# Analysis of the Role of Interfacial Tryptophan Residues in Controlling the Topology of Membrane Proteins†

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ABSTRACT: Tryptophans have a high affinity for the membrane−water interface and have been suggested to play a role in determining the topology of membrane proteins. We investigated this potential role experimentally, using mutants of the single-spanning Pf3 coat protein, whose transmembrane topologies are sensitive to small changes in amino acid sequence. Mutants were constructed with varying numbers of tryptophans flanking the transmembrane region and translocation was assessed by an in vitro translation/translocation system. Translocation into *Escherichia coli* inner membrane vesicles could take place under a variety of experimental conditions, with co- or posttranslational assays and proton motive force-dependent or -independent mutants. It was found that translocation can even occur in pure lipid vesicles, under which conditions the tryptophans must directly interact with the lipids. However, under all these conditions tryptophans neither inhibited nor stimulated translocation, demonstrating that they do not affect topology and suggesting that this may be universal for tryptophans in membrane proteins. In contrast, we could demonstrate that lysines clearly prefer to stay on the cis-side of the membrane, in agreement with the positive-inside rule. A statistical analysis focusing on interfacially localized residues showed that in single-spanning membrane proteins lysines are indeed located on the inside, while tryptophans are preferentially localized at the outer interface. Since our experimental results show that the latter is not due to a topology-determining role, we propose instead that tryptophans fulfill a functional role as interfacially anchoring residues on the trans-side of the membrane.

Nearly all integral membrane proteins have a unique orientation in the membrane, which is achieved by a combination of biosynthetic and membrane integration processes. The topology of membrane proteins can in most cases be described by the so-called positive-inside rule: positively charged segments that flank transmembrane helices tend to stay at the cis-side of the membrane (*1*). This is partly due to interaction of lysines and arginines with the negatively charged membrane (*2*), since lowering the amount of anionic lipids in the membrane facilitates the passage of positively charged loops (*3, 4*). However, other residues might play additional roles in controlling the topology of transmembrane segments. For example, in *Escherichia coli* anionic amino acids may promote translocation in the presence of a membrane potential, due to an electrophoretic effect (*5*).

Tryptophan is also a potential candidate for being a topological determinant, because of all amino acids it has the highest affinity for the membrane−water interface (*6*). Its aromaticity causes it to have favorable interactions with the interface region, where it is positioned close to the carbonyls (*7*−*9*). These Trp residues are thought to anchor proteins to the membrane interface (*10, 11*). Such an anchoring function would suggest that tryptophans may hinder translocation of protein segments. Indeed, studies of a Trp-containing gramicidin analogue suggested that the tryptophans might prevent transfer of the peptide across a pure lipid bilayer (*12*).

However, there are also some indications that Trp has the opposite effect. In structures of integral membrane proteins such as the photosynthetic reaction center (*13, 14*), bacteriorhodopsin (*15*), porins (*16, 17*), cytochrome *c* oxidase (*18*), and the bacterial potassium channel (*19*), belts of tryptophans and tyrosines are located near the membrane−water interface. As was first shown for the photosynthetic reaction center (*13, 14*), in some proteins these aromatic amino acids are more abundant in the periplasm (*20*−*22*). Therefore it was proposed that they may in fact be preferentially translocated across the membrane (*20*).

In this study, we investigated the potential influence of tryptophan on controlling the topology of membrane proteins, and we compared the effects of tryptophans with those of

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lysine residues, which are known to be topological determinants. For this purpose, we selected the major coat protein of the filamentous bacteriophage Pf3 as model protein. This is a small protein that has a single transmembrane segment that inserts directly into the *E. coli* inner membrane without using the Sec machinery (*23*). Therefore, the topology of this protein will be determined by direct interactions with the membrane, rather than by recognition by a translocation machinery. Knowledge on the mode of insertion of Pf3 into the bilayer has been obtained from biophysical studies of reconstituted systems (*24*). More recently, in vitro assays have been developed that allow us to determine the topology of this protein under a variety of translocation conditions (*5*). Most importantly, mutants of this protein have been constructed that can translocate their N- and C-terminus equally well (*5*, *25*). Since these mutants apparently lack a dominant topological determinant, they can be used as sensitive tools for the study of topogenic factors.

Pf3 mutants were constructed with different numbers of tryptophan or lysine residues bordering both sides of the transmembrane segment. The N-terminal translocation of these mutants was analyzed in in vitro translocation systems with *E. coli* inner membrane vesicles (IMVs)[1] as well as pure lipid vesicles. The results indicated that lysines stay on the cis-side of the membrane, whereas tryptophans do not have a preference for the cis- or trans-side in any of these systems. Therefore we conclude that, in contrast to lysines, tryptophans are not able to act as topological determinants. A statistical analysis showed that tryptophans nevertheless have a preference for the outer interface, which is particularly prominent for single-spanning membrane proteins. We propose that this statistical outside preference is due to the ability of tryptophan residues to stabilize proteins in a transmembrane state.

