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Membrane Insertion and Orientation of Polyalanine Peptides: A ^{15}N Solid-State NMR Spectroscopy Investigation

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ABSTRACT Polyalanine-based peptides were prepared by solid-phase peptide synthesis, labeled with ^{15}N at selected sites, reconstituted into oriented phosphatidylcholine bilayers, and investigated by proton-decoupled ^{15}N solid-state NMR spectroscopy. The anisotropic ^{15}N chemical shift is a direct indicator of helix alignment with respect to the membrane normal. The in-plane to transmembrane equilibrium is the focus of this study. Time- and solvent-dependent transmembrane alignments of $K_3A_{18}K_3$ have been obtained, and these are stabilized when a few alanine residues are replaced with leucine. The results are discussed in the context of a model where polyalanines adopt a variety of configurations, which are interconnected by multiple equilibria. The data indicate hydrophobicity values of alanine close to zero when studied in the context of helical polypeptides (≥24 residues) and phospholipid bilayers.

INTRODUCTION

The organization of integral membrane proteins is characterized by the separation of hydrophobic membrane-spanning and hydrophilic membrane-flanking regions (reviewed in von Heijne, 1996; Garavito, 1998; Tsukihara and Lee, 1999). Only a few membrane protein structures have been determined at high resolution, and structural information on this class of proteins therefore remains sparse (e.g., Garavito, 1998; Buchanan, 1999; Tsukihara and Lee, 1999; Palczewski et al., 2000; Toyoshima et al., 2000). On the other hand, DNA sequence analysis is fast and routine; therefore, structure prediction algorithms that identify membrane-spanning domains from primary sequence information remain an important source of information. These protocols involve a first step in which the primary sequence is searched for hydrophobic, putative transmembrane helical domains (Kyte and Doolittle, 1982). However, the reliability of the results is to a large extent dependent on the quality of the underlying data base that assigns hydrophobicity values to individual amino acids (von Heijne, 1981; Kyte and Doolittle, 1982; Engelman et al., 1986; Wimley et al., 1996). Many of the amino acid hydrophobicity scales rely on studies of single residues or small polypeptides in isotropic solvents (Kyte and Doolittle, 1982; Engelman et al., 1986; Wimley et al., 1996). Importantly, a more elaborate scale differentiating between the membrane interface and the membrane interior has also been presented (Wimley and White, 1996).

In most of the established hydrophobicity scales alanine is considered moderately apolar (von Heijne, 1981; Kyte and Doolittle, 1982; Engelman et al., 1986). More recently, investigations of helix propensities of polyalanine-based peptides in the presence of membranes seem to confirm that alanine exceeds the hydrophobic threshold for membrane insertion (Liu et al., 1996; Liu and Deber, 1998). In addition, these studies indicate that alanine exhibits a high propensity for α-helical conformations in nonpolar environments.

However, when the hydrophobicity of alanine has been reconsidered by taking into account hydrophilic contributions from the peptide backbone it became obvious that alanine might in fact not be as apolar as previous studies have suggested (Wimley and White, 1996). Free energy calculations support such notions by predicting the occurrence of polyalanine in both transmembrane or surface-oriented configurations with close to similar probability (Ben-Tal et al., 1996). Furthermore, strong membrane interactions were absent when $A_{27}YK_6$ has been studied in the presence of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) bilayers (Chung and Thompson, 1996). Instead, most of this peptide remains associated with the water phase. However, when three alanines close to the N-terminus were replaced by leucines the peptide exhibits multiple equilibria and lipid bilayer association (Chung and Thompson, 1996).

As alanine is an abundant amino acid in soluble and in membrane proteins, it is of considerable interest to determine the properties of this amino acid in the context of membrane-associated polypeptides in more detail. Mixed alanine-leucine sequences, like $K_2(LA)_xK_2$, or lysine-flanked polyleucine peptides have been used in several previous studies to represent simplified versions of transmembrane helical domains (Zhang et al., 1992, 1995; de Planque et al., 1999; Ren et al., 1999; Harzer and Bechinger, 2000). During the design of these peptides it is assumed that leucine and alanine residues favorably interact with the hydrophobic interior of lipid bilayers, where they adopt

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α-helical conformations. Furthermore, the amino acid hydrophobicities were taken to suggest transmembrane alignments of the resulting peptides. Although these assumptions seem valid for leucine, recent experimental studies have raised doubts about the correct hydrophobicity value of alanine (Moll and Thompson, 1994; Wimley and White, 1996; Chung and Thompson, 1996). According to White and Wimley (1999) the transfer energies of alanine from water to octanol (being used as a mimic of the membrane interior) and from water to the membrane interface are +2.1 and +0.7 kJ/mol, respectively. The corresponding values for leucine are −5.2 and −2.3 kJ/mol.

