Properties of a LacY Efflux Mutant[†]

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ABSTRACT: Crystal structures of lactose permease from *Escherichia coli* (LacY) exhibit two six-helix bundles with 2-fold pseudosymmetry separated by a large hydrophilic cavity. The cavity is open only on the cytoplasmic side and contains the side chains important for both sugar and H^+ binding at the apex in the middle of the protein; the periplasmic side is tightly closed. A plethora of biochemical and biophysical data strongly support an alternating access mechanism in which both the sugar- and H^+-binding sites are exposed alternatively to either side of the membrane by reciprocal opening and closing of cytoplasmic and periplasmic cavities. Here we describe a unique mutation that results in an increase in sugar efflux. Asp240 (helix VII), which interacts with Lys319 (helix X), also comprises part of a salt-bridge/H-bond network that is critically involved in the mechanism of sugar/H^+ symport. The mutant, which contains Glu in place of Asp240, exhibits a marked decrease in active lactose transport and an enhanced rate of downhill lactose/H^+ efflux. Transport is increased to normal levels when the sugar concentration is increased 10-fold, consistent with the decrease in sugar affinity observed for this mutant. Taken as a whole, the results suggest that the primary defect induced by the mutation may involve a decrease in affinity for H^+.

LacY[1], a paradigm for the major facilitator superfamily (MFS), carries out the tightly coupled symport of a galactoside with an H^+, utilizing the free energy released from downhill translocation of H^+ in the presence of a H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$, interior negative and/or alkaline) to drive accumulation of galactopyranosides against a concentration gradient (reviewed in refs *1* and *2*). Notably, in the absence of $\Delta\tilde{\mu}_{H^+}$, LacY catalyzes the converse reaction, utilizing free energy released from downhill translocation of sugar to drive uphill translocation of H^+ with the generation of $\Delta\tilde{\mu}_{H^+}$, the polarity of which depends upon the direction of the substrate concentration gradient (*3*–*5*). LacY also catalyzes exchange or counterflow of sugar without translocation of H^+, and these reactions are unaffected by $\Delta\tilde{\mu}_{H^+}$. Therefore, it is likely that the primary driving force for the global conformational change is concurrent occupancy of cosubstrate binding sites.

X-ray crystal structures of a conformationally constrained mutant (*6*, *7*), as well as wild-type LacY (*8*), reveal two six-helix bundles with 2-fold pseudosymmetry surrounding a large hydrophilic cavity open only to the cytoplasmic side. The side chains important for both sugar and H^+ binding are at the apex of the cavity, which is in the middle of protein, and inaccessible from the periplasmic side of LacY (Figure 1A). The sugar-binding site is located primarily in the N-terminal bundle on helices IV (Glu126), V (Arg144, Cys148, and Trp151), and VIII (Glu269).

In contrast, the residues involved in H^+ translocation/coupling are in the C-terminal bundle and include helices VII (Tyr236, Asp240), VIII (Glu269), X (Lys319, His322, and Glu325), and IX (Arg302). These side chains do not represent a pathway through the membrane for either sugar or H^+ but form two distinct binding sites located at the approximate middle of the LacY molecule across the cavity near the apex (*2*).

A mechanism has been proposed for LacY in which both sugar- and H^+-binding sites become alternatively accessible to either side of the membrane as a result of reciprocal opening and closing of periplasmic and cytoplasmic cavities (the alternating access model) (*2*, *6*, *8*). Several independent lines of evidence, which include site-directed alkylation (*9*–*13*), single-molecule Förster resonance energy transfer (*14*), double electron–electron resonance (*15*), and site-directed thiol cross-linking (*16*) indicate that sugar binding or to a lesser extent $\Delta\tilde{\mu}_{H^+}$ (interior negative) (*10*) increases the open probability of a hydrophilic cavity on the periplasmic side of LacY. Moreover, site-directed thiol cross-linking (*16*) also shows that the periplasmic cavity must close, as well as open, for transport to occur. Although the physiological role of LacY is to take up sugar from periplasmic space, the probability of opening the periplasmic cavity is very low, and the great majority of LacY molecules in the membrane appear to be in an inward-facing conformation, as observed in the crystal structures (*6*–*8*).

