Sulfhydryl Oxidation of Mutants with Cysteine in Place of Acidic Residues in the Lactose Permease†

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Received February 3, 1998; Revised Manuscript Received March 24, 1998

ABSTRACT: To examine further the role of charge-pair interactions in the structure and function of lactose permease, Asp237 (helix VII), Asp240 (helix VII), Glu126 (cytoplasmic loop IV/V), Glu269 (helix VIII), and Glu325 (helix X) were replaced individually with Cys in a functional mutant devoid of Cys residues. Each mutant was then oxidized with H_2O_2 in order to generate a sulfinic and/or sulfonic acid at these positions. Due to the isosteric relationship between aspartate and sulfinate, in particular, and the lower pK_a of the sulfinic and sulfonic acid side chains, oxidized derivatives of Cys are useful probes for examining the role of carboxylates. Asp237→Cys or Asp240→Cys permease is inactive, as shown previously, but H_2O_2 oxidation restores activity to an extent similar to that observed when a negative charge is reintroduced by other means. Glu126→Cys, Glu269→Cys, or Glu325→Cys permease is inactive, but oxidation does not restore active lactose transport. The data are consistent with previous observations indicating that Asp237 and Asp240 are not critical for active lactose transport, while Glu126, Glu269, and Glu325 are irreplaceable. Although Glu269→Cys permease does not transport lactose, the oxidized mutant exhibits significant transport of β,D-galactosylpyranosyl 1-thio-β,D-galactopyranoside, a property observed with Glu269→Asp permease. The observation supports the idea that an acidic residue at position 269 is important for substrate recognition. Finally, oxidized Glu325→Cys permease catalyzes equilibrium exchange with an apparent pK_a of about 6.5, more than a pH unit lower than that observed with Glu325→Asp permease, thereby providing strong confirmatory evidence that a negative charge at position 325 determines the rate of translocation of the ternary complex between the permease, substrate, and H^+.

The lactose permease (lac permease)[1] of *Escherichia coli* is a paradigm for secondary active transport proteins that transduce free energy stored in electrochemical ion gradients into work in the form of a concentration gradient (reviewed in *1*, *2*). This hydrophobic, polytopic, cytoplasmic membrane protein which catalyzes the coupled, stoichiometric translocation of β-galactosides and H^+ has been solubilized, purified, reconstituted into artificial phospholipid vesicles, and shown to be solely responsible for β-galactoside transport (reviewed in *3*) as a monomer (see *4*). All available evidence indicates that the protein has 12 transmembrane domains in α-helical configuration that traverse the membrane in zigzag fashion connected by hydrophilic loops with the N and C termini on the cytoplasmic face of the membrane (Figure 1; reviewed in *5*−*8*).

Site-directed and Cys-scanning mutagenesis of each residue in the permease indicates that only six residues—Glu126, Arg144, Glu269, Arg302, His322, and Glu325, each of which is thought to be charge-paired or H-bonded (Figure 2)—play an irreplaceable role in the mechanism (see *9*; reviewed in *10*). In addition, Asp237 and Asp240 are charge-paired with Lys358 and Lys319, respectively, but neither the residues nor the charge pairs play an obligatory role in the mechanism (*11*−*16*). Based on a variety of site-directed approaches which include second-site suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance, thiol cross-linking, and identification of discontinuous mAb epitopes, a helix packing

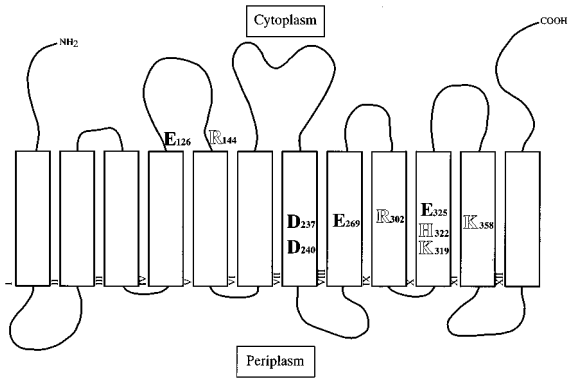

FIGURE 1: Secondary structure model of lac permease. Rectangular boxes indicate transmembrane helices. Residues dicussed in the text are highlighted.

