Journal of Biomolecular NMR, 9 (1997) 329–335
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J-Bio NMR 443

Isotropic solutions of phospholipid bicelles: A new membrane mimetic for high-resolution NMR studies of polypeptides

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Received 22 January 1997
Accepted 4 March 1997

Keywords: Phospholipid bicelle; Membrane-bound peptide; Mastoparan

Summary

In order to illustrate the utility of phospholipid bicelles [Sanders, C.R. and Schwonek, J.P. (1992) *Biochemistry*, **31**, 8898–8905] as a membrane mimetic for high-resolution NMR studies, we have recorded two-dimensional ^{1}H NMR spectra of the tetradecameric peptide mastoparan *Vespula lewisii* in an isotropic aqueous solution of dimyristoyl and dihexanoyl phosphatidylcholine. Mastoparan is largely unstructured in water, but assumes a well-defined helical conformation in association with the bilayers. A pronounced periodicity of the sequential NH chemical shifts provides strong evidence that the helix axis of this short peptide is parallel, rather than perpendicular, to the bilayer plane. The bicellar solutions still require in-depth morphological characterization, but they appear to be ideal media for NMR determination of the mode of binding and the structure of membrane-associated peptides and proteins.

It has been a longstanding lament that uni- or multilamellar phospholipid vesicles are not suitable for high-resolution NMR studies of membrane-associated peptides and proteins. Because overall vesicular reorientation rates are too low for high-resolution NMR, detergent micelles, usually composed of sodium dodecylsulfate or dodecylphosphocholine, have served in the role of membrane mimetic since the early days of NMR structure determination of biopolymers (Brown and Wüthrich, 1977; Wüthrich et al., 1980). However, detergent micelles are far from ideal in this role: their extreme curvature is a liability when structural conclusions are to be made, and enzymes are rarely, if ever, active in micellar solution. To circumvent such limitations, researchers have resorted to the use of transferred nuclear Overhauser effect (TrNOE) experiments (Clore and Gronenborn, 1982) for structural studies of e.g. mastoparans in unilamellar vesicles (Wakamatsu et al., 1992) and other membrane-associated peptides and proteins (Wang et al., 1993). However, the successful application of the TrNOE experiment requires that the peptide be present in relatively high concentration and that it exchanges rapidly between the free and vesicle-bound states.

Attempts were made in the 1960s (Lawson and Flautt, 1967) and 1970s (Forrest and Reeves, 1981) to develop magnetically aligned lyotropic liquid crystals that could be used for structural studies of peptides and other substances of biological interest. However, in all cases the appeal of the oriented solutions was reduced by the fact that several components were hostile to biologically interesting materials, and the effort to develop these systems for NMR studies was curtailed nearly 20 years ago. In view of these difficulties, the recent recognition by Prestegard and co-workers (Ram and Prestegard, 1988; Sanders et al., 1994) that discoid phospholipid/detergent micelles would orient in the magnetic field was greeted with enthusiasm within the NMR community. Even more exciting was the development by the Sanders group (Sanders and Schwonek, 1992) of discoid bilayered micelles consisting solely of phospholipids, e.g. a mixture of dimyristoyl phosphatidylcholine (DMPC) and dihexanoyl phosphatidylcholine (DHPC), and their subsequent demonstration (Sanders and Landis, 1994) that embedded proteins yielded structural information while retaining their enzymatic activity in such 'bicelles' (Sanders and Landis, 1995). Since then, a handful of preliminary studies (DiNatale et

