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Information Content of Amino Acid Residues in Putative Helix VIII of the *lac* Permease from *Escherichia coli*

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ABSTRACT: Mutants in putative helix VIII of lactose permease that retain the ability to accumulate lactose were created by cassette mutagenesis. A mutagenic insert encoding amino acid residues 259–278 was synthesized chemically by using reagents contaminated with 1% each of the other three bases and ligated into a *KpnI/BclI* site in the *lacY* gene in plasmid pGEM-4. Mutants that retain transport activity were selected by transforming a strain of *Escherichia coli* containing a wild-type *lacZ* gene, but deleted in *lacY*, with the mutant library and identifying colonies that transport lactose on indicator plates. Sequencing of the mutated region in *lacY* in 129 positive colonies reveals 43 single amino acid mutations at 26 sites and 26 multiple mutations. The variable amino acid positions are largely on one side of the putative α-helix, a stripe opposite Glu269. This mutable stripe of low information content is probably in contact with the membrane phospholipids.

The lactose (*lac*) permease of *Escherichia coli* is a polytopic cytoplasmic membrane protein that catalyzes the simultaneous translocation of a single β-galactoside with a single H^+ (i.e., β-galactoside/H^+ symport or cotransport) (Kaback, 1989, 1990). The permease has been solubilized from the membrane, purified to homogeneity, and reconstituted into phospholipid vesicles (Newman et al., 1981; Viitanen et al., 1985) and is probably completely functional as a monomer (Costello et al., 1987). The *lacY* gene, which encodes the permease, has been

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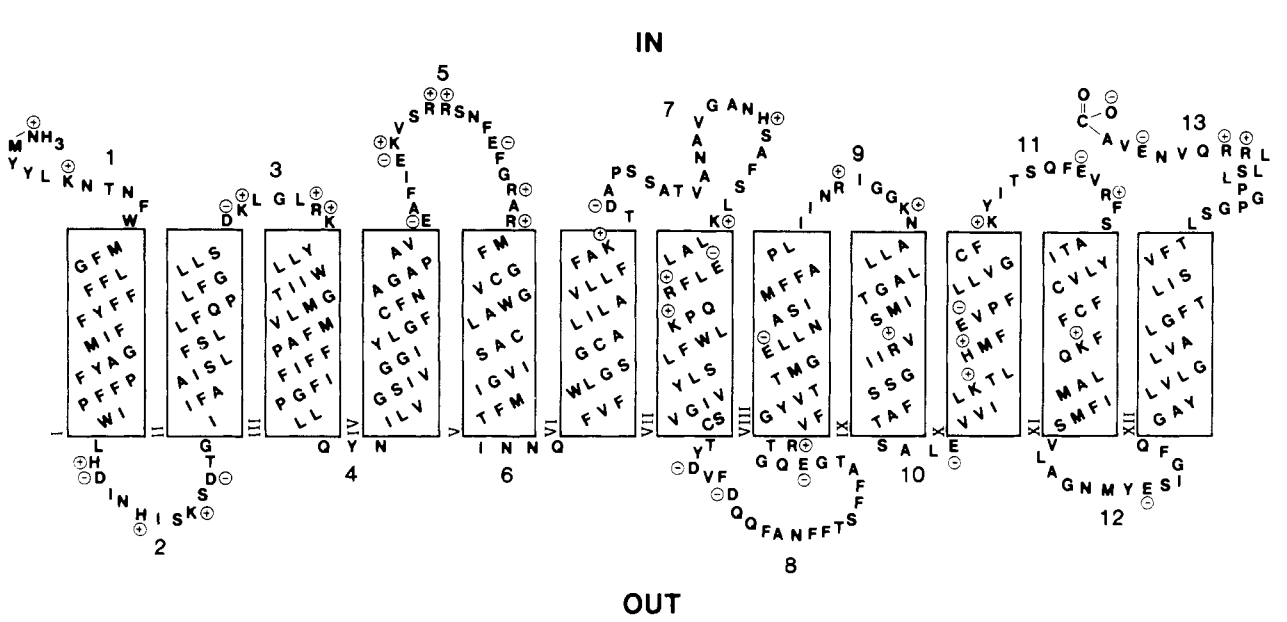

