
Fusogenic Alzheimer's peptide fragment A β (29–42) in interaction with lipid bilayers: Secondary structure, dynamics, and specific interaction with phosphatidyl ethanolamine polar heads as revealed by solid-state NMR

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Abstract

The interaction of the native Alzheimer's peptide C-terminal fragment A β (29–42), and two mutants (G33A and G37A) with neutral lipid bilayers made of POPC and POPE in a 9:1 molar ratio was investigated by solid-state NMR. This fragment and the lipid composition were selected because they represent the minimum requirement for the fusogenic activity of the Alzheimer's peptide. The chemical shifts of alanine methyl isotropic carbon were determined by MAS NMR, and they clearly demonstrated that the major form of the peptide equilibrated in membrane is not in a helical conformation. ²H NMR, performed with acyl chain deuterated POPC, demonstrated that there is no perturbation of the acyl chain's dynamics and of the lipid phase transition temperature. ²H NMR, performed with alanine methyl-deuterated peptide demonstrated that the peptide itself has a limited mobility below and above the lipid phase transition temperature (molecular order parameter equal to 0.94). MAS ³¹P NMR revealed a specific interaction with POPE polar head as seen by the enhancement of POPE phosphorus nuclei T₂ relaxation. All these results are in favor of a β -sheet oligomeric association of the peptide at the bilayer interface, preferentially recruiting phosphatidyl ethanolamine polar heads.

Keywords: Alzheimer; membrane fusion; POPE; ³¹P NMR relaxation times; lipid dynamics; ²H NMR

Alzheimer's disease (AD) is a neurodegenerative disease leading to a progressive loss of memory and a cognitive decline. It is affecting an increasing number of people in the world (~40 million in 2003) (Antzutkin 2004), and although it is one of the most studied pathologies, it is not yet fully understood at the molecular level. One of the AD features is extracellular amyloid deposits within patients' brains (Kumar-Singh et al. 2002). The major component of these

plaques is the amyloid β -peptide (A β). This peptide is generated through the proteolytic cleavage of the β -amyloid precursor protein (APP), a glycoprotein of 695–770 amino acids (Evin and Weidemann 2002). After hydrolysis of APP at its N-terminus by a β -secretase, the A β peptide is released by γ -secretase as a 39–43 residues peptide (Hardy and Selkoe 2002). Missense mutations within genes of APP and/or presenilins (PS1 and PS2) lead to an increased production and accumulation of A β (1–42), which is more hydrophobic and has a larger propensity to aggregate than the A β (1–40).

Several studies have demonstrated that A β (1–40) and A β (1–42) are neurotoxic (Pillot et al. 1999; Butterfield and

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Kanski 2002). This property can be linked to experimental data showing, on the one hand, the interactions of A β peptides with lipids and cholesterol (Terzi et al. 1997; Waschuk et al. 2001; Eckert et al. 2003) and, on the other hand, the destabilizing effects of membranes by amyloid peptides, such as the fusion of small unilamellar vesicles (Pillot et al. 1996), changes in membrane fluidity, and permeability (Xiaocui et al. 2002), lipids peroxidation (Butterfield and Kanski 2002), or formation of a calcium-permeable ion-channel (Lin et al. 2001).

β -Amyloid peptides have an extracellular hydrophilic N-terminus (residues 1–28) and a membrane-inserted C-terminus region (residues 29–40 or 29–42). Destabilizing and fusogenic properties are likely to be due to the carboxy terminus of A β (1–40) and A β (1–42) (Mingeot-Leclercq et al. 2002). Based on energetic considerations, Mingeot-Leclercq et al. have proposed that A β (29–42) is a tilted peptide: it induces membrane permeabilization and fusion, and it could penetrate membranes as an α -helix whose axis forms an angle of 70° with the bilayer plane (Mingeot-Leclercq et al. 2002). Several solid-state NMR experiments performed on A β peptides in regard to fibril formation revealed a β -sheet structure (Petkova et al. 2002; Tycko 2003). Fewer NMR experiments have been addressed to the A β (1–40) and A β (1–42) peptide structures in interaction with membranes (Terzi et al. 1997; Bokvist et al. 2004).

The present study aimed specifically at providing a better understanding on A β (29–42)–membrane interactions at equilibrium. Specific questions were addressed using a combination of solid-state NMR approaches: (1) What is the major stable secondary structure of this peptide in a membrane environment, (2) where is it located in the membrane and what are the stabilizing interactions, (3) what is the peptide's and lipid's dynamics in this complex, and (4) what are the lipid perturbations which could explain its activity of fusion peptide. These NMR techniques were: (1) determination of peptide's carbon chemical shifts as an indication of the secondary structures (Wishart and Sykes 1994; Saito et al. 2000), (2) ^2H NMR on labeled peptides and labeled phospholipids providing a direct measure of order parameters, i.e., magnitudes of motion of labeled C-D bonds (Davis 1983), (3) ^{31}P NMR isotropic chemical shift and chemical shift anisotropy determinations related to the lipid's head group orientation and dynamics (Seelig et al. 1977; Bokvist et al. 2004) and relaxation time analyses, which are more directly linked to head group dynamics (Dufourc et al. 1992).

