Characterization of Glu126 and Arg144, Two Residues That Are Indispensable for Substrate Binding in the Lactose Permease of *Escherichia coli*†

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ABSTRACT: Glu126 and Arg144 in the lactose permease are indispensable for substrate binding and probably form a charge-pair [Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci.* U.S.A. *95*, 9802−9807]. Mutants with Glu126→Ala or Arg144→Ala do not bind ligand or catalyze lactose accumulation, efflux, exchange, downhill lactose translocation, or lactose-induced H^+ influx. In contrast, mutants with conservative mutations (Glu126→Asp or Arg144→Lys) exhibit drastically different phenotypes. Arg144→Lys permease accumulates lactose slowly to low levels, but does not bind ligand or catalyze equilibrium exchange, efflux, or lactose-induced H^+ influx. In contrast, Glu126→Asp permease catalyzes lactose accumulation and lactose-induced H^+ influx to wild-type levels, but at significantly lower rates. Surprisingly, however, no significant exchange or efflux activity is observed. Glu126→Asp permease exhibits about a 6-fold increase in the K_m for active transport relative to wild-type permease with a comparable V_{max}. Direct binding assays using flow dialysis demonstrate that mutant Glu126→Asp binds *p*-nitrophenyl-α,D-galactopyranoside. Indirect binding assays utilizing substrate protection against [^{14}C]-*N*-ethylmaleimide labeling of single-Cys148 permease reveal an apparent K_d of 3−5 mM for lactose and 15−20 μM for β,D-galactopyranosyl-1-thio-β,D-galactopyranoside (TDG). The affinity of Glu126→Asp/Cys148 permease for lactose is markedly decreased (K_d > 80 mM), while TDG affinity is altered to a much lesser extent (K_d ca. 80 μM). The results extend the conclusion that a carboxylate at position 126 and a guanidinium group at position 144 are irreplaceable for substrate binding and support the idea that Arg144 plays a major role in substrate specificity.

The lactose permease (lac permease)[1] of *Escherichia coli* is representative of 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 polytopic membrane protein catalyzes the coupled stoichiometric translocation of β-galactosides and H^+. The *lac Y* gene which encodes the permease has been cloned and sequenced, and the product of the *lac Y* gene has been solubilized, purified, reconstituted into proteoliposomes (reviewed in *3*), and shown to be solely responsible for β-galactoside transport as a monomer (see *4*). All available evidence indicates that the permease consists of 12 hydrophobic, membrane-spanning helices connected by hydrophilic loops with both the N and C termini on the cytoplasmic face of the membrane. Although the permease has not been crystallized, application of a battery of site-directed techniques which include second-site suppressor analysis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance, thiol cross-linking, and identification of discontinuous monoclonal antibody epitopes has led to the formulation of a tertiary structure model of the protein (reviewed in *5*−*7*). In addition, experimental observations from structural and extensive mutational analysis have led to a proposed mechanism for energy coupling between sugar and H^+ transport (*7*, *8*).

Site-directed and Cys-scanning mutagenesis of each residue in the permease (reviewed in *7*) reveals that only six residues are irreplaceable with respect to active transport: Glu126 (helix IV) and Arg144 (helix V), which are indispensable for substrate binding; and Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix X), which are involved in H^+ translocation and coupling with substrate translocation. In addition, Cys148 (helix V), which is protected by substrate against alkylation by *N*-ethylmaleimide (NEM), interacts weakly and hydrophobically with the galactosyl moiety of the substrate (*9*, *10*), and Met145, which is on the same face of helix V as Cys148, interacts even more weakly with the glucosyl moiety (*10*).

Recently, the substrate binding properties of Glu126 and Arg144 mutants were studied by site-directed NEM labeling of Cys148 (*11*). Replacement of either Glu126 or Arg144 with Ala markedly decreases Cys148 reactivity, while interchanging the residues, double-Ala replacement, or replacement of Arg144 with Lys or His does not alter reactivity, providing a strong indication that Glu126 and Arg144 are charge-paired. Importantly, although alkylation

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[1] Abbreviations: lac permease, lactose permease; TDG, β,D-galactopyranosyl-1-thio-β,D-galactopyranoside; DM, *n*-dodecyl-β,D-maltopyranoside; NPG, *p*-nitrophenyl-α,D-galactopyranoside; NEM, *N*-ethylmaleimide; KP_i, potassium phosphate; MIANS, 2-(4-maleimidoanilino)naphthalene-6-sulfonic acid.

