Estimating Loop−Helix Interfaces in a Polytopic Membrane Protein by Deletion Analysis†

Christopher D. Wolin and H. Ronald Kaback*

Howard Hughes Medical Institute, Departments of Physiology and Microbiology & Molecular Genetics, Molecular Biology Institute, University of California Los Angeles, Los Angeles, California 90025-1662

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ABSTRACT: Insertions of amino acids into transmembrane helices of polytopic membrane proteins disrupt helix−helix interactions with loss of function, while insertions into loops have little effect on transmembrane helices and therefore little effect on activity [Braun, P., Persson, B., Kaback, H. R., and von Heijne, G. (1997) *J. Biol. Chem. 272*, 29566−29571]. Here the inverse approach, amino acid deletion, is utilized systematically to approximate loop−helix boundaries in the lactose permease of *Escherichia coli*. Starting with deletion mutants in the periplasmic loop between helices VII and VIII (loop VII/VIII), which has been defined by immunological analysis and nitroxide-scanning electron paramagnetic resonance spectroscopy, it is shown that mutants with single or multiple deletions in the central portion of the loop retain significant transport activity, while deletion of amino acid residues near the loop−helix boundaries or within the flanking helices leads to complete inactivation. Results consistent with hydropathy analysis are obtained with loops VI/VII, VIII/IX, and IX/X and the flanking helices. In contrast, deletion analysis of loops III/IV, IV/V, and V/VI and the flanking helices indicates that this region of the permease differs from hydropathy predictions. More specifically, evidence is presented supporting the contention that Glu126 and Arg144 which are charge paired and critical for substrate binding are within helices IV and V, respectively.

The lactose permease (lac permease)[1] of *Escherichia coli* which is encoded by the *lacY* gene of the *lac* operon (*1*) is an important paradigm for membrane transport proteins that transduce free energy stored in electrochemical ion gradients into solute concentration gradients. The permease utilizes free energy released from downhill translocation of H^+ in response to an H^+ electrochemical gradient to drive accumulation of galactosides against a concentration gradient (symport) with a stoichiometry of unity (reviewed in ref *2*). Lac permease has been solubilized and purified in a completely active state (reviewed in ref *3*) and functions as a monomer (see ref *4*).

Extensive use of site-directed and Cys-scanning mutagenesis with wild-type permease and a functional mutant devoid of Cys residues shows that only six side chains are irreplaceable with respect to active transport: Glu126 (helix IV) and Arg144 (helix V) which are essential for substrate binding and Glu269 (helix VIII), Arg302 (helix IX), and His322 and Glu325 (helix X) which are involved in H^+ translocation and coupling (reviewed in ref *5*). Moreover, by using the library of mutants in conjunction with a battery of techniques which include second-site suppressor analysis, site-directed mutagenesis and chemical rescue, excimer fluorescence, engineered divalent metal binding sites, chemical cleavage, electron paramagnetic resonance (EPR), thiol cross-linking, and identification of discontinuous monoclonal antibody epitopes, a helix packing model has been formulated (reviewed in refs *5*−*8*).

Hydropathy analysis (*9*) indicates that lac permease contains 12 transmembrane 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). A large amount of experimental evidence which includes studies on an extensive series of lac permease−alkaline phosphatase fusions (*10*) supports the general features of the model (reviewed in ref *11*). However, with the exception of the periplasmic loop between helices VII and VIII (loop VII/VIII) (*12*−*14*) and the C-terminal tail (*15*−*17*), the helix−loop boundaries are based almost entirely on hydropathy analysis. In this context, it is important that hydropathy analysis does not take into account charge pairing. Thus, when mutagenesis and chemical rescue experiments indicated that Asp237 and Asp240 in helix VII are charge paired with Lys358 (helix XI) and Lys319 (helix X), respectively (*18*−*24*), the secondary structure was altered to include Asp237 and Asp240 within helix VII (*18*), and subsequently, more direct evidence for the modification was presented (*12*, *14*, *25*, *26*). In a similar vein, Glu126 (helix IV) and Arg144 (helix V) were predicted to be at the membrane−water interface at the cytoplasmic face of the membrane by hydropathy analysis. However, it has been shown recently (*27*−*30*) that in addition to playing a critical role in substrate binding, these residues are charge paired and therefore probably within the membrane.

While integral membrane proteins appear to tolerate point mutations relatively well (*5*, *31*), insertion mutations appear to be disruptive in transmembrane helical domains, but not

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* Corresponding author. Tel: (310) 206-5053. Fax: (310) 206-8623. E-mail: RonaldK@HHMI.UCLA.edu.

[1] Abbreviations: lac, lactose; EPR, electron paramagnetic resonance; Δ, deletion.

