Monoclonal Antibody 4B1 Alters the pK_a of a Carboxylic Acid at Position 325 (Helix X) of the Lactose Permease of *Escherichia coli*†

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ABSTRACT: A carboxylic acid at position 325 in helix X is obligatory for lactose/H^+ symport at a step corresponding to deprotonation of lactose permease [Carrasco, N. et al. (1989) *Biochemistry 28*, 2533−2539]. In this paper, pH profiles for active transport, efflux, and equilibrium exchange are analyzed for wild-type permease and mutant Glu325 → Asp. With respect to active transport and efflux down a concentration gradient, both of which involve net H^+ translocation and are defective in the mutant, the wild-type and the mutant exhibit similar profiles, and at no pH is the mutant stimulated relative to the wild-type. Strikingly, exchange which does not involve H^+ translocation is comparable in the wild-type and the Glu325 → Asp mutant below pH 7.5. Above pH 7.5, however, the exchange activity of the mutant is progressively and reversibly inhibited with a midpoint at about pH 8.5; while the exchange activity of wild-type permease is only mildly decreased above pH 9.5, and exchange by Glu325 → Ala or Glu325 → Gln permease is comparable to wild-type and unaffected by pH. The findings are consistent with the idea that translocation of the ternary complex between the permease, lactose, and H^+ does not tolerate a negative charge at position 325. In wild-type permease, the electrostatic interaction between Glu325 (helix X) and Arg302 (helix IX) is sufficiently strong that the carboxylate is unaffected by pH. In contrast, with Asp at position 325, the electrostatic interaction is broken, the carboxylate becomes protonated, and the acid exhibits a pK_a of about 8.5. Monoclonal antibody 4B1 binds to the periplasmic loop between helices VII and VIII of the permease [Sun, J. et al. (1996) *Biochemistry 35*, 990−998] and mimics the Glu325 mutants. Dramatically, 4B1 shifts the apparent pK_a for exchange from about pH 8.5 to 7.5 in the Glu325 → Asp mutant with little or no effect on the wild-type or the Glu325 → Ala mutant. The findings are consistent with the conclusion that the uncoupling effect of 4B1 involves a conformational change in helix VII and/or VIII that secondarily alters the pK_a of the essential carboxylic acid at position 325.

The lactose (lac)[1] permease of *Escherichia coli* is a polytopic membrane transport protein encoded by the *lacY* gene. The permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for the coupled stoichiometric translocation of β-galactosides and H^+ as a monomer [reviewed in Kaback et al., (1994) and Kaback (1996)]. A variety of experimental approaches indicate that the permease is composed of 12 α-helical rods that traverse the membrane, with both N and C termini in the cytosolic side, and unequivocal support for the 12-helix motif has been obtained from analysis of fusions between lac permease and alkaline phosphatase (*lacY*−*phoA*) (Calamia & Manoil, 1990). Moreover, site-directed excimer fluorescence, site-directed mutagenesis, and second-site suppressor studies have led to a model describing the packing of helices VII−XI [see Kaback et al. (1994) and Kaback (1996)]. The model has been confirmed and extended recently by engineering divalent metal binding sites (bis- or tris-His residues) within the permease (He et al., 1995a,b; Jung et al., 1995), site-directed chemical cleavage (Wu et al., 1995), and site-directed spin labeling (Wu et al., 1996).

Site-directed mutagenesis of wild-type permease and Cys-scanning mutagenesis of a functional mutant devoid of Cys residues (C-less permease) reveals that only four out of over 400 residues mutated are irreplaceable with respect to active lactose transport and/or ligand binding: Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X). In addition, there are two pairs of interacting Asp and Lys residues in transmembrane domains [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] that are not essential for activity [reviewed in Kaback et al. (1994) and Kaback (1996)]. Differences in the properties of the mutants with respect to various translocation reactions have led to the suggestion that Arg302, His322, and Glu325 may function in a H^+ translocation pathway or alternatively may form part of a coordination site for H_3O^+ (Kaback, 1987, 1990). In this context, it is important that the three residues are in close physical proximity, as shown by site-directed excimer fluorescence (Jung et al., 1993) and engineering of divalent metal binding sites (i.e., bis- or tris-His residues) (He et al., 1995a).

The properties of permease molecules mutated at Arg302, His322, or Glu325 can be rationalized by the kinetic scheme shown in Figure 1 (Kaczorowski & Kaback, 1979). Accordingly, lactose efflux down a concentration gradient

† Preliminary descriptions of some of these findings have been published (Kaback, 1990, 1992).

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[1] Abbreviations: lac, lactose; mAb, monoclonal antibody; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RSO, right-side-out; KP_i, potassium phosphate.