Mutational analysis indicates that Asp240 (helix VII) and Lys319 (helix X) are salt-bridged (*17*, *18*). Thus, single replacement of Asp240 or Lys319 with neutral side chains leads to inactivation of transport, while LacY mutants with double neutral replacement of both side chains exhibit decreased, but significant transport activity. In addition, the polarity of the interaction is critical, as mutant D240K/K319D is inactive,

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[1]Abbreviations: LacY, the lactose permease; $\Delta\tilde{\mu}_{H^+}$, H^+ electrochemical gradient; $\Delta\psi$, membrane potential; KP$_i$, potassium phosphate; NaP$_i$, sodium phosphate; RSO, right side out; NEM, *N*-ethylmaleimide.
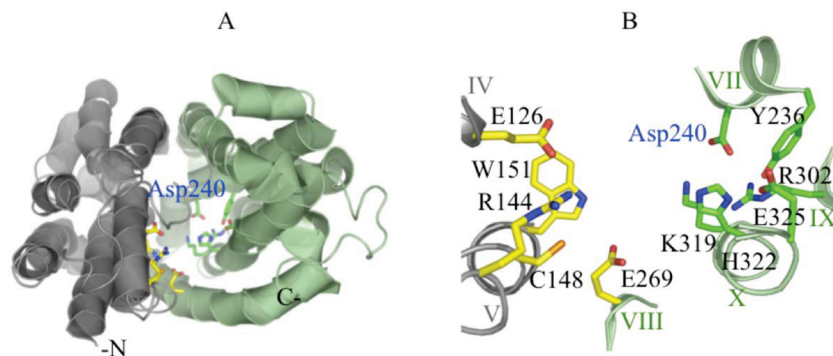
FIGURE 1: Crystal structure of LacY viewed from the cytoplasm. (A) A cartoon presentation of the crystal structure of wild-type LacY (PDB ID 2v8n) in an inward-facing conformation. (B) Cosubstrate binding sites. The N- or C-terminal six helices are colored as gray or green, respectively. The side chains important in the sugar binding or H^+ translocation are shown as yellow or green sticks, respectively.

unlike the Asp237 (helix VII)−Lys358 (helix XI) pair, which can be interchanged with no loss in activity (17, 19, 20). Although replacement of Lys319 with Arg maintains high transport activity, replacement of Asp240 with Glu abolishes active transport (20).

Cross-linking studies with homobifunctional, thiol cross-linking agents indicate that Asp240 is in close proximity to the Lys319 (21). In the crystal structures (6−8), Asp240 is ~5 Å from Lys319, and both residues participate in a complex salt-bridge/H-bond network that plays a central role in H^+ translocation/coupling. Although interaction between Asp240 and Lys319 is clearly important for lactose/H^+ symport and it has been shown recently that Asp240 mutants have decreased affinity for sugar (22), the role of Asp240 in the transport mechanism is unknown. In this study, we show that the defect in active transport by the D240E mutant is likely due to a decrease in affinity for sugar, as well as an increase in the rate of lactose/H^+ efflux down a concentration gradient. Both defects can be explained by a decrease in affinity for H^+.

EXPERIMENTAL PROCEDURES

Materials. [^{14}C]Lactose was obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Integrated DNA Technologies synthesized oligodeoxynucleotides. Restriction endonucleases, T4 DNA ligase, and Vent polymerase were from New England Biolabs (Beverly, MA). All other materials were of reagent grade and obtained from commercial sources.

Mutant Construction. Oligonucleotide-mediated, site-directed mutagenesis with pT7-5/WTLacY/CH10 (8) as a template and two-step PCR were used to construct plasmid pT7-5/D240E/CH10, which encodes LacY D240E with a 10-His tag at the C-terminus. DNA sequencing of the entire *lacY* gene confirmed the sequence of the construct.

