ELSEVIER

Biophysical Chemistry 119 (2006) 115 – 120

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Deuteration can affect the conformational behaviour of amphiphilic α-helical structures

Sarah R. Dennison [a], Thomas Hauβ [b], Silvia Dante [b], Klaus Brandenburg [c], Frederick Harris [d], David A. Phoenix [a,*]

[a] *Faculty of Science, University of Central Lancashire, Preston, PR1 2HE, UK*
[b] *Berlin Neutron Scattering Centre, Hahn-Meitner Institute, Germany*
[c] *Forschungszentrum Borstel, Leibniz-Center for Medicine and Biosciences, Parkallee 10, D-23845 Borstel, Germany*
[d] *Department of Forensic and Investigative Science, University of Central Lancashire, Preston, PR1 2HE, UK*

Received 5 July 2005; received in revised form 5 September 2005; accepted 6 September 2005
Available online 20 October 2005

Abstract

The replacement of hydrogen with deuterium is frequently used in conjunction with neutron diffraction to investigate peptide–membrane interaction. This isotopic substitution in an amino acid residue radically changes the neutron scatter pattern of the peptide, thereby allowing its localisation within the bilayer with the aid of derived Fourier maps. Nonetheless, this technique relies on the generally held assumption that normal and isotopically enriched protein species do not differ significantly in structure or biological activity. Recently, this assumption has been questioned and here, diffraction data from studies on a membrane interactive peptide clearly challenge the reliability of this assumption.

Keywords: Peptide; Deuteration; α-helix; Membrane; Neutron diffraction; Fourier transform infrared spectroscopy

1. Introduction

Over the last 15 years, site specific deuterium labelling has been used in conjunction with neutron lamellar diffraction to investigate the membrane interactions of a variety of biologically relevant peptides and protein segments [1] ranging from adenosine diphosphate ribosylation factors [2] to the β-amyloid peptide associated with Alzheimers disease [3]. Essentially, this latter methodology compares the measured diffraction of a neutron beam, which has passed through a crystallised lipid bilayer with that measured from a second crystallised membrane that has been allowed to interact with a peptide [4]. Using these neutron diffraction data, a bilayer map, normal to the plane of the membrane is reconstructed and provides a low-resolution, time and sample averaged profile of the bilayer cross section based on neutron scattering length. The subsequent isotopic substitution of some of the peptide's hydrogen atoms with deuterium radically changes the neutron scatter pattern of the peptide, yielding a higher resolution, time and sample averaged profile in the location of the label, thereby enabling the degree of bilayer penetration to be determined from resultant Fourier maps. Such analysis is clearly dependent on the assumption that deuteration does not affect the structure function profile of the peptide under investigation, although there is a considerable body of evidence that challenges this assumption, particularly the pioneering work of Crespi, Hattori and co-workers [5,6].

The present study was intended to test the prediction that a membrane interactive α-helix 'GTAMRILGGVI' was oblique orientated [7] using neutron diffraction. Unexpectedly, a variety of methods indicated that deuteration had reproducibly affected the structure and function of this peptide.

2. Materials and methods

2.1. Reagents

Peptide homologues of the GTAMRILGGVI segment were synthesised: one using normal amino acid residues (VP1) and the other (dVP1) where leucine had been substituted with a

* Corresponding author. Tel.: +44 1772 893481; fax: +44 1772 892903.
E-mail address: daphoenix@uclan.ac.uk (D.A. Phoenix).

0301-4622/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2005.09.002

deuterated form of the residue (GTAMRIL(d10)GGVI). The peptides were supplied by Albachem Ltd (UK), synthesised by solid state synthesis and purified by HPLC to purity greater than 99%. Palmitoyloleyl phosphatidylcholine (POPC) and palmitoyloleyl phosphatidylserine (POPS) were purchased from Avanti Polar Lipids (USA). Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylserine (DMPS) and all other reagents were purchased from Sigma (UK). Buffers and solutions for monolayer experiments were prepared from Milli-Q water.