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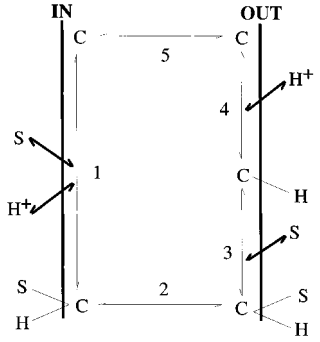

FIGURE 1: Schematic representation of reactions involved in lactose efflux, exchange, and counterflow. C represents lac permease; S is substrate (lactose). The order of substrate and H^+ binding at the inner surface of the membrane is not implied [from Kaczorowski Kaback, (1979)].

consists of a minimum of five steps: (1) binding of substrate and H^+ on the inner surface of the membrane (order unspecified), (2) translocation of the ternary complex to the outer surface, (3) release of substrate, (4) release of H^+, and (5) return of the unloaded permease to the inner surface. Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1−3 only. All steps in the mechanism that require protonation or deprotonation appear to be blocked in His322 (Padan et al., 1985; Püttner et al., 1986, 1989) or Arg302 mutants (Menick et al., 1987; Sahin-Tóth & Kaback, 1993), suggesting that these residues may be involved in protonation (step 1). In marked contrast, permease mutated at Glu325 is defective in all steps that involve net H^+ translocation but catalyzes exchange and counterflow as well as or better than wild-type permease (Carrasco et al., 1986, 1989), strongly indicating that the Glu325 is involved in step 4 (i.e., Glu325 is required for deprotonation of the permease). Interestingly, the Glu325 mutations are mimicked by D_2O (Viitanen et al., 1983) or monoclonal antibody (mAb) 4B1 (Carrasco et al., 1984).

In order to examine the role of Glu325 more thoroughly, the effect of pH on the translocation reactions catalyzed by the wild-type and E325D[2] permease primarily have now been examined. The mutant is partially uncoupled with respect to active transport, exhibiting about 20% of the activity of wild-type permease (Carrasco et al., 1989; Franco & Brooker, 1994), and markedly defective with respect to carrier-mediated efflux down a concentration gradient. Strikingly, E325D permease catalyzes equilibrium exchange as well as the wild-type at pH 7.5 or below, but as ambient pH is increased, exchange is progressively and reversibly inhibited with a midpoint at about pH 8.5. In contrast, the exchange activity of wild-type, E325A, or E325Q permease is insensitive to pH over the same range. Interestingly, mAb 4B1 shifts the apparent pK_a for exchange from about pH 8.5 to about pH 7.5 with E325D permease but has little or no effect on exchange by wild-type or E325A permease. The results support the ideas that a carboxylic acid at position 325 plays an essential role in lactose-coupled H^+ translocation and that the uncoupling effect of mAb 4B1 involves a change in the pK_a of this carboxylic acid.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. E. coli HB101 [*hsdS20* (r^-_B, m^-_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (*Sm*^{r}), *xyl-5*, *mtl-1*, *supE44*, l^-/F^-] (Boyer & Roulland-Dussoix, 1969) was used as a carrier for the plasmids described and for detection of lac permease activity on MacConkey indicator plates (Difco Laboratories) containing 25 mM lactose. *E. coli* T184 [$lacI^+O^+Z^-Y^-$(*A*), *rspL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR/F′*, $lacI^qO^+Z^{D118}$ (Y^+A^+)] (Teather et al., 1980) harboring plasmid pT7-5/*lacY* with given mutations was used for expression of lac permease from the *lacZ* promoter/operator by induction with iso-propyl 1-thio-β-D-galactopyranoside (IPTG). A cassette *lacY* gene encoding wild-type permease under the control of the *lacZ* promoter/operator (EMBL-X56095) was used for all *lacY* manipulations.

Table 1: Properties of Glu325 Mutants

*lac*Y	codon change	phenotype[a]	active transport[b]	membrane expression[c]
wild-type		red	100%	100%
E325D	GAA → GAC	red	15%	70%
E325A	GAA → GCA	red	0%	60%
E325Q	GAA → CAA	red	0%	ND[d]

[a] *E. coli* HB101 ($lacZ^+Y^-$) expressing each mutant was plated on MacConkey agar containing 25 mM lactose and incubated at 37 °C for 15 h. [b] *E. coli* T184 ($lacZ^-Y^-$) expressing each mutant was incubated with 0.4 mM [1-^{14}C]lactose at pH 7.5 and assayed by rapid filtration. Given are initial rates of lactose transport measured at 1 min relative to the wild-type. [c] Membranes prepared from *E. coli* T184 expressing each mutant were subjected to immunoblot analysis as described in Materials and Methods. [d] Not determined in this study; expression is comparable to that of E325A permease (54% of wild-type) (Franco & Brooker, 1994).

Oligonucleotide-Directed Site-Specific Mutagenesis. Replacement of Glu325 with Asp, Ala, or Gln in wild-type lac permease (Carrasco et al., 1986, 1989) was performed by two-stage PCR (overlap−extension; Ho et al., 1989) using pT7-5/ cassette *lacY* as a template. The PCR products were digested with *Kpn*I and *Spe*I restriction endonucleases and ligated to the similarly treated pT7-5/cassette *lacY* vector. Mutations were verified by sequencing the length of the inserted DNA fragment through the ligation junctions by dideoxynucleotide termination (Sanger et al., 1977) after alkaline denaturation (Hattori & Sakaki, 1986). The codon changes introduced by the mutations at position 325 are given in Table 1.

