Interfacial Positioning and Stability of Transmembrane Peptides in Lipid Bilayers Studied by Combining Hydrogen/Deuterium Exchange and Mass Spectrometry*

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Nano-electrospray ionization mass spectrometry (ESI-MS) was used to analyze hydrogen/deuterium (H/D) exchange properties of transmembrane peptides with varying length and composition. Synthetic transmembrane peptides were used with a general acetyl-GW$_2$(LA)$_n$LW$_2$A-ethanolamine sequence. These peptides were incorporated in large unilamellar vesicles of 1,2-dimyristoyl-sn-glycero-3-phosphocholine. The vesicles were diluted in buffered deuterium oxide, and the H/D exchange after different incubation times was directly analyzed by means of ESI-MS. First, the influence of the length of the hydrophobic Leu-Ala sequence on exchange behavior was investigated. It was shown that longer peptide analogs are more protected from H/D exchange than expected on the basis of their length with respect to bilayer thickness. This is explained by an increased protection from the bilayer environment, because of stretching of the lipid acyl chains and/or tilting of the longer peptides. Next, the role of the flanking tryptophan residues was investigated. The length of the transmembrane part that shows very slow H/D exchange was found to depend on the exact position of the tryptophans in the peptide sequence, suggesting that tryptophan acts as a strong determinant for positioning of proteins at the membrane/water interface. Finally, the influence of putative helix breakers was studied. It was shown that the presence of Pro in the transmembrane segment results in much higher exchange rates as compared with Gly or Leu, suggesting a destabilization of the α-helix. Tandem MS measurements suggested that the increased exchange takes place over the entire transmembrane segment. The results show that ESI-MS is a convenient technique to gain detailed insight into properties of peptides in lipid bilayers by monitoring H/D exchange kinetics. Important structural and dynamic features, such as stability of the transmembrane segments or their precise positioning at the lipid/water interface, will be determined not only by intrinsic properties of the transmembrane segments, but also by their interaction with surrounding lipids. A convenient way to gain insight into how the special characteristics of transmembrane segments and their interaction with lipids may influence the behavior of membrane proteins is by studying model systems of artificial transmembrane peptides with desired properties in well defined lipid bilayers.

Recently, we have described a new method using nano-ESI-MS (1) to study the properties of transmembrane protein segments in model systems by analyzing the kinetics of hydrogen/deuterium (H/D) exchange. The results showed that various populations of amide hydrogen atoms can be distinguished that are characteristic for different regions of the transmembrane segments. These populations are fast exchanging amide hydrogens located in the peptide termini that are exposed to the aqueous phase, immediately exchanging hydrogens of the residues located in the bilayer/water interface, and slowly exchanging hydrogens located in the hydrophobic core of the lipid bilayer. The results suggest that measurement of exchange properties of peptides by ESI-MS is a convenient method to investigate factors that determine interfacial positioning and/or stability of transmembrane protein segments. In the present study, we have investigated the influence of several factors. Hereby, special emphasis is given to the length of the α-helical hydrophobic core with respect to the bilayer thickness, the role of potential anchoring residues at the lipid/water interface, and the influence of α-helix breaking residues in the transmembrane segment.

As models for protein transmembrane segments, we have used WALP peptides that already have been used successfully to investigate various aspects of peptide/lipid interactions (2–4). These peptides have a hydrophobic core of alternating Leu and Ala, which is flanked on both sides by Trp residues (see Table I), and they have been shown to form α-helical transmembrane helices (4). Since in membrane proteins Trp residues are highly enriched near the membrane/water interface (5–8), they are thought to resemble a consensus sequence for

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1 The abbreviations used are: nano-ESI-MS, nano-electrospray ionization mass spectrometry; CID, collision-induced dissociation, DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; H/D, hydrogen/deuterium; LUVET, large unilamellar vesicles prepared by extrusion; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio.
transmembrane α-helical segments of intrinsic membrane proteins.

The extent to which the hydrophobic length of transmembrane segments matches the hydrophobic bilayer thickness can significantly influence membrane protein structure and function (9). To investigate whether this is related to changes in membrane protein interfacial positioning and/or stability, we first analyzed the effects of increasing the hydrophobic length of WALP peptides on the H/D exchange kinetics of peptides with different lengths of the Leu-Ala core in bilayers of DMPH. This lipid was chosen because it forms well-defined bilayers with different lengths of the Leu-Ala core in membranes of DMPH. Since this lipid is also important for membrane protein interfacial anchoring, its thickness that approximately matches the hydrophobic length of the shortest peptide used (3).

