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Self-Assembly of Flexible β-Strands into Immobile Amyloid-Like β-Sheets in Membranes As Revealed by Solid-State ^{19}F NMR

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Supporting Information

ABSTRACT: The cationic peptide $[KIGAKI]_3$ was designed as an amphiphilic β-strand and serves as a model for β-sheet aggregation in membranes. Here, we have characterized its molecular conformation, membrane alignment, and dynamic behavior using solid-state ^{19}F NMR. A detailed structure analysis of selectively ^{19}F-labeled peptides was carried out in oriented DMPC bilayers. It showed a concentration-dependent transition from monomeric β-strands to oligomeric β-sheets. In both states, the rigid ^{19}F-labeled side chains project straight into the lipid bilayer but they experience very different mobilities. At low peptide-to-lipid ratios ≤1:400, monomeric $[KIGAKI]_3$ swims around freely on the membrane surface and undergoes considerable motional averaging, with essentially uncoupled ϕ/ψ torsion angles. The flexibility of the peptide backbone in this 2D plane is reminiscent of intrinsically unstructured proteins in 3D. At high concentrations, $[KIGAKI]_3$ self-assembles into immobilized β-sheets, which are untwisted and lie flat on the membrane surface as amyloid-like fibrils. This is the first time the transition of monomeric β-strands into oligomeric β-sheets has been characterized by solid-state NMR in lipid bilayers. It promises to be a valuable approach for studying membrane-induced amyloid formation of many other, clinically relevant peptide systems.

Self-assembly of monomeric peptides or proteins into oligomeric β-sheets plays a crucial role in numerous amyloid diseases. The aggregation process is often accelerated by the presence of lipid membranes and by surfaces in general.[1] That is because the prealignment of monomers in a 2D plane reduces the entropic cost of assembly, and the formation of intermolecular H-bonds may be favored. Despite the vested interest in monitoring such monomer−oligomer transitions of membrane-bound peptides, no suitable methods exist which permits this in a nonperturbing and quantitative manner. An underlying challenge in discriminating monomeric and oligomeric β-conformations is the need to not only characterize their structures with quasi-atomic resolution but also take their dynamic behavior into account on an appropriate time scale.

Solid-state NMR is ideally suited to determine the conformation, alignment, and dynamic behavior of membrane-bound peptides.[2−5] Especially the use of selective CF_3 reporter groups has allowed the characterization of numerous ^{19}F-labeled peptides in a highly sensitive manner,[3−8] even at very low concentrations or when bound to native biomembranes.[9] A recent ^{19}F NMR study on a designer-made amphiphilic α-helical model peptide demonstrated its unexpected conversion into β-sheets in the presence of lipid bilayers.[5] To further explore the fundamental properties of membrane-bound β-structures, we focus here on a model sequence named $[KIGAKI]_3$, designed as an amphiphilic β-strand by Blazyk et al.[10] CD and IR analysis had shown a transition from random coil in aqueous solution to a β-conformation in lipid bilayers.[10−12] The thermodynamics of membrane binding and folding were studied by ITC, and the ensuing membrane perturbation was monitored by ^{31}P and ^{2}H NMR of the lipids.[12−14] However, no direct structural insights have been gained yet about the possibility that a peptide like $[KIGAKI]_3$ may bind to membranes as a monomeric β-strand and that it may self-assemble into oligomeric β-sheets. Such a transition is fundamental to understand the biophysics of amyloid formation, as often triggered by membranes. An interesting biological aspect of the $[KIGAKI]_3$ model system is the fact that this peptide exhibits a pronounced antimicrobial activity against bacteria, while hemolytic side effects are low.[10,12−14] These functions suggest that peptide oligomerization and/or aggregation may play an important role in membrane perturbation and cell damage. Very often small oligomers are implicated as the primary toxic species in amyloid diseases.[15]

The amidated $[KIGAKI]_3$ carries 7 positive charges and is known to bind to anionic lipid vesicles.[10] We thus recorded CD spectra of the peptide in the presence of small unilamellar vesicles of dimyristoylphosphatidylcholine/dimyristoylphosphatidylglycerol (DMPC/DMPG, 4:1), as shown in Figure 1A. The peptide is disordered in aqueous buffer. With the addition of lipid it binds electrostatically, and the random coil is gradually converted into a β-conformation. CD analysis in uncharged DMPC vesicles suffered from heavy light scattering artifacts due to extensive vesicle aggregation. No such artifacts were observed using oriented CD in macroscopically aligned DMPC samples, prepared with limiting hydration as in the case of the ^{19}F NMR analysis below. The inset of Figure 1A

