Journal of Biomolecular NMR, 1 (1991) 167–173
ESCOM

J-Bio NMR 015

Orientations of amphipathic helical peptides in membrane bilayers determined by solid-state NMR spectroscopy

B. Bechinger[a], Y. Kim[a], L.E. Chirlian[a], J. Gesell[a], J.-M. Neumann[a,*], M. Montal[b], J.Tomich[c], M. Zasloff[d] and S. J. Opella[a,**]

[a]*Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.*
[b]*Departments of Biology and Physics, University of California, San Diego, La Jolla, CA 92093, U.S.A.*
[c]*Department of Biochemistry, University of Southern California Medical School, 4650 Sunset Blvd., Los Angeles, CA 90027, U.S.A.*
[d]*Departments of Pediatrics and Genetics, Division of Human Genetics and Molecular Biology, Children's Hospital of Philadelphia, Philadelphia, PA 19104, U.S.A.*

Received 5 February 1991
Accepted 2 March 1991

Keywords: Channel structure; Magainin; Acetylcholine receptor; Lipid bilayer; Amphiphilic α-helix; ^{15}N chemical shift; Solid-state NMR

SUMMARY

Solid-state NMR spectroscopy was used to determine the orientations of two amphipathic helical peptides associated with lipid bilayers. A single spectral parameter provides sufficient orientational information for these peptides, which are known, from other methods, to be helical. The orientations of the peptides were determined using the ^{15}N chemical shift observed for specifically labeled peptide sites. Magainin, an antibiotic peptide from frog skin, was found to lie in the plane of the bilayer. M2δ, a helical segment of the nicotinic acetylcholine receptor, was found to span the membrane, perpendicular to the plane of the bilayer. These findings have important implications for the mechanisms of biological functions of these peptides.

INTRODUCTION

Amphipathic helical peptides are structural and functional entities by themselves, as oligomers, and as protein domains in membrane environments. Amphipathic helical peptides are thought to form trans-membrane ion channels by aggregating in oligomeric bundles with their hydrophilic residues on the inside, facing the lumen of the pore, and their hydrophobic residues on the out-

* Permanent address: Département de Biologie, Centre d'Etudes Nucleaires de Saclay, 91191 Gif-Sur-Yvette Cedex, Saclay, France.
** To whom correspondence should be addressed.

side, interacting with the hydrocarbon chains of the lipids. Each of the peptides in these channels contributes a single membrane spanning helix perpendicular to the plane of the bilayer. Amphipathic helical peptides also associate with the interface region of phospholipid bilayers in the plane of the bilayer. Solid-state NMR spectroscopy is well suited for differentiating between trans-membrane and in-plane orientations of helical peptides with many different orientationally dependent spectral parameters available for this purpose. In this paper we demonstrate that the ^{15}N amide chemical shift provides a convenient parameter for determining the orientation of amphipathic helical peptides in lipid bilayers.

Magainins are a family of 21–26 residue peptides first found in the skin of frogs (Zasloff, 1987). They exhibit a broad spectrum of antibacterial and other biological activities, possibly resulting from the formation of oligomeric peptide ion channels across membranes, or some other mechanism for disrupting the electrochemical ionic gradient across cell membranes (Urrutia et al., 1989). There is substantial evidence for strong interactions of magainins with bacterial membranes. However, these peptides do not lyse red blood cells, which suggests that they may interact quite differently with vertebrate membranes than with bacterial membranes. Two-dimensional solution NMR experiments indicate that these peptides are predominantly helical in detergent micelles (Shon et al., 1991d) and trifluoroethanol/water mixtures (Marion et al., 1988) and unfolded in aqueous solution. The 23-residue Magainin2 peptide, GIGKFLHSAKKFGK$\underline{\underline{A}}$FVGEIMNS-amide, was synthesized with Ala^{15} labeled with ^{15}N for these studies.