Predictions of the localization of polyalanine in a bilayer/water system, therefore, range from being associated with the water phase (Chung and Thompson, 1996; White and Wimley, 1999) to being inserted into the membrane interior (von Heijne, 1981; Liu et al., 1996), depending on the experimental results and theoretical estimates that have been obtained in different laboratories. To test these predictions, the in-plane (IP) to transmembrane equilibria (TM) of a series of long polyalanine-based peptides ($\geq$24 residues) has therefore been investigated in the context of phospholipid bilayers.

Solid-state NMR spectroscopy is a powerful method to study structural and topological aspects of polypeptides reconstituted into lipid bilayers (reviewed in McDowell and Schaefer, 1996; Cross, 1997; Griffin, 1998; Bechinger, 1999; Davis and Auger, 1999; de Groot, 2000). In previous studies oriented samples of membrane-associated peptides, isotopically labeled with ^{15}N, have been investigated by proton-decoupled ^{15}N solid-state NMR spectroscopy. The resulting ^{15}N chemical shift is a sensitive indicator of the approximate membrane alignment of α-helical peptides (Bechinger, 1996). Whereas transmembrane alignments correlate with ^{15}N chemical shifts of $>$190 ppm, values $<$100 ppm are observed for amphipathic peptides oriented along the membrane surface. This technique has been used to characterize in detail charged amphipathic α-helices that orient perpendicular to the membrane normal (e.g., Bechinger et al., 1999a; Henklein et al., 2000). In contrast, many hydrophobic sequences, including the channel-forming viral proteins Vpu of HIV-1 (Wray et al., 1999), M2 of influenza A (Kovacs and Cross, 1997; Bechinger et al., 1999a), the peptide antibiotic alamethicin (North et al., 1995; Bechinger et al., 2001), or the $\beta^{6.3}$-helix of gramicidin A (Cross, 1997), adopt transmembrane alignments.

In a similar manner, proton-decoupled ^{31}P NMR spectroscopy of lipid membranes has been used to analyze the orientational order of the phospholipid headgroup. Whereas in pure phosphatidylcholine membranes chemical shifts of 30 ppm are observed when the long axis of the phospholipid molecules are aligned parallel to the magnetic field direction, macroscopic deviations or changes in the phosphatidylcholine headgroup conformation result in chemical shift frequencies of $<$30 ppm (Bechinger et al., 1999a).

TABLE 1 Amino acid sequences of the peptides investigated

Peptide	Sequence
$K_3A_{18}K_3$	KKKAA AAAAA AA$\underline{\underline{A}}$AA AAAAA AKKK
$K_3A_{17}L_1K_3$	KKKAA AAALA AA$\underline{\underline{A}}$AA AAAAA AKKK
$K_3A_{16}L_2K_3$	KKKAA AAALA AA$\underline{\underline{A}}$AA ALAAA AKKK
$K_3A_{14}L_4K_3$	KKKAA AALLA AA$\underline{\underline{A}}$AA ALLAA AKKK
$A_{18}K_6$	AA AAAAA AA$\underline{\underline{A}}$AA AAAAA AKKKK KK
$K_2A_{20}K_2$	KKAAA AAAAA AA$\underline{\underline{A}}$AA AAAAA AAKK
$K_3A_{25}K_3$	KKKAA AAAAA AAAAA $\underline{\underline{A}}$AAAA AAAAA AAAKK K
A_{25}	AA AAAAA AAAAA $\underline{\underline{A}}$AAAA AAAAA AAA
$A_{25}K_4$	AA AAAAA AAAAA $\underline{\underline{A}}$AAAA AAAAA AAAKK KK

At the underlined position the central alanine is labeled with ^{15}N.

