Solid-State NMR Spectroscopy

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A Native-Like Conformation for the C-Terminal Domain of the Prion Ure2p within its Fibrillar Form**

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The tertiary and quaternary structures of infectious proteins, termed prions, are of particular interest because according to the "protein-only" hypothesis, they hold the information responsible for prion trait self-propagation.[1] The nature of the structural changes at the origin of a class of neurological prion disorders in mammals[1] and several prion phenotypes in yeast and fungi[2] remains mysterious in the absence of information at atomic resolution. Such information can be provided by solid-state NMR spectroscopy,[3] as it has recently been shown that full-length fibrils lead to well-resolved spectra, which contain detailed structural information.[4] Yeast prions are a convenient tool to study the molecular arrangement of prion proteins as they are innocuous. Herein, we present for the Ure2 prion (Ure2p) the measurement of structural probes specific for over 175 residues spread throughout the Ure2p C-terminal domain, and we show that the globular domain of the yeast prion Ure2p is largely identical in isolation (in crystalline form) and in the context of the full-length prion fibrils, obtained in vitro under physiological conditions. Interestingly, we find that the Ure2p globular domain, as seen on the NMR time scale of milliseconds and longer, adopts in full-length Ure2p fibrils a single conformation, as opposed to the conformation within crystals.

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This is astonishing when considering that, when proteins assemble into crystals, the conformational variability (sometimes termed the structural flexibility) of the polypeptide chain is often reduced.[5] In contrast, for fibrils, a form of conformational variability called polymorphism is often found upon fibrilization.

Ure2p is a 354-residue two-domain protein from the yeast *Saccharomyces cerevisiae* that assembles in vitro under physiological conditions into approximately 20 nm wide fibrils (see Supporting Information, Figure S1 a) and shows prion properties (Figure S1 b). The Ure2p N-terminal domain, rich in Gln and Asn, spans residues 1–93 and is essential for the propagation of the prion phenotype. The N-terminal domain, in isolation, forms approximately 5 nm wide fibrils. The C-terminal domain of the protein spans residues 94–354. In its soluble form, it exhibits enzymatic activities and is involved in protein–protein interactions that regulate nitrogen catabolism.[6] The X-ray crystal structure of the C-terminal domain revealed a GST-like fold.[7] The Ure2p C-terminal domain retains its enzymatic activity upon prion fibril formation.[8] However, it loses its ability to interact with protein partners, which leads to the [URE3] phenotype in yeast cells.[9] We reported the first solid-state NMR studies on full-length Ure2 prion fibrils grown under physiological conditions.[10] The 2D spectral fingerprints of Ure2p fibrils and Ure2p70–354 crystals hinted at a well-defined, highly similar structure for the C-terminal domain within the fibrils and the crystals. NMR experiments using a selective measurement of isoleucine resonances[11] have recently suggested results along the same lines. With the sequential assignment of the isolated C-terminal domain now available,[12] a comparison on a residue-by-residue basis between the conformation (and the conformational variability) of the isolated domain and the domain in the context of the full-length protein fibrils becomes possible.

NMR chemical shifts are a very sensitive probe of the conformation of a polypeptide chain. To compare the Ure2p C-terminal domain in its crystalline form and within full-length Ure2p fibrils on a residue-by-residue basis, resonance assignments of the NMR spectra were performed using a set of three-dimensional NMR spectra,[12,13] including NCACB, CAN(CO)CA, NCOCA, CANCO, and CCC for fibrillar Ure2p. The strategy used is described in detail elsewhere.[12,13] These results can then be compared with the sequential assignment of the Ure2p C-terminal domain in its crystalline form (BMRB entry 17499),[12] obtained under similar experimental conditions. A comparison of the backbone and side-chain resonances shows that chemical shifts of the vast majority of Ure2p C-terminal domain residues in Ure2p70–

