Interactions Involved in the Realignment of Membrane-associated Helices

AN INVESTIGATION USING ORIENTED SOLID-STATE NMR AND ATTENUATED TOTAL REFLECTION FOURIER TRANSFORM INFRARED SPECTROSCOPIES

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A series of histidine-containing peptides (LAH4nXn) was designed to investigate the membrane interactions of selected side chains. To this purpose, their pH-dependent transitions from in-plane to transmembrane orientations were investigated by attenuated total reflection Fourier transform infrared and oriented solid-state NMR spectroscopies. Peptides of the same family have previously been shown to exhibit antibiotic and DNA transfection activities. Solution NMR spectroscopy indicates that these peptides form amphipathic helical structures in membrane environments, and the technique was also used to characterize the pK values of all histidines in the presence of detergent micelles. Whereas one face of the amphipathic helix is clearly hydrophobic, the opposite side is flanked by four histidines surrounding six leucine, alanine, glycine, tryptophan, or tyrosine residues, respectively. This diversity in peptide composition causes pronounced shifts in the midpoint pH of the in-plane to transmembrane helical transition, which is completely abolished for the peptides carrying the most hydrophilic amino acid residues. These properties open up a conceptually new approach to study in a quantitative manner the hydrophobic as well as specific interactions of amino acids in membranes. Notably, the resulting scale for whole residue transitions from the bilayer interface to the hydrophobic membrane interior is obtained from extended helical sequences in lipid bilayers.

To date, high resolution three-dimensional structural information on membrane proteins remains sparse. Therefore, prediction and computational methods that allow identifying membrane-spanning segments from their amino acid sequence remain an important tool to establish a first topological model. To this purpose, a number of “hydrophobicity scales” have been developed and later improved using computational algorithms (1). These are either based on experimental data (2, 3) or mixed experimental and educated guesses (4–7). Furthermore, from the few known three-dimensional structures as well as from biochemical experiments that determine the topology of membrane proteins (8), knowledge-based statistical scales have been established (9–12).

A good algorithm should reliably identify all transmembrane helices and differentiate membrane-spanning domains from sequences that compose a hydrophobic helix within the interior of a soluble protein (13–17). Furthermore, the membrane exhibits very different properties in the interior, at the interface or within the regions directly next to the surface (11, 18–21). These marked differences in physico-chemical environment are also important for the alignment of polypeptide sequences either parallel or perpendicular to the membrane surface (22). How peptides orient relative to the membrane normal is of considerable importance for the activity and regulation of helical sequences such as antibiotic peptides, DNA transfectants, or signal sequences (23, 24).

Furthermore, biophysical studies indicate that some membrane-inserted proteins may exhibit a more loosely folded structure, thereby resembling a tethered assembly of individual helices. These include some of the colicin channel domains (25) or the antiapoptotic Bcl-xL protein (26). The interactions of these proteins with the membrane are therefore governed by the same mechanisms as those important for smaller peptide sequences.

A reliable prediction of membrane-spanning protein domains requires good knowledge of the free energies (or a parameter that is correlated to that) that are involved when placing amino acid side chains in the hydrophobic interior of the membrane. Bilayer insertion involves the transfer from the aqueous buffer to the membrane interface and then on into the hydrophobic interior. Most hydrophobicity scales therefore monitor the transition from the aqueous to a low dielectric environment (4–7). A series of model peptides is presented in this paper that provide experimental access to the free energies associated with the transitions from in-plane to transmembrane helical alignments and thus from residue localizations at the membrane interface to the bilayer interior. The LAH4 peptide (27), which was used as a design template, exhibits pronounced antimicrobial activity (23) and functions as a potent DNA transfectant (24). Interestingly, “mutagenesis” experiments indicate that this latter activity is strongly dependent on the capacity of the peptide to change its alignment relative to the membrane normal (24, 28). Here we investigate in a more systematic manner how the amino acid composition of LAH4-type peptides can be used to modulate the transition pH.