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of Cys148 is blocked by ligand in wild-type permease, no protection is observed with any of the Glu126 or Arg144 mutants. Further evidence for the essential role of Glu126 and Arg144 in substrate binding was obtained from site-directed fluorescence studies with 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS) in mutant Val331→Cys. In marked contrast to V331C permease, ligand does not alter MIANS reactivity in mutant E126A/V331C, R144A/V331C, or R144K/V331C and does not cause either quenching or a shift in the emission maximum of the MIANS-labeled mutants. These and other results demonstrate that Glu126 and Arg144 are irreplaceable for both high- and low-affinity substrate binding and indicate that alterations in the substrate translocation pathway at the interface between helices IV and V are transmitted conformationally to the H^+ translocation pathway at the interface between helices IX and X.

Based on the observations, the following model for substrate binding was postulated (*7*, *11*): (i) One of the guanidino NH_2 groups of Arg144 forms an H-bond with the OH group at the C4 and/or C3 position(s) of the galactosyl moiety of the substrate, an interaction that plays a key role in substrate specificity. (ii) The other guanidino NH_2 of Arg144 forms a salt-bridge with Glu126, and the interaction holds Arg144 and Cys148 in an orientation that allows specific interaction with the galactosyl moiety. One of the oxygen atoms of the carboxylate at position 126 could also act as an H-bond acceptor from the C6-OH of the galactosyl moiety. (iii) Cys148 interacts weakly and hydrophobically with the galactosyl end of lactose and other galactosides. (iv) Met145 interacts even more weakly with the glucosyl part of lactose.

In this study, the properties of mutants in Glu126 and Arg144 are characterized in more detail. Active lactose transport, equilibrium exchange, efflux, lactose-induced H^+ influx, and ligand binding are studied with neutral or conservative replacements. The data support the conclusion that Glu126 and Arg144 are indispensable for substrate binding and highlight a pivotal role for Arg144 in substrate recognition.

EXPERIMENTAL PROCEDURES

Materials. N-[*ethyl*-1-^{14}C]Maleimide (40 mCi/mmol) was purchased from DuPont NEN (Boston, MA). [^{125}I]Streptavidin and horseradish peroxidase-conjugated avidin were from Amersham (Arlington Heights, IL). Immobilized monomeric avidin was from Pierce (Rockford, IL). *p*-Nitrophenyl-α,D-[6-^{3}H]galactopyranoside (NPG) was kindly provided by Gérard LeBlanc. All other materials were reagent grade and obtained from commercial sources.

Growth of Bacteria. E. coli T184 [*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$*(A),rpsL, met*$^-$*,thr*$^-$*,recA,hsdM,hsdR/F′,lacI*^{q}*O*$^+$*Z*^{D118}*(Y*$^+$*A*$^+$*)*] (*12*) expressing given permease mutants was grown aerobically at 37 °C in LB broth with ampicillin (100 μg/mL). Fully grown cultures were diluted 10-fold and allowed to grow for 2 h at 37 °C before induction with 1 mM isopropyl 1-thio-α,D-galactopyranoside. After additional growth for 2 h at 37 °C, cells were harvested by centrifugation.

Preparation of Right-Side-Out (RSO) Membrane Vesicles. Right-side-out (RSO) membrane vesicles were prepared by lysozyme−ethylenediaminetetraacetic acid treatment and osmotic lysis as described (*13*, *14*). The vesicles were suspended in 100 mM potassium phosphate (KP_i; pH 7.5)/10 mM $MgSO_4$ at a protein concentration of 10−15 mg/mL, frozen in liquid N_2, and stored at −80 °C until use.

Lactose 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 600 nm (0.7 mg of protein/mL). Transport was initiated by addition of [1-^{14}C]lactose (5 mCi/mmol specific activity, 0.4 or 2.0 mM final concentration), and samples were quenched at given times with 100 mM KP_i (pH 5.5)/100 mM LiCl and assayed by rapid filtration. Efflux and equilibrium exchange were carried out with RSO vesicles as described (*15*). Vesicles were washed with 100 mM KP_i at a given pH, and resuspended in the same buffer at a protein concentration of 20−25 mg/mL. [1-^{14}C]Lactose (10 mCi/mmol specific activity, 10 mM final concentration) was added, and equilibration was accomplished by incubating the samples at 4 °C overnight in the presence of valinomycin (50 μM) and nigericin (0.5 μM). To initiate efflux or exchange, aliquots (2 μL) were rapidly diluted into 0.4 mL (200-fold excess) of 100 mM KP_i at a given pH containing 10 mM nonradioactive lactose (exchange) or no lactose at all (efflux). Reactions were quenched at given times with 100 mM KP_i (pH 5.5)/100 mM LiCl/10 mM $HgCl_2$ and assayed by rapid filtration.

Lactose-Induced H^+ Movements. Measurements were carried out essentially as described (*16*). Briefly, cells were washed twice in 1 mM KP_i (pH 7.5)/120 mM KCl and resuspended in 120 mM KCl/30 mM KSCN to an optical density of 80.0 at 420 nm (ca. 5.6 mg of protein/mL). An aliquot (0.5 mL) of the cells was placed in a closed, temperature-controlled (25 °C) glass cell equipped with a glass pH electrode, and the stirred suspension was equilibrated to an anaerobic state under a stream of N_2 gas. Lactose was added to a final concentration of 15 mM in a 30 μL volume, and pH changes were recorded. The signal was calibrated by adding 10 μL of 1 mM HCl which corresponds to 10 nmol of H^+.