2.2. Neutron diffraction studies

Neutron diffraction experiments were conducted using the V1 membrane diffractometer at the Berlin Neutron Scattering Centre (BENSC) according to techniques previously described [1–4]). Chloroform solutions of POPC/POPS (10:1 molar ratio) were prepared and used to solubilise either VP1 (final concentration 8 mM) or dVP1 (final concentration 8 mM) such that the lipid to peptide molar ratio was 100:1. Each peptide solution and the lipid solution alone were then separately sprayed onto a quartz microscope slide using an artist's airbrush (Aerobrush pro281) to form even layers. Under these conditions, lipid samples self assemble to form multi-lamellar structures, which may be considered as stacked membrane bilayers. These treated slides were placed in a vacuum desiccator for 12 h to remove all traces of chloroform. Samples were then individually placed in aluminium thermostatic sample cans and rehydrated for 24 h at 25 °C with relative humidity maintained at 98% using Teflon water baths containing saturated potassium sulphate solution. Potassium sulphate water solution at each of three isotopic compositions: 50%, 20% and 8% ^2H_2O were used to assist with phase assignment. After each completed neutron diffraction measurement, the sample slide was rehydrated with the water at the next isotopic composition with at least 24 h allowed for equilibration. The scattering intensities were recorded up to the 5th order and data analysis was performed according to Dante et al., [3].

2.3. Lipid monolayer studies

Monolayer studies were performed using Langmuir–Blodgett equipment supplied by NIMA (UK). These studies were conducted at a constant area using a Teflon trough with surface area dimensions of 5×5 cm and a volume of 10 ml. Monolayers were formed by spreading chloroform solutions of POPC/POPS (10:1 molar ratio) onto a buffer subphase (10 mM Tris, pH 7.5) to give an initial surface pressure of 30 mN m^{-1}. Either VP1 (10 mM) or dVP1 (10 mM) in 10 mM Tris (pH 7.5) was introduced to the subphase via an injection port to give a final subphase peptide concentration of 20 μM, equivalent to a monolayer lipid to peptide molar ratio of 100:1. The subphase was continuously stirred by a magnetic bar and changes in surface pressure monitored by the Wilhelmy method, using a paper plate and a microbalance [7].

2.4. FTIR conformational analyses

Using the methodology of Keller et al., [8], vesicular suspensions of DMPC/DMPS (10:1 molar ratio) were prepared in 10 mM Tris buffer (pH 7.5), formed using either H_2O or ^2H_2O. Each vesicular suspension was then used to solubilise either VP1 (final concentration 1 mM) or dVP1 (final peptide concentration 1 mM) such that the lipid to peptide molar ratio was 100:1. Solutions of VP1 and dVP1, respectively, in 10 mM Tris buffer (pH 7.5) were also prepared with a final peptide concentration of 1 mM in each case. All samples were spread onto a calcium fluoride, ATR plate as described by Goormaghtigh et al., [9] and FTIR conformational analysis of peptides was performed by monitoring the amide I vibrational bands as described by Brandenburg et al. [7]. The resulting spectra were used to compute the relative levels of secondary structure in VP1 and dVP1, as described by Brandenburg et al. [7].

2.5. Mass spectrometry analysis

Matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) analysis were performed using Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA). Acquisition was performed in the linear positive ion mode. The VP1 and dVP1 (10 μM) peptides were dissolved in 50% (v/v) acetonitrile and 0.1% (v/v) triflouroacetic acid (TFA). The matrix consisted of a mixture of α-cyano-4-hydroxy cinnamic acid (CHCA) (5 mg ml^{-1}) in 50% (v/v) acetonitrile and 0.1% (v/v) TFA. Approximately 10 pmol peptide stock solution (1 μl of a 10 μM stock solution) was then added to the matrix solution. Approximately 1 μl of the analyte/matrix solution was applied to the mass spectrometer target plate and allowed to air-dry. A scanning range of 883 to 1500 m/z was used. For conformation of the MALDI MS analysis, independent sequence analysis was determined using a nanoelectrospray (nanoES) quadrupole-time of flight (Q-ToF) MS (Micromass, Manchester, UK) with a nanospray source was used.

