Efficient Insertion of Odd-numbered Transmembrane Segments of the Tetracycline Resistance Protein Requires Even-numbered Segments*

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Functional membrane insertion elements in the pBR322 tetracycline resistance protein were identified by comparing the ability of odd-numbered transmembrane segments and their attached periplasmic loops to insert into the membrane individually or when combined with the next even-numbered segment in the tetracycline resistance protein sequence. The efficiency with which individual odd-numbered segments and periplasmic loops inserted was probed by treating proteins truncated at the distal ends of periplasmic loops P2–P6 with carboxypeptidases and endoproteases in inside-out membrane vesicles. Insertion of odd-numbered segments and attached loops is inefficient when they occupy a C-terminal position in the protein. The C-terminal odd-numbered segment and loop sequences of 34–54% of the molecules of periplasmic loop truncation mutants could be removed by carboxypeptidase Y. In contrast, odd-numbered segments and loops insert efficiently if the next even-numbered segment in the sequence is present. In such cytoplasmic loop truncation mutants, only the cytoplasmic tail sequences of the proteins could be removed by carboxypeptidases. Remarkably, insertion of individual odd-numbered segments and loops is inefficient even though free energies for insertion of these sequences are highly favorable. The results indicate that pairs of adjacent segments, possibly “helical hairpins,” are necessary for efficient membrane insertion of the tetracycline resistance protein.

The tetracycline resistance proteins (Tet)† of Gram-negative bacteria are a group of cyttoplasmic membrane proteins that catalyze active transport of tetracycline out of bacterial cells (1). Sequence (2), mutational (3), and topology (4–6) analyses conducted with the closely related Tet proteins encoded by transposon Tn10 and pBR322 place them in the major facilitator superfamily of transport proteins (7). Members of this family contain 12 hydrophobic α-helical transmembrane segments that may be organized into two helical bundle domains of six segments each. Segments 1–6 comprise Tet domain 1, and segments 7–12 comprise domain 2 (see Fig. 1).

Transmembrane segments of the pBR322 Tet protein are quite hydrophobic (average amino acid hydrophobicity, H = +0.58–0.73 units) (8), and loops connecting segments generally are short. Proposed periplasmic loops are about 2–8 residues long and appear not to project into the periplasm since they are resistant to proteolysis (9). The third and the sixth loops (P3 and P6) lack charged amino acids, and P2 and P5 each contain a single arginine, and P1 and P4 are the most polar with 3 or more charged residues. The cytoplasmic loops are longer (~9–30 residues) and contain more charged residues, and four of the 5 loops (C1–C4) can be cleaved by several endoproteases (6). Consistent with the “positive inside” rule for predicting bacterial inner membrane protein topology (10), cytoplasmic loops are enriched with arginines and lysines compared with periplasmic loops.

Due to the lengths of periplasmic loops and the distribution of positive charges across transmembrane segments (11), the pBR322 Tet protein could insert into and translocate across the membrane independently of the cellular sec machinery (12). sec-independent insertion has been demonstrated for proteins containing periplasmic loops shorter than ~25 amino acids such as M13 procoat protein (13) and inverted leader peptidase (14). For thermodynamic reasons, sec-independent insertion is thought to occur via “helical hairpins” composed of interacting, antiparallel α-helices formed from consecutive odd- and even-numbered segments and their short intervening polar loops (15). Formation of a helical hairpin may increase the net hydrophobicity of the sequence sufficiently to drive polar loop residues across the lipid bilayer and provide a means of masking interior hydrophilic residues via association of relatively polar faces of the helices.

Experimental evidence for the helical hairpin model is limited. Inverted leader peptidase and M13 procoat, both two-segment proteins, apparently insert into the membrane via helical hairpins since deletion of the even-numbered segments of the proteins blocks sec-independent translocation of their periplasmic loops (16). However, lactose permease, a twelve-segment transport protein, can assemble in the membrane in an active form starting from co-expressed, contiguous fragments separated within the first or fourth periplasmic loops (17), suggesting that intact hairpins are not required for insertion of all segments of the transporter.