† This research was supported in part by NIH Grant DK51131 to H.R.K.

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[1] Abbreviations: lac permease, lactose permease; C-less permease, functional lac permease devoid of Cys residues; KP_i, potassium phosphate; RSO, right-side-out; TDG, β,D-galactopyranosyl 1-thio-β,D-galactopyranoside.

S0006-2960(98)00266-9 CCC: $15.00
Published on Web 05/08/1998

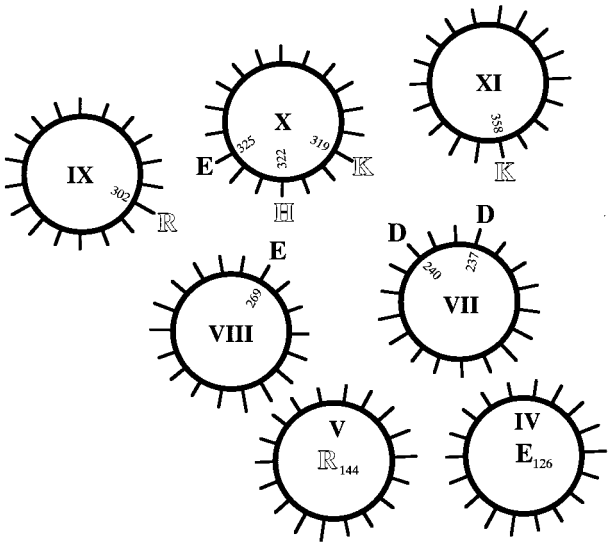

FIGURE 2: Helical wheel model of helices of lac permease showing the relationship between Asp237 (helix VII) and Lys358 (helix XI), Asp240 (helix VII) and Lys319 (helix X), Glu269 (helix VIII) and His322 (helix X), Arg302 (helix IX) and Glu325 (helix X), and Glu126 (putative loop IV/V) and Arg144 (putative loop IV/V). Glu126 and Arg144 are shown within helices IV and V, respectively, to indicate that their location in helices or loop IV/V is unclear at present. The Glu residues targeted for single cysteine replacement are given as emboldened dark letters.

model has been formulated (reviewed in *17*). The packing model in conjuction with the properties of mutants in the irreplaceable residues and their putative interactions has led to a proposed mechanism for coupling between lactose and H^+ translocation (*18*).

Chemical rescue (i.e., restoration of the activity of mutant enzymes by chemical agents that mimic the function of important side chains in the native enzyme) by noncovalent, exogenous compounds has been demonstrated in a variety of systems (*19*−*25*), including lac permease (*16*). In addition, rescue has been achieved by modifying Cys-replacement mutants either by covalent means (*14*, *15*) or by oxidation (*26*). In this study, chemical rescue of lac permease mutants with Cys in place of Asp237, Asp240, Glu126, Glu269, or Glu325 is examined by studying oxidation of the thiol to the sulfinic and/or sulfonic acid.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Lactose was purchased from Amersham (Arlington Heights, IL), Micro BCA protein determination kits from Pierce Chemical (Rockford, IL), and glass fiber filters (GF-75, 25 mm) from Osmonics (Livermore, CA). Thiodiglycol at the highest purity available (≥98%) was purchased from Sigma (St. Louis, MO). All other compounds were reagent grade and obtained from commercial sources.

Bacterial Strains and Plasmids. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A), rspL, met*$^-$*, thr*$^-$*, recA, hsdM, hsdR/F′, lacI*^{q}*O*$^+$*Z*^{D118} *(Y*$^+$*A*$^+$*)*] (*27*) harboring plasmid pT7-5/cassette *lacY* (EMBL X-56095) with given mutations was used for expression of lac permease from the *lacZ* promoter/operator. Construction and verification of mutants D237C,[2] D240C (*13*), E126C (*9*), E269C (*28*), and E325C (*29*) in a functional permease devoid of Cys residues (C-less permease) have been described previously.