The lipid environment which we selected was made of palmitoyl-oleoyl phosphatidylcholine (POPC) and palmitoyl-oleoyl phosphatidylethanolamine (POPE) (9:1 molar ratio). Indeed, it was observed previously that 10 mol % of POPE was necessary and sufficient to observe A β -induced fusion of liposomes (Mingeot-Leclercq et al. 2003).

Results and Discussion

The interaction between the N-terminally acetylated A β (29–42) peptide and zwitterionic membranes was characterized by using a complete set of NMR experiments with respect to the structure and dynamics of the peptide backbone and to the peptide location in the membrane bilayers.

In the first part, the study is focused on two $^{13}\text{C}_\beta\text{H}_3$ or C_βD_3 -Ala residues, which were introduced into two different A β (29–42) peptides at positions evenly distributed along the A β primary structure—i.e., Gly $_{33}$ \rightarrow Ala and Gly $_{37}$ \rightarrow Ala. By using the carbon ^{13}C -labeled peptides and high spinning frequency MAS NMR, isotropic chemical shifts were used to probe the local secondary structure of the A β peptide. The quadrupolar splittings of CD $_3$ segment of the deuterated peptides were used to establish the local backbone dynamics in the absence and in the presence of zwitterionic membrane.

In the second part, the effects of A β (29–42) on the lipid molecular structure and membrane supramolecular organization were monitored with static ^2H NMR and Magic Angle Spinning ^{31}P NMR.

^{13}C MAS NMR experiments: Structural implications of isotropic chemical shifts

Relations between chemical shifts and structure have been largely based on empirical rules, but the use of quantum chemical calculations is becoming increasingly important. Recently, significant advances in establishing the relationships between chemical shifts and protein backbone conformations have been made, driven by the large number of assignments now available, by empirical and semiempirical analysis of shifts in proteins of known structure, and by ab initio calculations on protein fragments (Saito et al. 2000; Sun et al. 2002). It is thus now well-recognized that ^{13}C chemical shifts of the backbone C_α and carbonyl carbons as well as side-chain C_β signals of any amino acid residues in polypeptides and proteins are significantly displaced depending upon their local peptide conformations, defined by the torsion angles Φ and Ψ . Therefore, the local conformation of a peptide chain can be conveniently determined from its ^{13}C chemical shift, with reference to accumulated data base. At least six kinds of different local conformations can be distinguished for the Ala residues as summarized in Saito et al. For instance, the resonance frequency 3- ^{13}C Ala residues is equal to 14.9 ppm for an α -helical local conformation while it is 18.9 ppm for a β -sheet secondary structure (Saito et al. 2000).

Chemical shifts and high spinning frequency MAS experiments have been used to obtain isotropic chemical shift of specifically ^{13}C labeled A β (29–42) peptides.

Figure 1 shows carbon MAS NMR spectra centered around the methyl region of A β (29–42) G33A($^{13}\text{C}_\beta$) di-

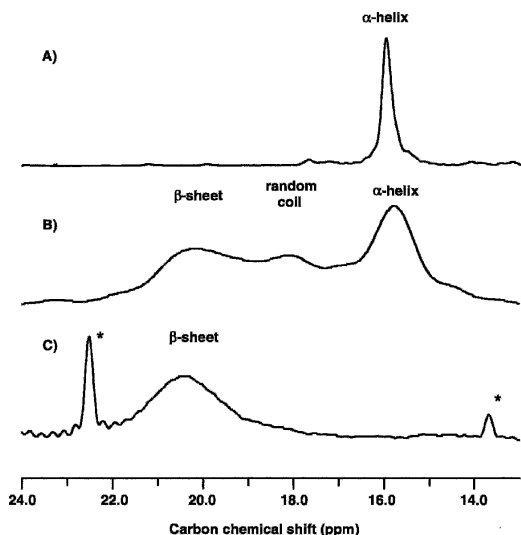


Figure 1. ^{13}C NMR spectra of A β (29–42), mutant G33 \rightarrow A33($^{13}\text{C}_\beta$), obtained by MAS NMR at 8.2-kHz spinning speed. (A) spectrum of an HFP solution at 303 K of the peptide, where it exists mainly in α -helical conformation; (B) spectrum of the same HFP solution, in the frozen state at 218 K; (C) spectrum of the peptide inserted into POPC/POPE 9:1 lipid bilayers at 303 K, where it is mainly in a β -sheet conformation. Peaks labeled with a * are due to natural abundance lipid resonances.

