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Biochemistry

Histidine-94 is the only important histidine residue in the melibiose permease of *Escherichia coli*

(transport/symport/bioenergetics/site-directed mutagenesis)

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ABSTRACT **Oligonucleotide-directed, site-specific mutagenesis has been utilized to modify the *melB* gene of *Escherichia coli* such that each of the seven His residues in the melibiose permease has been replaced with Arg. Replacement of His-213, His-442, or His-456 has no significant effect on permease activity, while permease with Arg in place of His-198, His-318, or His-357 retains more than 70% of wild-type activity. In striking contrast, replacement of His-94 with Arg causes a complete loss of sugar binding and transport, although the cells contain a normal complement of permease molecules. Thus, as shown previously with *lac* permease, only a single His residue is important for activity, but, in the case of *mel* permease, the critical His residue is present in the 3rd putative transmembrane helix rather than the 10th.**

The melibiose (*mel*) permease of *Escherichia coli* is a hydrophobic transmembrane protein, encoded by the *melB* gene, that mediates symport (i.e., cotransport) of α-D-galactopyranosides with monovalent cations (refs. 1–4; for recent reviews, see refs. 5 and 6). The *mel* permease is unusual because it uses H^+, Na^+, or Li^+ as the coupling cation depending on the sugar transported. Studies carried out with right-side-out (RSO) membrane vesicles suggest that sugar binding as well as membrane potential-driven active transport, equilibrium exchange, and sugar translocation down a concentration gradient differ greatly depending upon whether the coupling cation is H^+, Na^+, or Li^+.

The *melB* gene has been cloned (7) and sequenced (8), and it has been shown that the DNA encodes a 52,029-Da protein with 469-amino acid residues. By using the T7 RNA polymerase system, Pourcher *et al.* (9) demonstrated that the *melB* gene product migrates with an apparent molecular mass of 39 kDa in $NaDodSO_4$/polyacrylamide gels. Furthermore, a secondary structure model for *mel* permease based on hydropathy profiling (8) and a series of *melB–phoA* fusions (10) suggests that the polypeptide, like *lac* permease (11), contains 12 hydrophobic segments in an α-helical conformation that span the bilayer in a zigzag manner connected by hydrophilic charged segments with the amino and carboxyl termini on the inner surface (Fig. 1). Although *mel* permease and *lac* permease exhibit similar secondary structure motifs, share several sugars as substrate, and catalyze phenomenologically similar translocation reactions, no homology in the primary amino acid sequences is observed (8).

Histidine residues are important in many proteins (cf. ref. 12), and application of site-directed mutagenesis to *lac* permease has led to the notion that His-322 (putative helix X) may act in concert with Arg-302 (putative helix IX) and Glu-325 (putative helix X) as a component of a possible catalytic triad (cf. ref. 11 for recent review). Thus, *lac* permease mutated at His-322 is defective in active transport, efflux, equilibrium exchange, counterflow, and binding of *p*-nitrophenyl α-D-galactopyranoside (NPG) but catalyzes downhill lactose influx without H^+ translocation (13). In contrast, mutation of His residues at positions 35, 39, or 205 has no effect on *lac* permease activity. Moreover, *lac* permease with a single His residue at position 322 retains full transport activity as well as other properties of wild-type permease (14).

Although the mechanism of sugar/cation symport catalyzed by *mel* permease is unknown, studies with diethylpyrocarbonate suggest that His residues may also be important for this permease (3, 5). *mel* permease contains seven His residues (ref. 8; Fig. 1), and we have utilized site-directed mutagenesis to systematically replace each His with Arg. The results demonstrate that, of the seven His residues in *mel* permease, only His-94 is important for transport and binding.

MATERIALS AND METHODS

Materials. All materials were of reagent grade and were obtained from commercial sources (9, 15).