The LAH4-derived sequences are composed of four histidines interrupted by a hydrophobic stretch of alanines and leucines. Furthermore, several lysines at each terminus act as membrane anchors and increase the solubility of the peptides in polar solvents. Whereas the central core
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of the peptide sequence is sufficiently long and hydrophobic to be able to span the lipid bilayer, the histidines are arranged in such a manner as to allow the formation of an amphipathic α-helical structure (Fig. 1). The histidine side chains exhibit pKα values close to 6.0 when in an aqueous environment, thus being positively charged at acidic pH and polar but uncharged at pH ≥7 (Table 1). Histidines have been used previously to control the membrane interactions of model peptides (27, 29).

The helical wheel diagram of LAH6X6 indicates how six amino acids of variable composition (labeled X) are interspersed between the histidines (Fig. 1). In this design, hydrophobic residues at the X positions exert a strong driving force for membrane insertion. This is counteracted by the preference of the histidine side chain to remain in the aqueous environment, thus being positively charged at acidic pH and to allow the formation of an amphipathic α-helix. The pH-dependent in-plane to transmembrane transition can thus be used to directly test for the relative hydrophobicity of the X residues.

Previously, it has been shown that oriented solid-state NMR or ATR-FTIR3 spectroscopy can be used to follow the alignment of helical peptides in membranes. The 15N chemical shift (30) and the dichroic ratio (31) provide sensitive indicators of helical tilt angles when α-helices are incorporated in oriented phospholipid bilayers. When the 15N NMR technique is applied to membranes oriented with the normal parallel to the magnetic field direction, the measurement of 15N chemical shifts of <100 ppm is indicative of helix orientations parallel to the membrane surface, whereas resonances of >180 ppm agree with transmembrane helix alignments (30, 32).

On the other hand, ATR-FTIR spectroscopy of oriented membrane samples allows one to monitor the average peptide alignment and conformation by measurement of the dichroic ratio (33–35) and the frequencies of the amide bands (31, 36), respectively. The characteristic frequencies are 1662–1645 cm⁻¹ for α-helical peptides, 1689–1682 cm⁻¹ for β-sheet conformations, and 1644–1637 cm⁻¹ in the case of random coil sequences (36, 37). Furthermore, the dichroic ratio of the amide I band is a direct indicator of the average tilt angle of helical polyamides. Whereas in-plane oriented peptides exhibit R values around 1.3, this parameter augments by a factor of 2–3 for transmembrane helix orientations. Here we have used both approaches to investigate several peptides of the LAHnXα series.

THEORY

In order to analyze the data in quantitative detail, dynamic equilibria between membrane-associated states are considered. The underlying model and theory have been described in detail elsewhere (38). In short, the following membrane-associated states are considered: IPo → IP1 → IP2 → IP3 → IP4 (including hydrophobic energies). At acidic pH, the processes of membrane insertion (reorientation into the transmembrane configuration) and discharge are therefore tightly connected. The contribution of the hydrophobicity of individual helices can be quantitated. When the X amino acids exhibit a low degree of hydrophobicity, the in-planar state remains populated even when the histidines are charged. In order to take into account the gradual protonation of four histidine side chains, the in-planar states IP1⁻, IP2⁻, IP3⁻, and IP4⁻ are taken into consideration (38).

The equilibrium constant for the transition from IPo to TM is related to the Gibbs free energy ΔG gained by inserting the peptide into the membrane. The equilibrium constant is therefore defined as follows.

$$k_{TM} = \frac{[TM]}{[IPo]} = e^{-\Delta G/kT}$$  (Eq. 1)

Furthermore, the uncharged in-planar configuration is in equilibrium exchange with the series of charged in-planar states, none of these being able to insert in a transmembrane fashion. When combined into a single state, [IP*] = [IP1⁻] + [IP2⁻] + [IP3⁻] + [IP4⁻], the constant (1/kCh) for the transition IPo ↔ IP* is the sum of the individual equilibrium constants.

$$1/k_{Ch} = \frac{[IP*]}{[IPo]} = \sum 1/k_j$$  (Eq. 2)

By taking into consideration the number of possible states IPr+ and some algebraic transformation, the experimentally accessible ratio of the transmembrane over total peptide concentration, pTM, is given by the following (38),

$$p_{TM} = \frac{1}{1 + e^{\Delta G/RT}(1 + e^{2.3c(pKα - pH)})^4}$$  (Eq. 3)

with the meaning of c being discussed below. The resulting line shapes are shown in supplemental Fig. 8.