The major superfamilies of ion channels in biological membranes, such as the nicotinic acetylcholine receptor and voltage-gated cation channels, are large and complex proteins. A promising approach to their analysis is to synthesize and study peptide sequences corresponding to segments of the protein (Montal, 1990a, b). The peptide M2 was selected from the δ subunit of the *torpedo* acetylcholine receptor, because homology and model studies suggest that this sequence-specific motif is responsible for specific functions in the channel activity of the receptor (Oiki et al., 1988). M2δ differs substantially from Magainin2 in that it does lyse red blood cells (Kersh et al., 1989) and closely mimics the ion channel properties of the receptor in model membranes. M2δ and similar peptides have been shown to be helical by solution NMR spectroscopy (Brown et al., 1982, 1983; Lee et al., 1987; Holak et al., 1988; Mulvey et al., 1989; Wennerberg et al., 1990; Ikura et al., 1991; Shon et al., 1991b). The 23-residue M2δ peptide, EKMSTAISVLL$\underline{\underline{A}}$QAVFLLLTSQR, was synthesized with Ala^{12} labeled with ^{15}N for these studies.

METHODS

The peptides utilized in the NMR experiments were synthesized by automated solid-phase methods based on t-Boc chemistry with a single amide site specifically labeled with ^{15}N. The samples were prepared from peptides uniformly mixed with lipids in an organic solvent at a defined ratio, applied to thin glass plates where the solvent was evaporated, equilibrated at 93% relative humidity at a temperature above that for the lipid phase transition, and compressed between plates. ^{31}P NMR spectra of the peptide containing lipids indicate that the samples are highly oriented. Each sample was then placed in the doubly tuned coil of a probe of a solid-state NMR spectrometer and maintained at constant humidity and temperature. Coils with either planar geometry, suitable for a single pair of glass plates, or square geometry, suitable for a stack of glass plates, were used (Bechinger and Opella, 1991). ^{15}N NMR experiments were performed on a

home-built double-resonance spectrometer with an 8.5 T magnetic field. The spectra were obtained with spin-temperature-alternated and phase-cycled MOIST cross-polarization sequence (Pines et al., 1973; Levitt et al., 1986) with typical parameters of 1.6 ms mix time, 3 s recycle delay, 1.5 mT decoupling field, and signals acquired with 20 000 proton decoupled transients.

RESULTS AND DISCUSSION

Figure 1 compares experimental solid-state ^{15}N NMR spectra of the specifically labeled Magainin2 and M2δ peptides in oriented phospholipid bilayers, to a simulated ^{15}N amide chemical-shift powder pattern. Each oriented peptide has a relatively narrow single-line resonance from its one ^{15}N-labeled amide site. The resonance frequencies observed in these two samples are quite different, varying by nearly the full breadth of the powder pattern, indicating that the planes containing the labeled peptide groups have very different orientations relative to the direction of the applied magnetic field. In order to fully determine the orientation of a labeled peptide plane, it would be necessary to measure at least one other spectral parameter associated with that site

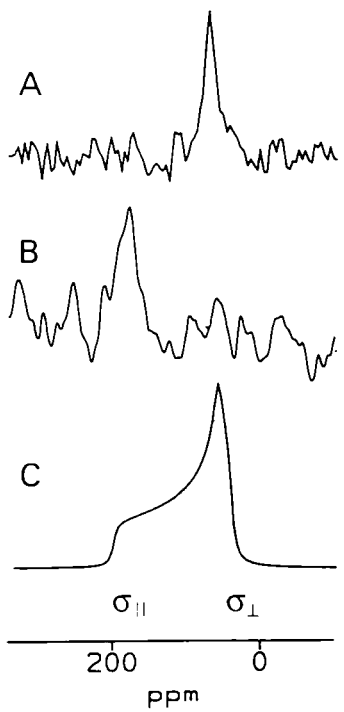

Fig. 1. ^{15}N NMR spectra of peptides. (A) Experimental spectrum of ^{15}N-Ala^{15} - Magainin2 (3 mol %) in POPC/POPG (3:1) (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine / 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol) bilayers oriented between glass plates at 40 ° C. (B) Experimental spectrum of ^{15}N-Ala^{12}-M2δ peptide (5 mol %) in DMPC/DMPG (4:1) (1,2 - dimyristoyl-*sn*-glycero-3-phosphocholine / 1,2 - dimyristoyl-*sn*-glycero-3-phosphoglycerol) bilayers oriented between glass plates at 30°C. In both samples, the glass plates were oriented so that the plane of the bilayers is perpendicular to the direction of the applied magnetic field. (C) Simulation of ^{15}N chemical-shift powder pattern for a peptide nitrogen, using the values given in Fig. 2. The positions associated with the N-H bond approximately parallel to the field and perpendicular to the field are marked.

