**Alanine Insertion Scanning Mutagenesis of Lactose Permease Transmembrane Helices**

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A priori, single residue insertions into transmembrane helices are expected to be highly disruptive to protein structure and function. We have carried out a systematic analysis of the phenotypes associated with Ala insertions into transmembrane helices in lactose permease, a multispanning *Escherichia coli* inner membrane protein. Insertion of alanine into the center of 7 transmembrane helices was found to abolish stable integration of lactose permease into the membrane or uphill lactose transport. A more detailed Ala insertion scan was made of transmembrane helix III. The results pinpoint a central region of 2 helical turns that is crucial for lactose permease stability and/or activity. A Trp scan in this region identified 2 residues essential for lactose permease stability. From these results, it appears that transmembrane helices have differential sensitivities to single residue insertions and that such mutations may be useful for identifying structurally and/or functionally important helix segments.

Like globular proteins, integral membrane proteins appear to be highly resilient to point mutations, both in their membrane-spanning and extra-membranous domains (1). Insertion mutants, on the other hand, are expected to be generally disruptive, at least when introduced into the transmembrane α-helices (2). However, the phenotypic effects of insertion mutants in transmembrane helices have not been systematically tested nor have their potential use as a general approach to discriminate between structurally and functionally important and unimportant parts of transmembrane helices been assessed.

In an initial study, we demonstrated that Ala insertion scanning could be used to identify segments in the glycophorin A transmembrane helix that contribute to the dimerization of this protein in vitro (3). To explore the phenotypes associated with insertion mutants in large multispanning membrane proteins, we have now performed a systematic Ala insertion scan analysis of lactose permease (*lac* permease; *LacY*), a sugar transporter that catalyzes the coupled translocation of a single β-galactoside molecule with a single proton through the plasma membrane of *Escherichia coli*.

Lactose permease was chosen for this study since it is one of the best characterized integral membrane proteins to date and has become a paradigm for mutagenesis-based structure-function analysis of membrane proteins. A two-dimensional topological model with 12 putative transmembrane domains in α-helical conformation connected by hydrophilic loops has been proposed (4) and confirmed by analyses of *LacY-PhoA* fusions (5) (Fig. 1). Extensive site-directed and cysteine-scanning mutagenesis studies in which each of the 417 residues in the permease have been mutagenized reveal that only 4 residues, Glu269 (helix VIII), Arg302 (helix IX), His322 (helix X), and Glu325 (helix X), are irreplaceable with respect to active lactose transport (reviewed in Refs. 6 and 7). Moreover, site-directed excimer fluorescence, site-directed mutagenesis, and secondary suppressor studies have led to a model describing the packing of helices VII to XI (6, 7). The model has been confirmed and extended recently by engineering divergent metal binding sites (bis- or tris-His residues) within the permease (8–10), site-directed chemical cleavage (11), and site-directed spin labeling (12).

We find that insertion of alanine into 7 different transmembrane helices in lactose permease results in one of three phenotypes: rapid degradation of the protein, lack of uphill lactose transport activity, or no effects on stability and function. A detailed Ala insertion scan of transmembrane helix III, a segment previously shown to be able to accommodate cysteine residues in every position without significant effects on permease expression or activity (13), shows that a central core of 9 residues is crucial for stability and/or activity. Ala insertions outside this central core have surprisingly mild effects, and the distal segments in helix III thus appear to play only a minor role in supporting protein stability and lactose uptake activity. A Trp scan of the core region identifies residues Ile92 and Gly96 as important for the stability of the permease.

Together with our previous results for the glycophorin A transmembrane helix dimer (3), these observations suggest that single residue insertions near the center of transmembrane helices are generally disruptive to protein structure and/or function, whereas more distal segments can be less sensitive. This not only has implications for our understanding of structural stability in membrane proteins, but also suggests that Ala insertion scanning may be used to identify critical segments in transmembrane helices that can then be further analyzed by, e.g., replacement mutagenesis.