The degree of hydrophobicity of the X amino acids and thus the ΔG of transfer from the in-plane to the transmembrane state is reflected by three properties of this function. First, the slope of the transition changes with ΔG, being steeper for high negative ΔG (very hydrophobic X). Second, the maximal amount of transmembrane orientation that is observed varies with ΔG. For moderately hydrophobic or hydrophilic peptides, the transmembrane state is never fully populated. Third, the

*The abbreviations used are: ATR, attenuated total reflection; FTIR, Fourier transform infrared; Fmoc, N-protected N-methylglycine proline; DPC, dodecyl phosphocholine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine; MALDI, matrix-assisted laser desorption ionization; HPLC, high pressure liquid chromatography; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy.
transition midpoint is shifted toward pH values higher than the pKₐ values of the histidines.

During the analysis of pH titration experiments, it should be considered that the local pH on the surface of a membrane can differ significantly from the pH in bulk solution. In addition, the protonation of the four histidines in the peptide are dependent on each other due to electrostatic interactions. We have, therefore, investigated the (de)protonation reactions of the LAHₓₐ histidines in membrane environments by monitoring the pH-dependent ¹H chemical shift changes of the histidines using solution NMR spectroscopy (see "Results" and Fig. 4). When analyzing the data, it becomes obvious that due to constraining the charges at biomolecular surfaces, the slope of the sigmoidal transition is reduced when compared with the Henderson-Hasselbach equation. The fitting procedure provides the averaged correction factor c, which is also used during the line-fitting analysis of the experimental transition curves using Equation 3.

Whereas the fraction of transmembrane-oriented peptide can be evaluated by integration of the appropriate spectral ranges of the ¹⁵N solid-state NMR spectra, the ATR-FTIR data were analyzed using Equation 4 (31).

\[
\frac{2p_{\text{TM}}(R_{a}^{\text{ATR}} - R_{b}^{\text{ATR}}) + R_{b}^{\text{ATR}}(R_{a}^{\text{ATR}} + 2)}{p_{\text{TM}}(R_{a}^{\text{ATR}} - R_{b}^{\text{ATR}}) + R_{b}^{\text{ATR}} + 2} \quad (\text{Eq. 4})
\]

Here \( R_{a}^{\text{ATR}} \) and \( R_{b}^{\text{ATR}} \) are the maximal and minimal dichroic ratio, respectively.

MATERIALS AND METHODS

The LAHₓₐ peptides of the primary structure KKKKALXHLHX-
LAXHHXLAXXXALKK-COOH were prepared by solid-phase peptide synthesis on a Millipore Corp. 9050 automatic peptide synthesizer using Fmoc chemistry (40, 41). The six X-amino acids at positions 7, 11, 14, 18, 21, and 22 represent alanine (LAHₓₐ), glycine (LAHₓₐ), leucine (LAHₓₐ), tyrosine (LAHₓₐ), or tryptophan (LAHₓₐ), respectively. The underlined alanine 13 position indicates the use of the ¹³C-labeled derivative of Fmoc-alanine (Promochem, Wesel, Germany) during the coupling step. Whenever the crude product was considered inadequate, the synthetic products were purified using reversed phase high performance liquid chromatography using an acetonitrile/water gradient and a Prontosil 300-5-C4 5.0-μm column (Bischoff Chromatography, Leonberg, Germany). The purity and composition of the peptides were controlled by reversed phase HPLC and MALDI mass spectroscopy.

For solution NMR spectroscopy, 5 mg of peptide and 70 mg of perdeuterated DPC (Promochem, Wesel, Germany) were dissolved in 450 μl of 11 mM citrate-d₆ (Campro, Emmerich, Germany) and 5 mM NaCl. The pH was adjusted using a 1 M NaOH stock solution.