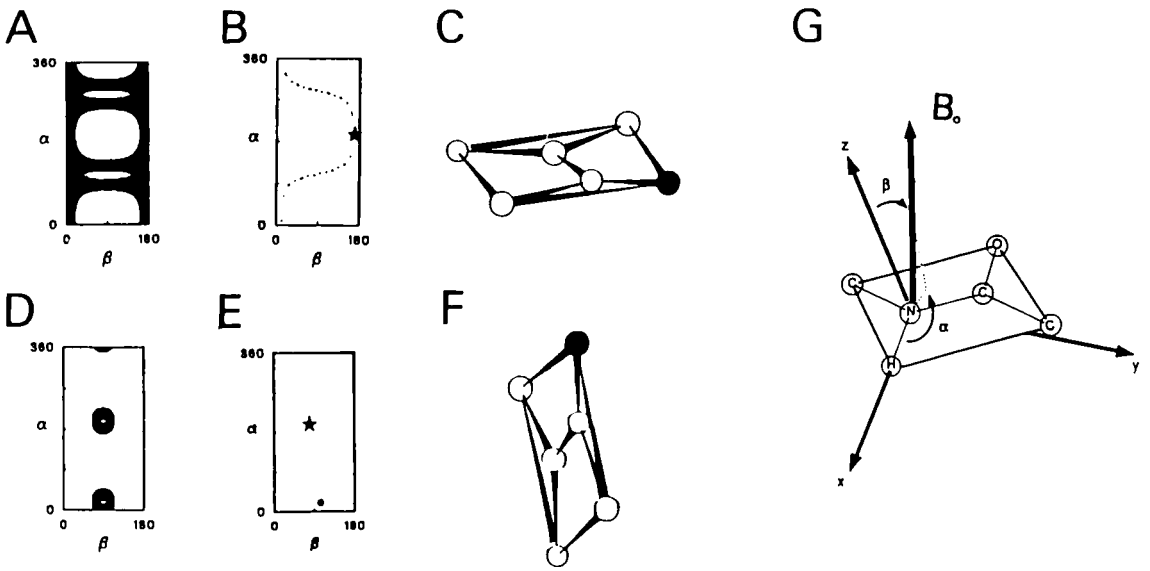

Fig. 2. (A) Restriction plot for a ^{15}N chemical shift measurement of 60 ± 5 ppm. The values for the chemical-shift tensor are: σ_{11} = 37.6 ± 6.5 ppm, σ_{22} = 55.4 ±5.2 ppm and σ_{33} = 200.6 ±5.5 ppm. The tensor orientation is 18.5° ±6.5°. (B). restriction plot showing the possible α, β pairs for an α-helix oriented perpendicular to the direction of the applied magnetic field, B_o. The star indicates the pair of angles corresponding to the orientation with the N-H bond perpendicular to B_o, α = 180°, β = 180°. (C) The peptide plane corresponding to the starred pair of angles in B. (D) Restriction plot for a ^{15}N chemical-shift measurement of 185 ±5 ppm. The tensor is the same as in A. (E) Restriction plot showing the possible α, β pairs for an α-helix oriented parallel to B_o. The two possibilities are symmetry related, corresponding to the α-helix being oriented with the N-H bonds pointing 'up' or 'down'. (F) The peptide plane corresponding to the orientation indicated by the star in E. (G) Molecular axis system for the peptide plane. The x-axis is coincident with the N-H bond, the z-axis is perpendicular to the plane, and the y-axis is perpendicular to both x and z. The orientation of the peptide plane with respect to B_o is given by the angles α and β. β is the angle between B_o and the z-axis and α is the angle between the x-axis and the projection of B_o in the xy plane.