**MATERIALS AND METHODS**

*Enzymes and Chemicals*—[1-14C]Lactose, [35S]methionine, and [α-35S]dATP were from Amersham Corp. Isopropyl-β-D-thiogalactopyranoside was from Boehringer Mannheim, and phenylmethylsulfonyl fluoride was from Sigma. All enzymes were from Promega, Boehringer Mannheim (Expand high fidelity polymerase chain reaction system), and Pharmacia Biotech Inc. (T7 DNA-polymerase). Oligonucleotides were provided by Kebo Lab (Stockholm, Sweden) and MedProbe (Oslo, Norway). Quantitative BCA protein assay reagent A was from Pierce (Rockford, IL). ECL Western blotting reagent was from Amersham (Buckinghamshire, United Kingdom). Rabbit polyclonal antiserum...
against a dodecapeptide corresponding to the C terminus of lactose permease (14) was prepared by BabCo (Richmond, California).

**Western blotting.** For detection. Equal amounts of protein, as determined using the protophoresis and autoradiography or Western blotting using an anti-

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| 1 mg of lysozyme per ml. After incubation on ice for 150,000 ylmethylsulfonyl fluoride), and resuspended in 150 m

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**DNA Techniques—Alanine residues were inserted into lactose permease using an adapted version of inverse polymerase chain reaction (20) where the entire pt7–5-cassette lacY plasmid was amplified by the Expand high fidelity polymerase chain reaction system, allowing the codon for alanine to be inserted anywhere in the plasmid. To limit the stretch of DNA that had undergone amplification, the amplified plasmids were restricted in the region of the insertion, and the restricted fragments were ligated into nonamplified pt7–5-cassette lacY plasmid. After propagation in E. coli HB101, recombinant plasmid DNA was isolated, and the insertions were verified by plasmid sequencing through the insertions using T7 polymerase. Except for the bases coding for the inserted alanines, all sequences were identical to that of Cys-less cassette lacY. For Trp scanning, residues 87–96 were individually replaced by Trp residues by the insertion of double-stranded oligonucleotides with appropriate sticky ends between the AccI and PstI restriction sites flanking helix III in Cys-less cassette lacY.

**Lactose Transport—Transport of [1-14C]lactose (19 mCi/mmol; 1 Ci

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| 25 GBq; 0.4 mM final concentration) was assayed in intact cells by rapid filtration as described (21). Transport measurements were initiated 2 h after induction where the steady-state level of lactose permease in the membrane has been reached (28), and unstable mutants are present in the membrane at amounts much lower than wild type. It is thus unclear whether the reduced activity of these mutants is due to the low permease content in the membrane only or if the mutant permease is also intrinsically inactive. Unfortunately, the quantitation of the protein levels and transport activities are not sufficiently precise to allow this point to be clearly resolved.

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| X:\]

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| 17] and a sensitive Cys scanning system (22) (Fig. 3). Using this combination of high rates of lactose permease synthesis and a highly sensitive detection system, we found that all the insertion mutants were expressed, albeit weakly in some cases (e.g. the helix III mutant).

**Ala Insertion Scanning of Transmembrane Helix III—**Previous Cys scanning mutagenesis indicated that none of the residues in helix III is essential with respect to insertion, stability, or activity but that replacement of helix III with a stretch of Ala, Leu, or Phe residues completely abolishes lactose transport (24). Also, as shown above, an Ala insertion in the middle of helix III results in an unstable protein. To dissect the critical region(s) in helix III further, alanine residues were inserted at 6 positions further in the helix (Fig. 1).

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| the Ala insertion in helix I resulted in similar levels of lactose permease as seen for the wild-type protein. The Ala insertion in the loop between helices III and IV had no effect on the steady-state level of the protein in the membrane.

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| this observation but note that the very high expression levels obtained with the T7 system may play a role.