Next, we investigated the importance of Trp as interfacial anchoring residues. It has been suggested that Trp residues prefer to be positioned at a well-defined site in the lipid headgroups (3, 11–15) and that thereby they can act as membrane anchors. Their abundance at the lipid/water interface in several membrane proteins (5–7) is therefore likely to be functionally important, e.g., for stabilization of transmembrane helices or precise positioning of such helices at the interface. The present mass spectrometric method offers the opportunity to investigate these effects by analyzing the H/D exchange kinetics of peptides of identical total length in which the position of Trp residues along the sequence is varied.

Finally, besides the length of the transmembrane helices and their interfacial anchoring behavior, the stability of the transmembrane segment is also important for membrane protein structure and function. In water-soluble proteins, the stability of the backbone of regular β-sheets has been found to undergo large changes when potent breakers of both α-helical and β-sheet structures, like Pro and Gly, are inserted (e.g. see Ref. 16). However, little is known about the effect on stability of these residues in transmembrane segments. Therefore, H/D exchange kinetics in transmembrane segments containing Pro and Gly residues are compared with those of peptides without these residues.

The results of this study show that peptides that are long with respect to the hydrophobic thickness of the bilayer are protected from H/D exchange to a relatively large extent, which is explained in terms of induced adaptation (thickening) of the bilayer and/or a tilting of the peptides. Furthermore, the positions of the Trp residues in the transmembrane sequence are shown to be a critical factor for H/D exchange kinetics. These results suggest that Trp side chains interact strongly with the membrane/water interface at specific sites. Finally, it is shown that peptides containing Pro, but not Gly, show a markedly different H/D exchange pattern than peptides lacking this residue. This suggests a significant effect of Pro on the stability of the transmembrane α-helix. The results are discussed in relation to existing literature data on related peptide/lipid interactions.

MATERIALS AND METHODS

Chemicals—Trifluoroacetic acid was obtained from Merck (Darmstadt, Germany), 2,2,2-trifluoroethanol from Sigma. Deuterium oxide (>99.9% D) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). D2O was stored under nitrogen at 4 °C. Sodium iodide was from OPG Farma Company (Utrecht, The Netherlands). Ammonium acetate was from Fluka (Switzerland). The phospholipid DMPH was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). The peptides WALP16, WALP19, WALP21, WALP23, WALP23inner, WALP23outer, WALP23Pro, and WALP23Gly were synthesized as described by Killian et al. (4), as modified by Greathouse et al. (17).

The WALP21 peptide was synthesized as described by De Planque et al. (9) for related peptides. The peptides were tested for purity by nano-ESI-MS and found to be pure.

Proteoliposome Preparation—Peptide incorporation into phospholipid vesicles was performed essentially as described previously (1). Shortly, peptides were dissolved in a small volume of trifluoroacetic acid (10 μl per mg of peptide) and dried under a nitrogen stream. To remove residual trifluoroacetic acid, the peptides were subsequently dissolved in TFE (1 mg/ml) followed by evaporation of the solvent in a rotavapor. Peptides were then again dissolved in TFE to a final concentration of 1 mg/ml. Dry mixed films of peptide and DMPH (peptide to lipid ratio of 1:25) were prepared as follows. Peptide solutions in TFE (1 ml; 0.46 mM) were added to DMPH solutions in methanol (1 ml; 12 mM) and vigorously vortexed. The solvent was removed by evaporation in a rotavapor. The mixed films were then dried for 24 h under vacuum. The films were hydrated at about 40 °C, well above the gel to liquid crystalline phase transition temperature of the phospholipid (24 °C (18)) in 0.5 ml of 10 mM ammonium acetate buffer (pH 7.5). Large unilamellar vesicles (LUVETs) were prepared by extrusion through a 400-nm filter at room temperature and kept at 4 °C until use.

