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Glycine and β-branched residues support and modulate peptide helicity in membrane environments

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Transmembrane (TM) segments of integral membrane proteins are putatively α-helical in conformation once inserted into the membrane, yet consist of primary sequences rich in residues known in soluble proteins as helix-breakers (Gly) and β-sheet promoters (Ile, Val, Thr). To examine the specific 2° structure propensities of such residues in membrane environments, we have designed and synthesized a series of 20-residue peptides with 'guest' hydrophobic segments – expected to provide three turns of incipient α-helix content – embedded in 'host' hydrophilic (Lys-Ser) matrices. Circular dichroism (CD) spectra of the model peptides in water showed that significant helical content was observed only for peptides with high Ala content; others behaved as 'random coils'. However, in the membrane-mimetic environment of sodium dodecylsulfate (SDS) micelles, it was found that Gly can be accommodated as readily as Ala, and Ile or Val as readily as Leu, in hydrophobic α-helices. Further subtleties of structural preferences could be observed in electrically-neutral lyso-phosphatidylcholine (LPC) micelles, where helical propensity decreased in the order Ala-Leu-rich > Gly-Leu-rich > Gly-Ile(Val)-rich hydrophobic segments. The results conjure a role of environment-dependent helix-modulation for Gly, Ile, and Val residues – and suggest that these residues may provide, in part, the structural basis for conformational transitions within or adjacent to membrane domains, such as those accompanying membrane insertion and/or required for transport or signalling functions.

Peptide; Conformation; Membrane; α-Helix; Circular dichroism; Hydrophobic segment

1. INTRODUCTION

The finding that short (<20-residue) peptides are able to form stable α-helices in aqueous media [1] has prompted re-examination of the helical preferences of commonly occurring amino acids in soluble proteins [2–5]. Although precise ranking orders differ in detail, Leu and Ala have been shown to rank among the best 'helix-promoters', while the conformationally flexible Gly residue is one of the strongest 'helix-breakers' [2–5]. β-branched residues such as Ile, Val and Thr are also widely accepted as potentially destabilizing to helical structures due to the steric effect of their bulky side chains [3,5]. While these findings have enriched our understanding of globular protein structure, their significance for integral membrane proteins remains obscure. Transmembrane (TM) segments within membrane proteins are generally assumed to be in an α-helical conformation [6,7], an assumption strongly influenced by the helical membrane-interactive structures determined crystallographically for the photosynthetic reaction center of *Rps. viridis* [8], and by cryo-microscopy for the proton pumping protein of purple membrane, bacteriorhodopsin (bR) [9]. Nevertheless, multiple Gly residues occur in several of the seven bR TM helices [10], while, for example, the 14 proposed membrane-spanning segments of the bovine mitochondrial transhydrogenase contain ca. 20% Gly content [11]. Single-spanning membrane proteins display similarly characteristic residue contents; thus, the coat protein of bacteriophage M13 contains three Gly and nine β-branched (Ile, Val, Thr) residues in its 19-residue effective TM segment [12], yet this segment has been demonstrated to be α-helical by extensive spectroscopic studies [13]. This 'GIVT' bias is not limited to the examples above, but rather appears as a general motif in essentially all categories of integral membrane proteins [14,15]. Conformational transitions in integral membrane proteins during membrane insertion [16,17] and/or function (i.e. transport, signalling) [18–20], are likely to require regions of protein structurally responsive to the immediate membranous or aqueous environment. In the present work, we use designed hydrophobic peptides [15] to inquire as to why these proteins recruit so many helix-destabilizing residues while maintaining helicity of their TM segments, and to what structural/functional role(s) may be implied for these residues.

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Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; ODhbt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; TFA, trifluoroacetic acid; SDS, sodium dodecylsulfate; HPLC, high performance liquid chromatography; CD, circular dichroism; TM, transmembrane; LPC, L-α-(myristoyl)-lysophosphatidylcholine.