[^{14}C]NEM Labeling. Reactivity of Cys148 with [^{14}C]NEM in situ was determined in the absence and presence of substrates (*17*). Single-Cys148 and E126D/single-Cys148 permeases used for these assays contain a biotin-acceptor domain at the C terminus and are biotinylated in vivo. RSO membrane vesicles were preequilibrated in a final volume of 50 μL (containing 0.5−0.7 mg of protein) with given sugar concentrations for 20 min at room temperature. Labeling was initiated by addition of 12 μL of [^{14}C]NEM to a final concentration of 0.5 mM (40 mCi/mmol), and the vesicles were incubated for 5 min at 25 °C. Reactions were quenched by addition of 10 mM dithiothreitol (final concentration). The vesicles were solubilized by 2% dodecyl maltoside (DM, final concentration), and the samples were mixed with immobilized monomeric avidin (avidin−Sepharose) equilibrated with 50 mM KP_i (pH 7.5)/0.1 M NaCl/0.02% DM (w/v). After a 15 min incubation at 4 °C, the resin was washed with 5 × 1 mL of equilibration buffer, and biotinylated permease was then eluted with 2 × 40 μL of equilibration buffer containing 5 mM *d*-biotin. After addition of 20 μL of electrophoresis sample buffer (concentrated 5×), an 80 μL aliquot was analyzed electrophoretically on a sodium dodecyl sulfate ($NaDoDSO_4$)/12% polyacrylamide gel. The gel was dried and exposed to a PhosphoImager

screen for 2−5 days. Incorporation of [^{14}C]NEM was visualized and quantitated by a Storm 860 PhosphoImager (Molecular Dynamics).

Flow Dialysis. Binding of [6-^{3}H]NPG was measured by flow dialysis as described previously (*18*). The upper chamber contained 250 μL of right-side-out vesicles in 0.1 M KP_i (pH 7.5) under constant stirring at a concentration of 20−22 mg of protein/mL. To ensure complete de-energization, 20 μM valinomycin and 0.4 μM nigericin (final concentrations) were included in the upper chamber. Buffer [0.1 M KP_i (pH 7.5)] was pumped through the lower chamber at a flow rate of 0.6 mL/min, and 1.2 mL fractions were collected. One milliliter aliquots were assayed for radioactivity by addition of 5 mL of ScintiSafe Econo 2 (Fisher Scientific) scintillation cocktail and liquid scintillation spectrometry.

Western Blot Analysis. An aliquot (20 μL) of purified [^{14}C]-NEM-labeled permease was electrophoresed on an Na-DoDSO$_4$/12% polyacrylamide gel, electroblotted to poly-(vinylidene difluoride) membranes (Immobilon-PVDF; Millipore), and treated with horseradish peroxidase-conjugated avidin. The PVDF membrane was subsequently developed with fluorescent substrate (Renaissance, DuPont NEN) and exposed to film. Films were scanned with an imaging densitometer (Molecular Dynamics), and the amount of permease was quantitated. Alternatively, protein on the PVDF membrane was treated with [^{125}I]streptavidin, autoradiographed, and visualized and quantitated with a Storm 860 PhosphoImager (Molecular Dynamics).

RESULTS

Active Lactose Transport. As shown (*19*), *E. coli* T184 expressing E126A or R144A permease does not transport lactose to any extent whatsoever. In contrast, E126D permease catalyzes active lactose transport at a relatively slow rate to the same steady-state level of accumulation as the wild-type at an external lactose concentration of 0.4 mM, while R144K permease catalyzes accumulation at a markedly diminished rate to about 25% of the control steady-state [Figure 2A; see (*19*) in addition]. Interestingly, when transport is assayed at 2 mM lactose (Figure 2B), the rate of transport in cells with E126D permease is significantly increased, and the steady-state level of accumulation is more than 2-fold higher that of the control. At the steady-state, the rates of influx and efflux are equal. Thus, the observation suggests that efflux with E126D permease is relatively more impaired than influx, leading to a higher steady-state level of accumulation. Finally, both the rate and steady-state level of lactose accumulation by cells expressing R144K permease are drastically decreased at both 0.4 and 2 mM lactose, which is consistent with the inability of the mutant to bind ligand (*11*).