(Opella et al., 1987; Opella and Stewart, 1989). However, since the ^{15}N-labeled residues in both Magainin2 (Marion et al., 1988; Shon et al., 1991d) and M2δ (Shon et al., 1991a) are known to adopt helical secondary structures in membrane environments from solution NMR experiments, the ^{15}N chemical shifts provide sufficient information to define the orientations of the helices.

The experimental spectra in Figs. 1A and 1B can be readily interpreted qualitatively, since the magnitudes and orientations of the principal values of the (nearly) axially symmetric ^{15}N amide chemical-shift tensor have been established in a variety of model peptides, and the observed resonance frequencies are near the discontinuities of the chemical-shift powder pattern; the chemical-shift tensor of a ^{15}N amide site is oriented such that an N-H bond that is approximately parallel to the direction of the applied magnetic field has a ^{15}N resonance frequency close to that of the principal element $\sigma_{||}$, and an amide N-H bond perpendicular to the field has a ^{15}N resonance frequency close to that of the principal element $\sigma_{\perp}$(Harbison et al., 1984; Hartzell et al., 1987; Oas et al., 1987; Teng and Cross, 1989). In Fig. 1A, the ^{15}N NMR spectrum of ^{15}N-Ala^{15}-labeled Magainin2 consists of a single line with a resonance frequency near $\sigma_{\perp}$, therefore the N-H bond of Ala^{15}, as well as those of the other residues in the helical peptide, are approximately perpendicu-

lar to the direction of the magnetic field and parallel to the plane of the bilayer. In Fig. 1B, the ^{15}N NMR spectrum of ^{15}N-Ala^{12}-labeled M2δ has its resonance intensity near $\sigma_{||}$, therefore the N-H bond of Ala^{12}, as well as those of the other residues in the helical peptide, are approximately parallel to the direction of the magnetic field and perpendicular to the plane of the bilayer.

The experimental spectra in Figs. 1A and 1B can be interpreted in a more detailed and quantitative manner by identifying all of the peptide plane orientations that are consistent with the observed resonance frequencies. The two polar angles α and β defined in Fig. 2G describe the orientation of a peptide plane with respect to the direction of sample orientation and the applied magnetic field, B_0. Peptide plane orientations consistent with an experimental measurement are represented as pairs of α and β angles. This method provides limitations or restrictions on the possible peptide plane orientations rather than determining a unique orientation from the experimental data. Successive applications of the restrictions from various experimental measurements reduce the number of allowed orientations to a manageable number (Chirlian and Opella, 1990a). The results are displayed graphically in restriction plots, where the dark areas represent the α- and β-angle pairs for all orientations consistent with the experimental measurement; orientations described by pairs of α and β angles in the white areas are ruled out by the measurement. The restriction plots in Figs. 2A and 2D correspond to the experimental data in Figs. 1A and 1B, respectively. In order to ensure that all plausible orientations are present in the restriction plots, the full range of ^{15}N chemical-shift tensor values which have been observed in proteins or peptides, and a very conservative estimate of experimental error, are utilized in the calculations (Chirlian and Opella, 1990b, 1991).

Many different peptide-plane orientations are consistent with each ^{15}N resonance frequency. If information about the secondary structure was not available, then the determination of orientation would be possible only through additional experimental measurements. However, since the secondary structure has been determined independently for both of the peptides, we can take advantage of the structural regularity of the α-helix to extend the results from the specifically labeled site to the entire peptide. The peptide planes in an α-helix oriented parallel to the field all have the

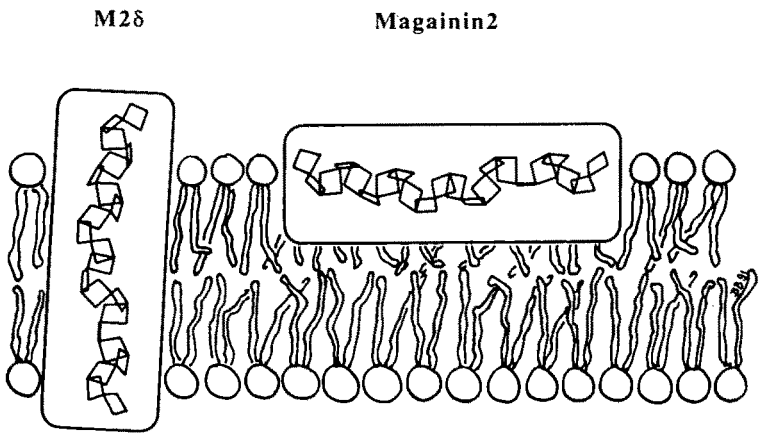

Fig. 3. Schematic drawing of in-plane (Magainin2) and trans-membrane (M2δ) orientations of 23-residue amphipathic helical peptides in lipid bilayers. The helical backbones are represented by their 22 peptide bond planes, whereas the white boxes indicate the extending side chains.

