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Use of Site-Directed Mutagenesis To Study the Mechanism of a Membrane Transport Protein

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Lac permease of *Escherichia coli* is a hydrophobic transmembrane protein encoded by the *lac Y* gene that catalyzes coupled translocation of β-galactosides with H^+ (i.e., H^+/substrate symport) [cf. Kaback (1986a,b) and Wright et al. (1986)]. Thus, when a H^+ electrochemical gradient ($\Delta\bar{\mu}_{H^+}$)[1] is generated across the cytoplasmic membrane (interior negative and alkaline), the permease utilizes free energy released from downhill translocation of H^+ in response to $\Delta\bar{\mu}_{H^+}$ to drive uphill accumulation of β-galactosides against a concentration gradient (Figure 1A). Conversely, when a concentration gradient of substrate is created in the absence of $\Delta\bar{\mu}_{H^+}$, the permease utilizes free energy released from downhill translocation of substrate to drive H^+ uphill with generation of $\Delta\bar{\mu}_{H^+}$, the polarity of which depends on the direction of the substrate concentration gradient (Figure 1B,C). As such, *lac* permease is a model for a wide range of biological machines that transduce the free energy stored in an electrochemical ion gradient into work in the form of a concentration gradient. Therefore, experimental approaches used to study the permease are applicable to a variety of other membrane proteins.

The β-galactoside transport system in *E. coli* was first described in 1955 [cf. Cohen and Monod (1957)] and is part of the *lac* operon. In addition to regulatory loci, the *lac* operon contains three structural genes: (a) *Z*, encoding β-galactosidase, a cytosolic enzyme that cleaves lactose upon entry into the cell; (b) *Y*, encoding *lac* permease; and (c) *A*, encoding thiogalactoside transacetylase, an enzyme of unknown physiological function that catalyzes acetylation of thio-β-galactosides.

Lac Y has been cloned and sequenced, and the permease has been purified to a single polypeptide species, reconstituted into proteoliposomes, and shown to be completely functional, thereby demonstrating that the *lac Y* gene product is solely responsible for β-galactoside transport. Secondary structure models for *lac* permease suggest that the polypeptide is organized into 12–14 hydrophobic α-helical segments that traverse the membrane in a zigzag manner, connected by more hydrophilic, charged segments [Figure 2; cf. Kaback (1986a,b)]. Evidence supporting certain general aspects of these models has been obtained from circular dichroic and laser Raman spectroscopy, from proteolysis studies, and from binding studies with monoclonal and site-directed polyclonal antibodies. In view of the subsequent discussion, however, it is clear that a three-dimensional crystal structure is required.

Although chemical modification of amino acid residues in proteins can provide important information, there are drawbacks to the technique. Thus, site-directed mutagenesis has been used to introduce amino acid changes into proteins (Zoller & Smith, 1983), and recently, this approach has been applied to *lac* permease (Sarkar et al., 1986a). The provocative implication of the studies is that important insight can be obtained at the level of individual amino acid residues despite lack of a high-resolution structure.

Site-Directed Mutagenesis of Cys Residues in Lac Permease. On the basis of substrate protection against *N*-ethylmaleimide (NEM) inactivation, Fox and Kennedy (1965) postulated an essential SH group in the permease located at or near the active site, and Cys-148 was shown to be the critical residue (Beyreuther et al., 1981). Trumble et al. (1984) and Viitanen et al. (1985) cloned *lac Y* into single-stranded M13 phage DNA and, using a synthetic deoxyoligonucleotide primer, converted Cys-148 in the permease into Gly. Cells bearing mutated *lac Y* transport lactose at about one-fourth the rate of cells bearing the wild-type gene (Table I), but accumulate lactose to normal steady states. Transport is less sensitive to NEM, although complete inactivation is observed eventually, and galactosyl 1-thio-β-D-galactopyranoside (TDG) affords no protection. Furthermore, permease with Ser in place of Cys-148 (Neuhaus et al., 1985; Sarkar et al., 1986b) catalyzes transport as well as wild-type permease (Table I) and exhibits the same properties as Gly-148 permease with respect to NEM inactivation and TDG protection. Thus, although Cys-148 is important for substrate protection against NEM

[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; NEM, *N*-ethylmaleimide; TDG, β-D-galactosyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; DEPC, diethyl pyrocarbonate; TMG, methyl 1-thio-β-D-galactopyranoside.

