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Published paper
Nakazawa, Y., Suzuki, Y., Williamson, M.P., Saito, H. and Asakura, T. (2009) *The interaction of amyloid A beta(1-40) with lipid bilayers and ganglioside as studied by P-31 solid-state NMR.* Chemistry and Physics of Lipids, 158 (1). pp. 54-60.

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The interaction of amyloid Aβ(1-40) with lipid bilayers and ganglioside as studied by ^{31}P solid-state NMR

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Abstract

Amyloid β-peptide (Aβ) is a major component of plaques in Alzheimer's disease, and formation of senile plaques has been suggested to originate from regions of neuronal membrane rich in gangliosides. We analyzed the mode of interaction of Aβ with lipid bilayers by multinuclear NMR using ^{31}P nuclei. We found that Aβ (1-40) strongly perturbed the bilayer structure of dimyristoylphosphatidylcholine (DMPC), to form a non-lamellar phase (most likely micellar). The ganglioside GM1 potentiated the effect of Aβ (1-40), as viewed from ^{31}P NMR. The difference of the isotropic peak intensity between DMPC/Aβ and DMPC/GM1/Aβ suggests a specific interaction between Aβ and GM1. We show that in the DMPC/GM1/Aβ system there are three lipid phases, namely a lamellar phase, a hexagonal phase and non-oriented lipids. The latter two phases are induced by the presence of the Aβ peptide, and facilitated by GM1.

Key Words: β-amyloid, Alzheimer's disease, NMR, GM1- ganglioside, lipid bilayer

Abbreviations: DMPC: dimyristoylphosphatidylcholine, Aβ: Amyloid β-peptide, AD: Alzheimer's disease, NMR: Nuclear Magnetic Resonance

1. Introduction

Alzheimer's disease (AD) is a devastating neurodegenerative disease affecting up to 15 million individuals worldwide. The brains of Alzheimer's disease patients are characterized by fibrillar amyloid plaques that are associated with progressive deposits (Iversen et al., 1995). The principal component of amyloid deposits is a 39-42 amino acid residue peptide, Aβ (Glenner et al., 1984; Masters et al., 1985), a product of proteolytic processing of a much larger amyloid precursor protein encoded by a gene on chromosome 21 (Kang et al., 1987). Several spectroscopic investigations have clarified the structure of the fibrils, which form a 'cross-β sheet' structure (Kirschner et al., 1986; Petkova et al., 2002; Petkova et al., 2006). Moreover, a link between Aβ and AD neuropathological lesions is demonstrated by the toxicity of aggregated Aβ to neuronal cells in culture (Lambert et al., 1998) and in aged brain (Geula et al., 1998). However, the molecular mechanisms of the neurotoxic action of Aβ remain unknown. A growing number of observations indicate that Aβ may alter the physicochemical properties of neuronal membranes, including membrane fluidity (Muller et al., 1995) and permeability to ions and nonelectrolytes (Arispe et al., 1993; de Planque et al., 2007; Lau et al., 2006). These findings strongly suggest that at least some of the pathophysiological effects of Aβ may be mediated by Aβ-membrane interactions. Indeed, a number of studies have shown that Aβ is able to perturb lipid bilayers (Arispe et al., 1993). Interest in studies of the interaction between Aβ and constituents of brain membranes has been further stimulated by the identification of an Aβ-GM1 ganglioside-bound form in AD brain (Chi et al., 2007; Kakio et al., 2002; Yanagisawa et al., 1995).

Gangliosides (Brocca et al., 1997; Kasahara et al., 2001) are sialic acid-containing glycosphingolipids and consist of two main components: a hydrophobic ceramide unit, which anchors the ganglioside to the plasma membrane, and a hydrophilic oligosaccharide chain, to which one or more sialic acid groups are attached. Gangliosides are abundant components of neuronal membranes and are involved in important neurobiological events such as neurodifferentiation, synaptogenesis, and synaptic transmission (Nagai, 1995). Aβ permeabilizes ganglioside-containing membranes and thus disturbs ion homeostasis (McLaurin et al., 1996; McLaurin et al., 1998a). Different research groups have reported extensive studies of Aβ-ganglioside interactions investigated by different methodologies (Choo-Smith et al., 1997; Kakio et al., 2003; Kakio et al., 2002; McLaurin et al., 1996; McLaurin et al., 1998b; Yanagisawa et al., 1998), but no conclusive results have been obtained. GM1 ganglioside-mediated accumulation of Aβ protein was proposed together with an effect of cholesterol (Kakio et al., 2003). Recently, we have demonstrated using solution state NMR on ^{15}N-labelled Aβ (1–40) and Aβ (1–42) that the interaction with ganglioside GM1 micelles is localized to the N-terminal region of the peptide, particularly residues His^{13} to Leu^{17}, which become more helical when bound, leaving the C-terminal region unstructured (Williamson et al., 2006). Further, insertion of Aβ peptide into lipid bilayer to cause micellar structure was proposed on the basis of a monolayer study (Bokvist et al., 2004; Brender et al., 2007; Chi et al., 2007). These studies imply that the main driving force for interaction is not coulombic, in accordance with the low net charge on the Aβ peptide.