## EXPERIMENTAL PROCEDURES

*Materials.* The lipids 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were supplied by Avanti Polar Lipids Inc. Monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG) were obtained from Larodan lipids. L-[$^{35}$S]-Methionine was supplied by Amersham Corp. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and L-amino acids were from Sigma, and puromycin dihydrochloride and proteinase K were from Boehringer Mannheim. Oligonucleotides for the construction of mutants came from Pharmacia, and the QuikChange site-directed mutagenesis kit was purchased from Stratagene.

*Construction of Mutants.* Mutants were constructed according to the QuikChange site-directed mutagenesis method from Stratagene, using an annealing temperature of 68 °C. A plasmid with the gene coding for the Pf3 mutant DDtag1 behind a T7 promoter (*5*) was used as template for constructing the tryptophan or lysine mutants (Figure 1). The resulting mutants were named $DW_n$-$W_n$ or $DK_n$-$K_n$, indicating that the protein is derived from the DDtag1-mutant and showing the amount of tryptophans or lysines bordering the hydrophobic putative transmembrane segment on the N- and C-terminal side. Similarly, a plasmid containing the 3L-4N mutant (*25*) was used for constructing the $NW_nL_3W_1$ series (Figure 1). All mutants contain the unique N-terminal methionine residue needed for labeling. All mutations were confirmed by DNA sequencing.

*Preparation of Cell Extract and Inverted Inner Membrane Vesicles.* An S-30 cell extract was prepared from *E. coli* MRE600 as described (*26*). Membrane-free S-135 extracts were prepared by centrifuging 500 $\mu$L of S-30 per tube in a Beckman TL ultracentrifuge for 15 min at 4 °C at 55 000 rpm in a TLA100.1 rotor and collecting the top 300−350 $\mu$L of the supernatant. The extract was rapidly frozen in 50 $\mu$L aliquots in liquid $N_2$ and stored at −80 °C. IMVs were prepared from *E. coli* MC4100 in IMV buffer (50 mM triethanolamine acetate, pH 7.5, 250 mM sucrose, and 1 mM dithiothreitol) as described previously (*27*).

*Preparation of Large Unilamellar Vesicles.* Dry lipid films were prepared by mixing the appropriate amounts of lipids dissolved in chloroform and evaporation of the solvent under a stream of nitrogen. These films were hydrated at room temperature with LUV buffer (104 mM $Na_2SO_4$ and 40 mM Hepes-NaOH pH 8.0) to a final concentration of 20 mM (phospho)lipid and vortexed. After 10 cycles of freeze−thawing, the vesicle suspensions were extruded 10 times through 0.4 $\mu$m polycarbonate filters.

*In Vitro Translation and Cotranslational Translocation. In vitro* transcriptions were performed with the T7 transcription kit from MBI Fermentas, supplemented with T7 RNA polymerase and transcription buffer from Epicentre Technologies. Translation reactions were performed in 50 $\mu$L total volumes as described (*5*) with minor changes, using 40 mM Hepes-KOH, pH 8.0, 104 mM potassium acetate, 10 mM ATP, 2 mM GTP, CTP, and UTP, 5 $\mu$Ci of $^{35}$S-methionine, 6 $\mu$L of S-135 extract, and 8 $\mu$L of transcription mix. For translocation experiments, 10 $\mu$L of IMVs or IMV buffer or 5 $\mu$L of LUVs or LUV buffer was added at the start of the translation. Where indicated, 25 $\mu$M CCCP was added to the reaction mixture to dissipate the proton motive force (pmf). Reactions were performed at 37 °C for 30 min, and subsequently an aliquot of 10 $\mu$L was withdrawn to serve as a 25% standard. The IMVs or LUVs in the reaction mixture were reisolated by centrifugation. The IMVs were pelleted by layering them on top of 200 $\mu$L of 0.5 M sucrose in buffer (50 mM triethanolamine acetate, pH 7.5) and centrifuging in a TLA100.3 rotor at 60 000 rpm for 30 min at 4 °C. To pellet the LUVs, the salt concentration was lowered by dilution with 60 $\mu$L of $H_2O$ and subsequently the mixture was centrifuged in a TLA100 rotor at 100 000 rpm for 60 min at 4 °C. The resulting pellets were resuspended in 40 $\mu$L of the original buffer (IMV or LUV buffer) and divided into two equal aliquots, one of which was treated with 2 volumes of proteinase K (1 mg/mL) for 30 min at room temperature. Where indicated, the vesicles were disrupted before proteinase K treatment with 1% (v/v) Triton X-100. All samples were trichloroacetic acid- (TCA-) precipitated and analyzed on SDS−tricine 16.5% polyacrylamide gels containing 6 M urea (*28*). Protein bands were quantified on a PhosphoImager (Molecular Dynamics).