The NMR spectra were acquired on a Bruker AMX 500 spectrometer at 300 K. For the determination of the pH-dependent behavior of the histidine resonances, TOCSY (42) and NOESY spectra (43) were acquired using data matrices of size 2048 × 512 or 4096 × 512. Water suppression was achieved using the WATERGATE sequence (44). Typically, the applied mixing times were 85 ms for NOESY and 60 ms for TOCSY spectra. Before Fourier transform, phase-shifted sine-square apodization functions and polynomial baseline corrections were applied. The processed matrix size was chosen between 2048 × 1024 and 4096 × 4096. Published methods were used to assign the resonances of the histidine side chains (45).

For solid-state NMR spectroscopy, 20 mg (−6.5 μmol) of peptide was dissolved in water/trifluoroethanol and mixed with 300 mg (≈400 μmol) of POPC (Avanti Polar Lipids, Birmingham, AL). Prior to the addition of organic solvent, the pH of the peptide solution was adjusted by the addition of 1 N NaOH. The mixtures were slowly applied onto 30 thin cover glasses (11 × 22 mm), dried in air, and exposed to high vacuum overnight. After the samples had been equilibrated in an atmosphere of 93% relative humidity, the glass plates were stacked on top of each other and sealed. The uniaxially oriented stacks of membranes were introduced into the flat coil of a home-built solid-state NMR probe head (46) with the bilayer normal parallel to the magnetic field direction. Proton-decoupled ¹⁵N solid-state NMR spectra were acquired on a wide bore Bruker AMX400 spectrometer using a cross-polarization pulse sequence (47). Typical acquisition parameters were as follows: spin lock time, 1.3 ms; recycle delay, 3 s; ¹H B₁-field, 1 millitesla; 254 data points; spectral width, 40 kHz. An exponential apodization function corresponding to a line broadening of 300 Hz was applied before Fourier transformation. The chemical shifts were referenced using ¹⁵N ammonium sulfate (27 ppm).

ATR-FTIR spectroscopy of oriented membrane samples was performed using a Bruker IFS 55 infrared spectrometer equipped with a liquid nitrogen-cooled MCT detector, as described previously (31, 38). In short, using trifluoroethanol/water solutions, 50 μg of peptide and 500 μg of POPC were spread onto an area of 5 × 1 cm of a carefully cleaned planar germanium plate (ACM, Villiers St. Frédéric, France). The crystal is characterized by an aperture angle of 45°, yielding 25 internal reflections. The organic solvent was evaporated under a stream of nitrogen. Oriented lipid bilayers spontaneously form along the surface of the crystal, as discussed in detail in Refs. 33 and 34. The pH was adjusted by covering the membranes with 200 μl of 66 mM phosphate buffer. After a few minutes, the buffer was carefully removed, and the sample was washed with 200 μl of MilliQ-water (Millipore). Although it remains possible that some of the peptide is washed away, thereby modifying the effective lipid-peptide ratio, this procedure has proven essential in order to obtain a well defined pH value without interference from the remaining salt. A fresh sample was prepared for every pH value. After the samples had been dried under a stream of nitrogen, the crystal was introduced into the ATR-FTIR spectrophotometer. During the measurements, the samples were kept dehydrated with a stream of nitrogen. During spectral acquisition, the spectrometer was continuously purged with dry air. For each spectrum, 64 scans were accumulated at a nominal spectral resolution of 2 cm⁻¹.

RESULTS

The peptides of the LAHₓₐ series were designed to form α-helical secondary structures in membrane environments, as has been observed for many other sequences of related composition (27, 31, 38, 48–51). This feature was confirmed by the large number of NH-NH nuclear Overhauser effect cross-peaks observed for the LAHₓₐ peptides in the presence of detergent micelles (Fig. 2). Furthermore, when reconstituted into oriented POPC phospholipid bilayers, the LAHₓₐ peptides exhibited absorptions at 1657 cm⁻¹ (amide I) and at 1542 cm⁻¹ (amide II) characteristic of helical structures (52) (Fig. 3). Some spectra of LAHₓₐ showed small additional resonances at 1688 cm⁻¹, suggestive of the presence of tiny amounts of β-sheet conformations.