In the present study, several polyalanine sequences labeled with ^{15}N at selected sites have been prepared (Table 1). Experimental results and theoretical estimates indicate that the length of a lysine-flanked 18-residue α-helix matches well the hydrophobic thickness of POPC bilayers (27 Å) (Harzer and Bechinger, 2000; Bechinger et al., 2001). Focus was therefore given to $K_3A_{18}K_3$ and its leucine derivatives. However, longer peptides more closely resembling those used in previous studies have also been investigated (Ben-Tal et al., 1996; Chung and Thompson, 1996). To reduce problems that would be associated with peptide aggregation, oriented membranes were reconstituted from clear homogeneous solutions of peptide and lipid. Furthermore, terminal lysines were attached as membrane surface anchors and helped to avoid peptide aggregation (Subczynski et al., 1998; de Planque et al., 1999). The equilibria of the peptides between surface-associated and transmembrane alignments and the orientational order of the phospholipids were investigated by proton-decoupled ^{15}N and ^{31}P solid-state NMR spectroscopy, respectively.

MATERIALS AND METHODS

Peptides were prepared by solid-phase peptide synthesis on a Millipore 9050 automated peptide synthesizer using Fmoc chemistry. The sequences and their abbreviations used in the text are indicated in Table 1. At the underlined positions ^{15}N-labeled Fmoc-protected amino acid analogs (Promochem, Wesel, Germany) were incorporated. The identity and high purity of the peptides were analyzed by reversed-phase high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).

Oriented samples for solid-state NMR spectroscopy were prepared by dissolving 10–20 mg of peptide in trifluoroethanol (TFE)/water (typically 2:0.35, v/v), or in hexafluoroisopropanol (HFIP) water (6:1, v/v). The final pH of the samples was adjusted to neutral by addition of the appropriate amounts of 1 N NaOH. Approximately 200 mg of POPC (Avanti Polar Lipids, Birmingham, AL) was added to the sample as a powder to yield a lipid-to-peptide ratio of 1/50 to 1/100. In additional experiments the lipid was added as a solution of dichloromethane in a volume matching that of TFE. The homogeneous mixtures were applied onto 30 thin cover glasses (9 × 22 mm), dried slowly, and exposed to high vacuum overnight. After the samples had been equilibrated in an atmosphere of 93% relative humidity for several days (controlled by a saturated KNO_3 solution), the glass plates were stacked on top of each other and sealed. The membrane stacks were inserted into the flat coil of a solid-state NMR probe head with the glass plate normal oriented parallel to the magnetic field direction. Proton-decoupled ^{15}N solid-state NMR spectra were acquired on Bruker wide-bore AMX400 or DSX400 spectrometers using a cross-polarization pulse sequence (Pines et al., 1973; Levitt et al., 1986). The samples were cooled during data acquisition with a stream of air at ambient temperature. Typical acquisition parameters were as follows: spin lock time, 1.6 ms; recycle delay, 3 s; ^1H B_1-field, 1 mT; number of data points, 128; spectral width, 40 kHz; and number of scans, 20,000. An exponential apodization function corresponding to a line broadening of 200 Hz was applied before Fourier transformation. Chemical shift values are referenced with respect to NH_4Cl (41.5 ppm).

^{31}P solid-state NMR spectra were recorded from all samples to analyze the orientational distribution of the phospholipids using a Hahn-echo pulse sequence with proton decoupling (Rance and Byrd, 1983). The ^{31}P 90° pulses were 3 μs, the echo delay 40 μs, and the recycle delay 1.5 s. Chemical shift values are given relative to the isotropic value of POPC.

Circular dichroism (CD) spectra were recorded at ambient temperature using a Jasco J715 CD spectropolarimeter and a 0.1-mm quartz cuvette. Small aliquots of 300 mg/ml dodecyl phosphocholine (DPC) (Avanti Polar Lipids) in 20 mM Tris, pH 7.2, were added in a step-wise manner to peptide solutions (1 mg/ml) in the same buffer. Two 190–250-nm scans were added.