3. Results and discussion

Neutron diffraction was used to investigate the ability of VP1 and dVP1 to penetrate lipid bilayers that were designed to be mimetic of naturally occurring mammalian cytoplasmic membranes. These lipid bilayers were formed from palmitoyloleyl phosphatidylcholine (POPC) and palmitoyloleyl phosphatidylserine (POPS) at a 10:1 molar ratio and neutron scattering density profiles were determined for: bilayers in the absence of peptides (Fig. 1A), bilayers in the presence of VP1 (lipid to peptide molar ratio 100:1) (Fig. 1B), and bilayers in the presence of dVP1 (lipid to peptide molar ratio 100:1) (Fig. 1C), all at 8% ^2H_2O contrast [3]. The horizontal scale in these figures represents a transect across the membrane where the two maxima correspond to the positions of the lipid head groups whilst the minima indicate the position of the bilayer core, corresponding to the lipid terminal methyl groups.

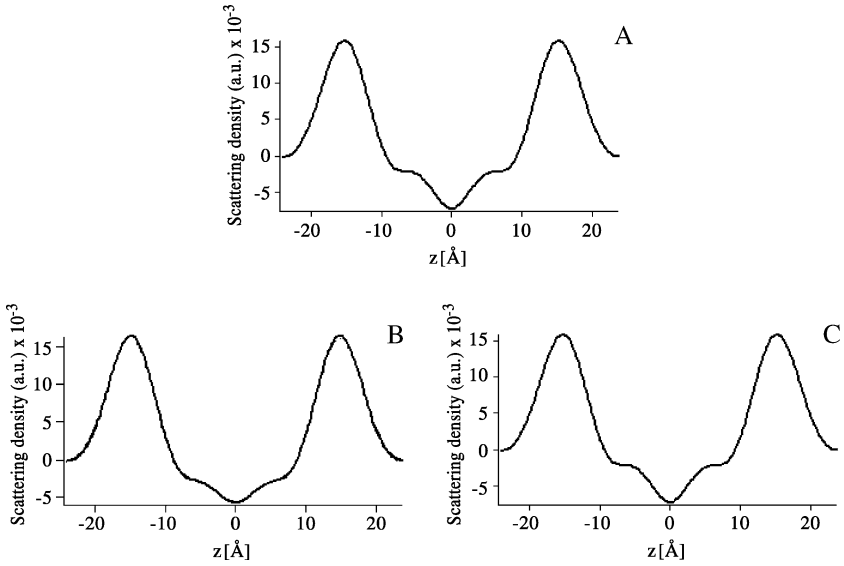

Fig. 1. Neutron scattering length density profiles were determined for lipid bilayers formed from POPC/POPS at a 10:1 molar ratio (A) or of these bilayers in the presence of either VP1 (8 mM) (B) or dVP1 (8 mM) (C) where the lipid to peptide molar ratio was 100:1. A, B and C respectively, show the neutron scattering density profile generated by a transect of the bilayer taken perpendicular to the bilayer surface. The two maxima of the profile indicate that the highest levels of neutron scattering are shown by the bilayer lipid head group regions whilst the minimum indicates that the lowest levels are generated by lipid terminal methyl groups, located at the centre of the bilayer hydrophobic core. Each profile was determined at 8% ^2H_2O contrast. It can be seen that there are no significant differences between the neutron scattering profiles shown in (A) and (C), suggesting that dVP1 does not penetrate POPC/POPS bilayers. In contrast the scattering profile of VP1 in (B) departs markedly from these latter profiles in the region of their minima, which represent the hydrophobic core of the bilayer.

The scattering density profiles for lipid bilayers alone (Fig. 1A) and for those bilayers in the presence of dVP1 (Fig. 1C) can be seen to follow identical profiles, which suggests that dVP1 does not penetrate the membrane. In contrast the profile of VP1 (Fig. 1B) departs markedly from these latter profiles in the region of the minima, clearly indicating that this peptide has penetrated the bilayer and interacted with its acyl chain region. These results were reproducible and contrasted strongly to those expected from such experiments where the neutron scattering profiles of the normal and the isotopically substituted peptides differ only in relation to these substitutions [10].