We have investigated the topology of the pBR322 Tet protein using N-terminal fusion proteins between MBP and Tet (6, 18). When a nonsecretable, signal peptide-deleted MBP322–26 domain (19) is fused at the N terminus of the Tet protein, the Tet sequence inserts normally into the inner membrane, and cells expressing the fusion protein (designated CMT10) are tetracycline-resistant. Segment and loop topology in the Tet portion of hybrid proteins can be established by protease digestion.

In this study, we investigate the nature of membrane insertion elements in pBR322 Tet using CMT10-related fusion pro-
Membrane Insertion of Tet Transmembrane Segments

**Fig. 1.** Sequence and topology of the Cmt10 fusion protein. The 12 putative transmembrane segments of the pBR322 Tet protein (bracketed residues) are connected by 6 periplasmic (P1–P6) and 5 cytoplasmic (C1–C5) loops. Cmt10-related truncation mutants analyzed by proteolysis are indicated in boxes. Fusion size standards (CMTARV, etc.; see Table I) are indicated with arrows. Cytoplasmic loops from which trypsin (T1, T2, and T3), chymotryptic (CT1), and endoproteinase LysC (KC1 and KC2) fragments are derived with arrows. The placement of amino acids is adapted from the N terminus of Tet (6).

**Experimental Procedures**

Bacterial Strains and Plasmids—MBP-Tet fusion proteins were expressed in Escherichia coli strain PR722 (F^+^ lacIZ deletion pro^+^ proC^+^ lacZ^+^) (New England Biolabs Inc.). Strains T1G1 (F^+^ traD36 proA^−^ lac^−^ lacZ^M15^ supE^3^ thi^−^ hsdR^D^ (Amersham Corp.) and JM109 (F^−^ traD36 proA^−^ lac^−^ lacZ^M15^ supE^3^ [lac-pro] thi^−^ hsdR^D^ (Amersham Corp.)) were used to propagate M13 bacteriophage. The construction of pBR322 was described elsewhere (6).

Mutagenesis of the Tet Sequence—Codon changes in tet DNA were introduced using a phosphorothioate in vitro mutagenesis procedure (21). pBR322 tet DNA (22) was cleaved in half by digestion at a SauI restriction site located in the C3 cytoplasmic loop coding sequence located between Tet domains 1 and 2 (see Fig. 1). The two halves of the tet coding sequence were cloned individually into bacteriophage M13mp18 to produce M13mp18 Tet-D1 and Tet-D2 vectors.

The Tet-D1 vector contains the HindIII-SalI fragment from plasmid pBR322, whereas the Tet-D2 vector contains a SalI-BglII fragment from the pBR322 derivative vector, pB9 (6).

Single-stranded M13 Tet-D1 and Tet-D2 DNAs were prepared and annealed to mutagenic oligonucleotides to prime synthesis of mutant DNA strands in vitro. The following oligonucleotides were used to introduce stop codons at the indicated tet codons: oligo 1104, 5'-GGACGCTAGGGCGCCGCGACT-3'; oligo P162, 5'-GCGAATTCGAT-TCTGTTG-3'; oligo W1243, 5'-TCTGCGGATGGAAGCGCAG-3'; oligo Q578, 5'-GCGAGAATGGAGCCATT-3'; oligo W501, 5'-GCGAGATCTGAAGCCATT-3'; oligo G338, 5'-AGCTCAATCTGACTGACTCTG-3'; and oligo W366, 5'-AGCAGATCTGACCGGGTT-3'. Underlined nucleotides differ from wild type. After completing in vitro mutagenesis, mutagenized double-stranded DNAs were transformed into strain T1G1. Mutations were confirmed by dideoxy chain-termination sequencing of single-stranded plasmid DNAs using the Sequenase procedure (U. S. Biochemical Corp.).

Construction of MBP-Tet Fusion Protein Expression Vectors—Tet domain 1 truncation mutant expression vectors (p1104 and pP162) were constructed by substituting BamHI-SalI fragments from mutagenized phage DNAs for the corresponding region of the pCMT10 expression vector (6). Tet domain 2 truncation mutant vectors (p2W243, pQ578, pW301, pG338, and pW366) were constructed by substituting SalI-EcoRI fragments from mutagenized phage DNAs for the corresponding region of the vector pCMT10RI. pCMT10RI was constructed from pCMT10 by converting a HindIII site downstream of the maltEU 265-tet fusion gene to an EcoRI site using EcoRI linkers (New England Biolabs Inc.).

