Acc. Chem. Res. **1999,** *32,* 805−813

What To Do while Awaiting Crystals of a Membrane Transport Protein and Thereafter‡

H. RONALD KABACK* AND JIANHUA WU†

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, Molecular Biology Institute, University of California—Los Angeles, Los Angeles, California 90095-1662

Received January 25, 1999

The lactose permease (lacP) of *Escherichia coli*, encoded by the *lacY* gene of the lac operon, is an important paradigm for membrane transport proteins that transduce free energy stored in electrochemical ion gradients into solute concentration gradients from *Archaea* to the mammalian central nervous system. LacP utilizes free energy released from downhill translocation of H^+ in response to an H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) to drive accumulation of galactosides against a concentration gradient (symport) with a stoichiometry of unity. In the absence of $\Delta\bar{\mu}_{H^+}$, lacP catalyzes the converse reaction, utilizing free energy released from downhill translocation of substrate to drive uphill translocation of H^+ with generation of $\Delta\bar{\mu}_{H^+}$, the polarity of which depends on the direction of the substrate concentration gradient. LacP has been solubilized and purified in a completely active state (reviewed in ref 1) and functions as a monomer.[2] The protein contains 12 α-helices that traverse the membrane in zigzag fashion, connected by relatively hydrophilic loops with both N and C termini on the cytoplasmic face (Figure 1) (reviewed in refs 3−5).

To obtain insight into this basic biological phenomenon, it is essential to identify the side chains that are "major players" in the mechanism, delineate their function and relationship to one another, and obtain structural information at least at the level of helix packing. Crystallization of membrane proteins is a major problem that accounts for the availability of only a handful of 3D structures, all of which are rigid molecules. Nonetheless, the use of molecular biological techniques to engineer lacP for site-directed biochemical and biophysical techniques has provided detailed information about structure and function. Furthermore, the methods that have been developed are being applied to many other pro- and eukaryotic membrane proteins.

Functional lacP devoid of eight native Cys residues has been constructed (C-less lacP) and used for Cys-scanning mutagenesis (reviewed in ref 5). Each residue in C-less lacP has been replaced with Cys or other residues in order to determine which functional groups play an obligatory role in the mechanism and to create a library of mutants with a single Cys residue at each position for structure/function studies. Analysis of the mutants has led to the following developments: (1) Almost all of the mutants are expressed normally in the membrane and exhibit significant activity. Only six side chains are clearly irreplaceable with respect to active transport: Glu126 (helix IV) and Arg144 (helix V), which are critical for substrate binding, and Glu269 (helix VIII), Arg302 (helix IX), His322, and Glu325 (helix X), which play essential roles in H^+ translocation and coupling. (2) Helix packing, tilts, and ligand-induced conformational changes are determined. (3) Positions accessible to solvent are identified. (4) Positions where the reactivity of the Cys replacement is increased or decreased by ligand are identified. (5) LacP is highly flexible. (6) Structural data and the properties of mutants in the irreplaceable residues have led to a working model for the transport mechanism.

Noncrystallographic Approaches to Structure. With a library of lacP mutants containing a single Cys residue at each position encoded by a cassette *lacY* gene with a unique engineered restriction site about every 100 bp (EMBL X-56095), construction of paired Cys mutants by restriction fragment replacement is a simple operation. Furthermore, incorporation of a biotin acceptor domain (see ref 6) or a His tag[7] allows rapid purification by affinity chromatography. By using the mutant library with techniques that include second-site-suppressor analysis and site-directed mutagenesis, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance (EPR), thiol cross-linking, and identification of discontinuous monoclonal antibody (mAb) epitopes, a helix packing model has been formulated (Figure 2).

A particularly powerful approach carried out in situ[8−13] involves expression of functional lacP in two contiguous,

H. Ronald Kaback received his M.D. from the Albert Einstein College of Medicine in 1962 and did research in membrane biochemistry at the National Heart Institute and the Roche Institute of Molecular Biology, where he chaired the Department of Biochemistry. He is currently an Investigator of the Howard Hughes Medical Institute, Professor of Physiology and of Microbiology & Molecular Genetics, and a Member of the Molecular Biology Institute at UCLA. Dr. Kaback is a Member of the National Academy of Science and the American Academy of Arts & Sciences and a Fellow of the American Academy of Microbiology.

Jianhua Wu received his Ph.D. in Biochemistry from Wayne State University School of Medicine in 1992. At present, he is a Senior Scientist at Genome Therapeutics Corp. in Waltham, Massachussetts.

† Present address: Genome Therapeutics Corp., 100 Beaver St., Waltham, MA 02453-8443.

‡ Abbreviations: lacP, lac permease; $\Delta\bar{\mu}_{H^+}$, the H^+ electrochemical gradient across the membrane; C-less permease, functional permease devoid of Cys residues; EPR, electron paramagnetic resonance; mAb, monoclonal antibody; split lacP, functional permease expressed in two contiguous, nonoverlapping fragments; NEM, *N*-ethylmaleimide; MTSES, methanethiosulfonate ethylsulfonate; MIANS, 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid; ATR-FTIR, attenuated total reflection Fourier transform infrared spectroscopy; D-galactopyranosyl, 1-thio-β,D-galactopyranoside; $\Delta\mu_{lac}$, the lactose concentration gradient across the membrane.