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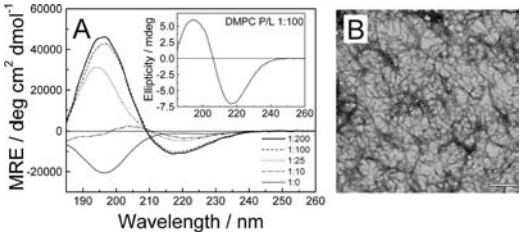

Figure 1. (A) CD spectra of $[KIGAKI]_3$ in DMPC/DMPG (4:1) vesicles at 30 °C for different peptide-to-lipid ratios. Inset: Oriented CD spectrum in DMPC; sample prepared the same way as for solid state ^{19}F NMR. (B) TEM image of aggregated $[KIGAKI]_3$ in water showing typical amyloid-like fibrils (scale bar = 200 nm).

confirms a pronounced β-sheet conformation, showing somewhat unusual intensities due to anisotropic alignment of the transition dipoles. The ability of $[KIGAKI]_3$ to self-assemble as β-sheets is further characterized in Figure 1B by transmission electron microscopy (TEM). These data demonstrate the formation of extended amyloid-like fibrils.

Next, we characterized the detailed structural and dynamic properties of $[KIGAKI]_3$ in lipid membranes using solid-state ^{19}F NMR. Five selectively ^{19}F-labeled analogues were synthesized, each carrying a single substitution with 3-(trifluoromethyl)-L-bicyclopent-[1.1.1]-1-ylglycine (CF_3-L-Bpg)[16] at position Ile-6, Ile-8, Ala-10, Ile-12, or Ile-14, (plus another set of five carrying the enantiomeric CF_3-D-Bpg label; see below). To check whether the stiff hydrophobic side chain induced any perturbation, the ^{19}F-labeled analogues were analyzed by CD. Like the wild type peptide, all of these L-analogues turned β-pleated in the presence of DMPC/DMPG vesicles, and in 50% trifluoroethanol they form a nascent α-helix (Figures S1 and S2, Supporting Information (SI)). The minimum inhibitory concentrations (MIC) against four bacterial strains were measured to assess a more subtle biological function. All ^{19}F-labeled analogues had similar MIC values as the wild type (Table S1). Only the CF_3-L-Bpg substitution at Ala-10 caused a slight decrease in activity, most likely due to the increase in hydrophobicity. TEM confirmed that the labeled peptide forms fibrils (for CF_3-L-Bpg at position Ile-8, data not shown). Overall, labeling with CF_3-L-Bpg significantly affects neither the secondary structure nor the biological activity of $[KIGAKI]_3$.

For solid-state NMR analysis, the ^{19}F-labeled analogues were reconstituted in oriented DMPC bilayers at different peptide-to-lipid molar ratios (P/L) at full hydration. Anionic lipids are not required in this kind of sample, since the peptides cannot escape into an excess of bulk water.[8] Representative ^{31}P NMR spectra of the phospholipids are illustrated in Figure 2A for samples with the membrane normal along the external magnetic field B_0 (0° tilt). The reconstituted membranes are seen to form a well oriented liquid crystalline lamellar phase at low P/L ≤ 1:200. With increasing concentration of $[KIGAKI]_3$, the oriented ^{31}P NMR signal is broadened, suggesting that the bilayers become more perturbed. Finally, at P/L = 1:25 the lipid spectra exhibit a pronounced fraction of unoriented powder. A previous ^{31}P NMR study of $[KIGAKI]_3$ was performed at the same concentration in multilamellar vesicles, reporting only a slight influence of the peptide on fast lipid headgroup rotation.[13] However, in those nonoriented multilamellar samples it was intrinsically impossible to detect the perturbed powder component.