luted in HFP (1,1,1,3,3,3-hexafluoro-2-propanol) solvent at 303 K (Fig. 1A) and 218 K (Fig. 1B). The ^{13}C chemical shifts of the A β (29–42) peptides were calibrated with respect to the ^{13}CO signal of an external ^{13}C -labeled glycine carbonyl (176.03 ppm, according to Saito et al. 2000) in all three spectra. As discussed above, isotropic chemical shifts are sensitive markers for secondary structure, and a chemical shift typical of α -helices is observed in HFP solution at room temperature. Similarly, the spectrum of a lyophilized powder of pure peptide displays a unique resonance around 19 ppm, typical of a β -sheet conformation, as expected for this amyloid peptide fragment (data not shown). The α -carbon and CO carbon natural abundance resonances were also observed and had chemical shifts in accordance with the expected secondary structures (i.e., α for HFP solutions and β for lyophilized powders). When freezing the HFP solution under magic-angle spinning conditions in the spectrometer different conformations were observed, as indicated by the spectrum of Figure 1B with a broad resonance, and three resolved maxima, one at 16 ppm (α -helix), one at 20.5 ppm (β -sheet), and one at 18 ppm (random coil). This indicates that, upon freezing, part of the peptide retains its initial α -helix conformation, while part is shifted to other conformations, namely random coil and β -sheet. Figure 1B illustrates very clearly the capacity of MAS ^{13}C NMR to probe the local secondary structure around the labeled carbon atom. When 5 mol % of the peptide was incorporated into the POPC/POPE (9:1) bilayers at 303 K, the MAS spectrum clearly showed that it is mostly in a β -sheet conformation

(Fig. 1C). The same results were obtained at 2.5 mol % of peptide, and with the Gly \rightarrow Ala mutant at positions 33 and 37. The spectra were also shown to be stable for 2 h after liposome preparation (minimum time of equilibration before the acquisition of data) and for 1 week, something important to stress, since slow equilibria have been described for amyloid peptide–membrane systems (Bokvist et al. 2004). These results do not demonstrate that the secondary structure is a β -sheet all along the peptide; however, the proposal seems reasonable, since the two positions analyzed are at one-third and two-thirds of the sequence.

One question arises while observing the same spectra for the peptide in water in the presence and in the absence of lipid bilayers: Are we looking at a β -sheet conformation of the peptide inserted into the membranes, or merely at a membrane-expulsed structure. We thus carefully checked that, after density gradient centrifugation a single band containing both the peptide and the lipids was observed on the sucrose gradient. This was shown to be the case, both for the 2.5 mol % and the 5 mol % conditions, and after 1 day and 3 days of equilibration.

Therefore, both G \rightarrow A mutants of A β (29–42) do exist in a stable β -sheet conformation in POPC/POPE 9:1 lipid bilayers in the liquid crystalline phase.

Static ^2H NMR experiments: Dynamical behavior of A β (29–42) in the absence and the presence of lipids

Dynamical information is complementary to structural information. For instance, where a monomeric tilted peptide in a fluid membrane is expected to be highly disordered, i.e., to have a small molecular order parameter, a large aggregate such as a β -amyloid fiber should have a much higher degree of structural order. We observed a high degree of structural order as demonstrated by the narrow ^{13}C and ^{15}N NMR lines detected under MAS conditions. Indeed, sharp resonances in solid-state NMR were shown to be characteristic of well-structured, homogeneous peptides (Tycko 2004).

^2H static NMR is an ideal probe of the local dynamics of peptides in lipids (Davis 1983). Static ^2H NMR spectra have been recorded for the $\beta\text{-CD}_3$ of G₃₃ \rightarrow A₃₃ and G₃₇ \rightarrow A₃₇ peptides, in a crystalline state, and in the presence of membranes, both below (218 K) and above (298 K), the gel to liquid–crystalline phase transition of the lipid mixture (τ_m); the gel to liquid crystalline phase transition temperatures are equal to 271 K (POPC) and 298 K (POPE) for pure lipids and to 277 K for a 1:1 mixture of POPC and POPE (Epanand Bottega 1988). A 9:1 POPC:POPE mixture is thus expected to have a gel-to-liquid crystalline phase transition temperature close to 271 K, and was actually found to occur between 271 and 272 K by ^2H NMR.

The characteristic parameter of these spectra is the quadrupole splitting ($\Delta\nu_Q$) as defined by the separation of the most intense peaks. Alanine CD_3 $\Delta\nu_Q$ provides a direct

measure of the $C\alpha-C\beta$ segment's order parameter ($S_{C-C} = 1/2 \langle 3\cos^2\theta - 1 \rangle$), which is directly related to the magnitude of motion of this bond. The results were identical for the two labeled positions 33 and 37. In the crystalline state, and in lipids below τ_m , a mean quadrupolar splitting of 39.5 ± 0.4 kHz was found, which is close to the rigid limit value for such a CD_3 quadrupolar splitting ($S = 1$). These groups perform fast threefold hopping motions and symmetric rotations about the $C-C$ bond axis, which scales the rigid value of quadrupolar splitting by a factor of 1/3.