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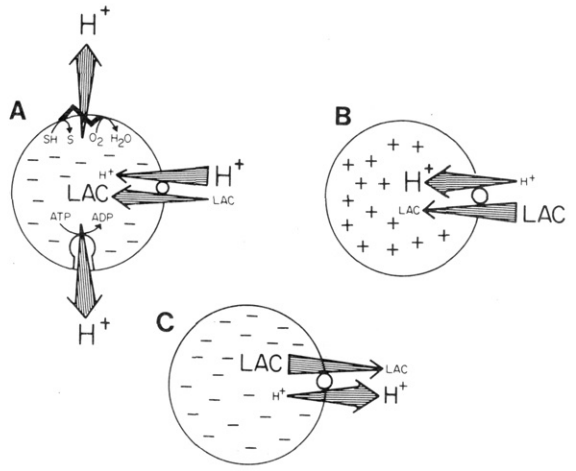

FIGURE 1: Lactose transport in *E. coli*. (A) Uphill lactose transport in response to $\Delta\bar{\mu}_{H^+}$ (interior negative and alkaline) generated either by respiration or by ATP hydrolysis: (heavy jagged line) membrane-bound respiratory chain with alternating H^+ and electron carriers; (keyhole) the H^+-ATPase; (O) the *lac* permease. (B) Uphill H^+ transport in response to an inwardly directed lactose gradient. (C) Uphill H^+ transport in response to an outwardly directed lactose gradient.

inactivation, it is not obligatory for lactose/H^+ symport, and another SH is required for activity.

Strikingly, site-directed mutagenesis of Cys-154 shows that an SH at this position is important (Menick et al., 1985; cf. Table I). Permease with Gly in place of Cys-154 exhibits no activity, while substitution with Ser also causes marked, though less complete, loss of activity. On the other hand, permease with either Gly-154 or Ser-154 binds the high-affinity ligand *p*-nitrophenyl α-D-galactopyranoside (NPG) normally. Recently, Brooker and Wilson (1986) have replaced Cys-176 or Cys-234 with Ser, and Menick et al. (1987) have replaced Cys-117, Cys-333, or Cys-353 and Cys-355 with Ser with less than 50% loss in activity (Table I). Overall, the results indicate that, of eight Cys residues in the permease, only Cys-154 is important.

In view of the attention paid to the importance of SH groups in the permease over the past 20 years, the conclusion is particularly interesting. In addition to the postulated essentiality of an SH group at or near the binding site, other hypotheses implicating Cys residues in permease function have been put forward. Specifically, it has been suggested that the permease might undergo SH/SS interconversion during turnover, either as a respiratory intermediate (Kaback & Barnes, 1971) or as a H^+ carrier in equilibrium with $\Delta\bar{\mu}_{H^+}$ (Konings & Robillard, 1982). In this context, results from site-directed mutagenesis of Cys residues place severe restrictions on any theory that invokes SS formation as part of the catalytic mechanism. Since Cys-154 alone appears to be important for activity, any postulated SS formation must occur between two permease monomers. Although evidence has been presented that is consistent with the notion that permease may dimerize in the presence of $\Delta\bar{\mu}_{H^+}$, it is indirect [cf. Menick et al. (1987)]. Furthermore, although Ser-154 permease is defective, it catalyzes accumulation at about 10% the rate of the wild-type molecule. On the basis of these considerations, it seems highly unlikely that SH/SS interconversion plays a role in the transport mechanism.