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 IPTG. After further growth for 2 h at 37 °C, cells were harvested and used for transport assays or preparation of membranes.

Preparation of RSO Membrane Vesicles. RSO membrane vesicles were prepared from 4 L cultures of *E. coli* T184 by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis (Kaback, 1971; Short et al., 1975). The vesicles were suspended at a protein concentration of 20−30 mg/mL in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and stored at −80 °C until use.

Transport Assays. For active transport, *E. coli* T184 was washed once with 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ and adjusted to an optical density of 10.0 at 420 nm

[2] Site-directed mutants are designated as follows. The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild-type lac permease and then a second letter denoting the amino acid replacement at this position.

(approximately 0.7 mg of protein/mL). Transport of [1-^{14}C]-lactose (2.5 mCi/mmol; final concentration of 0.4 mM) was assayed by rapid filtration, as described (Consler et al., 1991). When the effect of pH was tested, cells were washed in 100 mM KP_i (adjusted to a given pH by mixing 100 mM solutions of K_2HPO_4 and KH_2PO_4) containing 5 mM ethylenediaminetetraacetate (potassium salt) and assayed after addition of 0.2 μM nigericin (final concentration). Active lactose transport in RSO membrane vesicles was assayed under oxygen in the presence of 20 mM potassium ascorbate and 0.2 mM phenazine methosulfate (Konings et al., 1971).

Efflux and equilibrium exchange were performed with RSO membrane vesicles as described (Kaczorowski & Kaback, 1979). Briefly, RSO membrane vesicles were washed in 100 mM KP_i (pH given) and resuspended in the same buffer containing valinomycin (20 μM) and nigericin (0.2 μM) at a protein concentration of 20 mg/mL. [1-^{14}C]-Lactose (10 mCi/mmol; final concentration of 10 mM) was added, and the samples were incubated at 0−4 °C overnight. To initiate efflux or exchange, aliquots (2 μL) were rapidly diluted into a 200-fold excess (0.4 mL) of 100 mM KP_i (at a given pH) alone (efflux) or containing 10 mM nonradioactive lactose (equilibrium exchange) 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.

Effect of Monoclonal Antibody 4B1. To assess the effect of mAb 4B1 on lactose exchange, RSO membrane vesicles were diluted to a protein concentration of 2.0 mg/mL in 0.5 mL of 100 mM KP_i (pH 7.5) containing 50 μL of affinity-purified mAb 4B1 (5 mg/mL) (Sun et al., 1996) and incubated at room temperature for 45 min. Control vesicle samples were incubated in 0.5 mL of 100 mM KP_i (pH 7.5) alone. After incubation, the vesicles were washed in ice-cold 100 mM KP_i (pH given) and resuspended at a protein concentration of 20 mg/mL. Equilibration with [1-^{14}C]-lactose and equilibrium exchange assays were performed as described above.

Immunoblots. Membrane fractions were prepared from 10 mL cultures of *E. coli* T184 by osmotic lysis and sonication as described (Frillingos et al., 1994). Samples containing 10 or 50 μg of membrane protein were subjected to 12% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (Laemmli, 1970; Newman et al., 1981), and immunoblot analysis was carried out by using a site-directed polyclonal antibody against the C terminus of lac permease (Carrasco et al., 1984; Herzlinger et al., 1985). The amount of permease was quantitated with a Model 425F PhosphorImager (Molecular Dynamics) as described (Frillingos & Kaback, 1996; Sun et al., 1996).

Protein Determination. Protein concentrations were determined as described (Peterson, 1977) with bovine serum albumin as standard.

RESULTS

Expression of Glu325 Mutants. Immunoblot analyses demonstrate that E325D and E325A permeases are present in the membrane at about 70 and 60% of the wild-type level, respectively (Table 1). Mutant E325Q is also expressed to highly significant levels (Carrasco et al., 1989; Franco & Brooker, 1994).

Colony Morphology. All of the Glu325 mutants described grow as red colonies indistinguishable from wild-type when expressed in *E. coli* HB101 (*lacZ*$^+$*Y*$^-$) and plated on indicator plates containing 25 mM lactose (Table 1). Therefore, as shown previously (Carrasco et al., 1986, 1989), the mutants retain the ability to translocate lactose down a gradient at high concentrations of the disaccharide.

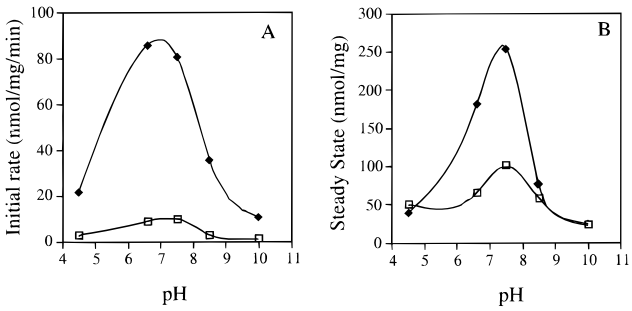

FIGURE 2: pH titration of active lactose transport by *E. coli* T184 expressing wild-type (◆) or E325D permease (□). Measurements were carried out in the presence of nigericin, as described in Materials and Methods. Initial rates of [1-^{14}C]lactose transport were measured at 1 min (A), and steady state levels of [1-^{14}C]lactose accumulation were measured at 60 min (B).