**RESULTS**

34–44% of the C-terminal Odd-numbered Segments and Loops of Periplasmic Loop Truncation Mutants Can Be Removed by Cyp4 Digestion—Truncation mutants I104, P162, W243, W301, and W366 were constructed by substituting stop codons for codons specifying amino acids at the distal ends of periplasmic loops P2–P6 (Fig. 1 and Table I). Termination sites were selected so that truncated proteins contained polar amino acids normally found in the loops. Membrane insertion of C-terminal odd-numbered segments and loops was analyzed by subjecting truncation mutants to Cyp4 digestion in IMVs. Carboxypeptidase digests were performed using minimum mass ratio of carboxypeptidase:IMV protein required to maximally digest fusion proteins. In some cases, digestion was maximal within ≤1 h, but in other cases, 18-h incubations were required to attain equilibrium digestion. Mock carboxypeptidase digests were conducted by incubating membranes in buffers without enzymes for up to 18 h at 37°C. Cyp4 maintains ~80% of its activity in an 18-h incubation at 37°C (24).

Proteolysis reactions were heated to 95°C for 5 min in sample loading buffer containing 4% SDS and were subjected to electrophoresis on 10% acrylamide-bisacrylamide SDS gels (25) and Western immunoblotting using anti-MBP antiserum, as described previously (18). Intensities (I) of stained bands were determined by scanning immunoblots with an LKB Ultrascan XL laser densitometer. Percentages of molecules accessible to carboxypeptidases were calculated using the formula: % accessible = I(protein)/(I(protein) + I(protein)) × 100. Intensities of background bands co-migrating with digestion products were determined by scanning mock-digested lanes and were subtracted from carboxypeptidase product intensities.

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Membrane Insertion of Tet Transmembrane Segments

TABLE I

| Fusion protein | Truncation region | Tet segments | Last Tet amino acid | No. of C α-amino acids |
|----------------|------------------|--------------|---------------------|------------------------|
| CMTΔRV        | P1               | 1            | Asp<sup>24</sup>     | 2                      |
| CMTΔB         | P2               | 1–3          | Ile<sup>95</sup>     | 2                      |
| I104          | P2               | 1–3          | Arg<sup>55</sup>     | 0                      |
| CMTΔE         | C2               | 1–4          | Gly<sup>124</sup>    | 4                      |
| P162          | P3               | 1–5          | Ala<sup>161</sup>    | 2                      |
| CMTΔS         | C3               | 1–6          | Arg<sup>342</sup>    | 0                      |
| W243          | P4               | 1–7          | Lys<sup>277</sup>    | 0                      |
| Q278          | C4               | 1–8          | Gly<sup>300</sup>    | 0                      |
| W301          | P5               | 1–9          | Gln<sup>337</sup>    | 0                      |
| G338          | C5               | 1–10         | Thr<sup>365</sup>    | 0                      |
| W366          | P6               | 1–11         | —                     | —                      |

* Location of truncation sites.
* Remaining segments.
* Last amino acid in the Tet sequence.
* Number of C-terminal amino acids added to the Tet sequence by construction procedures.

Mutants because the yeast enzyme is able to hydrolyze through proline residues (28), which are present in segments 3, 5, 7, and 11. Reactions were performed for 18 h to achieve maximal digestion of proteins (see “Experimental Procedures”). In most cases, digestion end points within the Tet polypeptide chain were determined by comparing the size of products to engineered fusion protein size standards (Table I).

Treatment of the I104 truncation mutant with CpY generated a digestion product that migrated comparably with a chymotryptic fragment of I104 (C<sub>T</sub>), which serves as a size marker for MBP-Tet proteins terminating in the C1 loop (Figs. 1 and 2). The size of the CpY product indicates that the enzyme removed the P2 loop and segment 3 sequences from I104 and stopped hydrolyzing the protein in the C1 loop. Only about half of the molecules of I104 were digested, and digestion reached a maximum level in 1 h (Table II).