Above τ_m , the CD_3 quadrupolar splittings in lipid membranes was slightly smaller. A motion averaged quadrupolar splitting equal to 37.4 ± 0.4 kHz was obtained corresponding to a bond vector order parameter S of 0.94 ± 0.01 . A small but significant difference was observed between lyophilized powder (or peptide/lipid complex in the gel phase) and peptide/lipid complex in the fluid phase, confirming again that the peptide is in contact with the membrane since its dynamics is sensitive to the lipid phase transition. On the other hand, an order parameter of 0.94 for the peptide chain (at the level of Alanine 33 and 37) is very high and close to the rigid limit value, which is again in favor of a β -sheet aggregate rather than a monomer freely diffusing in the bilayer. It may be stressed that, within the signal to noise resolution limits of this experiment, one single quadrupolar splitting is observed which shows that the population of peptides is homogeneous in terms of conformation and dynamics.

We now turn to the questions of the peptide's localization within the bilayer, and of its capacity to induce lipid perturbations, using solid-state NMR on lipid's spins, 2H and ^{31}P .

2H and ^{31}P NMR analyses of the lipid dynamics

In a first set of experiments, POPC deuterated on its sn1-palmitoyl acyl chain (POPC- d_{31}) was used to investigate the effects of $A\beta$ (29–42) on chain order, mobility, and gel to liquid crystalline phase transition of the lipid bilayer. 2H NMR is widely used to probe the conformational disorder of liquid crystalline lipid bilayers (Davis 1983). For instance, addition of cholesterol or amphiphilic drugs that penetrate into the lipid membrane leads to dramatic changes of the quadrupolar splitting of acyl chains. In the presence of $A\beta$ (29–40), no change in the quadrupole splittings or in the shape of the spectra was observed (Fig. 2B). Furthermore, the phase transition of POPC/POPE (9:1) lipid mixture MLVs occurs between 271 K and 273 K, both in the presence and in the absence of $A\beta$ (29–42), irrespective of the peptide/lipid molar ratio, i.e., for 2.5 mol % and 5 mol %. The unaffected phase transition temperature and quadrupolar splittings of the mixture in the presence of $A\beta$ (29–42) peptide indicates that the peptide does not penetrate deeply

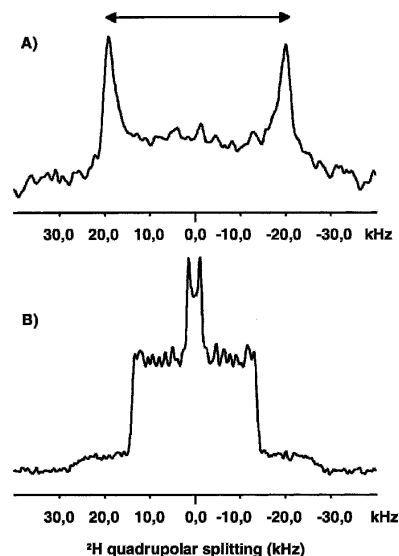


Figure 2. Static 2H NMR spectra of POPC/POPE (9:1) + 5 mol % $A\beta$ (29–42) $^{33}G \rightarrow ^{33}A$ mutant, at 303 K. (A) spectrum obtained using $Ala(C\beta D_3)$ deuterium-labeled peptide; (B) spectrum obtained using POPC- d_{31} perdeuterated on its palmitoyl chain.

into the hydrophobic core of the bilayer, but rather, binds superficially at the interface.

^{31}P NMR was then used to monitor changes in the phase behavior of the phospholipid bilayer upon interaction with $A\beta$ (29–42) (Ghosh 1988).

A typical static ^{31}P NMR spectrum from a POPC:POPE mixture (9:1 molar ratio) is shown in Fig. 3Ai). It is dominated by the chemical shift anisotropy of the phosphate from both PC and PE, partially averaged by fast axial rotation of lipid molecules. The spectrum is a superposition of two powder spectra originating from the phosphorus nuclei of POPC and POPE. The maximum chemical shielding anisotropy was 47 ppm. It was identical with and without $A\beta$ (29–42). On this spectrum, it was, however, not possible to distinguish between the CSAs contributions of PC and PE headgroups.

Mechanical rotation of the MLV suspensions at the magic angle (54.7°) partially averages the effective CSA and results in higher resolution spectra in which the phosphate resonances from all individual lipids are well resolved due to their different isotropic chemical shifts.

