with no change in K<sub>d</sub>. The affinity of protonated LacY for sugar has an apparent pK (pK<sup>app</sup>) of ~10.5, due specifically to the pK<sub>a</sub> of Glu325, a residue that plays an irreplaceable role in coupling. In this study, rates of lactose/H<sup>+</sup> efflux were measured from pH 5.0 to 9.0 in the absence or presence of a membrane potential ( $\Delta\Psi$ , interior positive), and the effect of the imposed  $\Delta\Psi$  on the kinetics of efflux was also studied in right-side-out membrane vesicles. The findings reveal that  $\Delta\tilde{\mu}_{H^+}$  induces an asymmetry in the transport cycle based on the following observations: 1) the efflux rate of WT LacY exhibits a pK<sup>app</sup> of ~7.2 that is unaffected by the imposed  $\Delta\Psi$ ; 2)  $\Delta\Psi$  increases the rate of efflux at all tested pH values, but enhancement is almost 2 orders of magnitude less than observed for influx; 3) mutant Glu325 → Ala does little or no efflux in the absence or presence of  $\Delta\Psi$ , and ambient pH has no effect; and 4) the effect of  $\Delta\Psi$  (interior positive) on the K<sub>m</sub> for efflux is almost insignificant relative to the 50- to 100-fold decrease in the K<sub>m</sub> for influx driven by  $\Delta\Psi$  (interior negative).

membranes | transport | permease | membrane proteins | efflux

*Escherichia coli* lactose permease (LacY), the prototype of the major facilitator superfamily (MFS), catalyzes coupled transport of lactose and an H<sup>+</sup> (lactose/H<sup>+</sup> symport). Thus, in the presence of an electrochemical H<sup>+</sup> gradient ( $\Delta\tilde{\mu}_{H^+}$ , interior negative and/or alkaline), LacY utilizes free energy generated by the energetically downhill flux of H<sup>+</sup> to drive uphill accumulation of lactose against a concentration gradient (aka active transport; Fig. 1A). Furthermore, in the absence of  $\Delta\tilde{\mu}_{H^+}$ , downhill transport of lactose in response to a concentration gradient drives uphill flux of H<sup>+</sup> with generation of  $\Delta\tilde{\mu}_{H^+}$ , the polarity of which depends on the direction of the lactose gradient (Fig. 1B, influx → interior positive and acid; Fig. 1C, efflux → interior negative and alkaline) (reviewed in ref. 1).

LacY is structurally and functionally a monomer (2) with 12 transmembrane  $\alpha$ -helices, many of which are shaped irregularly, arranged into N- and C-terminal 6-helix bundles with the N and C termini on the cytoplasmic side of the membrane (3). Two 3-helix inverted repeats are also observed within each 6-helix bundle (4), and there is a relatively long cytoplasmic loop between helix VI and VII that tethers the 2 6-helix pseudosymmetrical domains. Ten structures of LacY have been obtained by X-ray crystallography (3, 5–10). The first, a conformationally restricted mutant C154G, and the WT are inward (cytoplasmic)-open, apo conformers with a spacious, central aqueous cavity open on the cytoplasmic side and tightly sealed on the periplasmic side (3). This conformer appears to be the resting state of LacY in the membrane (11, 12). A second conformer obtained with double-Trp mutant G46W/G262W (LacY<sub>ww</sub>) (13) is a partially outward (periplasmic)-open, occluded conformer with either of 2 bound lactose homologs in the middle of the molecule and a sealed cytoplasmic side (9, 10). An additional

apo conformer was also obtained with LacY<sub>ww</sub> in complex with a nanobody (Nb) bound to the periplasmic side (8).

More than a half dozen independent biochemical/spectroscopic methods combined with the X-ray crystal structures of LacY provide virtually unequivocal evidence that conformational transitions between inward- and outward-facing forms result in sugar and H<sup>+</sup> transport across the membrane (14). In this manner, cytoplasmic and periplasmic cavities open/close reciprocally, thereby allowing alternating exposure of galactoside- and H<sup>+</sup>-binding sites to either side of the membrane (Fig. 2) (15, 16). Transmembrane exchange reactions (i.e., equilibrium exchange and counterflow), which reflect alternating access, occur without deprotonation, and  $\Delta\tilde{\mu}_{H^+}$  has no effect on these reactions. Therefore, the driving force for the conformational change(s) responsible for alternating access is not  $\Delta\tilde{\mu}_{H^+}$  but binding and dissociation of galactoside (17).