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; MGDG, monogalactosyl diglyceride; DGDG, digalactosyl diglyceride; IMVs, inner membrane vesicles; LUVs, large unilamellar vesicles; pmf, proton motive force; pK, proteinase K; TCA, trichloroacetic acid.

$DW_0$-$W_0$ MQSVITDVTGQLTAVQAD *ITTIGGAIIVLAAVVLGI* D IDAQLLHPVQLF

$DW_1$-$W_0$ MQSVITDVTGQLTAVQAD**W** *ITTIGGAIIVLAAVVLGI* D IDAQLLHPVQLF

$DW_2$-$W_0$ MQSVITDVTGQLTAVQAD**WW***ITTIGGAIIVLAAVVLGI* D IDAQLLHPVQLF

$DW_0$-$W_1$ MQSVITDVTGQLTAVQAD *ITTIGGAIIVLAAVVLGI* D**W**IDAQLLHPVQLF

$DW_1$-$W_1$ MQSVITDVTGQLTAVQAD**W** *ITTIGGAIIVLAAVVLGI* D**W**IDAQLLHPVQLF

$DW_2$-$W_1$ MQSVITDVTGQLTAVQAD**WW***ITTIGGAIIVLAAVVLGI* D**W**IDAQLLHPVQLF

$DW_0$-$W_2$ MQSVITDVTGQLTAVQAD *ITTIGGAIIVLAAVVLGI***W**D**W**IDAQLLHPVQLF

$DW_1$-$W_2$ MQSVITDVTGQLTAVQAD**W** *ITTIGGAIIVLAAVVLGI***W**D**W**IDAQLLHPVQLF

$DW_2$-$W_2$ MQSVITDVTGQLTAVQAD**WW***ITTIGGAIIVLAAVVLGI***W**D**W**IDAQLLHPVQLF

$DK_1$-$K_0$ MQSVITDVTGQLTAVQAD**K** *ITTIGGAIIVLAAVVLGI* D**W**IDAQLLHPVQLF

$DK_2$-$K_0$ MQSVITDVTGQLTAVQAD**KK***ITTIGGAIIVLAAVVLGI* D**W**IDAQLLHPVQLF

$DK_0$-$K_1$ MQSVITDVTGQLTAVQAD *ITTIGGAIIVLAAVVLGI* D**K**IDAQLLHPVQLF

$DK_0$-$K_2$ MQSVITDVTGQLTAVQAD *ITTIGGAIIVLAAVVLGI***K**D**K**IDAQLLHPVQLF

$NW_0L_3W_1$ MQSVITDVTGQLTAVQAD *ITTIGGAI***LLL***IVLAAVVLGI* D**W**IDAQLLHPVQLF

$NW_1L_3W_1$ MQSVITDVTGQLTAVQAD**W** *ITTIGGAI***LLL***IVLAAVVLGI* D**W**IDAQLLHPVQLF

$NW_2L_3W_1$ MQSVITDVTGQLTAVQAD**WW***ITTIGGAI***LLL***IVLAAVVLGI* D**W**IDAQLLHPVQLF

FIGURE 1: Amino acid sequences of the Pf3 mutants used in this study. The tryptophan and lysine residues are shown in boldface type and the hydrophobic transmembrane segment in italic type. The three additional leucines in the hydrophobic segment of the $NL_3$ mutants are also shown in boldface type. The aspartate residues in the D-mutants, changed to asparagines in the $NL_3$ mutants, are underlined.

*Posttranslational Translocation.* Translation was carried out in the absence of IMVs for 30 min at 37 °C in a 40 $\mu$L volume with the same composition as described above and terminated by the addition of 1 $\mu$L of 10 mM puromycin. After 2 min, 10 $\mu$L of IMVs or IMV buffer was added and the incubation was resumed for 20 min. Subsequently the samples were centrifuged and analyzed as described above. Proteinase K treatment of the pellet resulted in the same fragment as observed in cotranslational experiments. This translocated protein fragment did not appear when the IMVs were omitted. To verify translation termination, it was checked that no $^{35}$S-methionine could be incorporated into the protein after puromycin addition (*29*).

