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Sequential truncation of the lactose permease over a three-amino acid sequence near the carboxyl terminus leads to progressive loss of activity and stability

EDWARD MCKENNA, DOROTHY HARDY, JOHN C. PASTORE, AND H. RONALD KABACK*

Howard Hughes Medical Institute, Department of Physiology, Molecular Biology Institute, University of California, Los Angeles, CA 90024-1570

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ABSTRACT **Previous experiments are consistent with the notion that residues 396–401 (. . . SVFTLS . . .) at the carboxyl terminus of the last putative transmembrane helix of the lactose (*lac*) permease of *Escherichia coli* are important for protection against proteolytic degradation and suggest that this region of the permease may be necessary for proper folding. Stop codons (TAA) have now been substituted sequentially for amino acid codons 396–401 in the *lacY* gene, and the termination mutants were expressed from the plasmid pT7-5. With respect to transport, permease truncated at residue 396 or 397 is completely defective, while molecules truncated at residues 398, 399, 400, and 401, respectively, exhibit 15–25%, 30–40%, 40–45%, and 70–100% of wild-type activity. As judged by pulse–chase experiments with [^{35}S]methionine, wild-type permease or permease truncated at residue 401 is stable, while permease molecules truncated at position 400, 399, 398, 397, or 396 are degraded at increasingly rapid rates. The findings indicate that either the last turn of putative helix XII or the region immediately distal to helix XII is important for proper folding and protection against proteolytic degradation.**

The lactose (*lac*) permease of *Escherichia coli* is a hydrophobic, polytopic cytoplasmic membrane protein that catalyzes the coupled translocation of a single β-galactoside with a single H^+ (i.e., symport or cotransport). Encoded by the *lacY* gene, the permease has been solubilized from the membrane, purified, reconstituted into proteoliposomes, and shown to be solely responsible for β-galactoside transport (see refs. 1 and 2 for reviews). Based on circular dichroism studies and hydropathy analysis (3), a secondary structure for the permease was proposed (Fig. 1) in which the polypeptide has a short hydrophilic amino terminus, 12 transmembrane hydrophobic domains in α-helical configuration connected by more hydrophilic loops, and a 17-residue hydrophilic carboxyl-terminal tail. Evidence confirming some of the general features of the model and demonstrating that both the amino and carboxyl termini are on the cytoplasmic face of the membrane has been obtained from laser Raman (4) and Fourier transform infrared (P. D. Roepe, H.R.K., and K. J. Rothschild, unpublished data) spectroscopy, from limited proteolysis (5, 6), binding studies with monoclonal (7–10) and site-directed polyclonal antibodies (11–15), and chemical labeling (16). Moreover, exclusive support for the topological predictions of the 12-helix motif has been obtained recently by analyzing a large number of *lac* permease–alkaline phosphatase (*lacY–phoA*) fusion proteins (17).

Stochaj *et al.* (18) demonstrated that sequences within the first 170 amino acid residues of *lac* permease are important for insertion. Thus, a truncated permease containing only the amino-terminal 50 amino acid residues is inserted into the membrane, and it was proposed that this region contains an internal "start transfer" sequence. Conversely, Roepe *et al.* (19) showed that although the 17-amino acid carboxyl-terminal tail of the permease is not involved in insertion of the protein into the membrane, its stability, or its ability to catalyze transport, a 5-amino acid segment near the carboxyl terminus of putative helix XII (residues 396–400; Fig. 1) is important for stability and hence activity once the protein is inserted into the membrane. However, the question of whether one or more of the residues between 396 and 400 are responsible for the phenomena was not resolved. In any case, it appears that recognition sequences at the amino terminus of the permease are required for membrane insertion, whereas the carboxyl terminus of putative helix XII is necessary for proper folding and protection against proteolysis once the permease is inserted.

In this communication, we demonstrate that permease sequentially truncated at positions 396–401 exhibits a progressive increase in transport activity and lifetime after insertion into the membrane. The results are consistent with the idea that the interface between putative transmembrane helix XII and the carboxyl-terminal tail of the permease is important both for the folding of the permease into an active conformation and for protection against proteolysis.