RESULTS AND DISCUSSION

A wide variety of polyalanine as well as leucine-alanine peptides have been shown to adopt predominantly helical conformations in membrane environments (Oliver and Deamer, 1994; Zhang et al., 1995; Liu and Deber, 1998; Percot et al., 1999; Harzer and Bechinger, 2000). In particular, by using CD spectroscopy, polyalanine-based peptides such as $K_2(A_3XA_2)_2WA_2XA_3K_4$ ($X = A, V, F$) have been shown to exhibit fully helical conformations in SDS, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol, lysophosphatidylglycerol, or lysophosphatidylcholine environments (Liu et al., 1996). Correspondingly, in the presence of DPC the CD spectra of $K_3A_{18}K_3$ and $K_3A_{14}L_4K_3$ are characterized by strong negative intensities at 209 and 221 nm (not shown). In the presence of 100 mg/ml DPC both peptides exhibit ratios $R_1 = \Theta_{max}/\Theta_{209} \approx -2.35$ and $R_2 = \Theta_{221}/\Theta_{209} = 0.9$, respectively, in agreement with high degrees of helix formation (Bruch et al., 1991).

The proton-decoupled ^{15}N chemical shift spectra of $K_3A_{14}L_4K_3$ and $K_3A_{16}L_2K_3$ exhibit a single resonance at 206 and 209 ppm (± 5 ppm), respectively, thereby indicating stable transmembrane alignments of these polypeptides (Fig. 1, *A* and *B*). Similarly, lysine-flanked polyleucine or $(LA)_n$ sequences of sufficient length adopt transmembrane orientations (Huschilt et al., 1989; Axelsen et al., 1995; Bechinger et al., 1999b; Harzer and Bechinger, 2000). When $K_3A_{18}K_3$ or $K_3A_{17}LK_3$ is investigated, predominant resonances corresponding to transmembrane orientations are observed (210 ppm). However, significant additional contributions of in-plane oriented peptides (~70 ppm) are also present (Fig. 1, *C* and *D*). To gather information about the effect of peptide on the macroscopic order of the phospholipids, proton-decoupled ^{31}P NMR spectra of the samples shown in Fig. 1 were obtained. Major resonance intensities at 30 ppm indicate that the phospholipids are well oriented (not shown). Only a moderate degree of disorder of the phosphatidylcholine headgroups is apparent due to intensities in the range −15 to 30 ppm.

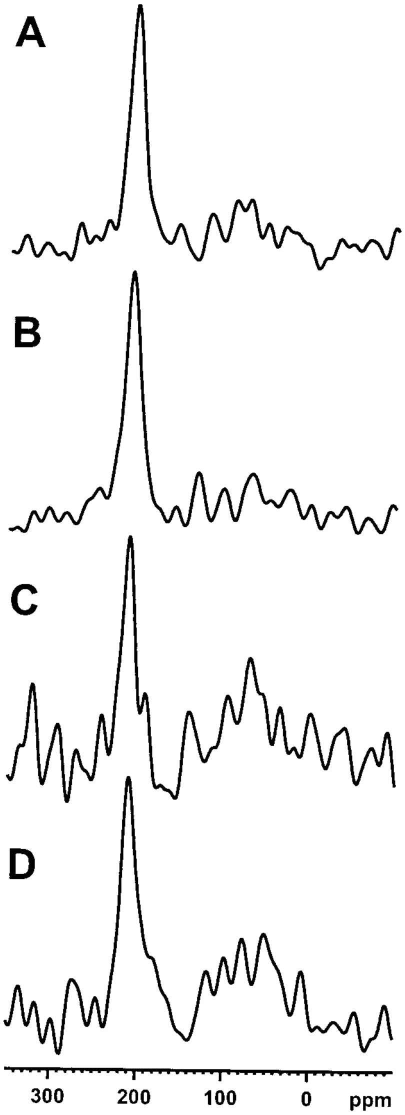

FIGURE 1 Proton-decoupled ^{15}N solid-state NMR spectra of 1.7 (± 0.2) mol % polyalanine-type peptides reconstituted into oriented POPC bilayers with and without leucine replacements. (*A*) $K_3A_{14}L_4K_3$; (*B*) $K_3A_{16}L_2K_3$; (*C*) $K_3A_{17}L_1K_3$; (*D*) $K_3A_{18}K_3$. Sample *D* was prepared from a solution of hexafluoroisopropanol/water, *A–C* were prepared from TFE/water mixtures. Only 8000 transients were accumulated for spectrum *C*.