Methods. *Bacterial strains and plasmids.* The following strains of *E. coli* K12 were used: JM-101 [*supE, thi,* Δ(*lac–pro*)/*traD36, proA*$^+$ *B*$^+$, *lacI*^{q}, *lacZ*Δ*M15*] and BMH71-18 mutL [*supE, thi,* Δ(*lac–pro*), *mutL*::Tn*10*/*traD36, proA*$^+$ *B*$^+$, *lacI*^{q}, *lac ZΔM15*] (cf. ref. 13). DW2 (*melA*$^+$, Δ*melB,* Δ*lacZY*) and JA 200/pLC 25-33 were a gift from T. H. Wilson (Harvard Medical School, Cambridge, MA). Plasmid pKK223-3 was from Pharmacia.

Construction of plasmids carrying wild-type or mutated melB. A recombinant plasmid carrying wild-type *melB* was constructed with a modified pKK223-3 vector (pK30). The *Eco*RI restriction site in the vector polylinker was suppressed by digesting the protruding 5′ ends of the *Eco*RI-linearized plasmid with mung bean nuclease and ligating the resulting blunt ends (pK30). A *Pst* I/*Pst* I DNA fragment (≈5 kilobase pairs) carrying *melA*, *melB*, and the putative *melC* gene was isolated from a recombinant plasmid in the Clarke and Carbon colony bank (pLC25-33; ref. 7) and ligated into the modified vector linearized with the same enzyme, yielding plasmid pK31 (Fig. 2). The resulting construction contains a unique *Eco*RI site about 100 base pairs downstream from the TGA termination codon of *melB*. An additional *Eco*RI site was first introduced a few bases upstream from the *melB* gene by exchanging a *Bgl* II/*Eco*RV DNA fragment (carrying

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Abbreviations: RSO, right-side-out; TMG, methyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; RF, replicative form.

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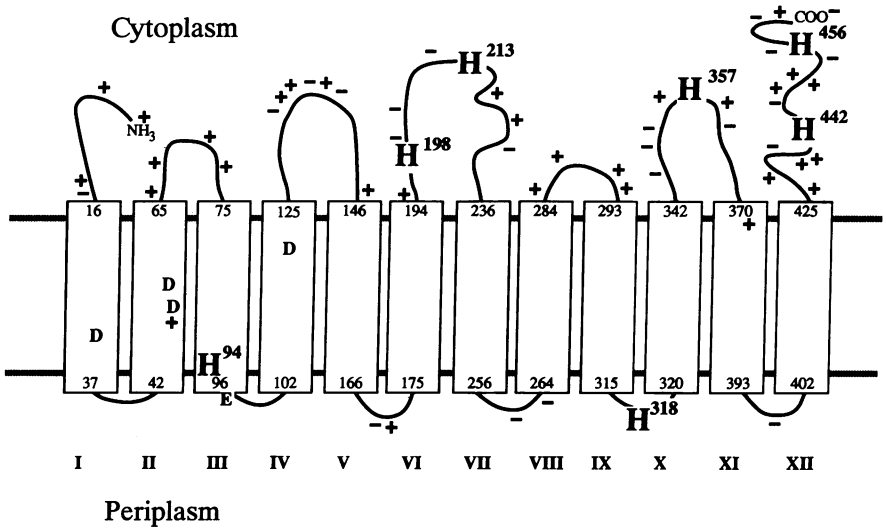

FIG. 1. Secondary structure model of *mel* permease with His residues highlighted. The model was derived from hydropathy profiling of the primary amino acid sequence of *mel* permease (6, 8) and analysis of a series of *melB–phoA* fusions (10). Helices are numbered with Roman numerals. H, histidine; D, aspartic acid; E, glutamic acid.

melA and most of the *melB* gene of plasmid pK31) with a homologous DNA fragment isolated from recombinant M13mp18 replicative form (RF) DNA containing the novel *Eco*RI site created by site-directed mutagenesis (13, 15) in the intergenic sequence between *melA* and *melB* (9). This strategy provides a means of selectively excising and replacing the entire *melB* gene. In addition, a mutagenic primer (5′-GAACAACTGGTACCTTATCCGCGT-3′) was used to introduce a unique *Kpn* I restriction site in *melB* by replacing the GTT triplet encoding Val-141 with GTA, which also encodes Val. In combination with a *Bgl* II site present in *melA*, the *Kpn* I site was used to exchange the first 420 base pairs of the coding sequence of *melB*. The modified recombinant plasmid (pK32) was used to analyze functional expression of all mutants.