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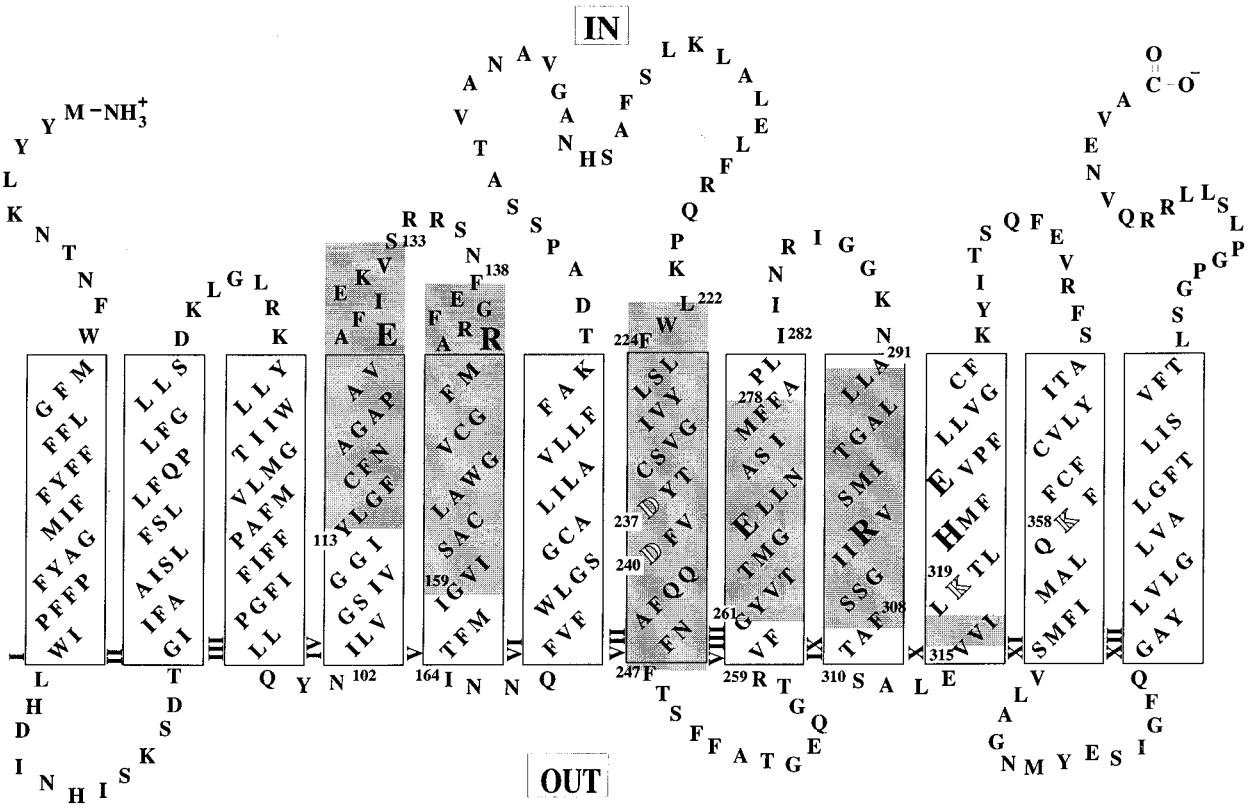

FIGURE 1: Secondary structure model of the lactose permease. The single-letter amino acid code is used, residues critical for active transport are highlighted in large bold-face type, and charge pairs Asp237/Lys358 and Asp240/Lys319 are shown in large open-face type. The shaded areas indicate modifications to the secondary structure from deletion analysis where the ends of the helices are placed at the first residues which show complete loss of active transport upon deletion.

in extramembraneous loops (*32*−*34*). On the basis of this idea, Braun et al. (*35*) studied lac permease by inserting Ala residues into transmembrane domain III in particular.

In this paper, amino acid deletions were introduced systematically into lac permease in order to approximate helix−loop boundaries, the general notion being that like insertions, deletions should be disruptive to transmembrane helices but relatively innocuous in loops. Beginning with loop VII/VIII, the N terminus of which has been clearly delineated (*12, 14*), it is shown that single or multiple deletions have relatively little effect on permease activity. On the other hand, deletion of amino acid residues over a narrow range of positions near the loop−helix boundary leads to complete loss of activity. On the basis of these observations within a defined region of the permease, other domains were studied. While findings generally consistent with hydropathy analysis are obtained in loops VI/VII, VIII/IX, and IX/X and the flanking helices, deletion analysis of loops III/IV, IV/V, and V/VI and the flanking helices indicates that Glu126 and Arg144 are located within helices IV and V, respectively, rather than at the membrane−water interface at the cytoplasmic face of the membrane (*9*). On the basis of the results, a modified secondary structure for the permease is proposed.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. [1-^{14}C]Lactose and ^{125}I-labeled Protein A were obtained from Amersham (Sunnyvale, CA). Restriction endonucleases, T4 DNA ligase, and appropriate reaction buffers were from New England Biolabs (Beverly, MA). Sequenase (modified T7 polymerase) and Sequence reaction kits were from United States Biochemicals (Cleveland, OH). Elongase Enzyme Mix and Buffer was obtained from Gibco BRL (Gaithersburg, MD). Site-directed rabbit polyclonal antiserum against a dodecapeptide corresponding to the C terminus of permease (*36*) was prepared by BabCo (Richmond, CA). Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. All other materials were reagent grade obtained from commercial sources.

Bacterial Strains and Plasmids. Subcloning grade *E. coli* XL-1blue (Stratagene Cloning Systems, Inc., La Jolla, CA) [F′:: Tn10 *proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)M15/ *recA*1 *endA*1 *gyr*A96 (Nal^r) *thi hsd*R17 ($r_k^-m_k^+$) *sup* E44 *rel* A1 *lac*] was used as a host for transformation of all PCR products and subcloning procedures. *E. coli* HB101 (Promega, Inc., Madison,WI) [F$^-$ Δ(*gpt-proA*) *leuB*6 *sup*E44 *ara*-14 *gal*k2 *lacY*1 Δ(*mcr*C-*mmr*) *rps*L20 *xyl*-5 *mtl*-1 *recA*13] was used for initial assessment of permease activity by growth on MacConkey indicator plates containing lactose. *E. coli* T184 [F′ *lacI*^{q}*O*$^+$*Z*^{U118} (*lacY*$^+$*lacA*$^+$)/*lacI*$^+$*O*$^+$*Z*$^-$*Y*$^-$(*A*$^+$), *rps*L *met thr recA hsd*MR] was used for expression of lac permease and quantitative transport assays. All deletion mutants were created in plasmid pT7-5/cassette *lacY* (EMBL-X56095) encoding wild-type lac permease.