P162 also was digested by CpY (Fig. 2). Comparison of the size of the P162 product with the CMTΔE size standard indicates that the enzyme removed loop P3 and segment 5 from the protein before stopping in the C2 loop (Fig. 1). The percentage of P162 digested reached a maximal level in 1 h and was ~9–14% less than that obtained by digestion of I104 (Table II).

The C-terminal segments and loops of domain 2 truncation mutants, W243, W301, and W366, also were susceptible to CpY digestion (Fig. 2). In each case, 18 h of treatment with enzyme was required for maximum hydrolysis of exposed sequences (Table II). Products from W301 and W366 migrated comparably with size standards Q278 and G338, respectively, that terminate in cytoplasmic loops immediately upstream of segments 9 and 11 (Fig. 1). Again, digestion stopped before the next even-numbered segment upstream, and not all truncated molecules could be cleaved.

Mutant W243 was unique in that two digestion products were produced during an 18-h incubation with CpY (Fig. 2, lane 11). The larger product migrated just faster than the undigested protein, and the smaller product co-migrated with an endogenous degradation product present in untreated W243 membranes. The smaller product also co-migrated with the CMTΔS size standard that terminates in loop C3 (Table I). The combined amount of the two products (54%) was calculated after subtracting the contribution of the endogenous band present in the undigested sample in lane 9. The data indicate that CpY removed P4 and segment 7 from some W243 molecules but stopped digesting others before reaching the C3 loop. Segment 7 is one of the most hydrophobic segments in the protein (H = +0.71) and may resist digestion by associating with the membrane.

Unchanged by Truncation—Periplasmic loop truncation mutants were subjected to partial endopeptidolysis in IMVs to probe the membrane topology of odd-numbered segments when they are not located at the C termini of the proteins. A range of enzyme concentrations was used to generate partial digests containing fragments from all cytoplasmic loops of the Tet protein that can be cleaved (6, 18). The nonsecretable MBP domain of the fusions is resistant to digestion at the enzyme concentrations used. As in the case of carboxypeptidase digestion, cytoplasmic loops cut by enzymes were identified by comparing fragment mobilities with those of the Q278 and CMTΔS standards used. Digestion of W243, W301, and W366 with endoproteinase LysC, which hydrolyzes peptide bonds on the C-terminal side of lysines, generated fragments KC<sub>1</sub> from W301 and W366 and KC<sub>2</sub> from all three proteins (Fig. 3A).<sup>2</sup> Comparison of the mobilities of these fragments with those of the Q278 and CMTΔS standards shows that the Tet domain is cleaved at Lys<sup>272</sup> and/or Lys<sup>277</sup> in loop C4 (KC<sub>C</sub>)) and at Lys<sup>186</sup> in loop C3 (KC<sub>C</sub>) (Fig. 1). A minor endoproteinase LysC fragment that migrated between 1- and 18-h digested samples. In the case of the W243 fusion, two digestion products are present (see “Results”). The CT<sub>C</sub>, chymotryptic fragment is the lower of the two bands in lane 4 (refer to Fig. 1). The digests shown are representative of three or more digestion experiments performed with each fusion protein.

<sup>2</sup>*Endopeptidolysis fragments are labeled according to the nomenclature used for fragments produced from the full-length MBP-Tet fusion protein, CMTΔ (6).*
Digestion fragments are labeled to the Arg204, and Arg207) and two potential sites in region C2 for produced is difficult to evaluate due to migration of an endog-
produced by trypsin digestion, but the amount of the band as described in the legend to Fig. 2. Size standards shown are 37°C, and digestion fragments were analyzed by Western immunoblot-
ogenesis as described in the legend to Fig. 2. Size standards shown are (panels A and B): CMTARV and CMTAB (lane 1), CMTAE (lane 2), and CMTAS and Q278 (lane 3). Mock digested samples shown are (panels A and B): W243 (lane 4), W301 (lane 8), and W366 (lane 12). Endoproteinase LysC (KC) digests shown are (panel A): 40 μg/ml (lanes 5, 9, and 13), 80 μg/ml (lanes 6, 10, and 14), and 120 μg/ml (lanes 7, 11, and 15). Trypsin (T) digests shown are (panel B): 25 μg/ml (lanes 5, 9, and 13), 50 μg/ml (lanes 6, 10, and 14), and 75 μg/ml (lanes 7, 11, and 15). Digestion fragments are labeled to the right of the blots.

of W243 and is thought to arise from cleavage at Lys2 located immediately after MBP.