The affinity of LacY for galactosides varies with pH, and the apparent pK (pK<sup>app</sup>) for galactoside binding is unexpectedly alkaline at ~10.5 (18–21). Direct measurements of Glu325 in situ by surface enhanced infrared absorption spectroscopy demonstrate that the side chain has a pK<sub>a</sub> of 10.5 ± 0.1 (21), which concurs with the pK<sup>app</sup> for galactoside affinity (18, 19). These and previous findings (reviewed in refs. 1 and 17) clearly indicate that LacY (i.e., Glu325) is protonated over the physiological pH range. Indeed, sugar binding to purified LacY in detergent does

## Significance

Protonation and deprotonation of Glu325 with a pK<sub>a</sub> of 10.5 is required for symport. Moreover, the H<sup>+</sup> electrochemical gradient ( $\Delta\tilde{\mu}_{H^+}$ ) accelerates deprotonation on the intracellular side with a 50- to 100-fold decrease in the K<sub>m</sub>. To probe the pK on the cytoplasmic side of the membrane, rates of lactose/H<sup>+</sup> efflux were determined from pH 5.0 to 9.0 without or with a membrane potential ( $\Delta\Psi$ , interior positive) in right-side-out membrane vesicles. WT lactose efflux has an apparent pK of ~7.2 that is unaffected by  $\Delta\Psi$ , mutant E325A is defective, and pH or  $\Delta\Psi$  (interior positive) has no effect. The effect of  $\Delta\Psi$  (interior positive) on the K<sub>m</sub> for efflux with WT LacY is insignificant relative to the marked effect on influx.

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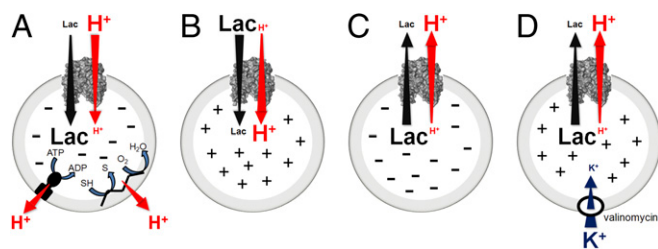
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**Fig. 1.** Transport reactions of LacY. (A)  $\Delta\mu_{H^+}$ -driven influx (i.e., active transport): free energy released from the downhill translocation of  $H^+$  in response to  $\Delta\mu_{H^+}$  (interior negative and/or alkaline) generated by the respiratory chain or  $F_1/F_o$  ATPase drives energetically uphill translocation of galactoside. (B) Influx: galactoside influx down a concentration gradient drives uphill  $H^+$  translocation with generation of  $\Delta\mu_{H^+}$  (interior positive and acidic). (C) Efflux: galactoside efflux down a concentration gradient with generation of  $\Delta\mu_{H^+}$  (interior negative and alkaline). (D) Imposition of  $\Delta\Psi$  (interior positive) during galactoside efflux: generated by influx of  $K^+$  down a concentration gradient in the presence of valinomycin.

not induce a change in ambient pH under conditions where binding or release of 1  $H^+$ /LacY can be measured (20).

Coupling of galactoside with  $H^+$  translocation is clearly central to the mechanism of galactoside/ $H^+$  symport. Site-directed and Cys-scanning mutagenesis of the 417 residues in LacY reveal 9 irreplaceable aminoacyl side chains with respect to  $\Delta\mu_{H^+}$ -driven lactose/ $H^+$  symport (22). Among the 9 residues, Glu325 is essential for  $H^+$  translocation since neutral replacement mutants at this position do not catalyze any reaction involving  $H^+$  translocation but bind galactosides with normal affinity and catalyze transmembrane sugar exchange reactions at least as well as WT LacY (23, 24). Strikingly, sugar binding becomes pH independent over a wide pH range in the mutant. This behavior is unique and indicates that Glu325 is directly involved in  $H^+$  binding and coupled  $H^+$  transport (17).