FIGURE 1: Secondary structure model of *lac* permease based on the hydropathy profile of the protein (Foster et al., 1983). The single amino acid code is used, and hydrophobic domains are shown in boxes as transmembrane α-helices connected by hydrophilic segments with the N-terminus numbered as hydrophilic segment 1. Putative transmembrane α-helices are labeled with Roman numerals and hydrophilic segments with Arabic numberals.

cloned and sequenced, and the amino acid sequence of the permease has been deduced from the DNA sequence (Büchel et al., 1980). On the basis of circular dichroic measurements and hydropathy analysis of the primary sequence, a secondary structure model was proposed (Foster et al., 1983) in which the polypeptide is organized into 12 hydrophobic domains in α-helical conformation that traverse the membrane in a zig-zag fashion connected by hydrophilic loops with the N- and C-termini on the cytoplasmic face of the membrane (Figure 1). The model is consistent with other spectroscopic measurements (Vogel et al., 1985),[1] as well as chemical modification (Page & Rosenbusch, 1988), limited proteolysis (Goldkorn et al., 1983; Stochaj et al., 1986), and immunological studies (Carrasco et al., 1982, 1984a,b; Seckler et al., 1983, 1984, 1986; Herzlinger et al., 1984), but none of these approaches is able to differentiate between the 12-helix structure and another proposed model (Vogel et al., 1985) containing 14 transmembrane helices. Recently, however, analysis of a series of *lacY–phoA* (*lac* permease–alkaline phosphatase) fusions (Calamia & Manoil, 1990) has provided strong exclusive support for the 12-helix motif.

Over 100 site-directed mutations have been made in *lac* permease, including all of the Cys [cf. Menick et al. (1987)], His [cf. Püttner et al. (1989)], Tyr (Roepe & Kaback, 1989), Trp (Menezes et al., 1990), and Pro (T. G. Consler, O. Tsolar, and H. R. Kaback, unpublished results) residues. This approach has identified Arg302, Lys319, His322, and Glu325 as residues that are essential for lactose/H^+ symport and/or substrate recognition, and it has been postulated that they may function in a type of charge-relay mechanism [cf. Kaback (1990) for a recent review].

Another approach to mutational analysis of *lac* permease has been to mutagenize the entire gene and select for interesting phenotypes. A partially uncoupled mutant was recently sequenced and found to have Cys in place of Gly262 (Brooker et al., 1989). Several mutants with decreased affinity for lactose have been isolated, and one has been identified as T266I[2] (Margraf et al., 1985). Thirty-six mutants with altered sugar specificity have been sequenced (Brooker & Wilson, 1985; Brooker et al., 1985; Collins et al., 1989), and all of the mutations occur at one of only six positions: Ala177, Tyr236, Ser306, Lys319, His322, and Ala389. Some of the sugar specificity mutants are also uncoupled, suggesting the pathways for H^+ and sugar translocation may overlap [cf. Menick et al. (1987) and Püttner et al. (1989) in addition]. Clearly, strategies for mutagenesis that allow patterns of change with respect to particular properties to be recognized can be most revealing.

We have now utilized saturation cassette mutagenesis on a limited region of *lac* permease, putative transmembrane α-helix VIII, in order to determine how much the region can be altered and yet maintain activity. A simple experimental design allows rapid sequencing of mutants with transport activity so that many such mutants can be characterized. The approach allows identification of amino acid residues with low "information content", as well as residues that are important for activity. A highly mutable stripe of residues on one side of the putative α-helix is observed, and it is suggested that this side is in contact with the membrane phospholipids in accordance with the ideas of Rees et al. (1989).

Experimental Procedures

Materials

[1-^{14}C]Lactose was synthesized by Yu-Ying Liu under the direction of Arnold Liebman (Isotope Synthesis Group at Hoffmann-La Roche Inc.). All other materials were reagent grade and obtained from commercial sources.