354 crystals and fibrillar full-length Ure2p are virtually identical. As an example, extracts of the NCACB spectra representing the resonances of the stretch V319–G324 are displayed in Figure 1 a. ^{15}N planes from the 3D NCACB spectra of crystalline Ure2p70–354 and fibrillar full-length Ure2p are superimposed in Figure S2 a, as well as planes from the 3D CCC spectrum in Figure S2 b, and from the 3D CAN(CO)CA spectrum in Figure S3. INEPT spectra of both proteins show similar resonances,[4a] and point to the presence of flexible parts. The residues from the Ure2p C-terminal domain that were assigned within the fibrillar form of the protein span 68 % of the polypeptide chain (BMRB entry 17570). The residues that are not assigned are colored in yellow in Figure 1 c (Ure2p structure, PDB code 1G6Y). The chemical-shift differences for the Cα and Cβ resonances in the isolated crystalline Ure2p C-terminal domain and fibrillar full-length Ure2p are plotted in Figure 1 b. The variation in ^{13}C atom chemical shifts, expressed as the sum of the absolute value of the difference of the chemical shift between crystal and fibril forms for the Cα and Cβ resonances, $|\Delta\delta C\alpha|+|\Delta\delta C\beta|$, are below 0.5 ppm for almost all residues, which is the practical limit below which the conformation can be considered identical.[14] The root mean square (RMS) value of the ^{13}C Cα and Cβ shifts in the crystals and fibrils amounts to only 0.15 ppm, well within the resonance line width. The chemical-shift changes in the ^{15}N dimension are mostly less than 1 ppm and show an RMS value of 0.43 ppm (Figure S4). The small chemical-shift changes clearly indicate that the conformations of the Ure2p C-terminal domain within its crystalline form and fibrillar full-length Ure2p are almost identical. The residues we assigned are distributed throughout the Ure2p C-terminal domain; a significant