MATERIALS AND METHODS

Materials. [1-^{14}C]Lactose, deoxyadenosine 5′-[α-[^{35}S]thio]-triphosphate, and L-[^{35}S]methionine were purchased from Amersham. Isopropyl β-D-thiogalactopyranoside (IPTG) was from Boehringer Mannheim. Deoxyoligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and were used without further purification. *Hin*dIII and *Sty* I restriction endonucleases, polynucleotide kinase, and T4 DNA polymerase were from New England Biolabs. Sequenase (modified T7 DNA polymerase) and Sequenase reaction kits were from United States Biochemical. All other materials were reagent grade and were obtained from commercial sources.

Bacterial Strains. The following strains of *E. coli* K-12 were used: T206 [$lacI^+O^+Z^-Y^-(A)$, *rpsL*, *met*$^-$, *thr*$^-$, *recA*, *hsdM*, *hsdR*/F′: $lacI^qO^+Z^{U118}(Y^+A^+)$] harboring plasmid pGM21 [$lac\Delta(I)O^+P^+\Delta(Z)Y^+\Delta(A)$, *tet*^{r}] (20); T184 (T206 cured of plasmid pGM21) (20); HB101, *hsdS20* (r_B^-, m_B^-), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(*Sm*^{r}), *xyl-5*, *mtl-1*, *supE44*, λ^-/F$^-$ (21); CJ236, *dut-1*, *unf-1*, *thi-1*, *relA-1* harboring plasmid pCJ105 (*Cm*^{r}); and MV1190, Δ(*lac-proAB*), *thi*, *supE*, Δ(*srl-recA*)*306*::Tn*10* (*tet*^{r})[F′: *traD36*, *proAB*, $lacI^qZ\Delta M15$] (Muta-Gene mutagenesis kit; Bio-Rad Laboratories).

Site-Directed Mutagenesis. All site-specific mutations were directed by synthetic oligodeoxynucleotide primers complementary to the antisense strand of the *lacY* gene cloned into

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Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

*To whom reprint requests should be addressed.