The results presented in Fig. 1, *A–D*, indicate that sequences composed of 18 alanine (and leucine) residues can be accommodated in a transmembrane fashion. This observation confirms that lysine-flanked 18-residue helices match the hydrophobic thickness of POPC bilayers. However, without the exchange of at least two alanines for leucines, additional signal intensities in the range 50–130 ppm are observed. In this study the maximum transmem-

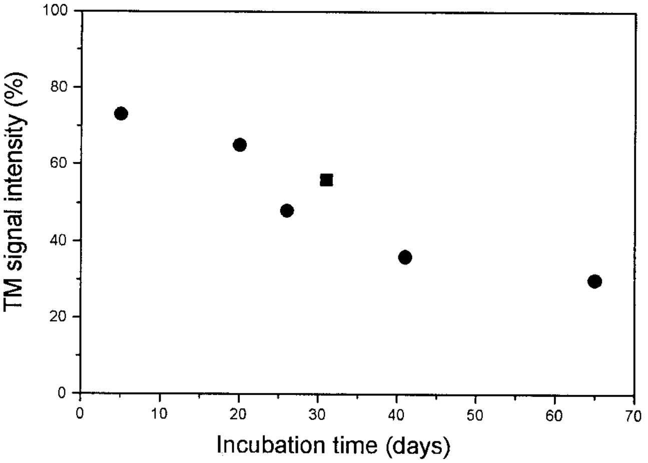

FIGURE 2 The proportion of transmembrane signal intensity of $K_3A_{18}K_3$ as a function of incubation time (●). The sample shown in Fig. 1 *D* was prepared, equilibrated for 5 days at 93% relative humidity at room temperature, and sealed. After the first NMR spectrum was recorded (Fig. 1 *D*, day 5) the sample was stored at 4°C. Additional spectra were recorded at ambient temperatures at the time points indicated. The result of integration of the spectrum of $K_3A_{17}LK_3$ (Fig. 1 *C*) is shown for comparison (■).

brane signal of $K_3A_{18}K_3$ was ~70–80% (Fig. 1 *D*). Changes in spectral appearance over time of this $K_3A_{18}K_3$/POPC sample were recorded over a period of several weeks (Fig. 2). Although major changes in the ^{31}P solid-state NMR spectra were absent (not shown), the 210-ppm signal intensity decreases in a continuous manner, indicating that other processes efficiently compete with stable transmembrane alignment (Fig. 2). Using mass conservation and Arrhenius's law, the maximal TM/IP ratio has been used to estimate a ΔG for the initial IP ↔ TM equilibrium of $K_3A_{18}K_3$ as ≥ -4 kJ/mol. Therefore, an average per-residue value very close to zero is obtained for 18 alanines in the sequence (≥ -0.2 kJ/mole).

Previous experiments demonstrate that in aqueous solutions many different conformations of polyalanine-type sequences are present and interconnected by multiple equilibria (Chung and Thompson, 1996). A simplified model including some of the peptide configurations discussed in this work is shown in Fig. 3. Importantly, some of these configurations correspond to aggregated forms of the peptide (Chung and Thompson, 1996). It is reasonable to assume that their size, shape, and existence are strongly affected by changes in the solvent system. Although the inherent hydrophobicity of alanine allows $K_3A_{18}K_3$ to exhibit transmembrane alignments, kinetically trapped configurations, such as for example small multimeric complexes, that do not efficiently insert into the lipid bilayer exist in a preparation- and time-dependent manner (Figs. 2 and 3). Similar observations have been made when other polyalanine peptides that vary in length or the position and number of lysine anchors, including $K_2A_{20}K_2$, $K_3A_{25}K_3$, $A_{18}K_6$, $A_{25}K_4$, and A_{25}, have been tested using TFE/water and/or HFIP/water as solvent systems. None of these sequences exhibit ^{15}N solid-state NMR spectra indicative of uniform and stable transmembrane alignments (not shown). Similarly, membrane insertion has been small or absent when other polyalanine sequences were added in aqueous buffer to preformed vesicles (e.g., Moll and Thompson, 1994).