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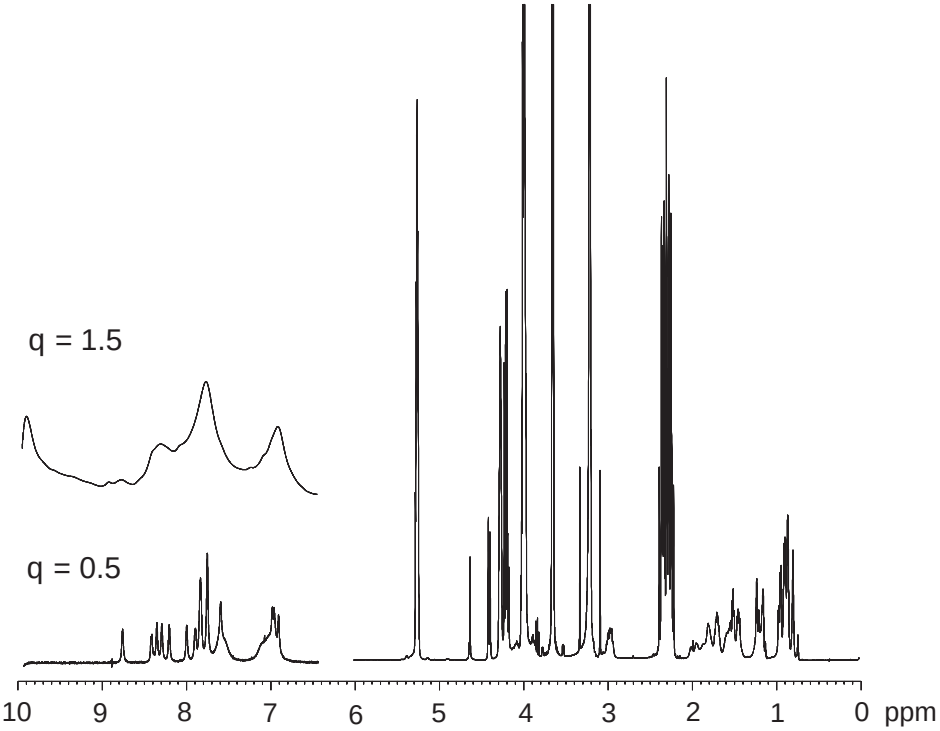

Fig. 1. 600 MHz proton NMR spectrum (NH peak intensities are multiplied by four) of mastoparan *Vespula lewisii* (INLKALAALAKKIL) in a bicellar solution (80% H_2O, 20% D_2O) containing 15% (w/v) q = 0.5 DMPC/DHPC bicelles loaded with 1.0 mol% peptide. All proton spectra were obtained at 37 °C and pH 4.9 on a Bruker DRX600 spectrometer, using the WATERGATE suppression technique (Piotto et al., 1992), 64 or 128 scans, a dwell time $\Delta t = 76.2$ μs, and a relaxation delay of 1.0 s. A Lorentzian line broadening of 0.2 Hz was applied prior to Fourier transformation, and the chemical shifts were referenced to H_2O at 4.623 ppm (Wishart et al., 1995). DMPC and DHPC were only partially deuterated and the ^1H resonances of the lipid head group were readily assigned with the aid of the TOCSY connectivities. (Glycerol protons: 1-CH_2, 4.40 and 4.19 ppm; 2-CH, 5.26 ppm; 3-CH_2, 3.99 ppm. Choline protons: 1-CH_2, 4.26 ppm; 2-CH_2, 3.64 ppm; $N(CH_3)^+$, 3.20 ppm.) The presence of a multiplet at 2.35 ppm shows that back-exchange of the α-deuterons took place during the preparation of DHPC-d_{22}. The subspectrum of a mastoparan/DMPC/DHPC solution with q = 1.5 illustrates the effect of higher viscosity and greater bicellar size on the amide proton line widths.

al., 1995; Czereski et al., 1996; Howard and Opella, 1996) appear to confirm the early promise of the bicelles for structural work, but so far their use has been confined to the magnetically aligned anisotropic phases.

We have now taken the next step, extending the use of bicelles into the isotropic phase, and report here the first high-resolution NMR conformational study of a peptide bound to phospholipid bicelles. By using a total phospholipid concentration of 15% (w/v) while increasing the content of short-chain phospholipid (DHPC) relative to that of long-chain phospholipid (DMPC) until a ratio q ≡ [DMPC]/[DHPC] = 0.5 was reached, we achieved a medium suitable for high-resolution NMR studies. It is important to emphasize at the outset that in *isotropic* solution the morphology of the DMPC/DHPC mixed micelles has yet to be unequivocally established. The shape of phospholipid/bile salt mixed micelles has, however, been shown to be disk-like in X-ray (Müller, 1981) and light scattering (Mazer et al., 1980) studies. In *anisotropic* solution, i.e. the magnetically oriented phase, X-ray diffraction studies (Hare et al., 1995) and diffusion measurements (Chung and Prestegard, 1993) of DMPC/bile salt bicelles, as well as ^{31}P (Sanders and Schwonek, 1992) and ^2H (Vold and Prosser, 1996) NMR studies of DMPC/DHPC bicelles yielded results consistent with the concept of the mesogenic unit being a planar DMPC bilayer section surrounded by a protective rim of the shorter amphiphiles. On this basis, we shall assume that the bicellar morphology persists into the isotropic phase, with the bicellar diameter shrinking as the ratio q is reduced (Vold and Prosser, 1996).