Observations demonstrating that inactivation of the permease by maleimides is enhanced by $\Delta\bar{\mu}_{H^+}$ (Cohn et al., 1981) and the finding that this property is retained when Cys-148

Table I: Summary of Transport Activities in *Lac Y* Cysteine Mutants[a]

Cys residue	substitution	initial rate of transport (% wild type)	reference
117	Ser	70	Menick et al., 1987
148	Gly	25	Trumble et al., 1984 Viitanen et al., 1985
148	Ser	100	Sarkar et al., 1986b
154	Gly	0	Menick et al., 1985
154	Ser	10	Menick et al., 1985
154	Val	30	unpublished information
176	Ser	80	Brooker & Wilson, 1986
234	Ser	70	Brooker & Wilson, 1986
333	Ser	100	Menick et al., 1987
353, 355	Ser	≥50	Menick et al., 1987

[a] Apparent initial rate of lactose transport in *E. coli* T184 transformed with the appropriate plasmid.

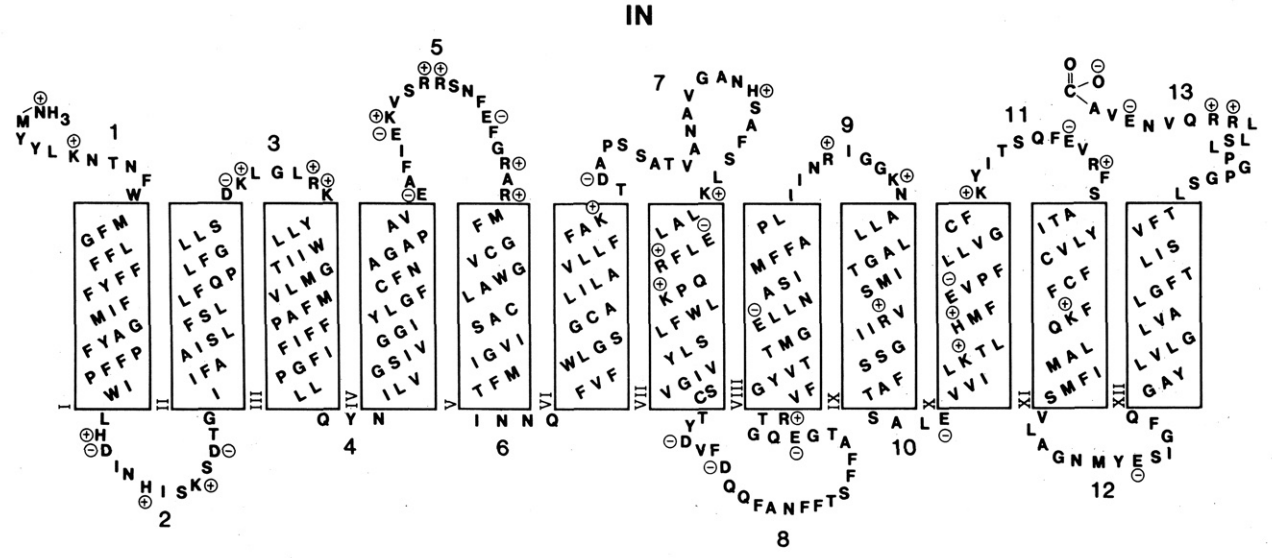

FIGURE 2: Secondary structure model of *lac* permease based on the hydropathy profile of the protein. Hydrophobic segments are shown in boxes as transmembrane, α-helical domains connected by hydrophilic segments. The carboxyl terminus and hydrophilic segments 5 and 7 (with the amino terminus as hydrophilic segment 1) have been shown to be on the cytoplasmic surface of the membrane [cf. Kaback (1986a,b)].