Segment topology in W243, W301, and W366 also was probed by partial digestion with trypsin (Fig. 3B). Four digestion products were produced from each mutant: 1) a doublet of fragments (T2) that migrated comparably with the C3 loop standard, CMTAS; 2) a fragment (T3) that migrated comparably with the C2 loop standard, CMTAE; and 3) a fragment (T4) that migrated between the CMTAB and CMTARV standards. There are five potential trypsin sites in region C3 for generation of the T1 doublet (Lys310, Arg210, Arg218, Arg219, and Arg327) and two potential sites in region C2 for generation of T2 (Arg322 and Arg324). The only amino acids that could give rise to the T3 fragment are Arg306 and Arg307 in region C1. It is unlikely that Arg312 in C1 or Arg319 in C3 can be cleaved because trypsin does not hydrolize peptide bonds in which arginine precedes proline (4). The combined endoproteinase LysC and trypsin digestion data indicate that segment topology is normal upstream of the C-terminal segments of the W243, W301, and W366 mutants.

Membrane topology of internal sequences in the 3- and 5-segment mutants, I104 and P162, was probed by partial digestion with trypsin and chymotrypsin. The C2 loop of P162 was cut by trypsin as indicated by production of the T5 fragment (Fig. 4). Possibly, a small amount of a T3 digestion fragment also was produced by trypsin digestion, but the amount of the band produced is difficult to evaluate due to migration of an endog-

![Fig. 3. Digestion of the W243, W301, and W366 mutants with endoproteinase LysC (panel A) and trypsin (panel B). IMVs (400 μg/ml) containing the fusions were treated with proteases for 1 h at 37 °C, and digestion fragments were analyzed by Western immunoblotting as described in the legend to Fig. 2. Size standards shown are (panels A and B): CMTARV and CMTAB (lane 1), CMTAE (lane 2), and CMTAS and Q278 (lane 3). Mock digested samples shown are (panels A and B): W243 (lane 4), W301 (lane 8), and W366 (lane 12). Endoproteinase LysC (KC) digests shown are (panel A): 40 μg/ml (lanes 5, 9, and 13), 80 μg/ml (lanes 6, 10, and 14), and 120 μg/ml (lanes 7, 11, and 15). Trypsin (T) digests shown are (panel B): 25 μg/ml (lanes 5, 9, and 13), 50 μg/ml (lanes 6, 10, and 14), and 75 μg/ml (lanes 7, 11, and 15). Digestion fragments are labeled to the right of the blots.](image)

![Fig. 4. Trypsin and chymotrypsin digestion of the P162 mutant. IMVs (400 μg/ml) were digested with enzymes and analyzed by Western immunoblotting as described in the legend to Fig. 2. Trypsin (T) digests shown are: 25 μg/ml (lane 6), 50 μg/ml (lane 7), and 75 μg/ml (lane 8). Chymotrypsin (CT) digests shown are: 37.5 μg/ml (lane 9), 75 μg/ml (lane 10), and 125 μg/ml (lane 11). A mock digested sample was run in lane 5. Size standards were analyzed in lanes 1–4. The T4 and CT3 digestion fragments are labeled to the right of the blot.](image)
Membrane Insertion of Tet Transmembrane Segments

**DISCUSSION**

We have demonstrated that five odd-numbered segments and their attached periplasmic loops in pBR322 Tet are inefficiently inserted into the lipid bilayer in the absence of downstream segments. Distal sequence deletions blocked membrane insertion of 34–54% of the C-terminal odd-numbered segments in these periplasmic loop truncation mutants. In contrast, odd-numbered segments and periplasmic loops inserted efficiently if the next even-numbered segment was present in the sequence, as discussed below.