2. MATERIALS AND METHODS

Peptides were synthesized by solid-phase techniques on a Biolynx 4170 automatic peptide synthesizer (LKB) using Fmoc chemistry [21]. Amino acids were coupled as their pentafluorophenyl esters with an equivalent amount of HOBt added, except for the case of serine in which Fmoc-Ser(O-*t*-butyl)-ODhbt was used. Cleavage of peptides from the resin was accomplished by treating peptide-resins for 3–5 h in TFA/mercaptoethanol/anisol (95:2.5:2.5) at ambient temperature. Crude peptide was dissolved to saturation into 10% HOAc or 0.5 N HCl and the resulting cloudy mixture was extracted with 1/3 volume of ethyl ether for three times to remove low molecular weight organic contaminants. Purification of peptides was carried out on a Delta-Pak C4 RCM column (25 mm × 100 mm, Waters), using a linear elution gradient of 10-25% of acetonitrile (containing 0.1% TFA) in the first 10 min, followed by 25-35% of acetonitrile in the second 10 min, and then 35-50% of acetonitrile in the final 10 min. Main peak fractions were collected and further chromatographed on a Vydac C4 analytical HPLC column (4.6 mm × 250 mm), from which a single peak was obtained for each peptide. Characterization of peptides was performed by amino acid analysis, analytical purity by liquid chromatography, and mass spectrometry.

CD measurements were made on a Jasco-720 spectropolarimeter equipped with a Neslab variable temperature control unit, using cuvettes of 1 mm path length. Peptide concentrations were typically 25–50 μM, as determined by quantitative amino acid analysis. CD spectra were generally independent of peptide concentration from 20 μM to 500 μM (data not shown), and it was thus assumed that peptides exist in a monomeric state through this range. The aqueous buffer used for CD experiments consisted of 10 mM sodium chloride and 10 mM sodium phosphate, pH 7.0. SDS and LPC micellar solutions were prepared by dissolving the desired amount of lipids into that buffer, and the resulting buffers were directly used for sample preparations. Micellar solutions were routinely checked for background absorbance, from which it was noted that light scattering was insignificant at concentrations up to 50 mM SDS or LPC. Any noise that did arise was averaged out by running several CD scans (usually six scans) for each peptide. Where constant concentration was required of a peptide in a series of measurements, the peptide was dissolved in double-distilled water at 500 μM, and the peptide stock solution thus obtained was then divided into equal parts using a micro-pipette and lyophilized. Aliquots of peptide powder could then be either stored at 4°C or dissolved in suitable buffers immediately before CD measurements.

3. RESULTS AND DISCUSSION

Although oligomerization and/or assembly of TM helices has crucial importance to membrane protein functioning, studies with bR [22], the β_2-adrenergic receptor [23], and channel-forming synthetic peptides [24], strongly suggest that individual TM helices can fold independently of each other, and subsequently assemble into the final membrane protein structure without major rearrangements [7,25]. We have designed and synthesized molecules which represent individual TM segments inserted between short 'soluble domains' [15]. The designed peptides contain prototypical sequence NH_2-(Ser-Lys)$_2$-Ala^5-Leu^6-x^7-Ala^8-Leu^9-y^{10}-Trp^{11}-Ala^{12}-Leu^{13}-z^{14}-(Lys-Ser)$_2$-OH, where x, y, and z have been varied from Ala to Gly in a stepwise manner (Table I). To examine the impact of β-branched residues on helical propensity, two additional peptides (3G3I and 3G3V) were produced by further replacement of Leu^6, Leu^9 and Leu^{13} in 3G by three Ile (3G3I) or three Val (3G3V) residues. Trial syntheses dictated the length of the hydrophobic stretch (10 residues vs. >18 residues in most natural TM segments) for optimal balance between membrane affinity and aqueous solubility in a monomeric state (up to 10 mg/ml in H_2O, data not shown). In peptide design, Trp was inserted to provide a measure of aromatic character typical of TM segments [14], as well as a spectroscopic probe. Positively charged Lys (and Arg) residues occur commonly at both N- and C-termini of natural TM segments, where they act as determinants of transmembrane topology [26]. As well, Lys residues at either terminus can interact electrostatically with the head groups of the lipids used as membrane-mimetics in conformational studies (vide infra).

Taking ellipticity at 222 nm as a direct measure of peptide helicity [27], peptide 3A (Table I) exhibits significant helicity (ca. 30%) in water, but this structure is rapidly lost through this series of peptides as Gly is introduced into the hydrophobic core, with a resultant fully 'random coil' structure for peptide 3G in aqueous solution (CD spectra shown for 3A and 3G in Fig. 1). These results parallel the findings of Baldwin and his associates [28], which indicate that Gly is a strong helix-breaker in H_2O. Peptide conformations were then examined in the membrane-mimetic environments of SDS and LPC. Although SDS stabilizes helical conformations in a wide variety of peptides and proteins, SDS can nevertheless discriminate among conformations promoted by individual sequences [29]. In contrast to our observations in aqueous media, *all peptides in Table I adopt essentially full helical structures in 10 mM SDS*