The NMR spectrum obtained for a pure POPC:POPE mixture at 2 kHz sample rotation is represented in Figure 3Aii). This spectrum is composed of a pair of central resonances flanked by a series of spinning sidebands separated by multiples of the spinning frequency. The narrow linewidths reflect long transverse relaxation time T_2 (18 msec) and high lipid mobility in the liquid crystalline bilayers at 293 K. The relevant isotropic ^{31}P NMR chemical shift difference between PC and PE without peptides is 0.63 ppm. At this spinning frequency, ^{31}P chemical shift anisotropy

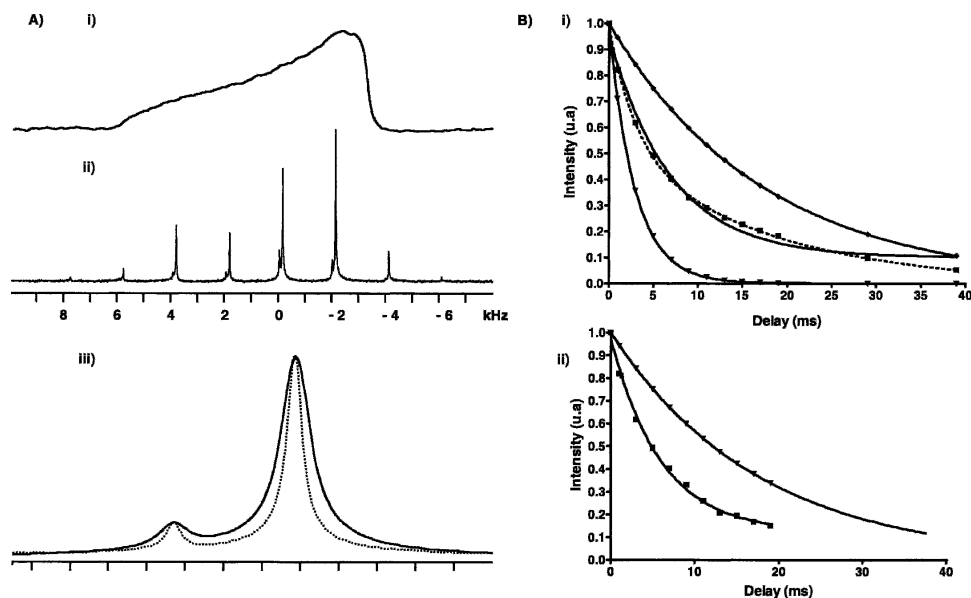


Figure 3. ^{31}P NMR spectra of $\text{A}\beta(29-42)$ in complex with POPC/POPE (9:1) lipid bilayers, at 293 K. (Ai) Static ^{31}P powder spectrum; (Aii) ^{31}P MAS spectrum at a spinning frequency of 2 kHz; (Aiii) expansion of the ^{31}P isotropic signal at a spinning frequency of 8 kHz, in the absence (dotted line) and in the presence (continuous line) of 5 mol % of $\text{A}\beta(29-42)$ $^{37}\text{G-A}$ mutant peptide (scale 1 ppm = 202 Hz per tic). (B) Variations of the signal intensity versus echo delay. The intensities have been normalized with respect to their value at 20 μsec echo delay. (Bi) PC resonance; the experimental data (squares) were fitted by a mono-exponential decay (solid line) and by a bi-exponential decay (dotted line). Note that the bi-exponential fitting is clearly required. Theoretical mono-exponential decays corresponding to each T_2 component are also shown below ($T_2 = 2.92$ msec, \bullet and above ($T_2 = 18$ msec, \blacklozenge) the experimental decay. (Bii) PE resonances; a single mono-exponential decay was sufficient to fit PE decay curves in the POPC:POPE 9:1 mixture, both in the absence (18 msec, \bullet) and in the presence (5.9 msec, \blacksquare) of peptide.

parameters of both lipid components are reflected in the intensities of the resulting spinning sidebands pattern. By using the standard Hertzfeld-Berger's method we found both CSA to be axially symmetric, as expected for fast axially diffusing lipids, with a span (i.e., $\sigma_{\parallel} - \sigma_{\perp}$) equal to 47 ± 0.3 ppm and 37.5 ± 0.6 ppm for PC and PE, respectively. At higher spinning frequencies, these sidebands disappear, while the central resonances remain unchanged in position. At this speed, a quantitative analysis can be carried out by integration of the individual resonance with an intensity ratio of 9:1 reflecting the chemical composition of the bilayer (Fig. 3Aiii, dotted line).

In the presence of peptide, the wide line ^{31}P spectra showed no deviation from the powder distribution (data not shown). This confirms that the lipid system remains in the liquid crystalline lamellar phase upon addition of $\text{A}\beta(29-42)$. In particular, there is no evidence of isotropic or H_{II} resonances, showing that the interaction with the peptide does not induce nonbilayer structures in these conditions.

Under MAS conditions, changes in the individual PC and PE lipid species within the bilayer caused by $\text{A}\beta(29-42)$ can be monitored simultaneously. The individual ^{31}P NMR resonances are not affected by the addition of peptide as observed by Bokvist et al. (2004) on $\text{A}\beta(1-40)$: the addition of peptide to PC neutral bilayer does not lead to any change in ^{31}P chemical shift associated with change in local

electrostatic environment. However, in our case, the interaction with $\text{A}\beta(29-42)$ leads to a significant and specific broadening of the PC and PE ^{31}P resonances as observed in Figure 3Aiii (continuous line).