10.1021/ar970256i CCC: $18.00
Published on Web 05/26/1999

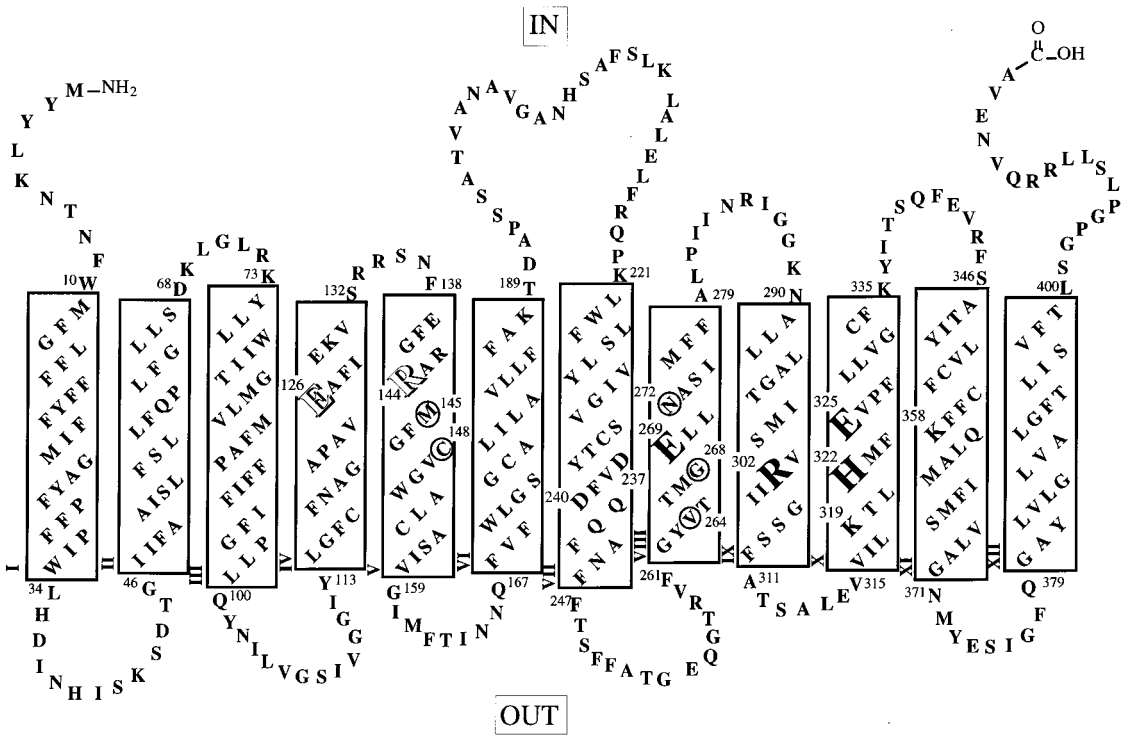

FIGURE 1. Secondary structure model of lacP. The one-letter amino acid code is used, and putative transmembrane helices are shown in boxes. Residues irreplaceable with respect to active transport are enlarged: those involved in substrate translocation are open [Glu126 (helix IV) and Arg144 (helix V)]; those involved in H^+ translocation and coupling are in black [Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X)]. Asp237 (helix VII) and Lys358 (helix XI), as well as Asp240 (helix VII) and Lys319 (helix X), which are charge paired, are slightly enlarged and numbered. Nonessential residues thought to be involved in substrate translocation [Met145 and Cys148 (helix V); Val264, Gly268, and Asn272(helix VIII)] are encircled.

nonoverlapping fragments (split lacP), each with a single Cys residue. Proximity of the paired Cys residues is then assayed on Western blots by disulfide or chemical cross-linking of the fragments. Alternatively, lacP with an engineered factor Xa protease site can be used for the same purpose.[14−17]

Scanning Accessibility of Single Cys Mutants. Single Cys mutants that are reactive with *N*-ethylmaleimide (NEM) are located primarily in helices I, II, IV, V, VIII, X, VII, and XI (Figure 3). These mutants, most of which react with the impermeant thiol reagent, methanethiosulfonate ethylsulfonate (MTSES),[18] cluster on one face of a helix, and the positions appear to line a path within the permease. The solvent-accessible crevice probably corresponds to the notch observed in 2D crystals of lacP.[19]

Substrate Effects on Alkylation. To elucidate the role of helical interfaces where single Cys replacement mutants are inactivated by NEM, the effect of substrate on inactivation of transport, as well as the reactivity of the mutants with NEM in situ, is studied (reviewed in ref 5). While the reactivity of many mutants increases in the presence of ligand, indicating widespread conformational changes, only a few mutants are protected, and they cluster on adjacent faces of helices V (Cys148, M145C) and VIII (V264C, G268C, N272C) (Figures 1 and 3). Native Cys148 is completely protected against alkylation, interacts weakly and hydrophobically with the galactosyl moiety of substrate,[20,21] and is accessible to solvent from both sides of the membrane.[22,23] Met145 interacts even more weakly with the non-galactosyl moiety of certain substrates. Cys replacements at positions 264, 268, or 272, which are on one face of helix VIII, exhibit transport activity that is inactivated by NEM, and both inactivation and reactivity with NEM are blocked partially by ligand.[24] In contrast, a Cys in place of Thr265 on the same face of helix VIII exhibits a dramatic ligand-induced increase in NEM reactivity, and the increase is nullified by MTSES. Therefore, the substrate-induced decrease in NEM reactivity exhibited by V264C, G268C, or N272C lacP probably reflects a conformational change, rather than direct interaction of the side chains with substrate. In any case, all five residues play a relatively minor role.