The mechanism of lactose/ $H^+$  symport with LacY may not be symmetrical. For instance, generation of  $\Delta\mu_{H^+}$  (interior negative and/or alkaline) dramatically stimulates the initial rate of lactose influx by decreasing  $K_m$  50- to 100-fold (25, 26). However, a  $\Delta\Psi$  (interior positive or negative) or a  $\Delta pH$  (interior acid or alkaline) does not affect the  $K_m$  for efflux dramatically (25). Recent stopped-flow experiments also demonstrate that the cytoplasmic side of LacY is able to open spontaneously when the periplasmic side is locked by a disulfide bond, indicating that an asymmetric conformational change can occur during the transport cycle (27). Nevertheless, there is strong evidence (reviewed in refs. 1 and 28) for a symmetrical ordered kinetic mechanism in which protonation precedes galactoside binding on one side of the membrane and follows sugar dissociation on the other side. A similar ordered mechanism, which also insures against futile  $H^+$  cycles, may be common to other members of the MFS (29–33). In an effort to study the problem more extensively, the present study was undertaken. However, because of the difficulty obtaining quantitative transport data from inverted vesicles due to their small internal volume (24), efflux from right-side-out (RSO) vesicles was utilized.

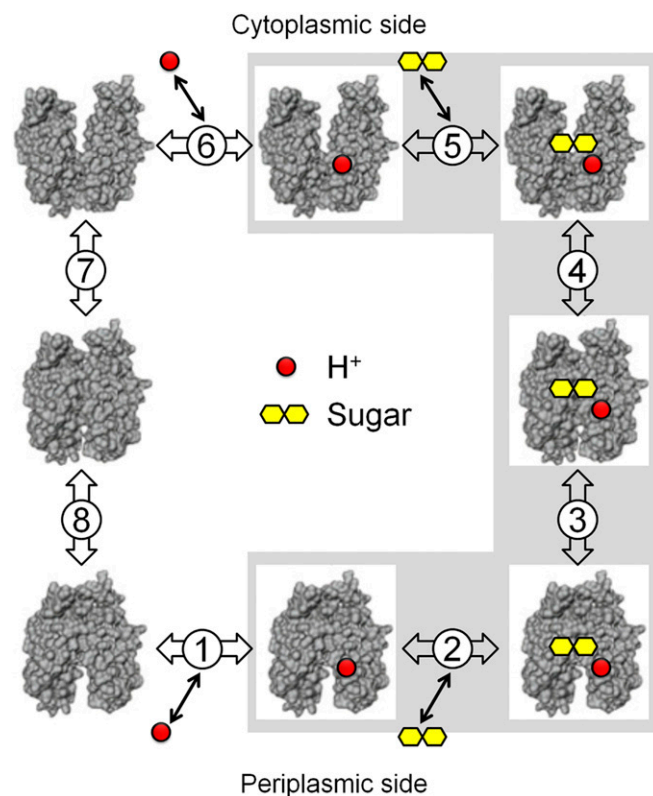
Initial rates of lactose efflux were measured from pH 5.0 to 9.0 in the absence or presence of a membrane potential ( $\Delta\Psi$ , interior positive) generated by  $K^+$  influx in the presence of valinomycin, a well-known  $K^+$  ionophore. Rates of efflux are pH dependent and exhibit a  $pK^{app}$  of  $\sim 7.2$ . Imposition of  $\Delta\Psi$  (interior positive) increases the initial rate of efflux by only 2-fold or less, while a  $\Delta\Psi$  (interior negative) causes a 50- to 100-fold decrease in  $K_m$ . Mutant E325A catalyzes efflux very slowly, and an imposed  $\Delta\Psi$  has no significant effect. Moreover, the  $K_m$  for efflux remains unchanged when a  $\Delta\Psi$  (interior positive) is imposed. The results demonstrate that the  $pK^{app}$  for efflux also

involves Glu325 but is hardly affected by  $\Delta\mu_{H^+}$ , which imposes a strong asymmetry on the symport cycle.