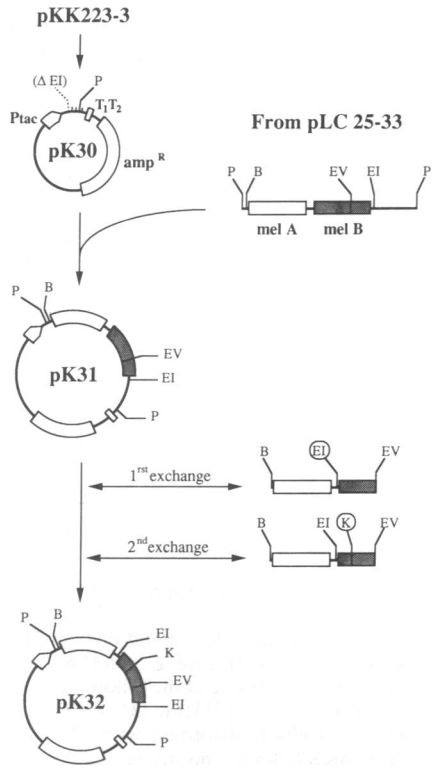

FIG. 2. Construction of plasmids carrying wild-type or mutated *melB* genes. Construction of pK32 containing wild-type *melB* is described in *Materials and Methods*. Plasmids carrying mutated *melB* genes were prepared by exchanging the *Eco*RI/*Eco*RI *melB* DNA fragment of pK32 for analogous DNA fragments isolated from recombinant M13mp18 RF DNAs carrying the desired mutation. B, *Bgl* II; EI, *Eco*RI; EV, *Eco*RV; K, *Kpn* I; P, *Pst* I.

Oligonucleotide-directed, site-specific mutagenesis. Site-directed mutagenesis was performed as described (13, 15). The *melB* gene contained in the *Eco*RI DNA fragment from pK32 (1.5 kilobase pairs) was inserted into M13mp18 RF DNA so as to place *melB* in reverse orientation with respect to the *lac* promoter. The recombinant single-stranded phage DNA used as a template contained the *melB* sense strand. The sequences of the mutagenic primers used to change each of the seven His codons (Fig. 1) into Arg codons are given in Table 1. Closed circular heteroduplex DNA with the desired mutation was synthesized *in vitro* and used to transfect *E. coli* BMH 71-18 mutL. The seven His → Arg codon changes were verified by dideoxynucleotide sequencing with seven synthetic deoxyoligonucleotide sequencing primers complementary to appropriate regions of *melB*.

Permease activity. The pK32 *Eco*RI/*Eco*RI DNA fragment containing wild-type *melB* was replaced with each of the seven analogous DNA fragments bearing given mutations, and the resultant plasmids, designated pH94R¶, pH198R, pH213R, pH318R, pH357R, pH442R, and pH456R, were used to transform *E. coli* DW2. Permease activity was assayed qualitatively on MacConkey plates containing 10 mM melibiose, and transport was measured quantitatively by determining the time course of [^{14}C]methyl 1-thio-β-D-galactopyranoside ([^{14}C]TMG) (3 mCi/mmol; 1 Ci = 37 GBq) accumulation in intact cells by filtration (16). Binding of [^{3}H]NPG to RSO membrane vesicles was assayed under nonenergized conditions by using flow dialysis (4).

Quantitation of mel *permease. In vivo* labeling of *mel* permease with [^{35}S]methionine was performed as described by Pourcher *et al.* (9).

Protein determinations. Protein was assayed according to Lowry *et al.* (17) with bovine serum albumin as standard.

RESULTS

Replacement of His Residues in *mel* Permease with Arg. The *melB* gene in each plasmid was cloned initially into the RF of M13mp18 DNA, and single-stranded phage DNA containing the sense strand of *melB* was isolated and used as a template to replace each His residue in *mel* permease at position 94,

¶Site-directed mutants are designated as follows. The one-letter amino acid code is used followed by a number indicating the position of the residue in the wild type. The sequence is followed by a second letter denoting the amino acid replacement at this position.