Methods

Bacterial Strains. The following strains of *E. coli* K-12 were used: HB101, *hsd S20* (r^-_B, m^-_B), *rec A 13*, *ara-14*, *pro A2*,

[1] In addition to circular dichroic and laser Raman spectroscopy, Fourier-transform infrared studies also show that purified *lac* permease is largely helical in conformation (P. D. Roepe, H. R. Kaback, and K. J. Rothschild, unpublished results).

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

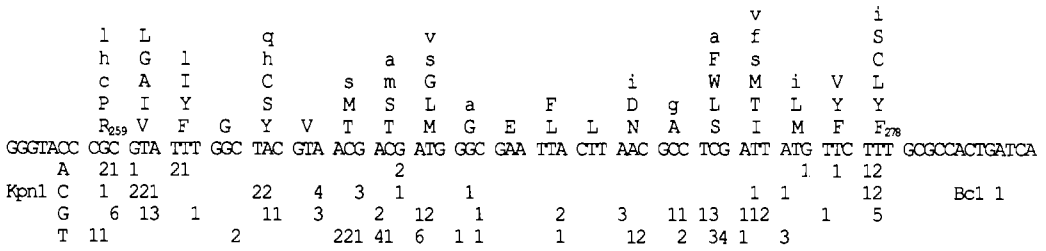

FIGURE 2: Mutations in putative helix VIII that yield active permease. The nucleotide sequence of the mutagenic oligonucleotide is shown. Below the sequence are the number of times the positions changed to the nucleotide shown. Above the sequence is the wild-type amino acid sequence from Arg259 to Phe278, and above each amino acid residue is the substitution deduced from DNA sequencing. Upper case letters indicate single mutations and lower case letters multiple mutations.

lac Y1, gal K2, rps L20 (*Sm*^{r}), *xyl-5, mtl-1, supE44*, λ^-/F^- (Boyer & Roulland-Dussoix, 1969); *T184*, *lac* I^+O^+, Z^-, $Y^-(A)$, *rpsL, met, thr, recA, hsdM, hsd R/F', lac* I^q O^+P^+ Z^{U118} (Y^+ A^+) (Teather et al., 1980); *AZ-1, recA1, thi* Δ*lac-proAB/F'* I^q, Z^+ ΔY, *pro*$^+$ (J. A. Lee and H. R. Kaback, unpublished experiments).

Construction of pGEM(lacY). The plasmid used for all steps in this study was pGEM-4 (Promega) into which the *lacY* gene, with a Shine–Delgarno site but no *lac* promoter/operator, was cloned from pGM21 (Teather et al., 1980). Routine molecular biology methods were as described by Maniatis et al. (1982). The insert was from the *Pvu*II site, 99 bases upstream from the start codon, to the *Eco*RI site at the end of the insert in pGM21. The *Pvu*II site was ligated into the *Hin*cII site in pGEM-4. The plasmid is called pGEM(*lacY*). The *lacY* gene used has been changed to introduce several unique restriction sites, including the *Kpn*I site shown in Figure 2 (J. C. Pastore, J. D. Larigan, and H. R. Kaback, unpublished results).

Synthesis of the Mutant Insert. The sequence shown in Figure 2 was synthesized on an Applied Biosystems Model 380B DNA synthesizer, using cyanoethyl phosphoramidite nucleotide reagents contaminated with 1% each of the other three bases between positions 7 (codon 259) and 66 (codon 278) and purified by urea–polyacrylamide gel electrophoresis. A complementary primer was also made to positions 67–81, 100 pmol of each was annealed, and the primer was extended with 1 μL of Sequenase (U.S. Biochemicals) in Sequenase buffer, dithiotheitol (10 mM), and dNTPs (0.5 mM) for 1 h at 37 °C. The reaction was stopped at 80 °C and slow-cooled, and a 10-pmol aliquot was restricted with *Kpn*I and *Bcl*I.