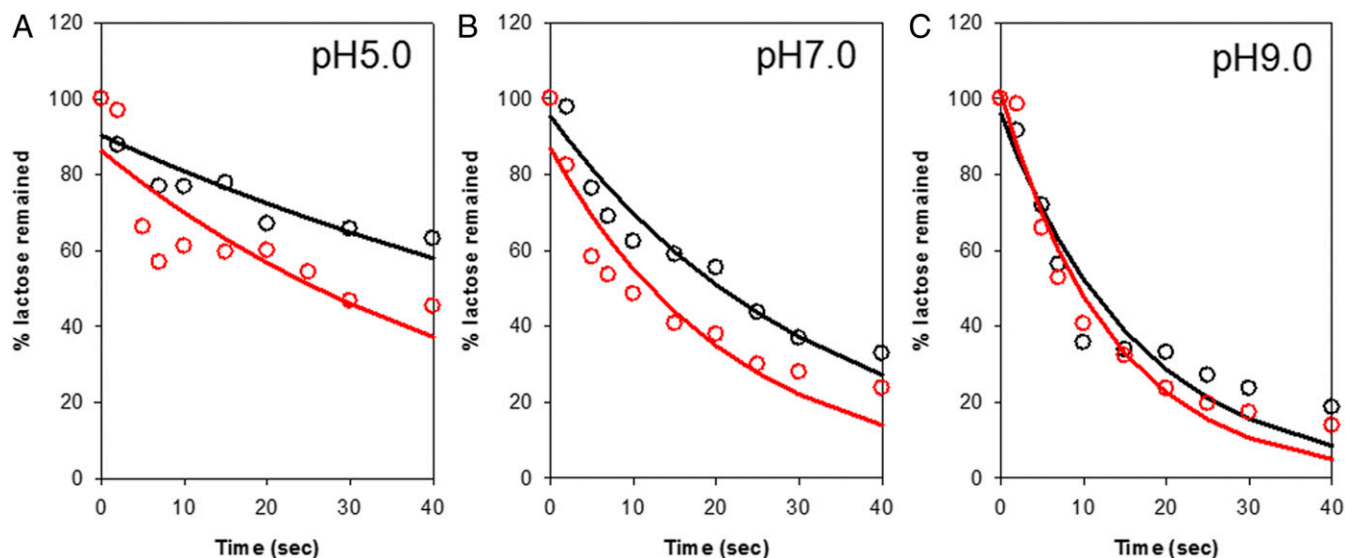
## Results

**WT LacY.** RSO membrane vesicles expressing WT LacY prepared in  $KP_i$  (for efflux without an imposed  $\Delta\Psi$ ) or  $NaP_i$  and valinomycin (for efflux with an interior positive  $\Delta\Psi$ ) were pre-equilibrated with [ $^{14}C$ ]lactose at a given pH and rapidly diluted 200-fold into  $KP_i$  at the same pH, and samples were rapidly filtered over the initial 40 s in order to measure initial rates of efflux (Fig. 3 and *SI Appendix*, Fig. S2). In the absence of an imposed  $\Delta\Psi$ , efflux is relatively slow at acidic pH and becomes more rapid with increasing pH. Thus, the half-time for efflux ( $t_{1/2}$ ) decreases from  $\sim 62$  s at pH 5.0 to  $\sim 22$  s at pH 7.0 to  $\sim 11$  s at pH 9.0 (Fig. 3 and *SI Appendix*, Fig. S2 and Table S1), as indicated previously (34).