To explore the utility of the bicelles for structural studies we used mastoparan *Vespula lewisii*, INLKALAALAKKIL-NH_2 (Sigma Chemical Co., St. Louis, MO, U.S.A.). The material (Cat. No. M-5280, lot 44H4951) was specified as having a peptide content of 75% and was used as received. Initially the ^1H NMR spectra of the peptide showed the absence of peptidic impurities above the 1–2% level, but a minor increase of impurity peaks in the NH region was observed to take place during a time span of several weeks. Dimyristoyl-d_{54} phosphatidylcholine and dihexanoyl-d_{22} phosphatidylcholine were obtained from Avanti Polar Lipids (Alabaster, AL, U.S.A.), DHPC as a custom synthesis with caproic-d_{11} acid supplied by CDN (Pointe Claire, PQ, Canada). A bicellar solution with q = 0.5 was prepared from 59 mg DMPC-d_{67}, 76 mg

DHPC-d_{22}, and 700 µl of 80/20 H_2O/D_2O. To this solution 3.5 mg (estimated minimum) of mastoparan (1479 Da) was added, yielding [DMPC]/[mastoparan] ≈ 40 and [total lipid]/[mastoparan] ≈ 120. The resulting NMR sample had pH 4.9, was perfectly clear, and had a viscosity of 1.7 mPa s (1 mPa s = 1 centipoise (cP) in the cgs system) at 35 °C, only twice that of pure water. Using numerical arguments presented earlier (Vold and Prosser, 1996), we estimate that the q = 0.5 DMPC/DHPC bicelles have a diameter of 80–100 Å. It is important to point out that, if a bicelle with d = 80 Å were a rigid object, its rotational correlation time τ_c would be 170 ns under the conditions of our experiments, while τ_c would be 26 ns for a regular spherical micelle with d ≈ 50 Å. The observation of narrow peptide lines in the NMR spectrum is a direct consequence of the high internal mobility of membrane constituents and the resulting low molecular order. Values of 0.2–0.3 are typical for S_{zz}^{local}, the reorientational order parameter of individual phospholipid molecules with respect to the bilayer director (Bloom et al., 1991). Furthermore, we have found good agreement between the line widths of carbon-bound proton resonances in NMR spectra of mastoparan and those of the phospholipid methylene deuterons, which for nematic q = 3.2 bicelles have an effective correlation time of 1.5 ns.

Proton NMR spectra of the bicellar solution, with and without mastoparan, were recorded at 600 MHz and 37 °C. A typical spectrum of the mastoparan-containing sample is presented in Fig. 1, and the amide, H^{α} and H^{β} chemical shifts are listed in Table 1. The water resonance at 4.623 ppm (Wishart et al., 1995) was well suppressed by the WATERGATE pulse sequence (Piotto et al., 1992), but the aliphatic region of the spectrum is clearly dominated by resonances from non-deuterated phospholipid headgroup positions and residual alkyl-chain protons. The peptide α-CH region is particularly well hidden under the resonances from the five glycerol protons, positions not deuterium labeled in either DMPC or DHPC, but the amide region is free from solvent interference. Also shown in Fig. 1 is the amide region recorded at 37 °C for a bicellar mastoparan sample with q = 1.5. At this DMPC/DHPC ratio, the bicellar solution was found to have a viscosity of 20 mPa s, the diameter of the bicelles is estimated (Vold and Prosser, 1996) to be 200 Å, and the NMR resonances observed under these conditions are too broad (>100 Hz) for useful structural work.