Table 1. DNA sequence analysis of His mutations in *melB*

Plasmid	Mutagenic primer	DNA sequence change	Amino acid sequence change
pH94R	5'-CTTTAGTGCGC***G***TCTGTTTGAAG-3'	CAT → CGT	His-94 → Arg
pH198R	5'-ACTCTGCGCAATGTGC***G***TGAAGTCTTTTCGTCA-3'	CAT → CGT	His-198 → Arg
pH213R	5'-GCTGAAGGAAGCC***G***TCTGACACTTAAAGCC-3'	CAT → CGT	His-213 → Arg
pH318R	5'-TTAATGAGCTATC***G***CAACGTCGTCCTC-3'	CAC → CGC	His-318 → Arg
pH357R	5'-GAATATAAACTGC***G***CGTACGCTGTGAA-3'	CAC → CGC	His-357 → Arg
pH442R	5'-AGGATCCAGATCC***G***TCTGCTGGATAAA-3'	CAT → CGT	His-442 → Arg
pH456R	5'-CCGAGCCTGTTC***G***TGCTGATATTCC-3'	CAT → CGT	His-456 → Arg

The altered base is in boldface italic type.

198, 213, 318, 357, 442, or 456 (Fig. 2) with Arg (13, 15). Subsequently, single-stranded phage DNA containing mutated *melB* was isolated and sequenced by using seven deoxyoligonucleotide primers to confirm that only the desired mutations were present. Table 1 summarizes the results of DNA sequence analyses, and it is apparent that each mutant contained a *melB* gene in which a given His codon (CAT or CAC) was replaced with an Arg codon (CGT or CGC). The seven DNA fragments containing *melB* with single Arg replacements were then restricted from the respective M13mp18 RF DNA and inserted in place of the analogous DNA fragment containing wild-type *melB* in a recombinant derivative of plasmid pKK223-3 (pK32, see *Materials and Methods*). The resulting plasmids (pH94R, pH198R, pH213R, pH318R, pH357R, pH442R, and pH456R) were then used to express the mutated *mel* permeases.

Effect of His Mutations on Permease Activity. *H198R, H213R, H318R, H357R, H442R, and H456R permeases.* When *E. coli* DW2 (*melA*$^+$, Δ *melB*, Δ*lac ZY*) is transformed with pH198R, pH213R, pH318R, pH357R, pH442R, or pH456R and grown on MacConkey agar containing melibiose, the cells form red colonies in a manner comparable to DW2/pK32 cells expressing wild-type *melB*. Qualitatively, therefore, *mel* permease containing Arg in place of His at position 198, 213, 318, 357, 442, or 456 transports melibiose as well as wild-type *mel* permease. This conclusion is confirmed by Na^+-dependent TMG transport studies with *E. coli* DW2 transformed with each plasmid (Fig. 3). The initial rate of TMG transport and the steady-state level of accumulation are identical within experimental error in *E. coli* DW2/pK32 (wild-type *melB* gene), DW2/pH213R, DW2/pH442R, and DW2/pH456R. On the other hand, with DW2/pH198R, DW2/pH357R, and DW2/pH318R, there is about a 30% decrease in the initial rate and steady-state level of Na^+-dependent TMG transport, which may be marginally significant. Although not shown, Li^+-dependent TMG uptake studies yield similar results. Furthermore, as observed with wild-type *mel* permease (3), Na^+-dependent TMG uptake in each mutant is markedly inhibited by diethylpyrocarbonate. Therefore, it is concluded that His residues at position 198, 213, 318, 357, 442, or 456 are not essential for activity.