**Taken together, the immunoblotting and pulse-chase analysis results indicate that the insertion of an alanine into helices III, V, VII, IX, and XII renders lactose permease susceptible to endogenous proteolysis. The Ala insertions in the center of helices I and X seem not to disrupt interactions essential for lactose permease stability.**

**The effect of the alanine insertions on lactose uptake are shown in Fig. 4. The rates and steady state levels of lactose accumulation were very low, except for the insertion in the loop between helices III and IV. For the Ala insertions in helices III, VII, IX, and X, [14C]lactose steady-state levels were comparable to that of pT7–5 with lacY deleted (<5% of the activity of wild-type lactose permease). The Ala insertions in helices I, V, and XII resulted in steady-state lactose levels of up to 10% of that of wild type, indicating that these mutants can still transport lactose but at a reduced rate. Transport measurements were initiated 2 h after induction where the steady-state level of lactose permease in the membrane has been reached (28), and unstable mutants are present in the membrane at amounts much lower than wild type. It is thus unclear whether the reduced activity of these mutants is due to the low permease content in the membrane only or if the mutant permease is also intrinsically inactive. Unfortunately, the quantitation of the protein levels and transport activities are not sufficiently precise to allow this point to be clearly resolved.

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| In Vivo Labeling With [35S]Methionine—In vivo labeling with [35S]methionine during pulse-chase experiments was carried out as described (22). Quantitations were carried out on a Fuji BAS1000 phosphimager using the MacBas software (version 2.31).

**Membrane Preparation—Cells were centrifuged, washed in buffer A (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1.0 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride), and resuspended in 150 mM of buffer A containing 1 mg of lysozyme per ml. After incubation on ice for >1 h, the lysates were sonicated briefly and cell debris was removed by low centrifugation. The membranes were recovered by centrifugation at 150,000 × g for 30 min and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography or Western blotting using an anti-serum directed against a C-terminal fragment of LacY and ECL reagent for detection. Equal amounts of protein, as determined using the protein assay reagent A, were loaded onto the gels for the analysis by Western blotting.

**RESULTS**

**Insertion of Alanine Into the Center of Transmembrane Helices I, III, V, VII, IX, and XII**—Based on the schematic topological model of lactose permease (6, 7), single alanine residues were inserted near the center of helices I, III, V, VII, IX, X, and XII, and in the loop between helices III and IV, Fig. 1. This includes helices IX and X, which have been shown to contain residues essential for transport (6, 7), helix VII, which has been shown to be important for the folding of the permease and insertion into the membrane (23), helices III and XII, which have been shown to be structurally important (19, 22, 24, 25), helix V, which has been shown to pack closely with helices VII and VIII but where no functionally critical residues have been found (11, 12), and helix I where no critical residues have been found by Cys scanning (26) and where half of the helix can be deleted without serious effects on permease function (27).

The steady-state amount of the membrane-embedded Ala insertion mutant was assessed by SDS-polyacrylamide gel electrophoresis and subsequent immunoblotting of purified membranes, Fig. 2. Ala insertions into helices III, V, VII, IX, and XII resulted in the complete absence of lactose permease in the membrane. On the other hand, the insertion into helix X only slightly reduced the lactose permease level in the membrane, and the Ala insertion in helix I resulted in similar levels of lactose permease as seen for the wild-type protein. The Ala insertion in the loop between helices III and IV had no effect on the steady-state level of the protein in the membrane.

**To further examine the mutants lacking detectable steady-state levels of lactose permease, protein turn-over was assayed by pulse-chase experiments in a T7 RNA polymerase expression system (22) (Fig. 3). Using this combination of high rates of lactose permease synthesis and a highly sensitive detection system, we found that all the insertion mutants were expressed, albeit weakly in some cases (e.g. the helix III mutant).