Before the start of H/D exchange, LUVETs were preincubated at 30 °C for at least 30 min. For accurate comparison of the exchange data, LUVET suspensions with different peptide composition were mixed prior to the start of the exchange. LUVET suspensions were then 50 times diluted in deuterated ammonium acetate buffer at 30 °C (10 mM, pH 7.5), containing 1 m NaCl. At selected time points, 2 μl of this diluted suspension was transferred into a gold-coated glass capillary and the measurement was started as quickly as possible, whereby the peptides were analyzed simultaneously. The dead-time between dilu-
TABLE I
Amino acid sequences of the peptides used and their number of exchangeable hydrogens

| Peptide | Sequence | Average Mass (Da) | No. of labile H's |
|---------|----------|------------------|------------------|
| WALP16  | Ac-GWWLAALALALALWWA-Etn ² | 1897.31          | 22               |
| WALP19  | Ac-GWWLAALALALALALWWA-Etn ² | 2194.71          | 25               |
| WALP21  | Ac-GWWLAALALALALALWWA-Etn | 2379.95          | 27               |
| WALP23  | Ac-GWWLAALALALALALWWA-Etn | 2563.18          | 29               |
| WALP21 ³ | Ac-GW W' LALALALALALW W' A-Am ³ | 2391.00          | 23               |
| WALP23inner | Ac-GWALALALALALALALWAA-Etn ³ | 2332.91          | 27               |
| WALP23outer | Ac-GWALALALALALALALWAA-Etn ³ | 2374.99          | 27               |
| WALP23Pro | Ac-GWWLAALAPALALALALWWA-Etn | 2547.14          | 28               |
| WALP23gly | Ac-GWWLAALAGALALALALLA-Etn | 2507.07          | 29               |

² Ac, acetyl.
³ Etn, ethanolamine.
³ Am, amide.
⁴ W’, Trp analog with N-methylindole side chain.

TABLE II
Numbers of fast, intermediate, and remaining hydrogens in the investigated WALP peptides

| Peptide | No. of fast H⁺ | No. of intermediate H⁺ | No. of remaining H⁺ |
|---------|----------------|------------------------|--------------------|
| WALP16  | 6.9            | 2.4                    | 12.7               |
| WALP19  | 8.0            | 2.9                    | 14.1               |
| WALP21  | 8.5            | 2.9                    | 15.6               |
| WALP23  | 9.1            | 3.5                    | 16.4               |

Standard errors are below 10%.

RESULTS

To study effects of the hydrophobic length of transmembrane peptides on H/D exchange properties, a series of WALP/DMPC systems with varying peptide lengths (see Table I) were prepared for nano-ESI-MS measurements.

To allow accurate detection of small differences in exchange properties of different peptides, all experiments were performed with mixtures of two or more peptide-lipid systems synchronically. Therefore, LUVET suspensions with different peptide composition were mixed prior to the start of the exchange. Because such combination experiments are performed under identical experimental conditions, the deuterium levels can be compared directly. Fig. 1 illustrates that this method works. This figure presents, as an example, ESI mass spectra derived from simultaneously incubated and injected LUVETs of WALP21/DMPC and WALP23/DMPC dispersed in buffer, recorded by the direct proteoliposome method. The extremely hydrophobic membrane peptides are almost exclusively detected as [M + Na⁺] ions, whereas the monomers, dimers, trimers, and tetramers of DMPC are observed as [M + H⁺] as well as [M + Na⁺] ions. The zoomed-in spectra in the inset show the isotope envelopes of the [M + Na⁺] ions of the non-deuterated reconstituted WALP21 and WALP23 peptides (top), as well as those of the WALP peptides reconstituted in bilayer dispersions that were incubated for 5 min in buffered D₂O (bottom). The shift in mass of the peptides indicates uptake of about 8.5–9 deuterium atoms in both cases. Moreover, the spectra indicate that the vesicles are disrupted during the ionization process in such a way that only monomers and small oligomers of phospholipid molecules are observed.

The effect of increasing the hydrophobic length of the peptides with respect to the hydrophobic thickness of the bilayer was tested using a series of WALP peptides with varying hydrophobic core lengths (see Table I). Fig. 2 shows the measured deuterium content as a function of incubation time in deuterated buffer. All WALP peptides incorporated in DMPC show a gradual increase in deuterium content with time, though at a much slower exchange rate (defined as intermediate exchange rate, Ref. 1).
Fig. 2 shows that all peptides have similar exchange kinetics curves in the first 20 min after incubation. All curves level off to a deuterium level, which is low as compared with the total number of labile hydrogens present in each peptide, which varies from 22 to 29 (see Table I). Furthermore, the deuterium content increases with increasing peptide length. However, the differences in deuterium levels are relatively small and represent only a fraction of the number of additional hydrogens that become available for exchange when the peptide length is increased.