Creation of a Mutant Library. The insert described above was ligated into the pGEM(*lacY*) vector, and the ligation mixture was desalted by drop dialysis on VSWP 025 filters (Millipore) floating on distilled water twice for 1 h. The desalted ligation mixture (2 μL) was transformed into strain HB101 by electroporation (Dower et al., 1988). After growth for 1 h in Luria broth (LB),[3] cells were diluted into 20 mL of LB/ampicillin (amp) and grown overnight. Aliquots were also plated on LB/amp agar, and it was determined that about 5000 cells were transformed to amp resistance during electroporation. An alkali–plasmid preparation was made from the overnight culture and used as the mutant library. The plasmid preparation had much higher transformation efficiency than the original ligation mixture and was used to transform *E. coli* AZ-1 which is less efficient than HB101 with respect to transformation. The random distribution of mutants obtained indicates that generation of sister colonies is not problematic.

Selection of Active Mutants. E. coli AZ-1, which is wild-type for β-galactosidase (*lacZ*$^+$) but deleted for *lac* permease (Δ*lacY*), was transformed with the mutant library and grown on MacConkey/Lactose (25 mM) plates. After 16–20-h growth, the colonies were either clearly red (active *lac* permease) or white. Colonies were picked and grown overnight in 3 mL of LB/amp for plasmid preparations. The overnight cultures were also replated on MacConkey/lactose to confirm the result.

Sequencing. The mutated region of *lacY* was sequenced directly from the plasmid as described (Hattori & Sakaki, 1986) or, in some cases, with substitution of Glass milk (Geneclean, Bio 101) for the poly(ethylene glycol) precipitation step in the plasmid preparation. Sequencing was with Sequenase using a sequencing primer containing codons 350–357 (Viitanen et al., 1985).

Transport Assays. E. coli AZ-1 or T_{184} containing the appropriate pGEM(*lacY*) plasmid was grown overnight in LB/amp, washed in 100 mM potassium phosphate (pH 7.5) and 10 mM $MgSO_4$, and resuspended in the same medium to an OD_{420} of 20. Aliquots (50 μL) of cell suspensions were incubated at room temperature with 4 mM lactate with or without 2 mM dinitrophenol, and transport was initiated by addition of 0.4 mM [1-^{14}C]lactose (3 mCi/mmol) or 0.3 mM [*methyl*-^{14}C]TMG (2.5 mCi/mmol). The reactions were stopped by addition of 3 mL of 100 mM potassium phosphate (pH 5.5), 100 mM LiCl, and 20 mM $HgCl_2$ and immediately filtered on Whatman GF/F glass fiber filters, and the filter was washed twice with 3 mL of the stopping solution. Radioactivity retained on the filter was assayed by liquid scintillation spectrometry.

Results and Discussion

The method used to generate mutants is described in detail under Experimental Procedures. It was observed that the *lacY* gene is expressed when inserted into the multiple cloning site of the high-copy plasmid pGEM-4. In the construct, pGEM(*lacY*), a Shine–Delgarno sequence is present for translation, but the *lac* promoter/operator is absent, and expression of *lacY* is under the control of the T_7 promoter. Expression of *lac* permease is lower than the usual induced levels in wild-type *E. coli*, but sufficient for screening colonies on MacConkey/lactose plates. The use of this simple selection procedure and plasmid sequencing allows a very simple protocol to be followed which allows sequencing of many mutants in a short time.

The results of sequencing the mutated region of the *lacY* gene from 129 positive colonies revealed 41, 55, 24, 7, and 2 sequences with 0, 1, 2, 3, and 4 or more base changes, respectively. This yielded 43 single amino acid mutants in 26 unique categories and 26 multiple mutants. All of the base and amino acid alterations found are shown in Figure 2.

[3] Abbreviations: LB, Luria broth; amp, ampicillin; TMG, methyl 1-thio-β-D-galactopyranoside.

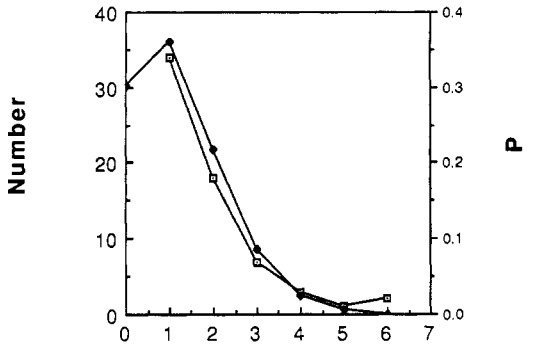

FIGURE 3: Distribution of the frequency of a given nucleotide alteration. Open symbols are taken from the data presented in Figure 2; closed symbols are a fit to the curve from eq 1.

