Fibril Formation

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Polymorphism in an Amyloid-Like Fibril-Forming Model Peptide**

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The conversion of peptides or proteins from their soluble forms into amyloid fibrils is frequently associated with pathological conditions ranging from neurodegenerative disorders to systemic amyloidoses.[1] Although amyloid fibrils and non-disease-associated amyloid-like fibrils can be formed by peptides and proteins that share no sequence identity,[2] they display several common properties. One hallmark of amyloid and amyloid-like fibrils is their highly ordered organization into a laminated cross-β structure, in which the β strands run perpendicular to the long fibril axis. Another characteristic is that the same protein or peptide can form fibrils of different morphologies. It has been suggested that the structural and morphological variability of fibrils is likely to form the molecular basis for the phenomenon of strains, and may play a role in amyloid diseases.[3–5] Although the basis of amyloid fibril polymorphism is not well understood, there is spectroscopic evidence that it is accompanied by specific changes in the conformation and packing of the individual polypeptide chains.[6–8] It has been shown that fibril polymorphism can partially be controlled by variation of the growth conditions[9,10] and that seeds from fibrils with a particular morphology can induce the sample to polymerize into fibrils of the same morphology. Elucidation of the factors that control the polymorphism of amyloid fibrils is therefore of major importance for understanding amyloid and prion diseases at the molecular level.[1]

Herein we address the molecular basis of polymorphism using the example of the de novo designed peptide ccβ-p as a model system.[11,12] Previous studies have shown that ccβ-p (Ac-SIRELEARIRELELRIG-NH_2) adopts a three-stranded α-helical coiled-coil structure in aqueous solution at low temperatures. However, the peptide forms amyloid-like fibrils spontaneously and irreversibly upon raising the temperature.[11] When formed from a solution buffered at pH 7.3, the β strands within the fibrils were shown to assume a laminated cross-β conformation[13] in which the extended β strands form antiparallel β sheets. The β strands were found to be shifted by three amino acid residues from an in-register arrangement (see Figure 1 b,d). We denote this arrangement as "+3 out-of-register" (+3-or).[26] It was suggested that, in addition to the clustering of hydrophobic residues, extensive salt-bridge formation between the charged side chains of Glu and Arg is a stabilizing factor for this arrangement.[11,12,14,15] Therefore, the protonation of the Glu side chains at low pH was suspected to potentially change the register.

As a result of its sensitivity to the inverse third power of the internuclear distance, solid-state NMR spectroscopy, and more specifically rotational echo double-resonance (REDOR) experiments,[16,17] are a powerful tool to unambiguously determine the register of constituent β strands within an amyloid fibril. The distance between the carbonyl carbon atom and the amide nitrogen atom is close to 4.2 Å if two amino acid residues are hydrogen-bonded partners, and larger than 5.5 Å otherwise. If the samples investigated are selectively labeled with a single ^{13}C and a single ^{15}N atom and the distance measured is about 4.2 Å, the corresponding register is unambiguously established.

To investigate the structure of ccβ-p amyloid-like fibrils at the atomic level, differently labeled peptides were prepared. Of particular interest in the following are the results from two compounds: for compound I the ^{15}N label was located on Ala7, and for compound II on Ile2. Both samples contained, in addition, a ^{13}C label on the carbonyl of Leu14. Compound I will lead to a strong REDOR effect for a +3-or antiparallel β-sheet structure, known to form at pH 7.3,[11] and sample II for a −2-or arrangement (see Figure 1), which will be shown to form at low pH.

Figure 2 shows the REDOR dephasing on fibrils of compound I prepared from solution at different pH values. The dephasing increases with increasing pH in the range from 2.0 to 7.3, indicating an increase of the abundance of the +3-or fibril polymorph, which indeed is the dominant structure at neutral pH.

Figure 3 shows the REDOR data obtained from samples of compound II. For samples prepared at low pH values, a strong REDOR effect is visible, attesting the existence of a −2-or structure.