In α-helical conformations, the hydrophobic face made up of leucines and alanines covers a hydrophobic angle of 160° (lower face in Fig. 1). The remaining part (upper face) contains six amino acids X, which are flanked by the four histidines. By changing the composition of the peptide (six variable X residues) and the pH of the environment (protonation of the four histidine side chains), large variations of the amphiphilic moment and the overall hydrophobicity of the peptide can...
thus be obtained. In related histidine-containing peptides, changes in pH and amino acid compositions have resulted in reorientation of the peptide helices relative to the membrane normal (27, 31, 38).

A quantitative analysis of the titration data requires knowledge of the protonation state of histidines in membranes. We therefore investigated the pK values of a representative selection of LAH4X6 peptides in DPC micellar environments using solution NMR spectroscopy. By following the 1H chemical shift of the imidazole protons, the pK values of these residues in membrane environments were obtained (Fig. 4). Although the three peptides LAH4G6, LAH4A6, and LAH4L6 showed a very different pH dependence when their topological alignment was tested in oriented phospholipid bilayers (see below; Figs. 5 and 6), the 12 histi-
dines investigated all exhibited pK values in a narrow range around 6 (Table 1). The line-fitting procedure also showed that the transitions are flattened when compared with the theoretical Henderson-Hasselbach equations of acid-base equilibria in solution. This is due to electrostatic (and other) interactions of charged peptides confined to a surface and has been taken into account by the factor $c$ in Equation 3. For the ensemble of LAH$_4X_6$ peptides, the averages observed were as follows: $c = 0.77 \pm 0.09$ and $pK_a = 5.95 \pm 0.12$ (Table 1), where differences in these values represent the differences in the position of individual histidines relative to the membrane surface, relative to each other, and with respect to the end of the helix dipoles (27) as well as experimental variations. Similar titration curves have also been observed for the related peptide LAH$_4X_6$ (27).

In a next step, the peptides were reconstituted into oriented POPC phospholipid bilayers, and their alignment was analyzed by proton-decoupled $^{15}$N solid-state NMR spectroscopy (Fig. 5). Samples from five different LAH$_4X_6$ peptides (where $X$ represents Ala, Leu, Trp, Gly, or Tyr) were prepared, and the pH of the solution was adjusted to a given value before the membranes were oriented along microscope coverslips. Inspection of Fig. 5A indicates that the $^{14}$N chemical shift value of LAH$_4A_6$ (labeled with $^{15}$N at the alanine 13 position) changes from 87 to 225 ppm when the pH is increased from 4 to neutrality. In the intermediate pH region, a broad distribution of signal intensities is observed, indicating in-plane ($<100$ ppm) and transmembrane-oriented helices ($>160$ ppm) in slow exchange on the time scale of the chemical shift anisotropy ($10^{-4}$ s).

When the pH of oriented membrane preparations encompassing the LAH$_4A_6$ peptide was increased, the $^{15}$N chemical shift changed from 90 to 225 ppm. Notably, the transition was shifted to lower pH values when compared with LAH$_4A_6$. The $^{15}$N NMR spectra of the LAH$_4W_6$ peptide...
exhibited a broad intensity of <110 ppm at low pH and a well defined resonance at 220 ppm when the pH was increased (Fig. 5C). In contrast, neither the LAH4G6 nor the LAH4Y6 peptide exhibited a clear transition to transmembrane orientations (Figs. 5D and 6A). At low pH, the peak maxima of the glycine peptide occurred at 93 ppm (i.e. only slightly elevated from the in-plane oriented alanine and leucine peptides of the same series).