Growth of Bacteria. E. coli T184 (*Z*$^-$*Y*$^-$) transformed with each plasmid described was grown aerobically at 37 °C in Luria−Bertani medium containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and allowed to grow for another 2 h before induction with 0.3 mM isopropyl 1-thio-β,D-galactopyranoside. After further growth for 2 h at 37 °C, cells were harvested and used for preparation of vesicles.

Preparation of Right-Side-Out (RSO) Membrane Vesicles. RSO membrane vesicles were prepared from 4 L cultures of *E. coli* T184 by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis (*30*, *31*). The vesicles were suspended at a protein concentration of 20−30 mg/mL in 100 mM potassium phosphate (KP_i, pH 7.5)/10 mM $MgSO_4$, frozen in liquid N_2, and stored at −80 °C until use.

Sulfhydryl Oxidation. Oxidation was carried out by incubating RSO membrane vesicles [2 mg of protein/mL in 20 mM KP_i (pH 7.0)] with 40 mM H_2O_2 at 20 °C. After a given incubation time, the reaction was quenched by adding thiodiglycol to a final concentration of 0.8 M. The vesicles were then harvested by centrifugation, washed once with 100 mM KP_i (at a given pH)/10 mM $MgSO_4$, and adjusted to a concentration of 2 mg of protein/mL in the same buffer.

Transport Assays. Initial rates of [1-^{14}C]lactose (2.5 mCi/mmol; final concentration 0.4 mM) transport by RSO membrane vesicles were assayed under oxygen in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (*32*) by rapid filtration on glass fiber filters (*33*).

Equilibrium exchange was assayed with RSO membrane vesicles as described (34). Briefly, RSO membrane vesicles were washed in 100 mM KP_i (at a given pH) and resuspended in the same buffer to a concentration of 20 mg/mL protein; valinomycin and nigericin were added to final concentrations of 20 μM and 0.2 μM, respectively. [1-^{14}C]Lactose (10 mCi/mmol; final concentration 10 mM) was then added, and the samples were incubated at 0−4 °C overnight. To initiate exchange, 2 μL aliquots were rapidly diluted into 400 μL of 100 mM KP_i (at the same pH) containing 10 mM cold lactose at 25 °C. Reactions were quenched at given times with 100 mM KP_i (pH 5.5)/100 mM lithium chloride and assayed by rapid filtration on glass fiber filters.

Protein Determinations. Protein was determined using the Micro BCA protein assay.

RESULTS

Oxidation of D237C Permease. Upon exposure to a strong oxidizing environment, free sulfhydryls are ultimately converted into sulfonic acids (−SO_3H). A stable intermediate in the reaction is the sulfinic acid (−SO_2H) which predominates early in the time-course of the oxidation (*26*). The sulfinic acid of Cys, alanine-3-sulfinic acid, is isosteric to aspartate, but more acidic by about 2 pH units. Thus, replacement of Cys with alanine-3-sulfinic acid mimics Asp, but has a lower pK_a. Oxidation of D237C permease with H_2O_2 for 3 min dramatically restores the initial rate of lactose

[2] Site-directed mutants are designated by the single-letter amino acid abbreviation for the targeted residue, followed by the sequence position of the residue in the wild-type lac permease and a second letter indicating the amino acid replacement.