The solid lines in Figures 2 and 3 indicate the best fit of the data by a model in which the dephasing is described by a superposition of the +3-or and the −2-or register dephasing curves. This approach is justified because compound I will

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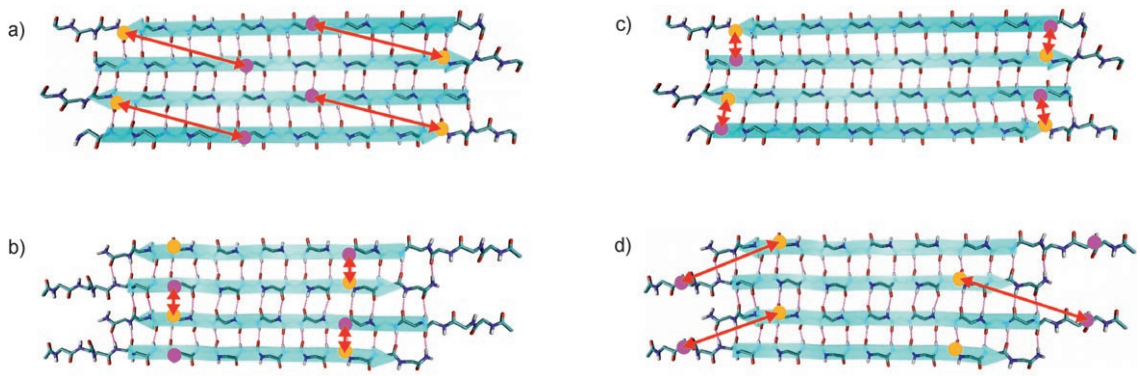

Figure 1. Models of two β-sheet structures (without side chains) of ccβ-p fibrils with schemes for isotopic labeling. a,b) ccβ-p with selective isotope labeling, with ^{15}N on the amide of Ala7 (magenta) and ^{13}C on the carbonyl of Leu14 (orange). c,d) ccβ-p with selective isotope labeling, with ^{15}N on the amide of Ile2 (magenta) and ^{13}C on the carbonyl of Leu14 (orange). The structures (a) and (c) show identical registers of the β sheet, which is the dominant form for fibrils formed at pH ≈ 2.0. Structures (b) and (d) show the register of the β sheet found in fibrils formed at neutral pH. The red arrows indicate the shortest distance between the ^{15}N and ^{13}C labels within one β sheet.

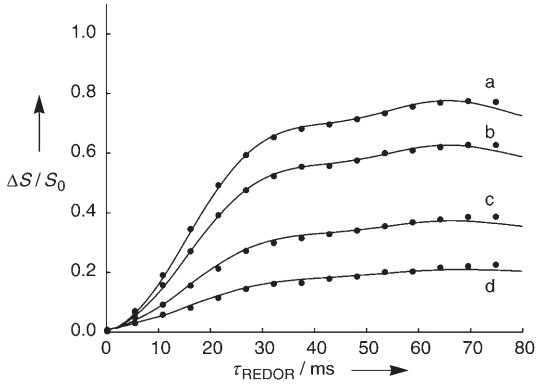

Figure 2. Experimental REDOR dephasing of fibrils of ^{15}N-Ala7- and ^{13}C′-Leu14-labeled ccβ-p (dots) and best fits based on numerical simulations of model structures of the β sheet (lines). The fibrils were prepared form solutions with pH 7.3 (a), pH 5.5 (b), pH 3.5 (c), and pH 2.0 (d).

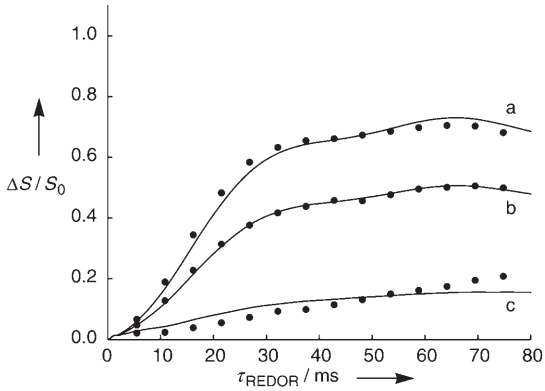

Figure 3. Experimental REDOR dephasing of fibrils of ^{15}N-Ile2- and ^{13}C′-Leu14-labeled ccβ-p (dots) and best fits based on numerical simulations of model structures of the β sheet (solid lines). The fibrils were prepared form solutions with pH 2.0 (a), pH 3.5 (b) and pH 7.3 (c).

give virtually no REDOR dephasing for a −2-or structure and compound II none for a +3-or structure (distance within sheet > 17 Å, between sheets > 9 Å). The only free parameter in the fit is the relative abundance of the two registers.[18]

The results of the analysis are listed in Table 1 for the different compounds and pH values. Notably, for fibrils assembled at a given pH, and under otherwise identical conditions, the two register fractions add up to values of 100 % in good approximation, indicating that the composition of the sample can be described by a mixture of these two registers only.