Rapid dilution of RSO membrane vesicles preloaded with  $NaP_i$  into equimolar  $KP_i$  in the presence of valinomycin generates a membrane potential ( $\Delta\Psi$ , interior positive) that can be monitored by the fluorescence change in bis(1,3-diethylbarbituric acid) [DiBAC<sub>4</sub> (3)] (Fig. 1D). The positive  $\Delta\Psi$  causes an increase in the fluorescence of DiBAC<sub>4</sub> (3), which is maintained for at least 1.0 min (*SI Appendix*, Fig. S1). Under these conditions, the rate of efflux also increases with pH, and  $t_{1/2}$  decreases (Table 1). Compared to the rates in the absence of  $\Delta\Psi$ , imposition of  $\Delta\Psi$  (interior positive)



**Fig. 2.** Kinetic scheme for galactoside/ $H^+$  symport, exchange, and counterflow. Symport starts with protonation of LacY (step 1 or 6 for influx or efflux, respectively), which is required for high-affinity binding of lactose. Sugar binding to protonated LacY (step 2 or 5) causes a conformational change to an occluded state (step 3 or 4), which can relax to either side where sugar dissociates first (step 2 or 5), followed by deprotonation (step 1 or 6) and return of unloaded LacY via an apo occluded intermediate (steps 7 and 8). Exchange or counterflow involves only steps 2 to 5 (gray shaded area). Since LacY catalyzes symport in both directions, when symport is in the influx direction—step 1, protonation—the  $pK$  is very alkaline ( $\sim 10.5$ ), and step 6—deprotonation—must have a much lower  $pK$  for deprotonation to occur. However, in the efflux direction, the  $pK$  values of these steps are reversed.



**Fig. 3.** Effect of pH on the rate of efflux in the absence or presence of  $\Delta\Psi$  (interior positive) by WT LacY. Typical examples of efflux experiments with WT LacY were carried out by equilibrating RSO membrane vesicles with 10 mM [ $^{14}\text{C}$ ]lactose at given values of pH followed by a rapid 200-fold dilution (*Materials and Methods*) in the absence (black line) or presence (red line) of  $\Delta\Psi$  (interior positive).  $\Delta\Psi$  (interior positive) was generated by diluting [ $^{14}\text{C}$ ]lactose-loaded RSO membrane vesicles in 100 mM  $\text{NaP}_i$  into 100 mM  $\text{KP}_i$  in the presence of 25  $\mu\text{M}$  valinomycin at given pH values. Efflux data of WT LacY obtained at pH 5.0, pH 7.0, and pH 9.0 are shown in A, B, and C, respectively. No effect was observed upon omission of valinomycin or dilution of vesicles loaded with  $\text{KP}_i$  into equimolar  $\text{KP}_i$  in the presence of the ionophore. Lines represent an exponential fit of the data.

clearly enhances the rate of efflux particularly below pH 7.0. For example, at pH 5.0,  $t_{1/2}$  decreases from  $\sim 62$  to  $\sim 33$  s; at pH 7.0, from  $\sim 22$  to  $\sim 15$  s; and at pH 9.0, from  $\sim 11$  to  $\sim 9$  s (Fig. 3, Table 1, and *SI Appendix*, Fig. S2). Enhancement of the rate is 2-fold at pH  $< 7.0$  and decreases as pH increases to 9.0.

The  $\text{pK}^{\text{app}}$  for efflux by WT LacY was determined by plotting the rate of efflux at each pH shown in the absence (Fig. 4, solid black curve) or presence of the imposed  $\Delta\Psi$  (interior positive) (Fig. 4, solid red curve). The imposed  $\Delta\Psi$  (interior positive) has no significant effect on the  $\text{pK}^{\text{app}}$  for efflux, which exhibits a value of  $\sim 7.2$  in the absence or presence of  $\Delta\Psi$  (interior positive).

**Mutant E325A LacY.** Although mutant E325A binds galactosides and catalyzes alternating access (i.e., equilibrium exchange and counterflow) at least as well as WT LacY, the mutant is defective in net downhill efflux (17, 23, 24). When rates of lactose efflux by mutant E325A LacY are measured at various pH values in the same manner as described for WT LacY, it is apparent that mutant E325A catalyzes relatively little efflux at any pH tested. Furthermore, imposition of  $\Delta\Psi$  (interior positive) has no effect (Fig. 4 and *SI Appendix*, Fig. S3).