This nonclassical behavior has been explored further by analyzing T_2 relaxation times of phosphorus nuclei of the two different polar heads. ^{31}P T_2 s of each resonance were extracted from the variation of the relative intensity versus echo time τ recorded with a classical spin echo experiment ($\pi/2-\tau-\pi-\tau-\text{Acq}$). For the PE ^{31}P resonance, the intensity decay could be fitted by a single exponential and the transverse relaxation time (5.9 msec) was clearly shorter than for PE in a pure POPC:POPE mixture (18 msec) as illustrated in Figure 3Bii. By contrast, a two-exponential decays model was required for PC phosphorus resonance. Figure 3Bi compares mono- and bi-exponential decays for the PC resonance and clearly shows that the mono-exponential fitting is not sufficient.

These results show that the entire population of PE is affected in the presence of $\text{A}\beta(29-42)$, whereas two PC populations can be distinguished, one characterized by a T_2 typical of pure lipids ($T_2 = 18$ msec), and the other having a much shorter T_2 ($T_2 = 2.9$ msec).

The same results were observed with 2.5 mol % of peptides, the respective T_2 s being 18 msec for unaffected POPC, 3 msec for POPC bound to $\text{A}\beta$, and 6.1 msec for

POPE. Therefore, in both cases (5 mol % and 2.5 mol %), the peptide is able to recruit the entire POPE population (respectively 2 and 4 POPE molecule per peptide) while leaving part of POPC molecules unaffected. The peptide interacting and noninteracting POPC molecules are on slow exchange at the ^{31}P NMR time scale (10^{-4} sec).

These results demonstrate a pronounced tendency of A β (29–42) to interact with PE head groups more than with PC head groups, in accordance with the necessity to add PE lipids to induce vesicle fusion.

Molecular modeling

The IMPALA procedure was used to check the influence of the peptide conformation on its insertion in a lipid bilayer (Lins et al. 2001). Briefly, this method simulates the interaction between a peptide and a simplified model membrane using a Monte Carlo minimization procedure coupled to an angular dynamics.

Figure 4 shows the most stable position in the model bilayer of A β (29–42) peptides mutants G33 \rightarrow A33 and G37 \rightarrow A37, in α -helical or β -sheet conformation. The A β (29–42) G33 \rightarrow A33 and G37 \rightarrow A37 mutants in a helical

conformation present an angle with the bilayer plane of 79.4° and 86.4° , respectively, whereas the wild-type peptide penetrates membranes with an angle of 66° . This could explain the partial loss of the fusogenic properties of the mutants peptides in agreement with the work of Martin et al. in 1994 (Martin et al. 1994). The A β (29–42) peptides in a β -sheet conformation are parallel to the lipid/water interface. This supports our NMR results that indicates that mutants peptides are adsorbed at the bilayer interface, and is in good agreement with the literature (Demeester et al. 2000; Bokvist et al. 2004).

Conclusions

In the present study, the steady-state interaction of the Alzheimer peptide C-terminal fragment (A β 29–42), and two mutants 33,37-(G \rightarrow A) with neutral lipid bilayers made of POPC and POPE in a 9-to-1 ratio was investigated by solid-state NMR. This peptide fragment and this lipid composition were selected because they are the minimum required for the fusogenic activity of the Alzheimer peptide. It has been postulated that the fusogenic activity could be due to the propensity of this peptide to be “tilted,” i.e., to insert in bilayers as an oblique α -helix, neither perpendicular nor parallel to the bilayer plane as many peptides do (Pillot et al. 1996; Mingeot-Leclercq et al. 2002). Isotropic carbon chemical shifts clearly demonstrate that the peptide equilibrated in the membrane is not helical. ^2H NMR demonstrates that there is no perturbation of the acyl chain dynamics and of the lipid phase transition temperature, and that the peptide itself has a limited mobility even in the fluid phase. Moreover, ^{31}P NMR reveals a specific interaction of the peptide with the POPE polar head as seen by the enhancement of the T_2 relaxation associated with a restricted mobility. All these results and molecular modeling support the conclusion that when the peptide has been inserted in the membrane, its steady-state structure is an oligomeric association of β -sheet peptides located at the bilayer interface, preferentially recruiting PE lipids. The selective recruitment of PE by A β aggregates further suggests the idea that the inaccessibility of this crucial lipid could be an aspect of the cellular disorders induced in the Alzheimer’s disease. It is in complete agreement with the observation that A β 40/42 binding is controlled by lipid head group interactions and leads to the production of interfacial packing defects (Waschuk et al. 2001).

