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A New Method for Determining the Local Environment and Orientation of Individual Side Chains of Membrane-Binding Peptides

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Protein−membrane interactions are the molecular basis of many biological processes. Among such protein−membrane interactions are membrane lysis by antibiotic peptides, fertilization facilitated by fusion peptides, and viral infections mediated by fusion peptides. Other biological processes that are based on protein−membrane interactions involve proteins that are intrinsically associated with membranes such as ion channels and receptors. Therefore, development of methods for the site-specific determination of protein−membrane interactions contributes to the understanding of the molecular basis of many such processes. The amide I band of polypeptides, which arises mainly from the amide C═O stretching vibration, has been used extensively to determine the conformation as well as orientation of transmembrane peptides in conjunction with polarized attenuated total reflection (ATR) infrared (IR) spectroscopy.[1] However, this method does not offer site specificity because the amide I band of peptides and proteins is broadened by both homogeneous and inhomogeneous mechanisms. Employing ^{13}C═Os may sometimes help to resolve backbone conformation of individual residues;[2] however, the local environment seen by individual side chains cannot be revealed by this approach. Here we describe a new method for studying the local environment of transmembrane peptides.

Recently, Getahun et al.[3] have shown that nitrile-derivatized amino acids could be used as infrared environmental probes, due to the sensitivity of the CN stretching vibration to hydration as well as other factors.[4] Here we show that nitrile-derivatized amino acids can further be used to provide detailed information regarding the interaction between peptides and membranes, especially the hydration state and orientation of individual side chains. To demonstrate the validity of this method, we employed a well-studied membrane-binding peptide, i.e., mastoparan x (MPx peptide with the sequence INWKGIAAMAKKLL). Seven nitrile-derivatized mutants of the MPx peptide, i.e., MPx−CNy peptides, with a Phe$_{CN}$ residue (Supporting Information) replacing different positions along the peptide sequence (i.e., $y = 5-11$), were synthesized and studied spectroscopically.[5] MPx is a typical amphipathic peptide, whose structure in lipid bicelles and SDS micelles has been determined by NMR spectroscopy;[6] its binding to lipids has also been studied by other NMR methods.[7]

The free MPx−CNy peptides in water exhibit a CN stretching vibration centered at ~2235 cm^{-1} with a Lorentzian bandwidth of ~13 cm^{-1} (Supporting Information and Table 1). These results indicate that the Phe$_{CN}$ side chain in these peptides is fully hydrated.[3] However, when bound to POPC phospholipid bilayers, the CN stretching vibration of these peptides shifts to lower wavenumbers and becomes narrower (Supporting Information and Table 1). In fact, its position and width are almost identical to those obtained on a Phe$_{CN}$ residue that is buried in a hydrophobic pocket,[3] indicating that the segment labeled with Phe$_{CN}$ (i.e., 5−11) in the MPx peptide is also buried in the hydrophobic region of the phospholipid bilayer and is dehydrated. Although previous studies have shown that, when MPx peptide interacts with zwitterionic lipids, it lies near the bilayer interface,[6] it is not clear if its polar side chains are still exposed to solvent. Our results nevertheless suggest that even the hydrophilic side of this amphipathic peptide lies likely beneath the phosphate headgroups of the lipids and is dehydrated.

Table 1. Band Position (ν) and Full Width at Half-Maximum ($\Delta\nu$) of the Stretching Vibration of CN in MPx−CNy Peptides.

Phe$_{CN}$ position	5	6	7	8	9	10	11
ν_F (cm^{-1})	2235.7	2234.9	2234.8	2235.6	2235.1	2235.2	2235.8
$\Delta\nu_F$ (cm^{-1})	12.8	14.1	13.8	11.0	12.0	13.3	12.8
ν_B (cm^{-1})	2229.6	2229.8	2229.4	2229.8	2228.9	2229.3	2228.5
$\Delta\nu_B$ (cm^{-1})	12.5	8.4	10.2	10.6	8.4	9.3	9.9
θ (deg)	39 ± 7	49 ± 8	41 ± 7	47 ± 6	67 ± 7	47 ± 6	68 ± 7
α (deg)	62 ± 7	57 ± 8	61 ± 7	58 ± 6	50 ± 7	58 ± 6	50 ± 7
θ_t (deg) − trans	53	53	53	53	53	53	53
α_t (deg) − trans	37.9	85.5	39.1	71.3	48.8	56.8	60.6
θ_g (deg) − gauche	48	48	48	48	48	48	48
α_g (deg) − gauche	81.8	44.8	68.8	54.8	55.6	66.1	46.8
fraction trans	0.45	0.30	0.26	0.19	0.82	0.87	0.23

[a] ν_F and $\Delta\nu_F$ are parameters for free peptides, whereas ν_B and $\Delta\nu_B$ are parameters for bound peptides. Also listed are angles of the CN transition dipole with respect to the helical axis, Θ, and with respect to the membrane normal, α. θ_t, α_t, θ_g, and α_g are the calculated values for θ and α obtained from an ideal 3.6 residue/turn helix aligned parallel to the membrane surface, with Phe$_{CN}$ built in the two most populous sidechain rotamer conformations at each of the seven positions.