**Kinetics of Efflux.** RSO vesicles expressing WT LacY were pre-equilibrated with [ $^{14}\text{C}$ ]lactose at given concentrations, and efflux was measured in the absence or presence of an imposed  $\Delta\Psi$  (interior positive) at pH 7.5 (*SI Appendix*, Fig. S4). The amount of lactose released in the first 10 s was used to estimate the velocity of efflux at each lactose concentration, and  $K_m$  and  $V_{\text{max}}$  for lactose efflux were determined with the Michaelis–Menten equation (*SI Appendix*, Fig. S5). The observed  $K_m$  in the absence or presence of  $\Delta\Psi$  (interior positive) is about 8.4 or 6.8 mM, respectively. Therefore,  $\Delta\Psi$  (interior positive) has only a borderline significant effect on the  $K_m$  for lactose efflux.  $V_{\text{max}}$  for lactose efflux is also similar in the absence or presence of the imposed  $\Delta\Psi$  (interior positive), 72 or 68  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg protein}^{-1}$ , respectively.

## Discussion

LacY catalyzes lactose/ $\text{H}^+$  symport across the cytoplasmic membrane chemiosmotically (17). However, rather than driving the

conformational change in LacY responsible for alternating access,  $\Delta\tilde{\mu}_{\text{H}^+}$  drives active transport by changing the rate-limiting step for turnover from deprotonation to reorientation of apo LacY (reviewed in refs. 1 and 28). Thus,  $\Delta\tilde{\mu}_{\text{H}^+}$  causes a kinetic asymmetry because of 1) a change in rate-determining step and 2) a decrease in the  $K_m$  from the outside by  $\sim 50$ - to 100-fold with little effect on  $K_m$  from the inside. The differences produce the kinetic asymmetry.

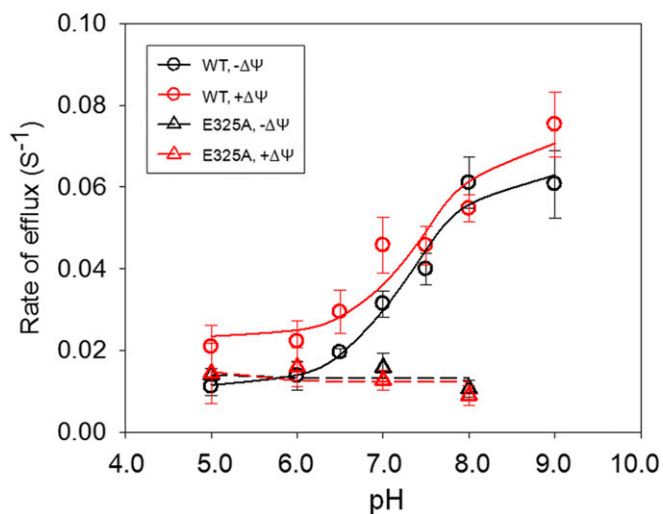
Thus, lactose/ $\text{H}^+$  symport catalyzed by LacY exhibits a kinetic asymmetry in the presence of  $\Delta\tilde{\mu}_{\text{H}^+}$ . Importantly, a recent study (27) demonstrates that the cavity can open on the cytoplasmic side to provide access to the galactoside-binding site when the periplasmic side is locked by cross-linking, thereby indicating that conformational changes on one side of LacY can occur independent of structural changes on the other side (27).

Transmembrane exchange does not involve  $\Delta\tilde{\mu}_{\text{H}^+}$  indicating that binding and dissociation of sugar, not  $\text{H}^+$  turnover, drives the alternating access conformational change (17). Since the driving force for accumulation against a concentration gradient is  $\Delta\tilde{\mu}_{\text{H}^+}$  and *E. coli* has a stable internal pH of 7.6 (35–37), a  $\text{pK}_a$  of 10.5 for binding (18, 21) suggests that decreasing the  $\text{pK}_a$  to  $\sim \text{pH } 7.2$  would deprotonate only  $\sim 50\%$  of the LacY molecules. Without decreasing the  $\text{H}^+$  concentration of the cytoplasm, how can deprotonation be increased? Neutral replacement mutants for Arg302 (helix IX), which is relatively

**Table 1.**  $t_{1/2}$  of efflux of WT LacY in the absence or presence of  $\Delta\Psi$  (interior positive) at given pH

