VIP Fibril Dissociation

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Dissociation of Amyloid Fibrils of α-Synuclein in Supercooled Water**

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Several neurodegenerative diseases, including Alzheimer's, Creutzfeldt–Jakob, and Parkinson's disease, are associated with the formation of amyloid fibrils.[1] Amyloid fibrils have a β-sheet-rich molecular architecture called a cross-β structure.[2] The β-sheet conformation imparts extremely high thermodynamic stability and remarkable physical properties to amyloid fibrils.[3] They are highly resistant to hydrostatic pressure and high temperature, whereas protofibrils and earlier aggregates are more sensitive to these extreme conditions.[4,5] The stability of mature amyloid fibrils exceeds that of globular proteins, thus suggesting that they may represent the global minimum in terms of free energy. In addition, they have a strength comparable to that of steel.[6] Nature exploits these unusual properties of amyloidgenic structures for a variety of physiological functions.[7] Moreover, fibrillar peptide structures might have great potential as structural or structuring elements in nanotechnology applications.[8]

The native state of proteins can be unfolded both by high temperature and by cooling. Cold denaturation is predicted by the Gibbs–Helmholtz Equation, and attributed to specific interactions between nonpolar protein groups and water: tightly packed structures unfold at sufficiently low temperature to expose internal nonpolar groups to the water.[9] Direct observation of cold denaturation is generally hard to achieve in the absence of denaturant, extreme pH values, or mutations, as the transition temperature for most proteins is well below 0 °C. Freezing, however, can be avoided down to temperatures as low as −20 °C by careful supercooling of small sample volumes.[10,11] Nevertheless, this is generally not sufficiently cold to induce denaturation in stable, native proteins.[9,11]

Here we demonstrate that amyloid fibrils of the protein α-synuclein (αS), which constitute the insoluble aggregates found in brains of patients suffering from Parkinson's disease, are highly sensitive to low temperature. Despite their remarkable stability to hydrostatic pressure and high temperatures, mature amyloid fibrils of αS rapidly dissociate in supercooled water at −15 °C.

^{15}N-Labeled αS amyloid fibrils were prepared in vitro by incubating 0.1 mM freshly prepared ^{15}N-labeled αS[12] in 20 mM tris(hydroxymethyl)aminomethane (Tris) and 100 mM NaCl at pH 7.4. Incubation was carried out under continuous stirring at 37 °C for up to 14 days until a steady state was reached, as judged by thioflavin-T (ThT) fluorescence.[13] Matured fibrils were pelleted by centrifugation at 215000 *g* for 2 h and then resuspended in 50 mM phosphate buffer. Transmission electron micrographs showed regular fibrils with a diameter of approximately 40 nm (Figure 1a). A strong ThT fluorescence signal was detected for the fibrils (see the Supporting Information). Previous X-ray diffraction and solid-state NMR measurements have shown that amyloid fibrils of αS adopt a cross-β structure.[14,15] No cross-peaks were visible in the ^{1}H-^{15}N HSQC spectra, which is in agreement with the large molecular weight of amyloid fibrils and their associated fast relaxation (Figure 1a).