Construction of Deletion Mutants. Amino acid residues were deleted using a modification of the inverse polymerase chain reaction (*37*). In cases where identical amino acids occur sequentially, only one codon was deleted unless stated

otherwise. Deletions were constructed by amplification of pT7-5/cassette *lacY* in the presence of increased concentrations of template and a lower number of cycles to decrease the likelihood of nonspecific mutations. In brief, unphosphorylated primers were designed where the 5′ ends flanked the codon(s) to be excluded. The entire pT7-5/cassette *lacY* (0.5 pmol) was amplified with 20 pmol of each primer and 250 μM of each deoxynucleotide by using 2 U of Elongase Enzyme Mix and 1X Buffer B as supplied by the manufacturer in a total volume 25 μL. The reaction was amplified through 12 cycles as described (*37*). After amplification, the entire reaction mixture was subjected to treatment with 20 U of DpnI for 30 min at 37 °C to selectively cleave the methylated parental template and 1.25 U of Pfu polymerase for 30 min at 72 °C to eliminate overhangs. The full-length PCR product was isolated by electrophoresis in low-melting-point agarose. The sample was kinased and ligated overnight at room temperature in a buffer containing 50 mM Tris-Cl (pH 7.7)/10 mM $MgCl_2$/7.5 mM DTT/0.3 mM ATP with the addition of 10 U of T4 polynucleotide kinase and 400 U of T4 DNA ligase. One-tenth of the ligated material was transformed into XL1-blue cells. The regions containing the desired deletions were restricted and subcloned into nonamplified pT7-5/cassette *lacY*. The sequences of the restricted fragments and ligation junctions were confirmed by double-stranded DNA sequencing after alkaline denaturation (*38*) using dideoxy chain termination (*39*).

Colony Morphology. For qualitative assessment of permease activity, *E. coli* HB101 (Y^-Z^+) was transformed with plasmid encoding a given mutant, and the cells were plated on MacConkey indicator media containing 25 mM lactose. The phenotypes were scored after a 24-h incubation at 37 °C as red (high activity), haloed (low activity), or white (no activity).

Active Transport. To assess transport activity quantitatively, *E. coli* T184 (Z^-Y^-) was transformed with a given plasmid and grown aerobically at 37 °C in Luria−Bertani broth containing streptomycin (10 μg/mL) and ampicillin (100 μg/mL). Overnight cultures of cells were diluted 10-fold and grown for 2 h. Permease expression was induced by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. After further growth for 2 h, cells were harvested by centrifugation, washed with 100 mM potassium phosphate (KP_i, pH 7.5/10 mM $MgSO_4$), and adjusted to an optical density of 10 at 420 nm in the same buffer (approximately 0.7 mg of protein/mL). Transport of [1-^{14}C]lactose (2.5 mCi/mmol; 1 mCi = 37 Mbq) at a final concentration of 0.4 mM was assayed by rapid filtration at 25 °C (*40*). Rates of transport were determined at 2 min, and the rate for cells transformed with vector containing no *lacY* insert (ca. 2.5 nmol/mg) was subtracted. The specific activities of mutants were calculated by dividing the rate of transport at 2 min (% wild-type) by expression (% wild-type). In cases where the rate was less than 2% or expression was less than 5% of wild-type, specific activities could not be calculated accurately. Steady-state levels of accumulation were determined at 60 min, and the average steady-state level of accumulation by *E. coli* T184 harboring pT7-5/cassette *lacY* was 123 nmol/mg of membrane protein. The data were corrected for accumulation by *E. coli* T184 transformed with pT7-5 with no *lacY* insert (ca. 8 nmol/mg of membrane protein). All values are expressed as percentage of the wild-type.

Quantitation of Permease. Crude membranes from the same cells utilized for active transport assays were prepared by osmotic lysis and sonication as described (*41*). Total membrane protein was assayed by a modified Lowry procedure (*42*). A sample containing 25 μg of membrane protein from each sample was separated by electrophoresis in sodium dodecyl sulfate/12% polyacylamide gels (*43*). Proteins were electroblotted onto polyvinylidene fluoride membranes (Immobilon-PVDF, Millipore) and probed with a site-directed polyclonal antibody against the C terminus of lac permease followed by treatment with [I^{125}]Protein A. After autoradiography, the permease was quantitated with a model 425F phosphorimager (Molecular Dynamics) (*12*).

RESULTS

Loop VII/VIII. A series of mutants was constructed in which codons 245−262 were deleted individually, and downhill lactose translocation was assayed qualitatively by transforming *E. coli* HB101 (Z^+Y^-) which expresses active β-galactosidase but carries a defective *lacY* gene on MacConkey indicator plates containing lactose. Cells transformed with plasmids encoding functional permease allow access of external lactose to cytosolic β-galactosidase, and subsequent metabolism of the monosaccharides causes acidification and the appearance of red colonies on indicator plates containing lactose. Cells devoid of permease activity appear as white colonies, and permease mutants with low activity form red colonies with a white halo. HB101 expressing deletions at positions 245−248 (Δ245 to Δ248) or 260−262 (Δ260 to Δ262) appears as white or haloed colonies, indicating no or little ability to translocate lactose downhill (Table 1). The remainder of the deletion mutants from Δ249 to Δ259 grow as red colonies indistinguishable from wild-type permease.