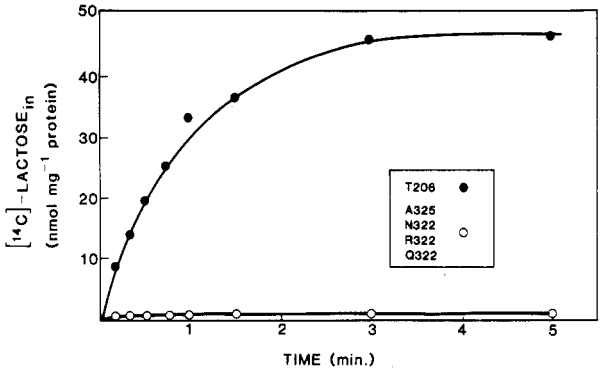

FIGURE 3: Lactose transport in *E. coli* T184 transformed with the plasmid pGM21 encoding the wild-type *lac* permease (strain T206) or plasmids encoding mutated *lac* permeases with given amino acid replacements (A, Ala; N, Asn; R, Arg; Q, Gln). The experimental data are taken from Püttner et al. (1986) and Carrasco et al. (1986).

is replaced with Gly (Viitanen et al., 1985) are also noteworthy. Since Cys-154 is the only Cys in the permease that is essential, the observations suggest that Cys-154 is the residue that exhibits enhanced reactivity in the presence of $\Delta\bar{\mu}_{H^+}$. The behavior of the permease in this respect suggests that $\Delta\bar{\mu}_{H^+}$ increases the nucleophilic character of Cys-154 and that this residue might be involved in H^+ translocation. The following considerations tend to exclude this notion. Permease with Ser in place of Cys-154 catalyzes lactose accumulation, albeit at 10% the rate of the wild type. Since Ser is similar to Cys in that the OH function might replace the SH to an extent, Cys-154 was replaced with Val [cf. Menick et al. (1987)]. Permease with Val-154 catalyzes transport about 3 times faster than permease with Ser in place of Cys-154 (cf. Table I). Therefore, although Cys-154 is the only Cys residue in the permease whose replacement leads to dramatic loss of activity, it is probably not directly involved in lactose/H^+ symport.

Role of His-322 in Lactose/H^+ Symport. Chemical modification with diethyl pyrocarbonate (DEPC) and rose bengal provided initial evidence that His residues play an important role in coupling H^+ and lactose translocation [cf. Kaback (1986a,b)]. Subsequently, each of the four His residues in *lac* permease was replaced with Arg by site-directed mutagenesis (Padan et al., 1985). Replacement of His-35 and His-39 with Arg has no effect on activity. In contrast, replacement of either His-205 or His-322 (cf. Figure 3) with Arg causes severe loss of activity, although the cell membrane contains a normal complement of permease molecules. While substitution of His-205 or His-322 with Arg results in loss of lactose/H^+ symport, permease molecules with Arg at residue 322 facilitate downhill lactose influx at high substrate concentrations. Furthermore, permease with Arg-322 that was purified and reconstituted into proteoliposomes catalyzes facilitated diffusion at about 50% the rate of wild-type permease without concomitant H^+ translocation; purified permease with Arg-205 has no transport activity.

Recently, Lowe et al. (1985) showed that the side chain of Asn may be superimposed on His in such a manner that the amide N of Asn occupies the same position as N1 in His, while the amide N of Gln may be superimposed on N3. By use of site-directed mutagenesis to replace His-48 with Lys, Asn, or Gln in tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*, it was demonstrated that enzyme with Asn in place of His-48 retains normal activity, while enzymes with Lys or Gln are markedly defective. Thus, it was concluded that N1 of His-48 hydrogen bonds with ATP.

In view of these observations, Püttner et al. (1986) replaced His-205 or His-322 in the permease with either Gln or Asn.