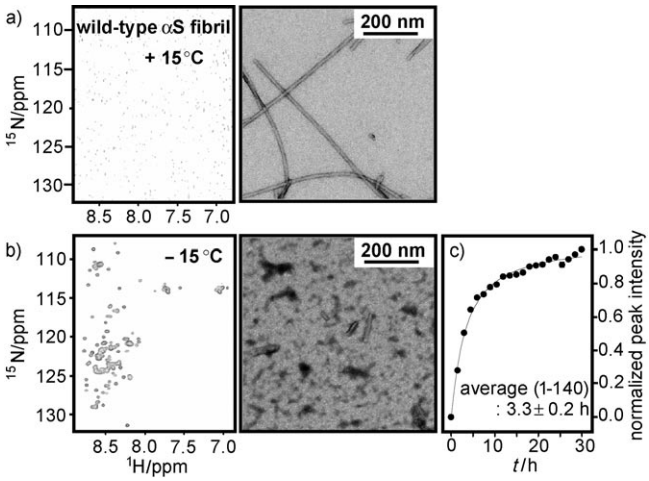

Figure 1. 2D ^{1}H-^{15}N HSQC spectra and electron micrograph of a sample containing ^{15}N-labeled αS fibrils in physiological buffer a) at +15 °C and b) after keeping it at −15 °C for 1 day. c) Average increase in the signal intensities of residues 1–140. The solid line indicates a sigmoidal function to the experimental data.

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We used supercooled water to probe the stability of αS fibrils at temperatures below 0 °C. ^{15}N-Labeled αS fibrils were injected into glass capillaries of 1.0 mm outer diameter, and the capillaries were placed in a 5 mm NMR tube.[11] Subsequently, the NMR tube was incubated at −15 °C for 1 day in the vibration-free environment of an NMR spectrometer. After incubation, transmission electron microscopy (TEM) images, ThT fluorescence, and 2D HSQC spectra were measured. The TEM images showed disordered aggregates and rare fibrillar structures (right panel in Figure 1b). The ThT fluorescence emission was decreased dramatically down to around 10% of the initial values (see the Supporting Information). In the HSQC spectrum, a large number of cross-peaks had appeared. The observed spectrum was very similar to the HSQC spectrum of freshly prepared ^{15}N-labeled monomeric αS under the same conditions (that is, in the same buffer at −15 °C). HSQC signals for each amide group in αS (except for proline residues) could be identified (left panel in Figure 1b). No significant differences in the chemical shifts or relative signal intensity were observed for the monomeric protein and the sample that originally contained amyloid fibrils and had been incubated in supercooled water for 1 day (see the Supporting Information). In addition, NMR diffusion experiments and ^{15}N T1ρ relaxation times were measured for the two samples (see the Supporting Information). No significant differences were observed, thereby indicating that the NMR signals detected after incubation of the amyloid fibrils at −15 °C originate from monomeric αS. Taken together, our measurements show that amyloid fibrils of αS dissociate into small aggregates and monomers in supercooled water at −15 °C, at which temperature many globular proteins remain folded.

We employed real-time NMR spectroscopy to obtain insight into the dissociation kinetics of αS fibrils. After cooling a sample of αS fibrils to −15 °C, a series of HSQC signals were acquired over 30 h (see the Supporting Information). Residue-specific changes in the intensities of the cross-peaks were extracted and fit to a sigmoidal function of time. From the fit, a half time of dissociation of 3.3 h averaged over all the residues was obtained (Figure 1c). The steady state was approached after more than 30 h. Similar results were obtained when analyzing the increase in the intensity of aliphatic signals in the 1D ^{1}H spectra (data not shown).

Why do low temperatures destabilize amyloid fibrils? Analyses of amyloidgenic peptides demonstrated that amyloid fibril structures are highly hydrogen-bonded, nearly anhydrous, and densely packed β sheets.[4,16,17] In addition, hydrophobic and electrostatic interactions can contribute to the stability of amyloid fibrils.[18] All of these interactions are strongly temperature-dependent: protonation of the protein groups increases as the temperature decreases (ionization of essential groups with decreasing temperature) and hydrophobic interactions decrease as the temperature decreases.[9] Additionally, the properties of water itself and the way it interacts with polypeptides are strongly temperature-dependent.[19]