Differences between the membrane interactivity of these two peptides were further investigated using POPC/POPS (10:1 molar ratio) monolayers at an initial surface pressure of 30 mN m^{-1}, thereby mimicking the surface pressure of biological membranes [11]. At a lipid to peptide ratio of 100:1, VP1 interacted strongly with these monolayers, achieving a maximal level of penetration of 3 mN m^{-1} but under corresponding conditions, dVP1 showed no evidence of monolayer penetration (Fig. 2). These combined results clearly suggested that deuteration had affected the ability of the GTAMRILGGVI sequence to interact with membranes and Fourier transform infra-red (FTIR) spectroscopy was used to investigate the possibility that this effect may be related to conformational differences between VP1 and dVP1. Analysis of the pure peptides in aqueous solution (Fig. 3A and B) showed each to exhibit a broad band in the range 1625 to 1650 cm^{-1}, indicating the superposition of α-helical with β-sheet structures, and band components in the range 1670 to 1695 cm^{-1}, corresponding to β-turns and anti-parallel β-sheets. However, the peptides showed clear differences between their structures with VP1 possessing circa 15% α-helicity (Fig. 3A) and dVP1 showing less than 5% α-helicity and high levels of β-type structures (Fig. 3B). Analysis of these peptides in the presence of lipid (DMPC/DMPS, 10:1 molar ratio) was undertaken. At a total lipid to peptide molar ratio of 100:1, VP1 possessed strong bands at 1649 and 1646 cm^{-1}, respectively (Fig. 3C and E), indicating that the peptide was predominantly α-helical (circa 55%), which is consistent with previous data [7,11]. However, Fig. 3D and F clearly shows that under corresponding conditions, dVP1 exhibits a markedly different conformational behaviour. In Fig. 3D, it can be seen that the spectrum of the peptide is dominated by a band at 1660 cm^{-1}, often indicating the superposition of bands representing unordered and α-helical structures. When these experiments were repeated in ^2H_2O these superposed bands were resolved

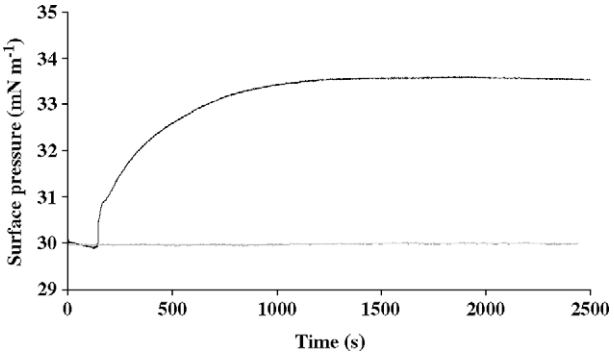

Fig. 2. Either VP1 (black) or dVP1 (grey) were introduced into the subphase of a POPC/POPS monolayer (10:1 molar ratio), set at an initial surface pressure of 30 mN m^{-1}, to give a lipid to peptide molar ratio of 100:1. VP1 showed clear evidence of monolayer interaction, inducing a surface pressure change of 3 mN m^{-1} but in contrast, dVP1 clearly showed no ability to interact with these monolayers.