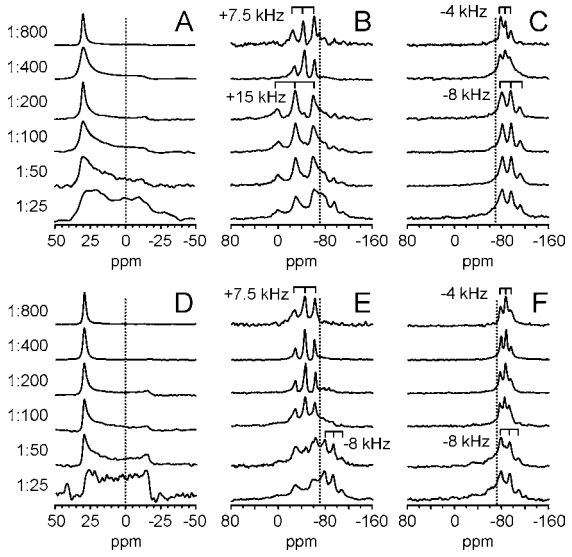

Figure 2. Solid-state NMR spectra of $[KIGAKI]_3$ labeled at position Ile-8 with CF_3-L-Bpg (A, B, C) or with the enantiomer CF_3-D-Bpg (D, E, F), measured in DMPC at 35 °C. (A, D) ^{31}P NMR spectra of oriented samples at 0° tilt. (B, E) ^{19}F NMR at 0° tilt. (C, F) ^{19}F NMR at 90° tilt. The peptide-to-lipid ratio is given in each row, the isotropic frequency is marked with a dashed line, and the splittings of the dipolar triplets are indicated.

The ^{19}F NMR approach relies fundamentally on the rigid scaffold of the CF_3-L-Bpg side chain (see Figure 3 below), in which the C−CF_3 bond is collinear with the C_α−C_β bond.[16,17] The time-averaged direction of the C−CF_3 bond vector thus reflects the orientation and mobility of the backbone segment to which it is rigidly attached. In an oriented sample, the NMR signal shows a triplet due to the ^{19}F−^{19}F dipolar coupling of the CF_3-group. The splitting of each individually labeled position

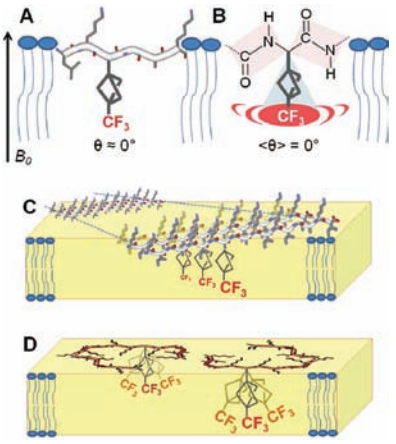

Figure 3. (A) At high peptide concentration (P/L ≥ 1:200) of $[KIGAKI]_3$ in liquid crystalline DMPC, all ^{19}F-labeled segments are rigidly oriented. The CF_3-L-Bpg side chains project into the lipid bilayer, parallel to the membrane normal, at a fixed angle of $\theta = 0°$. (B) At low peptide concentration (P/L ≤ 1:400), the dipolar splittings are reduced due to vigorous wobbling of the side chains around an orientation with a time-averaged angle $<\theta> = 0°$. (C) At high P/L, the β-strands are self-assembled into amphiphilic β-sheets, which are immobile and lie flat on the membrane surface, presumably as amyloid fibrils. (D) At low P/L, the amphiphilic β-strands are monomeric and intrinsically flexible, such that they can swim around freely in the 2D hydrophilic/hydrophobic interface of the membrane.

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provides one local orientational constraint for the overall structure calculation.[17] Representative ^{19}F NMR spectra of $[KIGAKI]_3$ substituted at Ile-8 with CF_3-L-Bpg are illustrated in Figure 2B for different peptide concentrations in DMPC at 0° tilt. The narrow triplets indicate that the peptides are well aligned in the membranes and suitable for structure analysis. Only at very high concentrations, an additional powder contribution emerges with the characteristic −8 kHz splitting of a rotating CF_3-group.[18] (Negative splittings occur to the right of the isotropic frequency.[19]) The prominent signals from the well oriented peptide molecules fall into two discrete regimes. At P/L ≤ 1:400, the dipolar couplings are ca. +7.5 kHz, while at P/L ≥ 1:200 they are close to +15 kHz. (Exact values are listed in Tables S2 and S3.) It is remarkable that, for any given P/L, all five different ^{19}F-labeled positions show the same splittings as the values given here for position Ile-8 (Figures S3 and S4).

