Mutant Membrane Protein Toxicity*

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This report describes an extensive mutational analysis of the most carboxyl-terminal membrane-spanning sequence of Escherichia coli lac permease (TM12). In addition to identifying residues important for lactose transport function, the analysis revealed that numerous mutations made lac permease highly toxic to cells. In the most extreme cases, production of such proteins at very low steady-state levels reduced cell viability greater than 104-fold. Both frameshift and missense mutations led to toxicity, with the frameshift mutations having the strongest effects observed. The toxic missense mutations corresponded to changes in TM12 expected to interfere with membrane insertion or folding, such as the introduction of charged residues or prolines in the putative helix. The results suggest that cellular toxicity may be a relatively common consequence of mutations altering integral membrane protein folding. An analogous toxicity might contribute to the pathogenesis of several degenerative diseases caused by mutant membrane proteins, such as retinitis pigmentosa, Charcot-Marie-Tooth syndrome, and Alzheimer's disease.

Several diseases caused by mutant integral membrane proteins show dominant inheritance and are associated with tissue degeneration. These diseases include autosomal dominant retinitis pigmentosa, Charcot-Marie-Tooth disease, and Dejerine-Sottas syndrome (1–3). A simple hypothesis to help account for the pathogenesis of such diseases is that they reflect a general phenomenon in which mutations render membrane proteins toxic to cells (4).

This report describes a mutational analysis of the 12th transmembrane segment (TM12) of Escherichia coli lac permease (5, 6). Our studies identify a set of TM12 residues that tolerate a variety of substitutions without loss of transport activity. These residues exhibit an α-helical periodicity and may correspond to a side of the TM12 helix which faces the lipid bilayer. A number of missense and frameshift mutations were also identified which render lac permease toxic to cells. The toxic missense changes did not cluster on either the tolerant or sensitive face of TM12 and corresponded to changes expected to cause misfolding of the mutant proteins.

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MATERIALS AND METHODS

Bacteria and Plasmids—The strains and plasmids used in this study are listed in Table I. Plasmid pCS112 is a pBR322-based plasmid derived from pCM472 (7) which carries a modified lacY with a UGA nonsense codon corresponding to codon 386 and a StyI restriction site at the NH2-terminal end of the sequence encoding TM12 (Table I). To construct pCS112, a SepI-SepI fragment that included phoA was first deleted from pCM472. Second, the StyI site in the tet gene was removed by StyI cleavage, followed by DNA polymerase I Klenow fragment and T4 DNA ligase treatments. Third, a StyI cleavage site was introduced at a position corresponding to the NH2-terminal end of TM12, and a UGA termination codon (codon 386) and SpeI cleavage site were introduced within the sequence corresponding to TM12 by site-directed mutagenesis. The new StyI and SpeI restriction sites are unique in pCS112, and the lacY it carries is inactive because of the termination codon. Plasmid pCS111 is identical to pCS112 except that it encodes active lac permease, with a glycine codon at position 386.

Growth Media—Cells were cultured on TYE agar (per liter: 10 g of tryptone, 5 g of yeast extract, 8 g of NaCl, and 15 g of Bacto-agar (Difco)), MacConkey lactose agar (Difco), or M63 minimal medium (8) containing 0.2% glucose, 0.5% lactose, or 0.5% lactose, 0.2% glycerol, or 0.5% lactose, 0.2% glycerol.