Kinetic analysis of active lactose transport reveals a 6-fold increase of K_m and a 1.4-fold decrease in V_{max} with the E126D mutant relative to wild-type (data not shown). Thus, K_m values of 0.96 ± 0.13 mM (SE, $n = 4$) and 6.1 ± 0.05 mM (SE, $n = 3$) are observed for the wild-type and the E126D mutant, respectively, with corresponding V_{max} values of 473 ± 53 nmol/mg of protein (SE, $n = 4$) and 341 ± 29 nmol/mg of protein (SE, $n = 3$). Low rates of transport with R144K permease preclude reliable kinetic studies.

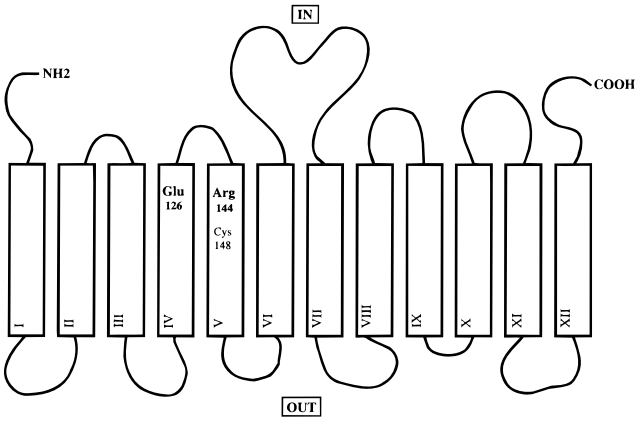

FIGURE 1: Secondary structure model of lac permease. Putative transmembrane helices are shown in boxes. The positions of Glu126, Arg144, and Cys148 are indicated. Glu126 and Arg144 were placed initially by hydropathy profiling at the membrane−water interface at the cytoplasmic ends of helices IV and V, respectively. Recent studies using single amino acid deletions (C. Wolin and H. R. Kaback, unpublished observations), nitroxide-scanning accessibility measurements (M. Zhao, J. Hernandez-Borrell, W. L. Hubbell and H. R. Kaback, unpublished observations), and lac permease fusions with the NG domain of FtsY (E. Bibi, personal communication) indicate that loop IV/V is much smaller and that Glu126 and Arg144 are within the membrane.

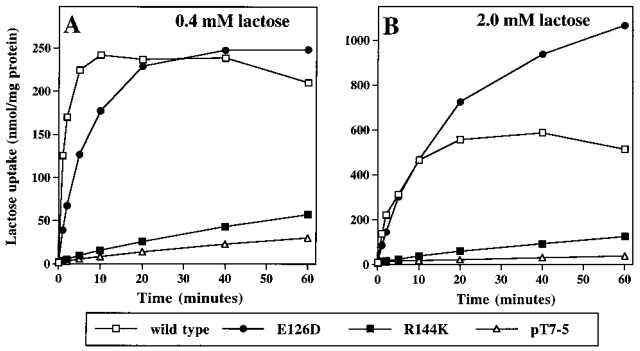

FIGURE 2: Time courses of active lactose transport by *E. coli* T184 expressing wild-type permease, no permease (pT7-5 vector only), or mutants E126D and R144K. Cells were grown at 37 °C, and aliquots of cell suspensions (50 μL, containing approximately 35 μg of protein) in 100 mM KP_i (pH 7.5)/10 mM $MgSO_4$ were assayed at 0.4 mM (A) or 2.0 mM (B) final external lactose concentrations as described under Experimental Procedures.

Lactose-Induced H^+ Influx. H^+ influx induced by downhill translocation of lactose under de-energized (anaerobic) conditions was monitored with a pH electrode (Figure 3). With *E. coli* T184 expressing wild-type permease, rapid alkalinization of the medium is observed upon lactose addition, and the pH tracing reaches maximum displacement in 1−2 min. Mutant E126D exhibits lactose-induced H^+ influx, but the rate is about one-fourth of that observed with the wild-type. In marked contrast, cells expressing E126A, E126Q, R144A, and R144K permease exhibit essentially the same response to lactose as the negative control with no permease, and slight acidification is observed.

Efflux and Equilibrium Exchange. Lactose efflux down a concentration gradient is permease-mediated and occurs in symport with H^+. In contrast, equilibrium exchange does not involve net H^+ translocation, and the permease recycles in the protonated state (*7*, *15*, *20*−*22*). When RSO membrane vesicles containing wild-type permease are equilibrated with 10 mM [1-^{14}C]lactose and diluted into medium devoid of

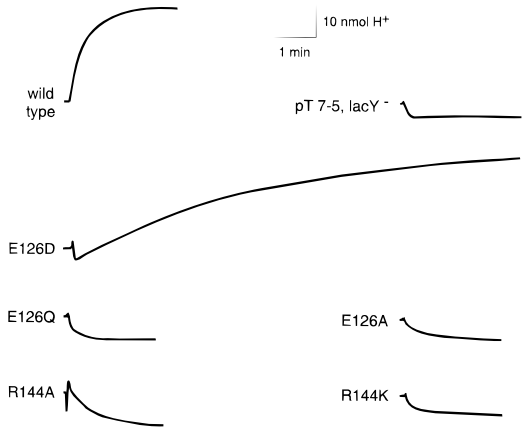

FIGURE 3: Lactose-induced H^+ movements in *E. coli* T184 expressing wild-type permease, no permease (pT7-5 vector only), or mutants E126A, E126Q, E126D, R144A, and R144K. Changes in external pH upon addition of 15 mM lactose (final concentration) were monitored with a pH electrode as described under Experimental Procedures. Upward displacement on the recordings indicates alkalinization of the external medium.