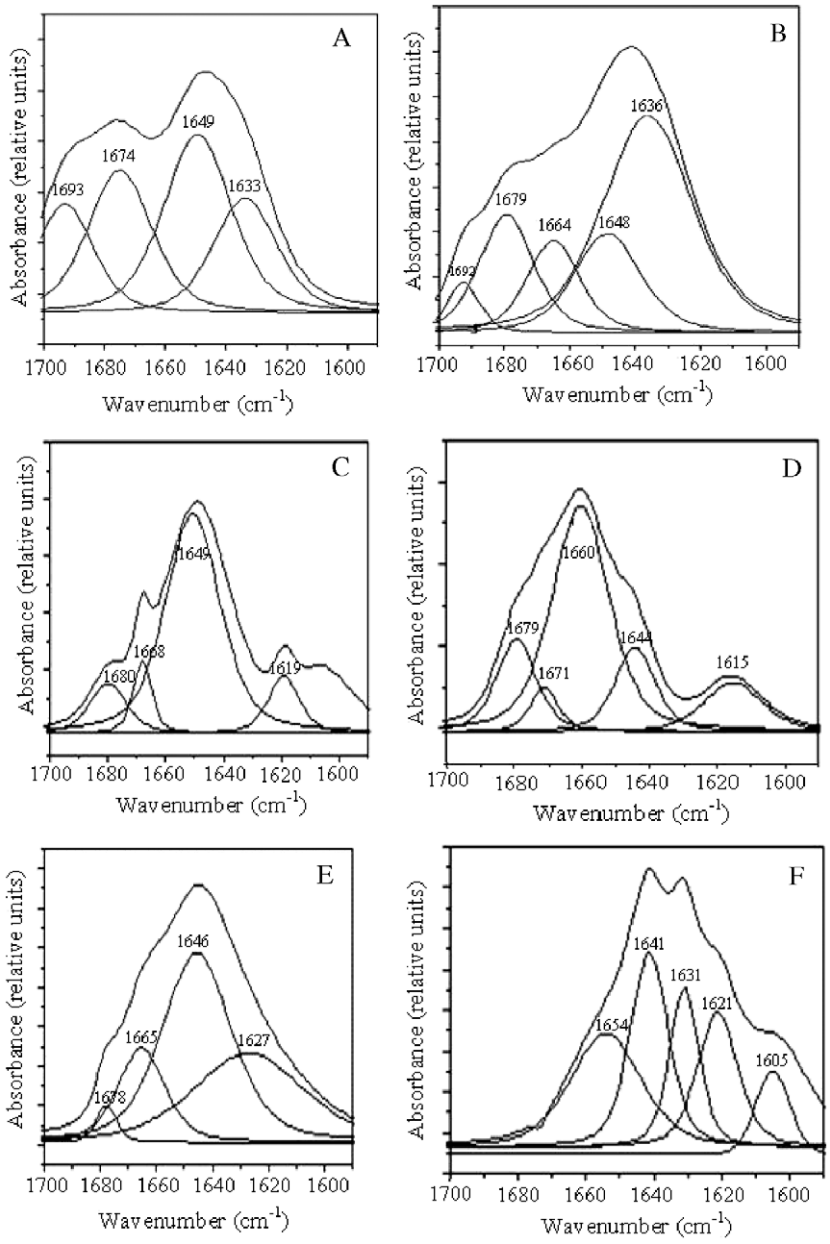

Fig. 3. FTIR conformational analyses of VP1 (1 mM) either in aqueous solution (A) or in the presence of lipid vesicles formed from DMPC/DMPS (10:1 molar ratio) to give a lipid to peptide molar ratio of 100:1 were performed. For these latter experiments, the peptide/vesicles were suspended in either H_2O (C) or ^2H_2O (E). The aqueous peptide showed a band at 1649 cm^{-1} consistent with circa 15% α-helicity, which was increased in the presence of lipid to approximately 55%, as indicated by corresponding bands in (C) and (E). The corresponding FTIR analyses for dVP1 (1 mM) are shown in (B), (D) and (F), respectively. The aqueous peptide showed only circa 5% α-helicity (B), which was not significantly affected by the presence of lipid. In (D), dVP1 exhibits a clearly defined peak at 1660 cm^{-1}, which represents the superposition of unordered and α-helical structures. In (D), dVP1 shows a peak at 1641 cm^{-1}, indicating high levels of unordered structure (>30%), and another peak at 1654 cm^{-1} representing circa 5% α-helical structures. These results clearly show that VP1 and dVP1 adopt dissimilar conformational structures, both in aqueous solution and in the presence of a DMPC/DMPS lipid matrix.

and dVP1 showed a peak at 1641 cm^{-1}, which corresponds to the presence of high levels of unordered structure (>30%), and another peak at 1654 cm^{-1} due to low levels of α-helical structure, which were circa 5%. There is the possibility that the sharpness of the underlying bands in Fig. 3C and F are indicative of restricted peptide solubility, nonetheless, these results clearly show that in the presence of a DMPC/DMPS lipid matrix, VP1 and dVP1 adopt dissimilar conformational structures. In combination these results suggest that deuteration has strongly reduced the ability of the GTAMRILGGVI segment to adopt α-helical structure and thereby its ability to penetrate membranes.