*Statistical Analysis of Transmembrane Segments.* Data of transmembrane segments were derived from TMbase (ftp://ulrec3.unil.ch/pub/tmbase), a SwissProt-derived database containing over 15 000 entries of transmembrane segments (*30*). For our analysis a data set of prokaryotic proteins was constructed. Only one protein per family (PAM200-reduced) was included, and the proteins were divided into single-spanning membrane proteins (58 proteins) and multispanning proteins (503 helices). A TMbase entry lists the putative transmembrane helix as well as the five residues preceding and following it. The interface region was defined as the terminal five residues of the transmembrane segment plus the five residues bordering it. The amino acid frequencies (as percentage of the total number of residues) at the inner and outer interfaces of the membrane could be determined from the topology of the proteins given in TMbase. The statistical significance of the observed differences in amino acid frequencies is evaluated by $\chi^2$ analysis.

## RESULTS

*Translocation Assay.* The topological roles of interfacially localized tryptophan and lysine residues were studied by analyzing their effect on the N-terminal translocation of the single-spanning protein Pf3. We employed the DDtag1 mutant (Figure 1), which has negatively charged N- and C-termini that can be translocated across the membrane equally well (*5*). This mutant was renamed $DW_0-W_1$ to indicate the aspartate-containing tails and the position of the tryptophan residues. The strategy of the in vitro translocation experiments is depicted in Figure 2 (top). The mutant is synthesized in an *E. coli* translation system in the presence of $^{35}$S-methionine, resulting in a protein that is radioactively labeled at the N-terminus. IMVs are present during translation (cotranslational assay) or added afterward (posttranslational assay) to allow the protein to insert into these vesicles, which are subsequently reisolated by centrifugation to increase the sensitivity of the assay. When the N-terminus