same values of α and β, as shown in the restriction plot in Fig. 2E. When the helix is oriented perpendicular to the field a regular pattern of α, β pairs exists, as is shown in Fig. 2B. Comparisons between these restriction plots based only on secondary structure, with those based on experimental results, enables the orientation of the helix to be determined by elimination. The correct peptide plane orientation (α, β pair) for a helix parallel to the magnetic field is only contained in the restriction plot for the 185-ppm chemical shift. No overlap with the α, β pairs for a helix oriented perpendicular to the field is observed. Similarly, common α, β pairs are present in Figs. 2A and 2B consistent with a helix oriented perpendicular to the field. These conclusions are strengthened by the absence of overlap between the restriction plots in Figs. 2B and 2E.

The qualitative and quantitative methods of analysis of the oriented ^{15}N chemical shifts are in agreement in describing the orientations of these two helical peptides in lipid bilayers. Magainin2 is oriented approximately parallel to the plane of the bilayer. The drawing in Fig. 3 places it at the interfacial region of the bilayer based on the expectation that an amphipathic peptide interacts with both the polar headgroups and the hydrocarbon chains of the lipids. M2δ is clearly a transmembrane peptide, as shown in Fig. 3, oriented perpendicular to the plane of the bilayer. Both of the peptides are visualized as monomers in Fig. 3; however, they are most likely associated as oligomers in the membranes, with each peptide having the same orientation relative to the plane of the membrane. These findings are consistent with the functional models of the M2δ peptide forming trans-membrane molecular channels. However, they suggest that the magainins, at least at equilibrium in this lipid system, do not cross the membrane, but rather reside in the interfacial regions.

α-Helices are the dominant structural features of membrane proteins (Deisenhofer, 1985; Rees et al., 1989; Henderson et al., 1990). Both hydrophobic and amphipathic helices longer than about 20 residues span the membrane and are aligned approximately perpendicular to the plane of the bilayer, and shorter amphipathic helices are associated with the polar headgroups of the lipids and are aligned along the plane of the bilayer. These results demonstrate that the combination of determining the secondary structure of proteins in micelles and the orientation of helices in bilayers that has been successfully demonstrated for membrane-bound fd (Shon et al., 1991c) and Pf1 (Shon et al., 1991b) filamentous bacteriophage coat proteins is applicable to isolated amphipathic helices in bilayers.

ACKNOWLEDGEMENTS

We thank P. McDonnell and H. Eck for help with peptide synthesis. The research of S.J.O. is supported by grants (AI-20770, GM-24266 and GM-29754) from the National Institutes of Health. The research of M.Z. is supported by grants from the G. Harold and Leila Y. Mathers Foundation. The research of M.M. is supported by grants (GM-42340 and MH-44638) and a Research Scientist Award (MH-00778) from the National Institutes of Health, grant (N00014-89-J-1469) from the Office of Naval Research, and grant (DAMD 17-89-C-9032) from the Department of Army Medical Research. The research of J. T. is supported by grant (GM-43617) from the National Institutes of Health. B.B. is supported by an EMBO long-term fellowship (ALTF 454-1989) and L.E.C. is supported by a postdoctoral fellowship (GM-13256) from the National Institutes of Health.

REFERENCES

Bechinger, B. and Opella, S.J. (1991) unpublished results.

Braun, W., Wider, G., Lee, K.H. and Wüthrich, K. (1983) *J. Mol. Biol.*, **169**, 921-948.

Brown, L.R., Braun, W., Kumar, A. and Wüthrich, K. (1982) *Biophys. J.*, **37**, 319-328.