A more quantitative analysis of the spectral transitions shown in Fig. 5, A and B, was obtained when the integrated signal intensities of >160 ppm were compared with the total signal intensity of the amide bonds and plotted as a function of pH (Fig. 6A). The fraction of transmembrane-oriented peptides was analyzed in detail using Equation 3, and the Gibbs free energies of reorientation (\( \Delta G \)) were thus obtained for the two peptides. When compared with the transition of LAH4L6 (\( \Delta G = -214 \pm 0.1 \) kJ/mol) the reorientation of LAH4A6 (\( \Delta G = -7.8 \pm 0.9 \) kJ/mol) occurred at higher pH, was less steep, and was less complete (Table 2). This behavior is in agreement with the more hydrophilic properties of alanine (7, 53) and with the corresponding theoretical simulations (38).

The FTIR spectra of LAH4L6, LAH4A6, and LAH4W6 are characterized by a well resolved lipid C=O peak at about 1736 cm\(^{-1}\) as well as amide I and amide II bands at 1657 and 1542 cm\(^{-1}\), respectively (Fig. 3). The amide I resonance of LAH4L6 broadens for pH values below 4.5 and exhibits an additional shoulder at 1630 cm\(^{-1}\). To analyze in more detail the spectral intensity at 1657 cm\(^{-1}\), the sample was consecutively irradiated with a light beam of parallel or perpendicular polarization, respectively. Within the peptide series investigated, the amide I band exhibited dichroic ratios between 1.2 and 2.7, these values reflecting in-plane and transmembrane helical orientations, respectively (Fig. 3). The dichroic ratio was calculated by dividing the intensity of the parallel by the intensity of the perpendicular spectrum. In a first experiment, the pH-dependent dichroic ratio of the amide I band was used to follow the topological changes of LAH4L6. With pK\(_a\) = 6.0 and c = 0.8, the data analysis according to Equations 3 and 4 provides \( R^{\text{ATR}}_a = 1.53 \pm 0.05, R^{\text{ATR}}_{b} = 2.33 \pm 0.07, \) and \( \Delta G = -10.5 \pm 1.8 \) kJ/mol (Fig. 6B).

The FTIR spectra of the LAH4W6 samples exhibit a well resolved lipid C=O peak at about 1737 cm\(^{-1}\). The amide I peak at about 1657 cm\(^{-1}\) is
very distinct and indicative of α-helical secondary structures of the membrane-associated peptide (Fig. 3). The amide II peak appears at 1542 cm⁻¹. Furthermore, the dichroic ratio defined by the spectral intensities of parallel and perpendicularly polarized light changes in a systematic manner as a function of pH (Figs. 3 and 6B). The in-plane to transmembrane transition curve is well defined between pH 4 and pH 6. With \( pK_a = 6.0 \) and \( c = 0.8 \), the data analysis provides \( R_{ATR}^a = 1.57 \pm 0.03 \), \( R_{ATR}^{b \phi} = 2.06 \pm 0.02 \), and \( \Delta G = -19.3 \pm 0.3 \) kJ/mol (Fig. 6B).

In contrast, the dichroic ratio of LAH₄X₆ does not show the profound pH-dependent alterations observed for the above peptides (Fig. 6B). By comparing this pH-dependent function with theoretical transition curves (supplemental Fig. 8), we estimate that under the given conditions, \( \Delta G \) for this peptide is \( \pm 2.5 \) kJ/mol.

**DISCUSSION**

Histidine-containing sequences have been shown to alter their alignment with respect to the bilayer surface in a pH-dependent manner (27). With the help of these peptides it has proven possible to test the interaction contributions that determine the membrane topology of membrane-associated helices (22). Here we have modified these sequences to create a helical surface composed of six variable \( X \) residues being flanked by four histidines (Fig. 1). This design of the LAH₄X₆ peptides is such that the effects of up to six \( X \) residues on the in-plane to transmembrane transition are tested. The large number of exchangeable sites allows one to increase the differences in the transition pH, thereby down-scaling the statistical errors associated with the per residue analysis of \( \Delta G \).