Glu126 and Arg 144. Glu126 (helix IV) and Arg144 (helix V) are irreplaceable residues placed initially in the cytoplasmic loop between helices IV and V (see ref 5). However, studies utilizing single amino acid deletions,[43] as well as nitroxide-scanning EPR and accessibility measurements (Zhao, M.; Zen, K.-C.; Hubbell, W. L.; Kaback, H. R., manuscript in preparation), indicate that both residues are within helices IV and V, respectively, rather than in the loop (Figure 1). Furthermore, E126H/R144H permease binds Mn^{2+} with a K_d of about 40 μM at pH 7.5, and the appropriately labeled double Cys mutant exhibits spin−spin interactions and excimer fluorescence.[44] Thus, positions 126 and 144 are in close proximity. Replacement of Glu126 or Arg144 with neutral amino acids completely abolishes transport, and activity is not observed with double neutral substitutions or when the

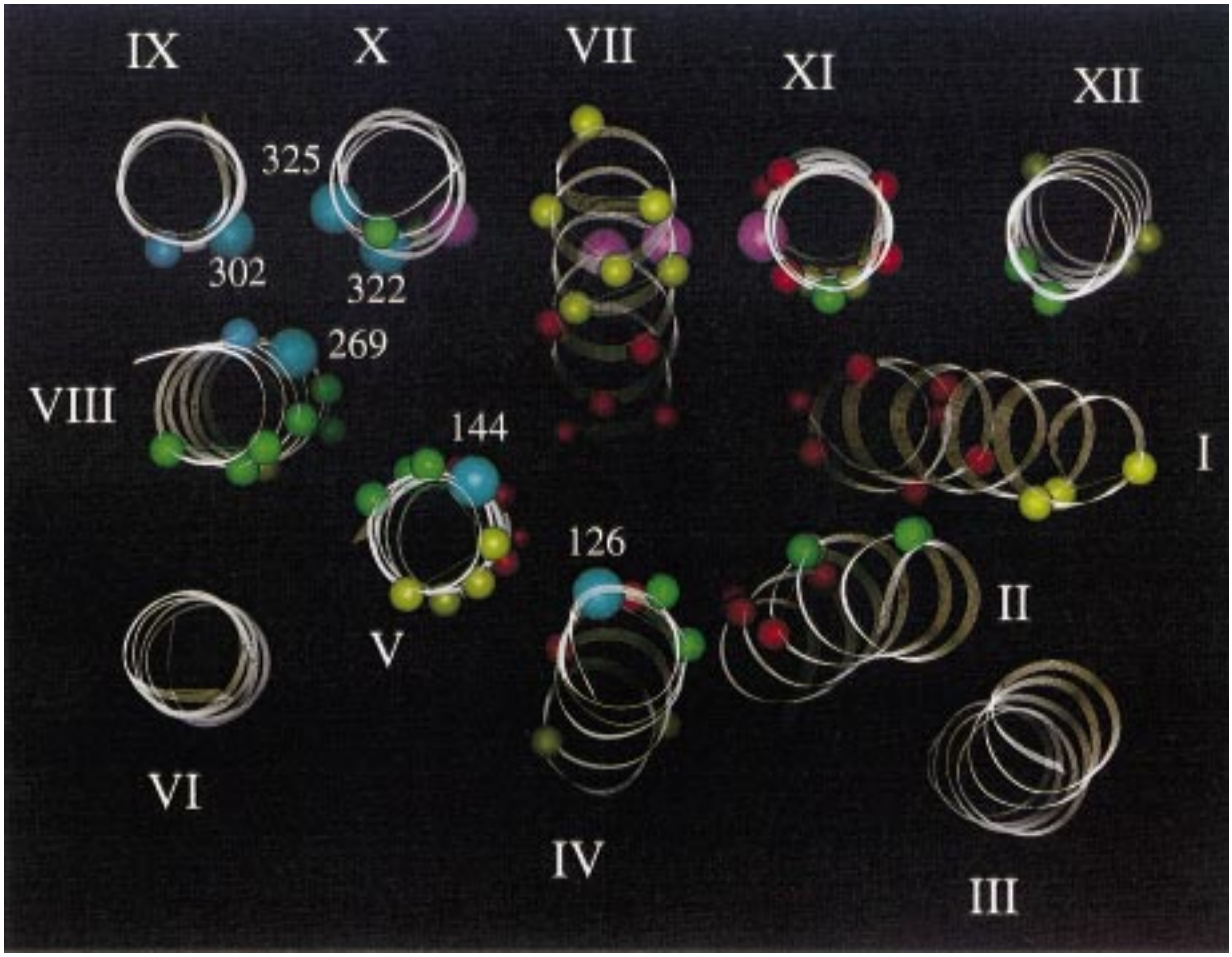

FIGURE 2. Helix packing of lacP viewed from the cytoplasmic face. The positions of the six irreplaceable residues [Glu126 (helix IV), Arg144 (helix V), Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X)] are enlarged and in blue. The smaller blue balls in helices VIII and IX at positions 273 and 299, respectively, are positions where appropriately labeled double Cys mutants exhibit excimer fluorescence and spin−spin interaction. Positions of the two interacting pairs of Asp-Lys residues [Asp237 (helix VII)/Lys358 (helix XI) and Asp240 (helix VII)/Lys319 (helix X)] are enlarged and in pink. Positions where Cys replacements exhibit cross-linking in the periplasmic half of the transmembrane helices are in red, those in the cytoplasmic half are green (except for helices V and VIII, which cross-link throughout their lengths), and those that do not cross-link are in yellow. Helices III, VI, IX, and X have yet to be examined for cross-linking.

residues are interchanged.[25] The only mutants that exhibit significant activity are E126D, which exhibits wild-type steady-state levels of accumulation but a K_m that is about 6-fold higher than that of the wild-type with a similar V_{max}, and R144K, which transports lactose at a negligible rate to only about 25% of the wild-type steady state.[26] In addition, lactose-induced H^+ translocation is observed at a slow rate with E126D permease, but not with any of the other Glu126 or Arg144 mutants.