It is instructive to first interpret the large splitting of +15 kHz in Figure 2B, which is observed at moderate to high $[KIGAKI]_3$ concentrations. The maximum possible splitting that can ever be observed for a CF_3-group is ca. +17 kHz, being directly related to the static powder splitting in frozen samples or lyophilized peptides (in which the CF_3-rotor is still spinning). The unique +17 kHz splitting represents a CF_3-group with θ = 0°, where θ is the angle between the C−CF_3 bond and B_0. In our horizontally aligned NMR samples, the observed +15 kHz splitting thus means that all CF_3-labeled side chains must be oriented almost parallel to the membrane normal, as illustrated in Figure 3A. This conclusion is fully supported by the intended design of $[KIGAKI]_3$ as an amphiphilic β-strand with alternating polar and apolar residues. It is intuitively clear that the backbone would come to lie flat on the membrane surface, so that the C_α−C_β bonds of all amino acids project alternatingly up and down, i.e. into the aqueous phase and into the bilayer with $\theta \approx 0°$. From the NMR data we can also conclude that the β-strands must have self-assembled into extended β-sheets, because the spectra are not affected by motional averaging to any significant extent. A perfectly aligned (θ = 0°) and completely immobile CF_3-group would exhibit a +17 kHz splitting; hence the slight reduction to +15 kHz reflects a very weak wobble or a minor deviation in alignment.

The same structural and dynamical conclusions are obtained also by a formal analysis of the ^{19}F NMR data based on the exact dipolar splittings of the five labels, as described in more detail in the SI.[7] We calculate a tilt angle $\tau \approx 90°$ for the extended backbone (using ϕ = −139° and ψ = 135°), an azimuthal rotation angle $\rho \approx 60°$ for the radial C_α vector, and a molecular order parameter of $S_{mol} \approx 0.96$ for the mobility. These values mean that membrane-bound $[KIGAKI]_3$ is assembled into untwisted β-sheets at moderate to high peptide concentration. This structural description holds unambiguously for the sequence of the labeled stretch from Ile-6 to Ile-14, and very likely also over the full length from N- to C-terminus. Based on the fact that the peptide tends to form amyloid-like fibrils, Figure 3C illustrates how these fibrils are arranged flat on the membrane surface. In this state, they cause significant perturbation to the lipid bilayer, according to ^{31}P NMR (Figure 2A).

The oriented NMR samples were also measured with a sample tilt angle of 90° with respect to B_0 (Figure 2C). This experiment is usually employed to find out whether a peptide is laterally mobile in the bilayer plane on a millisecond time scale corresponding to the kHz splittings. Such motional averaging reduces the effective dipolar splitting by a factor of −1/2 at 90° tilt, whereas immobilized peptides yield a powder-like line shape with a prominent splitting of −8 kHz. For $[KIGAKI]_3$ we see at low peptide concentrations of P/L ≤ 1:400 that the +7.5 kHz splitting (which will be discussed in detail below) at 0° tilt reduces to ca. −4 kHz at 90° tilt. This factor of −1/2 indicates that the peptide must be freely mobile in the bilayer plane and is therefore most likely monomeric under these conditions. At high P/L ≥ 1:200, we also see a halving of the +15 kHz splitting to ca. −7.5 kHz in Figure 2C, but in this particular case it is not possible to tell whether the peptides are mobile or not. That is because −1/2 of +15 kHz for a mobile case is roughly equal to the powder splitting of −8 kHz for an immobilized system. Here, the tilted sample cannot provide any direct information about lateral mobility in this particular situation. However, as noted above, the absence of molecular wobble supports the notion that $[KIGAKI]_3$ is immobilized in extended β-sheets at high P/L ratios.

Next, consider the dipolar splittings at low peptide concentration (P/L ≤ 1:400) in Figure 2B, for which a uniform value of +7.5 kHz is observed for all ^{19}F-labeled positions (Table S3). Compared to the +15 kHz situation at high concentration, the much smaller splitting can indicate (i) a dramatic change in orientation of the stationary C−CF_3 bonds, (ii) the onset of vigorous motional averaging, or (iii) a combination of both effects. For a motionless peptide according to scenario (i), the uniform splitting of +7.5 kHz would imply that all C−CF_3 bonds would have reoriented to an angle of $\theta \approx 38°$ with respect to the membrane normal (7.5 kHz/17 kHz = $[3\ \cos^2\ \theta\ -\ 1]/2$). However, such geometry is clearly incompatible with the designed amphiphilic sequence, and we had also concluded that the peptide is motionally averaged at low concentration. Scenario (ii) is more convincing, as it suggests that it is the time-averaged direction $\langle\theta\rangle$ of the C−CF_3 bond that remains oriented at 0° along the membrane normal, with $[KIGAKI]_3$ being highly dynamic. The dipolar splitting of the side chain is reduced due to a wobble in a cone, for which we estimate an opening angle of 38°, as illustrated in Figure 3B. Since all five ^{19}F-labeled positions give essentially the same +7.5 kHz splitting, we conclude that all hydrophobic membrane-immersed side chains are engaged in such vigorous local fluctuations, along the full sequence probed here.