The LAH₄X₆ peptides have been composed and designed to exhibit a high propensity for α-helix conformations in membrane environments. Indeed, the high abundance of nuclear Overhauser effect cross-peaks between amide protons of consecutive residues (Fig. 2) confirms this helical design (45). Furthermore, CD spectroscopy of LAH₄ and LAH₄X₆ peptides indicates a high propensity for helical secondary structures when investigated in membrane environments for these and related sequences (23, 38).

The Gibbs free energy difference between the in-planar and the transmembrane configuration is directly obtained from the corresponding transition curves (Table 2). These were established from oriented bilayer samples either by integration of solid-state NMR intensities or by measurement of the dichroic ratio in ATR-FTIR spectra (Fig. 6). The thermodynamic analysis is possible, since the reversibility of the in-plane to transmembrane transition has been demonstrated for LAH₄ and its derivatives (31).

In a previously presented model (27), the interplay of the pH-dependent energy of discharge of the histidine side chain as well as changes in hydrophobic, polar, and mismatch interactions (51, 54, 55) are all factors that determine the alignment of these helices parallel or perpendicular to the membrane normal. Furthermore, interactions that are dependent on the peptide-lipid ratio in the bilayer might have an effect on the peptide alignment, since increasing the peptide concentration has been suggested to be favorable to their transmembrane insertion (56, 57). Although the absolute value of \( \Delta G \) includes many different contributions, some of which also depend on the experimental set-up, the direct comparison of two peptides of the LAH₄X₆ series allows one to compare the specific and hydrophobic interaction contributions of the \( X \) residues. Therefore, when the transitions of LAH₄L₆ and LAH₄A₆ are compared with each other (Figs. 5, A and B, and 6) the transfer from the membrane interface to the bilayer interior of one leucine is 2.3 kJ/mol more favorable than that of a single alanine. Notably, this value represents the average over all six \( X \) positions in the sequences.

When reference is made to published hydrophobicity tables, membrane insertion of leucine is favored over alanine by about 3–7 kJ/mol (3, 4, 7, 58). However, these hydrophobicity tables are based on the transfer energies from the water phase to (a mimic of) the membrane interior. Furthermore, White and Whimley have systematically measured transfer energies of whole amino acids within a hexapeptide from the aqueous solvent to the membrane interface (58) and more recently verified this interfacial scale using four histidines (Fig. 1). This design of the LAH₄X₆ peptides indicates a high propensity for helical secondary structures when investigated in membrane environments for these and related sequences (23, 38).

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direct manner, considering long helical peptides that are associated with real bilayers.

The energy obtained for the transfer of one tryptophan within the LAH

X₆ peptide appears in average 1.5 kJ/mol more hydrophobic than leucine (Trp < Leu < Ala). This is in agreement with a high propensity of tryptophans to partition into hydrophobic environments (58). Furthermore, the tryptophan side chain has been suggested to potentially interact with the phospholipid interface (59, 60). It is therefore likely that the energy of transfer of LAH₆/W₆ is a composite of the change in the dielectric environment of these residues as well as breakages/formation of specific interactions involving some of these side chains.

Two experimental approaches have been used in this paper to follow the topological transitions of the LAH₆ peptides. On the one hand, oriented solid-state NMR spectroscopy of ¹⁵N labeled peptides reveals separate signals for the in-plane and transmembrane-oriented fraction, thereby indicating that exchange between the two is slow on the time scale of the ¹⁵N chemical shift anisotropy (10⁻⁴ s). Unfortunately, the NMR technique is relatively insensitive and requires considerable amounts (several mg) of labeled peptide as well as long measurement times on dedicated NMR spectrometers (several h/sample). On the other hand, the FTIR approach works with much smaller amounts of peptide and allows the acquisition of complete titration curves within a few hours. Whereas the wave number is correlated to the secondary structure (36, 37), the dichroic ratio is an indicator of the average alignment of the peptide (33–35). However, no details about orientational distributions and exchange are obtained by the infrared measurements.