Active Transport. When the mutants are expressed in *E. coli* T184 (*lacZ*$^-$*Y*$^-$) and active transport is monitored at pH 7.5, E325A or E325Q permease exhibits no lactose accumulation, while E325D permease transports lactose at a low rate (15% of wild-type) to a significant but low steady state (30−40% of wild-type) [Table 1 and Figure 2; see Carrasco et al. (1986, 1989) and Franco and Brooker (1994) in addition]. Studies on the initial rate (Figure 2A) and steady-state level of accumulation (Figure 2B) as a function of pH demonstrate that wild-type permease exhibits optimal activity at about pH 7.5 [see He et al. (1995b) in addition]. E325D permease exhibits much less activity, but similar pH optima, and at no pH tested is the mutant stimulated significantly relative to the wild-type. Although data are not shown, neither E325A nor E325Q permease exhibits a significant ability to accumulate lactose over the same pH range (Carrasco et al., 1986, 1989).

Efflux. Efflux of lactose down a concentration gradient occurs in symport with H^+, and the rate increases with increasing pH (Kaczorowski & Kaback, 1979; Viitanen et al., 1983). When efflux from RSO vesicles containing wild-type permease is measured from pH 4.5 to 10.3, it is apparent that the rate of efflux increases markedly, as reflected by at least a 10-fold decrease in $t_{1/2}$ (i.e., the time at which the internal lactose concentration decreases by one-half) over this pH range (Figure 3A). The observation is consistent with the idea that either deprotonation (step 4) or return of the unloaded carrier (step 5) is rate-determining for efflux (Figure 1) (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Viitanen et al., 1983). In contrast, RSO vesicles containing E325D permease catalyze efflux at a markedly defective rate (Figure 3B). The efflux activity of the mutant is minimal from 4.5 to 8.1 and increases slightly but significantly from pH 8.1 to 10.3. Minimal rates of efflux are observed for E325A or E325Q permease over the entire range of pH tested.

Equilibrium Exchange. Equilibrium exchange by wild-type permease is relatively insensitive to pH (Kaczorowski & Kaback, 1979; Kaczorowski et al., 1979; Garcia et al., 1983; Viitanen et al., 1983). Thus, rapid exchange with a $t_{1/2}$ of about 5 s which increases slightly above pH 8.0 is observed with RSO vesicles containing wild-type permease (Figure 4A). In contrast, vesicles treated with *p*-(chloromer-

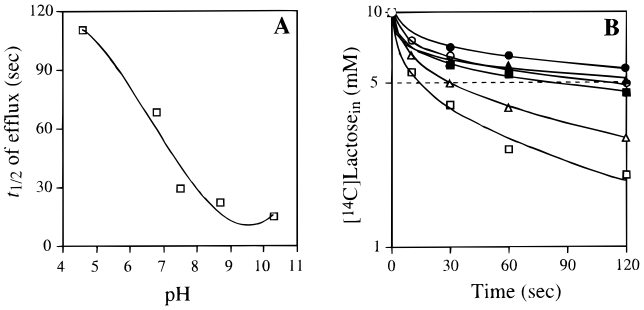

FIGURE 3: Effect of pH on lactose efflux by RSO membrane vesicles containing wild-type (open symbols) or E325D permease (closed symbols). RSO vesicles were equilibrated with 10 mM [1-^{14}C]lactose at given pH values and assayed by dilution into equilibration buffer without lactose, as described in Materials and Methods. (A) Effect of pH on $t_{1/2}$ for efflux by wild-type permease. (B) Time courses of efflux by wild-type permease at pH 4.5 (○), 7.5 (△), or 10.3 (□) or E325D permease at pH 4.5, 6.8, 7.5, or 8.1 (●), 8.7 (▲), or 10.3 (■). E325A or E325Q permease displays minimal efflux (●) from pH 4.5 to 10.0.

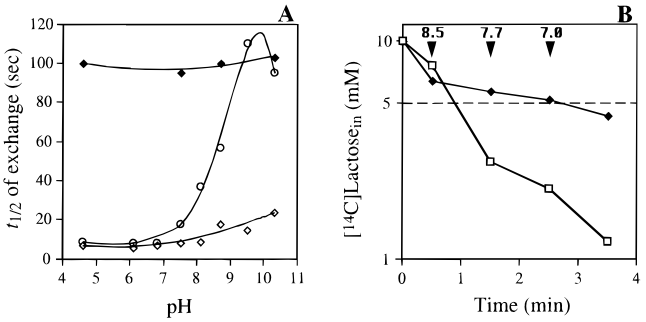

FIGURE 4: Effect of pH on equilibrium lactose exchange by RSO membrane vesicles containing wild-type or E325D permease. RSO vesicles were equilibrated with 10 mM [1-^{14}C]lactose at given pH values and assayed by dilution into equilibration buffer with 10 mM nonradioactive lactose, as described in Materials and Methods. (A) pH titration of exchange by wild-type (◇), E325D (○, or wild-type permease preincubated with 2 mM *p*-(chloromercurio)-benzenesulfonate (◆). (B) Reversibility of the pH effect on exchange by E325D permease. RSO vesicles containing E325D permease were equilibrated at pH 10.0. The exchange reaction was initiated by dilution into the same buffer (pH 10.0) at time zero. For the open symbols, at the indicated times, ambient pH was decreased stepwise to 8.5, 7.7, or 7.0, by adding appropriate aliquots of 1.0 M KH_2PO_4. For the closed symbols, in a control experiment, 1.0 M KP_i (pH 10.0) was added in place of 1.0 M KH_2PO_4.