Interestingly, the MPx−CNy peptides in the bilayer exhibit small but noticeable differences in the bandwidth of their CN stretching vibrations. For example, MPx−CN5 has a relatively broader bandwidth, whereas those of MPx−CN6 and MPx−CN9 are relatively narrower. This is due presumably to the subtle difference in the local environments seen by these CN groups. Taken together, these results suggest that the side chain of residue 5 in the MPx peptide samples on average a more inhomogeneous environment, whereas those of residue 6 and 9 see a less inhomogeneous surrounding. This difference may be explained by the amphipathic nature of the peptide. NMR studies have revealed that both residue 6 and 9 (i.e., I6 and M9) of the MPx peptide are buried deeply in the hydrophobic interior of the membrane. Thus, it is expected that the side chains of these residues would sample a less inhomogeneous environment. On the other hand, the Phe$_{CN}$ in MPx−CN5 probably has its side chain pointed toward the headgroups of the lipids; as a result, it exhibits a broader absorption bandwidth (see below). On a similar note, the phase of the helix facing the lower layer was determined by fitting the variation in the square root of the CN bandwidth as a function of position to a sinusoidal function. The phase computed from the bandwidth matches that calculated

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with hydrophobicity to within 6°. This further supports that the bandwidth relates to the degree of insertion into the membrane. It is worth noting that the free peptides in water also exhibit variations in their CN bandwidths, due probably to either peptide self-association or residual structures.

Further ATR polarization studies of the amide I band of the MPx peptide indicate that the peptide lies in the membrane plane, consistent with previous results.[6] For example (Supporting Information), the amide I absorbance of the MPx−CN5 peptide depends on the polarization of the linearly polarized IR light and the dichroic ratio gives rise to an angle of 14 ± 7° between the helical axis and the membrane plane.[8] Within experimental uncertainty, other peptides (labeled and unlabeled) give rise to the same angle, indicating that the incorporation of the Phe_{CN} residue does not alter the structure and orientation of the peptide significantly. Since the helix lies at a slight angle in the membrane, one of its termini would be buried slightly deeper into the membrane, whereas the other would be close to or at the interface. For MPx peptide, NMR data[6] have shown that it is the N-terminus that lies close to the membrane interface. Consistent with this picture is the broader bandwidth observed for the CN stretching vibration of the MPx−CN5 peptide. Because the side chain of residue 5 is closer to the more dynamic lipid headgroups, it would be expected that it experiences a more inhomogeneous environment and, thus, a broader vibrational transition.

In addition, polarized ATR-IR measurements were carried out for the CN stretching vibration (Supporting Information). Since the molecular axis of the CN group lies parallel to the phenyl group of the Phe_{CN} side chain, the direction of the vibrational transition dipole moment of the CN group, which can be obtained by measuring its dichroic ratio, directly reports the angle of the Phe_{CN} side chain with respect to either the membrane normal or the helical axis (Table 1). Because of the rigidity of the structure of the side chain between the Cβ and the nitrile group, the orientation of the CN group is defined by the torsional angle χ_1 (centered at the Cα−Cβ bond) and the orientation of the helix. Therefore, the polarization of the nitrile stretch provides information concerning both the orientation and the rotamer distribution of the Phe_{CN} side chain in the bilayer. As an example, we computed here the angles of the Phe_{CN} side chain relative to the helical axis and the membrane normal for a perfect helix formed by the MPx−CNy peptides aligned parallel to the membrane surface (Supporting Information). Assuming that the preferred χ_1 values of Phe_{CN} are the same as those of Tyr, the nitrile group should have an angle of approximately 53 or 48° relative to the helical axis for the two highly populated rotamer states of Phe_{CN} (trans and gauche, respectively). Interestingly, the observed angle of the CN group relative to the helical axis is more or less independent of position and has an average value of 51 ± 10° (Table 1). This finding suggests that the helical structure formed by the MPx peptide within the membrane is nearly perfect. Any slight differences in the observed angles can be accounted for by small distortions to the side chains due to the bilayer or mutations to Phe_{CN}. The latter may cause peptides to adopt slightly different conformations. Ideally, the CN group should be incorporated in the side chains of the native residues.

In contrast, the angle between the nitrile group and the membrane normal should depend both on the sequence position and the rotamer state of Phe_{CN}. Thus, the value of the experimentally determined α can be used to determine the relative populations of the two rotamer states of Phe_{CN}. As shown (Table 1), the fractional contribution of each conformer was found for each rotamer using the following relationship: $\alpha = F^*\alpha_t + (1 - F)^*\alpha_g$, where F is the fraction of the trans conformer. Interestingly, it appears that each position favors one conformation over the other with the exception of residue 5, which samples the two different conformations almost equally. This result is consistent with the inhomogeneity of the environment at this site suggested by the broader bandwidth of the CN stretching vibration of Phe_{CN} at position 5.

In summary, we demonstrated here the utility of using nitrile-derivatized amino acids to determine the hydration state of specific sites of membrane-interactive peptides (upon binding). We have also shown that polarization measurements can further be used to uncover information regarding the spatial orientation of individual side chains as well as their rotamer distributions.

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Supporting Information Available: Regular and polarized FTIR spectra of MPx−CNy peptides as well as methods (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) MPx−CNy peptides were synthesized by employing the standard Fmoc protocol, purified by reverse-phase HPLC, and verified by electrospray-ionization mass spectroscopy. For ATR-IR measurements, the MPx−CNy peptide (0.1 mM in MeOH) was added to POPC vesicles (15 mM in chloroform) in a ratio of 1:75 peptide/lipid. From this sample, 100 μL (~650 μg) was then placed on a Ge crystal (5 × 1 cm) and allowed to dry. Before the experiment, the peptide−lipid sample was rehydrated by placing the Ge crystal in a humidified chamber for 12 h (covered beaker with water at the bottom and the Ge crystal supported to avoid immersion in water). Alternatively, rehydration was accomplished by placing 20 μL of water dropwise directly on the peptide/lipid bilayer surface. Both methods yielded similar results. ATR-IR spectra were collected with a Harrick's Horizon multiple-reflection attachment in conjunction with a Magna-IR 860 spectrometer equipped with a MCT detector. An average of 128 scans were taken for each sample.

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