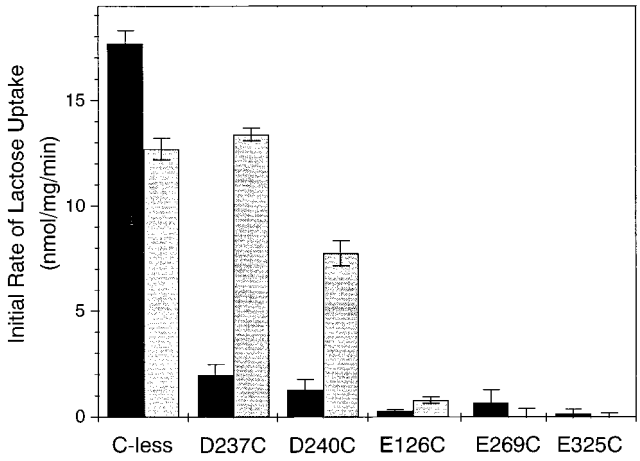

FIGURE 3: Initial rate of lactose transport by RSO membrane vesicles containing given mutants. Dark bars, control; light bars, vesicles exposed to 40 mM H_2O_2 for 3 min. Experiments were carried out as described under Experimental Procedures. Error bars represent the standard error of the mean ($n = 4$).

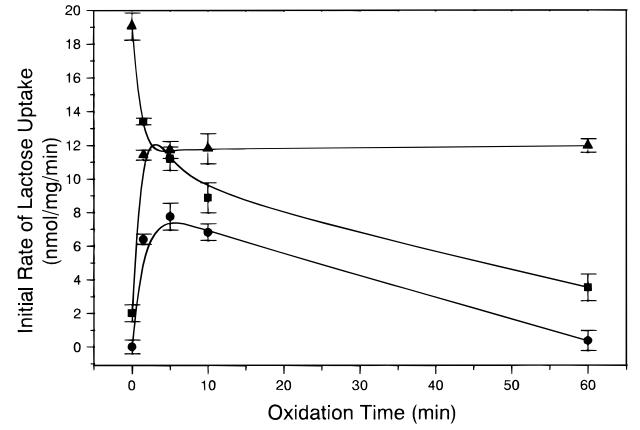

FIGURE 4: Effect of oxidation time on lactose transport. Shown are initial rates of lactose transport by RSO membrane vesicles containing C-less (▲), D237C (■), or D240C (●) permease after various incubation times with 40 mM H_2O_2. Experiments were carried out as described under Experimental Procedures. The error bars represent the standard error of the mean ($n = 4$), and the data were curve-fitted using a computer program (Origin; Microcal, Northhampton, MA).

transport to 75% of the nonoxidized C-less control sample (Figure 3). As shown in Figure 4, a rapid increase in the initial rate of transport to a maximum at 2−5 min is observed, followed by a slow decrease in activity, and by 60 min, activity is markedly decreased. In contrast, C-less permease exhibits no activation upon incubation with H_2O_2. Rather, a 30% decrease in the initial rate occurs within the first 3−5 min after exposure to H_2O_2, and the activity remains constant thereafter. Thus, activation of D237C permease by H_2O_2 and most of the subsequent slow inactivation involve the oxidation of the thiol group at position 237 specifically. Furthermore, since C-less permease is inactivated by about 30% due to an unknown mechanism over the same time-course as D237C permease is activated, the extent of activation is probably underestimated.

Oxidation of D240C Permease. Although negatively charged thiol reagents (*14*, *15*) or alkylsulfonates (*16*) rescue the activity of D240C permease, recovery is significantly lower than observed with D237C permease. In a similar

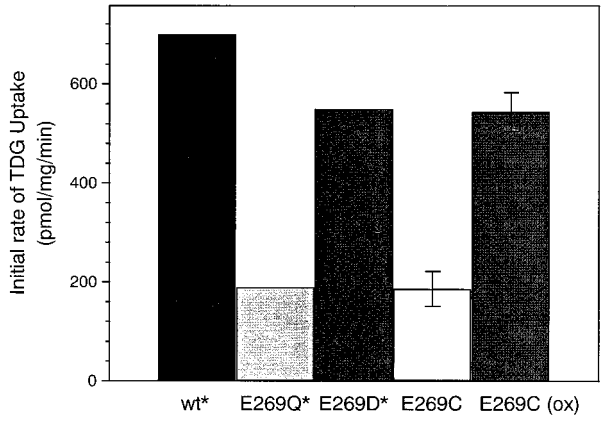

FIGURE 5: TDG transport by RSO membrane vesicles containing E325C before and after 3 min oxidation with 40 mM H_2O_2. Experiments were carried out as described under Experimental Procedures. The error bar represents the standard error of the mean ($n = 10$). *Data from Ujwal et al. (28) are shown for comparison.