By fitting the kinetic curves using a non-linear squares fitting to multiexponential functions in all peptides, three kinetic regions of exchange rates representing hydrophobic populations were distinguished. In the short time range from 0 to 20 min, the fast and intermediate exchangeable hydrogens exchange, whereas the remaining hydrogens exchange only after ~20 min. The number of hydrogens in specific populations are shown in Table II. As was shown previously (1), and will also be shown later in this work, the fast exchanging hydrogens are predominantly the hydrogens at the N and C termini of the peptides, sticking out of the bilayer, while the hydrogens with intermediate exchange rates are the ones close to the Trp in the interfacial region. The remaining hydrogens, including the ones in the hydrophobic core have rate constants of (much) smaller than 0.10 min⁻¹. These slow exchange rates are a result of both the extremely low partition coefficient of D₂O in a lipid bilayer (19) and the involvement of the amide protons in hydrogen bonding because of α-helix formation. The amount of fast exchanged hydrogens increases with the length of the peptides but does not increase quantitatively with the numbers of exchangeable hydrogens. For instance, only about one-third of the extra number of hydrogens of WALP23 compared with WALP16 exchanges rapidly and, therefore, the majority of the extra hydrogens of WALP23 are protected against exchange.

These results may be explained by several possible mechanisms. First, the number of protected hydrogens may be higher due to a larger core region of the α-helix in the longer peptides. Second, the longer peptides may be more protected by the bilayer itself, as a result of tilting of the longer helices, or due to adaptation of the lipids to the peptide/lipid mismatch by stretching of the acyl chains (2). These possibilities will be discussed in more detail below (see “Discussion”). In principle, an alternative possible explanation for the protection of part of the peptides against H/D exchange would be the formation of peptide aggregates, which would also hamper access of D₂O molecules to the amide hydrogens. However, several observations argue against this. First, no restricted component was observed in electron spin resonance (ESR) measurements in these peptide/lipid systems, suggesting that WALP peptides are most probably present as monomeric transmembrane helices (2). Second, nano-flow ESI-MS experiments on the non-soluble WALP23 in buffer showed that when this dispersion was injected, not even a trace amount of WALP23 could be detected after extensive sonication (data not shown). Remarkably, after addition of TFE (1:1; volume ratio), which presumably breaks up the aggregates, the peptide could again be easily detected in the spectrum. It is therefore concluded that it is not possible to measure aggregated peptides directly by ESI-MS, indicating that the WALP peptides of which the mass spectra are analyzed in the present study do not originate from aggregates.

It has been proposed that Trp residues have strong interactions with the interface. To gain more insight into the potential anchoring role of the Trp residues, the positions of this aromatic amino acid were varied in peptides of invariable length. Moreover, to make the putative interaction zone less broad, the number of Trp residues was reduced to one at each terminus of the peptide (see Table I). WALP23inner has Trp residues at the 3 and 21 positions, whereas WALP23outer has Trp residues at positions 2 and 22. Fig. 3 shows the deuterium incorporation of both peptides reconstituted in DMPC after synchronous dilution in deuterated buffer and subsequent mass spectrometric analysis. Although both peptides have the same number of exchangeable hydrogens, they clearly show different H/D exchange behavior. The deuterium level curve for WALP23inner lies well above the one of WALP23outer, both in the short term region as well as after longer incubation periods. Comparing the average m/z values of the isotope clusters of the peptides in both undeuterated and deuterated buffer results in a 1.6 (± 0.2) Da higher deuterium content for WALP23inner. This suggests that WALP23inner has a larger population of fast exchangeable hydrogens. Consequently, WALP23inner incorporated in a DMPC bilayer seems to have a shorter protected region that could be due to a shorter inter-Trp distance. The deuteration levels of WALP23inner are also on the long term about 2 Da higher than for WALP23outer. These results suggest that the position of the Trp side chain determines the extent of protection in the bilayer environment, consistent with the idea that Trp forms a strong interfacial anchor (3, 12, 14, 15).