Cassette Mutagenesis—A degenerate mixture of 80-nucleotide oligomers corresponding to LacY TM12 was synthesized (5’-ggt tca ggc GCT TAT CTg TGT CGT GGT CGG CCG Tgg CAC ACC TTA ATT Tcc GTg TGC ACC ctg ggc cc-3’, with 2.5% contamination of an equimolar mixture of all 4 bases at the positions shown in capital letters). These oligomers span two restriction sites in pCS112: the engineered StyI site (5’-CAGG), corresponding to the NH2-terminal end of TM12, and a naturally occurring BlpI site (GCTTAGC), corresponding to the COOH-terminal end of TM12. A double-stranded DNA cassette corresponding to the 80-mer mixture was synthesized essentially as described by Lim and Sauer (9). The resulting DNA was purified by elution from an 8% non-denaturing polyacrylamide gel and digested with StyI (15 units) and BpuI1102 (7.5 units) or BlpI (30 units). This fragment was mixed with pCS112 that had also been digested with StyI and BlpI and treated with T4 DNA ligase. The mixture was then digested with SpeI to render uncleavable parental pCS112 linear (and therefore poorly transformable), and the DNA was then precipitated and transformed by electroporation into CCS15. Transformants were selected on TYE agar containing 100 μg/ml ampicillin and 0.2% glucose. (The glucose is included to minimize expression of lacY.) Colonies were replica printed onto MacConkey lactose agar containing ampicillin to assess LacY activity and the efficiency of mutagenesis (cells receiving parental plasmids formed white colonies, whereas cells receiving active mutant plasmids formed red colonies) and onto TYE agar containing ampicillin and 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to detect toxic phenotypes associated with induction of LacY synthesis.

Sequence changes in TM12 were identified by the dideoxynucleotide termination DNA sequencing of plasmid DNA using a primer (5’-TAA GCA ACT GCT GAT GA-3’) hybridizing to the 5’-side of the sequence encoding TM12.

Quantitation of Lactose Permease Transport Activity—Radioactive lactose uptake assays were carried out using the ΔlacZ-lacY strain CCS19 carrying mutant plasmids. The lacZ mutation prevents metabolism of transported lactose. CCS19 maintains the lacY plasmids at reduced copy number because of a pcnB mutation, leading to a level of lac permease production such that uptake of [14C]lactose under assay conditions is linear with respect to the amount of protein/cell (10). 2 Uptake of [14C]lactose (Amersham Pharmacia Biotech) was assayed as
described (10). Cultures were grown overnight at room temperature, diluted to A$_{600}$ = 0.1 and grown at 37 °C to A$_{600}$ ~ 0.9. IPTG was then added (2 mM final), and the cells were harvested after a 45-min incubation at 37 °C. Cells were then rinsed twice in buffer (100 mM potassium phosphate, pH 7.0, 10 mM MgSO$_4$), resuspended at A$_{600}$ ~ 5.8, and [14C]lactose uptake into 50-µl cell aliquots measured as a function of time at room temperature.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Cells were harvested from cultures grown as described above for [14C]lactose uptake assays. Whole-cell protein was prepared by centrifuging 1.5 ml of culture (5 min at 5,000 × g) followed by resuspension of the cell pellet in 0.25 ml of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS) and heating to 55 °C for 10 min. Protein samples were diluted 1:20 in distilled water and quantified by Lowry assay (Sigma). Immediately before loading samples for SDS-polyacrylamide gel electrophoresis, β-mercaptoethanol was added to 5%, and the samples were again heated at 55 °C for 10 min.

Equal amounts of total protein from each sample (usually 50 µg) were subjected to SDS-polyacrylamide gel electrophoresis (9–12% gel) and heating to 55 °C for 10 min. Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, β-mercaptoethanol was added to 5%, and the samples were again heated at 55 °C for 10 min.