curi)benzenesulfonate which inactives the permease exhibit a $t_{1/2}$ of ca. 100 s over the entire pH range. Remarkably, vesicles containing E325D permease catalyze equilibrium exchange at rates comparable to that of wild-type permease up to about pH 7.5. As ambient pH is increased, however, exchange activity is markedly decreased in a sigmoidal fashion between 7.5 and 9.5 with a midpoint at about pH 8.5, and essentially no exchange activity is observed above pH 9.5 (Figure 4A). Importantly, the effect of alkaline pH on the mutant is completely reversible. When RSO vesicles containing E325D permease are assayed first at pH 10.0 and the pH is subsequently decreased stepwise to pH 8.5, 7.7, and 7.0, exchange activity is restored to high levels (Figure 4B). Finally, the rate of equilibrium exchange by vesicles containing E325A or E325Q permease exhibits a $t_{1/2}$ of about 5 s over the pH range tested (Figure 5).

Effect of mAb 4B1. mAb 4B1 mimics the effects of mutations at Glu325, binds to the periplasmic loop between helices VII and VIII, and is thought to alter the pK_a values of residues critical for lactose-coupled H^+ translocation (Sun et al., 1996). Therefore, the effect of 4B1 on equilibrium exchange was examined as a function of pH. Exchange activity catalyzed by wild-type permease which is relatively insensitive to pH and is only moderately inhibited above pH 9.5 is essentially unaffected by 4B1; exchange catalyzed by E325A permease which is completely insensitive to pH is also unaffected by 4B1 over the same pH range (Figure 5). As shown in Figures 4A and 5, the exchange activity of E325D permease is sensitive to pH, exhibiting normal activity up to ca. pH 7.5 and decreasing sigmoidally between pH 7.5 and 9.5 with a midpoint at about pH 8.5. Strikingly, in the presence of mAb 4B1, the midpoint of the pH titration for E325D permease exchange activity is observed at about pH 7.5, indicating that binding of the mAb causes an acidic shift in the pK_a of Asp325 by approximately 1 pH unit. Although data are not shown, binding of mAb 4B1 to RSO vesicles containing wild-type, E325D, or E325A permease is comparable and unaffected by pH from 4.5 to 10.5 (J. Sun, S. Frillingos, and H. R. Kaback, unpublished results).

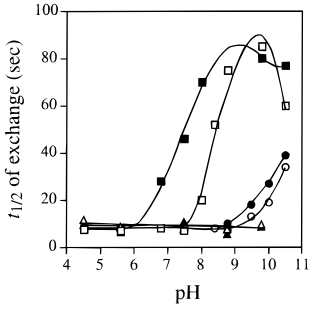

FIGURE 5: Effect of mAb 4B1 on the pH titration of equilibrium lactose exchange by wild-type (○, ●), E325D (□, ■), or E325A permease (△, ▲). RSO membrane vesicles were incubated at pH 7.5 in the absence (open symbols) or presence of 4B1 (closed symbols), equilibrated with 10 mM [1-^{14}C]lactose and assayed at the appropriate pH as described in Materials and Methods.

DISCUSSION

Glu325 (helix X) is one of only four amino acid residues in lac permease that is irreplaceable with respect to active transport [reviewed in Kaback et al. (1994) and Kaback (1996)]. Replacement of Glu325 with Ala, Gln, His, Cys, Val, Trp (Carrasco et al., 1986, 1989), Ser, or Gly (Franco & Brooker, 1994) results in permease that mediates downhill influx of lactose without H^+ translocation but does not catalyze either active transport or efflux. Remarkably, however, all of the Glu325 mutants catalyze normal exchange and counterflow at saturating lactose concentrations, and counterflow is enhanced at least 3-fold relative to wild-type when the external lactose concentration is below the apparent K_m (Carrasco et al., 1989). In addition, binding studies with *p*-nitrophenyl α-D-galactopyranoside show that E325A permease has a K_D approximating that of wild-type permease (Carrasco et al., 1989). The observations are entirely consistent with the kinetic scheme in Figure 1 and imply that replacement of the carboxylic acid at position 325 leads to blockade of step 4 (deprotonation) without affecting steps 1−3 of the cycle (Carrasco et al., 1986, 1989). In the present study, attention is focused on mutant E325D in which the essential carboxylate is retained but the side chain is one methylene group shorter relative to wild-type.