The time course for exchange of WALP23inner appears to match that of WALP23, when comparing the data in Figs. 2 and 3. However, if one assumes that the tryptophan side chain hydrogens exchange fast, then WALP23inner and WALP23outer would have two fast exchangeable hydrogens less than WALP23, and therefore the data would suggest that it is the backbone exchange of WALP23outer that matches well that of WALP23. Measurements with WALP21, which contains methylated Trp residues (see Table I), and thus has no exchangeable hydrogen atom in the indole side chain moiety, suggested that these indole hydrogens indeed exchange rapidly. It was found that the H/D exchange curve of this peptide lies close to 4 Da lower than that of WALP21 after both short and longer incubation times (data not shown). The apparent similarity in backbone exchange kinetics of WALP23 and WALP23outer is consistent with the idea that in the unfavorable case when the peptide is long with respect to the hydrophobic thickness of the bilayer (as is the case for the WALP23/DMPC system), the outer Trp will be furthest away from the favorable interaction site and therefore will be more important.
for determining the interfacial positioning of the peptide.

As shown previously, the proteoliposome spraying technique enables study of not only H/D exchange of the fast and intermediate exchangeable hydrogens, but also extremely slowly exchanging transmembrane amide hydrogens (1). The stability of the backbone of regular α-helices has been found to undergo large changes when potent breakers of α-helical structures in soluble (globular) proteins, like Pro and Gly, are inserted (e.g. Ref. 16). To investigate the effect of such amino acids on the stability of transmembrane α-helices, modified WALP23 peptides with a Pro or Gly residue in the middle of the peptide (see Table I) were incorporated in DMPC model membranes, and peptide mass increase was monitored. Generally, the time courses for exchange of WALP23, WALP23Gly, and WALP23Pro shown in the inset in Fig. 4A are quite similar at short incubation times (fast and intermediate exchange). This indicates that the positioning of the termini and the residues located in the interfacial region of all peptides is similar. However, after longer incubation times the deuterium level of WALP23Pro increases at a higher rate than that of WALP23 and WALP23Gly. In this time period, the amide hydrogens in the transmembrane region exchange (1). To probe H/D exchange rate constants the data were converted to plots of the natural logarithm of the number of remaining exchangeable hydrogens (H) with time, showing linear segments representing populations of hydrogens exchanging at the same rate (see Fig. 4B).

The rate of exchange for the remaining 13 hydrogens in WALP23Pro as determined from these logarithmic plots is about seven times higher than in WALP23 (Table III), but for WALP23Gly this rate is almost similar to that of WALP23. This suggests that the amide hydrogens in the transmembrane helix of WALP23Pro are more accessible to exchange and thus less protected, probably due to destabilization of the α-helix. In our studies Gly does not significantly disturb the α-helix character of the transmembrane part.

To further investigate the differences in exchange kinetics between WALP23Pro and WALP23, fragmentation studies were performed, which allow determination of the sites of the exchanged hydrogens (1). Collision-induced fragmentation (CID) of Na+ cationized hydrophobic peptides generates mostly N-terminal A, fragment ions and C-terminal Y, fragment ions (see Fig. 5A). The deuterium levels in series of fragments were determined from differences in the centroids of the isotope envelopes and are represented by bar diagrams (Fig. 5, B and C). Briefly, the smaller the slope in these bar diagrams, the less deuterium uptake takes place in this part of the peptide. Fig. 5, B and C show a series of deuterium content plots of the fragment ions of WALP23 and WALP23Pro, respectively, both incorporated in DMPC and after various incubation times in deuterated buffer. WALP23 (as well as WALP16, WALP19, and WALP21; data not shown) has a transmembrane part that is quite stable against exchange even after very long incubation times, indicated by the flat region in the bar diagrams, while both the C-terminal ends (higher A fragments) and the N-terminal ends (higher Y fragments) of the peptide exchange fast. Also the termini of WALP23Pro (Fig. 5C) exchange fast, and, like in WALP23, after short incubation times there is no exchange in the segment corresponding to the transmembrane part. However, after longer incubation times the flat region in the center of WALP23Pro disappears, indicating that a significant part of the H/D exchange also takes place in the transmembrane part.

These results suggest that Pro destabilizes the α-helical structure. From the CID MS experiments it then can be concluded that destabilization is not centered around the Pro residue but that it takes place over the entire transmembrane region.

**DISCUSSION**

In this study, the recently developed proteoliposome nanoflow ESI-MS technique (1) has been exploited to study the H/D exchange kinetics of a series of synthetic transmembrane peptides. These peptides were designed to gain insight into several crucial factors that can influence the positioning and stability of transmembrane peptides in lipid bilayers.

First, the effect of elongation of the hydrophobic part of transmembrane peptides with respect to the hydrophobic thickness of the bilayer was investigated. The peptides were incorporated in a DMPC bilayer and deuterium exchange kinetics of peptides that were only differing in the hydrophobic part of the transmembrane peptides in lipid bilayers.