H94R permease. In striking contrast, when the His codon at position 94 is replaced by an Arg codon and pH94R is used to transform *E. coli* DW2, the cells grow as white colonies on MacConkey/melibiose plates, suggesting that H94R permease is severely defective. Generation of a unique *Kpn* I restriction site about 420 base pairs from the 5' end of *melB* allows verification that loss of activity in H94R permease is due to a mutation lying within the 5' third of the mutated *melB* gene. Thus, restriction of the mutated DNA with *Bgl* II/*Kpn* I and replacement with the analogous fragment from wild-type *melB* results in a gene that confers normal transport to *E. coli* DW2 (data not shown). It was then established by dideoxynucleotide sequencing of the *Eco*RI/*Kpn* I restriction fragment that loss of transport activity in H94R is due specifically to the A → G mutation in codon 94 (Table 1).

More direct evidence demonstrating that H94R permease is unable to catalyze active transport was obtained by measuring Na^+-dependent [^{14}C]TMG accumulation in DW2/pH94R. Initial rates of Na^+/TMG (Fig. 3) and Li^+/TMG (data not shown) symport and steady-state levels of accumulation are negligible. It is also noteworthy that H94R *mel* permease is unable to catalyze melibiose symport with Na^+, H^+, and Li^+ (data not shown).

Comparison of Na^+-dependent NPG binding in wild-type and H94R RSO membrane vesicles demonstrates that the ability of H94R permease to bind ligand is also severely impaired (Fig. 4). At equivalent protein concentrations, the dialyzable concentration of [^{3}H]NPG is significantly higher with H94R vesicles relative to wild-type vesicles. Upon addition of excess melibiose, NPG is competitively displaced from wild-type vesicles, and the dialyzable NPG concentration increases to the same level as that observed in H94R vesicles. No change is observed when excess melibiose is added to H94R vesicles.

The possibility that the transport and binding defects observed with H94R permease are due to a defect in insertion of the permease into the membrane or enhanced proteolysis is ruled out by [^{35}S]methionine labeling experiments (Fig. 5). The product of *melB*, which migrates with a molecular mass of 39 kDa (9), is of comparable intensity in membranes prepared from cells expressing wild-type or H94R permease.

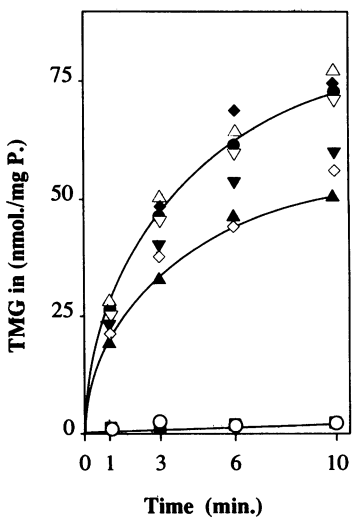

FIG. 3. Effect of individual His mutations on Na^+-dependent uphill TMG transport by *mel* permease. *E. coli* DW2 (*melA*$^+$, Δ*melB*, Δ*lac ZY*) was transformed with one of the following plasmids: pH94R (■), pH198R (▼), pH213R (△), pH318R (▲), pH357R (◇), pH442R (◆), or pH456R (▽). Cells transformed with pK32 (●) containing wild-type *melB* or pKK223-3 (○, no insert) were used as controls. Cells were grown in LB medium, washed, and resuspended in 100 mM potassium phosphate, pH 6.6/10 mM magnesium sulfate/10 mM sodium chloride. Time courses of transport were measured in 50-μl cell suspensions (100 μg of protein) containing 0.2 mM [^{14}C]TMG (3 mCi/mmol) by using a rapid filtration method (16). For clarity, the curves fitting the time points of TMG accumulation by mutants H198R, H213R, H357R, H442R, and H456R were omitted. P., cell protein.