Chirlian, L. E. and Opella, S. J. (1990a) *Adv. Magn. Reson.*, **14**, 183-202.

Chirlian, L. E. and Opella, S. J. (1990b) *New Polymeric Mater.*, **2**, 279-290.

Chirlian, L. E. and Opella, S. J. (1991) unpublished results.

Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) *Nature*, **318**, 618-624.

Harbison, G. S., Jelinski, L. W., Stark, R. E., Torchia, D. A., Herzfeld, J. and Griffin, R. G. (1984) *J. Magn. Reson.*, **60**, 79-82.

Hartzell, C. J., Whitfield, M., Oas, T. G. and Drobny, G. P. (1987) *J. Am. Chem. Soc.*, **109**, 5967-5969.

Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, N., Beckmann, E. and Downing, K.H. (1990) *J. Mol. Biol.*, **213**, 899-929.

Holak, T.A., Engström, Å., Kraulis, P.J., Lindeberg, G., Bennich, H., Jones, T.A., Gronenborn, A.M. and Clore, G.M. (1988) *Biochemistry*, **27**, 7620-7629.

Ikura, T., Gō, N. and Inagaki, F. (1991) *Proteins*, **9**, 81-89.

Kersh, J., Tomich, J. and Montal, M. (1989) *Biochem. Biophys. Res. Commun.*, **162**, 352-356.

Lee, K.H., Fitton, J.E. and Wüthrich, K. (1987) *Biochim. Biophys. Acta*, **911**, 144-153.

Levitt, M. H., Suter, D. and Ernst, R. R. (1986) *J. Chem. Phys.*, **84**, 4235-4255.

Montal, M. (1990a) in *Ion Channels*, Vol 2 (Ed. Narahashi, T.) Plenum Press, New York, pp. 1-31.

Montal, M. (1990b) *FASEB J.*, **9**, 2623 - 2635.

Marion, D., Zasloff, M. and Bax, A. (1988) *FEBS Lett.*, **227**, 21 - 26.

Mulvery, D., King, G.F., Cooke, R.M., Doak, D.G., Harvery, T.S. and Campbell, I.D. (1989) *FEBS Lett.*, **257**, 113-117.

Oas, T. G., Hartzell, C. J., Dahlquist, G. W. and Drobny, G. P. (1987) *J. Am. Chem. Soc.*, **109**, 5962-5966.

Oiki, S., Danho, W., Madison, V. and Montal, M. (1988) *Proc. Natl. Acad. Sci.*, USA, **85**, 8703-8707.

Opella, S. J. and Stewart, P. L. (1989) *Methods Enzymol.*, **176**, 242-275.

Opella, S. J., Stewart, P. L. and Valentine, K. G. (1987) *Q. Rev. Biophys.*, **19**, 7-49.

Pines, A., Gibby, M.G. and Waugh, J.S. (1973) *J. Chem. Phys.*, **59**, 569-590 .

Rees, D.C., Komiya, H., Yeates, T. O., Allen, J. P. and Feher, G. (1989) *Annu. Rev. Biochem.*, **58**, 607-633 .

Shon, K., Gesell, J., Montal, M., Tomich, J. and Opella, S. J. (1991a) unpublished results.

Shon, K., Kim, Y., Colnago, L. A. and Opella S. J. (1991b) *Science*, in press.

Shon, K., Schrader, P., Kim, Y., Bechinger, B., Zasloff, M. and Opella, S. (1991c) in *Biotechnology: Bridging Research and Applications* (Eds. Kamely, D., Chacrabarty, A. and Kornguth, S.) Kluwer Academic, Dordrecht.

Shon, K., Zasloff, M. and Opella, S. J. (1991d) unpublished results.

Teng, Q. and Cross, T. A. (1989) *J. Magn. Reson.*, **85**, 439-447.

Urrutia, R., Cruciani, R. A., Barker, J. L. and Kuchar, B. (1989) *FEBS Lett.*, **247**, 17-21.

Wennerberg, A.B.A., Cooke, R.M., Caarlquist, M., Rigler, R. and Campbell, I.D. (1990) *Biochem. Biophys. Res. Commun.*, **166**, 1102-1109.

Zasloff, M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5449-5453.