vein, double neutral replacements for Asp240 and Lys319 lead to lower activity than observed with analogous double mutants in Asp237 and Lys358 (*12*, *13*), and reversal of Asp240 and Lys319 leads to inactivation, while D237K/K358D permease retains high activity (*13*, *15*). As shown in Figure 3, oxidation of D240C permease for 3 min reactivates the initial rate of transport to 60−70% of oxidized C-less permease, which corresponds to about 40% of the untreated control. Similar to D237C permease, recovery of activity is maximal within 3−5 min exposure to H_2O_2, and longer exposure leads to inactivation (Figure 4).

Irreplaceable Glu Residues. (A) E126C Permease. Recent experiments (*9*) reveal that Glu126 which is presumably located in the cytoplasmic loop connecting helices IV and V and may be charge-paired with Arg144 (see Figures 1 and 2) is irreplaceable with respect to active transport and lactose influx down a concentration gradient. Incubation of RSO vesicles in the presence of H_2O_2 for 3 min has little or no effect on the initial rate of lactose transport (Figure 3).

(B) E269C Permease. Glu269 is essential for active lactose transport, and all replacements tested exhibit essentially no transport activity toward the dissacharide (see *18*). However, with Asp at position 269, the analogue β,D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) is accumulated in a partially uncoupled fashion with an increase in H^+:TDG stoichiometry (*28*, *35*). Consistently, while oxidation of E269C permease does not reactivate lactose active transport (Figure 3), TDG transport is restored to a level similar to that reported when Glu269 is replaced with Asp (Figure 5).

(C) E325C Permease. Although permease with neutral replacements for Glu325 is completely unable to catalyze all translocation reactions involving net H^+ translocation, the mutants catalyze equilibrium exchange and counterflow at least as well as wild-type permease (see *18*). With Asp in place of Glu325, the permease is partially uncoupled, accumulating lactose about 20% as well as wild-type. More interestingly, however, equilibrium exchange catalyzed by E325D permease is sensitive to pH (*33*). As opposed to wild-type permease or the E325A mutant, where equilibrium exchange is essentially constant from pH 4.5 to 9.5, E325D

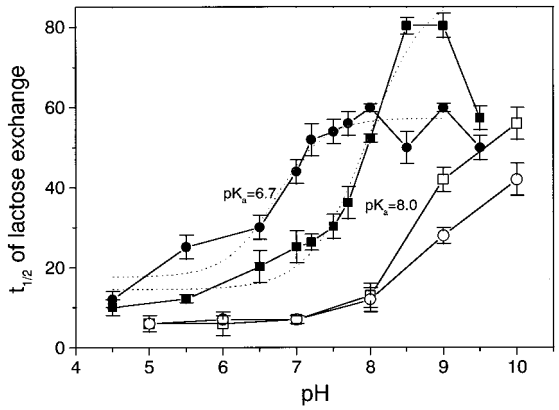

FIGURE 6: pH dependence of lactose equilibrium exchange by RSO membrane vesicles containing single-Cys E325C permease (■), E325C permease oxidized with 40 mM H_2O_2 for 3 min (●), C-less permease (□), or C-less permease treated with 40 mM H_2O_2 for 3 min (○). Experiments were carried out as described under Experimental Procedures. The error bars represent the standard error of the mean ($n = 4$), and the dotted lines are Lorenzian sigmoidal functions.

exhibits normal exchange activity from pH 4.5 to about 7.5, but is completely inactivated in a reversible fashion between pH 7.5 and 9.5 with a midpoint at about pH 8.5. The observations indicate that a carboxylic acid at position 325 has a perturbed pK_a and that a negative charge at position 325 limits translocation of the ternary complex between the permease, substrate, and H^+ across the membrane (see *18*).