In the I104 truncation mutant, segment 3 and the P2 loop are not inserted in the membrane in 48% of the molecules based on the observation that I104 could be digested by CpY as far upstream as loop C1. In contrast, the membrane topology of both segments 3 and 4 appears to be normal in the five-segment mutant, P162. This conclusion is based on observations that loop C1 of P162 could be cleaved by chymotrypsin, loop C2 could be cleaved by trypsin, and CpY digestion of P162 molecules did not proceed upstream of loop C2. Segments 3 and 4 of P162 are able to insert into the membrane despite the fact that segment 5 is not inserted in 34% of P162 molecules. Therefore, insertion of segment 4, but not segment 5, correlates with insertion of segment 3.

Based on the results of proteolysis of the P162, W243, and CMTΔS mutants, segment 6 also appears to facilitate insertion of segment 5. Although segment 5 inserts into the membrane in only 66% of P162 molecules, the topology of both segments 5 and 6 is normal in W243. In W243, the C2 and C3 loops flanking segments 5 and 6 were susceptible to trypsin digestion, and CpY digestion of the protein did not proceed upstream of loop C3. Thus, insertion of segments 5 and 6 occurs despite the fact that segment 7 is not inserted in 54% of W243 molecules. In further support of the role of segment 6 in segment 5 insertion, it was observed that both segments are membrane embedded in the C3 loop truncation mutant, CMTΔS. Only the short, cytoplasmic C3 tail of CMTΔS was removed by CpA and CpB digestion.

Similar conclusions regarding the effects of segment 8 on segment 7 insertion and segment 10 on segment 9 insertion are drawn based on proteolysis of Tet domain 2 truncation mutants. Although segment 7 inserts into the membrane in only 46% of W243 molecules, insertion of both segments 7 and 8 occurs in the nine-segment W301 and eight-segment Q278 mutants. These conclusions are based on observations that endoprotease LysC cut W301 in its C3 and C4 loops, CpY stopped digesting W301 in loop C4, and CpW removed only the C4 tail sequence from Q278. Similarly, although segment 9 inserts in only 63% of W301 molecules, the segment is inserted in the eleven-segment W366 and ten-segment G338 mutants. In this regard, CpY could not digest W366 upstream of loop C5, and only the C5 tail sequence of G338 could be removed by CpA and CpB. That segment 11 is able to insert into the membrane in the presence of segment 12 is inferred from observations that the CMT10 fusion confers tetracycline resistance to host cells (6), and only the C-terminal tail of CMT10 can be trimmed by CpY in IMVs.3

Taken together, the data suggest that the most efficient membrane insertion elements in the pBR322 Tet protein are helical hairpins formed from consecutive pairs of odd- and even-numbered segments. The distribution of positive charges across segments and the lengths of periplasmic loops in the protein are compatible with the hairpin mechanism of insertion (11). Clearly, hairpins are not absolutely required for segment insertion because substantial percentages of individual C-terminal odd-numbered segments and loops are able to integrate into the lipid bilayer, probably because of their hydrophobicity. The inability of deletions to disrupt insertion of internal hairpins indicates that hairpins can integrate into the membrane independently of one another. This may explain why perturbation of the orientation of N-terminal Tet segments does not affect insertion of segments downstream (6, 18).

In some respects, results obtained here with Tet truncation mutants are similar to those obtained with M13 procoat and coat proteins. It has been shown recently that the two-segment M13 procoat protein adopts two topologies when incubated with preformed phospholipid vesicles in vitro (30). About 20% of procoat molecules integrate into the lipid bilayer as hairpins and assume a proteinase K-resistant, transmembrane orientation. The remaining molecules, although bound to lipid vesicles, probably insert only into the interfacial region of the bilayer since they are accessible to the protease. In contrast, coat protein, which contains just the second segment of the procoat hairpin, adopts only the protease-accessible, interfacial conformation when incubated with vesicles. Both procoat and Tet protein studies show that individual hydrophobic segments cannot efficiently establish a transmembrane topology.