Glu126 and Arg144 are charge-paired and appear to play a direct role in substrate binding.[27] Replacement of either residue with Ala in lacP containing a single native Cys residue at position 148 markedly decreases reactivity of Cys148 with NEM, but the double Ala mutant exhibits normal reactivity. Thus, an unpaired charge decreases reactivity of Cys148 just as neutral replacement of one partner in either the Asp237 (helix VII)−Lys358 (helix XI) or Asp240 (helix VII)−Lys319 (helix X) charge pair abolishes accumulation, while the double neutral replacement mutants catalyze active transport (reviewed in refs 3−5). Consistently, normal reactivity is observed when Glu126 and Arg144 are interchanged or when Arg144 is replaced with Lys. Strikingly, however, no substrate protection against NEM is observed with any of the mutants, a finding that is particularly salient with respect to mutant R144K/single Cys148.

Site-directed fluorescence with 2-(4-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS) in mutant V331C (helix X) was also studied.[27] In contrast to V331C lacP, ligand does not alter MIANS reactivity in mutant E126A/V331C, R144A/V331C, or R144K/V331C and does not cause quenching or a shift in emission maximum of the MIANS-labeled mutants. However, mutations E126A and R144A and, to a lesser extent, R144K cause a red shift in the emission spectrum and render the fluorophore more accessible to iodide. The results demonstrate that Glu126 and Arg144 are indispensable for substrate binding and suggest a model for the substrate binding site (Figure 4). In addition, alterations in the substrate translocation

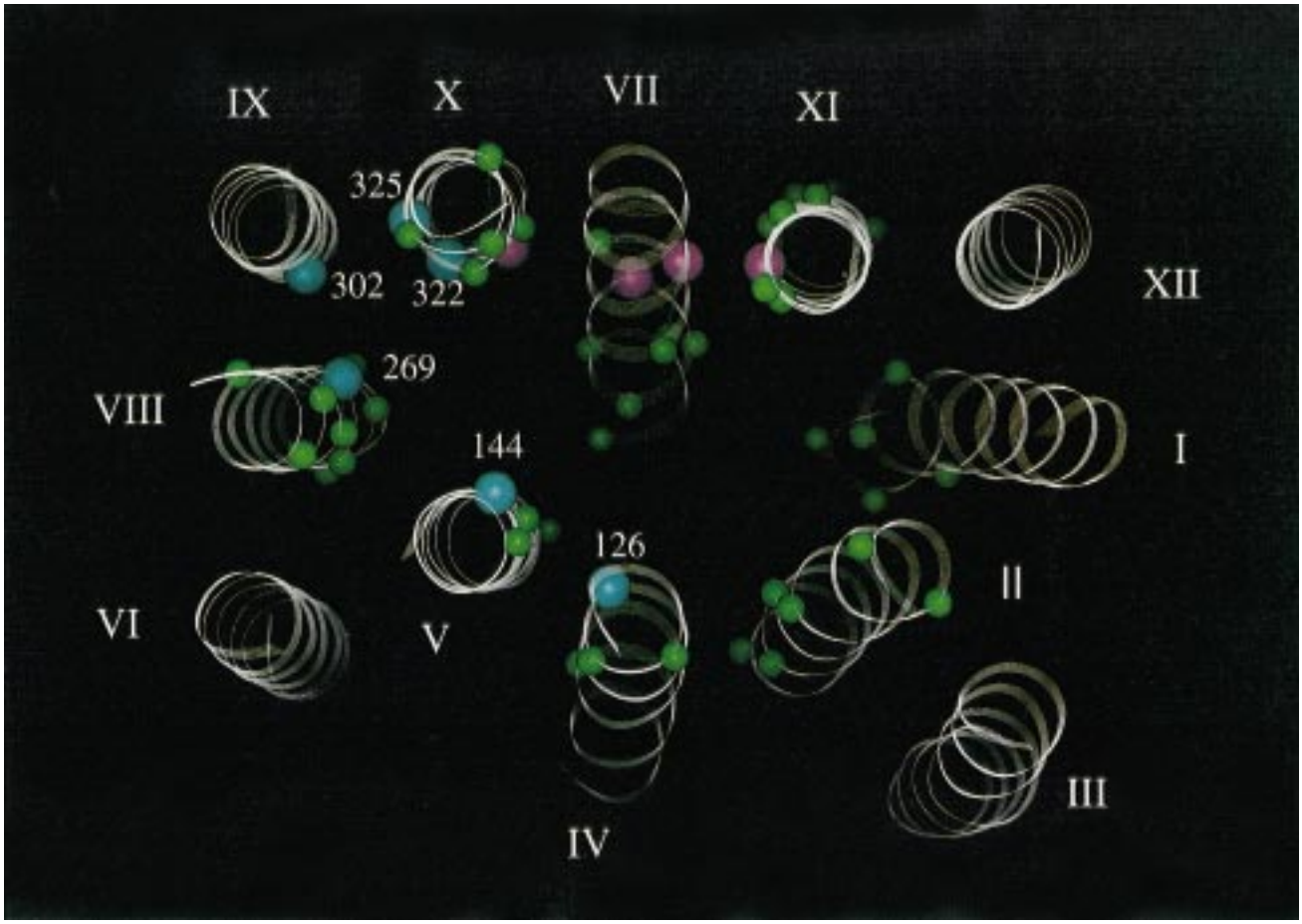

FIGURE 3. Helix packing model of lacP viewed from the cytoplasmic face showing positions of NEM reactive single Cys replacement mutants. Irreplaceable residues are enlarged and in blue. Positions of the two interacting pairs of Asp-Lys residues are enlarged and in pink. Positions where Cys replacement mutants react with NEM are in green.

pathway at the interface between helices IV and V are transmitted to the H^+ translocation pathway between helices IX and X (see below).