The wild-type protein was not very degraded during the extended chase period, (cf. Refs. 19 and 22). In contrast, all the Ala insertion mutants except the one in helix XII had a more rapid turnover rate than the wild-type permease, consistent with their low steady-state levels. The result for the helix XII mutant was unexpected since it could not be detected by immunoblotting; at present, we have no explanation for this observation but note that the very high expression levels obtained with the T7 system may play a role.

**Taken together, the immunoblotting and pulse-chase analysis results indicate that the insertion of an alanine into helices III, V, VII, IX, and XII renders lactose permease susceptible to endogenous proteolysis. The Ala insertions in the center of helices I and X seem not to disrupt interactions essential for lactose permease stability.**

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In any case, these results suggest that all helices tested are required for the assembly of active lactose permease. Interestingly, not all of the alanine insertions result in an unstable protein, indicating either different roles for the helices or that the phenotypic effect of an insertion may depend on its exact location in the helix. To address this point, further Ala insertions were made in one of the transmembrane helices, helix III.

**Ala Insertion Scanning of Transmembrane Helix III—**Previous Cys scanning mutagenesis indicated that none of the residues in helix III is essential with respect to insertion, stability, or activity but that replacement of helix III with a stretch of Ala, Leu, or Phe residues completely abolishes lactose transport (24). Also, as shown above, an Ala insertion in the middle of helix III results in an unstable protein. To dissect the critical region(s) in helix III further, alanine residues were inserted at 6 positions further in the helix (Fig. 1).

The effect of the insertions on the steady-state amount of lactose permease in the membrane was found to be position-dependent. Insertions at amino acid position 79, 83, 87, or 96 in helix III all resulted in essentially stable mutant proteins (albeit with somewhat varying steady-state levels), whereas in-
sertions at positions 90, 91, or 93 completely eliminated the expression of lactose permease in the membrane as judged by immunoblots (Fig. 5).

The stability of the latter 3 mutants was further assayed by pulse-chase experiments. As already shown in Fig. 3, the insertion at position 90 dramatically decreased the amount of lactose permease initially inserted into the membrane relative to the wild-type protein, and the half-lives of the mutants with Ala insertion in positions 91 and 93 were clearly reduced (Fig. 6).

The effect of the Ala insertions on lactose permease activity was determined by measuring [14C]lactose uptake (Fig. 7). Uptake was abolished by insertions at positions 90, 91, and 93, whereas insertions at positions 79, 83, 87, and 96 permitted significant lactose uptake (15–50% steady-state level of that of the wild-type protein).

Loss of lactose permease activity correlated with loss of expression for alanine insertions at positions 90, 91, and 93 completely eliminated the expression of lactose permease in the membrane as judged by immunoblots (Fig. 5).

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Loss of lactose permease activity correlated with loss of expression for alanine insertions at positions 90, 91, and 93. Insertion at position 96 reduced both the level of lactose permease in the membrane and the activity to about 50% of the wild-type level. Insertion at position 97 resulted in reduced amounts of permease in the membrane (about 30% compared with wild type) and a corresponding reduction in activity (about

![Fig. 1. Schematic model of lactose permease outlining the Ala insertions.](image)

A, current model of lactose permease topology. Alanine insertions (indicated by ●) were made in central positions of helices I, V, VII, IX, X, and XII, in the loop connecting helices III and IV, and at 7 different positions in helix III. B, close-up of helix III. Residue numbers and the positions of the Ala insertions are shown. All insertions were made in the so-called cassette LacY construct lacking cysteines.

![Fig. 2. Immunoblot of Ala insertion mutants.](image)