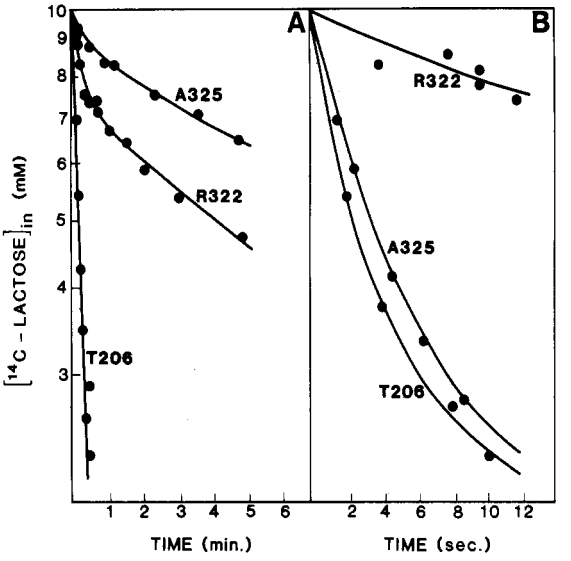

FIGURE 4: Lactose efflux (A) and exchange (B) in right-side-out membrane vesicles from *E. coli* T206 (which contains wild-type permease), R322 (which contains permease with Arg in place of His-322), and A325 (which contains permease with Ala in place of Glu-325). The experimental data are taken from Püttner et al. (1986) and Carrasco et al. (1986).

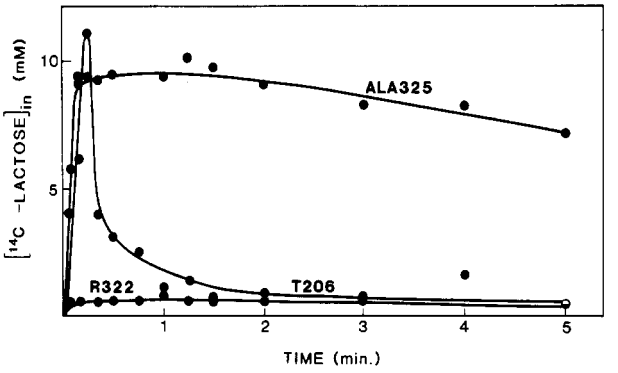

FIGURE 5: Lactose counterflow in right-side-out membrane vesicles from *E. coli* T206 (which contains wild-type permease), R322 (which contains permease with Arg in place of His-322), and A325 (which contains permease with Ala in place of Glu-325). The experimental data are taken from Püttner et al. (1986) and Carrasco et al. (1986).

Dramatically, His-205 can be replaced with either Asn or Gln without loss of activity. The findings, in conjunction with the considerations of Lowe et al. (1985), suggest that His-205 plays a role in hydrogen bonding, either to substrate or within the tertiary structure of the permease. In marked contrast, permease with Arg, Asn, or Gln in place of His-322 does not catalyze lactose/H^+ symport (Figure 3). Therefore, the results are consistent with the proposal that the imidazole ring in His-322 must be able to be protonated in order to catalyze symport.

Efflux, exchange, and counterflow are useful strategems for studying permease turnover because they can be utilized to delineate specific steps in the overall catalytic cycle (Kaback, 1986a,b; cf. below). It is clear from results shown in Figures 4 and 5 that permease with Arg in place of His-322 is grossly defective in each mode of translocation.

His-322 and Glu-325 as Components of a Charge-Relay System. Given the evidence that His-322 may be involved in H^+ translocation and a secondary structure model suggesting that this residue is located in transmembrane α-helix X, attention focused on Glu-325 [Figure 2; cf. Bieseler et al. (1985) for more direct evidence that this portion of the permease is in α-helical conformation]. If His-322 and Glu-325 are in an

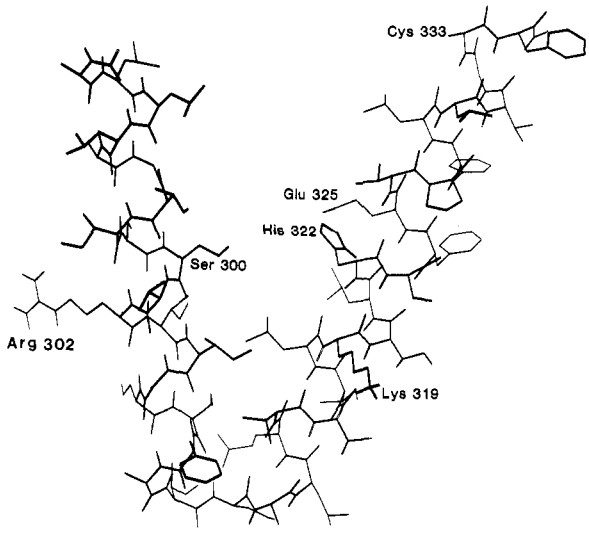

FIGURE 6: Computer modeling of helices IX and X in *lac* permease. The study was performed by Dr. Vincent Madison on an Evans–Sutherland computer.