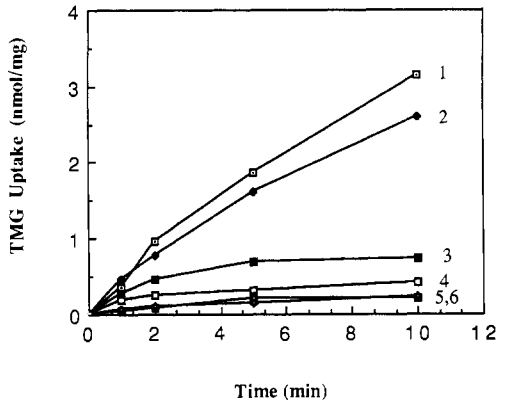

FIGURE 4: Transport of [*methyl*-^{14}C]TMG by *E. coli* AZ-1 transformed with pGEM(*lacY*) containing various mutations. 1, Wild type; 2, M267G; 3, L270F; 4, A273G/S274A; 5, G262V; 6, untransformed AZ-1.

The number of mutant positions, P, found a given number of times, r, is a function of the probability of a single base change, p, and the number of nucleotides in the segment mutagenized, n, according to the equation (Mellor, 1955):

$$P = \frac{(np)^r}{r!}e^{-np} \qquad (1)$$

The distribution of the mutations described was fitted to the equation (Figure 3). The value of np was 1.2, and the number of positions not identified ($r = 0$) was about 28, or 30% of the total.

Transport Activity. Although analysis on MacConkey plates indicates that the red colonies have lactose transport activity, the assay is not quantitative and is a poor indicator of whether or not transport is coupled to H^+ influx [cf. Padan et al., (1985)]. For a more quantitative characterization of transport, [^{14}C]methyl 1-thio-β-D-galactopyranoside ([^{14}C]-TMG) uptake was measured in transformed *E. coli* AZ-1. The data presented in Figure 4 represent typical transport measurements with *E. coli* AZ-1 expressing the wild-type permease or representative mutants. As shown, permease with M267G transports TMG almost as well as wild-type permease, and L270F permease retains about 25% of wild-type activity. In contrast, A273G/S274A and G262V permeases have little or no activity as judged by the observation that cells expressing these permeases accumulate lactose to about the same extent as cells harboring pGEM-4 with no *lacY* insert.

TMG is significantly permeant and is accumulated to a steady state that represents active transport followed by passive efflux (Maloney & Wilson, 1973). Therefore, at low permease activities, it is difficult to determine the sensitivity of TMG

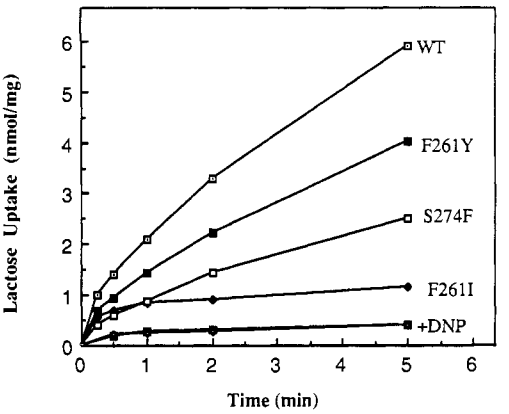

FIGURE 5: [1-^{14}C]Lactose transport by *E. coli* T184 transformed with pGEM(*lacY*) containing various mutations. Experimental conditions are described under Experimental Procedures. The lowest curve is transformed cells in the presence of 2 mM dinitrophenol. There was no significant difference between the wild type and the mutants under these conditions.