Growth of Cells. *Escherichia coli* T184 (Δ*lacZY*) or HB101 (*lacZ$^+$Y$^-$*) containing a given plasmid was grown in Luria−Bertani broth with 100 mg/L ampicillin. Overnight cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After additional growth for 2−3 h at 37 °C, cells were harvested by centrifugation.

Preparation of Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared from *E. coli* T184 (Δ*lacZY*) by osmotic lysis as described (23, 24), suspended in 100 mM potassium phosphate (KP$_i$) (pH 7.2)/10 mM MgSO$_4$ at a protein concentration of about 12−20 mg/mL, frozen in liquid N$_2$, and stored at −80 °C until use.

Transport Assays. *E. coli* T184 (Δ*lacZY*) containing given LacY was washed with 100 mM KP$_i$ (pH 7.2)/10 mM MgSO$_4$ and adjusted to an OD$_{420}$ of 10.0 (0.7 mg of protein/mL). Transport was carried out with [^{14}C]lactose (10 mCi/mmol) at a final concentration of 0.4 mM, unless described otherwise. Lactose transport in RSO membrane vesicles was assayed at a protein concentration of 3.5 mg/mL in the presence of 20 mM ascorbate/0.2 mM phenazine methosulfate under oxygen with 0.4 or 4.0 mM [^{14}C]lactose at a specific activity of 10 or 2.5 mCi/mmol, respectively (25).

Efflux and Exchange. RSO membrane vesicles containing a given LacY at a protein concentration of ca. 30 mg/mL in 100 mM KP$_i$ (pH 7.5) were preequilibrated overnight on ice with 10 mM [^{14}C]lactose (10 mCi/mmol) in the presence of 55 μM valinomycin and 1 μM nigericin. Aliquots (2 μL) were diluted 200-fold into the same solution in the absence (efflux) or presence (exchange) of 10 mM unlabeled lactose, and reactions were terminated by dilution and rapid filtration at given times, as described (3).

Δμ̃$_{H^+}$ Effect on Efflux. RSO membrane vesicles containing D240E LacY at a protein concentration of ca. 29 mg/mL were washed with 100 mM KP$_i$ (pH 7.5), preequilibrated with 10 mM [^{14}C]lactose (10 mCi/mmol) in the presence of 55 μM valinomycin, and diluted 200-fold into 100 mM NaP$_i$ at pH 5.5. At given times, reactions were terminated by dilution and rapid filtration (3).

RESULTS

Crystal structures of LacY (6−8) exhibit a complicated salt-bridge/H-bond network in the C-terminal helix bundle composed of residues Tyr236 and Asp240 (helix VII), Lys319, His322, and Glu325 (helix X), and Arg302 (helix XI) (Figure 1B). These residues are involved primarily in coupling and/or H^+ translocation (26), but they also participate in the affinity of the sugar-binding site (22).