Unlike other Glu325 mutants, E325D permease retains a low but significant ability to catalyze active transport (Carrasco et al., 1989; Franco & Brooker, 1994). Moreover, while E325A or E325Q permease has no activity at any pH

tested, E325D permease displays a pH titration profile similar to that of wild-type (Figure 2). Therefore, a carboxylic acid is essential at position 325 for active lactose transport, and the proper length of the side chain is important for optimal activity. In addition, lactose efflux down a concentration gradient which also involves concomitant H^+ translocation is severely defective in mutant E325D at all pH values tested but exhibits some activity at alkaline pH (Figure 3). The findings are consistent with the idea that Glu325 forms part of a H bond network that is involved in lactose-coupled H^+ translocation (Kaback, 1987, 1990) and that replacement of Glu with Asp alters the interaction between the carboxylate at position 325 and Arg302.

Analysis of pH effects on efflux and exchange in proteoliposomes reconstituted with wild-type permease (Viitanen et al., 1983) demonstrates that in H_2O the initial rate of efflux increases sigmoidally with pH, exhibiting a sharp rise between pH 7.5 and 9.5 with a midpoint at about 8.3. The rate is maximal at pH 9.5 and above and more than 6 times greater than the rate at pH 7.5. In D_2O, the pD profile for efflux is shifted to the right by 0.4−0.5 pH (pD) unit and is essentially parallel to the pH profile. Over the same extended range of pH (pD) values, exchange remains constant and unaffected by D_2O. Moreover, the rate of membrane potential-driven uphill transport is identical in H_2O and D_2O, while entrance counterflow is stimulated at alkaline pH when the external lactose concentration is subsaturating. These results also provide strong support for the model presented in Figure 1 and are consistent with the notion that D_2O acts at step 4 (i.e., slows the rate of deprotonation).

Remarkably, equilibrium lactose exchange catalyzed by E325D permease is pH dependent (Figure 5). Below pH 7.5, exchange is rapid and occurs at a rate comparable to wild-type. As ambient pH is increased above 7.5, the rate decreases progressively with a midpoint at about 8.5 and is almost completely inhibited above pH 9.5. The effect of pH is reversible and, therefore, is not due to denaturation of the protein at high pH. The simplest interpretation of the observation is that translocation of the ternary complex between the permease, lactose, and H^+ does not tolerate a negative charge at position 325. In wild-type permease, the electrostatic interaction between Glu325 (helix X) and Arg302 (helix IX) (Figures 6 and 7A) is sufficiently strong that the carboxylate is unaffected by pH. In contrast, with Asp at position 325, the electrostatic interaction between Asp325 and Arg302 is broken, the carboxylate is protonated (Figure 7B), and the acid exhibits a pK_a of about 8.5. Interestingly, Asp96 in bacteriorhodopsin, a residue that is clearly involved in H^+ translocation during the photocycle, exhibits an anomalously high pK_a (Bashford & Gerwert, 1992). In addition, Asp61 which functions in H^+ translocation in subunit c of F_1F_0 H^+-ATP synthase also exhibits an unexpectedly high pK_a (Zhang & Fillingame, 1994; Assadi-Porter & Fillingame, 1995). Although it is possible that inhibition of exchange in E325D permease at high pH reflects deprotonation of another dissociable side chain such as His322 (helix X) or Arg302 (helix IX), this is unlikely because neutral replacements for Glu325 do not lead to pH sensitive exchange activity.

It is particularly interesting in the context of this paper that mAb 4B1 uncouples lactose and H^+ translocation in a manner that mimics the effects of D_2O and mutations at Glu325. Thus, 4B1 inhibits all steps that involve net H^+ translocation with no effect on equilibrium exchange or