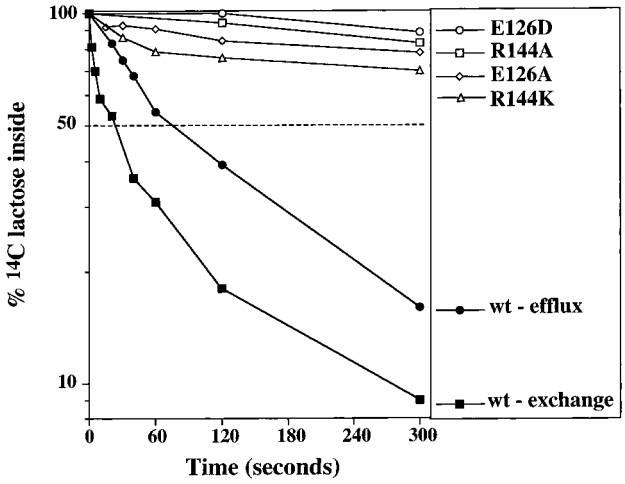

FIGURE 4: Lactose efflux and equilibrium exchange by RSO membrane vesicles containing wild-type permease, E126A, E126D, R144A, or R144K permease mutants. RSO vesicles were equilibrated with [1-^{14}C]lactose at pH 7.5, and assayed by dilution into equilibration buffer without lactose (efflux) or with 10 mM lactose (exchange) as described under Experimental Procedures. Efflux and exchange were identical in the different mutants, and both activities are represented by the same symbol. Although not shown, vesicles containing no permease or NEM-treated vesicles with wild-type permease exhibit no significant efflux or exchange over the indicated time period.

lactose (efflux) or into medium containing 10 mM unlabeled lactose (equilibrium exchange), loss of intravesicular radioactivity occurs at rapid rates ($t_{1/2}$ ca. 20−25 s for exchange and 70−75 s for efflux). In contrast, vesicles containing R144A, R144K, E126A, or E126D permease are markedly defective with respect to both efflux and exchange, and all the mutants exhibit $t_{1/2}$ values from 20 to 60 min (Figure 4). In control experiments that are not shown, similar $t_{1/2}$ values are obtained with vesicles containing wild-type or mutant permeases treated with NEM, vesicles containing no permease, or vesicles loaded with [^{14}C]sucrose. In particular, E126D permease catalyzes NEM-sensitive exchange or efflux with a $t_{1/2}$ of about 30 min, a rate that is approximately 70−90 times slower than wild-type permease. As discussed above, the observation is in agreement with the finding that

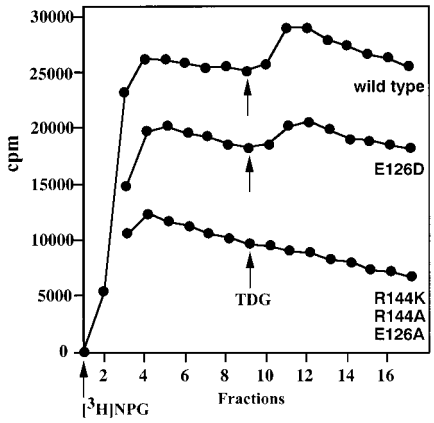

FIGURE 5: Binding of NPG to right-side-out (RSO) membrane vesicles. Binding of [^{3}H]NPG to nonenergized RSO vesicles was assayed by flow dialysis as described under Experimental Procedures. An aliquot of RSO vesicles (250 μL) in 0.1 M KP_i (pH 7.5) at a concentration of 20−22 mg of protein/mL was placed in the upper chamber. Five microliters of [^{3}H]NPG [0.95 mCi/mmol, approximately 4−4.5 × 10^6 cpm] was added to a final concentration of 20 μM at fraction 1. Where indicated by the arrow, 2.5 μL of TDG was added to the upper chamber to a final concentration of 10 mM. For clarity, curves are displayed in decreasing order; however, the actual levels of radioactivity in the dialysate were identical in fractions 1 through 9 in each case (i.e., the curves should be superimposed).

E126D permease accumulates lactose to higher steady-state levels than wild-type at relatively high external lactose concentrations. Finally, rates of lactose exchange or efflux by E126D permease are indistinguishable at pH 4.5, 7.5, or 9.5.