Finally, direct binding assays demonstrate that mutants E126A, R144A, and R144K do not bind the high-affinity analogue *p*-nitrophenyl-α,D-galactopyranoside.[26]

Dynamics and Flexibility. NEM reactivity of single Cys residues in many transmembrane domains is altered in the presence of ligand, implying that transport involves widespread changes in tertiary structure (see refs 3−5). More specifically, ligand increases the distance between position 245 (helix VII) and positions 52 and 53 (helix II) equally by up to 4 Å.[9] Moreover, proximity relationships between periplasmic loops, as well as cytoplasmic loops, are altered by ligand. Most importantly, ligand induces changes in the tilt of certain helices.[9−13]

The notion that lacP, a 12-helix bundle without prosthetic groups or metals, is highly flexible and metastable may partially explain the difficulty in crystallizing this protein. At a lipid:protein ratio of 100:1 (mol/mol), the average helix tilt of lacP relative to the bilayer normal is 51°, as determined by polarized attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR).[28] However, the average tilt angle decreases to about 33° maximum as the lipid:protein ratio is increased to about 800:1 in a manner that correlates with increases in activity and in the lipid order parameter.

ATR-FTIR spectroscopy also demonstrates that lacP exhibits unusually fast H/D exchange to 90−95% completion.[28,29] Accessibility of the backbone to water, which is also observed with the glucose transporter GLUT1,[30] is consistent with the ability of intramembrane bis-His residues to bind divalent metal with a pK_a similar to that of unperturbed imidazole (see refs 3−5). Other membrane proteins such as EmrE, a multidrug transporter for hydrophobic substrates,[31] bacteriorhodopsin, a light-driven H^+ pump,[32] or the prokaryotic K^+ channel SliK,[29] exhibit much lower rates and extents of amide H/D exchange.

Turnover Begins with Protonated Glu325. Single Cys148 lacP is completely protected by 10 mM D-galactopyranosyl 1-thio-β,D-galactopyranoside (TDG) against NEM labeling at neutral or alkaline pH.[22] With Asp in place of Glu325, a marked decrease in protection is observed at high pH relative to the wild-type or mutant E325A (Venkatesan, P.; Kaback, H. R., unpublished observations). A reasonable explanation is that the pK_a of a Glu at position 325 is high (>pH 10) due to the low dielectric of the phospholipid bilayer in which the carboxylic acid is embedded. With Asp325, the carboxylic acid