The sequence corresponding to the TM12 of lac permease (Fig. 1A) was mutagenized using an efficient “cassette” method (Fig. 1B) (9). The lac permease gene (lacY) gene was present in a small plasmid (pCS112) and carried a mutation creating a termination codon (UGA) corresponding to a site within TM12 (codon 386). The nonsense codon inactivates the lac permease, and pCS112 transformants of a lacZ+ lacY− strain therefore produce white (LacY−) colonies on MacConkey lactose indicator agar. To generate mutations, double-stranded oligonucleotide fragments corresponding to TM12 and derived from a degenerate mixture were inserted into pCS112 plasmids, replacing the pCS112 sequence. The mixture was transformed into CS815, a lacZ+ ΔlacY− strain that maintains pCS112 at reduced copy number because of a penB mutation (12). CS815 produces about three times as much lac permease as a strain containing a single chromosomal copy of lacY (10).3 Transformant cells that formed LacY− (red) colonies on indicator agar (MacConkey lactose) had acquired plasmids in which the inactive pCS112 TM12 sequence had been replaced by functional sequences. Transformant cells that formed LacY+ (white) colonies on the indicator agar carried either mutated plasmids encoding nonfunctional TM12 sequences or pCS112 parent plasmid that escaped mutagenesis. The parent plasmid DNA could be distinguished from mutant by the presence of a restriction site (SpeI), and cleavage by SpeI before transformation reduced the amount of parent plasmid in the mixture (Fig. 1B). Using these procedures, 35 different LacY+ and 28 different LacY− mutant plasmids were identified for analysis (Tables II and III).

Transformants resulting from the cassette mutagenesis of pCS112 were also screened for toxicity associated with mutant lac permease synthesis (see “Materials and Methods”). Transformant colonies (on MacConkey lactose or TYE agar) were replica printed onto TYE agar supplemented with IPTG and X-gal to induce high level lac expression. This medium allows the detection of LacY− cells that arise during further growth because such cells do not hydrolyze X-gal as efficiently as do LacY+ cells and therefore form light blue sectors and papillae. Toxicity was detected as decreased growth accompanied by abundant papillation of LacY− cells. 18 mutants exhibiting growth inhibition associated with lac induction were identified (Table IV).

Plasmid DNAs were sequenced to identify TM12 changes, and representatives of all mutant classes were assayed to determine rates of [14C]lactose uptake (Tables II–IV).

**Nontoxic Mutants That Retain lac Permease Activity**—The

### Table I

| Strain   | Genotype                                      | Description                                      |
|----------|-----------------------------------------------|--------------------------------------------------|
| CC815    | ΔlacY4700 · cat Δara, leu7697 phoA20 galE galK thi rpsE rpoB argE(am) penB pyrG C3512 · Tn10Δkan |                                                                                     |
| CS819    | F lacI ΔlacZ-lacY4784 · cat ΔlacX74 Δara, leu7697 phoA20 galE galK thi rpsE rpoB argE(am) penB tac z∆G |                                                                                     |
| CC1006   | F128 lacI lacZ ΔlacY4700 · cat ΔlacU169 araD139 rpsL thi penB penB tac z∆G |                                                                                     |
| CC1351   | F128 lacI lacZ ΔlacY4700 · cat ΔlacU169 araD139 rpsL thi penB penB tac z∆G |                                                                                     |
| Plasmid  | Description                                   |                                                   |
| pCM701   | pBR322-derived plasmid encoding LacY with a 31-codon insert corresponding to residue 38 (11) |                                                                                     |
| pCS311   | pBR322-derived plasmid encoding wild-type LacY |                                                                                     |
| pCS112   | pBR322-derived plasmid encoding LacY with a nonsense codon (UGA) corresponding to amino acid 386 |                                                                                     |

3 C. Stewart, unpublished results.
TM12 sequences of the mutants exhibiting significant lactose permease activity are listed in Table II. 25 of these mutants carry single amino acid substitutions, and 10 carry changes at two sites. Most of the changes are relatively conservative. These mutants formed red colonies on MacConkey lactose agar, grew significantly better than a Δ(lacY) strain on minimal lactose agar, and (when assayed) exhibited at least 54% of the wild-type rate of [14C]lactose transport. These mutants thus carry sequence changes that are tolerated without major effects on the membrane insertion, folding, or transport activities of the proteins.