For quantitative transport assays, *E. coli* T184 (Z^-Y^-) was transformed with plasmid encoding a given mutant and assayed for [1-^{14}C]lactose accumulation. Mutants Δ250 to Δ259 exhibit highly significant specific activities and/or steady-state levels of lactose accumulation relative to wild-type permease (Figure 2). Deletion of residue 249 or 248 causes a progressive decrease in activity, and deletion of residues 245−247 completely abolishes activity. As shown previously, Phe247 is the primary epitope determinant for monoclonal antibody 4B1 (*12*), and nitroxide-scanning EPR indicates that position 247 is at the membrane−water interface, while positions 245 and 246 are in helix VII within the membrane (*14*). Similarly, deletion of residue 259 or 260 at the C terminus of loop VII/VIII causes progressive loss of activity, and mutants Δ261 and Δ262 are completely inactive. In general, levels of permease expression in the membrane exhibit a similar profile; however, activity decreases more acutely at the N and C termini of the domain relative to expression.

Two series of constructs containing sequential multiple deletions were also created between residues 252 and 258. One set contains sequential deletions starting from residue 252 and proceeding toward the C terminus; the second set contains sequential deletions starting from residue 258 and proceeding toward the N terminus (Table 2). Mutants Δ(252−253) or Δ(252−254) and Δ(257−258) or Δ(256−258) grow as red colonies and exhibit essentially wild-type

Table 1: Phenotype of Deletion Mutants[a]

position deleted	phenotype
Loop III/IV	
110/111 G	red
112 I	red
113 Y	halo
114 L	halo
Loop IV/V	
132 V	halo
133 S	halo
134/135 R	red
136 S	red
137 N	red
138 F	halo
139 E	halo
Loop V/VI	
158 V	halo
159 G	halo
160 I	red
161 M	red
162 F	red
Loop VI/VII	
219 Q	red
220 P	red
221 K	red
222 L	halo
223 W	halo
Loop VII/VIII	
245 N	white
246/247 F	white
248 T	halo
249 S	red
250/251 F	red
252 A	red
253 T	red
254 G	red
255 E	red
256 Q	red
257 G	red
258 T	red
259 R	red
260 V	halo
261 F	halo
262 G	halo
Loop VIII/IX	
276 M	halo
277/278 F	halo
279 A	halo
280 P	halo
281 L	halo
282/283 I	halo
284 N	halo
285 R	halo
286 I	red
287/288 G	halo
289 K	halo
290 N	halo
291 A	halo
292 L	halo
Loop IX/X	
308 F	halo
309 A	halo
310 T	halo
311 S	halo
312 A	halo
313 L	red
314 E	red
315/316 V	halo
317 I	halo

[a] See Experimental Procedures.

expression and activity. Further sequential deletion in either set causes progressive loss of expression and activity. Thus, mutants Δ(252−255), Δ(252−256), and Δ(255−258) exhibit

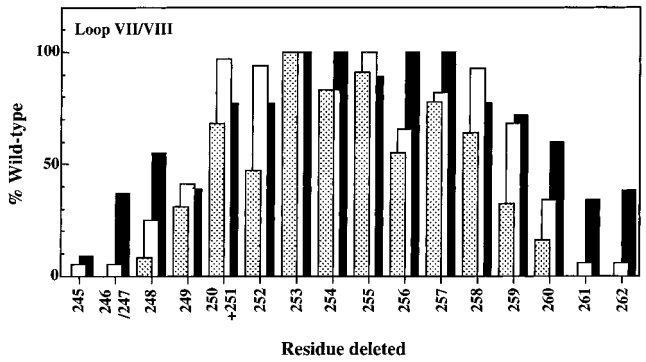

FIGURE 2: Specific activity (black stippled bars), steady-state levels of lactose transport (open bars), and expression levels of lac permease (closed bars) for deletion mutants in loop VII/VIII. All assays were performed as described in Experimental Procedures. The amino acid deleted is denoted by the position within the primary sequence. In cases where identical amino acids occur sequentially, deletion of one of the residues is indicated by listing both positions separated by a slash. All mutants are single deletions with the exception of the double deletion of residues 250 and 251 (250 + 251). Mutants with single deletion of either position 250 or 251 exhibit a specific activity of 57%, a steady-state accumulation of 26%, and an expression level of 49% relative to wild-type.

Table 2: Properties of Multiple Deletion Mutants within Loop VII/VIII[a]

residues deleted	steady state	expression (% wild-type)	specific activity	phenotype
Δ(252−253)	95	118	69	red
Δ(252−254)	96	100	84	red
Δ(252−255)	82	71	41	red
Δ(252−256)	60	64	22	halo
Δ(252−257)	3.5	not detected		halo
Δ(257−258)	90	98	59	red
Δ(256−258)	100	70	99	red
Δ(255−258)	62	42	41	red
Δ(254−258)	4.8	not detected		white

[a] See Experimental Procedures

lower expression and activity, and mutants Δ(252−257) and Δ(254−258) are not expressed to a significant level and are inactive.