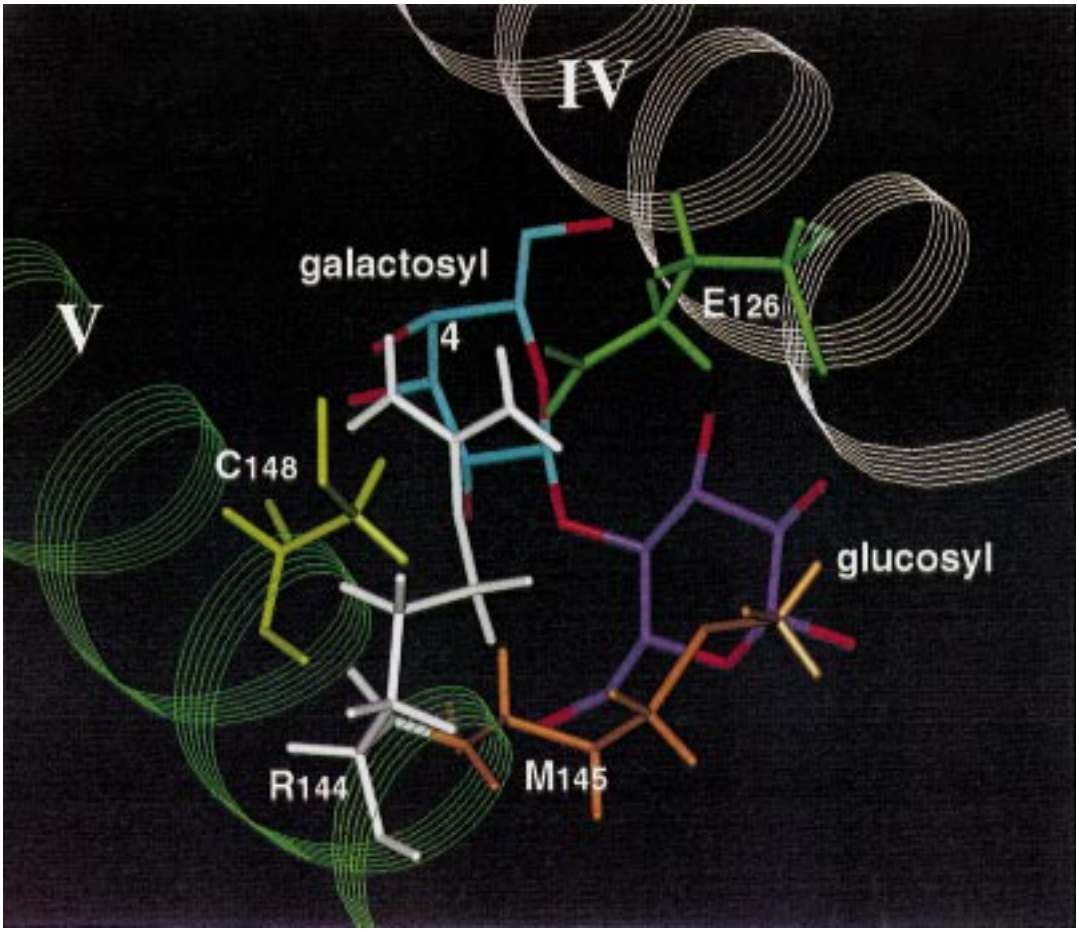

FIGURE 4. Putative binding site interactions in *E. coli* lacP. Helices IV (white) and V (green) are shown with Glu126 (green), Arg144 (white), Met145 (orange), and Cys148 (yellow). The galactosyl and glucosyl moieties of lactose are in light and dark blue, respectively. As indicated, one of the guanidino N atoms of Arg144 interacts with the hydroxyl group at the 4 position of the galactosyl pyranose ring, and the other guanidino N atom interacts with Glu126. Cys148 interacts weakly and hydrophobically with the galactosyl moiety, and Met145 interacts even more weakly with the glucosyl moiety.

is less accessible to the low dielectric, which lowers the pK_a. Therefore, at neutral pH, Asp325 is protonated, and lacP binds ligand with high affinity, but at high pH, Asp325 is negatively charged which markedly decreases affinity.

Glu269, Arg302, His322, and Glu325 Are Irreplaceable for H^+ Translocation and Coupling. Mutants in Arg302, His322, and Glu325 are unable to catalyze active transport but exhibit downhill lactose influx without H^+. Because individual steps in the translocation cycle cannot be delineated by studying active transport, lacP-mediated efflux down a chemical gradient, equilibrium exchange, and entrance counterflow are used to probe the mechanism (Figure 5). Efflux, exchange, and counterflow with wild-type permease are explained by a simple kinetic scheme (Figure 6). Efflux consists of six steps: (1) protonation of lacP; (2) binding of lactose at the inner surface of the membrane; (3) a conformational change that results in translocation of lactose and H^+ to the outer surface; (4) release of substrate; (5) release of H^+; and (6) a conformational change corresponding to return of the binding site in unloaded lacP to the inner surface. Exchange and counterflow, which occur without H^+ translocation, involve steps 2−4 only.

Exchange and counterflow are blocked in His322 and Arg302 mutants, although the mutants catalyze lactose influx down a concentration gradient. Except for E269D, which catalyzes TDG accumulation with increased H^+:TDG stoichiometry, Glu269 mutants are completely defective in all translocation reactions with lactose.[33,34] In contrast, Glu325 mutants are specifically defective in all steps involving H^+ translocation but catalyze exchange and counterflow as well as or better than wild-type. Glu325 appears to be the only residue in lacP directly involved in H^+ translocation (see below) and is required for release of H^+ (step 5).

Importantly, the effects of Glu325 mutations are mimicked by D_2O[35] or mAb 4B1,[36] and substrate binding appears to be unaffected. Replacement of Arg302 with Lys or His322 with Arg yields permease that does not catalyze active transport, exchange, or counterflow. Therefore, it is unlikely that disruption of charge pairs between the irreplaceable residues per se leads fortuitously to the properties described.

E325D lacP exhibits about 20% of wild-type active transport and is grossly defective with respect to efflux down a concentration gradient. Remarkably, exchange of

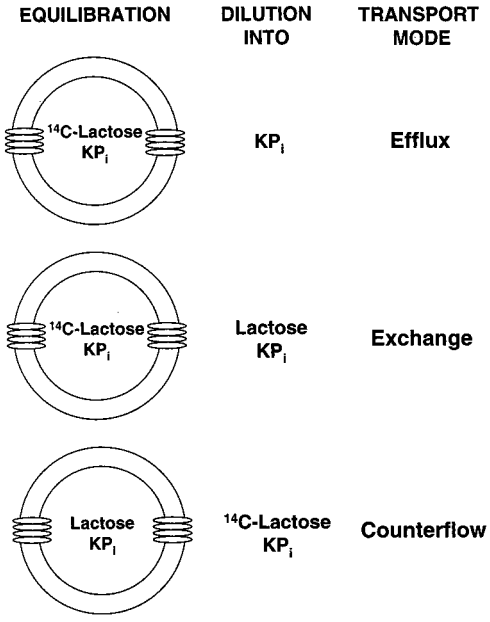

FIGURE 5. Efflux, exchange, and counterflow. To study efflux or equilibrium exchange, concentrated suspensions of right-side-out membrane vesicles are equilibrated passively with radioactive lactose at a final concentration of 10 mM. Aliquots are then diluted 200-fold or more into buffer without (efflux) or with 10 mM cold lactose (equilibrium exchange). For counterflow experiments, concentrated suspensions are equlibrated passively with cold lactose at a final of 10 mM, and aliquots are diluted 200-fold or more into buffer containing radioactive lactose at a final concentration of 0.4 mM. Initially, internal cold lactose exchanges rapidly with external radioactive lactose until all of the internal cold is exchanged, at which point the reaction ceases, the gradients dissipate, and the signal returns to baseline. During each of these operations, care is taken to collapse any $\Delta\bar{\mu}_{H^+}$ that may be generated.