Direct Binding Assays. To obtain direct evidence that mutations in Glu126 and Arg144 cause a defect in substrate binding, RSO membrane vesicles containing wild-type, E126D, E126A, R144K, or R144A permeases were assayed for binding of the high-affinity ligand *p*-nitrophenyl-α,D-galactopyranoside (NPG) by flow dialysis under nonenergized conditions (Figure 5). At the inception of the experiments, [^{3}H]NPG (20 μM final concentration) is added to the upper chamber containing membrane vesicles, and radioactivity in the dialysate increases linearly (fractions 1−3), reaching a maximum (fraction 4) which then decreases at a slow rate. When saturating concentrations of TDG (10 mM final concentration) are added to the upper chamber containing RSO membranes with wild-type or E126D permease, bound [^{3}H]NPG is displaced, and the concentration of dialyzable radioactivity increases. No displacement is observed with an equal concentration of sucrose which is not a ligand for the permease (data not shown). In contrast, no change is observed in the dialyzable ligand concentration upon addition of 10 mM TDG to vesicles with E126A, R144K, or R144A permease, demonstrating that the mutants do not bind NPG to a significant extent.

Sugar Binding by E126D Permease. Since the reactivity of single-Cys148 permease with NEM is blocked by ligand in a manner that reflects the affinity of the permease (i.e., TDG ≫ lactose > galactose), Glu126 was replaced with Asp in single-Cys148 permease in order to assess ligand binding (*11*, *17*). The time course of alkylation of single-Cys148 permease with NEM at pH 7.5 reveals that after a 2−5 min linear phase, the reaction rapidly saturates at 10−15 min (Figure 6). In contrast, at pH 5.0, the reaction proceeds at a

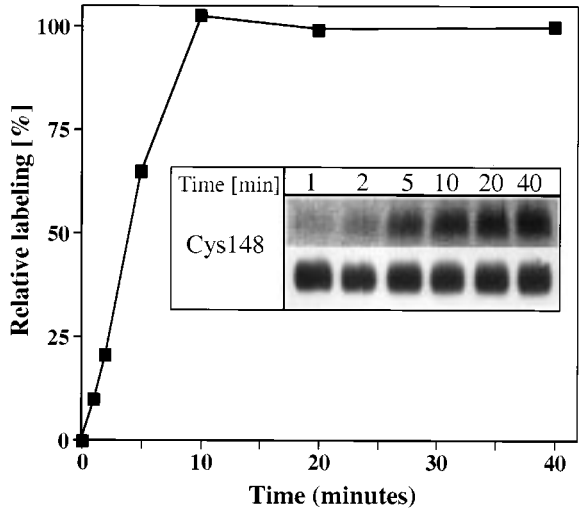

FIGURE 6: Time course of alkylation of Cys148 by [^{14}C]NEM. RSO membrane vesicles were incubated in 100 mM KP_i (pH 7.5) with 0.5 mM [^{14}C]NEM at 25 °C for the indicated times. Reactions were quenched by dithiothreitol, and biotinylated permease was solubilized and purified as described under Experimental Procedures. Aliquots of protein were separated by a 12% $NaDoDSO_4$−polyacrylamide gel, and ^{14}C-labeled protein was visualized by autoradiography (insert, upper panel). A fraction of the protein was analyzed by Western blotting in order to ascertain the content of permease in each sample (insert, lower panel). Incorporation of [^{14}C]-NEM was quantitated by a Storm 860 PhosphoImager machine, and values obtained were corrected for protein content.