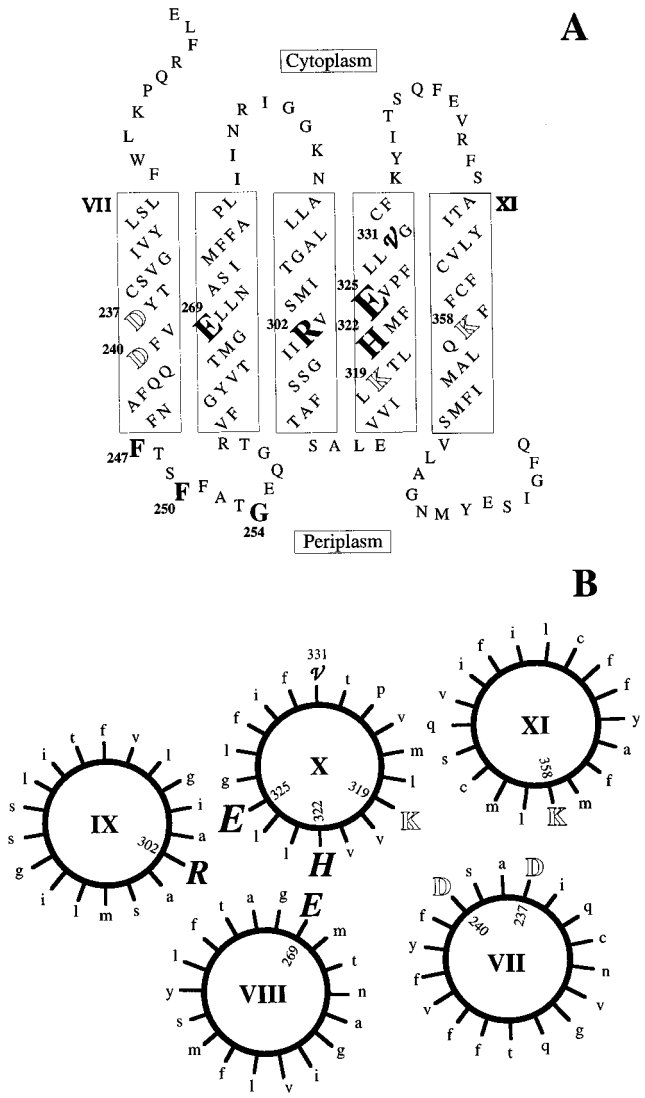

FIGURE 6: Secondary structure (A) and helix-packing model (B) of helices VII−XI of lac permease (Jung et al., 1993; Kaback et al., 1994; Kaback, 1996). Residues Glu269, Arg302, His322, and Glu325 that are obligatory for lactose/H^+ transport are bold. Charge pairs Asp237/Lys358 and Asp240/Lys319 are outlined. mAb 4B1 binds to an epitope in the loop between helices VII and VIII in which Phe247, Phe250 and Gly254 are determinants (Sun et al., 1996). Val331 (helix X) which responds to a conformational change induced by 4B1 binding is italicized.

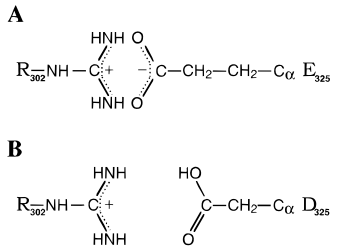

FIGURE 7: Interaction between Arg302 (helix IX) and either Glu325 (A) or Asp325 (B) (helix X). Note that increasing the proximity between Arg302 and Glu325 will decrease the pK_a of the carboxylic acid part *B*.

entrance counterflow, as well as ligand binding (Carrasco et al., 1982, 1984; Herzlinger et al., 1984). Recently, (Sun et al. (1996) demonstrated that the mAb 4B1 binds to an epitope in the periplasmic loop between helices VII and VIII which is in close proximity to Glu269, Arg302, His322, and Glu325 (Figure 6). Furthermore, the uncoupling effect of 4B1 involves highly specific interactions with three amino

acid residues (Phe247, Phe250, and Gly254) on one face of a region that is probably a short helix. The interaction has been suggested to exert a torsional effect, resulting in a conformational change of helix VII and/or VIII that alters the pK_a(s) of a residue(s) involved in lactose-coupled H^+ translocation. Consistent with this idea, mAb 4B1 binding shifts the midpoint of the pH profile for exchange activity of mutant E325D from about pH 8.5 to about pH 7.5. Since the midpoint probably reflects the pK_a of Asp325 and binding of the mAb is unaffected over the pH range tested, it seems reasonable to conclude that 4B1 binding secondarily causes the shift in the pK_a of Asp325. If binding of mAb 4B1 allows helix IX to come into closer proximity to helix X by interfering with the interaction between Asp240 (helix VII) and Lys319 (helix X), for example (Figure 6B), the interaction between Arg302 and a protonated carboxylate at position 325 (i.e., Asp; Figure 7B) will cause the proton to become more acidic (i.e., the pK_a of Asp325 will decrease). In any case, the general conclusion is supported by the findings that mAb 4B1 has no effect on the exchange activity profile of E325A permease where there is no carboxylic acid at position 325 or on wild-type permease where the carboxylate electrostatically interacts with Arg302 and is insensitive to pH. In addition, 4B1 binding markedly alters the reactivity of a Cys residue in place of Val331 (helix X) [see Wu et al. (1994)] and the fluorescence of V331C permease after labeling with 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (J. Wu, S. Frillingos, and H. R. Kaback, unpublished results).

The results presented here are relevant to the question of whether the carboxylic acid at position 325 of the permease is directly involved in lactose-coupled H^+ translocation. Since mutants in Glu325 are blocked at a step in the kinetic scheme (Figure 1) that corresponds to deprotonation of the permease and the effects of mutations at this position are mimicked by D_2O, one possibility is that deprotonation of the carboxylic acid *per se* or a conformational change that leads to deprotonation at this position (Kaczorowski & Kaback, 1979) corresponds directly to step 4. Another possibility is that Glu325 is not directly involved in H^+ translocation but is salt bridged to either Arg302 or His322 and that disruption of the salt bridge by replacement of Glu325 fortuitously leads to the properties described. The latter possibility is unlikely, however, since replacement of Arg302 with Lys (D. Menick, L. Patel, S. Frillingos, and H. R. Kaback, unpublished information) or His322 with Arg (Padan et al., 1985; Püttner et al., 1986, 1989) yields permease that only catalyzes downhill translocation of lactose without H^+.