At pH 2.0 and 7.3, one of the two registers is dominant (>80 %), namely the −2-or at pH 2.0 and the +3-or at pH 7.3. Between these pH values, there is a gradual transition from one register to the other. The coexistence of the two registers is explicit for the samples prepared at pH 3.5 in which a significant fraction of both is present (0.38 and 0.55). The characteristic shape of the REDOR dephasing curves for intermediate pH values supports a model in which each register segregates into different fibrils or, alternatively, populates larger domains within the same fibril. Although the present data cannot distinguish between these two possibilities, they clearly exclude a fully random mixing of the two registers.

Table 1: Fraction of each register as a function of pH during fibril formation.[a]

pH	Labeling Scheme for ccβ-p[b]	
	Compound I ^{15}N-Ala7, ^{13}C′-Leu14 (fraction of +3-or)	Compound II ^{15}N-Ile2, ^{13}C′-Leu14 (fraction of −2-or)
2.0	0.167 ± 0.020	0.840 ± 0.040
3.5	0.381 ± 0.021	0.550 ± 0.012
5.5	0.771 ± 0.019	–[c]
7.3	0.896 ± 0.017	0.102 ± 0.058

[a] As determined by fitting of the data of each compound. [b] Each labeling scheme is sensitive to only one of the two registers of the β-sheet structures. [c] Not determined.

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The register shift as a function of the solution pH during fibril formation is likely to be a consequence of the protonation state of the glutamic acid side chain. At neutral pH, these side chains are negatively charged, and the combination of charge compensation between the glutamic acid and arginine side chains and an optimum hydrophobic clustering of the leucine, isoleucine, and alanine side chains likely promotes a +3-or β-sheet structure. This arrangement, as seen in Figure 4b, leads to β sheets with two identical faces

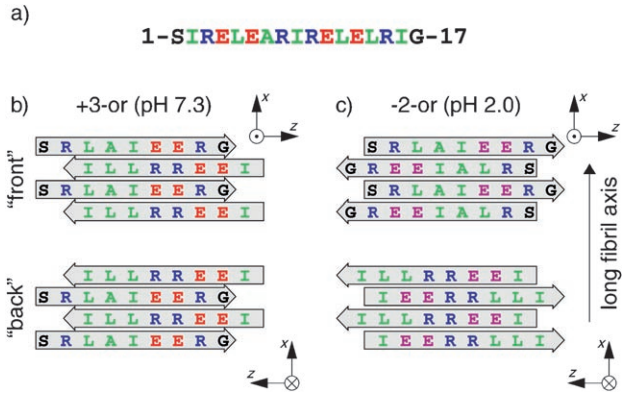

Figure 4. Representation of ccβ-p β-sheet structures. a) Single-letter representation of the primary structure of ccβ-p. Colors indicate the physicochemical character of the side chains (red: negatively charged; blue: positively charged; green: hydrophobic; black: other). b,c) Schematic representations of the antiparallel β-sheet structure of ccβ-p for the +3-or (b) and −2-or (c). Only those residues are shown which have their side chains above the plane of the β sheet with the top and bottom panels showing the two faces of the β sheets. The glutamic acid residues in (c) are shown in purple to emphasize their protonation state at pH 2.0 and hence the change of character from negatively charged to polar. The coordinate systems emphasize that a rotation around the *x* axis was used to show the two faces.

for infinitely long sheets. At pH 2.0, the glutamic acid side chains are uncharged, and the alternative arrangement with the different hydrophobic clustering shown in Figure 4c is found experimentally to be more favorable. For this structure, there is a clear difference between the two faces (the "front" and "back" sides of the β sheets, with a central hydrophobic patch along the middle of the front side and hydrophobic patches along the edges of the back side.

Steric zipper motives which form cross-β spine fibrils can be classified according to a recently proposed scheme.[19] Such a classification also requires the knowledge of the packing of β sheets in the fibril (along the *y* axis in Figure 4). Our REDOR experiments do not provide information about this packing. Based on X-ray diffraction data, Kammerer et al.[11] suggested that for the +3-or structure, the β strands between β sheets pack in an antiparallel manner. An identical packing was proposed by Steinmetz et al.[15] for fibrils assembled at pH 7.3 of the closely related ccβ-Met variant of the original ccβ-p peptide. Based on these assumptions, ccβ-p fibrils with a +3-or β-sheet structure fall into class 8 of the classification scheme.[19] This class is defined by β sheets composed of antiparallel β strands, where the two faces of the β sheets are identical and the individual β strands stack in an antiparallel manner. The alternative parallel stacking (class 7) is highly disfavored for energetic reasons, in addition to being inconsistent with the X-ray data. In contrast, in the −2-or structure (predominant at pH 2.0) there is a clear difference between the "front" and "back" side of the β sheets. It therefore falls either into category 5 or 6,[19] depending on the way the sheets stack. Neither of these two classes has been observed by X-ray crystallography, but evidence for their existence is available from solid-state NMR spectroscopy.[20,21] We have no experimental data to distinguish between these classes at present, but, considering the distribution of hydrophobic side chains on both sides, a face-to-face packing (class 5) seems to be most likely.