**Table III**

| Peptide    | No. of hydrogens | Exchange rate \( \times 10^7 \text{s}^{-1} \) |
|------------|------------------|---------------------------------------------|
| WALP23     | 13.8             | 1.5                                         |
| WALP23Gly  | 13.0             | 1.7                                         |
| WALP23Pro  | 13.3             | 10.0                                        |

**Fig. 4.** A, time-dependent change in deuterium contents of WALP23 (○), WALP23Pro (▲) and WALP23Gly (■), all incorporated in a DMPC bilayer after exposure to deuterated buffer. The inset shows the uptake in the time region between 0 and 20 min. The plot in B shows the converted data in which the time-dependent decrease in the natural logarithm of the number of protected hydrogens is shown. Best-fit regression lines are shown, and hydrogen populations with similar exchange rates were probed from these plots (see text for details).
be 24 Å if one assumes that it forms an ideal α-helix with a net length of 1.5 Å per amino acid. WALP23 on the other hand, would be too long for this relatively thin bilayer and would extend into the aqueous phase. If it is assumed that the part that extends out of the hydrophobic part of the bilayer exchanges fast, as was shown to occur for polar extensions on both sides of a WALP16 analog (1), then peptides that are longer than WALP16 should have a larger population of fast exchangeable hydrogens. This indeed was found to be the case. However, the number of fast exchangeable hydrogens in the longer peptides was much less than the total number of additional exchangeable hydrogens, suggesting that longer peptides somehow are more protected against exchange. There are several possibilities to explain this observation. A likely explanation is that the membrane environment itself provides increased protection, for instance by increasing its hydrophobic thickness by stretching of the phospholipid acyl chains. Such an interpretation would be in agreement with earlier results from 2H NMR experiments (2), which show that the phospholipid acyl chains are more ordered in the presence of relatively long peptides. This interpretation is schematically depicted in Fig. 6A. An alternative or additional mechanism by which the membrane environment could provide increased protection of longer peptides would be by accommodating the peptides in a tilted orientation. Infrared experiments (10) indicate that such a tilt may indeed occur, albeit only to a minor extent. Finally, one might argue that the longer core region is responsible for the larger extent of protection in longer α-helices, because more backbone amide protons will be involved in hydrogen bonding. However, the results with peptides of identical total length, but with the Trp at different positions, argue against this possibility. These peptides, WALP23inner and WALP23outer (see Table I), are expected to have an equal length of the stable core region, yet they show distinct differences in H/D exchange kinetics. It was found that the part that exhibits extremely slow exchange corresponds closely to the Trp–Trp distance in these peptides. Because tryptophans have a strong interaction with the lipid/water interface (3, 11–15), these results suggest that it is the position of the helix with respect to the interface that determines the extent of exchange. Thus, it is likely that the affinity of Trp for the lipid/water interface causes the larger protection from exchange for WALP23outer as compared with WALP23inner, by inducing the bilayer to adapt at least partially to the Trp–Trp distance by either stretching of the acyl chains, as schematically depicted in Fig. 6B, or by accommodating the peptide in a tilted orientation.

Remarkably, WALP23 which contains two Trp residues at each side of the peptide, showed similar backbone amide exchange kinetics as WALP23outer. This suggests that the outer Trp residues are the determinants for interfacial positioning of this relatively long peptide and hence that localization of Trp residues further away from the interface toward the aqueous phase is unfavorable. Together, the results show that Trp has strong membrane-anchoring properties and interacts with a
acyl chains are able to partially adapt their hydrophobic thickness (4).

When a peptide that is too long is incorporated in the bilayer, the lipid one of the possible mechanisms to explain the observed data. DMPC bilayer, depicting adaptation of the bilayer thickness as

Therefore, the regular transmembrane and show faster exchange over the whole transmembrane region.

with a Pro residue are involved in hydrogen bonding to a lesser extent (5). Amide hydrogens in a transmembrane segment B face region, because WALP23inner exhibits more H/D exchange than does WALP23outer (B). Amide hydrogens in a transmembrane segment with a Pro residue are involved in hydrogen bonding to a lesser extent and show faster exchange over the whole transmembrane region. Therefore, the regular transmembrane α-helix is destabilized, perhaps involving introduction of a kink in the helix (C).

well defined region in the bilayer interface. This supports the idea that Trp residues, which have shown to be clustered at the border between the hydrophilic and hydrophobic zones both in single-spanning (8) and multispansing integral membrane proteins (e.g. Refs. 5, 7) are important for stabilizing integral membrane proteins in a lipid bilayer (3, 11–15). The observation that Trp residues may determine the precise interfacial position of transmembrane segments of proteins also is functionally relevant, because the interfacial positioning of e.g. receptor proteins or channel-forming proteins can determine their accessibility to enzymes, substrates, or ligands (21).