In this paper, we have recorded ^{1}H decoupled ^{31}P solid-state NMR spectra of the neutral lipid dimyristoylphosphatidylcholine (DMPC) and DMPC/GM1 ganglioside in a

mechanically oriented system in the presence of Aβ(1-40) in order to clarify the behavior of Aβ in the presence of GM1 ganglioside. DMPC is a commonly used model lipid for neutral membranes. 2-Oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) is also frequently used, and is very similar except that the phase transition temperature of the former is higher than that of the latter.

We also recorded ^{13}C NMR spectra of ^{13}C-labeled Aβ (1–40) incorporated into liquid crystalline DMPC bilayer, to gain insight into conformational features of this peptide.

2. Results

First, we recorded the orientational behavior of DMPC lipid bilayers as a function of Aβ peptide using angular-dependent solid-state ^{31}P NMR experiments. The ^{31}P chemical shift tensors of phospholipids are represented graphically by an ellipsoid. The three main axes of the ellipsoid represent the main tensor elements $\delta_{11} \geq \delta_{22} \geq \delta_{33}$. The chemical shift that is measured at a certain orientation of the molecule corresponds to the length of a vector parallel to the magnetic field direction, and the dependence of shift on orientation (ie the chemical shift anisotropy, CSA, given by $\Delta = \delta_{//} - \delta_{\perp}$) is highly sensitive to electrostatic interactions and dynamics near the lipid surface (Ramamoorthy et al., 2006b; Santos et al., 2004). Thus the chemical shifts indicate the tilt of the phospholipids with respect to the magnetic field and/or the mobility of the lipid bilayers. In the case of a completely oriented lipid bilayer, ^{31}P NMR peaks obtained with aligned samples are narrow and there is only a small powder resonance under the aligned signal.

In a first set of experiments, we observed ^{31}P solid-state NMR spectra of powder and glass-plate aligned DMPC samples as shown in Fig. 1. These results indicate that the

DMPC lipid bilayer was completely oriented on the glass plates. The ^{31}P chemical shift values of DMPC move to high field on changing the alignment axis to the magnetic field B_0 from 0° to 90°. Fig. 1(d) and (e) show angular-dependent ^{31}P NMR spectra of a DMPC/GM1 ganglioside system. The behavior of this system is similar to the result in a DMPC lipid bilayer alone, implying that the addition of GM1 ganglioside does not significantly perturb the structure of the DMPC bilayer.

Fig. 2 illustrates the ^{31}P NMR spectra of a mechanically oriented DMPC bilayer as a function of the angle (θ) between the applied magnetic field and bilayer normal. The left, central and right traces correspond to the ^{31}P NMR spectra of DMPC/Aβ(1-40) molar ratios of 30:1, 20:1 and 10:1, respectively. Four peaks can be distinguished whose positions vary with the angle, while an isotropic peak remains almost at the same position independent of orientation angle. In particular, the outermost pairs among the four peaks exhibit an orientation-dependent displacement of peaks. In addition, the separation of the outer and inner peaks varies with orientation, corresponding to the CSAs of the phosphodiester moieties, as summarized in Table 1. The anisotropy values of the outer and inner pairs decrease from 43.4 and 30.9 ppm, respectively, at a low proportion of Aβ (1-40) to 38.0 and 22.9 ppm at a higher ratio (Table 1), suggesting that the alignment of the DMPC bilayers is reduced in the presence of Aβ peptide. The most anisotropic component (ie, most ordered) is attributed to a lamellar bilayer, in agreement with the observed CSA values. The component with reduced anisotropy is assigned to a different and therefore non-lamellar structure, most likely the hexagonal H_{II} form (Cullis et al., 1979) based on its orientation dependence and the fact that the observed CSA value is approximately half the value measured for the bilayer structure (Smith et al., 1984;

Thayer et al., 1981). The same structure was recently proposed to be induced by antimicrobial peptides (Ramamoorthy et al., 2006a). The isotropic peak is assigned to a disordered component showing rapid isotropic averaging. The most likely origin of this peak is from micellar components. We note that the chemical shift of the 'isotropic' signal varies with alignment. This is not inconsistent with micellar structure, because micelle chemical shifts will still show orientation dependence because of differences in bulk magnetic susceptibility.