Given our observation that the strong, uniform fluctuations affect the whole monomeric peptide, there are in principle two possible explanations. Either (a) the entire $[KIGAKI]_3$ monomer could be stiff and undergo vigorous concerted motions *en bloc*, affecting all atoms the same way, or (b) the amino acid residues are essentially "uncoupled" from one another, as implied in Figure 3B, D. An elegant way to discriminate between these different scenarios is to analyze some peptide analogues prepared with the D-enantiomer of CF_3-Bpg. In the unnatural D-configuration, the labeled side chain projects out from the backbone in a very different direction compared to the L-epimer; hence it should give a different dipolar splitting.[19] The ^{19}F NMR spectra for the CF_3-D-Bpg label in position Ile-8 of $[KIGAKI]_3$ are illustrated in Figure 2E. They show a splitting of +7.5 kHz up to a P/L ratio of 1:50, which changes to +15 kHz at very high peptide concentrations, where the spectra become dominated by a powder component with a splitting of −8 kHz. It is remarkable that all these splittings are essentially the same as those observed for the L-epimer in Figure 2B, yet the transition from +7.5 to +15 kHz is shifted to much higher P/L ratios. Also at

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90° tilt the D-epimers in Figure 2F show the same pattern as the L-epimers in Figure 2C, yet again with the transition shifted from P/L ≥ 1:200 up to P/L ≥ 1:50. According to steric considerations, CF_3-D-Bpg should indeed prevent self-assembly of $[KIGAKI]_3$ into β-sheets up to high peptide concentrations, because the rigid side chain sticks out at an angle that prevents H-bonding.[5] We have thus demonstrated that the D-epimeric peptides are highly mobile and monomeric up to much higher concentrations than the L-forms.

From the ^{19}F NMR data of the D-epimers we can now discriminate the aforementioned scenarios (a) and (b). It is clear that the CF_3-D-Bpg side chains are aligned in the bilayer in the same way as the side chains of L-epimers, i.e. with a time-averaged angle of $\langle\theta\rangle = 0°$ (as depicted in Figure 3B). This finding implies that the ϕ/ψ backbone torsion angles of $[KIGAKI]_3$ must be very flexible in the monomeric form, allowing the sterically maladjusted D-enantiomeric side chain to flip over and immerse fully in the hydrophobic membrane. Each backbone segment thus appears to be uncoupled from the neighboring peptide planes, as proposed in scenario (b). In conclusion, the entire peptide must have an essentially unstructured conformation. Figure 3D illustrates how the amphiphilic sequence is constrained in the 2D membrane plane only by the requirement that every other, hydrophobic residue must be anchored to the lipid bilayer. When the D-epimeric peptides eventually start to aggregate at a very high concentration (P/L ≥ 1:50), the formation of regular amyloid-like fibrils is much less favorable due to the inability of the CF_3-D-Bpg substituent to form H-bonds.

Overall, we conclude that at low concentration the amphiphilic $[KIGAKI]_3$ peptide binds to membranes as a highly flexible β-strand, without engaging in intra- or intermolecular H-bonds. This monomer swims around freely in the 2D membrane plane. It may be regarded as analogous to intrinsically unstructured proteins that have been described in aqueous solution.[20] Such an "unstructured" behavior has also been postulated for a membrane-bound peptide derived from the ras protein, and it has been also shown qualitatively for disordered regions in other proteins, based on magic angle spinning NMR techniques.[21] The decisive criterion to differentiate between the flexible monomeric β-strands and the immobilized β-sheeted fibrils is the ^{19}F NMR dipolar splitting of a selectively labeled CF_3−Bpg side chain. A dramatic change in mobility − rather than an orientational realignment − provided the ultimate information about the structural transition of the molecule. Solid-state ^{19}F NMR can thereby provide unique structural and dynamic details, not only for α-helices as in past applications, but also for flexible β-strands and for rigid β-sheets. Using the amphiphilic $[KIGAKI]_3$ model peptide, we have elucidated for the first time the ^{19}F NMR characteristics of an intrinsically disordered membrane-bound peptide and monitored its concentration-dependent self-assembly. It will be possible now to study other amyloid-forming peptides using this new approach.

■ ASSOCIATED CONTENT

Supporting Information

Material and methods, NMR data, biological activity, CD spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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