Growth on Indicator Plates. *E. coli* HB101 (*lacZ$^+$Y$^-$*) expressing D240E LacY grows as red colonies on McConkey indicator plates indistinguishable from cells expressing wild-type LacY (data not shown). In contrast, cells transformed with empty vector grow as white or light pink colonies. Thus, cells expressing wild-type or D240E LacY are able to catalyze downhill lactose transport and metabolism of the incoming lactose in a similar manner.
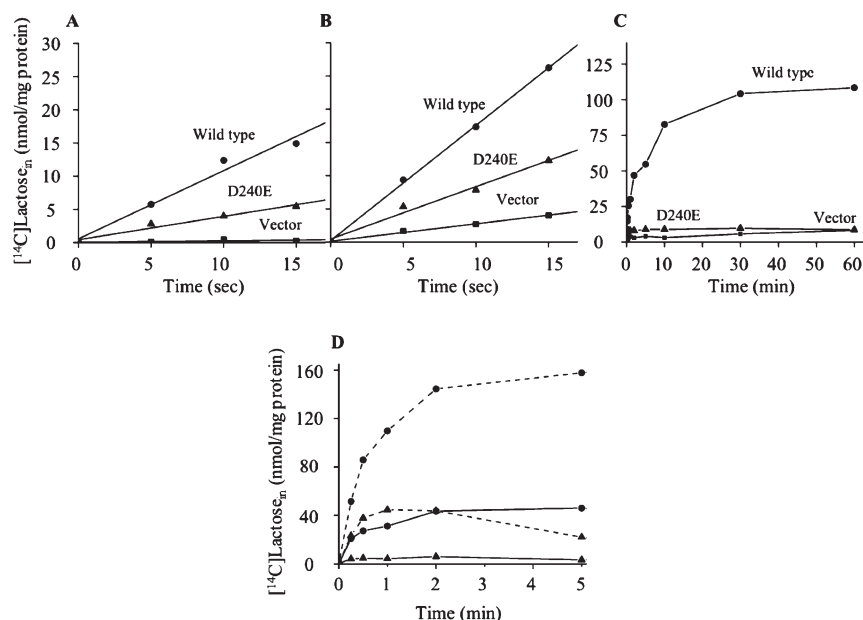
FIGURE 2: Lactose active transport. (A, B) Initial rates of lactose transport for *E. coli* T184 (Δ*lacZY*) or HB101 (*lacZ*⁺*Y*⁻) intact cells, respectively. Key: (●) wild type; (▲) D240E; (■) empty vector. (C) Time course of lactose accumulation for *E. coli* T184 intact cells. Key: (●) wild type; (▲) D240E; (■) empty vector. (D) Active transport by RSO membrane vesicles prepared from *E. coli* T184 expressing wild-type (●) or D240E (▲) LacY. *E. coli* T184 or HB101 intact cells were adjusted to an OD_{420} of 10.0 (0.7 mg of protein/mL). Transport was carried out with [¹⁴C]lactose (10 mCi/mmol) at a final concentration of 0.4 mM (solid line). The intracellular lactose concentration is plotted as a function of time. Lactose transport in RSO membrane vesicles in panel D was assayed at a protein concentration of 3.5 mg/mL in the presence of 20 mM ascorbate/ 0.2 mM phenazine methosulfate under oxygen with [¹⁴C]lactose at a final concentration of 0.4 mM (10 mCi/mmol; solid line) or 4.0 mM (2.5 mCi/ mmol; dashed line) as described in Experimental Procedures. The intravesicular lactose concentration after correction for RSO vesicles transformed with empty vector is plotted as a function of time.

Active Transport. The rate of $\Delta\tilde{\mu}_{H^+}$-driven uphill lactose accumulation by *E. coli* T184 (*lacZ*⁻ *Y*⁻), a *lacZY* deletion strain, transformed with a plasmid encoding wild-type LacY is linear for at least 15 s (Figure 2A) and reaches a steady state in ∼10 min (Figure 2C). *E. coli* T184 expressing the D240E mutant transports at about a third of the rate (Figure 2A) but accumulates lactose very poorly, almost as poorly as cells transformed with empty vector (Figure 2C). The initial rate of downhill lactose influx was also assayed in *E. coli* HB101 (*lacZ*⁺*Y*⁻) containing β-galactosidase, which hydrolyzes lactose to glucose and galactose as soon as it enters the cell (Figure 2B). Correcting for transport by cells transformed with empty vector, uphill and downhill initial rates of transport are similar for cells expressing wild type or mutant D240E (compare panels A and B of Figure 2).

RSO vesicles containing D240E LacY also exhibit poor transport activity relative to vesicles with wild-type LacY when incubated with a relatively low lactose concentration under conditions that generate $\Delta\tilde{\mu}_{H^+}$ (interior negative) (Figure 2D). However, when the sugar concentration is increased 10-fold, the mutant transports at a rate and to a steady-state level of accumulation similar to RSO vesicles containing the wild type at the lower lactose concentration (Figure 2D). The finding is consistent with the observation that the affinity of mutant D240E for β-D-galactopyranosyl-1-thio-β-D-galactopyranoside is ∼one-tenth of that obtained for wild-type LacY (*22*).