As shown in Figure 3, oxidation of RSO vesicles containing oxidized E325C single-Cys permease has no significant effect on active lactose transport. To further explore translocation of the ternary complex as a function of charge at position 325, equilibrium exchange catalyzed by control and oxidized E325C permease was studied as a function of pH (Figure 6). Like wild-type permease (33), equilibrium exchange by C-less permease is relatively insensitive to pH. Thus, rapid exchange with a $t_{1/2}$ of about 5 s which increases only above pH 8.0 is observed with RSO vesicles containing C-less permease. Furthermore, little difference is observed after the vesicles are exposed to H_2O_2. Equilibrium exchange by E325C single-Cys permease is also relatively insensitive to pH from pH 4.5 to about pH 7.0. As the ambient pH is increased above 7.0, however, exchange activity decreases markedly (i.e., $t_{1/2}$ increases) in a sigmoidal fashion between pH 7.0 and 8.5 with a midpoint slightly above pH 8.0. Importantly, after oxidation, the titration of E325C single-Cys permease is clearly shifted to a more acidic pH. Exchange activity is inhibited mildly from pH 4.5 to about pH 6.0, and decreases relatively sharply above pH 6.5 to low activity at pH 7.5 and above with a midpoint at about pH 6.7. Relative to permease with Asp in place of Glu325 which exhibits a midpoint at about pH 8.5 (33), the midpoint of the exchange titration for the oxidized E325C single-Cys permease is acid-shifted by approximately 1.8 pH units.

DISCUSSION

As demonstrated previously, Cys substitution for Asp237 yields inactive permease due to disruption of a salt bridge between Asp237 and Lys358 (*14*, *15*, *36*). Accordingly, replacement of Asp237 with Glu, carboxymethyl-Cys, or sulfonylethylthio-Cys restores permease activity (*14*, *15*). In addition, the activity of D237A permease is restored by *n*-alkylsulfonates (*16*). As opposed to D237E permease, replacement of Asp240 with Glu inactivates. In addition, Ala replacement for Asp240 cannot be chemically rescued by *n*-alkylsulfonates (*16*), and covalent modification by anionic alkylating agents is less effective in restoring activity (*14*, *15*). These observations, as well as the finding that reversal of Asp237 and Lys358 yields active permease while D240K/K319D is inactive, indicate that the properties of the two charge pairs are different.

Oxidation of Asp→Cys mutants offers advantages over other means of reintroducing a negative charge. The sulfinic acid derivative of Cys, alanine-3-sulfinic acid, preserves the charge, H-bonding potential, H^+ binding, and side-chain volume of Asp, but reduces the pK_a of the side chain by approximately 2 pH units. Oxidation times that result in optimal rescue of the D237C and D240C mutants are within 2−5 min, and at subsequent times, inactivation is observed. While it cannot be ruled out that a significant population of the oxidized species is the sulfonic acid, activation followed by inactivation with increasing oxidation time has also been observed with an Asp→Cys mutant in ketosteroid isomerase (*26*). The predominant oxidized species present in the activated and inactivated isomerase was identified by mass spectroscopy as the sulfinic and sulfonic acid derivatives, respectively.

Why a sulfonic acid derivative of D237C or D240C permease might fail to rescue activity is not clear, since rescue of D237A permease by *n*-alkysulfonates (*16*) or D237C permease modified with methanethiosulfonate ethylsulfonate (*14*, *15*) demonstrates that the sulfonate group is an effective counterion. On the other hand, the proximity of the sulfonic acid to the Lys side chain at position 358 is likely to be substantially closer in the previous studies. In any case, although C-less permease is inactivated by 30% during the first 3−5 min incubation with H_2O_2, further inactivation is not observed with longer exposure to H_2O_2. In contrast, D237C and D240C permeases are both inactivated almost completely by long-term exposure. Taken as a whole, the observations suggest that stepwise oxidation of the thiol at position 237 or 240 predominantly is responsible for both activation and inactivation of the Cys-replacement mutants.