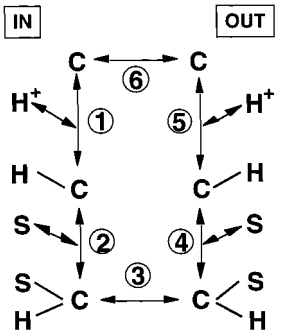

FIGURE 6. Schematic representation of reactions involved in lactose efflux, exchange and counterflow. C represents lacP; S is substrate (lactose).

lactose across the membrane is pH dependent: below pH 7.5, exchange is rapid and the rate is comparable to that of wild-type; above pH 7.5, the rate decreases and is nil at pH 9.5 with a midpoint at about 8.5; and inhibition at alkaline pH is completely reversible.[37] In contrast, wild-type exchange is only mildly inhibited above pH 9.5, and exchange by mutant E325A is comparable to that of wild-type and unaffected by pH. Moreover, mAb 4B1, which binds to an epitope (Phe247, Phe250 and Gly254) in the periplasmic loop VII/VIII,[38] causes a shift in the midpoint for exchange by E325D permease to pH 7.5.[37] Also,

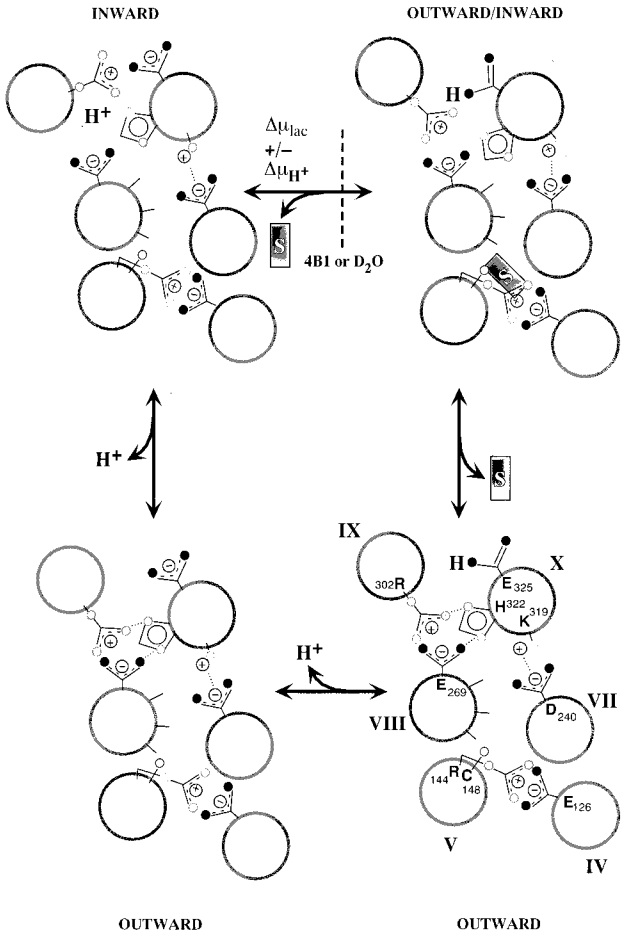

FIGURE 7. Proposed mechanism for lacP. See text for discussion.

oxidation of a Cys residue at position 325 probably to the sulfinic acid, which is isosteric with Asp but 2 pH units more acidic, causes the midpoint for exchange to shift to pH 6.5.[39] Thus, a negative charge at position 325 seems to be prohibitive for substrate binding and exchange, even though substrate does not interact with this residue.

Although none of the Glu269, Arg302, or His322 mutants catalyze active transport, evidence has been presented (see refs 3−5), indicating that these residues are not directly involved in H^+ translocation but in the coupling between substrate and H^+ translocation.

A Mechanism for Lactose/H$^+$ Symport. The mechanism postulated is based upon four conditions: (1) in the absence of substrate, lacP does not catalyze significant H^+ translocation; (2) substrate concentration gradients generate $\Delta\bar{\mu}_{H^+}$, the polarity of which depends on the direction of the concentration gradient; (3) Glu325 is the key and possibly the sole residue directly involved in H^+ translocation; and (4) the catalytic cycle must start with protonated Glu325.

Six helices are thought to play a central role (Figure 7). Critical for substrate binding are Arg144, one turn above Cys148 in helix V, and Glu126 in helix IV. Cys148, which makes direct contact with the galactosyl moiety of the substrate, is on the same face as Met145, and on the adjoining face of helix VIII are Val264, Gly268, and Asn272, where the reactivity of single Cys replacements with NEM

is decreased by ligand, and Thr265, where Cys reactivity is enhanced due to increased solvent accessibility. Thus, the interface between helices IV and V plays a major role in substrate recognition and translocation, but it is likely that the face of helix VIII with Asn272, Gly268, Thr265, and Val264 is also important for substrate translocation, possibly by coupling conformational changes at the interface between helices IV and V to the interface between helices IX and X, and vice versa. Helices VIII, IX, and X contain Glu269, Arg302, and His322, respectively, which are postulated to interact, forming a stable, neutral triad. In this configuration, Glu325 in helix X faces the low dielectric interior of the membrane and must be protonated. The H^+ might also be shared between Glu325 and His322, acting to increase the pK_a even further, with Arg302 paired with Glu269. As a result of these relationships, substrate-induced structural changes at the interfaces between helices IV, V, and VIII are transmitted through the network of interacting residues to the interfaces between helices VIII, IX, and X. Conversely, changes between helices VIII, IX, and X are transmitted to the interfaces between helices IV, V, and VIII.