To gain insight into the importance of hydrophobic and electrostatic interactions for the stability of αS fibrils we exposed ^{15}N-labeled fibrils to 8 M urea and 0.6 M NaCl. TEM and NMR spectroscopy showed that the αS fibrils are fully dissociated into monomeric protein in 8 M urea (see the Supporting Information). In 0.6 M NaCl, the morphology of the fibrils was changed: TEM images showed that the fibrils were fragmented and less regular. Also, the cross-peaks of some residues (mainly at the C terminus; for example, N^{122}, E^{137}, E^{139}, and A^{140}) appeared in the HSQC spectrum, which indicates that the αS fibrils are destabilized by increased ionic strength. It is noteworthy that keeping the αS fibrils at 95 °C for 16 h did not reduce the ThT fluorescence (data not shown), while the TEM images showed a comparable amount of fibrils before and after incubation (see the Supporting Information), thus indicating that mature amyloid fibrils of αS are highly resistant to high temperatures.[4,5]

To obtain evidence for the attenuation of electrostatic and hydrophobic interactions at low temperatures we investigated the conformational properties of monomeric αS in supercooled water by using NMR paramagnetic relaxation enhancement (PRE).[20] By incorporating paramagnetic spin labels into different regions of the sequence, we previously showed that the ensemble of conformations populated by monomeric αS at + 15 °C is stabilized by intramolecular long-range interactions.[12,21] In particular, transient long-range interactions exist between the positively charged N terminus and the negatively charged C terminus (Figure 2a, left panel). In addition, signal broadening was observed in the hydro-

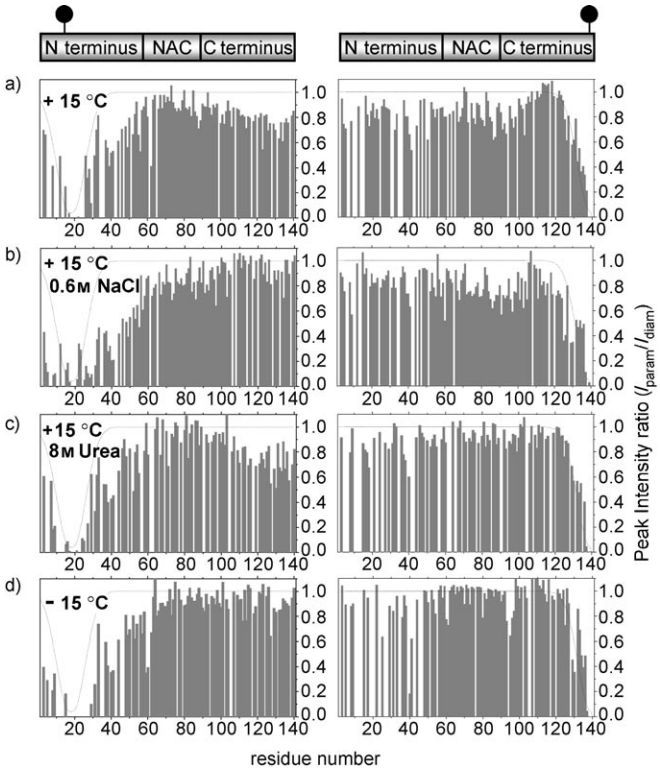

Figure 2. Intensity ratios (I_{param}/I_{diam}) of cross-peaks in the ^{1}H-^{15}N HSQC spectra recorded at + 15 °C in buffer in the presence (paramagnetic) and the absence (diamagnetic) of the spin label MTSL attached to position A18C or A140C (a), in the presence of 0.6 M NaCl at + 15 °C (b), upon addition of 8 M urea at + 15 °C (c), and in buffer at −15 °C (d). Black lines indicate intensity ratios expected for a fully extended structure.

phobic central domain between residues 70–105 when a spin label was attached to residue 140 (Figure 2a, right panel). We then studied changes in the PRE profile upon addition of 0.6 M NaCl (Figure 2b), addition of 8 M urea (Figure 2c), and on decreasing the temperature to −15 °C (Figure 2d). In the presence of 0.6 M NaCl, the spin label at position 18 no longer caused signal broadening in the C terminus (Figure 2b left panel), which suggests that the interaction between the N- and the C-terminal domains is electrostatically driven. On the other hand, the broadening that we observed for residues 70–105 when the spin label was attached to residue 140 was not reduced (Figure 2b, right panel). The opposite effect was observed when 8 M urea was added: the PRE profile observed for A18C was not changed, whereas signals in the central domain were no longer broadened (Figure 2c right panel). This finding suggests that long-range interactions probed by the A140C-MTSL protein are mainly related to hydrophobic interactions. Both long-range broadening effects were removed at −15 °C, which indicates that hydrophobic and electrostatic interactions were attenuated (Figure 2d). Taken together, our data suggest that—although we currently cannot estimate the relative importance of the various contributions—the temperature dependence of hydrophobic and electrostatic interactions contribute to the cold denaturation of αS fibrils.