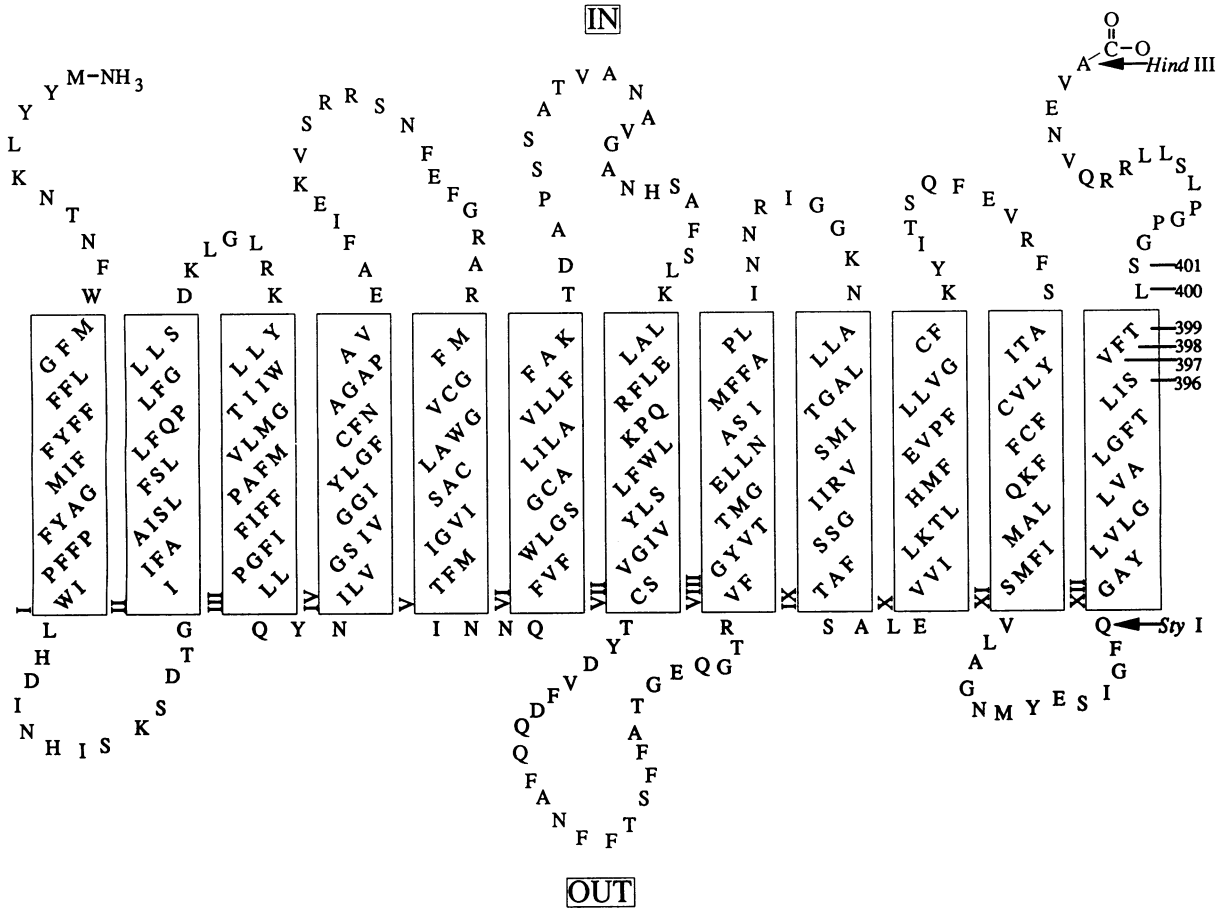

FIG. 1. Secondary-structure model of *lac* permease based on the hydropathy plot of the deduced amino acid sequence (3). Positions of amino acid residues 396–401 are indicated at the carboxyl terminus of putative transmembrane helix XII. The approximate locations of the *Sty* I and *Hin*dIII restriction endonuclease sites are indicated as they would appear in the protein sequence.

M13mp18. In general, the length of the primers was 30–35 nucleotides, and the region of the mismatch was in the center of the sequence (Table 1). Premature stop codons (TAA) were substituted for amino acid codons 396, 397, 398, 399, 400, or 401 (Fig. 1). A cassette version of the *lacY* gene (cassette *lacY*) containing the *lac* promoter/operator (J. C. Pastore, J. D. Larigan, T. G. Consler, and H.R.K., unpublished data) was used for all manipulations. The cassette *lacY* gene has a unique *Sty* I site in the DNA segment encoding the base of the last putative transmembrane helix (XII) and a unique *Hin*dIII site immediately after the stop codon of *lacY* (see Fig. 1).

Mutations were made as described (22). The replicative form of M13mp18 (cassette *lacY*) was isolated from transfectants that sequenced positive for a given mutation, and the DNA fragment containing a given *lacY* truncation mutation was digested with *Hin*dIII and *Sty* I, purified by agarose gel electrophoresis and ligated into pT7-5 (cassette *lacY*) that had been restricted with *Sty* I and *Hin*dIII. The resulting plasmids were transformed into *E. coli* HB101 (Z^+Y^-) and, as a qualitative assay of permease activity, the transformants were plated on MacConkey (Difco) indicator medium containing 1% lactose. Selected colonies were grown overnight on Luria broth, and plasmid DNA was isolated by alkaline lysis.

All mutations were verified by dideoxynucleotide sequencing (23, 24) of single-stranded M13mp18 phage DNA containing the mutated *lacY* genes. Because a cassette version of *lacY* was used, only the segment excised from the replicative form of M13mp18 (cassette *lacY*) and the ligation junctions were subjected to deoxynucleotide sequencing.