Table I

Sequences and α-helical propensities of peptides NH_2-S-K-S-K-A-L-x^7-A-L-y^{10}-W-A-L-z^{14}-K-S-K-S-K-S-OH[a]

Peptide (x y z)	Hydrophobic segment (5–14)	$-[\theta]_{222}$ (cm^2·deg·dmol^{-1})[b]	
		Aq. buffer	10 mM SDS
AAA (3A)	-ALAALAWALA-	10,200[c]	31,400[c]
GAA	-ALGALAWALA-	5,810	31,000
AGA	-ALAALGWALA-	4,720	30,800
AAG	-ALAALAWALG-	4,660	31,200
GGA	-ALGALGWALA-	3,000	30,800
GAG	-ALGALAWALG-	2,300	30,000
AGG	-ALAALGWALG-	1,580	30,500
GGG (3G)	-ALGALGWALG-	740	28,900
3G3I[c]	-AIGAIGWAIG-	770	29,400
3G3V[c]	-AVGAVGWAVG-	760	29,100

[a] Each peptide is 20 residues in length; the hydrophobic segment 5–14 only is given in the table. Peptide termini are unblocked. Abbreviations: A, Ala; S, Ser; K, Lys; L, Leu; G, Gly; W, Trp; I, Ile; V, Val.

[b] Values of $-[\theta]_{222}$, the mean residue ellipticity at 222 nm, are averaged over 6 scans, with background subtracted (25°C). Independent runs typically gave ellipticity values within 2% of values shown.

[c] Peptide 3G3I contains x=y=z=Gly, with Leu^{6,9,13} changed to Ile. Peptide 3G3V contains x=y=z=Gly, with Leu^{6,9,13} changed to Val.

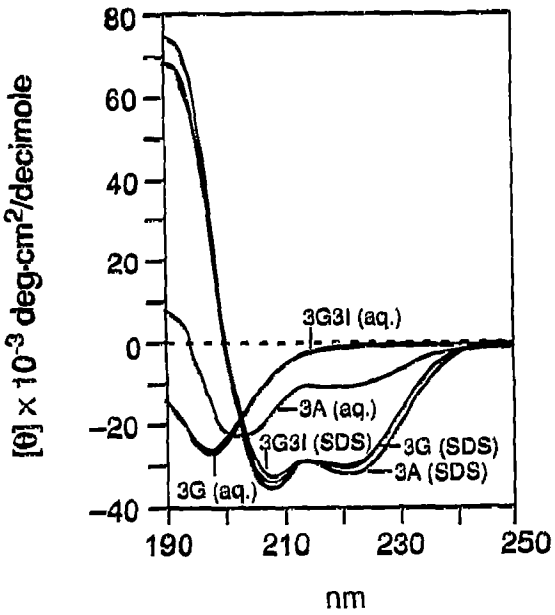

Fig. 1. Circular dichroism spectra for peptides 3A, 3G, and 3G3I (see Table I) in aqueous buffer, and in 10 mM SDS at 25°C. Curves were obtained by averaging over 6 scans with background subtracted. Spectra are as indicated in the diagram. CD spectra of peptide 3G3V were observed to be largely superimposable upon those of 3G3I, and have been omitted for clarity.

regardless of the differences in their primary sequences (spectra shown for 3A, 3G and 3G3I in Fig. 1). The net difference in helicity in SDS micelles between the eight peptides 3A through 3G (Table I) is only about 8%. This result is obtained despite the 100-fold helix-stabilizing propensity of Ala vs. Gly in water [28]. Gly, which dictates random coil structure in water [28], cannot do so in a non-polar environment where the energy cost of exposing non-hydrogen-bonded polar NH and C=O groups, as in a random structure, is apparently too high [7].

In further experiments, replacement of helix-promoting Leu residues [3,4] in peptide 3G by three helix-destabilizing Ile (or Val) residues [5] maintains the overall hydrophobicity of the peptide but as discussed above, might have been expected to decrease the helical propensity of peptides 3G3I and 3G3V. Nevertheless, both of these peptides, which now contain 60% of 'helix-destabilizing' residues [30] within the hydrophobic core, retain helicity >90% of that for peptide 3A in SDS micelles (Table I; shown for 3G3I in Fig. 1). It should be noted, though, that Ile and Val do not necessarily have identical structural propensities under all conditions, and the fine distinctions between them are currently under further investigation.