The present study demonstrates that amyloid fibrils formed by the protein αS are rapidly denatured, that is, dissociated and lose the conformation of the constituent protein molecules, in supercooled water at −15 °C. Thus, the stability of αS fibrils towards low temperature is low compared to globular proteins. This finding resembles the situation found for other supramolecular aggregates, such as microtubules, that dissociate upon cooling.[22]

Experimental Section

Sample preparation for fibrilization and dissociation of αS fibrils: Recombinant αS was expressed and purified as described.[12] ^{15}N-Labeled αS amyloid fibrils were prepared by incubating 0.1 mM freshly prepared ^{15}N-labeled αS in a solution of 20 mM Tris and 100 mM NaCl at pH 7.4 in the presence of 0.01 % sodium azide in glass vials. Incubation was carried out under continuous stirring with a microsized stir bar at 37 °C for up to 14 days until a steady state was reached according to a stained thioflavin-T fluorescence assay.[13] Matured fibrils were centrifuged at 215000 × *g* with a TL 100 ultracentrifuge (Beckman Coulter). For measurements in supercooled water at −15 °C, monomeric αS (0.1 mM protein concentration) and fibrillar αS were suspended in 50 mM phosphate buffer (pH 7.4) and 300 mM NaCl, and injected into glass capillaries of 1.0 mm outer diameter (Wilmed-Labglass, USA) using a 25 μM syringe (Hamilton Syringe, USA). The capillaries were placed in a 5 mm NMR tube. Prior to inserting the sample into the NMR spectrometer, the temperature was set to −15 °C.

Preparation of spin-labeled αS: A single cysteine residue was introduced into αS at positions 18 (A18C) and 140 (A140C) by using the QuikChange site-directed mutagenesis kit (Stratagene), and the introduced modification was verified by DNA sequencing. Labeling with 1-oxy-2, 2, 5, 5-tetramethyl-D-pyrroline-3-methyl)-methanethiosulfonate (MTSL; Toronto Research Chemicals, Toronto, Ontario, Canada) was performed as described previously.[12]

NMR spectroscopy: NMR experiments were recorded on Bruker Avance 600 and 700 MHz NMR spectrometers. Dissociation of the amyloid fibrils was followed by recording a series of 1D ^{1}H and 2D ^{1}H-^{15}N HSQC spectra. Pulse field gradient NMR experiments were measured for the determination of the hydrodynamic radii as described elsewhere.[21] The ^{15}N $T_{1\rho}$ values were obtained by collecting five 2D spectra using relaxation delays of 8, 32, 48, 88, and 176 ms for monomeric αS, and by collecting two 1D spectra using a delay of 2 and 100 ms for denatured fibrils. An on-resonance spin-lock pulse of 2.5 kHz was used. PRE profiles were derived from the measurement of the ratios of the peak intensity between two 2D HSQC spectra in the presence (I_{para}) and absence (I_{dia}) of the nitroxide radical.

TEM: Amyloid fibers, resuspended from the pellet, were prepared on a glow-discharged carbon foil and stained with 1 % uranyl acetate. The samples were evaluated with a CM 120 TEM (FEI, Eindhoven, The Netherlands). Images were taken with a 2048 × 2048 TemCam 224 A camera (TVIPS, Gauting, Germany) in spot mode at 195000-fold magnification at −1.15 μm defocus.

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