From the collection of active substitutions identified, it appears that some sites in TM12 tolerate a greater variety of substitutions than do others (Table II). When TM12 is diagrammed as a helical wheel, the residues tolerating the greatest variety of substitutions cluster on one face (centered on Ile-395) (Fig. 2). Opposite this face is a cluster of 10 residues (centered on Thr-393) for which either no active substitutions or only relatively conservative active substitutions were identified. (The only exception was Y382C.) The angle of the net variation moment for the helical wheel sequences corresponds approximately to the angle of the net hydrophobic moment (Fig. 2) (13). By analogy to studies of the Rhodobacter photosynthetic reaction center (see “Discussion”), we suspect that the substitution-tolerant, hydrophobic side of TM12 faces membrane phospholipids in folded lac permease.

Nontoxic Mutants with Reduced LacY Activity—The TM12 sequences of substitution mutants exhibiting reduced lactose permease activity are shown in Table III. Although the majority of mutants that lacked all detectable lactose transport activity carried frameshift or deletion mutations, such mutants were not studied in detail.4 The substitution mutants ranged from those that appeared to be completely inactive (i.e. indistinguishable from a ΔlacY strain in lactose utilization on different media and in lactose uptake activity) to those exhibiting considerable activity (e.g. up to 19% of the rate of [14C]lactose uptake of wild-type control cells). Nearly all of the missense mutants that expressed greatly reduced activity had suffered changes that either altered intolerant residues or introduced charged residues (Table III).

Toxic Mutants—The TM12 sequences of the mutants that were toxic to cells are shown in Table IV. To quantify the growth defects of different mutants, the efficiencies of colony formation under lac-inducing conditions were measured. The tests were carried out using strains maintaining plasmids at normal (PcnB") or reduced (PcnB") copy number. (The penB mutation reduces the pBR322-based plasmid copy number approximately 10-fold (12).) Both LacY" and LacY" toxic mutations were identified (Table IV). Again, most of the completely inactive mutants carried frameshift mutations. The recovery of frameshift mutants was a surprise because the cassette mu-

4 M. Jurica and C. Stewart, unpublished data.
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**TABLE II**
Active lac permease substitution mutants

The sequence changes in different mutants are indicated. The wild-type TM12 sequence is shown, with residues that appear relatively intolerant of substitution (based on this analysis) shown in boldface type.

| TM12 | [\(^{14}\)C]Lactose uptake \(^a\) |
|------|-------------------------------|
| Wild-type | 100 |
| A | Y | L | V | L | G | L | V | A | A | A | A | A | L | G | F | T | L | I | S | V | F | T | T | L |  |
| P | C | C | F | V | Q | A | L | G | V | V | A | L | S | V | I | F | V | S | L | F | S | S |  |
| 381 | 382 | 383 | 384 | 385 | 386 | 387 | 388 | 389 | 390 | 391 | 392 | 393 | 394 | 395 | 396 | 397 | 398 | 399 | 400 |  |
| 71 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 |  |

*\(^{14}\)C*Lactose uptake was determined for mutant plasmids in strain CC819 as described under “Materials and Methods.” An isogenic Δ(lacY) mutant showed 1% the lactose transport activity of the lacY \(^a\) strain. ++++, estimated to correspond to ~54% wild-type activity based on the behavior of mutants on MacConkey lactose and M63 lactose media. Dots correspond to positions with wild-type residues.

Levels of Toxic and Nontoxic Proteins—The steady-state cellular levels of missense mutant proteins were measured by Western blotting using an antibody recognizing a COOH-terminal epitope of lac permease (Fig. 3A; Tables III and IV) (see “Materials and Methods”). The amounts of protein detected from toxic missense mutants varied from being comparable to wild-type to undetectable (i.e., less than approximately 2% of wild-type) (Fig. 3A and Table IV). The results imply that the COOH-terminal epitope is susceptible to cellular degradation in some of the mutants; it is possible that the rest of the protein is degraded in such mutants as well. Because the antisera used to detect missense proteins could not recognize frameshift mutant proteins due to the altered COOH-terminal sequence, toxic frameshift mutations were subcloned into a plasmid (pCM701) that encodes an epitope-tagged lac permease (11). The tag is a 31-amino acid insertion in the most NH2-terminal periplasmic segment of the protein, and the insert does not significantly reduce lactose transport or the steady-state cellular level of the protein (11). Using antisera recognizing the epitope, we observed that all of the toxic frameshift products were either undetectable or were detected at a greatly reduced level relative to the parent (Fig. 3B and Table IV). Nontoxic frameshift mutant proteins were also undetectable in analogous tests (not shown). Earlier studies of COOH-terminal truncation mutants using a method that did not involve antibody binding showed that loss of TM12 residues resulted in cellular proteolysis of the mutant proteins (14, 15).
DISCUSSION