Efflux and Equilibrium Exchange. Concentrated RSO vesicles were preequilibrated with 10 mM [¹⁴C]lactose in the presence of valinomycin and nigericin as described in the Experimental Procedures. Then efflux or equilibrium exchange was carried out by rapid 200-fold dilution into buffer without (efflux) or with 10 mM unlabeled lactose (equilibrium exchange), respectively (*3*). The rate of efflux for vesicles with wild-type LacY is first order, with a $t_{1/2}$ of ∼11 s (Figure 3A). Efflux with
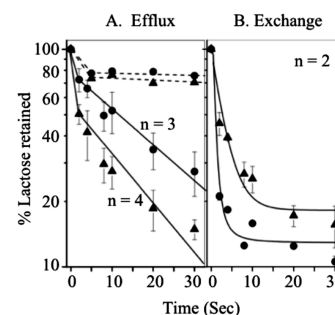

FIGURE 3: Efflux and equilibrium exchange. RSO membrane vesicles containing wild type (●) or D240E LacY (▲) in 100 mM KP_i (pH 7.5) were preequilibrated with 10 mM [¹⁴C]lactose (10 mCi/mmol) in the presence of 55 μM valinomycin and 1 μM nigericin and diluted 200-fold into the same buffer in the absence (efflux) or presence (exchange) of 10 mM unlabeled lactose, and reactions were terminated by quenching and rapid filtration at given times, as described. Efflux with *N*-ethylmaleimide-treated RSO vesicles (10 mM, final concentration for 20 min) is indicated by broken lines. The average percentage of lactose retained is plotted as a function of time.

RSO vesicles containing D240E LacY is faster than vesicles containing the wild type, with a $t_{1/2}$ of ∼4 s (Figure 3A). The reason for the apparent initial rapid loss of lactose is unknown and varies from one experiment to another. However, it is clear that the phenomenon is unrelated to LacY-mediated efflux since it is also observed with vesicles treated with *N*-ethylmaleimide (Figure 3A) or with vesicles devoid of LacY (*3*). The rate of exchange is too fast to measure accurately in vesicles with the wild type (Figure 3B). However, it is apparent that exchange in vesicles with D240E LacY is significantly reduced, which is consistent with decreased affinity of the D240E mutant for sugar.

Sugars were also assayed by nuclear magnetic resonance after incubation of intact *E. coli* HB101 (lacZ⁺Y⁻) with lactose.
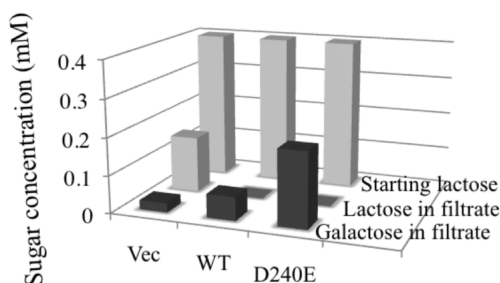
FIGURE 4: Galactose efflux by intact *E. coli* HB101. Intact *E. coli* 101 (*lacZ*$^+$*Y*$^-$) expressing wild type, mutant D240E, or empty vector were adjusted to an OD$_{420}$ of 10.0 (0.7 mg of protein/mL). Cells were incubated with 0.4 mM lactose (light gray column) for 1 h. After filtration, the filtrates were assayed for galactose (dark column) and lactose (light gray column) by nuclear magnetic resonance.
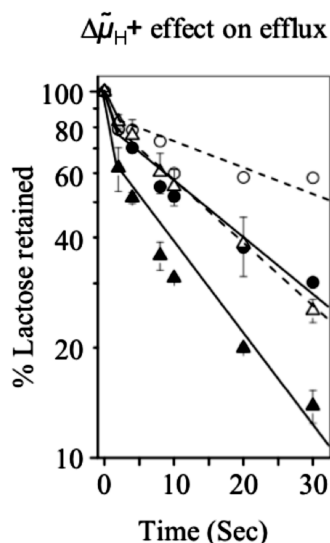