Loop VI/VII. Site-directed thiol cross-linking (*44*−*50*) indicates that helix VII is in close proximity to helices I, II, and V in the periplasmic third of the transmembrane domain and close to helices IV, X, and XI in the middle third. Thus, helix VII must be tilted in order to accommodate these contacts and should be longer than 20 residues in order to span the membrane. Therefore, a series of deletions at the putative C terminus of loop VI/VII was constructed. On indicator plates, mutants Δ219, Δ220, and Δ221 grow as red colonies (Table 1), permease expression is reasonable, and significant specific activities, as well as steady-state levels of lactose accumulation, are observed (Figure 3). In contrast, mutants Δ222 and Δ223 grow as haloed colonies, exhibit very low levels of expression, and show little or no activity. Therefore, as judged by deletion analysis, the N terminus of helix VII is probably located near Leu222, and since the C terminus is located at Phe247, helix VII likely contains 26 residues.

Loops VIII/IX and IX/X. The topology of these two loops is based almost exclusively on hydropathy analysis (*9*), although single lac permease−alkaline phosphatase fusions in each of these regions are consistent with placement on

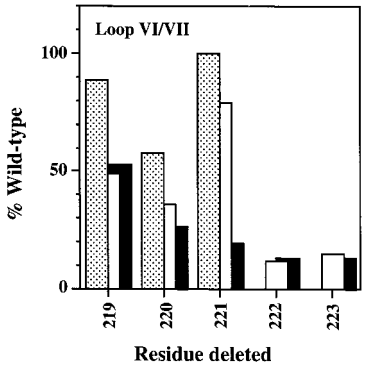

FIGURE 3: Specific activity (black stippled bars), steady-state levels of lactose transport (open bars), and expression levels of lac permease (closed bars) for deletion mutants at the C terminus of loop VI/VII. All assays were performed as described in Experimental Procedures.

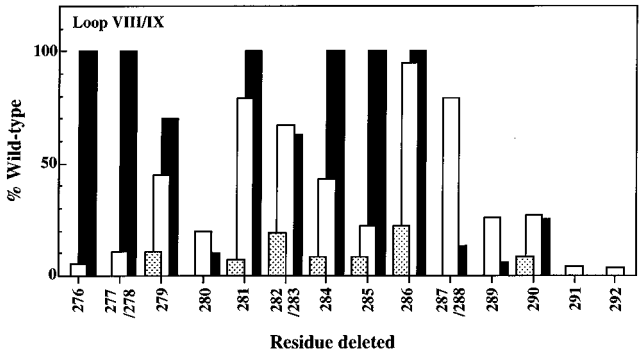

FIGURE 4: Specific activity (black stippled bars), steady-state levels of lactose transport (open bars), and expression levels of lac permease (closed bars) for deletion mutants in loop VIII/IX. All assays were performed as described in Experimental Procedures. Where identical amino acid residues occur sequentially, deletion of one of the residues is indicated by listing both positions separated by a slash.

the cytoplasmic and periplasmic faces of the membrane, respectively (*10*). Importantly, the helices connected by these loops contain four residues essential for H^+ translocation and coupling, and insertional mutagenesis causes a marked decrease in transport activity (*32*).

With the exception of mutant Δ286 which grows as red colonies on indicator plates, mutants with deletions in loop VIII/IX (Δ276 to Δ292) display a halo phenotype (Table 1). Furthermore, all of the deletion mutants within the domain exhibit markedly diminished rates of transport, and in some instances (Δ280, Δ287/288, and Δ289 to Δ292), expression is too low to allow accurate measurements of specific activity (Figure 4). However, mutants Δ279 through Δ290 accumulate lactose to significant steady-state levels of accumulation. Thus, complete loss of activity is observed only with mutants Δ276 and Δ277/278 and with the Δ291 and Δ292 mutants. Taken together with the results from loop VII/VIII, the findings are consistent with the interpretation that helix VIII contains approximately 18 residues extending from position 261 to 278.

Except for mutants Δ313 and Δ314 in loop IX/X which grow as red colonies on indicator plates, deletion of a single residue at positions 308−317 yields haloed colonies (Table 1). However, all of the mutants with the exception of Δ314 are expressed poorly and exhibit low initial rates which precludes accurate measurements of specific activity. On the other hand, mutants Δ311 to Δ314 accumulate lactose to steady states of 50% or more of wild-type (Figure 5). Mutant Δ310 catalyzes accumulation to ca. 30% of wild-type and mutant Δ309 to ca. 20% of wild-type, and mutants Δ308, Δ315/316, and Δ317 are completely devoid of activity. When considered with the findings from loop VIII/IX, the results suggest that helix IX contains 18 residues extending from position 291 to 308.

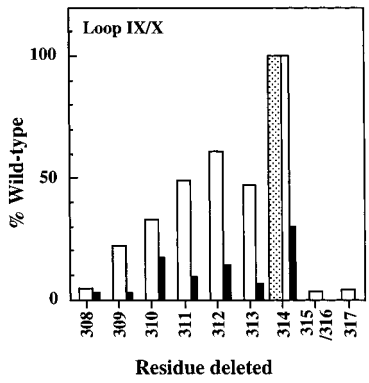

FIGURE 5: Specific activity (black stippled bars), steady-state levels of lactose transport (open bars), and expression levels of lac permease (closed bars) for deletion mutants in loop IX/X. All assays were performed as described in Experimental Procedures. Where identical amino acid residues occur sequentially, deletion of one of the residues is indicated by listing both positions separated by a slash.