Given the unexpected nature of the results, the peptides were re-synthesised and the possibility of racemisation in the deuterated leucine of dVP1 eliminated by the manufacturer. Subsequent independent analysis of VP1 peptide by MALDI-MS revealed a mass of 1087.53 kDa (Fig. 4A), however, in comparison the dVP1 peptide showed a mass increase of 10 kDa (Fig. 4B). Independent sequence analysis using a Q-ToF mass spectrometer (data not shown) indicated that deuteration

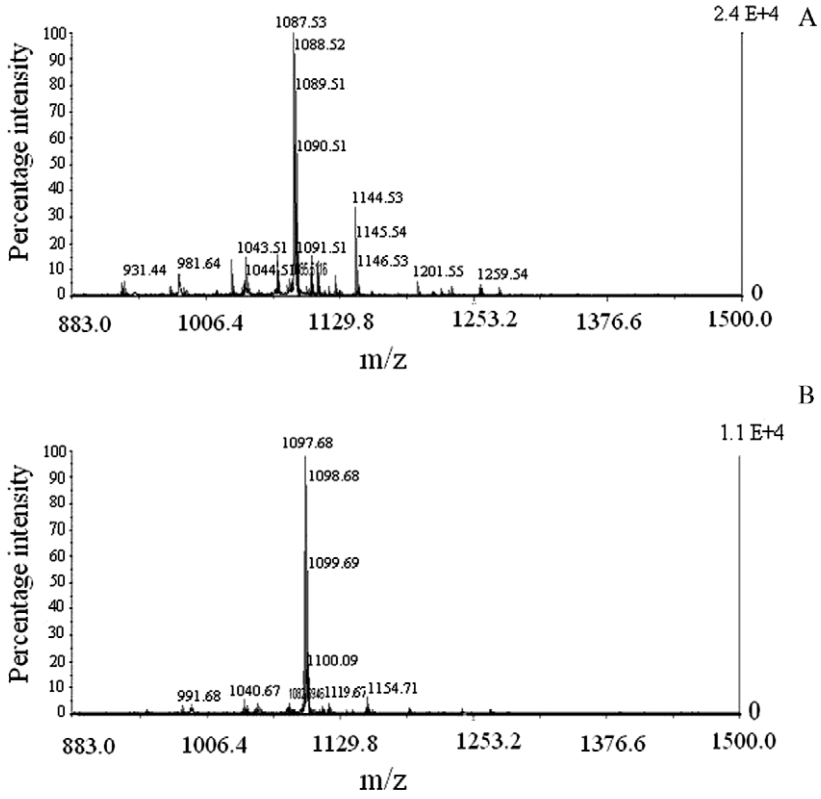

Fig. 4. Mass spectrometry analysis of the peptides VP1 (A) and dVP1 (B) obtained by MALDI-MS. The mass of VP1 is seen to be 1087.53 and 1097.68 Da for deuterated VP1.

had occurred on the leucine amino acid residue on the primary sequence. This provided clear evidence that the sequence is consistent with the manufacturer's specifications. Using the re-synthesised peptides, the results of this study were reproducible and clearly, these findings have far-reaching implications, requiring questions to be asked when deuteration is used in protein structure/function analysis. For the majority of peptides deuteration is unlikely to impact on structure but as seen here can have an impact on structure-formation. It has previously been shown that the 'GTAMRILGGVI' segment tends to adopt β-sheet/random coil in solution and that α-helical structure is stabilised at the lipid interface indicating that there is a delicate energy balance between the soluble random coil form and the membrane interactive α-helical form [7]. Melittin is also random coil in solution at neutral and acidic pH, however, like the 'GTAMRILGGVI' segment, it changes its secondary structure in the presence of a lipid membrane to form an amphiphilic α-helix [12]. It may well be that peptides with this conformational change are more susceptible to deuteration affecting the peptides ability to form a helix at the membrane lipid interface if it perturbs the energies of α-helix formation. As noted in recent studies, the deuteration of residues leads to less bulky side chains, which may disturb inter/intra molecular interactions and possibly impair the correct folding of proteins and peptides [13] such as that shown here.