This report describes an extensive mutational analysis of the most COOH-terminal transmembrane sequence (TM12) of E. coli lac permease. There were two principal findings of the study. First, about half of the sites in TM12 tolerated a variety of residues without loss of transport activity. These tolerant sites were arranged with a helical periodicity and may make up part of the lipid-facing surface of the protein. Second, a number of mutations made the altered lac permeases highly toxic to cells. The frameshift and missense changes leading to toxicity are expected to cause protein folding defects, a result suggesting that integral membrane protein misfolding may, in many cases, inhibit growth.

A number of TM12 substitutions had small effects on lac permease transport activity. When TM12 is represented as an α-helix, the sites most tolerant of substitutions cluster on one face, which is also the most hydrophobic face of the helix (Fig. 2). Studies of sequence polymorphisms of the Rhodobacter photosynthetic reaction center have shown that residues facing the lipid bilayer are less conserved and more hydrophobic than residues internal to the folded protein (13). By analogy, we assume that the substitution-tolerant, hydrophobic side of TM12 faces the surface in folded lac permease. Studies of lac permease TM8 (16) and TM10 (17) also identified substitution-tolerant sites showing helical periodicity which were proposed to face the surface of the protein.

Substitutions in TM12 which reduced lactose transport activity affected both tolerant and intolerant sites in the putative helix (Table III). The substitutions affecting tolerant sites were generally highly nonconservative (such as the introduction of charged residues in V384E and G391D) and are expected to cause generalized defects in membrane insertion and/or folding. Most of the inactivating substitutions markedly decreased the steady-state recovery of protein (Table III), suggesting that defective folding frequently leads to cellular proteolysis. Few inactivating changes altering TM12 have been identified previously (18–20). The earlier studies found that L385R, G386C, and L400C inactivated lac permease, whereas all of the other residues of TM12 could be converted individually into cysteine without decreasing transport greatly (19, 20).

The most surprising finding of this study was the number and variety of mutations affecting TM12 which made lac permease toxic to cells. The toxic effects were in some cases quite dramatic, reducing plating efficiencies of cells carrying mutant plasmids at low copy number greater than 104-fold. In addition, many of the toxic mutant proteins were present in cells at very low levels (undetectable in Western blotting), implying very high toxicity per molecule. The most toxic changes were frameshift mutations at sites corresponding to the sixth periplasmic domain (P6) of lac permease. Because nonsense mutations (and some frameshift mutations) corresponding to P6 did not cause dramatic growth inhibition (Table IV and data not shown), it appears that the frameshift sequence synthesized in place of TM12 contributes to whether the resulting mutant protein is toxic or not. The substitution mutations that were toxic corresponded to changes expected to favor unfolding of the protein.
The hydrophobic moment ($m$) and variation moment were calculated according to Rees et al. (13). The introduction of charged residues or proline, changes of glycine, or substitutions at “intolerant” sites (Table IV). Many of the toxic substitution mutant proteins retained significant transport activity (Table IV), suggesting that in some cases, toxicity is a more sensitive measure of misfolding than is loss of transport activity.