Transport Assays. Active transport of [1-^{14}C]lactose was measured in *E. coli* T184 (Z^-Y^-) transformed with each of the pT7-5(cassette *lacY*) plasmids described and plasmid pPG1-2 (25). Cells were grown as described (20) from overnight cultures to an A_{420} of 0.5–0.75 (3–4 hr). After a 20-min heat shock treatment at 42°C, IPTG was added to a final concentration of 0.2 mM, and the cells were grown for an additional 90 min. Cells were harvested by centrifugation, washed in 100 mM potassium phosphate (KP_i; pH 7.5)/10

Table 1. DNA sequence analysis of *lac* permease truncation mutants

Plasmid	Mutagenic primer	Codon change
pS396t	3′-AGTGGAATTAAA**TT**CACAAGTGCGA-5′	TCC → TAA
pV397t	3′-AATTAAAGG**ATT**AAGTGCGAA-5′	GTG → TAA
pF398t	3′-TAAAGGCAC**ATT**TGCGAATCG-5′	TTC → TAA
pT399t	3′-AGGCACAAG**ATT**GAATCGCCG-5′	ACG → TAA
pL400t	3′-CACAAGTGC**ATT**TCGCCGGGC-5′	CTT → TAA
pS401t	3′-ACAAGTGCGAA**ATT**CCGGGGCCGGG-5′	AGC → TAA

The stop codon (t) is indicated by boldface letters and underlining denotes altered nucleotides.

mM $MgSO_4$, concentrated by centrifugation, diluted to an A_{420} of 10.0 in the same solution (≈0.7 mg of protein per ml), and kept on ice until use. Individual aliquots (50 μl) of the cell suspension were used for each time point, and the reactions were carried out at room temperature in plastic tubes. The assay was initiated by addition of [1-^{14}C]lactose (10 mCi/mmol; 1 Ci = 37 GBq) to a final concentration of 0.4 mM and was stopped at given times by addition of 3.0 ml of 100 mM KP_i, pH 5.5/100 mM LiCl, followed by rapid filtration through glass fiber filters (Whatman GF/F). The filters were washed once with stop solution, and radioactivity trapped on the filters was determined by liquid scintillation spectrometry. Nonspecific adsorption of radioactivity was determined by adding stop solution to a cell suspension prior to addition of radioactive lactose, followed by rapid filtration and washing as described. All transport assays with cells expressing mutant permeases were carried out in parallel with cells harboring pT7-5 (the plasmid expression vector with no *lacY* insert) and with cells harboring pT7-5(cassette *lacY*) as negative and positive controls, respectively.

***In Vivo* Labeling with [^{35}S]Methionine.** Cloned DNA was overexpressed as described (19, 25). Briefly, *E. coli* T184 harboring plasmid pGP1-2 encoding T7 polymerase and plasmid pT7-5 with a given *lacY* gene were grown at 30°C in Luria broth supplemented with streptomycin (10 μg/ml), ampicillin (50 μg/ml), and kanamycin (10 μg/ml). Overnight cultures were diluted 1:7.5 with fresh medium at 30°C and growth was continued for 4 hr. Cells were washed three times in M9 salts/1.0 mM $MgSO_4$/$FeSO_4$ (0.55 μg/ml) prewarmed to 30°C and resuspended in the same medium supplemented with 0.5% glycerol, 0.01% thiamine, and 0.005% amino acids except methionine and cysteine. The cells were grown under these conditions for 1 hr at 30°C and then heat shocked at 42°C for 15 min. Rifampicin and IPTG were added to final concentrations of 200 μM and 0.4 mM, respectively, and incubation at 42°C was continued for an additional 15 min. Labeling was initiated by adding [^{35}S]methionine (1000 Ci/mmol) to a final concentration of 25 pM, and the cells were returned to 30°C for 10 min. An aliquot of the suspension, to which phenylmethylsulfonyl fluoride (PMSF, final concentration, 0.4 mM) was added, was quick-frozen as the zero time point, excess unlabeled methionine (200 μM) was added, and, at given times, aliquots were removed and rapidly frozen.