α-helix, the functional groups in these residues must be on the same side of the helix and in close enough proximity to be ion paired (Figure 6). In addition, structure/function studies on serine proteases, chymotrypsin in particular (Blow et al., 1969), have led to the idea that Asp and His may function with Ser as components of a charge-relay system, a mechanism that might readily be adapted in part to H^+ translocation in transport enzymes such as *lac* permease. Thus, Glu-325 was replaced with Ala (Carrasco et al., 1986).

As demonstrated for the His-322 mutations (Padan et al., 1985; Püttner et al., 1986), permease with Ala in place of Glu-325 catalyzes downhill lactose influx at high substrate concentrations without H^+ translocation. However, permease with Ala-325, like permease containing Arg-322, catalyzes neither active lactose transport (Figure 3) nor efflux (Figure 4A), both of which involve net H^+ translocation. Remarkably, the rate of exchange in vesicles containing Ala-325 permease is identical with that observed in vesicles containing wild-type permease (Figure 4B). Moreover, membrane vesicles with Ala-325 permease catalyze counterflow at the same rate and to the same extent as vesicles containing wild-type permease, but the internal concentration of [1-^{14}C]lactose is maintained at high levels for a prolonged period (Figure 5). The effect is consistent with a defect in efflux combined with unimpaired exchange activity [cf. Kaback (1986a,b)].

Mechanistically, the results are rationalized by the kinetic scheme shown in Figure 7. Accordingly, efflux down a concentration gradient 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^+; (5) return of unloaded permease to the inner surface. Alternatively, exchange and counterflow with external lactose at saturating concentrations involve steps 1–3 only [cf. Kaback (1986a,b)]. Since all steps in the mechanism that involve protonation or deprotonation appear to be blocked in the His-322 mutants, it seems reasonable to suggest that protonation of His-322 is involved in step 1. In contrast, replacement of Glu-325 with Ala results in a permease that is defective in all steps involving net H^+ translocation, but catalyzes exchange and counterflow normally. Therefore, permease with Ala-325 is probably blocked in step 4 (i.e., it is unable to lose H^+).

There are a number of Ser and Thr residues in the vicinity

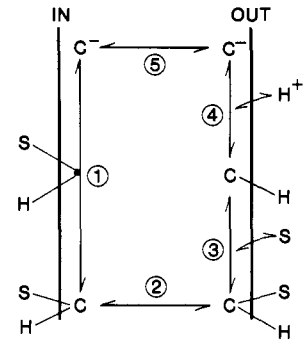

FIGURE 7: Schematic representation of reactions involved in efflux, exchange, and counterflow. C represents the *lac* permease; S, the substrate (lactose). The order of substrate and H^+ binding at the inner surface of the membrane is not implied [from Carrasco et al. (1986)].

of His-322/Glu-325, particularly in helix IX where six Ser and Thr residues are present (cf. Figure 2). Furthermore, when the amino acid sequence of putative helices IX and X is subjected to computer modeling (Figure 6), it is apparent that Ser-300 is the most likely OH-containing amino acid residue to be implicated if a triad like that postulated for chymotrypsin (Blow et al., 1969) is involved in H^+ translocation. However, when Ser-300 is replaced with Ala, the permease is unimpaired (N. Carrasco, L. Antes, and H. R. Kaback, unpublished information). Another residue in the vicinity of His-322/Glu-325 that might play an important role is Lys-319; however, replacement of this residue with Leu also has no effect on activity (N. Carrasco, L. Antes, and H. R. Kaback, unpublished information). Although these mutations, and in addition replacement of Cys-333 with Ser, have no effect on lactose/H^+ symport, the results are important for at least two reasons: (1) they highlight the specificity of His-322/Glu-325, and (2) they support the contention that single amino acid changes do not cause drastic conformational alterations even within a relatively localized portion of the permease.