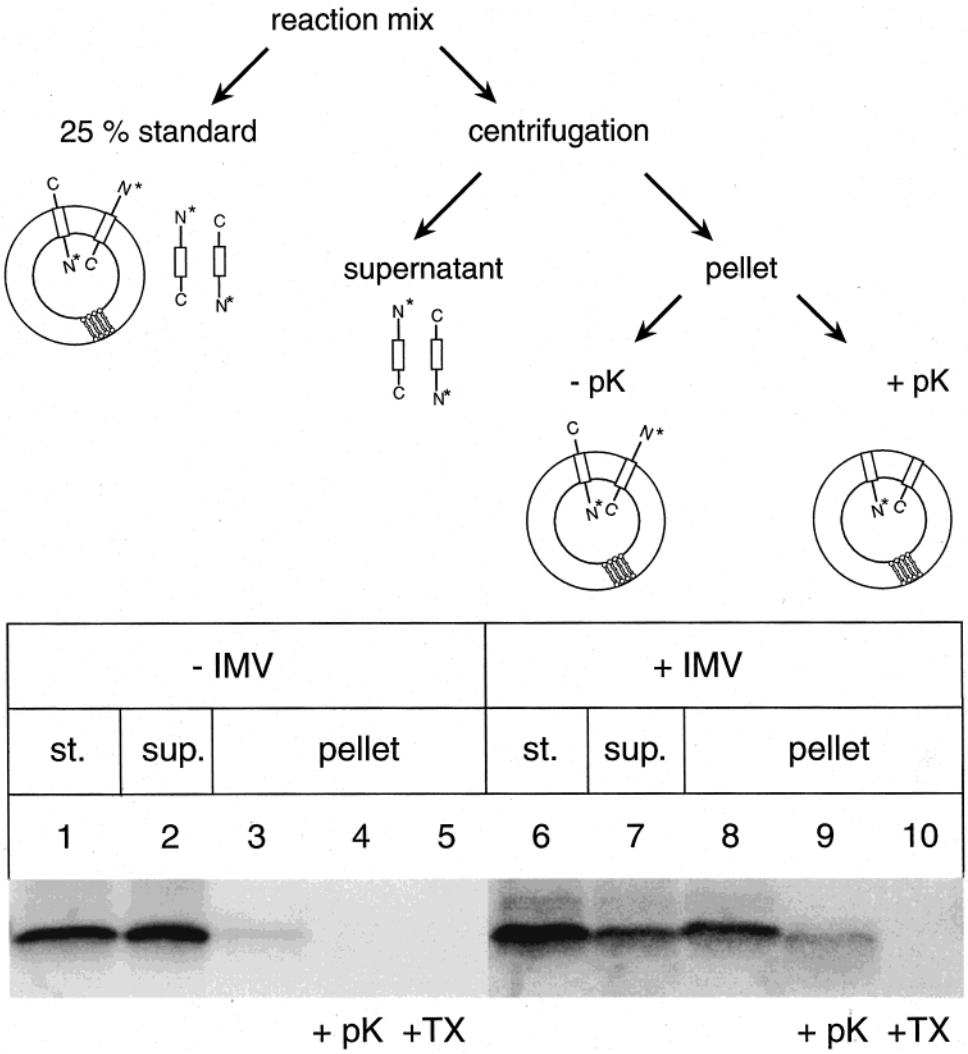


FIGURE 2: (Top) Schematic representation of the translocation assay. See text for details. For the sake of clarity only transmembrane orientations of the proteins are drawn. pK, proteinase K. (bottom) Cotranslational translocation of the $DW_0-W_1$ mutant in inner membrane vesicles. Translation was carried out in the absence (lanes 1−5) or presence of IMVs (lanes 6−10) and treated as described under Experimental Procedures. Lanes 1 and 6 show 25% standards, lanes 2 and 7 the supernatants, and lanes 3 and 8 the IMV pellets. The pellets were resuspended in IMV buffer and a fraction was treated with proteinase K (+pK), either without (lanes 4 and 9) or with 1% Triton X-100 (+TX) added to disrupt the vesicles (lanes 5 and 10). Samples were TCA-precipitated and analyzed by tricine−SDS−PAGE and autoradiography.

of the protein translocates to the inside of a vesicle, treatment with proteinase K produces a labeled fragment with a slightly lower molecular weight due to cleavage of the C-terminus (*5*). Part of the resulting pellet is therefore treated with proteinase K to determine which fraction of the vesicle-associated protein has translocated its N-terminus. A typical cotranslational experiment is shown in Figure 2 (bottom). When the $DW_0-W_1$ mutant is synthesized in the absence of IMVs (lane 1), most of the protein remains in the supernatant (lane 2) with only a small amount ending up in the pellet (lane 3) in which the protein can be completely degraded by proteinase K (lane 4), also in the presence of detergent (lane 5). In the presence of IMVs a considerable fraction, quantified as 40% of the synthesized protein, is pelleted together with the vesicles (lane 8), showing the membrane association of the protein. In all experiments described in this study, a similar extent of association with IMVs was observed. When the pellet is treated with proteinase K, a fragment with a slightly lower molecular weight is observed (lane 9). This fragment can be completely degraded when the vesicles are solubilized with the detergent Triton X-100 (lane 10) and thus corresponds to protein inserted with its N-terminus into the vesicles and its C-terminus cleaved off by proteinase K (*5*). This assay allows us to investigate the potential role of tryptophan in determining membrane protein topology under a variety of experimental conditions.

*Translocation of D-Mutants.* Starting from the $DW_0-W_1$ mutant we constructed mutants containing zero, one, or two tryptophans or lysines at either or both ends of the hydrophobic transmembrane segment (Figure 1). These D-mutants were analyzed in the translocation assay described above. As shown in Figure 3, proteinase K treatment resulted in a protected fragment with a slightly lower molecular weight, indicating that these mutants are capable of N-terminal translocation into IMVs. The translocation efficiency was determined as the fraction of protein bound to the IMVs (Figure 3, lane 1) that is cleaved by proteinase K to a molecule with a lower molecular weight (lane 2). The translocation efficiencies of the D-mutants are given in Table

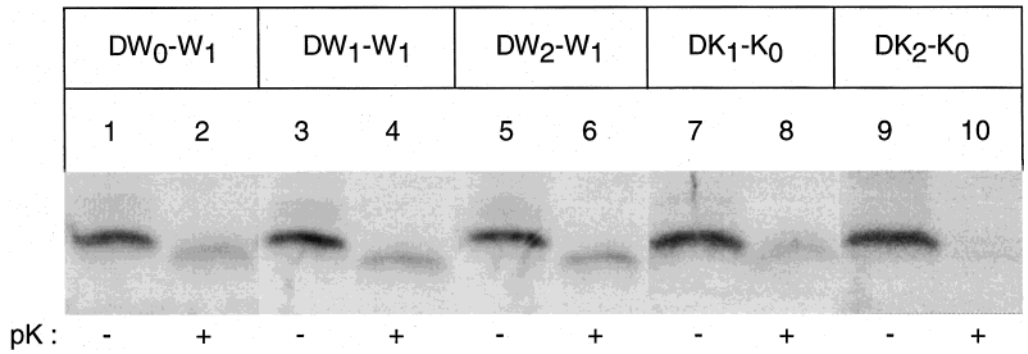


FIGURE 3: Cotranslational translocation of D-mutants with different numbers of tryptophan or lysine residues. Translocation of the mutants $DW_0-W_1$, $DW_1-W_1$, $DW_2-W_1$, $DK_1-K_0$, and $DK_2-K_0$ in IMVs was carried out as described in Figure 2. Only the pellet fractions are shown, which were either untreated (lanes 1, 3, 5, 7, and 9) or treated (lanes 2, 4, 6, 8, and 10) with proteinase K (pK).