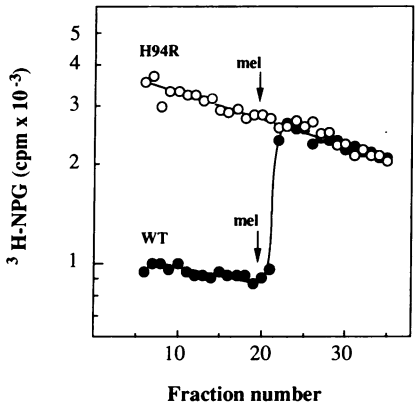

FIG. 4. [^{3}H]NPG binding to membrane vesicles containing wild-type or H94R permease. Osmotic lysis (16) was used to prepare RSO membrane vesicles from *E. coli* DW2 expressing wild-type (WT) or H94R permease. Flow dialysis was carried out with 0.4 ml of deenergized membrane vesicles (35 mg of protein per ml) suspended in 100 mM potassium phosphate (pH 6.6) in the presence of 10 mM NaCl, 0.75 μM monensin, and 10 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) as described (4). Radioactivity recovered in the dialysate is plotted as a function of the fraction number. The following additions were made to the upper chamber containing the vesicle suspensions. [^{3}H]NPG (800 mCi/mmol) was added at zero time to a final concentration of 1 μM; melibiose was added to a final concentration of 20 mM (arrows; fraction 20).

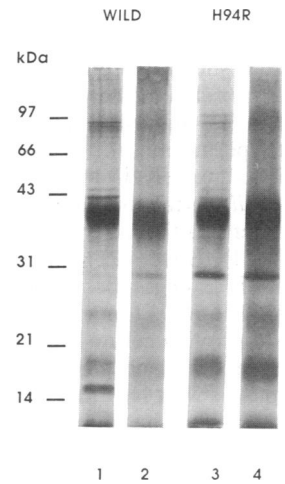

FIG. 5. Autoradiographic analysis of gene products encoded by pT7-(SD/B) (wild-type *melB*) and pT7-(H94R). *E. coli* DW2 (*melA*$^+$, Δ*melB*, Δ*lac ZY*) was cotransformed with plasmid pGP1-2 containing the T7 polymerase gene and either plasmid pT7-(SD/B) containing the wild-type *melB* gene (9) or plasmid pT7-(H94R) bearing *melB* H94R placed under the control of the T7 promoter. DW2[pT7-(SD/B)/pGP1-2] or DW2[pT7-(H94R)/pGP1-2] cells were grown at 28°C in M9 medium supplemented with Difco methionine assay medium to starve the cells for methionine. The cultures were transferred at 42°C to induce synthesis of T7 RNA polymerase. Rifampicin (200 μg/ml) was added to inhibit *E. coli* RNA polymerase activity. The cells were then incubated with [^{35}S]methionine, and labeling was terminated by adding an excess of unlabeled methionine (10 mM, final concentration). Cytoplasmic membranes were isolated by osmotic lysis (16). All samples were solubilized in 1% $NaDodSO_4$ and incubated for 1 hr at 37°C. Cell protein (25 μg) or membrane protein (2.5 μg) was resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis (18) using acrylamide and bisacrylamide at final concentrations of 12% and 0.32%, respectively. Gels were silver stained, dried, and autoradiographed. Lanes 1 and 3, total cell protein; lanes 2 and 4, inner membrane fractions.

DISCUSSION

The observations reported in this paper confirm and extend the argument that a His residue is critical for the activity of melibiose permease, a contention based on the previous demonstration (3, 5) that ethoxycarbonylation of RSO membrane vesicles with diethylpyrocarbonate inactivates Na^+, Li^+, or H^+/sugar symport by means of the melibiose permease. Thus, it is clear that replacement of His-94 with Arg completely abolishes both transport and binding, while replacement of His-198, His-213, His-318, His-357, His-442, or His-456 with Arg has little or no effect.

The demonstration that a single His residue in *mel* permease is essential for symport and binding parallels observations with *lac* permease (11, 13, 14) where replacement of His-322 alone is sufficient to inactivate H^+/lactose symport and markedly decrease affinity for NPG. Since both permeases catalyze cation/sugar symport and the role of His-322 in H^+/lactose symport has been suggested to involve a H^+-relay type mechanism (11), the role of His-94 in *mel* permease will be discussed in the context of this hypothesis.