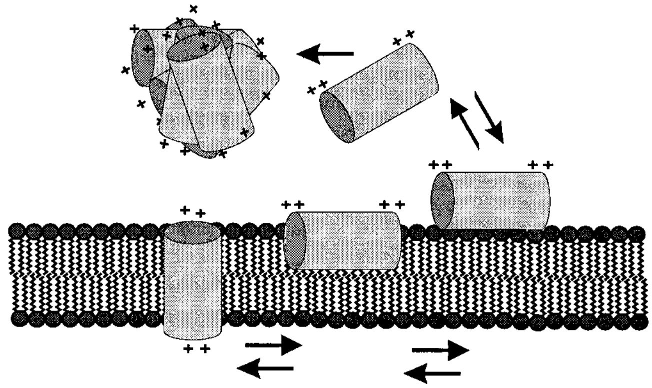

FIGURE 3 Working model showing various configurations and topologies of polyalanine-type peptides in the presence of lipid bilayers. In bilayers oriented with their normal parallel to the magnetic field direction, transmembrane peptides (*left*) exhibit ^{15}N chemical shifts around 200 ppm and in-plane oriented helices (*right*) at <100 ppm.

Introducing a few leucines into a polyalanine sequence considerably stabilizes transmembrane orientations of $K_3A_{16}L_2K_3$ or $K_3A_{14}L_4K_3$. In a related manner, mutagenesis experiments indicate that LA_{20} is insufficient to act as a stop transfer sequence whereas $(LA)_3LA_{13}L$ anchors alkaline phosphatase in *Escherichia coli* membranes (Chen and Kendall, 1995). Replacing an alanine by leucine increases the transfer energy from water to hydrophobic solvents, such as octanol, by ~5–7.5 kJ/mol (Engelman et al., 1986; White and Wimley, 1999). However, when transfer of peptides from interfacial to transmembrane locations are studied, the corresponding free energy differences are only 60% of those from water to octanol (White and Wimley, 1999; Vogt et al., 2000).

In this paper the total change in free energy during the transfer of α-helical peptides from surface-associated to transmembrane alignments has been tested directly, and the average contribution per alanine residue has been found to be close to zero. This is in agreement with theoretical investigations of the free-energy contributions that govern the transfer of helical polyalanine from water into a hydrophobic slab (Ben-Tal et al., 1996). Two energetic minima corresponding to in-plane and transmembrane insertions have been observed in these calculations. Previously established scales for water → oil transitions would predict a Gibbs free energy change of -120 kJ/mol for $K_3A_{18}K_3$ during membrane insertion (Engelman et al., 1986). The data presented here and in previous studies indicate that this value is much reduced (Chen and Kendall, 1995; White and Wimley, 1999). Recent studies suggest that hydrophilic contributions of the polypeptide backbone (C═O···N—H) amount to ~4.5–7 kJ/mol per H-bond (Ben-Tal et al., 1996; White and Wimley, 1999, and references cited therein). Although this by itself would fully explain the observed differences, other factors might contribute as well (cf. below).

When Deber and co-workers investigated the hydrophobic effect of amino acids that were placed within the scaffold of polyalanine, alanine was found to marginally exceed the threshold required for the formation of stable transmembrane α-helices (Liu et al., 1996). This is in good agreement with the results presented in Fig. 1. However, it should be noted that due to the presence of the hydrophobic fluorescence reporter tryptophan in their sequence (Liu et al., 1996), the hydrophobicity of this peptide is most closely related to $K_3A_{17}LK_3$ or $K_3A_{16}L_2K_3$ (Figs. 1, *B* and *C*).

The transmembrane alignments of polyalanines (Fig. 1) in conjunction with their time-dependent topological changes (Fig. 2) accommodate seemingly contradictory data of various laboratories. The solid-state NMR investigation presented here indicates that a close-to-zero free energy characterizes the transfer of polyalanine from surface-oriented to transmembrane alignments. The value measured in the experiment presented in Fig. 1 *D* constitutes the average gained from a helical peptide that consists of lysines and 18 alanines within a membrane-spanning peptide. During peptide insertion residues are transferred from the interface and the aqueous environment to locations at the interface as well as deeply buried in the membrane interior. As the value estimated is based on a two-state equilibrium between in-plane and transmembrane helical polypeptides (Fig. 3), other changes during the process such as alterations of lipid bilayer packing and/or peptide conformation also contribute. Possible interaction contributions during the transfer from in-plane to transmembrane oriented peptide are discussed in more detail in previous publications from this and other laboratories (Bechinger, 1996; Harzer and Bechinger, 2000, and references cited therein).

The hydrophobicity of alanine being on the borderline between that needed for membrane insertion of helical polypeptides and that for soluble protein domains makes this simple amino acid very versatile. This might be the reason that alanine is more often found in proteins than any other amino acid.

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