FIGURE 5: Effect of $\Delta\tilde{\mu}_{H^+}$ (interior negative and alkaline) on the lactose efflux. RSO membrane vesicles in 100 mM KP$_i$ (pH 7.5) were preequilibrated with 10 mM [^{14}C]lactose (10 mCi/mmol) in the presence of 55 μM valinomycin and diluted 200-fold into 100 mM KP$_i$ at pH 7.5 [wild-type LacY ($\bullet$, $n = 1$) or D240E mutant ($\blacktriangle$, $n = 2$)] or 100 mM NaP$_i$ at pH 5.5 [wild-type LacY ($\circ$, broken line, $n = 1$) or D240E mutant ($\triangle$, broken line, $n = 3$)]. Reactions were terminated by quenching and rapid filtration at given times. The average percentage of lactose retained is plotted as a function of time.

No lactose is found in the filtrate (extracellular solution) with either the wild type or the D240E mutant after a 1 h incubation; however, galactose is detected (Figure 4). Moreover, a much higher concentration of galactose is found in the filtrate from D240E LacY than from the wild type. Thus, external lactose is transported into *E. coli* HB 101 (*lacZ*$^+$*Y*$^-$) expressing wild-type LacY where it is hydrolyzed into galactose and glucose, and the galactose effluxes from the cells via LacY. With cells containing the D240E mutant, considerably more galactose is found in the external medium, which is consistent with the argument that efflux occurs more rapidly.

Previous studies with RSO vesicles (*4*) and proteoliposomes (*5*) demonstrate that the rate of lactose efflux in the wild-type LacY is inhibited by imposition of a pH gradient (ΔpH; interior alkaline) and a membrane potential ($\Delta\psi$; interior negative) or by acidic pH, indicating that the lactose efflux down a concentration gradient is coupled with efflux of H$^+$. In order to generate a $\Delta\tilde{\mu}_{H^+}$ (interior negative and alkaline), RSO vesicles with D240E LacY prepared in KP$_i$ (pH 7.5) were preequilibrated

with 10 mM [^{14}C]lactose in the presence of valinomycin and rapidly diluted into NaP$_i$ (pH 5.5). A $\Delta\tilde{\mu}_{H^+}$ generated in this manner also decreases the rate of efflux, increasing $t_{1/2}$ in vesicles with wild-type LacY from ~13 to > 30 s and in vesicles with the D240E mutant from ~5 to ~13 s (Figure 5). In addition, the effect of pH 5.5 on the rate of efflux was tested. RSO vesicles extensively washed with KP$_i$ (pH 5.5) before equilibration with 10 mM [^{14}C]lactose were diluted into KP$_i$ (pH 5.5). Relative to the rate of efflux at pH 7.5, the rate of efflux for D240E LacY at pH 5.5 is markedly reduced. Taken together, efflux of sugar is coupled with release of H$^+$ in the D240E mutant, as with wild-type LacY.

DISCUSSION

A six-step kinetic model for lactose transport has been proposed for lactose/H$^+$ symport (*2–4*, *26*, *27*). Efflux and exchange by wild-type LacY are explained by this simple kinetic scheme (Figure 6). Thus, H$^+$-coupled efflux consists of a minimum of six steps: (1) binding of H$^+$ and (2) binding of lactose to LacY at the inner surface of the membrane; (3) a conformational change to the outward-facing conformation; (4) release of sugar; (5) release of H$^+$; and (6) a conformational change(s) corresponding to return of unloaded LacY to the inner surface of the membrane. Alternatively, during exchange and counterflow, LacY does not deprotonate, and only steps 2, 3, and 4 are involved.