An expansion of the NH region is presented in Fig. 2 above the downfield region of a NOESY spectrum obtained at 37 °C. Two amide resonances (Ala^5 and Ile^{13}) are superimposed at 7.84 ppm and overlap with Leu^9 at 7.85 ppm, and at 7.76 ppm the Leu^6 and Ala^8 amide resonances overlap with one of the Asn^2 δ-NH_2 lines. Nevertheless, dispersion of the αN and βN cross peaks permitted unequivocal assignment of all NH resonances in the TOCSY and NOESY spectra. The following points are worth noting: (i) the line widths of the amide resonances are 11–12 Hz, except for that of Leu^{14}, which is 15 Hz. (The line widths of the carbon-bound proton resonances are 3–5 Hz.) In the 1D spectrum the scalar coupling to the α-protons can only be observed in the case of the Asn^2 NH resonance at 8.42 ppm, which is a poorly resolved doublet with $J_{\alpha N} \approx 7$ Hz. This agrees with the NOE data, which show that residues 3–14 form an α-helix, while the N-terminal end is unstructured and Leu^{14} is less rigidly fixed. (ii) As discussed further below, the NH resonances shift both upfield (which is normal for α-helices) and downfield relative to appropriate references (Wüthrich, 1986; Wishart et al., 1991; Merutka et al., 1995). (iii) Broad NH_3^+ resonances are observed between 7.0 and 7.7 ppm, with a total intensity corresponding to 6–7 protons out of the theoretical 12. This could mean that proton exchange with water is slowed down for some NH_3^+ groups by partial insertion of the peptide into the bilayer surface.

NOESY spectra were acquired with mixing times τ_m = 150 ms (shown in Fig. 2) and 250 ms. We found no evidence of positive peptide NOEs, which indicates that the dominating peptide reorientation times are long. The τ_m = 250 ms NOESY spectrum showed additional peptide–peptide cross peaks, but whether these were due to spin diffusion or weaker direct connectivities was not established. On the other hand, the absence of peptide–phospholipid intermolecular NOEs shows that specific peptide/phospholipid interactions are quenched, because the lipid motion and/or the peptide motion relative to a specific lipid molecule is fast.

TABLE 1
^{1}H CHEMICAL SHIFTS OF MASTOPARAN *Vespula lewisii* IN BICELLAR SOLUTION AT pH 4.9 AND 37 °C

Residue	Chemical shift (ppm)[a,b]			NH line width
	NH	$C^{\alpha}H$	$C^{\beta}H$	
Ile^1	–	3.94	2.00	–
Asn^2	8.42	4.89	3.04, 2.94[c]	15
Leu^3	8.77	4.06	1.80	13
Lys^4	8.21	3.98	1.93, 1.86[c]	12
Ala^5	7.84	4.20	1.45	[d]
Leu^6	7.76	4.07	1.76, 1.67[c]	[d]
Ala^7	8.30	3.96	1.49	12
Ala^8	7.76	4.08	1.51	[d]
Leu^9	7.85	4.12	1.80	[d]
Ala^{10}	8.36	3.81	1.37	11
Lys^{11}	8.01	3.89	1.87, 1.91[c]	12
Lys^{12}	7.61	4.09	2.00	~13
Ile^{13}	7.84	3.91	1.85	[d]
Leu^{14}	7.90	4.21	1.82, 1.76[c]	15

[a] The chemical shifts are referenced to H_2O at 4.623 ppm (Wishart et al., 1995).
[b] The experimental uncertainty is generally slightly better than ±0.01 ppm.
[c] Diastereotopic methylene protons are not separately identified.
[d] Overlapping amide peaks.