The postulated mechanism is as follows (Figure 7): (1) In the "outward-facing" conformation (lower right), Glu325 (helix X) is protonated and Glu269 (helix VIII), Arg302 (helix IX), and His322 (helix X) form an electroneutral triad. (2) Ligand binding at the interface between helices IV and V induces a conformational change, disrupting the triad (upper right). With saturating substrate concentrations at both surfaces of the membrane, the protonated form of Glu325 is stabilized, and lacP can oscillate between outward- and inward-facing conformations, thereby catalyzing exchange and counterflow with no H^+ translocation. Moreover, mAb 4B1 stabilizes lacP in this configuration, as does D_2O or neutral amino acid replacements for Glu325. (3) In the presence of a substrate concentration gradient ($\Delta\mu_{lac}$) with or without $\Delta\bar{\mu}_{H^+}$, dissociation of substrate leads to a marked decrease in the pK_a of Glu325 and ultimately its deprotonation by bringing the carboxylic acid into proximity with Arg302 (upper left). Since bis-His residues in this region of the permease form divalent metal binding sites with apparent pK_as that approximate an unperturbed imidazole, it seems likely that there is a water-filled crevice between helices VIII, IX, and X. Thus, when H^+ is released from Glu325 between helices IX and X, it can be acted upon equally by either the electrical potential or the pH gradient across the membrane, particularly if the changes postulated are accompanied by appropriate changes in helix tilt, resulting in the opening and closing of crevices on respective sides of the membrane with transient accessibility to both sides. The order of release is always sugar first and H^+ second. However, in the presence of $\Delta\mu_{lac}$, deprotonation of Glu325 is rate-limiting, while in the presence of $\Delta\bar{\mu}_{H^+}$, dissociation of sugar is limiting (see ref 35). In any case, the stable, uncharged triad between Glu269, His322, and Arg302 is re-formed in the outward-facing conformation, but Glu325 is negatively charged and in contact with the low dielectric of the membrane, which is thermodynamically unfavorable (lower left). (4) Glu325 is reprotonated, substrate is again able to bind, and the cycle can be repeated.

In addition to providing a rationale for coupling substrate and H^+ translocation and an explanation for why the electrical potential and the pH gradient across the membrane have the same mechanistic and thermodynamic effects, the model is consistent with many observations, some of which are listed here: (i) Although Asp237 (helix VII) and Lys358 (helix XI) can be reversed without adversely affecting permease activity, reversal of Asp240 and Lys319 inactivates.[40] According to the postulated mechanism, placement of a carboxylate at position 319 competes with Glu269 for His322. On the other hand, reversal of Asp237 (helix VII) and Lys358 (helix XI) has no effect on activity[41] because neither residue is sufficiently close to the residues directly involved to influence H^+ translocation or coupling. (ii) According to the proposed mechanism, Arg302, His322, and Glu269 are not directly involved in H^+ translocation, but Arg302 is important for decreasing the pK_a of protonated Glu325, while Glu269 and His322 play important roles in the coupled structural changes postulated. In addition, Glu269 and His322 lie close to the interface between helices IV and V, where substrate binding occurs and may be important for stabilizing the interface.[42] (iii) Mutant E325D is partially uncoupled, and exchange is pH dependent. Also, a negative charge at position 325 may prohibit substrate binding. In wild-type lacP, the pK_a of Glu325 may be perturbed to very alkaline pH because the carboxylic acid is exposed to a low dielectric. With Asp at 325, the side chain may be less accessible to the hydrophobic phase of the membrane and more accessible to water, which decreases the pK_a relative to that of Glu325. By stabilizing lacP in a conformation in which Glu325 is protonated, mAb 4B1 arrests the molecule in a form that catalyzes exchange and counterflow, thereby blocking reactions that involve H^+ translocation. Furthermore, with Asp at 325, the carboxylic acid is more accessible to water relative to the wild-type Glu side chain. Thus, mAb 4B1 may decrease the apparent pK_a of Asp325, as measured by exchange, but the pK_a of Glu325 would not be perturbed because the side chain protrudes further into the hydrophobic phase of the membrane. (iv) A Cys residue in place of Thr265 (helix VIII) undergoes a marked increase in solvent accessibility upon ligand binding,[24] indicating that substrate binding induces a conformational change in helix VIII.

How is substrate released from the permease? Since Glu126 is charge-paired with Arg144 and disruption of the interaction by neutral replacement of Glu126 clearly abolishes ligand binding and transport, protonation of Glu126 may play a role in the release of substrate from the permease. However, binding studies with TDG and lactose fail to exhibit a significant pH dependence of binding in single Cys148 or E126D/single Cys148 permease from pH 4.5 to 9.5.[26] The observations do not completely rule out the notion, but they clearly do not support it. On the other hand, inspection of Figure 2 suggests that Arg144 may be close enough to Glu269 to interact at some point during turnover. Thus, ligand binding might destabilize

the Arg144−Glu126 interaction sufficiently to allow Glu269 to compete with Glu126 for Arg144, thereby leading to release of ligand. Experiments are presently in progress to determine whether Cys or His side chains at positions 269 and 144 are sufficiently close to interact.

This work was supported in part by NIH Grant DK51131. We thank Qingda Wang for help with computer modeling and Kerstin Stempel for preparing the figures.

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AR970256I