Glutamate 269 plays an irreplaceable role in the permease, and mutation renders the permease unable to transport lactose (*28*, *35*). Interestingly, however, E329D permease catalyzes the accumulation of TDG in a partially uncoupled fashion with an increase in H^+:TDG stoichiometry. The findings suggest that Glu269 may be involved in substrate recognition, a notion supported by subsequent studies (*37*). The effect of oxidation on E269C single-Cys permease described here extends understanding of the chemical and spatial requirements at position 269 with respect to substrate translocation and recognition. Since oxidized E269C permease transports TDG in a manner similar to the E269D mutant, it seems likely that there is a requirement for a longer side chain such as Glu at position 269 to confer proper recognition of lactose. Specifically, substrate is suggested to bind at the interface between helices V and VIII which is stabilized by the

interaction between Glu269 and His322 (*37*). Moreover, ligand binding is postulated to disrupt the H-bond between these residues, leading to a conformational change that is transmitted to the interface between helices IX and X where H^+ translocation may take place (*18*). Thus, a shortened side chain at position 269 may destabilize the helixV/VIII interface in such a manner that only a high-affinity substrate such as TDG can be accumulated, but in an uncoupled manner.

A number of observations indicate that Glu325 plays a direct role in H^+ translocation (reviewed in *18*). Some of the strongest evidence in this regard stems from the properties of Glu325 mutants (*33*, *38*, *39*). Neutral replacement mutants at Glu325 are completely defective in all translocation reactions that involve net H^+ movement (lactose:H^+ symport), but catalyze equilibrium exchange and counterflow at least as well as wild-type permease. Furthermore, equilibrium exchange with wild-type or E325A permease is essentially insensitive to ambient pH from pH 4.5 to 9.5. With Asp in place of Glu, the permease catalyzes accumulation only about 20% as well as wild-type. Remarkably, however, E325D is pH-sensitive, exhibiting normal equilibrium exchange activity from pH 4.5 to about 7.5, and a marked decrease between pH 7.5 and 9.5 with a midpoint at about pH 8.5. The results are consistent with the interpretation that translocation of the ternary complex between the permease, lactose, and H^+ will not, tolerate a negative charge at position 325 and that by replacing Glu325 with Asp the carboxylic acid becomes more accessible to solvent, thereby decreasing the pK_a (see *18*). The experiments presented in this paper provide strong support for this hypothesis. Thus, H_2O_2 oxidation of E325C single-Cys permease causes an acidic shift in the pH titration of equilibrium exchange such that the midpoint is acid-shifted by almost 2 pH units, which correlates well with the difference in pK_a between Asp and a sulfinic or sulfonic acid.

The pH dependence of exchange displays midpoints at pH 8.5 or pH 6.7 for E325D (33) or oxidized E325C single-Cys permease, respectively. Position 325 is in helix X near the middle of the membrane, and it is postulated that substrate binding results in protonation of Glu325 and exposure to the low dielectric of the membrane, leading to a markedly elevated pK_a and no effect of pH on equilibrium exchange (*18*). With Asp in place of Glu325, the pK_a is less perturbed, but still exhibits an apparent pK_a of 8.5 because of exposure to a low dielectric. However, equilibrium exchange with untreated E325C permease exhibits a midpoint at about pH 8 which is close to the pK_a of a cysteinyl side chain in aqueous environment. In this regard, thiols are particularly sensitive to local electrostatics, which may lower the pK_a of a Cys residue exposed to a low dielectric. For example, Cys47 in human placental glutathione transferase is buried in a hydrophobic cleft, and its pK_a of 4.2 is attributed to the electrostatic influence of a lysyl ϵ-amino group approximately 5 Å from the sulfur atom (*40*). The effect of basic residues on the pK_a of a thiol side chain is also demonstrated in thiol proteases, where a pK_a below 5.0 is due to the presence of a thiolate−imidazole ion pair (*41*). Such an interaction may also be responsible for the apparently unperturbed pK_a of a Cys residue at position 325 in the permease, since His322 lies on the same face of helix X one turn removed toward the periplasm.

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BI9802667