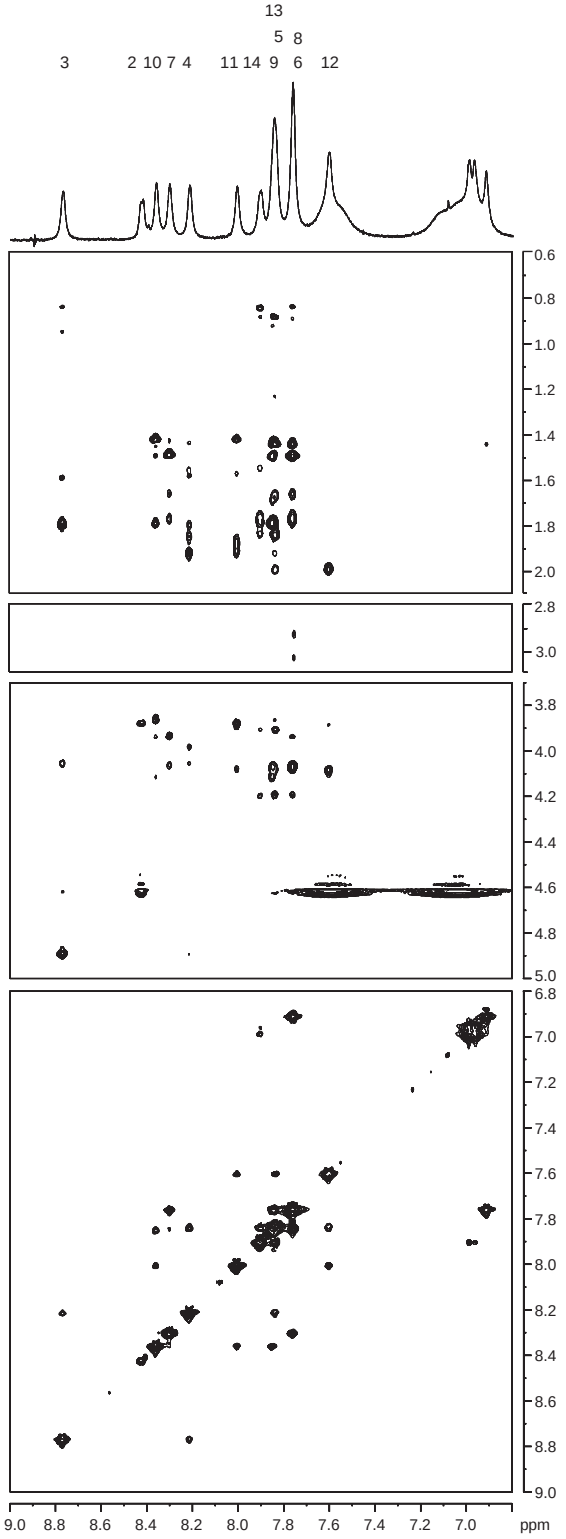

Fig. 2. Low-field regions of a NOESY spectrum obtained at 600 MHz and 37 °C using $\tau_m = 150$ ms, WATERGATE for suppression of the H_2O signal, TPPI (Drobny et al., 1979; Bodenhausen et al., 1980; Marion and Wüthrich, 1983), 64 or 128 scans, and 2048 and 256 complex points in F2 and F1, respectively. The spectrum was processed using the Bruker software package XWINNMR on a Silicon Graphics INDY 5000 workstation. Forward linear prediction was applied in the F1 domain, followed by sine-squared apodization with a 90° phase shift.

TOCSY spectra (not shown) were recorded with mixing times of 83 and 110 ms in order to provide the intraresidue N-α-β connectivities necessary for assignment. Intraresidue scalar coupling patterns were of minimal use, because the phospholipid resonances dominated the aliphatic region. No significant differences were observed between the two TOCSY spectra. A double-quantum-filtered COSY experiment was attempted, but – again – interference from strong lipid cross peaks and ridges limited its utility.

Sequential assignment (Wagner and Wüthrich, 1982) of the proton resonances relied on the identification of the NN(i,i+1), αN(i,i+1) and βN(i,i+1) NOESY cross peaks. Some overlap of cross peaks hampered the analysis, but i,i+3 cross peaks were prominent in the αN and αβ regions for the whole peptide except Ile[1] and Asn[2]. The NOE results are summarized in Fig. 3. It is clear that the bicelles induced an α-helical conformation, which is hardly surprising in view of results from earlier NMR studies of short peptides from insect and frog venoms in detergent micelles (Bairaktari et al., 1990; Seigneuret and Levy, 1995), in liposomal suspension (Wakamatsu et al., 1992), or on mechanically aligned phospholipid bilayers (Bechinger et al., 1993).

The helical conformation of bicelle-bound mastoparan was also evident in the circular dichroism (CD) spectra (see Fig. 4) obtained for bicellar and aqueous solutions of the peptide. The CD spectrum of a more dilute bicellar solution (actually, this was the q = 0.5 NMR sample diluted by a factor of five) showed the presence of ~80% α-helix, based on the mean residue molar ellipticity at 220 nm (Chen et al., 1974), while the spectra of four aqueous solutions (pH 6.5) proved mastoparan to be largely unstructured in water.