Experimental demonstration of the structure and location of tilted peptides in membranes is a difficult task, although several evidences do exist in the literature (Bradshaw et al. 2000; Han et al. 2001). One limitation results from the reasonable hypothesis that the tilted state of the peptide fragment actually responsible for the fusogenic activity is transient and is an intermediate form toward a stable membrane-inserted conformation as it could be for the Alzhei-

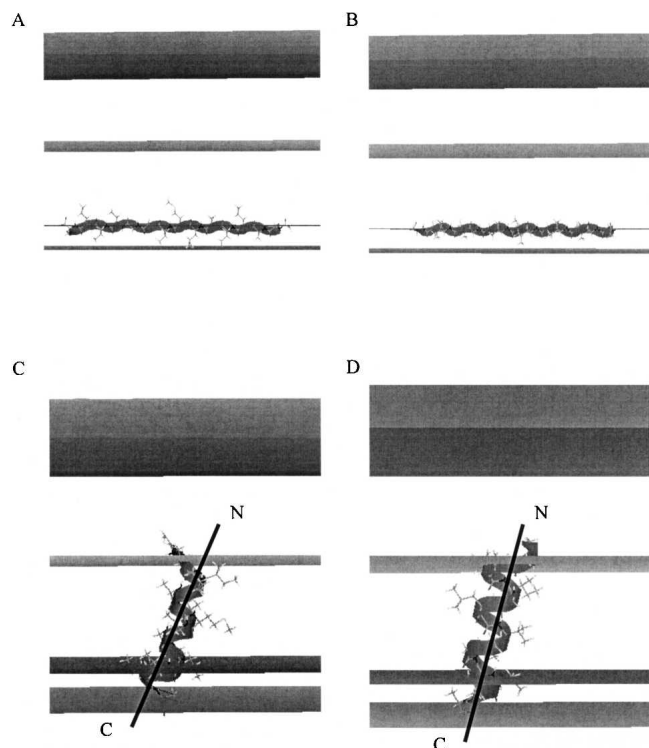


Figure 4. Best configurations of A β (29–42) peptides in ribbon representation after Monte Carlo simulation. Middle plane = bilayer center ($z = 0$); first plane = lipid acyl chain/polar head interface at 13.5 \AA from the center; second plane = lipid/water interface ($z = 18 \text{ \AA}$). (A,C) A β (29–42) G33 \rightarrow A33 peptides as a β -sheet and an α -helix, respectively. (B,D) A β (29–42) G37 \rightarrow A37 peptides as a β -sheet and an α -helix, respectively.

mer peptide. In such a case, most of the available methods such as solid-state NMR, neutron diffraction, or polarized ATR-IR (Martin et al. 2003) will detect the major membrane-inserted conformation after stabilization. Our results demonstrate firmly that after fusogenic events have occurred, the A β peptide is not a tilted peptide but merely a major β aggregated structure.

Our strategy, combining ^{13}C isotropic chemical shift for conformational analyses, ^2H NMR on labeled peptides, and on lipids for dynamical analyses and MAS ^{31}P NMR including relaxation time measurements for the detection of peptide-lipid-specific interactions provided a clear view on peptide's and lipid's structure and dynamics in POPC/POPE mixture. It could now be applied on the entire Alzheimer peptides A β (1–40) and A β (1–42) and in different experimental conditions: it has been shown that cholesterol is an important factor affecting the membrane insertion of A β (1–40), which drastically increases the helical content of A β (1–40) in DMPC, DPPC, and DPPC + sphingomyelin monolayers and bilayers (Ji et al. 2002). The role of gangliosides is increasingly stressed on Alzheimer fibrils formation and pathogenicity (Kakio et al. 2001; Tashima et al. 2004). In particular, it has been recently proposed that asialoGM1 lipids may stabilize the peptide in a monomeric form, thus preventing the formation of fibrils (Mandal and Pettegrew 2004). If lipid binding states, different from the occurrence of the β -sheet at the bilayer surface are found in some lipid mixtures relevant for the peptide's activity, other solid-state NMR approaches will be required to refine the peptide's structure and orientation, both using MAS and distance determinations (Tycko 2004) and using anisotropic constraints in oriented lipid bilayers (Mo et al. 2004).

Materials and methods

Materials

β -Amyloid peptides 29–42, A β (29–42), GAIIGLMVGGVVIA, and the two labeled mutants G33A and G37A were synthesized by Polypeptides Laboratories. The mutants were synthesized either in ^{13}C labeled form or in ^2H labeled form, both labels being introduced on the alanine methyl group, using labeled alanine during peptide synthesis. All peptides were subsequently N-terminally acetylated by treatment with acetic anhydride-pyridine overnight at room temperature. Their molecular weights were controlled by mass spectrometry.

1-Palmitoyl(d_{31})-2-Oleoyl-*sn*-glycero-3-phosphocholine (POPC- d_{31}) and 1-Palmitoyl(d_{31})-2-Oleoyl-*sn*-glycero-3-phosphatidylethanolamine (POPE) were purchased from Avanti Polar Lipids. Deuterium-depleted water was obtained from Aldrich. Salts and solvents were of analytical grade.

Proteo-liposomes preparation

Briefly, the peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) then dried under a stream of nitrogen to obtain a film.