Most significantly, the anisotropies of the ordered components are reduced with increased proportion of Aβ (1-40) (Fig 2, (a) to (b) to (c)). This observation is consistent with insertion of Aβ (1-40) into the membrane, because the observed anisotropies are averages of the large anisotropy arising from free lipids and the smaller anisotropy arising from lipids in direct contact with the peptide.

Next, we measured the ^{31}P angular-dependent NMR spectra of a range of DMPC/ GM1 ganglioside systems in the presence of Aβ (1-40), as shown in Fig. 3. No orientational distribution for DMPC molecules was observed at a DMPC/GM1 ganglioside ratio of 5:1 (data not shown). Therefore, we decided to perform an experiment using a ratio of DMPC to GM1 ganglioside of 10:1. In this case, it is notable that the relative intensity of the isotropic peak was substantially increased. With increasing concentration of Aβ (1-40) peptide, the intensity of the isotropic signal gradually increased further. This result indicates that the lipid bilayer was gradually disrupted with increasing Aβ (1-40) peptide concentration, again highly suggestive of insertion of Aβ into the membrane. The fraction of the lamellar structure of DMPC was decreased with respect to that of DMPC without Aβ (1-40) peptide. A similar decrease in

the proportion of oriented phospholipids on glass plates has also been reported in the presence of other membrane-bound peptides (Moll et al., 1990; Nicholson et al., 1987). As expected, the chemical shifts of the two oriented components are dependent on their angle relative to the magnetic field. In the case of the DMPC/GM1/Aβ system, the oriented components make up only about 60% of the observed DMPC signal. It appears that the fraction of isotropic component in DMPC/ Aβ (1-40) peptide samples depends on the amount of Aβ (1-40) peptide present. However, the proportion of the isotropic lipids is almost independent of lipid/peptide ratio when GM1 is present, as shown in Fig. 4.

We recorded ^{13}C NMR spectra of ^{13}C labeled Aβ (1-40) incorporated into liquid crystalline DMPC bilayers by DD- and CP-MAS methods, as shown in Figs 5A and 5B, respectively, in order to gain insight into conformational features of the peptide. In DD-MAS, mobile regions of the sample are more intense, whereas in CP-MAS, mobile regions are much less intense. ^{13}C NMR signals could not be detected for [2-^{13}C]G^{29} because of low S/N ratios. Local peptide conformations can be readily characterized by their ^{13}C chemical shifts with reference to the corresponding conformation-dependent chemical shifts (Saito, 1986), permitting assignment of Val^{18} C=O signals from α-helical and β-sheet conformations. The intensities of these signals differ substantially between the DD-MAS and CP-MAS NMR methods. We conclude that the α-helical peptide conformation is significantly flexible and is approximately equally partitioned between components penetrated into lipid bilayer and in liquid phase, whereas the β-sheet peptide conformation is rigid and is presumably deposited and stacked at the bilayer surface. Indeed, the latter peak alone can be detected by ^{13}C CP-MAS NMR (Fig. 5B).

3. Discussion

We have shown in this paper that the bilayer structure is strongly perturbed by the presence of Aβ (1-40) peptide and also GM1, leading to a fraction of anisotropic but non-lamellar structure suggested to be the hexagonal H_{II} structure. At the same time, the proportion of an isotropic lipid phase such as micelle or single vesicle increases, as determined from ^{31}P NMR spectra. Such perturbation to the bilayer was significantly more prominent when GM1 was present. The assignment of the non-lamellar phases to H_{II} and micellar structures is based partly on the observation that lipid polymorphic structure is strongly related to the shape of lipids: a cylindrical lipid such as phosphatidylcholine favors bilayer structure, while cone-like lipids such as (unsaturated) phosphatidylethanolamine, cardiolipin and phosphatidic acid-Ca^{2+} prefer to take a hexagonal H_{II} form (Cullis et al., 1979). Further, an inverted cone such as GM1 favors a micellar structure.

Non-lamellar lipid structures, such as that revealed here by ^{31}P NMR, have been reported for a number of biological membranes and reconstituted P-450 systems (De Kruijff et al., 1978; Stier et al., 1978). Several peptides including gramicidin, alamethicin, α-helical peptide and antimicrobial peptide also were shown to perturb bilayer structure to form non-lamellar hexagonal H_{II} lipids by peptide-induced changes in lipid phases (Cornell et al., 1988; Gasset et al., 1988; Keller et al., 1996; Killian et al., 1985), in addition to the effect of lipid molecules themselves as mentioned above. The hydrophobicity and conformational flexibility of transmembrane peptides in lipid bilayers can affect their propensity to induce the formation of inverted non-lamellar phases by mechanisms not primarily dependent on lipid-peptide hydrophobic mismatch (Liu et al.,

2001), although we note that a theoretical interpretation of a mechanism of the lamellar-to-inverted hexagonal phase transition was also proposed, in relation to the membrane fusion process (Siegel, 1986; Siegel et al., 1997). For this reason, it is likely that the effect of Aβ (1-40) arises from interaction with the lipid bilayer, following an initial penetration into the bilayer. The effect of ganglioside, however, on modulation of lamellar-inverted micelle (H_{II}) phase transition has been examined in relation to biomembranes and shown to vary depending upon the relative concentration of ganglioside to lipids (Siegel, 1986).