$t_{1/2}$ (s), pH	$-\Delta\Psi$	$+\Delta\Psi$
5	$62.4 \pm 9.9$	$33.2 \pm 6.7$
6	$50.2 \pm 10.2$	$31.2 \pm 5.8$
6.5	$35.5 \pm 1.7$	$23.6 \pm 3.6$
7	$22.1 \pm 2.0$	$15.2 \pm 2.0$
7.5	$17.4 \pm 1.5$	$15.2 \pm 1.4$
8	$11.4 \pm 1.1$	$12.7 \pm 0.7$
9	$11.4 \pm 1.4$	$9.2 \pm 0.9$

Rate constant of efflux was obtained by fitting experimental data with exponential decay as described in *Materials and Methods*, and  $t_{1/2}$  of efflux was calculated by  $\ln 2/\text{rate constant}$ .



**Fig. 4.** Effect of  $\Delta\Psi$  (interior positive) on the pH dependence of efflux. Rates of efflux are plotted as a function of pH. The error bar on each pH value indicates the SE of the rate constant.

close to Glu325 (~6 Å), are unable to catalyze active transport but exhibit transmembrane exchange (38), and evidence has been presented (39, 40) in support of the idea that  $H^+$  may be extracted from Glu325 by spatial fluctuations in the position of Arg302 that expel  $H^+$  by moving near Glu325 or vice versa.

However, of course, LacY must also deprotonate for turnover to occur. One possibility is that the  $pK_a$  of Glu325 (helix X) in a hydrophobic pocket between helices IX and X (21) may decrease by becoming more accessible to water. In this regard, galactosidic sugars are hydrated in aqueous solution. Although current structures do not diffract at a resolution sufficient to visualize water, it is likely that galactosides become dehydrated when they interact with side chains in the binding site. Since helices IX and X are next to each other in the tertiary structure, Arg302 (helix IX) is irreplaceable with respect to active transport and mutants R302A or R302S exhibit properties similar to mutants with neutral replacements for Glu325, positively charged Arg302 (helix IX) may be important with regard to deprotonation (41). Evidence supporting this idea has been presented (41, 42). Most recently, side chains in the vicinity of Glu325 were mutated with the rationale that interaction with Glu325 should alter its  $pK_a$ . Remarkably, only mutant R302K of the sites tested causes the  $pK_a$  to decrease and by over 2 pH units, thereby lending further support to the idea that  $H^+$  is displaced from Glu325 by spatial fluctuations in the position of Arg302 moving near Glu325 or vice versa (40). The possibility will be tested with a suppressor t-RNA approach using arginine homologs with side chains of varied lengths (43).

## Materials and Methods

**Materials.** [ $D$ -glucose- $^{14}C$ -(U)]Lactose was purchased from Moravak Biochemicals. Valinomycin was purchased from Calbiochem-Novabiochem Corporation. Isopropyl- $\beta$ -D-1-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology. Bis(1,3-dibutylbarbituric acid) trimethine oxonol was obtained from Sigma-Aldrich. All other materials were reagent grade and obtained from commercial sources. The genes of WT and mutant E325A were sequenced in their entirety.

**Preparation of RSO Membrane Vesicles.** *E. coli* T184 [ $lacI^+ O^+ Z^- Y^-$  (A)  $rpsL met^- thr^- recA hsdM hsdRIF lacI^q O^+ Z^{D118}$  ( $Y^+ A^+$ )] cells transformed with

plasmid pT7-5 encoding a cassette *lacY* gene with WT LacY or mutant E325A with a 10-His tag at the C terminus were grown in 1.0 L of LB broth at 37 °C. After 2 h induction with 1 mM IPTG, cells were harvested by centrifugation, and RSO membrane vesicles were prepared as described (44, 45). The vesicles were resuspended in 100 mM sodium or potassium phosphate ( $NaP_i$  or  $KP_i$ , as indicated; pH 7.5)/10 mM  $MgSO_4$  at a protein concentration of 10 to 15 mg/mL. Aliquots were flash frozen in liquid nitrogen and stored at -80 °C until use.