Table 1: Translocation Efficiencies of the D-Mutants with Different Numbers of Tryptophan or Lysine Residues[a]

| mutant | translocation (% of bound protein) |
|---|---|
| $DW_1-W_0$ | 28 |
| $DW_2-W_0$ | 28 |
| $DW_0-W_1$ | 33 |
| $DW_1-W_1$ | 33 |
| $DW_2-W_1$ | 34 |
| $DW_0-W_2$ | 21 |
| $DW_1-W_2$ | 24 |
| $DW_2-W_2$ | 28 |
| $DK_1-K_0$ | 18 |
| $DK_2-K_0$ | 5 |
| $DK_0-K_1$ | 48 |
| $DK_0-K_2$ | 63 |

[a] Translocation efficiencies were determined at least twice and the average values were calculated. The estimated error is about 20% of the value.

1. All mutants have similar translocation efficiencies, regardless of the number or the location of the tryptophan residues. Thus it can be concluded that the presence of tryptophans on either side of the transmembrane segment does not affect the N-terminal translocation of the protein and hence that under these experimental conditions tryptophan does not play a role in determining topology. In contrast, lysine residues have a significant effect, as shown in Figure 3 and Table 1. Insertion of one lysine in the N-terminus reduces translocation only slightly, probably because it is located next to the negatively charged aspartate and the charges cancel each other (*31*). The presence of two lysines in the N-terminus almost completely blocks N-terminal translocation. Moreover, insertion of lysines in the C-terminus results in an increase in the amount of N-terminal translocation, because now translocation of the C-terminus is hindered. The results of the lysine-mutants demonstrate the sensitivity of the system to topogenic signals.

*Translocation of $NL_3$-Mutants.* It is possible that the effect of tryptophan residues depends on the translocation mechanism used by the protein. Therefore, we also studied their effect on the N-terminal translocation of the $NW_0L_3W_1$ mutant (see Figure 1 for used constructs). In contrast to wild-type Pf3, insertion of this mutant is independent of the pmf (*25*), which we verified in our translocation assay (data not shown). This $NW_0L_3W_1$ mutant has no charged amino acids and its transmembrane segment is elongated by three leucines. Like the $DW_0-W_1$ mutant, it can insert in two orientations (*25*) and is therefore sensitive to topogenic factors. Tryptophans were introduced at the N-terminal side

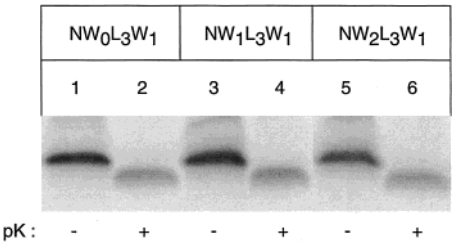


FIGURE 4: Cotranslational translocation of $NL_3$ mutants with different numbers of tryptophan residues. Translocation of the mutants $NW_0L_3W_1$, $NW_1L_3W_1$, and $NW_2L_3W_1$ was carried out as described in Figure 2. Only the pellet fractions are shown, which were either untreated (lanes 1, 3, and 5) or treated (lanes 2, 4, and 6) with proteinase K (pK).

Table 2: Translocation Efficiencies of the $NL_3$-Mutants[a]

| | translocation (%) | | |
|---|---|---|---|
| mutant | IMVs cotranslational | IMVs posttranslational | LUVs DOPE/DOPG (3:1) cotranslational |
| $NW_0L_3W_1$ | $43 \pm 14$ ($n$=18) | $7 \pm 1$ ($n$=5) | $17 \pm 1$ ($n$=2) |
| $NW_1L_3W_1$ | $45 \pm 15$ ($n$=9) | $12 \pm 3$ ($n$=4) | $18 \pm 3$ ($n$=2) |
| $NW_2L_3W_1$ | $38 \pm 5$ ($n$=7) | $14 \pm 3$ ($n$=4) | $25 \pm 5$ ($n$=2) |

[a] Translocations were performed for the indicated number of times and the average values are given. For the co- and posttranslational experiments, standard deviations are shown, while for the translocation into LUVs, the variation in the values is indicated.

of the transmembrane segment of this mutant and translocation experiments with these mutants were performed, as shown in Figure 4. The results show that all three proteins are able to translocate into IMVs but that again insertion of tryptophans in the N-terminus does not significantly affect the extent of translocation (Table 2).