Finally, the effect of inserting specific amino acid residues that are generally known to destabilize a regular α-helix in water-soluble proteins, like Pro and Gly, was studied. The frequent occurrence of Pro in the putative transmembrane helices of integral membrane proteins, particularly transport proteins and G-coupled receptors, has led to the suggestion that the presence of this conserved amino acid residue might play a role in folding and/or assembly of integral membrane proteins in general and that it may be essential for functional activity of transport proteins and ion channels (16, 22, 23), because of its supposed ability to introduce kinks into transmembrane α-helices. Our results show that the presence of a Pro residue in the transmembrane helix of WALP23 leads to a significant increase in exchange after longer incubation times, whereas MS/MS measurements demonstrated that exchange in the transmembrane helix region is increased. The similar exchange kinetics in the short term region of WALP23 peptides with and without Pro suggest that the peptides are anchored in the lipid bilayer interface in a similar way. Therefore, it is suggested that the presence of a Pro residue may cause a less stable helix fold of the hydrophobic core of WALP23, as schematically depicted in Fig. 6C.

There may be some other possibilities to interpret the increased H/D exchange rates for WALP23Pro as compared with WALP23. One such possibility would be that because of a Pro-induced kink in the peptide the lipid acyl chains in the vicinity of the transmembrane helix may be perturbed. This would allow an increase of water permeation in the bilayer, and, therefore, could lead to somewhat higher exchange rates specifically in the transmembrane region. In principle, an alternative possibility could be that the peptide does not have a transmembrane orientation, but instead is located on the surface of the bilayer, which also would result in higher exchange rates. However, in this case one would expect much faster exchange rates than the ones observed, because the peptide would be in direct contact with bulk deuterated water. Indeed, control measurements on WALP23 in TFE/D2O buffer (1:1; volume ratio), in which the peptide forms an α-helix (data not shown), demonstrated that under these conditions H/D exchange was already complete within less than 60 min at 30 °C. Another argument against a surface localization of WALP23Pro would be the observed similarity between the numbers of fast exchangeable protons with those in WALP23, which by various techniques has been shown to adopt a transmembrane orientation (3, 10). Hence we exclude this possibility. Finally, one might consider the possibility of a slow equilibrium between surface and bound states of WALP23Pro. In this case, however, several populations of deuterated WALP23Pro (i.e. fast and slow exchanging) should be present in the mass spectra at the same time. Because this is not observed, we exclude this latter possibility as well.

In contrast to WALP23Pro, WALP23Gly shows similar exchange kinetics as WALP23, indicating that Gly does not have a strong effect on the stability of the transmembrane helix in the systems studied here. Gly has been suggested to play a role in environment-dependent α-helix modulation (24) and to mediate helix-helix interactions in membrane proteins (25). Conformational studies on model peptides in a membrane mimetic environment (24) and analysis of dihedral backbone angles in crystal structures of several membrane proteins (26) suggested that Gly does not disrupt the secondary structure of α-helical transmembrane segments. The results of the present study support this. In summary, our results show that insertion of a Pro residue but not of Gly results in significant destabilization of a transmembrane α-helix in a lipid bilayer. The structural changes in Pro-containing transmembrane segments might be important for functional properties of membrane proteins.

This study has shown that the effect of variations in hydrophobic length, in the positions of flanking residues and of insertion of putative helix breakers in transmembrane peptides, can be studied in great detail by H/D exchange combined with mass spectrometry, whereby the positions of the exchanged hydrogens can be derived from MS/MS experiments. Moreover, it was shown that it is possible to measure H/D exchange in two or more peptides simultaneously, allowing determination of very small differences in deuterium levels. Detailed information about helix stability and interfacial positioning, as obtained in the present study, not only provides insight into the factors that determine the way in which membrane proteins are integrated into a lipid bilayer but also may be useful for predictions of transmembrane segments from...
amino acid sequences. In future studies we plan to extrapolate the method to larger integral membrane proteins and membrane-bound proteins.

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