In summary, the data of the present study support the growing body of evidence, which shows that deuteration is able to affect the physiochemical properties of proteins and peptides [5,6] and adds to the caveats that must be applied to neutron diffraction and other work relying on this form of structural modification.

Acknowledgements

*We would like to thank Dr. Frank Grunfeld (NIMA, UK) for his technical advice with the monolayer studies. This work was supported by the European Commission for European Access to BENSC Neutrons.

References

[1] J.P. Bradshaw, M.K. Duncan, P.J. Gilchrist, A.M. Saxena, in: B.P. Schoenborn, R.B. Knott (Eds.), Neutrons in Biology, Neutron Diffraction Studies of the Amphipathic Helices in Phospholipid Bilayers, vol. 44, Plenum Press, NY, 1996, pp. 35–55.
[2] T.A. Harroun, J.P. Bradshaw, K. Balali-Mood, J. Katsaras, A structural study of the myristoylated N-terminus of ARF1, Biochim. Biophys. Acta 1668 (2005) 138–144.
[3] Dante, T. Hauβ, N.A. Dencher, Beta-amyloid 25 to 35 is intercalated in anionic and zwitterionic lipid membranes to different extents, Biophys. J. 83 (2002) 2610–2616.
[4] J.P. Bradshaw, M.J. Darkes, T.A. Harroun, J. Katsaras, R.M. Epand, Oblique membrane insertion of viral fusion peptide probed by neutron diffraction, Biochemistry 39 (2000) 6581–6585.
[5] A. Hattori, H.L. Crespi, J.J. Katz, Effect of side-chain deuteration on protein stability, Biochemistry 4 (1965) 1213–1225.
[6] A. Hattori, H.L. Crespi, J.J. Katz, Association and dissociation of phyocyanin and the effect of deuterium substitution on the processes, Biochemistry 4 (1965) 1125–1138.
[7] K. Brandenburg, F. Harris, S. Dennison, U. Seydel, D. Phoenix, Domain V of *m*-calpain shows the potential to form an oblique-orientated alpha-

helix, which may modulate the enzyme's activity via interactions with anionic lipid, Eur. J. Biochem. 269 (2002) 5414–5422.
[8] R.C. Keller, J.A. Killian, B. de Kruijff, Anionic phospholipids are essential for alpha-helix formation of the signal peptide of prePhoE upon interaction with phospholipid vesicles, Biochemistry 31 (1992) 1672–1677.
[9] E. Goormaghtigh, V. Cabiaux, J.M. Ruysschaert, Secondary structure and dosage of soluble and membrane proteins by attenuated total reflection Fourier-transform infrared spectroscopy on hydrated films, Eur. J. Biochem. 193 (1990) 409–420.
[10] J.P. Bradshaw, S.M. Davies, T. Hauβ, Interaction of substance P with phospholipid bilayers: a neutron diffraction study, Biophys. J. 75 (1998) 889–895.
[11] S.R. Dennison, S. Dante, T. Hauβ, K. Brandenburg, F. Harris, D.A. Phoenix, Investigations into the membrane interactions of *m*-calpain domain V using neutron diffraction, Biophys. J. 88 (2005) 3008–3017.
[12] R. Strom, F. Podo, C. Crifo, G. Zaccai, Structural aspects of the binding of melittin to phospholipid bilayers, as a model for protein–lipid interactions in membranes, Adv. Exp. Med. Biol. 148 (1982) 195–207.
[13] S.J. Cooper, D. Brockwell, J. Raftery, D. Attwood, J. Barber, J.R. Helliwell, The X-ray crystal structures of perdeuteriated and protiated enzymes elongation factor Tu are very similar, Chem. Commun. 10 (1998) 1063–1064.