*Posttranslational Translocation.* Next, we investigated whether tryptophans could act as topological determinants in posttranslational insertion. Posttranslational translocation experiments with the tryptophan mutants were performed and the translocation efficiencies are listed in Table 2. In all cases, the translocation efficiency was less than in the cotranslational assay. This has been observed for other membrane proteins as well (*27, 29*) and is probably due to aggregation of the hydrophobic protein in the absence of vesicles. The results suggest slightly higher translocation efficiencies for the mutants with one or two tryptophans in the N-terminus in this posttranslational assay. However, this effect might be only apparent, since it could simply be caused by small differences in the extent of aggregation of the tryptophan mutants before insertion.

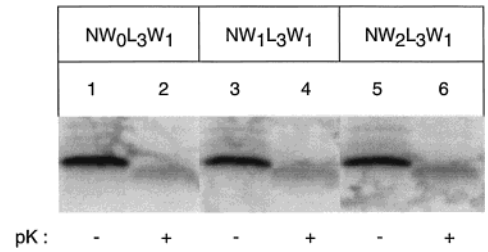


FIGURE 5: Cotranslational translocation of the $NL_3$ mutants in liposomes. Translation of the mutants $NW_0L_3W_1$, $NW_1L_3W_1$, and $NW_2L_3W_1$ was carried out in the presence of LUVs composed of 75% DOPE and 25% DOPG. The reaction mixtures were then processed as described for Figure 2. Only the pellet fractions are shown, which were either untreated (lanes 1, 3, and 5) or treated (lanes 2, 4, and 6) with proteinase K (pK).

*Translocation of $NL_3$ Mutants in LUVs.* In all studies with IMVs it is possible that proteins present in the membrane are involved in the translocation process. If interactions of tryptophans with lipids at the interface play a role in determining membrane protein topology, a clear effect should be observed when pure lipid vesicles are used as the accepting membrane. This can be conveniently studied with the $NL_3$ mutants because of their pmf-independent translocation.

We first used large unilamellar vesicles (LUVs) composed of 75% DOPE and 25% DOPG to mimic the lipid composition of the *E. coli* inner membrane. Similar to the IMVs, these LUVs were added cotranslationally to the translation reaction and reisolated by centrifugation. In this case, more than 80% of the synthesized protein could be pelleted together with the liposomes (data not shown), demonstrating efficient association of the protein with the lipid vesicles. Treatment with proteinase K resulted in a translocated fragment similar to the one seen with IMVs (Figure 5, lane 2). This demonstrates that this mutant is able to insert into a bilayer without any membrane proteins. However, the translocation efficiency was less than in IMVs (Table 2). This might indicate that the presence of membrane proteins stimulates insertion of the Pf3 mutants, or it could be due to other differences between the two systems, such as surface area or lipid composition. When the same experiment was also performed with the tryptophan mutants $NW_1L_3W_1$ (lanes 3 and 4) and $NW_2L_3W_1$ (lanes 5 and 6), again similar translocation efficiencies were observed (Table 2), unambiguously demonstrating that direct interaction of tryptophan with these lipids does not inhibit translocation.

Finally, tryptophans might affect membrane protein insertion through specific interactions with glycolipids that are not present in *E. coli* but are abundant in other bacterial membranes. Weik et al. (*32*) have found specific stacking arrangements of the aromatic residues of bacteriorhodopsin and the glycolipids in purple membranes. To test this possibility, we determined translocation efficiencies of these trypophan mutants in LUVs containing 20% of the glycolipids MGDG or DGDG (Table 3). In 100% DOPC the translocation efficiencies of all three mutants are similar, in agreement with the results obtained with the DOPE/DOPG LUVs. When the glycolipids MDGD or DGDG is incorporated into the LUVs, the translocation efficiencies do not change and again no significant differences are observed between the three tryptophan mutants. Therefore we conclude that tryptophans also have no influence on the N-terminal translocation efficiency of this protein when glycolipids are present in the membrane.

Table 3: Influence of Glycolipids on the Translocation of $NL_3$-Mutants into LUVs

| | translocation (%) | | |
|---|---|---|---|
| mutant | 100% DOPC | 80% DOPC/20% MGDG | 80% DOPC/20% DGDG |
| $NW_0L_3W_1$ | $22 \pm 3$ ($n$=8) | 24 | 25 |
| $NW_1L_3W_1$ | 21 | 20 | 29 |
| $NW_2L_3W_1$ | 17 | 28 | 26 |

Table 4: Statistical Analysis of Interfacial Residues of Prokaryotic Multi- and Single-Spanning Membrane Proteins[a]

| | W | Y | F | K | R |
|---|---|---|---|---|---|
| | | | Multispanning Proteins | | |
| out | 3.6 | 4.3 | 7.0 | 2.8 | 2.8 |
| in | 3.3 | 3.7 | 7.1 | 4.6 | 6.1 |
| | | | Single-Spanning Proteins | | |
| out | 5.3 | 4.1 | 6.7 | 3.6 | 2.4 |
| in | 1.8 | 3.1 | 5.9 | 5.7 | 5.5 |

[a] The frequency (in percent) with which certain residues occur in the outer (out) or inner (in) interface was determined. This interface region was defined as 10 amino acids around the end of a transmembrane segment (see Experimental Procedures).