Recently, a pK_a of ~10.5 was determined for sugar binding to purified LacY (*28*), which supports the contention that at physiological values of pH LacY is protonated before sugar binds during lactose/H$^+$ symport. But notably, affinity for sugar binding on either side of the membrane is unaffected by $\Delta\tilde{\mu}_{H^+}$ (*29*). However, acidic ambient pH or imposition of $\Delta\psi$ (inside negative), ΔpH (interior alkaline) or $\Delta\tilde{\mu}_{H^+}$ (inside negative and alkaline) across the membrane decreases the rate of efflux (*3*, *4*). In addition, deuterium oxide inhibits the rate of downhill efflux or influx at neutral pH, while equilibrium exchange, counterflow, and $\Delta\tilde{\mu}_{H^+}$-driven active transport are unaffected (*30*). These data imply strongly that deprotonation (Figure 6, step 5) is the rate-limiting step for downhill lactose/H$^+$ symport and that this step is no longer rate limiting in the presence of $\Delta\tilde{\mu}_{H^+}$, i.e., when there is a driving force on the H$^+$ (*2*, *26*). Mutants E325A (*27*), K319L (*31*), and R302A (*32*), which are also part of the salt-bridge/H-bond network (Figure 1B), bind sugar with high affinity and catalyze equilibrium exchange as well as the wild type. However, the mutants are unable to catalyze any mode of lactose translocation involving H$^+$ translocation (i.e., active transport, efflux, and influx down a concentration gradient), and they are clearly defective in H$^+$ release (Figure 6, step 5).

Remarkably, as opposed to mutants E325A, K319L, and R302A, the D240E mutant exhibits a faster rate of efflux than the wild type with a reduced rate of equilibrium exchange. Moreover, it has been shown (*33*) that mutant E240A exhibits enhanced H$^+$ influx in the presence of melibiose. The results are consistent with the conclusion that Asp240 mutations cause a faster release of H$^+$ than the wild type in response to a downhill sugar gradient. Notably, each of these mutants, E325A, R319L, R302A, and D240E, is located in the salt-bridge/H-bond network, and they apparently perturb the same step in a different manner.

Although the D240E mutant catalyzes lactose transport at an initial rate of ~one-third of the wild type, little if
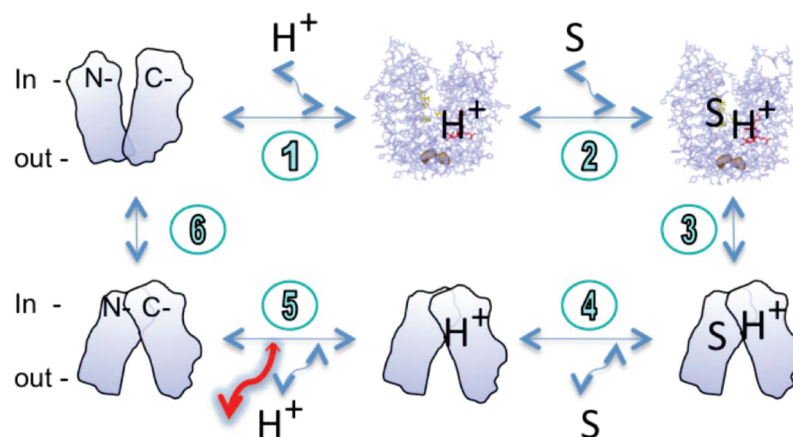
FIGURE 6: A kinetic model for lactose efflux. Two X-ray crystal structures without (PDB ID 2v8n) or with bound sugar (PDB ID 1pv7) represent the inward-facing conformation. The sugar substrate is shown as the capital letter S. Both N- and C-termini are indicated. The orientation of the membrane is marked as in or out. The closest residues, Ile32 (helix I) and Asn245 (helix VII), between two helix bundles are shown in surface model and light brown color. The larger red arrow indicates that step 5 may be enhanced in the D240E mutant.

any accumulation is observed, which is likely due to the fast rate of lactose efflux. Furthermore, by using a 10-fold higher concentration of lactose, the initial rate of transport, as well as the steady-state level of accumulation, approximates the wild type at the lower lactose concentration. Since affinity for sugar is dependent upon the protonation state of LacY, both the decrease in affinity for sugar and a rapid rate of efflux in the D240E mutant may be explained by a decrease in affinity for H^+.

ACKNOWLEDGMENT

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