Membrane Preparation. The cells were centrifuged, washed twice in buffer A (100 mM Tris·HCl, pH 8.0/50 mM NaCl/1.0 mM EDTA/0.4 mM PMSF), and resuspended in a small amount of buffer A containing 1 mg of lysozyme per ml. After incubation on ice for 20 min, the lysates were sonicated briefly, treated with 100 μg of DNase I in 50 mM $MgSO_4$ at room temperature for 30 min, and cell debris was removed by low-speed centrifugation. The membranes were then recovered by centrifugation at 150,000 × g_{max} for 30 min. Gel electrophoresis and autoradiography were performed as described (19).

Protein. Protein was assayed by either the method of Schaffner and Weissmann (26) or the method of Lowry *et al.* (27).

RESULTS

Verification of Mutations. As indicated in Table 1, each of the truncation mutants contained a TAA stop codon in place of a given amino acid codon, as determined by dideoxyoligonucleotide sequencing with a primer complementary to a region of pT7-5 located ≈100 nucleotides downstream from the mutations. The remainder of the *Sty* I/*Hin*dIII fragment and the ligation junctions were identical to the sequence observed in the cassette *lacY* construct (J. C. Pastore, J. D. Larigan, T. G. Consler, and H.R.K., unpublished data).

Colony Morphology. As a preliminary, qualitative test of *lac* permease activity, *E. coli* HB101 (Z^+Y^-) was transformed with pT7-5 encoding each of the truncation mutants and grown on MacConkey indicator plates. HB101 is a "cryptic" strain that produces active β-galactosidase but carries a defective *lacY* gene. Thus, cells transformed with plasmids encoding functional *lac* permease allow access of external lactose to the cytosolic β-galactosidase, and subsequent metabolism of the monosaccharides causes acidification and the appearance of red colonies. In contrast, cells unable to transport lactose appear as white colonies. HB101 expressing permease with S396t† or V397t grow as faintly pink colonies, while the other truncation mutants (F398t, T399t, L400t, and S401t) yield a red phenotype similar to wild-type *lacY*.

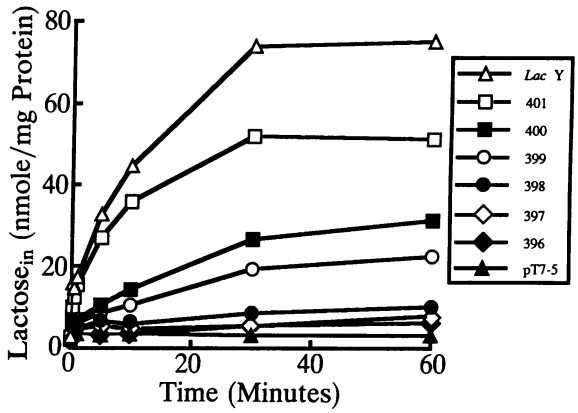

FIG. 2. Lactose transport in *E. coli* T184 harboring plasmids pGP1-2 and pT7-5 (no insert) or pT7-5 (cassette *lacY*) containing no stop codons (wild type) or given stop codons. Experiments were carried out as described, and results represent the average of three independent experiments.

Lactose Transport. Time courses of [1-^{14}C]lactose transport in *E. coli* T184 (Z^-Y^-) transformed with pGP1-2 and pT7-5 (negative control), pT7-5(cassette *lacY*) (positive control), and pT7-5(cassette *lacY*) encoding each of the truncation mutants are presented in Fig. 2. As shown, the initial rate of transport and the steady-state level of accumulation with S396t or V397t permease approximate the negative control, while F398t permease exhibits marginal activity. On the other hand, the transport activity of T399t, L400t, or S401t increases progressively from 30–40% to 40–45% to 70–100%, respectively, of the positive control (average percent initial rate and steady state of wild-type activity). In other words, as the length of the permease is extended from residue 398 to 401 (i.e., over the last turn of putative α-helix XII; Fig. 1), activity increases from essentially zero to almost full activity.