As pointed out already, this possibility was proved by the conformational study of the peptide by ^{13}C NMR spectra as shown in Fig. 5, which demonstrates that not all the Aβ (1-40) peptides necessarily interact with lipid molecules, because there remains a fraction of the Aβ population deposited upon the bilayer surface as a rigid β-sheet structure, although the relative proportion may be varied by several parameters such as peptide-lipid ratio, degree of hydration, temperature, etc.

The results described here show that Aβ (1-40) is capable of inserting into lipid membranes, and eventually disrupting the membrane structure, which is enhanced by the presence of GM1. It has previously been shown that Aβ (1-42) causes leaky peptide-lipid supramolecular structures, as a result of its membrane disrupting effect (Ambroggio et al., 2005). Indeed, the aggregation propensity of Aβ (1-42) was shown to be higher than that of Aβ (1-40) (Bateman et al., 2007). Consequently, it was demonstrated that the former peptide disrupts the membrane bilayer. Nevertheless, it is emphasized that the very long timescale for development of Alzheimer's disease supports the argument that any interaction that is thermodynamically feasible will eventually occur, and therefore that

insertion of Aβ into the membrane as an α-helical form and subsequent membrane disruption may be physiologically relevant. By contrast, the β-sheet peptides on the membrane surface are rigid (as seen from the ^{13}C data) and are therefore probably not interacting strongly with the lipid, and thus not involved in promotion of either micellar nor isotropic phase formation.

4. Materials and Methods

4.1 Materials

DMPC and GM1 ganglioside were purchased from Sigma (St. Louis, MO, USA). Unlabeled and ^{13}C labeled Aβ(1-40) (DAEFRHDSGY^{10}EVHHQKLVFF^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40} and DAEFRHDSGYEVHHQKL[1-^{13}C]V^{18}FF[3-^{13}C]A^{21}EDVGSNK[2-^{13}C]G^{29}AIIGLMVGGVV) were synthesized in a stepwise fashion on Fmoc-Val-PEG-PS resin (PE Biosystems) by a Pioneer^{TM} peptide synthesizer (PE Biosystems) using Fmoc amino-acids, including ^{13}C labeled ones. After synthesis, the peptides were cleaved from the resin by treatment with a mixture of TFA, phenol, triisopropylsilane and water (88:5:2:5 vol%) for 2 hours at room temperature. The precipitated peptide was washed repeatedly with cold diethylether. Then, the crude peptide was purified by preparative liquid chromatography. Peptides were > 95% pure by chromatography.

For the preparation of oriented samples, various ratios of DMPC/Aβ mixtures were prepared; for DMPC/Aβ (10:1), 7.0mg (8.6 μmol) of DMPC was dissolved in 100 μl of methanol/chloroform (1:1) solution and 3.7mg of Aβ peptide (0.85 μmol) was dissolved in 300 μl of benzene. Then, these solutions were mixed and spread onto 0.1

mm thick glass plates (5×9 mm), followed by complete drying under vacuum for 24 h. The dry plates thus prepared were stacked in a 10 ϕ NMR tube (20 mm length), together with ultra-pure water in order to achieve 60% (w/w) hydration (6.4 μl of water in Aβ peptide/DMPC(1:10)). Then, the sample tubes were sealed with a Teflon cap and epoxy resin, and incubated at 45 °C for 4 days. The DMPC/GM1/Aβ peptide systems were prepared by a method similar to that described above. The molar ratios of these samples were achieved to satisfy the following conditions:

DMPC:Aβ=10:1, 20:1, 30:1 (molar ratio),

DMPC:GM1:Aβ=10:1:0.5, 10:1:1, 10:1:1.5 (molar ratio)

Multilamellar vesicles were prepared by dissolving DMPC in methanol/chloroform (1:1) and Aβ peptide in benzene solution as described previously (peptide: lipid= 1:10 molar ratio). The solvents in the DMPC /Aβ peptide samples were removed by nitrogen gas, followed by pumping under vacuum until the lipid film was completely dry. The dried lipid thus obtained was suspended in 65.6 μl of ultra-pure water in order to achieve 80%(w/w) hydration. and homogenized by six cycles of successive freezing, thawing and vortexing for 5 min each and freezing. Then, the sample was incubated at 35 ºC for 24 h.