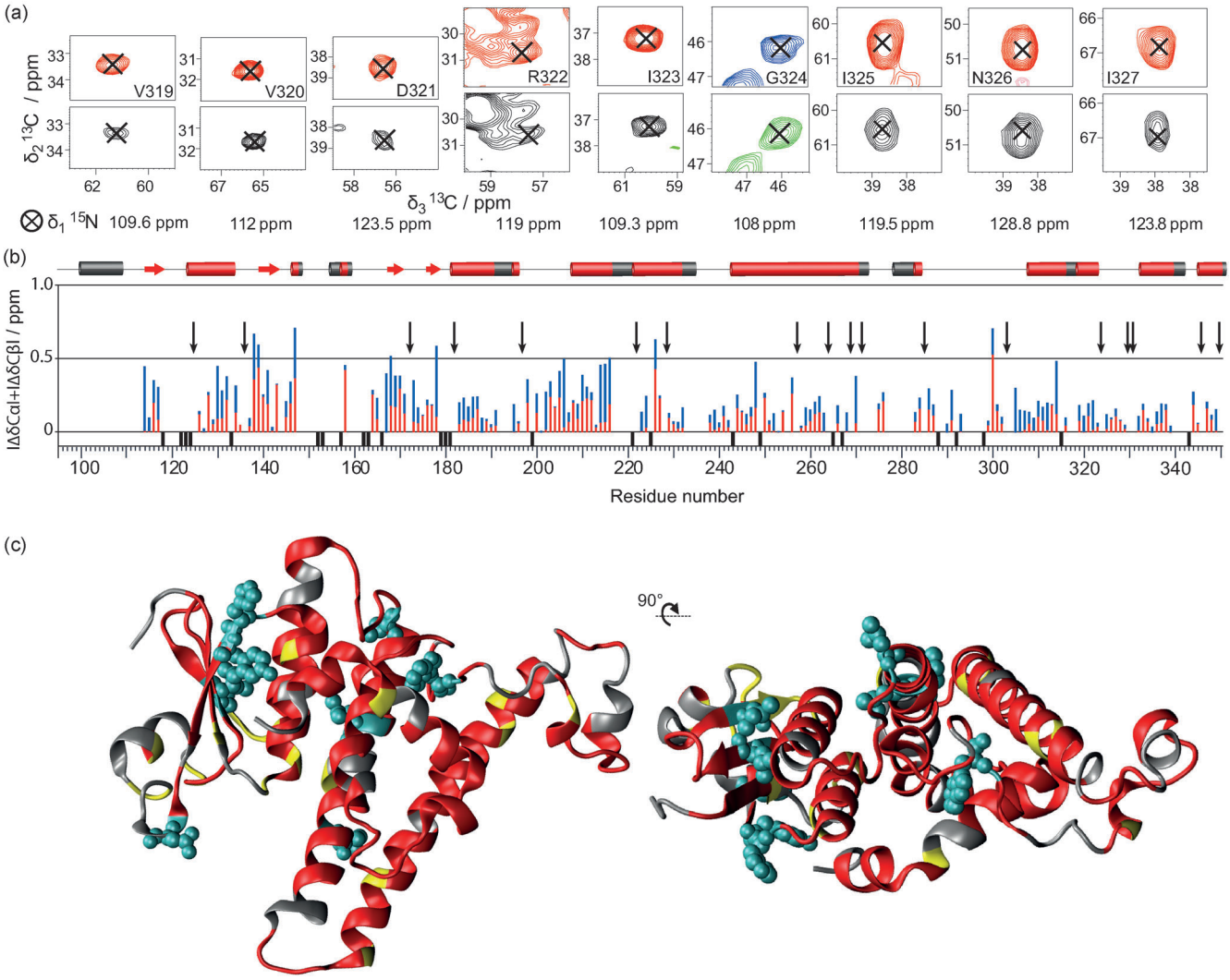

Figure 1. a) Comparison of the resonances corresponding to the Val319–Ile327 stretch in the 3D NCACB spectrum of Ure2p C-terminal-domain crystals (top row; red for negative contours, violet for positive) and Ure2p fibrils (bottom row; black for negative contours, green for positive). b) Sum of absolute value chemical-shift differences between Ure2p70–354 and Ure2p for the Cα and Cβ ($|\Delta\delta C\alpha|+|\Delta\delta C\beta|$), as a function of residue number. The individual contributions are marked in red for Cα and blue for Cβ. Negative bars mark assigned residues that are not found in the fibrillar full-length Ure2p spectrum. Arrows indicate residues with resonance peaks in crowded regions, mostly corresponding to Gly and Ala, where no comparison was possible. The RMS value in each ^{13}C dimension (Cα and Cβ) amounts to 0.15 ppm. c) Classification of the NMR properties plotted onto the X-ray structure: red = assigned and conserved residues ($|\Delta\delta C\alpha|+|\Delta\delta C\beta|<0.5$ ppm); yellow = residues that are not detected in fibrillar full-length Ure2p NCACB spectrum; cyan = assigned residues with a chemical-shift difference between crystalline Ure2p C-terminal and fibrillar full-length Ure2p ($|\Delta\delta C\alpha|+|\Delta\delta C\beta|$) larger than 0.5 ppm; gray = unassigned residues.

proportion of these residues are exposed at the surface and thus are particularly sensitive to the conformation this domain adopts within the fibrils, and to the interactions it establishes (see Figure 1 c where assigned residues are in red). The eight residues with somewhat larger shift differences between crystalline Ure2p C-terminal domain and fibrillar full-length Ure2p, $|\Delta\delta C\alpha|+|\Delta\delta C\beta|>0.5$ ppm, are in cyan in Figure 1 c. Despite their small size, these shift differences show a clear pattern and are mainly located around the β sheet, and in the loop following β strand 2. Further changes are located in two long loops (see Figure 1 c), indicating minor conformational changes in these regions. There are only a few side-chain resonances that vary by over 0.3 ppm (plotted on the structure in Figure S5). This implies that no major conformational changes affect the side chains of the amino-acid residues. Not only the peak position, but also the peak intensities and line widths are similar for the domain in isolation and in the fibrils. It is worth noting that the line width of the assigned peaks in the 3D NCACB spectrum is identical (within the error) for the crystalline C-terminal domain of Ure2p or the domain in the context of the fibrils, namely 130 ± 30 Hz. Our findings contrast remarkably with those for the assembly of HET-s prion into fibrils, where the globular domains lose most of their native tertiary structure and exhibit dynamic behavior, resulting in broad lines and a poor signal/noise ratio.[4b]