E. coli T184 (Z-Y) was transformed with the pT7–5 vector harboring no lactose permease gene (–lacY, lane 1), the wild-type lac permease gene (wt, lane 2), or the lactose permease gene with Ala insertions in the loop connecting helices III and IV (between Asn101 and Ile102; lane 3) or near the center of helices I (between Met23 and Gly24; lane 4), III (between Pro89 and Phe90; lane 5), V (between Cys154 and Ala155; lane 6), VII (between Gly231 and Val232; lane 7), IX (between Ser304 and Val305; lane 8), X (between Phe324 and Glu325; lane 9), and XII (between Ala389 and Leu390; lane 10). Cells were harvested 16 h after induction with isopropyl-β-D-thiogalactopyranoside, and the membrane fraction was collected for analysis. Each lane was loaded with equal amounts of protein, and the blotted gel was decorated with an antiserum directed against a C-terminal fragment of lactose permease.
20% compared with wild type). In contrast, insertion at position 87 did not affect the amount of permease in the membrane but reduced the uptake activity (15% compared with wild type), indicating that residues in the N-terminal half of helix III may have an important effect on permease activity but no significant effect on permease stability.

Trp Scanning of Helix III—The Ala insertion scan indicates that the central region of helix III takes part in interactions required for the stability of lactose permease. To identify individual residues that participate in those interactions, a Trp-replacement scan of region 87 to 96 was carried out.

Trp scanning has previously been used to obtain structural information on helix-helix interactions in integral membrane proteins (29, 30). Trp is chosen because of its large size and moderately hydrophobic character with the expectation that it will be well tolerated in positions facing the lipid, but it is likely to significantly perturb helix-helix interfaces inside the protein.

The effect of the single Trp replacements in the region outlined by the Ala insertion scan is shown in Fig. 8. Interestingly, the replacement Ile92 Trp completely eliminated the stable expression of lactose permease in the membrane as judged by immunoblots (Fig. 8A), and the Trp replacement in position 96 reduced the amounts of permease in the membrane to about 10% compared with wild type. The replacement in positions 87, 88, 91, and 95 reduced lactose permease levels to about 50%, whereas the replacements in positions 89, 90, 93 and 94 did not significantly affect the lactose permease levels in the membrane (Fig. 8A). Lactose uptake measurements showed that the
Ala insertions near the center of helices III, V, VII, IX, and XII give rise to unstable mutant proteins as assessed by immunoblotting and pulse-chase analysis (the helix XII mutant is stable when highly overexpressed but not otherwise, Figs. 2 and 3), demonstrating that these helices contain packing interfaces required either for proper membrane insertion or for the formation of a membrane-embedded structure that is stable to endogenous proteolysis. Due to their instability, it cannot be determined whether the mutants with insertions in helices V and IX are intrinsically nonfunctional as well. The helix V and helix XII mutants retain marginal activity, suggesting that the Ala insertions affect stability rather than specific activity in these cases.

The detailed Ala insertion scan of helix III indicates that this helix is divided into segments, with each having distinct effects on the stability or function of the permease. Insertions in the N-terminal part of helix III, up to position 87, result in stable molecules with somewhat reduced lactose transport activity. Thus, this part of the helix does not seem to be involved in critical packing interactions but may contain a functionally important interface, as suggested previously (24). Mutants with insertions in positions 90–93 are unstable and do not carry out uphill lactose transport. This segment thus seems to contain residues indispensable for the stability (and hence function) of lactose permease. The segment after residue 96 seems to be of little importance for the packing of helix III and does not seem to contain functionally essential residues, since the mutant with an Ala insertion in position 96 is stable and has nearly wild-type lactose transport activity.

The Trp scan of region 87–96 identifies 2 residues, Ile92 and Gly96, as critical for the assembly of lactose permease. The Ile92 → Trp mutant is undetectable by Western blotting, and the level of mutant Gly96 → Trp is only 10% of the wild-type protein. It should be noted, however, that Trp may not be sufficiently different from Phe, which comprises 5 out of the 9 residues tested, to disrupt critical interactions mediated by such residues.

Gly96 in helix III of the lactose permease has been shown to be more accessible to N-ethylmaleimide in the presence than in the absence of substrate, indicating that Gly96 is not lipid exposed and that interactions involving Gly96 change during transport-induced conformational changes (24). Interestingly, Gly96 is on the same face of helix III as Ile92 (Fig. 1). Gly residues are mostly found buried between transmembrane helices in membrane proteins of known structure (35). Taken together, this suggests that Ile92 and Gly96 are part of the buried face of helix III.