A comparison of the chemical shifts, δ(VPD), of the amide protons in Table 1 with shifts, δ(ref), for the 'random coil' peptides GGXA (Wüthrich, 1986) and GGXGG (Merutka et al., 1995) as well as average NH shifts from 70 proteins (Wishart et al., 1991) reveals a diagnostically useful 3–4-residue periodicity in Δδ. The differences, $\Delta\delta \equiv [\delta(\text{VPD}) - \delta(\text{ref})]$, are presented in Fig. 5. The secondary shift Δδ is most often negative (upfield shift), as normally observed for α-helical amide protons, while the amide shifts of Leu[3], Ala[7] and Ala[10] are positive (downfield). The largest downfield shifts are those referenced to the average NH shifts δ(WSR,α) in α-helical protein domains tabulated by Wishart et al. (1991).

The periodicity of Δδ is most simply rationalized by assuming that this mostly helical peptide is bound with the helix axis parallel to the bilayer surface – a conclusion anticipated from the amphipathic index (= 0.34) (Eisenberg et al., 1984) of mastoparan *V. lewisii*. A parallel orientation has been clearly demonstrated from the observed chemical shift anisotropy (Bechinger et al., 1991) of ^{15}N-labeled magainin in mechanically oriented phos-

pholipid bilayers, and from fluorescence and CD spectra combined with spin label broadening of proton resonances in phospholipid-bound signal peptides (Wang et al., 1993). However, our data do not exclude the possibility that mastoparan penetrates the membrane in a perpendicular fashion and then aggregates to form an amphiphilic channel. Mastoparan is too short, ~20 Å, to span the DMPC bilayer, ~45 Å, but concentration-dependent measurements and experiments performed in oriented bicellar solution should elucidate whether this is a possibility.

The downfield amide shifts associated with Leu[3], Ala[7] and Ala[10] and the smaller upfield shift of the Leu[14] amide resonance most likely result from this side of the helix being buried in the hydrophobic part of the phospholipid bilayer. Downfield shifts are commonly observed for NH protons on the more hydrophobic side of α-helices in proteins (Kuntz et al., 1991; Zhou et al., 1992). But such amide protons shift further downfield when the solvent is changed from water to a trifluoroethanol/water mixture (Reymond et al., 1997), where the opportunities for hydrogen bonding to water are reduced. It is important to note that the effects of the environment on hydrogen-bonded protons and their chemical shifts are still poorly understood (Rothemund et al., 1996), but for bilayer-bound peptide the strongly hydrophobic environment of the alkyl chains excludes water and may stabilize a slightly bent (Kuntz et al., 1991; Zhou et al., 1992) helical structure by reducing the length and increasing the strength of the intramolecular hydrogen bonds. Still, the isotropic amide shifts are clearly indicative of the mode of binding and may serve as a confirmation of conclusions reached by measuring the components of anisotropic tensor interactions in macroscopically ordered bicellar samples. We have yet to detect an environmental hydrophobic effect on the α- and side-chain protons. In aqueous solution, the hydrophobic sides of helices appear not

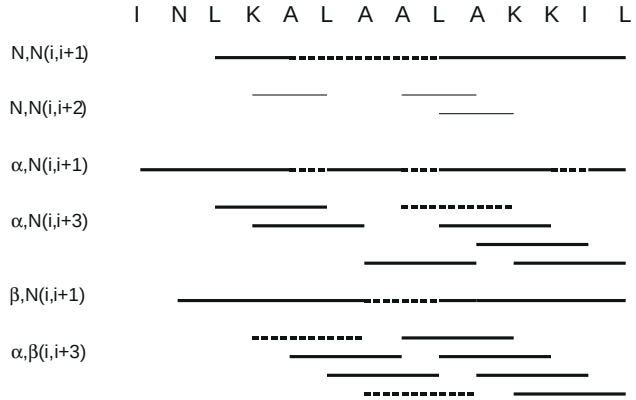

Fig. 3. Dipolar connectivities observed in the NOESY spectrum (Fig. 2) of mastoparan. The approximate cross peak intensity is indicated by the thickness of the lines, and dashed lines are used to indicate overlapping cross peaks of the same kind.