If most chemical shifts indicate that the Ure2p C-terminal domain shows a virtually identical structure in the two proteins, the comparison nonetheless also points out some significant differences: for residues Ala167–Leu168, Ile178, and Thr196–Leu200 two sets of peaks could sequentially be assigned in the crystalline C-terminal domain.[12] These residues are shown in green on the Ure2p structure in Figure 2 b, and the 2D spectra highlighting several of the peak doublings are given in red in Figure 2 a for the crystalline C-terminal domain. The spectra of the fibrils are shown in black. The peak doubling in the crystalline form is indicative of a significant conformational variability of the amino-acid residues involved. Chemical-shift differences of up to 3 ppm have been measured for these spins.[12] We have shown that the two conformations most probably correspond to the two inequivalent Ure2p molecules in the crystalline asymmetric unit, which means that the phenomenon is therefore unrelated to disorder.[15]

Interestingly, no peak doubling is found in the fibrillar Ure2p spectra, neither in the 2D (Figure 2 a) nor in the 3D spectra. Indeed, virtually every assigned signal found exclusively in the spectra of the crystalline C-terminal domain, which vanish in the spectra of full-length Ure2p fibrils, can be explained by signals arising from peak doubling. The signals from fibrillar Ure2p all coincide exactly with those of one of the conformers in the crystal (see Table S2). This observation implies that the conformational variability (as defined by the conformational space accessed by the protein in a given physicochemical state)[16] of Ure2p94–354 is smaller when Ure2p assembles into fibrils, as compared to the crystalline form. This result is surprising as the majority of NMR spectra of fibrillar proteins published so far show significant multiplicity and/or broadening of resonance lines, indicative of a conformational variability that is higher than in typical crystals.[17] Considerable conformational variability has been postulated in most prion models and, in particular, for Ure2p.[18]

In conclusion, our assignment reveals with residue-by-residue resolution, that the compactly folded C-terminal domain of Ure2p retains its native structure within the