4.2 NMR measurements

High power proton-decoupled ^{31}P NMR spectra at ambient temperature were performed on a Chemagnetics CMX 400 spectrometer operating at a resonance frequency of 397.79 MHz for ^{1}H and 161.03 MHz for ^{31}P. A static double-resonance probe equipped with a goniometer was used. ^{31}P NMR spectra were recorded for the oriented

samples. For the ^{31}P NMR experiments, typical NMR parameters were 7~8μs (90°) pulse length, 3 s recycle delay and the number of transients ranged from 1000 to 2000. The ^{31}P chemical shifts in ppm were referenced to H_3PO_4. A Gaussian line broadening of 200 Hz was applied to all static ^{31}P NMR spectra.

High resolution ^{13}C NMR spectra of unoriented ^{13}C labeled Aβ (1-40) incorporated into fully hydrated DMPC bilayer of liquid crystalline phase were recorded on a Chemagnetics CMX-400 NMR spectrometer, using ^{13}C CP/MAS NMR and dipolar decoupled magic angle spinning (DD/MAS) with a single pulse excitation method, at 38°C. The π/2 pulse for carbon and proton nuclei was 5 μs and a ^{1}H decoupling frequency of 60 kHz was used. Free induction decays were usually acquired with 512 data points, using a 3 s recycle delay and 2 ms contact time. The number of transients for the ^{13}C CP/MAS and ^{13}C DD/MAS spectra are 16,000 and 60,000, respectively. ^{13}C chemical shifts were referred to adamantane [(28.8ppm from tetramethylsilane (TMS)] and then expressed as relative shifts from the value of TMS. A Gaussian line broadening of 40 Hz was applied to CP/MAS and DD/MAS NMR spectra.

Acknowledgments.

This work has been supported by a Grant-in-Aid for Creative Scientific Research of the Japan Society for the Promotion of Science (JSPS) from 2004 to 2007. We are grateful for their support.

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Table 1 Chemical shift difference between the parallel and perpendicular components of the ^{31}P shift of DMPC lipid bilayer in the presence of Aβ (1-40).

moler ratio of DMPC/Aβ		Chemical shift difference	
		Outer peak	Inner peak
10/1	Chemical shift (ppm)	38.0	22.5
	Fraction		
20/1	Chemical shift	41.7	27.0
	Fraction		
30/1	Chemical shift	43.4	30.9
	Fraction		

Figure legends

Fig. 1 1D ^{31}P ^{1}H-decoupled solid-state NMR spectra of non-oriented and oriented DMPC samples at 35°C. θ is the angle between the bilayer normal and B_0 (see insert). (a) non-oriented DMPC sample. The dotted line is the spectral simulation, which gives the following average chemical shift anisotropy (CSA) principal values for ^{31}P of DMPC: σ_{11} = -16 ppm, σ_{22} = -16 ppm, σ_{33} = 31 ppm and σ_{iso} = -1 ppm. (b) Oriented DMPC bilayer sample with the order axis parallel to the magnetic field. (c) Oriented DMPC bilayer sample with the order axis perpendicular to the magnetic field. (d) and (e) Oriented bilayers of DMPC:GM1-ganglioside in molar ratio 10:1, with the order axis respectively parallel and perpendicular to the magnetic field.

Fig. 2 ^{31}P solid state NMR spectra of macroscopically oriented DMPC samples incorporating varying ratios of Aβ (1-40) at 35ºC as a function of the angle between the bilayer and the magnetic field. DMPC/Aβ (1-40) molar ratios were (a) 30:1, (b) 20:1, (c)10:1 respectively. θ is the angle between the bilayer normal and B_0. The dashed lines indicate the positions of the outer (long dashes) and inner (short dashes) lines at 0 and 90°: from their intensities these are identified as the parallel and perpendicular components respectively. The asterisks identify the isotropic component.

Fig. 3 ^{31}P solid state NMR spectra of macroscopically oriented DMPC/GM1 samples incorporating varying ratios of Aβ (1-40) at 35ºC as a function of the angle between the bilayer and the magnetic field. DMPC/GM1/Aβ (1-40) molar ratios were (a) 10:1:0.5 (b) 10:1:1 (c) 10:1:1.5 respectively. θ is the angle between the bilayer normal and B_0. The asterisks identify the isotropic component.

Fig. 4 Changes of isotropic signal fraction of DMPC/Aβ and DMPC/GM1/Aβ as a function of lipid/Aβ ratio. Triangles are DMPC/Aβ and squares are DMPC/GM1/Aβ. The error in the isotropic fraction is approximately 5%.

Fig. 5 ^{13}C DD-MAS(a) and CP-MAS NMR(b) spectra of ^{13}C-labeled Aβ (1-40) [DAEFRHDSGYEVHHQKL[1-^{13}C]V^{18}FF[3-^{13}C]A^{21}EDVGSNK[2-^{13}C]G^{29}AIIGLMV GGVV] incorporated into liquid crystalline DMPC bilayer. ^{13}C NMR signals of Gly^{29} Cα were of too low signal-to-noise ratio to be useful. Comparison with spectra of pure lipid shows that the signal at approximately 171.3 ppm is from the lipid carbonyl groups.