HFP lipid solutions were added to the peptide film in a POPC/POPE/peptide molar ratio of 86:9:5 (5 mol % of peptide), or 87.25:10.25:2.5 (2.5 mol % of peptide), and the mixture was evaporated overnight under high vacuum. Subsequently, the dry peptide/lipid film was resuspended with an excess of deionized water under extensive vortexing to produce proteoliposomes. It was then lyophilized and rehydrated with deuterium-depleted water for NMR experiments. Peptide sample homogeneity and final peptide/lipids ratio were checked by sucrose density gradient centrifugation, by the modified Lowry's procedure and by phosphorus analysis, respectively.

Fusogenic activity of A β (29–42) and its ala mutants

Each experiment was carried out with Small Unilamellar Vesicles (SUVs) of POPC/POPE: 9/1. The vesicles were prepared in Tris buffer pH 7.4 (Tris 10 mM, NaCl 150 mM, EDTA 0.1 mM, NaN_3 1 mM). The final phospholipid concentration was checked by phosphorus assay, and liposomes were used within the day following their preparation. The fusion assays were performed as described earlier (Van Bambeke et al. 1995), using a mixture of labeled and unlabeled vesicles (final concentration in lipids: 5 μM), by recording the increase in fluorescence due to the dequenching of octadecylrhodamine B chloride (R18) upon dilution in nonlabeled liposome. Labeled and unlabeled liposomes were mixed at a ratio of 1:4. Peptides were added and fluorescence was immediately recorded at room temperature for 25 min, using an excitation wavelength at 560 nm and an emission wavelength at 590 nm (Perkin-Elmer LS-30, Perkin-Elmer Ltd.). Fusion experiments were achieved with four different peptides/lipid ratio of each N-acetylated peptide. All labeled mutants and N-acetylated peptides induced the fusion of lipidic phases, although with fusogenic properties that were about four times less efficient than the wild-type peptide.

Homogeneity of proteo-liposomes

Sucrose gradients were 0%–30% sucrose. The samples (15% sucrose) were added in the tube and the linear gradient was made over. The tubes were centrifuged in a SW41 Ti rotor (Beckman ultracentrifuge) for 20 h at 103,847g. Fractions of 1 mL were collected for protein and lipid assays. To minimize the amount of sucrose in the samples (which interferes with protein and lipid assays), 100 μL of each fraction was diluted into 900 μL of Milli-Q water and centrifuged in a TLA 100.4 rotor in a Beckman ultracentrifuge at 104,300g for 1 h. The pellets were then diluted in 300 μL of Milli-Q water. Phospholipid content was determined by phosphorus assay, and peptide amount using standard Lowry protocol.

Solid-state NMR experiments

The NMR experiments were performed with a Bruker DMX narrow bore spectrometer operating at ^1H Larmor frequency of 500.13 MHz.

^{13}C and ^{31}P spectra were obtained with a doubly-tuned DOTY XC5-MAS probe equipped with a 5-mm spinning module. The ^1H RF field strength for heteronuclear two-pulse phase modulation (TPPM) decoupling was equal to 66 kHz. ^{13}C cross polarization (CP) MAS spectra were acquired using a ^1H excitation pulse length of 5 μsec and a CP spin-lock field strength of about 50 kHz. The CP contact time was 2 msec; the relaxation delay was equal to

3 sec. ^{31}P MAS spectra were acquired using a hahn echo pulse sequence, and echo delays were varied from 0.1 to 40 msec during T_2 measurement experiments. ^2H NMR spectra were recorded on a Bruker 7 mm probe with a solenoid coil oriented at 90° with respect to the magnetic field. Quadrupolar splittings were recorded by using a standard quadrupolar echo sequence. Deuterium $\pi/2$ pulses were equal to 5 μsec , the refocusing delay and the repetition time were set to 30 μsec and 1 sec, respectively.

Typically, the NMR spectra were obtained on 2 mg of labeled peptide, 23.2 mg of phospholipids, and 25 μL of deuterium-depleted H_2O .

Molecular modeling

Construction of the two helical peptides and two β -sheet peptides was carried out as previously described (Brasseur et al. 1992) with the Hyperchem program. Side-chain conformations were optimized by Polak-Ribiere conjugate gradient.

The IMPALA procedure allows the study of interactions between a peptide and a modeled membrane using simple restraint functions designed to mimic properties of the membrane as described elsewhere (Lins et al. 2001). Briefly, The interface is described by a $C(z)$ function. $C(z)$ can be considered as the water concentration with a value of 1 outside the membrane and 0 in the membrane core. $C(z)$ varies between 1 and 0 in the region corresponding to the lipid/water interface ($z \in [13.5\text{--}18]$ Å). Two restraints are used to mimic membrane properties, mainly the hydrophobic effect (named Eint) and the lipidic perturbation (named Elip), depending upon the accessible surface of the peptide atoms. Modification of the internal structure was performed using an angular dynamic procedure of 6000 steps with a maximal torsion angle speed of $1.5^\circ/\text{step}$.

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