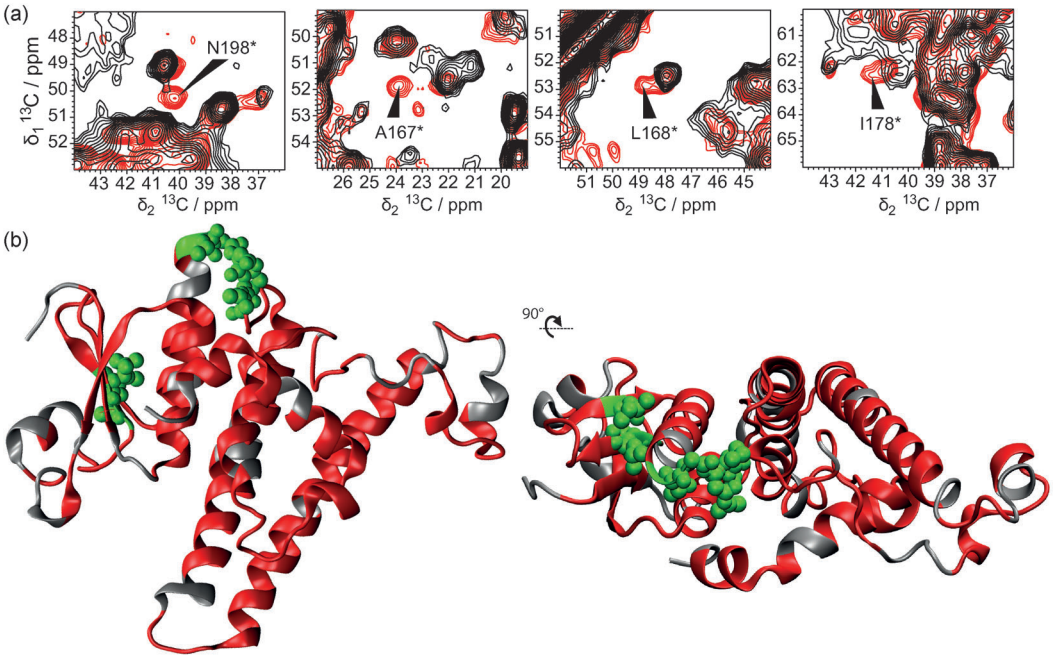

Figure 2. a) Extracts of 2D DARR spectra with regions where peak doubling occurs in crystalline Ure2p70–354 (red) and of fibrillar full-length Ure2p (black). The assignments of the doubled peaks are labeled. b) Residues showing peak doubling (green) on Ure2p C-terminal domain crystal structure (PDB entry 1G6Y). Red = assigned residues in the crystalline Ure2p C-terminal domain; gray = unassigned residues.

fibrillar form of the prion assembled under physiological conditions. The low conformational variability and the rigidity of the globular domain in the fibrils indicate that the compactly folded domain of Ure2p is highly ordered and regularly packed within the fibrils. We showed that the conformational variability of amino-acid stretches within the Ure2p C-terminal moiety is, surprisingly, lower in the fibrils than within crystals of the isolated C-terminal domain. This is—to our knowledge—the first time that such a result has been found, underlining the important role of the globular domains in Ure2p fibril formation. The finding that Ure2p C-terminal domain conformation is highly conserved in a native state within the fibrils suggests that the [URE3] phenotype is not merely the consequence of a conformational change within Ure2p but that of the inability of Ure2p to interact with its partners. Indeed, the assembly of Ure2p into fibrils might either involve the surface interfaces critical for the interactions between Ure2p molecules and their partners or make unavailable these surfaces, leading to the [URE3] phenotype. The assignment of the resonances originating from the Ure2p C-terminal domain within fibrillar Ure2p revealed over 50 additional narrow resonances, which most probably stem from the N-terminal moiety of fibrillar Ure2p prion. The assignment of the N-terminal resonances still poses a formidable challenge and will require higher-sensitivity spectra, for example, in higher magnetic fields.

Experimental Section

Protein expression, purification, and analysis: The *E. coli* strain BL21-Codon Plus (DE3+)-RIL (Stratagene, La Jolla, CA) was transformed with the expression vectors (pET3a) encoding full-length Ure2p. The bacteria were grown at 37 °C to an $OD_{600\,nm}$ of 0.8. The cells were spun at 3500 rpm in a Beckman JLA8.1000 rotor and washed twice. The final pellet was resuspended in half the original culture volume of M9 medium containing ^{15}N ammonium and ^{13}C uniformly labeled glucose. Protein expression was induced after 1 h incubation at 37 °C by addition of 0.5 mM isopropylthio-β-D-galactoside to the labeled M9 medium. Ure2p and Ure2p70–354 were purified as described.[19] Ure2p concentration was determined spectrophotometrically using an extinction coefficient of 0.67 mg cm^{-2} at 280 nm and a molecular mass of 40.2 kDa for Ure2p. The samples (4 mg mL^{-1}) were stored at −80 °C in buffer A (75 mM KCl, 20 mM Tris, pH 7.5, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol).

Assembly of Ure2p into fibrils: Soluble Ure2p was assembled at 8 °C in buffer A. The protein fibrils were directly centrifuged into the rotor for 30 min at 85000 g.[20]

Solid-state NMR spectroscopy: All spectra of Ure2p fibrils were recorded on a Bruker Avance II + 850 MHz spectrometer. A 3.2 mm Bruker triple resonance MAS probe equipped with an LLC coil was used. Sample temperatures were about 7 °C.[20] The pulse sequences are described in Ref. [19] and the experimental parameters given in Table S1. The spectra were referenced to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). All spectra were processed in TopSpin 2.0 (Bruker Biospin) and apodized using a squared cosine function. Spectra were analyzed and annotated using CCPNmr Analysis.[21] Protein structures were visualized using the program VMD.[22]

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