**Measurement of  $\Delta\Psi$  (Interior Positive).** The imposed  $\Delta\Psi$  (interior positive) generated by influx of  $K^+$  in the presence of valinomycin was estimated by measuring the fluorescence increase of Bis(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC $_4$  (3)] at 25 °C. RSO membrane vesicles (2 mg/mL total protein, in 100 mM  $NaP_i$  at a given pH) were incubated with 4  $\mu$ M DiBAC $_4$  (3) at 25 °C for 30 min. An aliquot (50  $\mu$ L) of RSO vesicles was harvested at 14,000 rpm for 10 min. The pellet was immediately resuspended in 50  $\mu$ L 100 mM  $NaP_i$  and diluted into 2 mL of 100 mM phosphate buffer at a given  $Na^+/K^+$  ratio and the same pH in a quartz cuvette. Fluorescence of DiBAC $_4$  (3) was monitored in a SLM-Aminco 8100 spectrofluorimeter (modified by OLIS, Inc.) at excitation and emission wavelengths of 490 and 516 nm, respectively. After 0.5 and 1.5 min, valinomycin and CCCP were added to a final concentration of 25  $\mu$ M, respectively, and measurement of fluorescence was continued for a total of 5 min.

**Efflux.** Efflux was carried out with RSO membrane vesicles as described (34, 46) with minor modifications. RSO membrane vesicles expressing WT LacY or mutant E325A were harvested by centrifugation, resuspended to a protein concentration of 25 mg/mL in 100 mM  $KP_i$  with 0.5% DMSO at a given pH, and equilibrated with 10 mM [ $^{14}C$ ]lactose (5 mCi/mmol) at 25 °C for 3 h. Aliquots (2  $\mu$ L) were rapidly diluted 200-fold into 100 mM  $KP_i$  at the same pH. For efflux with  $\Delta\Psi$  (interior positive), RSO membrane vesicles were harvested by centrifugation, washed twice with 100 mM sodium phosphate ( $NaP_i$ ) at a given pH (15-min incubation each time), and resuspended in the same  $NaP_i$  buffer to a protein concentration of 25 mg/mL. After 3-h equilibration with 10 mM [ $^{14}C$ ]lactose (5 mCi/mmol) in the presence of 25  $\mu$ M valinomycin (dissolved in 0.5% DMSO final concentration) at 25 °C, an aliquot of 2  $\mu$ L was rapidly diluted 200-fold into 100 mM  $KP_i$  at the appropriate pH. Reactions were terminated by addition of 3 mL of termination buffer (100 mM  $KP_i$ /100 mM LiCl, pH 5.5) and rapid filtration at given times. Radioactivity of the samples was determined by liquid scintillation spectrometry, and rate constants for efflux were obtained by fitting data with the equation for exponential decay ( $y = ae^{-bx}$ ).

**Kinetics of Efflux.** RSO membrane vesicles expressing WT LacY were resuspended to a protein concentration of 25 mg/mL in 100 mM  $KP_i$  (pH 7.5) with 0.5% DMSO and equilibrated with given concentrations of [ $^{14}C$ ]lactose overnight at 4 °C. An aliquot of 2  $\mu$ L was rapidly diluted 200-fold into 100 mM  $KP_i$  (pH 7.5). Reactions were terminated at given times by addition of termination buffer and rapidly filtered. For measurements in the presence of  $\Delta\Psi$  (interior positive), RSO vesicles were washed twice with 100 mM  $NaP_i$  (pH 7.5) and concentrated to 25 mg protein/mL in the same buffer. After equilibrating with given concentrations of [ $^{14}C$ ]lactose in the presence of 25  $\mu$ M valinomycin (dissolved in DMSO; final concentration of DMSO was 0.5%) overnight at 4 °C, an aliquot of 2  $\mu$ L was rapidly diluted 200-fold into 100 mM  $KP_i$  (pH 7.5). Efflux was terminated by addition of termination buffer and rapid filtration at given times. The amount of lactose lost in the first 10 s was calculated for each efflux reaction, and the  $K_m$  and  $V_{max}$  of efflux were determined by fitting the data with the Michaelis-Menten equation.

**Data Availability.** All of the data are included in the manuscript.

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