Interestingly, we have observed pronounced differences when the transition curves obtained by solid-state NMR and by ATR-FTIR spectroscopy are compared with each other. In the case of LAH₆L₆, the transition curves obtained by solid-state NMR and by ATR-FTIR spectroscopy are compared with each other. In the case of LAH₆L₆, the transition of the LAH₆A₆ peptide is virtually absent in the ATR-FTIR experiment when compared with the NMR samples (Fig. 6). Comparison of the ATR-FTIR experiment to calculated transition curves yields a difference of >10 kJ/mol also for this peptide. It therefore appears that the relative difference observed between the peptides within the LAH series remains approximately the same regardless of the method used.

Although both sample preparations seem very similar on first view, nonnegligible differences exist. First, the solid-state NMR samples contain considerable amounts of water, having been equilibrated at 93% relative humidity. In contrast, the ATR-FTIR samples have been dehydrated using a stream of nitrogen gas in order to avoid background signals from the water molecules in the spectral range of interest. Notably, the topological transitions of LAH₄ peptides have been monitored using either technique, thereby indicating that the peptides retain the information on the pH-dependent ionization state even after the amount of solvent has been reduced (27, 31). However, our data indicate that changes in the preparation method, including water activity, can shift the absolute interaction scales. Second, in order to adjust the pH accurately and without accumulating salts in the sample, the ATR-FTIR samples are first incubated in buffer and then in deionized water. It is possible that during this procedure, some of the peptide is washed away, thereby decreasing the peptide/lipid ratio of the sample. Lowering the peptide concentration might stabilize the in-plane oriented helix configuration (57). Third, the sample temperature might be different in the two experimental set-ups.

In our hands, it has thus proven useful to combine both techniques as we did in this paper. NMR approaches were used to characterize the pK₅ values of the histidines of membrane-associated peptides and to obtain a first view on the alignment and exchange properties of the peptides in bilayers (Figs. 2, 4, 5, and 6A). Full titration curves are then recorded using ATR-FTIR spectroscopy (Figs. 3 and 6B). The data complement each other and provide an internal control, and more viable results are thus obtained.

Potential experimental errors are introduced during the determination of pH (±0.5 units for individual solid-state NMR samples; ±0.1 units for FTIR) or the integration of the fraction of transmembrane peptide in solid-state NMR spectra (±0.1). However, during the data-fitting analysis, averages are taken over a large number of data points, thereby much improving the quality of the final results.

Here we have tested the relative hydrophobicity and the change in interactions of five different types of amino acid side chains during the transfer from the membrane interface to the membrane interior. It has been demonstrated that histidine-containing peptides can be used to test for the interaction contributions in the context of helical peptides and in lipid bilayers. Since NMR and FTIR spectroscopies work under different environmental conditions (e.g. hydration), the two techniques have provided differences in absolute values of ΔG. Nevertheless, the data agree on the relative differences of the energies of transfer measured for individual amino acids.

Previously, the transitions from an aqueous phase to hydrophobic environments, which are thought to mimic the membrane interior, have been studied. These investigations led to the hydrophobicity scales commonly used during the identification of membrane-inserting sequences (4–7, 61). These tables are insufficient when different locations within the membrane are to be tested (21), or when the alignment of a helical polypeptide is to be determined. The study presented here, therefore, extends previous investigations where the transition of small peptides from the water to the membrane interface has been measured (2, 29). The amino acid-dependent hydrophobicity of extended peptide chains has furthermore been analyzed previously when HPLC retention times have been analyzed in a systematic manner (62) or when the composition of transmembrane segments is analyzed by statistical methods (11, 12, 63).

In summary, a conceptually new approach is presented, where we take advantage of the pK₅ value of the histidine side chains in the physiological range. This opens up the possibility to manipulate the membrane topology of amphipathic helices carrying histidines at well chosen positions by adjusting the protonation force of the environment (27). The topological behavior of such peptides has been used to investigate by experimental methods interfacial preferences, “whole residue hydrophobicities,” and/or specific interactions within the membrane. In contrast to many previous studies, the present approach tests the transition from the membrane interface to its interior of amino acid residues within helical peptides and in the context of lipid bilayers. To our knowledge, for the first time, the relative energies of transition are thus obtained experimentally from studying polypeptides long enough to span a real membrane.

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