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REFERENCES

Bashford, D., & Gerwert, K. (1992) *J. Mol. Biol. 224*, 473−486.
Boyer, H. W., & Roulland-Dussoix, D. (1969) *J. Mol. Biol. 41*, 459−472.
Calamia, J., & Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937−4941.
Carrasco, N., Tahara, S. M., Patel, L., Goldkorn, T., & Kaback, H. R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6894−6898.
Carrasco, N., Viitanen, P., Herzlinger, D., & Kaback, H. R. (1984) *Biochemistry 23*, 3681−3687.
Carrasco, N., Antes, L. M., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4486−4488.
Carrasco, N., Puttner, I. B., Antes, L. M., Lee, J. A., Larigan, J. D., Lolkema, J. S., Roepe, P. D., & Kaback, H. R. (1989) *Biochemistry 28*, 2533−2539.
Consler, T. G., Tsolas, O., & Kaback, H. R. (1991) *Biochemistry 30*, 1291−1298.
Franco, P. J., & Brooker, R. J. (1994) *J. Biol. Chem. 269*, 7379−7386.
Frillingos, S., & Kaback, H. R. (1996) *Biochemistry 35*, 5333−5338.
Frillingos, S., Sahin-Toth, M., Persson, B., & Kaback, H. R. (1994) *Biochemistry 33*, 8074−8081.
Garcia, M. L., Viitanen, P., Foster, D. L., & Kaback, H. R. (1983) *Biochemistry 22*, 2524−2531.
Hattori, M., & Sakaki, Y. (1986) *Anal. Biochem. 152*, 1291−1297.
He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995a) *Biochemistry 34*, 15667−15670.
He, M. M., Voss, J., Hubbell, W. L., & Kaback, H. R. (1995b) *Biochemistry 34*, 15661−15666.
Herzlinger, D., Viitanen, P., Carrasco, N., & Kaback, H. R. (1984) *Biochemistry 23*, 3688−3693.
Herzlinger, D., Carrasco, N., & Kaback, H. R. (1985) *Biochemistry 24*, 221−229.
Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51−59.
Jung, K., Jung, H., Wu, J., Privé, G. G., & Kaback, H. R. (1993) *Biochemistry 32*, 12273−12278.
Jung, K., Voss, J., He, M., Hubbell, W. L., & Kaback, H. R. (1995) *Biochemistry 34*, 6272−6277.
Kaback, H. R. (1971) *Methods Enzymol. 22*, 99−120.
Kaback, H. R. (1987) *Biochemistry 26*, 2071−2076.
Kaback, H. R. (1990) *Philos. Trans. R. Soc. London, Ser. B 326*, 425−436.
Kaback, H. R. (1996) in *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., & Kaback, H. R., Eds.) Elsevier, Amsterdam (in press).
Kaback, H. R., Frillingos, S., Jung, H., Jung, K., Privé, G. G., Ujwal, M. L., Weitzman, C., Wu, J., & Zen, K. (1994) *J. Exp. Biol. 196*, 183−195.
Kaczorowski, G. J., & Kaback, H. R. (1979) *Biochemistry 18*, 3691−3697.
Kaczorowski, G. J., Robertson, D. E., & Kaback, H. R. (1979) *Biochemistry 18*, 3697−3704.
Konings, W. N., Barnes, E., Jr., & Kaback, H. R. (1971) *J. Biol. Chem. 246*, 5857−6581.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804−11808.
Padan, E., Sarkar, H. K., Viitanen, P. V., Poonian, M. S., & Kaback, H. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6765−6768.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346.
Püttner, I. B., Sarkar, H. K., Poonian, M. S., & Kaback, H. R. (1986) *Biochemistry 25*, 4483−4485.
Püttner, I. B., Sarkar, H. B., Padan, E., Lolkema, J. S., & Kaback, H. R. (1989) *Biochemistry 28*, 2525−2533.
Sachs, G., Shin, J. M., Besancon, M., Munson, K., & Hersey, S. (1992) *Ann. N.Y. Acad. Sci. 671*, 204−216.
Sanger, F., Nicklen, S., & Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Short, S. A., Kaback, H. R., & Kohn, L. D. (1975) *J. Biol. Chem. 250*, 4291−4296.
Sun, J., Wu, J., Carrasco, N., & Kaback, H. R. (1996) *Biochemistry 35*, 990−998.
Teather, R. M., Bramhall, J., Riede, I., Wright, J. K., Furst, M., Aichele, G., Wilhelm, V., & Overath, P. (1980) *Eur. J. Biochem. 108*, 223−231.
Viitanen, P., Garcia, M. L., Foster, D. L., Kaczorowski, G. J., & Kaback, H. R. (1983) *Biochemistry 22*, 2531−2536.
Wu, J., Frillingos, S., Voss, J., & Kaback, H. R. (1994) *Protein Sci. 3*, 2294−2301.
Wu, J., Perrin, D., Sigman, D., & Kaback, H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 9186−9190.
Wu, J., Voss, J., Hubbell, W. L., & Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Zhang, Y., & Fillingame, R. H. (1994) *J. Biol. Chem. 269*, 10221−10224.

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