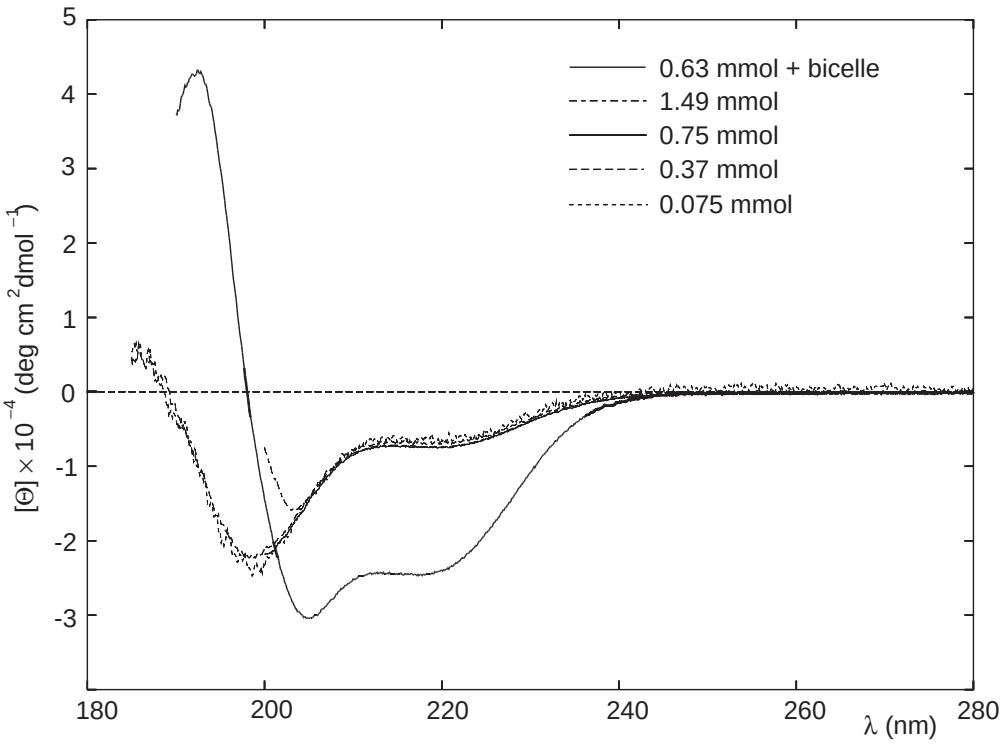

Fig. 4. Circular dichroism spectrum (——) obtained on a Cary 61 spectrometer at 20 °C for the mastoparan/DMPC/DHPC NMR sample diluted by a factor of five (0.63 mM peptide). Also shown are CD spectra of four aqueous solutions of mastoparan recorded at 20 °C and pH = 6.5. The CD spectrum of the 1.5 mM solution suggests the onset of some α-helix formation, presumably stabilized by peptide aggregation. The ordinate represents mean residue ellipticity [Θ] in deg cm^2 dmol^{-1}. [Θ] was calculated using a mean molar mass of 1479/14 = 106 g mol^{-1} per residue.