Stability of Membrane-Inserted Permeases. As shown previously (19), wild-type permease and permease devoid of the carboxyl-terminal tail (S401t) are stable, whereas permease truncated at or prior to residue 396 is proteolyzed at a rapid rate. To assess the stability of wild-type permease encoded by the cassette *lacY* gene and the truncated mutants described here, permease constructs were labeled with [^{35}S]methionine during expression from pT7-5 and membrane insertion, excess unlabeled methionine was added to the cells, and incubation was continued for up to 12 hr (Fig. 3*A*). The intensity of the radioactive bands corresponding to wild-type or S401t permease remains constant within experimental error for the entire time course of the experiment. In contrast, the radioactive bands corresponding to L400t, T399t, F398t, V397t, and S396t decrease in intensity over the same time

†Site-directed truncation mutants are designated as follows: the single-letter amino acid code is used followed by a number indicating the position of the residue in wild-type *lac* permease. The sequence is followed by a t denoting replacement of a termination codon (TAA) at this position.

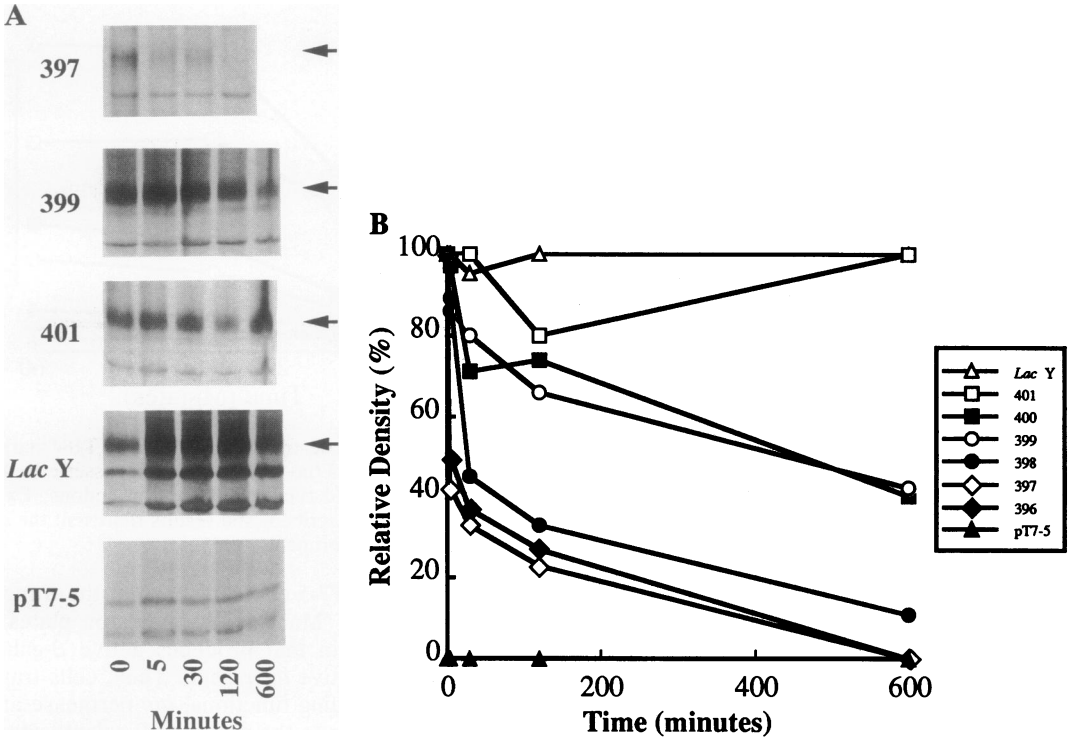

FIG. 3. Stability of wild-type *lac* permease or truncated *lac* permeases during pulse–chase experiments with [^{35}S]methionine. (*A*) Representative autoradiograms of truncation mutants. For each sample, time points were taken 0, 5, 30, 120, and 600 min after addition of excess unlabeled methionine as described. The exposure times for the autoradiograms varied in different experiments. However, it should be emphasized that the intensity of the zero-time band for *lac* permease in each of the truncation mutants is similar to that observed for the wild type when the gels are exposed for the same period of time (19). Arrow on left signifies the position of *lac* permease. The lower band (arrowhead on right) is β-lactamase, which is also observed in cells harboring plasmid pT7-5 with no *lacY* insert (lowest set of samples). (*B*) Densitometric quantitation of [^{35}S]methionine-labeled wild-type or truncated *lac* permeases. Bands corresponding to *lac* permease in autoradiograms similar to those shown in *A* were quantitated by densitometry, and the data were normalized to the zero time point in each set of samples, which was taken as 100%.

period at increasing rates. Although it is difficult to quantitate the data precisely, as indicated by densitometry (Fig. 3*B*), the half-time for disappearance of L400t, T399t, F398t, V397t, and S396t permease is about 12 hr, 12 hr, 0.5 hr, 5 min, and 5 min, respectively. It is also noteworthy that proteolytic intermediates are not observed, although peptides of <5–7 kDa are not resolved under the conditions used here for electrophoresis (i.e., 12% acrylamide).

DISCUSSION