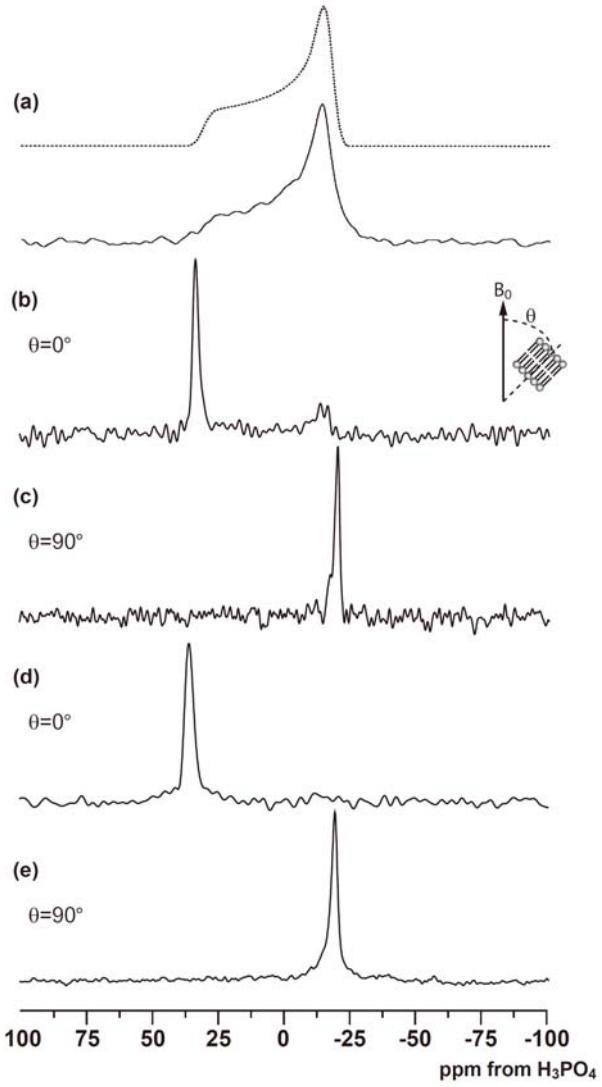

Fig. 1

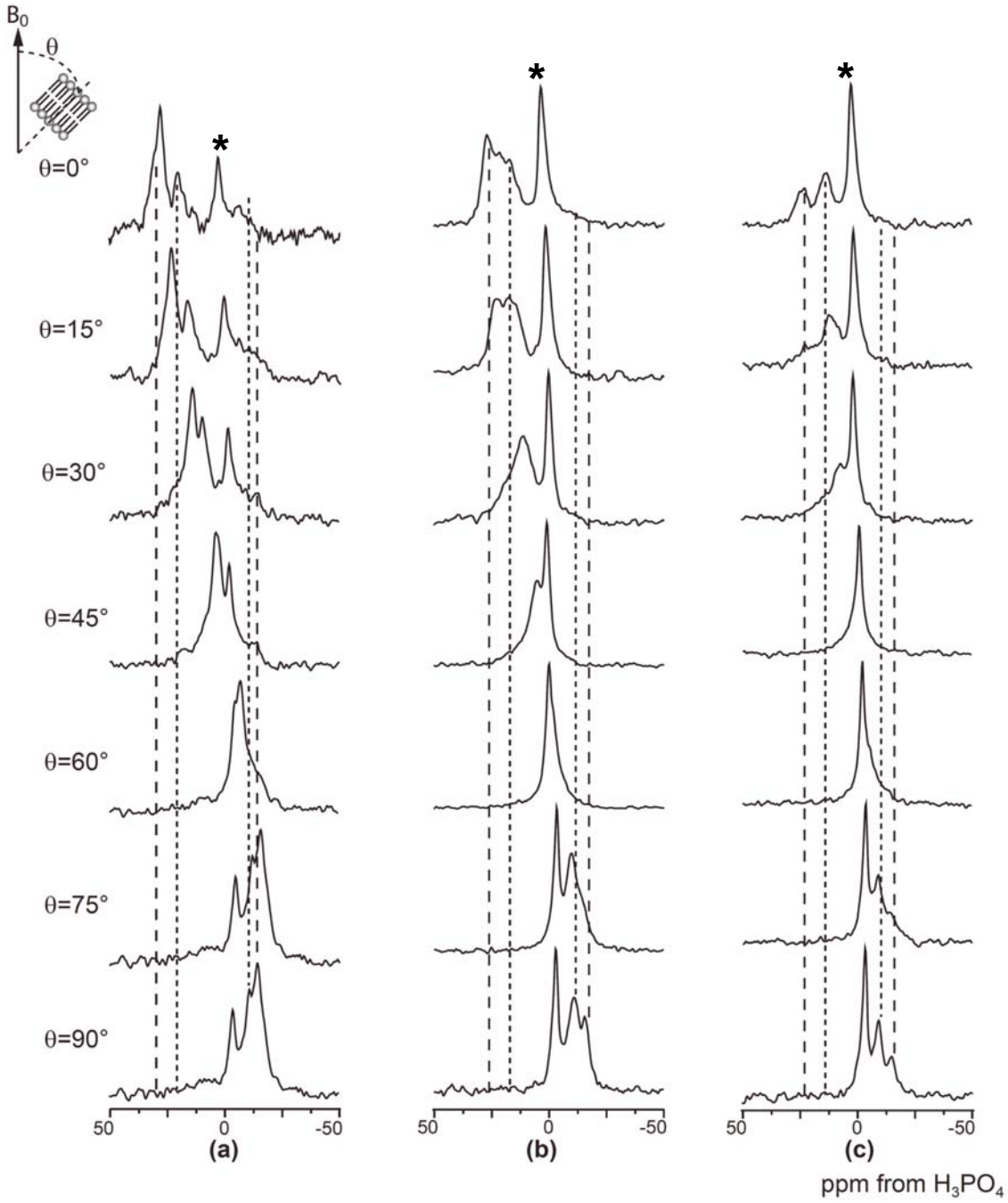

Fig. 2

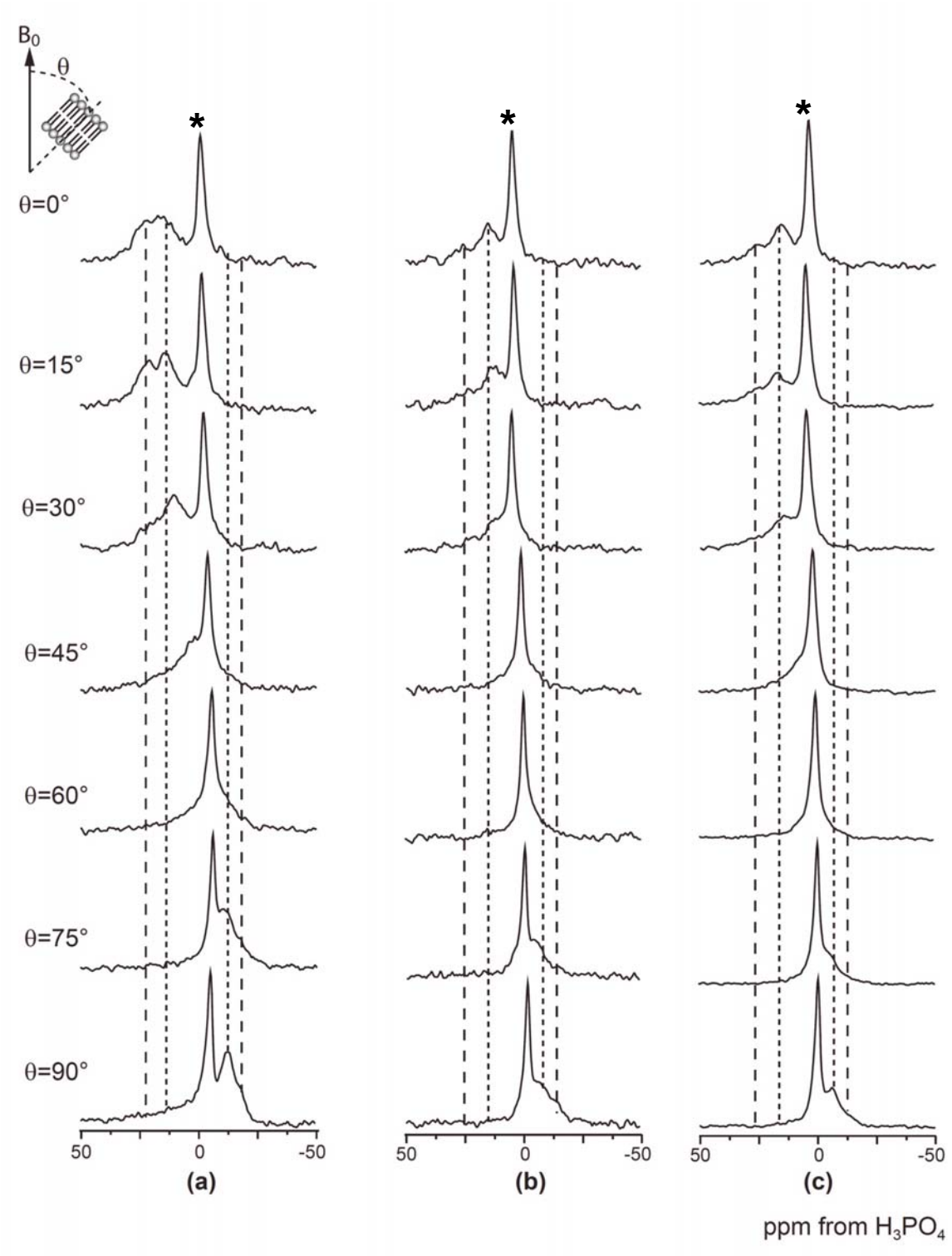

Fig. 3