Table I: Lactose Transport Activity of Single Active Mutants[a]

residue	ΔV (Å^3)	single mutants (% wild-type activity)
R_{259}	51	P(20)
V	107	I(96), L(31), A(65), G(63)
F	27	I(8), Y(68)
G		
Y	105	S(52), C(66)
V		
T	47	M(23)
T	27	S(93)
M	107	L(78), G(90)
G		
E		
L	23	F(20)
L		
N	7	D(78)
A		
S	139	L(83), F(42), W(57)
I	51	M(78), T(60)
M	4	L(44)
F	54	V(40), Y(70)
F_{278}	105	L(66), Y(31), S(50), C(57)

[a] The single mutants are listed followed by the percent wild-type activity, based on the uptake of [1-^{14}C]lactose for 10 min. The difference in side-chain volume between the largest and smallest residue at each position is also shown.

accumulation to dinitrophenol (i.e., whether or not transport is coupled to H^+ translocation). For this reason, lactose accumulation in transformed *E. coli* T184, which is devoid of β-galactosidase, was also measured (Figure 5). Lactose transport in *E. coli* T_{184}/pGEM(*lacY*) occurs at a rate about 5–10% of that observed in cells with a single chromosomal copy of *lacY* because of the unusual expression system used here. In any case, representative permease mutants containing F261Y, S274F, and F261I transport lactose about 75%, 50%, and 25%, respectively, as well as wild-type permease. Moreover, in the presence of dinitrophenol, each of the single amino acid mutants takes up lactose to an internal concentration that approximates that of the medium. Quantitative estimates of the rates of lactose transport in each of the mutants are given in Table I. Tabulation of multiple mutants which screened positively on MacConkey/lactose plates but were not further characterized and six inactive mutants are shown in Table II.

Pattern of Mutability. Some amino acids are clearly more mutable than others, but a pattern is not obvious from Figure 2. However, the pattern of mutable amino acids is clearly visible when the replacements are plotted on a helical-wheel

Table II: Multiple and Inactive Mutants[a]

multiple replacement active mutants		inactive mutants
A273G, S274A	F261L, N272D	G262V
T266M, S274A	M267L, G268A, I275V	G268A, L270Y, A273V
V260L, T266A	R259H, I275S	G268V
T265S, T266S	T266A, F278Y	A273D
Y263Q, M267V, N272I	V260A, M267S	E269V
	R259L, M276L	M267V, F277S
I275F, F278C	T266S, F278I	
R259C, S274W	Y263H, F278C	
Y263H, F278C	R259H, M276I	

[a] Listing of the active multiple mutants and inactive mutants obtained, based on whether colonies were red or white, respectively, on MacConkey plates.

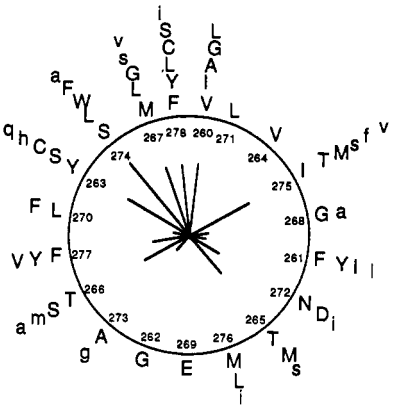

FIGURE 6: Helical-wheel plot of active mutations in putative helix VIII. The lines radiating from the center are proportional to the difference in volume between the largest and the smallest amino acid residue at that position.