Several previous studies have documented instances of mutant membrane protein toxicity. For example, it was observed that a number of hybrid lac permease-alkaline phosphatase proteins inhibited growth, with toxicity increasing with the length of the hybrid (7). Substitution and in-frame insertion mutations of lac permease and the $E. coli$ serine chemoreceptor have also been observed previously to inhibit growth (11, 18, 28). Table IV presents in vivo transport assays for hybrid lac permease proteins expressed in $E. coli$ strain CC1351 carrying pCS111 (encoding wild-type LacY), pBR322 (vector plasmid), or pCS112 encoding the indicated substitution mutants. Whole-cell protein ($\sim 80 \mu$g) derived from the PcnB$^+$ strain CC1006 carrying pCM701 (encoding epitope-tagged wild-type LacY), pBR322 (vector plasmid), or pCM701 with the indicated frameshift mutations was analyzed by Western blotting using antibody directed against the COOH-terminal region of lac permease (see “Materials and Methods”). An isogenic $\Delta$(lacY) mutant showed 1% the lac permease activity of the lacY$^+$ strain.

FIG. 2. Helical wheel representation of active lac permease substitutions. The sequence changes presented in Table II are shown. The hydrophobic moment ($\mu$H) and variation moment ($\mu$V) are indicated. The hydrophobic moment was calculated according to Eisenberg et al. (26) using the hydrophobicity scale of Engelman et al. (27). The variation moment was calculated according to Rees et al. (19).

A. lysogene $\times$ wild-type strain; B. frameshift mutants. Whole-cell protein ($\sim 80 \mu$g) derived from the PcnB$^+$ strain CC1351 carrying pCS111 (encoding wild-type LacY), pBR322 (vector plasmid), or pCS112 encoding the indicated substitution mutants was analyzed by Western blotting using antibody directed against the COOH-terminal region of LacY. Panel A, frameshift mutants. Whole-cell protein ($\sim 80 \mu$g) derived from the PcnB$^+$ strain CC1351 carrying pCS111 (encoding wild-type LacY), pBR322 (vector plasmid), or pCM701 with the indicated frameshift mutations was analyzed by Western blotting using antibody directed against the 31-amino acid epitope. *, an unidentified protein that cross-reacts with the epitope antibody.

FIG. 3. Cellular levels of toxic lac permeases. Panel A, missense mutants. Whole-cell protein ($\sim 80 \mu$g) derived from the PcnB$^+$ strain CC1351 carrying pCS111 (encoding wild-type LacY), pBR322 (vector plasmid), or pCS112 encoding the indicated substitution mutants was analyzed by Western blotting using antibody directed against the COOH-terminal region of LacY. Panel B, frameshift mutants. Whole-cell protein ($\sim 80 \mu$g) derived from the PcnB$^+$ strain CC1351 carrying pCM701 (encoding epitope-tagged wild-type LacY), pBR322 (vector plasmid), or pCM701 with the indicated frameshift mutations was analyzed by Western blotting using antibody directed against the 31-amino acid epitope. *, an unidentified protein that cross-reacts with the epitope antibody.
21), as have overproduced substitution mutants of yeast proton ATPase (22).

A simple model for mutant membrane protein toxicity is that it results from disruption of the lipid bilayer by polypeptides that insert into the bilayer but are unable to fold correctly. The resulting breakdown of the permeability barrier would kill cells or slow their growth. The effect might be analogous to that observed when cells are exposed to natural or synthetic amphipathic peptides that disrupt membranes (4, 23, 24).

Several degenerative diseases in humans are known to be caused by mutant integral membrane proteins, including retinitis pigmentosa and Charcot-Marie-Tooth syndrome (1–3). Many of the mutations associated with these diseases are analogous to those that make lac permease toxic. For example, mutations in the rhodopsin gene which introduce charged residues or prolines in transmembrane segments or cause frameshifts may lead to retinitis pigmentosa (1, 2). Recent studies have also suggested that frameshift derivatives of \( \beta \)-amyloid protein may contribute to Alzheimer’s disease (25). These similarities suggest that the pathogenesis of such degenerative diseases could include toxic effects analogous to those observed with mutant lac permeases.

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