Given the propensity for anionic lipids to promote helical conformations [31], the negative charge of SDS micelles may, in part, mask the inherent structural diversity in this series of peptides. The subtleties in their structural preferences became more conspicuous in the medium of electrically-neutral LPC micelles, where the peptides may be in equilibrium between water and the micellar phase [32]. As well, LPC more closely resembles natural membranes structurally, and thus may provide a better mimicry of native lipid bilayers. As seen in Fig. 2, peptides 3A, 3G and 3G3I are found to be capable of forming helical structures in LPC micelles, but respond differentially to LPC concentration. Thus, peptide 3G3I displays the lowest helical propensity among the three peptides (ca. 50% of 3A in 10 mM LPC), requiring 50 mM LPC to attain its maximum

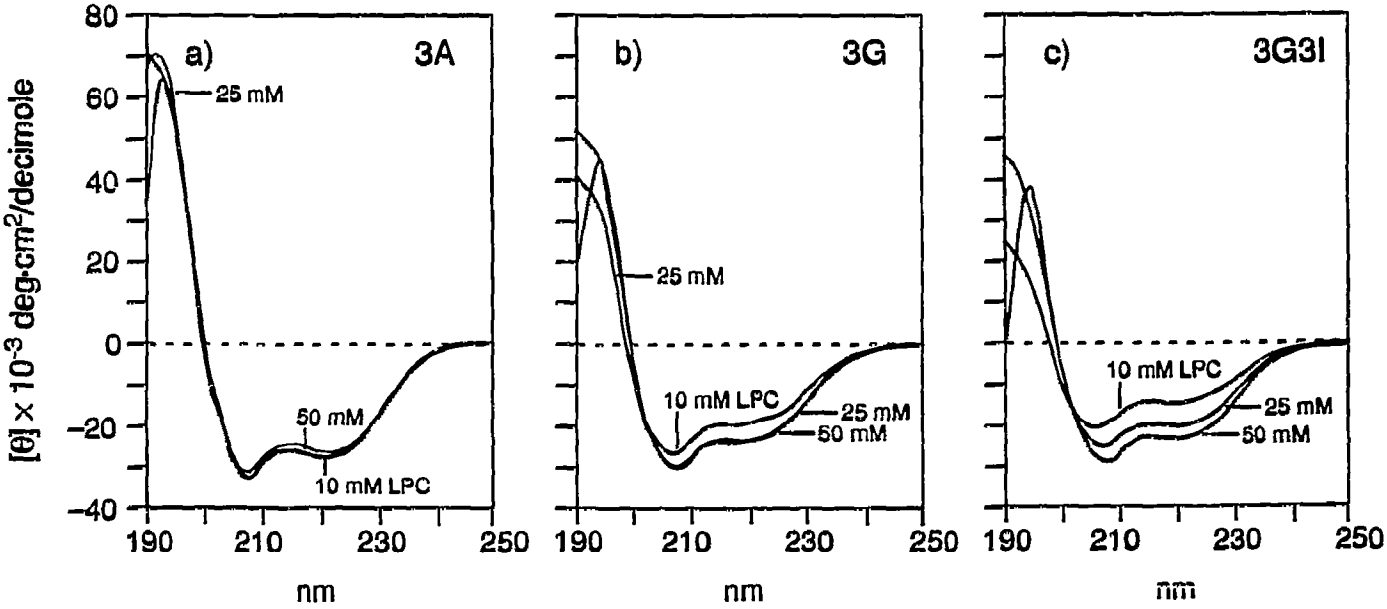

Fig. 2. Circular dichroism spectra of peptides 3A, 3G and 3G3I (see Table I) upon titration with lysophosphatidylcholine (LPC) micelles at 25°C. Peptide 3G3V displayed similar behavior to 3G3I in parallel experiments.

helicity (Fig. 2c). Since peptides 3G and 3G3I are essentially equivalent in net 'hydrophobicity', this conformational sensitivity to LPC concentration must therefore be explained by the *combined* effects of peptide intrinsic hydrophobicity and structural proclivity: the hydrophobicity of segment 5–14 provides the sufficient condition for the peptide to form a helix once in the lipid micelle, but in the mixed water/membrane medium, residue composition ultimately directs this transition.

As modulators of environment-dependent helicity, Gly, Ile and Val residues within hydrophobic segments represent suitable targets for mutagenesis in explorations of detailed structure/function relationships; for example, mutations of Gly residues in the TM domain of the Ca^{2+}-ATPase of sarcoplasmic reticulum directly affect Ca^{2+} ion affinity and translocation [18]. The conformational plasticity imparted by these residues may similarly govern structural transitions required in membrane proteins during their biosynthesis and membrane insertion, and/or subsequently in the performance of their specified functions.

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