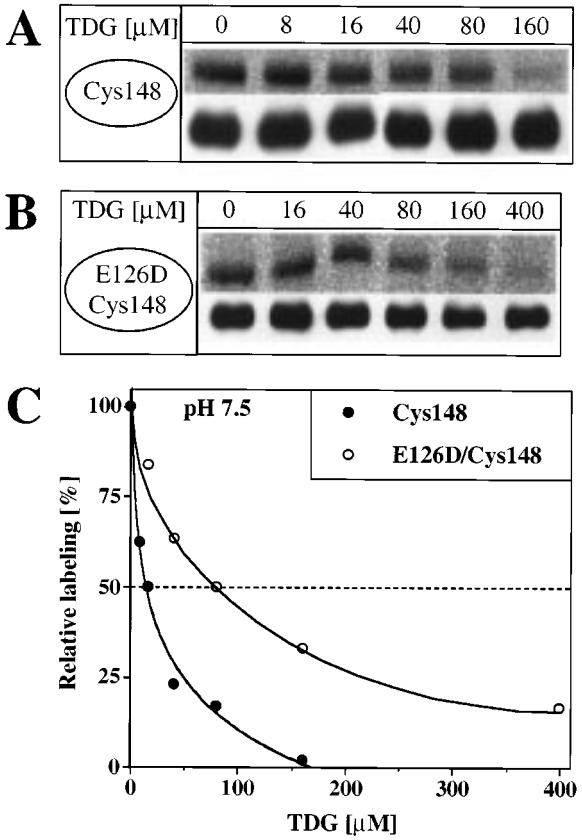

FIGURE 7: Effect of TDG on the reactivity of Cys148 in single-Cys148 and E126D/single-Cys148 permeases at pH 7.5. RSO membrane vesicles were incubated with 0.5 mM [^{14}C]NEM at pH 7.5, 25 °C, for 5 min in the absence or presence of the indicated concentrations of TDG. Reactions were quenched by dithiothreitol, and biotinylated permease was solubilized and purified as described under Experimental Procedures. Aliquots of protein were separated on a 12% $NaDoDSO_4$−polyacrylamide gel, and ^{14}C-labeled protein was visualized by autoradiography (A and B, upper panels). A fraction of the protein was analyzed by Western blotting in order to ascertain the content of permease in each sample (A and B, lower panels). Incorporation of [^{14}C]NEM was quantitated by a Storm 860 Phosphoimager machine, and values obtained were corrected for protein content. The figure shows autoradiograms of representative labeling experiments (A and B) and the average of three experiments (C).

much slower rate, and is linear for at least 20 min (data not shown).

As expected, increasing concentrations of TDG progressively block the reactivity of single-Cys148 permease (Figure 7), and half-maximal protection is observed at 15−20 μM TDG, a value similar to that obtained previously (*17*). E126D/single-Cys148 permease exhibits qualitatively similar TDG binding characteristics, but the apparent K_d is increased at least 4-fold to about 80 μM. In contrast to a relatively modest decrease in TDG affinity, the apparent affinity of E126D/single-Cys148 permease for lactose is drastically reduced (Figure 8). Thus, lactose protection against NEM labeling occurs with an apparent K_d of 3−5 mM in single-Cys148 permease, and, strikingly, no significant protection is observed with E126D/single-Cys148 permease at lactose concentrations up to 80 mM.

To test whether the protonation state of the carboxylate at 126 might be important for substrate binding (*11*, *17*), TDG protection of Cys148 against NEM labeling was also assayed at pH 5.0. As shown in Figure 9, single-Cys148 permease, as well as E126D/single-Cys148 permease, exhibits a relatively small decrease in affinity at pH 5.0 relative to pH 7.5. Thus, apparent K_d values for TDG increase about 4-fold in single-Cys148 permease to 70−75 μM and almost 2.5-fold in E126D/single-Cys148 permease to 180−200 μM, a difference that is within the experimental error of the measurements.

DISCUSSION

This paper describes a more complete characterization of mutants in Glu126 (helix IV) and Arg144 (helix V), two of six irreplaceable residues in lac permease which have been discovered recently to be obligatory for substrate binding and transport (*11*, *19*). According to the model postulated for the binding site, charge-pairing between Asp126 and one of the guanidino NH_2 groups of Arg144 positions the second guanidino NH_2 of Arg144 in such a manner as to allow H-bonding with the C4 and/or the C3 OH of the galactosyl moiety of lactose and other galactosides, thereby providing substrate specificity. Disruption of the putative salt-bridge by neutral replacements for either Glu126 or Arg144 leads to complete loss of binding (*11*) and, as a result, inability to catalyze active transport or downhill substrate translocation (*19*).

While neutral replacements for Glu126 or Arg144 yield similar phenotypes, conservative substitutions result in mutants with very different properties which is consistent with the notion that the two side chains play different roles in substrate binding. Thus, R144K permease does not bind TDG (*11*) or NPG to a demonstrable extent and accumulates lactose at a very poor rate to only about 25% of wild-type levels at both low and high external lactose concentrations (*19*; see Figure 2 in addition). The mutant is also completely defective in downhill lactose translocation (*19*), efflux,