The H^+-relay hypothesis for *lac* permease suggests that Arg-302 (putative helix IX), His-322 (putative helix X), and Glu-325 (putative helix X) are sufficiently close to form a hydrogen-bond network that plays a direct role in lactose-coupled H^+ translocation. In addition to the postulated geometric relationship between the three residues, the hypothesis is based on observations that *lac* permease mutagenized at Arg-302 or His-322 catalyzes downhill lactose translocation without concomitant H^+ translocation but is unable to catalyze equilibrium exchange or counterflow. Permease mutated at Glu-325 is also uncoupled but retains the ability to catalyze exchange and counterflow. For these reasons, it was suggested that protonation of His-322 is required for exchange and counterflow and that transfer of H^+ between His-322 and Glu-325 is required for H^+/lactose symport.

Replacement of His-94 in *mel* permease or His-322 in *lac* permease with Arg results in inactivation of active transport and loss of ability to bind ligand; however, additional considerations suggest that the analogy may be superficial. Thus, mutation of His-322 in *lac* permease uncouples lactose from H^+ translocation, and the mutated permease catalyzes downhill lactose influx without concomitant H^+ translocation (11, 13). For this reason, cells expressing H322R *lac* permease grow as red colonies on lactose indicator plates and utilize lactose as a sole carbon source at high concentrations. In contrast, *E. coli* DW2/H94R grows as white colonies on MacConkey agar containing 10 mM melibiose, indicating that H94R *mel* permease is completely unable to catalyze melibiose translocation. Finally, replacement of Glu-97 (Fig. 1) with Gln has no effect on *mel* permease activity (unpublished observations), while replacement of Glu-325 in *lac* permease with various amino acid residues including Gln results in an uncoupled permease that retains the ability to catalyze exchange and counterflow. Although Arg replacement for His-94 in *mel* permease may inactivate downhill sugar translocation in a secondary manner, at face value, the data suggest that His-94 may not be involved in coupling between cation and sugar translocation and that the defect observed may be due specifically to a loss in ability to bind substrate.

Although H^+- and Na^+-coupled symport are conceptually and thermodynamically analogous, it is unclear whether the two types of transport occur by the same general mechanism. The data available for *lac* permease (cf. ref. 11) are consistent with the idea that a H^+-relay may be important. However, it is not obvious how the same mechanism can be directly involved in Na^+ symport unless N or O atoms that can coordinate with Na^+ are present in the pathway in addition to the minimal structures necessary for a "H^+-wire" of the type suggested by Onsager (19). Despite certain marked

kinetic differences between H^+- and Na^+-coupled sugar translocation (compare refs. 3–6 and 11), a few lines of evidence suggest that substrate-coupled Na^+ and H^+ translocation may occur by related mechanisms: (*i*) NPG binding studies suggest that H^+ and Na^+ compete for a common binding site on *mel* permease (4), (*ii*) Na^+ is a competitive inhibitor of melibiose-coupled H^+ translocation by means of *mel* permease, and (*iii*) certain mutations in *melB* have been isolated and characterized that alter the cation specificity of *mel* permease (20, 21). Boyer (22) suggested recently that H_3O^+, rather than H^+, might be the symported species. Appropriately placed N or O atoms in symporters like *lac* or *mel* permease could provide cation-binding domains akin to those in the crown ethers or cryptates, both of which form coordination complexes with Na^+ and H_3O^+ (22, 23). In this context, however, some of the translocation reactions catalyzed by *lac* permease exhibit a significant ^2H_2O effect (24), which is not expected if coordination with H_3O^+ were the rate-limiting step in translocation. In summary, therefore, although the contrast between H^+- and Na^+-coupled symport is of singular importance, the mechanistic relationship between the two is presently unclear.

Finally, it is noteworthy that His-94, the critical His residue in *mel* permease, is located in putative helix III (Fig. 1), while His-322 in *lac* permease is located in putative helix X (11). Furthermore, in *mel* permease, the charged amino acid residues in putative transmembrane helices are present in the N-terminal half of the polypeptide, while the charged residues in putative transmembrane helices in *lac* permease are present in the C-terminal half. The observations suggest that hypothetical functional domains in the two proteins may reside in opposite ends of the proteins, which could have important implications for the construction of functional chimeras between *mel* and *lac* permeases.

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