Previous observations (19) demonstrate that the 17-amino acid carboxyl-terminal tail of *lac* permease is not important for membrane insertion, stability, or transport activity. Thus, permease truncated immediately after putative helix XII at residue 401 (see Fig. 1) is inserted into the membrane to the same extent as wild-type permease whether it is synthesized at low or high rates. In contrast, permease molecules truncated at or prior to Ser-396 are not present in the membrane when expressed at low rates from plasmid pACYC; however, when they are synthesized at high rates from the T7 promoter, the molecules are present in the membrane but are rapidly proteolyzed. In addition, permease truncated at or prior to Ser-396 is completely defective with respect to lactose/H^+ symport. Based on these observations, it was suggested that loss of activity is secondary to a defect in folding, which also results in proteolysis, and that a five-residue sequence near the carboxyl terminus of putative helix XII is required for stability. The results presented here confirm and extend these conclusions.

When *lac* permease is truncated sequentially at residues 396, 397, 398, 399, 400, and 401, a progressive increase in transport activity is observed such that activity increases from essentially zero to full activity as the protein increases in length from residue 398 to residue 401. Moreover, as the position of the truncation is moved over the same four residues, the polypeptide becomes increasingly stable in the membrane, as evidenced by the increase in half-life from ≈30 min to >12 hr. A variety of spectroscopic techniques indicate that the permease is ≈80% helical (refs. 3 and 4; P. D. Roepe, H.R.K., and K. J. Rothschild, unpublished data) and studies on an extensive series of *lac* permease–alkaline phosphatase fusion proteins (17) bear out the topological predictions of the secondary-structure model (Fig. 1). Moreover, it has been demonstrated (17) that about half of putative helices III and V (i.e., 8–11 amino acid residues) is needed to transfer alkaline phosphatase from the inner to the outer face of the membrane, thereby implying that the model may be accurate to within 3 amino acid residues. Thus, it seems reasonable to conclude that the last turn of helix XII is responsible for the properties of the truncated molecules described. It must be conceded, however, that the 3-amino acid sequence may lie immediately distal to helix XII, comprising the initial portion of the carboxyl-terminal tail of the permease. In one sense, this possibility is attractive, as incomplete transmembrane helices would be expected intuitively to be unstable and therefore inconsistent with the graded behavior of the truncation mutants described here.

Clearly, it is difficult to speculate meaningfully on the mechanism underlying the phenomena without knowledge of the folded tertiary structure of the permease in the membrane. Nonetheless, whether amino acid residues 398–400 represent the last turn of helix XII or the proximal portion of the carboxyl-terminal tail, it seems likely that this region of the protein interacts with another part of the permease in the

tertiary structure and that it is this interaction that stabilizes the polypeptide.

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