Interestingly, recent experiments (D. R. Menick, L. Patel, and H. R. Kaback, unpublished information) demonstrate that replacement of Arg-302 (cf. Figures 2 and 6) with Leu causes the permease to manifest properties similar to those of the Arg-322 mutant. Thus, Arg-302 may be on the right side of helix IX facing the putative His-322/Glu-325 ion pair (cf. Figure 6; Arg-302 is moved from the left to the right side of helix IX simply by transferring two amino acid residues from the helix to the loop connecting the helices), and it may also be part of the putative charge relay.

If His-322 and Glu-325 are ion paired and function as components of a charge-relay system, polarity of the residues and distance between the imidazole and carboxyl groups should be critical. To test polarity, His-322 and Glu-325 were interchanged (I. B. Püttner and H. R. Kaback, unpublished information). Permease altered in this manner is defective in active transport, efflux, exchange, and counterflow. To test the importance of distance, Glu-325 was first replaced with Asp, thereby shortening the side chain containing the carboxylate by about 1.5 Å. Permease with Asp-325 catalyzes lactose/H^+ symport as well as wild-type permease (N. Carrasco, L. Antes, and H. R. Kaback, unpublished information). Since the pK of Asp is about 0.5 pH unit lower than that of Glu and side chains of amino acids in proteins are thought to move to the extent of 1.5–2.0 Å, the result is not surprising. Interestingly, however, preliminary experiments indicate that the "pK" for efflux [cf. Viitanen et al. (1983)] is decreased by about 0.5 pH unit with Asp in place of Glu-325. More

recently, Glu-325 was interchanged with Val-326, and permease with this configuration is inactive (J. A. Lee and H. R. Kaback, unpublished information). Computer modeling of the involved residues indicates that distance per se between the imidazole of His-322 and the carboxyl of Glu is altered to a relatively minor extent when Glu is moved from position 325 to position 326. However, the imidazole ring must be rotated significantly in order to accommodate the putative ion pair. Finally, replacement of Glu-325 with Gln yields permease that does not catalyze lactose/H^+ symport (I. R. Püttner, J. Lolkema, and H. R. Kaback, unpublished information). These preliminary results support the contention that His-322 and Glu-325 must have a specific polarity and configuration in order to support lactose-coupled H^+ transport and that the amino acid residue at position 325 must be acidic; ability to hydrogen bond is insufficient.

Although the evidence is consistent with the notion that His-322 and Glu-325 are involved in lactose-coupled H^+ translocation, direct evidence that H^+ moves physically from one residue to another is lacking. In hope of obtaining such evidence, the permease has been "engineered" so as to be able to assess the pK of His-322 under various conditions. A *lac* permease molecule has been constructed in which Arg is substituted for His-35 and His-39 and Gln for His-205 (I. B. Püttner and H. R. Kaback, unpublished information). This molecule has a single His residue at position 322 and is completely active. In addition, an analogous molecule has been constructed in which Ala replaces Glu-325. Since DEPC and rose bengal react with the unprotonated form of His and both permeases catalyze counterflow, these reagents can be used to determine the apparent pK of His-322 [cf. Garcia et al. (1982)]. It will be particularly interesting to determine the effects of substrate and the presence or absence of Glu-325 on the pK of His-322. Furthermore, it should be revealing to determine the effect of $\Delta\bar{\mu}_{H^+}$ on the pK of His-322, especially in the absence of Glu-325. If H^+ moves from His-322 to Glu-325 in the presence of $\Delta\bar{\mu}_{H^+}$, when Glu-325 is replaced with Ala, the pK of His-322 may be significantly elevated. It is also apparent that functional permease molecules with single His residues, which can be enriched with ^{13}C or ^{15}N, might be amenable to NMR if sufficiently high concentrations can be obtained.