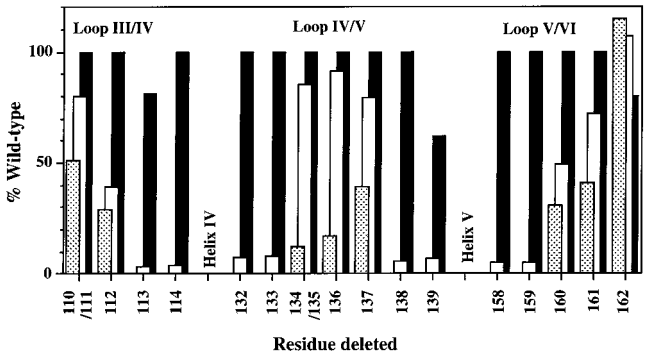

FIGURE 6: Specific activity (black stippled bars), steady-state levels of lactose transport (open bars), and expression levels of lac permease (closed bars) for deletion mutants in loops III/IV, IV/V, and V/VI. All assays were performed as described in Experimental Procedures. Where identical amino acid residues occur sequentially, deletion of one of the residues is indicated by listing both positions separated by a slash.

Loops III/IV, IV/V, and V/VI. Hydropathy analysis places Glu126 and Arg144 in loop IV/V at the cytoplasmic ends of helices IV and V, respectively, (*9*). However, the two residues are charge paired and therefore probably within the membrane (*27*−*30*). As shown in Table 1 mutants Δ134/135, Δ136, and Δ137 grow as red colonies on indicator plates, while mutants Δ132, Δ133, Δ138, and Δ139 grow as haloed colonies. Consistently, mutants Δ134/135, Δ136, and Δ137 transport lactose to steady-state levels of accumulation that approach wild-type levels, and mutants Δ132, Δ133, Δ138, and Δ139 exhibit no significant activity. In each mutant, the permease is expressed at a highly significant level (Figure 6). Thus, loop IV/V is probably shorter than predicted from hydropathy analysis, containing as few as four residues, and Glu126 and Arg144 are located within helices IV and V, respectively.

If loop IV/V is much shorter than predicted, either the flanking helices are longer or the periplasmic ends of helices IV and V differ from prediction. At the predicted N terminus of helix IV (*9*), deletion of residue 110/111 or 112 results in red colonies on indicator plates, and the mutants display significant specific activities and steady-state levels of accumulation. In sharp contrast, mutants Δ113 and Δ114 grow as haloed colonies (Table 1) and exhibit no significant activity (Figure 6). At the predicted C terminus of helix V (*9*), mutants Δ158 and Δ159 grow as haloed colonies (Table 1) with no significant activity, while mutants Δ160 to Δ162 grow as red colonies and exhibit good specific activities and steady-state levels of lactose accumulation (Figure 6). Furthermore, all of the mutants are expressed at essentially wild-type levels. The findings suggest that helix IV contains about 21 residues (from position 113 to 133) and that helix V contains about 22 residues (from position 138 to 159).

DISCUSSION

In lieu of a high-resolution 3D structure of lac permease, this laboratory has developed a battery of site-directed biochemical and biophysical techniques for determining proximity relationships that has led to a helix packing model which includes helix tilts (reviewed in refs *5, 7, 8*). In this article, amino acid deletion analysis is introduced as a technique that appears to be useful for approximating loop−helix boundaries. A simple method for such an approximation is important because hydropathy analysis does not take into account charge pairing which can result in incorrect placement of charged residues (*18*−*24, 26*−*28, 30*). Since amino acid insertions are disruptive in transmembrane helices, but relatively nondisruptive in extramembaneous loops (*32*−*34*), the same should hold for deletions, and we have chosen to use the latter approach to test the notion in detail.

Sixty single-amino-acid-deletion mutants in lac permease were constructed which cover 11 putative loop−helix boundaries. In addition, two series of multiple deletions in loop VII/VIII were studied. Generally, deletion of residues within a given loop does not abolish the ability of the permease to catalyze lactose accumulation against a significant concentration gradient, although with certain deletions, expression of the permease and/or specific activity is compromised. In contrast, deletion of amino acids at or near loop−helix boundaries causes complete loss of activity. The efficacy of the approach is most clearly demonstrated at the periplasmic interface between helix VII and loop VII/VIII where it has been demonstrated clearly by immunological studies (*12, 13*) and nitroxide-scanning EPR (*14*) that Phe247 is the primary determinant for a monoclonal antibody epitope and the first position accessible to solvent at the periplasmic end of helix VII. Consistently, mutant Δ246/247 is devoid of activity, while mutants Δ248 and Δ249 exhibit progressive increases in activity, and mutants Δ250/251 to Δ259 accumulate lactose at least 50% as well as wild-type. Deletion analysis was then extended to the N terminus of helix VII, both ends of helices VIII and IX, and the C terminus of helix X, and the results are in general agreement with those proposed by hydropathy profiling. The only significant difference is that the cytoplasmic boundary of helix VII is extended from position 216 to 222, thereby extending helix VII to include 26 residues which is consistent with the indication that the helix is tilted (*44*−*50*). On the basis of the results, the following modifications to the secondary structural model are proposed (Figure 1, shaded regions): helix VII contains 26 residues extending approximately from Leu222 to Phe246; helix VIII contains 18 residues extending approximately from Gly260 to Phe278; helix IX contains 19 residues extending approximately from Ala291 to Phe310; the N terminus of helix X approximates Val315.