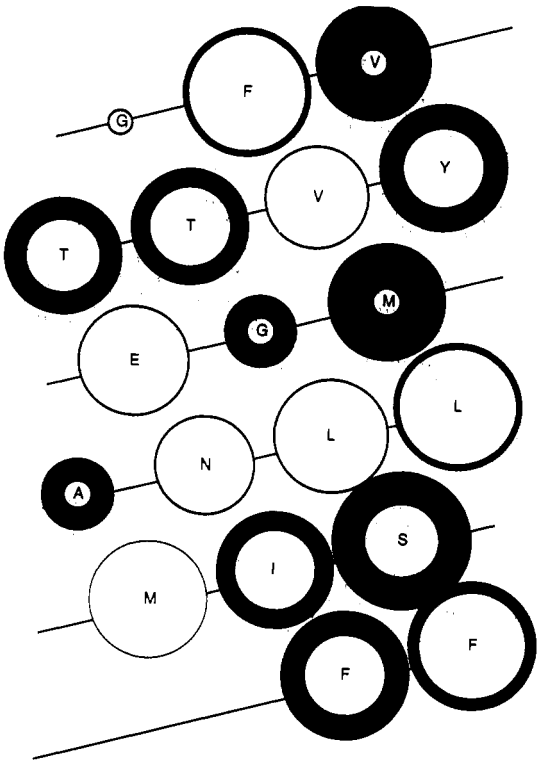

FIGURE 7: Helix plot of putative helix VIII. The letters are the wild-type amino acid sequence. The circles have radii proportional to the cube root of the side chain volume. The dark areas represent the differences between the largest and smallest amino acid residues at that position.

plot (Figure 6) or a helix plot (Figure 7). A stripe of mutable positions at the upper left side of the wheel plot consisting of positions Val260, Tyr263, Met267, Ser274, and Phe278 is the most striking feature of the results. To evaluate the extent of change tolerated more quantitatively at a given position, we have also graphed range of side-chain volumes (Zamyatnin, 1972) that occur at each position as the length of the line radiating from the center of Figure 6 and the black areas of Figure 7. When considering the range of changes observed, it should be recalled that most of the changes are caused by single base substitutions and that multiple base changes in the same codon are very unlikely.

The four positions that did not mutate to yield active permease (G262, V264, E269, and L271) are both hydrophilic and hydrophobic, and it is likely that the residues are involved in structure or catalysis (i.e., substrate and/or H^+ binding). Glu269 has been shown to be essential for activity by site-directed mutagenesis (L. Patel and H. R. Kaback, unpublished results). Gly262 was identified (Brooker et al., 1989) as the position changed to Cys in an uncoupled mutant, and G262V permease was among the negative mutants identified in this study (Table II). The two other positions at which mutations were not observed, Val264 and Leu271, are hydrophobic residues that flank the mutable stripe and may participate in structural interactions with neighboring helices. It should be noted, however, that failure to observe active permease mutants with amino acid replacements at these positions may result from insufficient screening.

Changes that occur on the less mutable face of helix VIII are relatively minor with respect to volume or hydrophobicity. The most significant changes are Asn272 to Asp, which introduces a negative charge near the middle of the membrane, and Asn272 to Ile, which is less polar and cannot participate in H bonding. Three positions, Thr265, Thr266, and Ile275, undergo significant volume changes. Position 265 remains partially polar when Thr is replaced with Met, and position 275 is adjacent to the most variable region of the helix and presumably near the inner surface of the membrane. The remaining positions exhibit only minor changes, as illustrated by the observation that the permease is active when Gly268 is replaced with Ala, but inactive when changed to Val (Figure 1 and Table II). Thus, the pattern seen is consistent with an orientation of putative helix VIII such that the mutable stripe is in contact with the membrane lipids and the positions near the mutable stripe interacting with neighboring helices.

The amino acid changes at positions 259–278 in the *lac* permease from *Klebsiella pneumoniae* relative to *E. coli* are Y263F, M267G, S274L, and F278C (McMorrow et al., 1988). These changes are all in the mutable stripe identified in this study. The amino acid changes in *Raf* permease from *E. coli* relative to *lacY* are V260A, Y263F, V264A, M267A, L270I, L271C, S274I, and F278C (Aslanidis et al., 1989). These changes are contiguous on the helical wheel plot (Figure 6) from L270 to V264, which covers the entire variable stripe identified in this study, and two flanking positions, L271 and V264, which did not change in this study. the volume changes at these two positions were quite small, 58 and 28 Å^3, respectively. moreover, theoretical analysis of helix orientation as described by Rees et al. (1989) is consistent with the experimental results described here (T. A. Consler, M. Wesson, H. R. Kaback, and D. Eisenberg, unpublished results). Thus, the major conclusion from this work, that there is a variable stripe of amino acid residues in putative helix VIII which interacts with the membrane lipids, provides experimental support for the proposal (Rees et al., 1989) that such variable regions in putative transmembrane helices may provide significant information about the structure of membrane proteins.

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