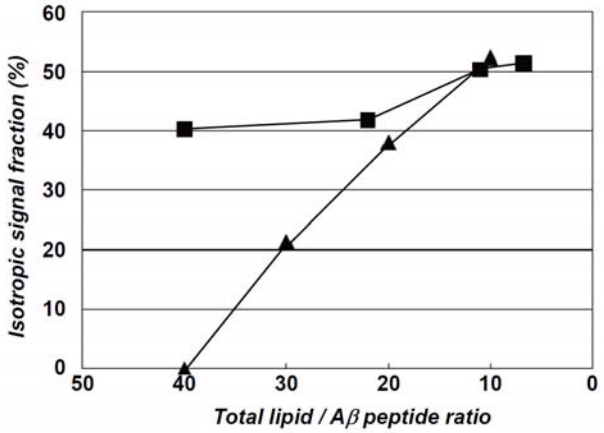
60
50
40
30
20
10
0
Isotropic signal fraction (%)
50
40
30
20
10
0
Total lipid / Aβ peptide ratio

Fig. 4

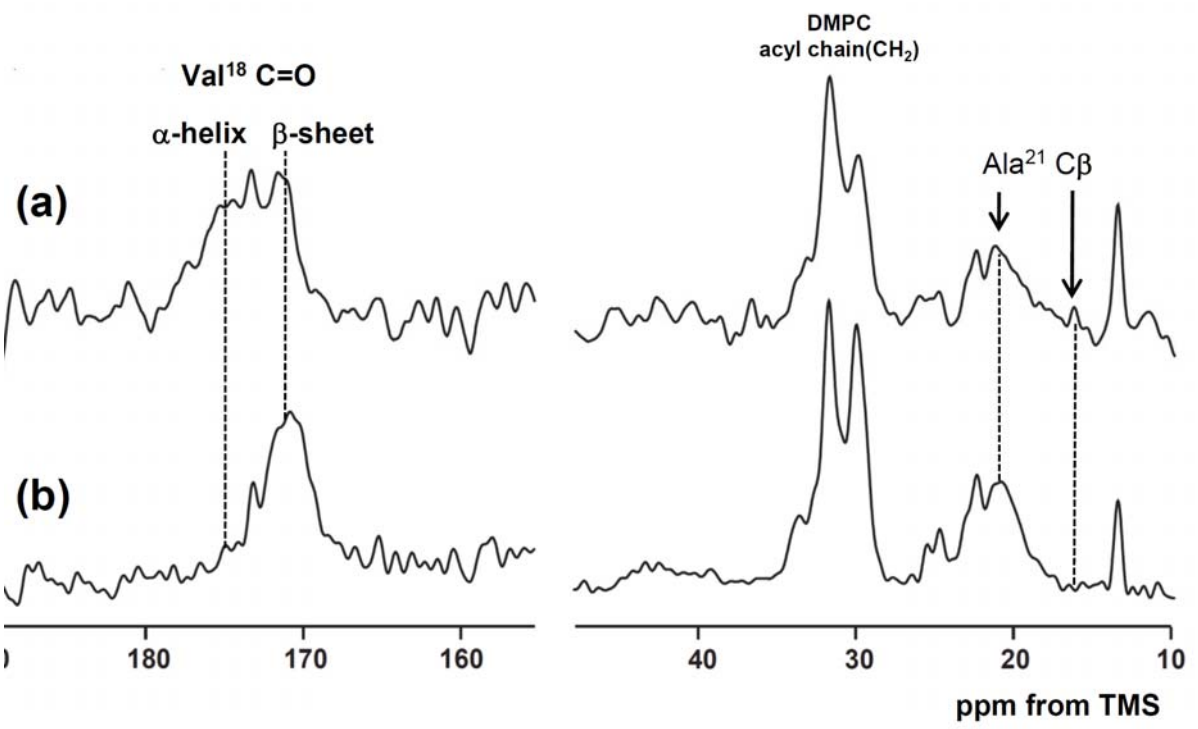
Val18 C=O
α-helix
β-sheet
(a)
(b)
180
170
160
DMPC
acyl chain(CH2)
Ala21 Cβ
40
30
20
10
ppm from TMS

Fig. 5