With the information that position 92 is critical for the assembly of lactose permease, it may be rationalized that the Ala insertions in positions 90 and 91 result in unstable mutants because in both cases the bulky side chain of Phe is moved into the membrane at high levels but then rapidly turned over (Fig. 8B). The Trp scan thus identified 2 critical residues, Ile92 and Cys96, in the 87–96 region of helix III.

**Discussion**

How do integral membrane proteins react to single residue insertions into their transmembrane helices? Our analysis of Ala insertions in the multispansing inner membrane protein lactose permease from *E. coli* suggests that insertions near the center of the transmembrane helices are generally highly disruptive to protein structure and/or function (7 helices out of 8 tested), but that the more distal segments of a transmembrane helix can be less sensitive. A series of Leu and Val insertions in helix X of lactose permease confirms this conclusion (31), although expression levels were not reported for these mutants.

Insertions near the center of transmembrane helices I and X give rise to structurally stable proteins unable to carry out uphill lactose transport. Helix I has previously been shown to be important for the membrane insertion of lactose permease (32, 33), but no functionally essential residues have been found in this helix by replacement (26) or deletion mutagenesis (27). Our results, nevertheless, show that mutations in helix I can affect the activity of the permease. Interestingly, it has been reported that helix I undergoes a ligand-induced conformational change (34). Alanine was inserted at only one position in this helix, and thus it cannot be excluded that helix I is important for structural stability as well.

Given that helix X contains 2 functionally essential residues (His322 and Glu325), the inactivity of the Ala insertion mutant. The Trp scan of region 87–96 identifies 2 residues, Ile92 and Gly96, as critical for the assembly of lactose permease. The Ile92 → Trp mutant is undetectable by Western blotting, and the level of mutant Gly96 → Trp is only 10% of the wild-type protein. It should be noted, however, that Trp may not be sufficiently different from Phe, which comprises 5 out of the 9 residues tested, to disrupt critical interactions mediated by such residues.

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With the information that position 92 is critical for the assembly of lactose permease, it may be rationalized that the Ala insertions in positions 90 and 91 result in unstable mutants because in both cases the bulky side chain of Phe is moved into position 92. Likewise, the insertion in position 93 moves a Phe residue into the position normally occupied by Gly96, which may be the reason for its instability. The stable and active Ala insertion in position 96, in contrast, places a much smaller residue and thus less perturbing Ala residue in position 96.

The observation that Ala insertions in the N-terminal half of helix III are tolerated quite well, whereas those in the C-terminal half are not, may also be interpreted to mean that the effect of a given insertion is to displace the part of the helix nearest to the surface of the membrane (or that contributes less to the overall stability of the protein) relative to the other helices in the protein while leaving the remainder of the helix in its original position. This is similar to the structural perturbations caused by Ala insertions in an α-helix in the globular protein lysozyme, which in most cases are accommodated.
within the helix with the residues N- and C-terminal of the insertion shifted by 100° relative to each other (36).

Based on these results, we suggest that Ala insertion scans can be used to distinguish between helix sections that contain structurally important residues, which contain functionally important residues, and those that contain neither structurally nor functionally important residues. After an initial Ala insertion scan, residues in the identified critical regions may be individually targeted by replacement mutagenesis.

From an evolutionary point of view this study appears to convey an important message; that amino acid insertions in the central portion of transmembrane helices are generally highly disruptive to protein function, as the results imply. This is in contrast to substitution mutations, which are well accommodated in all parts of an integral membrane protein without significant effects on the protein’s stability and function (1, 6, 7), and to insertions into loop regions protruding outside the membrane (2, 25). In sequence alignment studies, high gap penalties should thus be imposed on transmembrane helices, similar to the position-dependent gap penalties often used in the alignment of globular proteins (37, 38).

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