In summary, we have investigated the factors determining the balance between two antiparallel β-sheet conformations that can arise from a single peptide sequence. At low pH, an −2 out-of-register alignment is observed. At neutral pH, a +3 out-of-register is dominant. The solid-state NMR spectra of fibrils obtained in the range between pH 2.0 and pH 7.3 can be explained by a mixture of these two types of fibrils. The pH dependence of the quaternary structure of the fibrils shows that small changes at the molecular level, such as side-chain protonation, can have a large effect on the final fibril structure. The understanding of structural polymorphism at the atomic level may contribute to our understanding of amyloid and prion diseases.

Experimental Section

The two specifically $^{15}N/^{13}C$ labeled variants of the ccβ-p peptide (compounds I and II) were synthesized on an Applied Biosystems 433 A automated batch peptide synthesizer. In both cases the raw product was purified by reversed-phase HPLC. Product mass of the products was determined to be within 0.1% of the expected mass by MALDI-TOF mass spectrometry. The purity was above 95% as determined by analytical HPLC.

Fibrils were prepared by dissolving ccβ-p peptide in water at 4°C to a concentration of approximately 5 mM. The pH was adjusted to the desired value by adding 0.1M NaOH or 0.1M HCl. All samples were prepared with pure water as solvent except for the samples prepared at pH 7.3, for which a 20 mM sodium phosphate buffer was used to aid adjustment of the pH. The solution was then centrifuged to separate any undissolved material. The supernatant was incubated at (43 ± 3)°C for at least 6 h. The sample was subsequently heated to 90°C for 3 minutes to fibrilize any remaining material. The fibrils were sedimented by centrifugation. Finally the pellet was dried under a N_2 gas stream.

All solid-state MAS NMR experiments were performed on a Varian/Chemagnetics Infinity Spectrometer equipped with a 7 T magnet and a triple resonance Varian/Chemagnetics MAS probe. The temperature was set to −80°C to increase the transverse relaxation time. The spinning frequency was stabilized at (6000 ± 2) Hz. A REDOR pulse sequence with a single π pulse per rotor period on both the ^{15}N and ^{13}C channels was employed.[16,17] The pulses on both channels were offset by half a rotor period with respect to each other and were phase-cycled according to a XY-8 scheme[22] to reduce the effect of pulse errors. The REDOR dephasing period was incremented from 0 to 74.67 ms in steps of 5.33 ms.

Free induction decays for the dephased and non-dephased signals (^{15}N pulse amplitude set to zero) were acquired in an interleaved manner. Processing of the data was done with custom-written Matlab scripts. Simulations were calculated using a combination of Matlab scripts and C++ programs using the GAMMA environment.[23]

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Model structures on which the simulations are based were generated with CYANA.[24] Visualisation of the model structures in Figure 1 was carried out with the VMD (Visual Molecular Dynamics) software package.[25] Full experimental details are provided in the Supporting Information.

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[26] The nomenclature of the register is relative to an in-register antiparallel β sheet. The sign of the shift gives the direction in which the neighboring β strands are offset. This is based on the fact that the sum of the position number of two residues which are aligned on neighboring β strands in an antiparallel β sheet are a constant for any given β sheet register. For example, the in-register antiparallel β sheet of ccβ gives a sum of 18 (Ser1 is aligned with Gly17, Ile2 aligned with Ile16, etc.). The +3-or gives a sum of 21 (e.g. Ala7 aligned with Leu14), $18+3=21$. The −2-or gives a sum of $18-2=16$ (e.g. Ile2 aligned with Leu14). Incidentally, this also indicates which residues form hydrogen bonds and which are outside of the aligned region. For example, in the +3-or, Ser1 cannot be aligned, because to reach a sum of 21 it would need a residue 20, which does not exist.

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