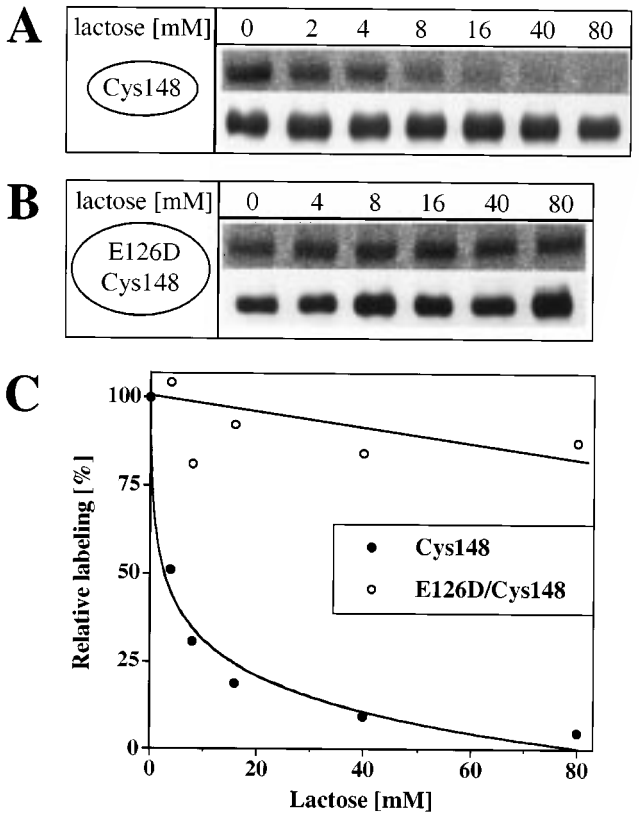

FIGURE 8: Effect of lactose on the reactivity of Cys148 in single-Cys148 and E126D/single-Cys148 permeases. Labeling experiments were carried out in the absence or presence of the indicated lactose concentrations exactly as described in the legend to Figure 7. Autoradiograms of representative labeling experiments (A and B) and the average of three experiments plotted (C) are shown.

exchange, and lactose-induced H^+ influx. In contrast, E126D permease catalyzes active lactose transport and lactose-induced H^+ influx at lower rates to wild-type levels. Interestingly, however, at a higher external lactose concentration, E126D permease achieves a steady-state level of accumulation that is more than 2-fold greater than wild-type, suggesting that efflux is relatively more impaired than influx. Indeed, efflux and exchange experiments demonstrate that E126D permease catalyzes these reactions at rates that are 70−90-fold lower than wild-type, while the K_m for active transport is increased only 6-fold with little change in V_{max}.

It is striking that wild-type permease binds TDG with almost 3 orders of magnitude higher affinity than lactose and that E126D permease exhibits a 4-fold decrease in apparent affinity for TDG and more than a 20-fold decrease in apparent affinity for lactose. While the precise mechanism for this difference is not readily apparent, the observation emphasizes the important role of the non-galactosyl moiety (e.g., lactose) and possibly the symmetry (e.g., TDG) of the dissacharide in contributing to binding affinity, although specificity is clearly directed toward the galactosyl end of the substrate (*9*, *10*). It is also possible that replacement of Glu126 with Asp elicits a subtle change in the conformation of the binding site such that lactose protection against alkylation is more affected than TDG. That is, protection of Cys148 against alkylation, an indirect assay, may no longer reflect differences in the ability of specific sugars to bind to the permease in a quantitative manner, a possibility that is currently being studied by using direct binding assays. In

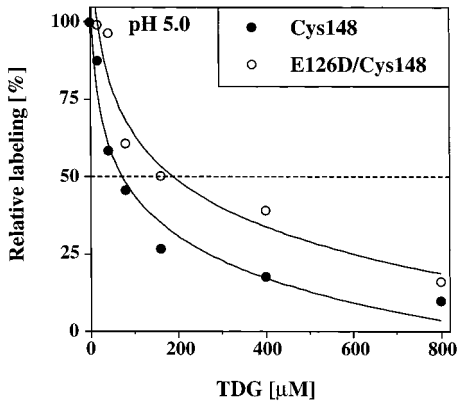

FIGURE 9: Effect of TDG on the reactivity of Cys148 in single-Cys148 and E126D/single-Cys148 permeases at pH 5.0. Labeling experiments were carried out in the absence or presence of the indicated TDG concentrations exactly as described in the legend to Figure 7. The graph shows the average of three independent experiments.

any event, the differences between the properties of E126D and R144K permease are consistent with two important aspects of the model for the binding site: (i) The primary role of Glu126 is to interact with Arg144, and this residue makes little, if any, contribution to binding interactions with the sugar. (ii) The presence of a guanidino group at position 144 is essential for binding, one NH_2 group making direct contact with the galactosyl moiety of the dissacharide and the other ion pairing with Glu126 so as to maintain the side chain in the proper position to interact with the galactosyl moiety (*7*, *11*).

Since Glu126 appears to be ion-paired with Arg144 and disruption of the interaction by neutral replacement of the carboxylate side chain clearly abolishes ligand binding and transport, it has been suggested (*19*) that protonation of Glu126 may play a role in the release of substrate from the permease. Although this may be an attractive notion, binding studies with TDG and lactose fail to exhibit a significant pH dependence of binding in single-Cys148 or E126D/single-Cys148 permease over the pH range from 4.5 to 9.5 (Figures 7 and 9; P. Venkatesan and H. R. Kaback, unpublished observations). Similarly, wild-type (*23*) or E126D exchange activity is insensitive to pH. Although the observations do not completely rule out the possibility that protonation of Glu126 may play a role in substrate release, they provide no evidence to support such a mechanism.

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