Steady-state levels of accumulation observed with mutants Δ279 to Δ290 in the loop VIII/IX are significant, and mutants Δ277/278 and Δ291 are devoid of activity (Figure 4). Within this region, it is not surprising that deletion of Arg285 or Lys289 decreases activity, as these residues occupy the first and last positions of the conserved motif (R/K)-X-G-[X]-(R/K) (*51*) in which certain point mutations have been shown to compromise activity (*52*). In addition, Pro280 has been included in an expanded version of the motif, and mutagenesis experiments show that replacements for Pro280 result in decreased activity (*52*). Deletion of Pro280, Gly287, Gly288, or Lys289 within the conserved motif also causes low expression as does replacement with Cys (*53*). The specific activity of each deletion mutant in loop VIII/IX is markedly lower than observed with loop VII/VIII deletions, an effect that cannot be attributed to low expression. Insertion of two or six His residues between positions 288 and 289 impairs activity, and functional complementation of nonoverlapping permease fragments (split permease) by introducing a discontinuity between positions 282 and 283 or 287 and 288 is not observed (C. Wolin, J. Wu, and H. R. Kaback, unpublished information). Taken together, the data suggest that factors other than the length of loop VIII/IX are important for optimal activity.

Since helices IX and X contain Arg302, His322, and Glu325, residues that are irreplaceable and play a role in H^+ translocation and coupling, as well as Val315 which is conformationally active (*54*), it is noteworthy that deletions in loop IX/X exhibit any activity whatsoever. However, the ability of deletion mutants in loop IX/X to catalyze accumulation follows a regular pattern with significant steady states observed for deletion of positions 311−314, progressively decreasing activity from position 311, and a sharp decrease in activity when Val315/316 is deleted. It is also noteworthy that deletions in this short loop result in generally low levels of permease expression which lead to low rates of transport.

In contrast to the observations discussed above, the findings resulting from deletion analysis of loop IV/V and the flanking helices lead to a radical change in secondary structural model for this region of the permease. Among the last residues in the permease to be subjected to Cys-scanning mutagenesis (*27*), Glu126 (helix IV) and Arg144 (helix V) were found to be irreplaceable for activity, and subsequent studies (*28*−*30*) demonstrate that these residues are charge paired and play an essential role in substrate binding. Since charge pairs are more stable in a low dielectric, it was postulated (*28*) that Glu126 and Arg144 are within the membrane, and the data presented here support this conclusion. Thus, mutants Δ134/135, Δ136, Δ137, and Δ138 catalyze lactose accumulation to highly significant steady states, while deletions at the N or C terminus of this stretch of residues are devoid of activity, indicating that loop IV/V is considerably shorter than predicted originally (*9*). In

addition, mutant E126H/R144H binds Mn(II) at pH 7.5 with high affinity, and the analogous double-Cys mutant exhibits excimer fluorescence after labeling with pyrene, shows spin−spin interactions after nitroxide labeling (*30*), and forms a disulfide bond spontaneously (C. D. Wolin and H. R. Kaback, in preparation). Given the evidence that Glu126 and Arg144 are located within helices IV and V, respectively, the periplasmic ends of these helices were also examined by deletion analysis, and the data presented is consistent with the suggestion that helix IV contains 21 residues extending from about position 113 to 133 and helix V contains 22 residues extending from about position 138 to 159 (Figure 1).

The data as a whole are generally consistent with the idea that deletions within helical regions cause loss of function, while deletions in loops do not. However, other interpretations are possible. For instance, a residue outside the bilayer and important for the folding of a region critical for function might be essential for activity. More specifically, with respect to loop IV/V, it is possible that deletion of residues within the loop disrupts substrate binding, since the major determinants are at the interface between the flanking helices (*28*, *29*). However, the ends of helices IV and V, as estimated by deletion analysis, are consistent with the length needed to span the lipid bilayer in helical conformation. Furthermore, the inability of Glu126, Glu130, Arg142, and Arg144 (Figure 1) to react with hydrophilic amino acid-specific reagents (*55*) and cross-linking of a Cys residue at position 129 (helix IV) or 140 with Cys replacements in helix XI (*49*) or in helices VIII and X (*50*), respectively, as well as nitroxide-scanning EPR studies (M. Zhao, K.-C. Zen, W. L. Hubbell, and H. R. Kaback, unpublished observations), argue in favor of the modified secondary structure with respect to helices IV and V (Figure 1). Finally, although the possibility seems unlikely, it must be conceded that deletion of a residue from a transmembrane helix shielded from the membrane by other parts of the protein in the folded structure may make the region less sensitive to deletions than more surface-exposed spanning segments.