The results of our translocation experiments with tryptophan mutants clearly show that tryptophans do not inhibit nor stimulate translocation of Pf3. This holds for many different experimental conditions, suggesting that it may be universal for interfacially localized tryptophans in membrane proteins.

*Statistical Analysis of Transmembrane Segments.* Previous studies suggested that tryptophans might have a preference for the periplasmic side. However, this was based mainly on their increased statistical occurrence in periplasmic loops (*1, 21, 22*). No data are available that are focused on the occurrence of aromatic amino acids at the interface region on either side of the membrane. For this reason we thought it of interest to perform a statistical analysis on the distribution of aromatic amino acids in the interface region. Using a database of predicted transmembrane segments containing both single- and multispanning proteins, we determined the frequency with which certain residues are present in a stretch of 10 amino acids corresponding to the interfacial region (see Experimental Procedures). As shown in Table 4, we find that tryptophan residues are enriched in the interface region [their abundance is 1.2% in soluble proteins (*1*)]. In multispanning membrane proteins they do not show a significant preference for the outer or inner interface. In contrast, in single-spanning proteins tryptophans are three times more abundant at the outer interface ($P < 0.05$). This preference for the outside seems to be a specific property of tryptophan, since for tyrosine and phenylalanine no significant difference is found. The positively charged residues lysine and arginine clearly prefer the inner interface in both multispanning ($P < 0.005$) and single-spanning proteins ($P < 0.05$), in agreement with the positive-inside rule and their topology-determining role (*1, 33, 34*). Since tryptophans do not appear to play a topological role, the clear preference of tryptophans for the outer interface in single-spanning membrane proteins suggests that these residues have a functional role. This role then should be one that is preferentially

executed at the trans-side of the membrane.

## DISCUSSION

In this study, we have compared the topological roles of interfacial tryptophans and lysines in membrane proteins by using mutants of the single-spanning Pf3 coat protein that are able to insert in two orientations and therefore are highly sensitive to topogenic factors. We used a versatile in vitro assay, using *E. coli* IMVs as well as pure lipid vesicles of different compositions, in which we investigated translocation of pmf-dependent and pmf-independent mutants.

It was shown that the presence of lysine residues severely affects the topology of the protein, demonstrating that positively charged residues indeed act as topological determinants in a single-spanning membrane protein, as was shown before (*5*, *25*). In contrast, we have not found any significant differences in translocation efficiencies between the different tryptophan mutants. This appeared to be independent of the translocation pathway. Therefore we conclude that tryptophans have no intrinsic preference for either side of the membrane. Hence, tryptophan residues are not able to act as topological determinants in this single-spanning membrane protein.

If tryptophans are no topological determinants, what is then the reason for the outside preference of interfacially localized tryptophans in single-spanning membrane proteins? A likely possibility is that the location of tryptophan residues is conserved because they have a function that is mainly executed on the outside of the membrane. Our hypothesis is that this function is to stably anchor transmembrane helices at the trans-side of the membrane. Such an anchoring role of tryptophans was for instance suggested in a molecular dynamics simulation of isolated helices of bacteriorhodopsin, where the α-helices containing aromatic residues at the interface were more stable than those without (*35*). It is also consistent with experimental work on tryptophan-flanked transmembrane model peptides (*9*) and with recent results by Braun and von Heijne (*36*), who showed that tryptophans have sufficient affinity for the interface to be able to readjust the vertical position of a transmembrane helix relative to the membrane-water interface in a biological membrane.

Why would this stabilization be required specifically on the trans-side of the membrane? On the cis-side proteins in general are anchored by positively charged lysine and arginine residues (*1*), which determine the topology of membrane proteins (*3*, *33*; this study). When this final topology is achieved, a transmembrane helix thus may need stabilization only at the trans side. This can be provided by tryptophan residues, since these can readily pass through the membrane.

Finally, a stabilizing role of tryptophans could also explain why enrichment of aromatic amino acids at the membrane−water interface (*37*, *38*) and their outside preference are more pronounced in single than in multispanning membrane proteins. Multispanning proteins are rather rigid, and their structure and orientation in the membrane will be mainly determined by protein−protein interactions between their transmembrane segments. Single-spanning membrane proteins, on the other hand, can be expected to have more structural flexibility. Thus, it is possible that in particular single-spanning transmembrane segments require the additional stabilization at the trans-side obtained by the interaction between tryptophans and the interface.

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