If these considerations are correct, they also provide the basis for a strategy to delineate other residues involved in lactose-coupled H^+ translocation both before and after His-322 and Glu-325. By functional analyses of a population of uncoupled mutants, it should be straightforward to determine which mutants do and do not catalyze exchange and counterflow. Alterations in residues in the pathway before His-322 should not catalyze exchange or counterflow, while alterations in residues after Glu-325 should catalyze both reactions, and DNA sequence analyses in the two subclasses of mutants should reveal the residues involved.

Implications for Other Cation Gradient Driven Transport Systems. Since *lac* permease is a model for cation/substrate symport, it is of interest to examine other symport proteins, particularly with regard to the presence of potential His/Glu(Asp) ion pairs in positions analogous to His-322/Glu-325 in *lac* permease. One such system is the melibiose (*mel*) permease of *E. coli*, encoded by the *mel B* gene that has been cloned and sequenced by Tsuchiya and co-workers (Hanatani et al., 1984; Yazyu et al., 1984). This permease catalyzes symport with Na^+, Li^+, or H^+, depending on the substrate [e.g., symport of methyl 1-thio-β-D-galactopyranoside (TMG) occurs with Na^+ or Li^+, while symport of melibiose occurs with either H^+ or Na^+, but not with Li^+]. From the nucleotide sequence of *mel B*, *mel* permease is predicted to consist of 469 residues (*lac* permease contains 417 residues), resulting in a protein with a molecular weight of 52 029 (*lac* permease has a molecular weight of 46 504). Like *lac* permease, *mel* permease is very hydrophobic, and the hydropathy profile is similar to that obtained for *lac* permease. Despite predicted structural similarities, amino acid sequence homology between *lac* and *mel* permeases is virtually nonexistent, and there is no homology in nucleotide sequence in the structural genes for the two permeases. Nonetheless, in the carboxyl-terminal third of *mel* permease, there are two potential His/Glu(Asp) ion pairs, His-357/Glu-361 and His-442/Asp-445, and the acidic residues have been subjected to site-directed mutagenesis (H. K. Sarkar and H. R. Kaback, unpublished information).

Replacement of Asp-445 with Asn or His has no effect on either Na^+-dependent TMG transport or H^+-dependent melibiose transport. In contrast, substitution of Glu-361 with Gly or Asp inactivates Na^+/TMG symport, as well as H^+/melibiose symport. Although the results are hardly conclusive, it does not seem presumptuous to conclude tentatively that a His/Glu ion pair may also play an important role in the mechanism of *mel* permease.

In addition to *lac Y* and *mel B*, the genes for a few other symporters have been cloned and sequenced, thereby allowing deduction of primary amino acid sequences and hydropathic profiling of the encoded permeases. Included are the phosphoglycerate permease of *Salmonella typhimurium* (Saier et al., 1975; G. Yu, D. Goldrick, H. R. Kaback, and J. Hong, unpublished information) and the arabinose and xylose permeases of *E. coli* (Maiden et al., 1986). None of these molecules exhibits significant amino acid homology with *lac* permease or *mel* permease, but all exhibit similar hydropathy profiles. Furthermore, each of these permeases has at least one possible His/Glu(Asp) ion pair in the carboxyl-terminal third. It will be interesting to assess the effects of site-directed mutagenesis on appropriate residues in these proteins.

In summary, the intention of this contribution is to emphasize recent applications of oligonucleotide-directed, site-specific mutagenesis to ion gradient driven transport with *lac* permease of *E. coli* as a model. Although use of site-directed mutagenesis in itself is unlikely to solve the complete mechanism of lactose/H^+ symport, the technique has provided what appears to be an exciting initial glimpse into the chemistry of substrate-coupled H^+ translocation. Thus, His-322 and Glu-325, neighboring residues in putative helix X of the permease, play an important role in lactose/H^+ symport, possibly as components in a charge-relay mechanism. Moreover, Cys residues, long thought to play a critical role in the mechanism of the permease, are probably not directly involved in either substrate binding or H^+ translocation. Finally, it is particularly noteworthy that site-directed mutagenesis has now been utilized to introduce 20–30 individual amino acid changes into *lac* permease, and the results suggest that single amino acid changes do not lead to marked conformational alterations.

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