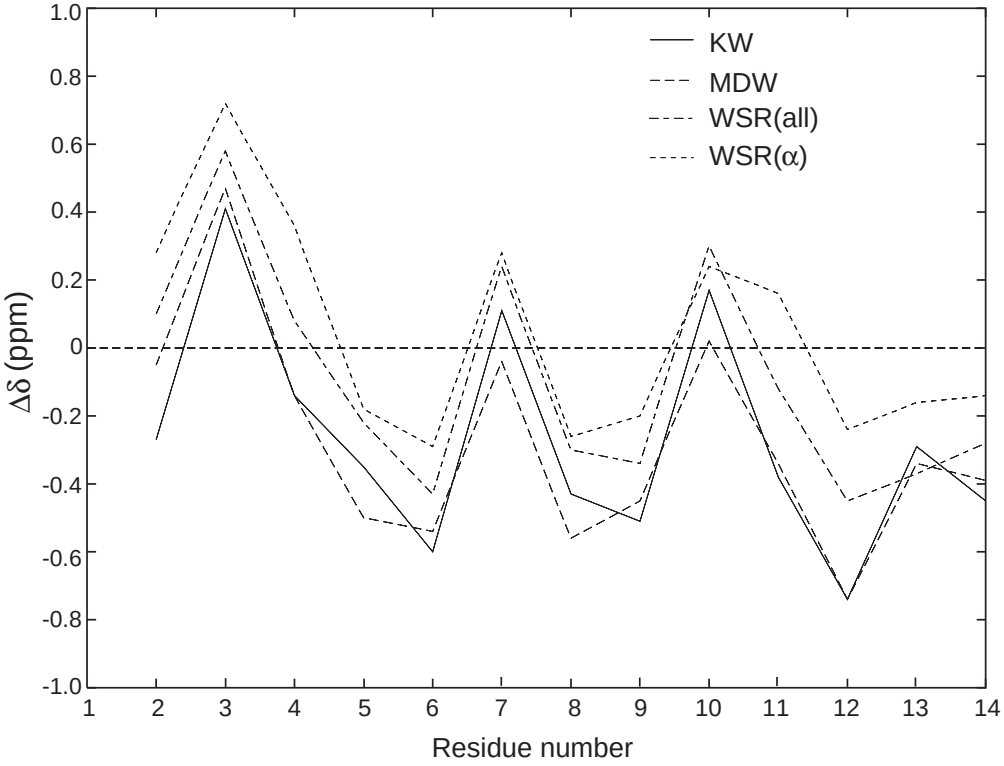

Fig. 5. Secondary chemical shifts $\Delta\delta \equiv [\delta(\text{VPD}) - \delta(\text{ref})]$ of the mastoparan amide protons relative to four sets of amide shift databases. Since the shifts of H_2O and NH protons are relatively strongly dependent on temperature with different values of $d\delta/dT$, and since the reference shifts are from NMR data collected at different temperatures, only the relative values of $\Delta\delta$ for each trace have significance. The experimental uncertainty is estimated to be on the order of ±0.02–±0.03 ppm. The four traces refer to shifts from δ(ref) for (i) KW: the peptides Gly-Gly-X-Ala (Wüthrich, 1986), where X refers to one of the 20 naturally occurring amino acids; (ii) MDW: the peptides Gly-Gly-X-Gly-Gly (Merutka et al., 1995); (iii) WSR(all): all NH protons associated with that particular amino acid in approximately 70 proteins (Wishart et al., 1991); and (iv) WSR(α): all NH protons in α-helical domains of the same set of proteins (Wishart et al., 1991).

to exhibit special chemical shifts for the carbon-bound protons (Kuntz et al., 1991; Zhou et al., 1992). For the bicelle-bound peptides, the analysis of CH shifts awaits the availability of fully deuterated phospholipids.

The partial analysis of the NMR results presented here illustrates the potential of isotropic phospholipid bicellar solutions in spectroscopic studies of polypeptides, proteins and other substances strongly bound to biological membranes. In conjunction with studies of magnetically ordered bicelles, this presents an opportunity for determining both the atomic resolution structure and the orientation of peptides and protein segments relative to the bilayer. Furthermore, by conducting relaxation experiments in both phases we expect to gain valuable information about the rate, amplitude and symmetry of internal motion (Vold and Vold, 1991), which should provide a test of models currently popular with interpreters of polypeptide relaxation data.

At present, the magnetically ordered phase has been characterized far more thoroughly than the isotropic phase, and the morphology of the bicelles in the isotropic phase should be determined urgently. For this purpose, dynamic light scattering and diffusion experiments are in progress, and morphological information may be obtainable from freeze fracture electron microscopy. Studies of other membrane-bound peptides and proteins are underway in our laboratory, with particular focus on the anchoring of myristoylated protein kinases and selected membrane-spanning protein units. Needless to say, it is important to determine binding constants and the on/off rates of peptide exchange with different types of phospholipid bicelles. We have very recently found that the replacement of up to 25% of the DMPC with DMPS (dimyristoyl phosphatidylserine) does not prevent the formation of well-ordered bicelles (J. Struppe and R.R. Vold, to be published). This opens the door for studies of peptide binding to both neutral and acidic phospholipid bilayers.

Acknowledgements

This work was supported by Grant CHE9632618 from the National Science Foundation and by a postdoctoral fellowship to R.S.P. from the Medical Research Council of Canada. We are particularly grateful to Murray Goodman and Joseph Taulane (UCSD) for allowing us to use their Cary 61 CD spectrophotometer, and thank Elizabeth Komives and Patricia Jennings (UCSD), Jane Dyson

and Peter Wright (The Scripps Research Institute), and Brian Sykes (University of Alberta) for their patience and friendly advice.

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