While some protease-resistant Tet hairpins may be located in the interfacial region of the bilayer, it is more likely that hairpins span the membrane because periplasmic loops, such as

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3 D. Guo, and K. W. Miller, unpublished data.
as P2, P4, and P5, which contain arginines, cannot be cleaved by trypsin from the cytoplasmic side of the membrane. It is also likely that the CpY-resistant fraction of C-terminal odd-numbered segments and loops of periplasmic loop truncation mutants span the bilayer. In this regard, noncleavable odd-numbered segments did not convert to accessible forms even during prolonged CpY digestion, and levels of digestion of the segments were not increased by raising the concentration of CpY by 3-fold.3 Inserted transmembrane segments should not oscillate readily back and forth across the membrane due to the large amount of free energy required to transfer inserted hydrophobic sequences out of, and polar loop sequences back through the bilayer (15).

To gain insight into why individual odd-numbered segments and periplasmic loops fail to insert into the membrane, we have calculated standard free energy changes for bilayer insertion of these sequences (Table III). Free energy changes were calculated using the Engelman free energy scale for partitioning of amino acids into membranes in a-helical conformation (29). For simplicity, periplasmic loop residues were treated as though they are a-helical during translocation, and this assumption leads to a slight overestimation of the magnitude of the free energy changes for membrane insertion of the hairpins (29). Experiments performed with M13 procot protein indicate the Engelman scale is a good predictor of free energy changes for membrane integration of hydrophobic segments (30).

Standard free energy changes for membrane insertion of odd-numbered segments and loops range from −6.1 kcal/mol for segment 7 and loop P4 of W243 to −51.3 kcal/mol for segment 5 and loop P3 of P162 (Table III). Because calculated ΔGs for insertion of these sequences are highly favorable, we conclude that insertion of individual segments is not at equilibrium. Possibly, unpaired segments are not long enough to adopt the a-helical conformation required for insertion of polypeptides into phospholipid bilayers (15). In this regard, short peptides often cannot form stable a-helices (31). Alternatively, the kinetic energy barrier for insertion may be higher for individual segments than for helical hairpins. In either case, even-numbered segments may serve as chaperones for the folding and/or insertion of odd-numbered segments. Experiments currently being performed to determine if specific sequence requirements for formation and insertion of hairpins can be identified.

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### TABLE III

| Mutant | Tet | Sequence | No. of Tet amino acids | $H^\circ$ | $\Delta G^\circ$ | $\Delta G^\circ_f$ |
|--------|-----|----------|------------------------|--------|----------------|----------------|
| I104   | TM3 | Val^74^-Leu^99 | 25      | +0.65 | −38.1          |                |
| P2     | P2  | Tyr^100^-Arg^133 | 4       | −0.29 | +10.4          | −27.7          |
| P162   | TM5 | Phe^133^-Ile^137 | 25      | +0.70 | −49.3          |                |
| P3     | P3  | Ser^158^-Ala^161 | 4       | +0.27 | −2.0           | −51.3          |
| W243   | TM7 | Ala^214^-Gly^237 | 24      | +0.71 | −44.4          |                |
| P4     | P4  | Glu^238^-Arg^242 | 5       | −1.10 | +38.3          | −6.1           |
| W301   | TM9 | Gln^278^-Thr^298 | 21      | +0.60 | −24.7          |                |
| P5     | P5  | Arg^299^-Gly^300 | 2       | −1.03 | +11.3          | −13.4          |
| W366   | TM11| Leu^340^-Ala^361 | 22      | +0.58 | −37.2          |                |
| P6     | P6  | Ser^362^-Thr^365 | 4       | +0.05 | −4.0           | −41.2          |

* Tet transmembrane segments and periplasmic loops.
* Segment and loop residues are assigned based on the topology model in Fig. 1.
* Number of amino acids.
* Average sequence hydrophobicity ($H$) (8).
* Standard free energy changes (kcal/mol) for membrane insertion of the indicated regions (29). Calculations assume inserted sequences are $\alpha$-helical.
* Standard free energy changes for membrane insertion of C-terminal odd-numbered segments plus periplasmic loops. Calculations are explained under footnote e and in the text.