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REFERENCES

1. Müller-Hill, B. (1996) *The lac Operon: A Short History of a Genetic Paradigm*, Walter de Gruyter, Berlin, New York.
2. Kaback, H. R. (1986) *Physiology of Membrane Disorders*, Vol. I, Plenum, New York.
3. Viitanen, P., Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1986) *Methods Enzymol. 125*, 429−452.
4. Sahin-Tóth, M., Lawrence, M. C., and Kaback, H. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5421−5425.
5. Frillingos, S., Sahin-Tóth, M., Wu, J., and Kaback, H. R. (1998) *FASEB J. 12*, 1281−1299.
6. Kaback, H. R., Voss, J., and Wu, J. (1997) *Curr. Opin. Struct. Biol. 7*, 537−542.
7. Kaback, H. R., and Wu, J. (1997) *Q. Rev. Biophys. 30*, 333−364.
8. Kaback, H. R., and Wu, J. (1999) *Acc. Chem. Res.* (in press).
9. Foster, D. L., Boublik, M., and Kaback, H. R. (1983) *J. Biol. Chem. 258*, 31−34.
10. Calamia, J., and Manoil, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4937−4941.
11. Kaback, H. R. (1996) in *Handbook of Biological Physics: Transport Processes in Eukaryotic and Prokaryotic Organisms* (Konings, W. N., Kaback, H. R., and Lolkema, J. S., Eds.) pp 203−227, Elsevier, Amsterdam.
12. Sun, J., Wu, J., Carrasco, N., and Kaback, H. R. (1996) *Biochemistry 35*, 990−998.
13. Sun, J., Frillingos, S., and Kaback, H. R. (1997) *Protein Sci. 6*, 1503−1510.
14. Voss, J., Hubbell, W. L., Hernandez, J., and Kaback, H. R. (1997) *Biochemistry 36*, 15055−15061.
15. Roepe, P. D., Zbar, R. I., Sarkar, H. K., and Kaback, H. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3992−3996.
16. McKenna, E., Hardy, D., Pastore, J. C., and Kaback, H. R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2969−2973.
17. McKenna, E., Hardy, D., and Kaback, H. R. (1992) *J. Biol. Chem. 267*, 6471−6474.
18. King, S. C., Hansen, C. L., and Wilson, T. H. (1991) *Biochem. Biophys. Acta 1062*, 177−186.
19. Sahin-Tóth, M., Dunten, R. L., Gonzalez, A., and Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10547−10551.
20. Lee, J. L., Hwang, P. P., Hansen, C., and Wilson, T. H. (1992) *J. Biol. Chem. 267*, 20758−20764.
21. Dunten, R. L., Sahin-Tóth, M., and Kaback, H. R. (1993) *Biochemistry 32*, 3139−3145.
22. Sahin-Tóth, M., and Kaback, H. R. (1993) *Biochemistry 32*, 10027−10035.
23. Frillingos, S., and Kaback, H. R. (1996) *Biochemistry 35*, 13363−13367.
24. Voss, J., Sun, J., and Kaback, H. R. (1998) *Biochemistry 37*, 8191−8196.
25. Zen, K. H., McKenna, E., Bibi, E., Hardy, D., and Kaback, H. R. (1994) *Biochemistry 33*, 8198−8206.
26. Ujwal, M. L., Jung, H., Bibi, E., Manoil, C., Altenbach, C., Hubbell, W. L., and Kaback, H. R. (1995) *Biochemistry 34*, 14909−14917.
27. Frillingos, S., Gonzalez, A., and Kaback, H. R. (1997) *Biochemistry 36*, 14284−14290.
28. Venkatesan, P., and Kaback, H. R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9802−9807.
29. Sahin-Tóth, M., le Coutre, J., Kharabi, D., le Maire, G., Lee, J. C., and Kaback, H. R. (1998) *Biochemistry 38*, 813−819.
30. Zhao, M., Zen, K., Hubbell, W., and Kaback, H. R. (1999) *Biochemistry* (in press).
31. Wen, J. A., Chen, X., and Bowie, J. U. (1996) *Nature Struct. Biol. 3*, 141−148.
32. McKenna, E., Hardy, D., and Kaback, H. R. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11954−11958.
33. Mingarro, I., Whitley, P., Lemmon, M. A., and von Heijne, G. (1996) *Protein Sci. 5*, 1339−1341.
34. Manoil, C., and Bailey, J. (1997) *J. Mol. Biol. 267*, 250−263.
35. Braun, P., Persson, B., Kaback, H. R., and von Heijne, G. (1997) *J. Biol. Chem. 272*, 29566−29571.
36. Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., and Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672−4676.
37. Weiner, M. P., Costa, G. L., Schoettlin, W., Cline, J., Mathur, E., and Bauer, J. C. (1994) *Gene 151*, 119−123.
38. Hattori, M., and Sakaki, Y. (1986) *Anal. Biochem. 152*, 1291−1297.
39. Sanger, F., Nicklen, S., and Coulsen, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−7.
40. Kaback, H. R. (1974) *Methods Enzymol. 31*, 698−709.
41. Sahin-Tóth, M., and Kaback, H. R. (1993) *Protein Sci. 2*, 1024−1033.
42. Peterson, G. L. (1977) *Anal. Biochem. 83*, 346−356.
43. Newman, M. J., Foster, D. L., Wilson, T. H., and Kaback, H. R. (1981) *J. Biol. Chem. 256*, 11804−11808.
44. Wu, J., Voss, J., Hubbell, W. L., and Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10123−10127.

45. Wu, J., and Kaback, H. R. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 14498−14502.
46. Wu, J., and Kaback, H. R. (1997) *J. Mol. Biol. 270*, 285−293.
47. Wu, J., Hardy, D., and Kaback, H. R. (1998) *J. Mol. Biol. 282*, 959−967.
48. Wu, J., Hardy, D., and Kaback, H. R. (1998) *Biochemistry 37*, 15785−15790.
49. Wu, J., Hardy, D., and Kaback, H. R. (1999) *Biochemistry 38*, 1715−1720.
50. Wu, J., Hardy, D., and Kaback, H. R. (1999) *Biochemistry 38*, 2320−2325.
51. Henderson, P. J. (1990) *J. Bioenerg. Biomembr. 22*, 525−569.
52. Pazdernik, N. J., Jessen-Marshall, A. E., and Brooker, R. J. (1997) *J. Bacteriol. 179*, 735−741.
53. Frillingos, S., Ujwal, M. L., Sun, J., and Kaback, H. R. (1997) *Protein Sci. 6*, 431−437.
54. Jung, H., Jung, K., and Kaback, H. R. (1994) *Protein Sci. 3*, 1052−1057.
55. Page, M. G., and Rosenbusch, J. P. (1988) *J. Biol. Chem. 263*, 15906−15914.

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