Solid-State NMR Spectroscopy

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The Conformation of the Prion Domain of Sup35p in Isolation and in the Full-Length Protein**

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The yeast protein Sup35p has prion properties,[1] and it aggregates into fibrillar assemblies.[2] It is at the origin of the [*PSI*$^+$] trait in baker's yeast, *Saccharomyces cerevisiae*.[3] The Sup35p yeast prion is an important model system to investigate the structure–function relationship of prions. To reduce the complexity of the problem, the Sup35pNM fragment is often used as a convenient model to document the assembly and infectious properties of the full-length prion, as fibrillar Sup35pNM is biologically relevant in the sense that it induces [*PSI*$^+$] when introduced into [*psi*$^-$] cells.[4] The notion that fibrillar Sup35pNM perfectly mimics Sup35p fibrils suggests that the N and M domains of Sup35p adopt identical conformations in Sup35pNM and Sup35p fibrils.[5] We question this assumption in the following: Using solid-state NMR measurements performed on Sup35pNM and full-length Sup35p fibrils assembled under identical physiological conditions, and both inducing [*PSI*$^+$] strains (Supporting Information, Figure S1), we demonstrate that fibrillar Sup35pNM and full-length Sup35p show significant structural differences, although both have a high β-sheet content.

Sup35p is a three-domain polypeptide (Supporting Information, Table S1); the N-terminal domain is critical for prion propagation, while the C-terminal domain has GTPase activity and is involved in translation termination.[6] The middle domain connects the two domains. The N domain alone or together with the M domain, and also the full-length Sup35p, assemble into amyloid fibrils.[5a,b,7] Fibrils of both Sup35p and Sup35pNM give rise to high-resolution solid-state NMR spectra, as can be seen in the 2D spectra in Figure 1 for Sup35pNM and Sup35p (overlaid in the Supporting Information, Figure S2), with the corresponding electron micrographs. The narrow and resolved resonances points to the presence of highly ordered structures in both fibrils. To increase spectral resolution, we recorded 3D NCACB spectra in which each amino acid residue shows a (N, C_α, C_β) frequency triple. Surprisingly, although 253 and 685 signals for Sup35pNM and Sup35p, respectively would be expected, the number of intense peaks in the 3D spectra is only 35 and 25, respectively (see Figure 2a for a representative plane). A set of 3D experiments[8] allowed us to assign the spin systems, and deposit the chemical shifts for 22 residues from Sup35p and Sup35pNM in the BMRB (accession codes 18407 and 18406, respectively).[9] All of the assigned residues are located within the 30 N-terminal residues in both Sup35p and Sup35pNM. We refer to this region in the following as the core amyloid region, following the term proposed by Weissman.[4] Seventeen out of the 22 assigned residues appear in the spectra of both proteins and allow for comparison of chemical shifts (Figure 2b). The ^{13}C chemical shift is known to be sensitive to the local conformation, with backbone (and C_β) chemical shifts being strongly correlated to the dihedral angles ϕ and

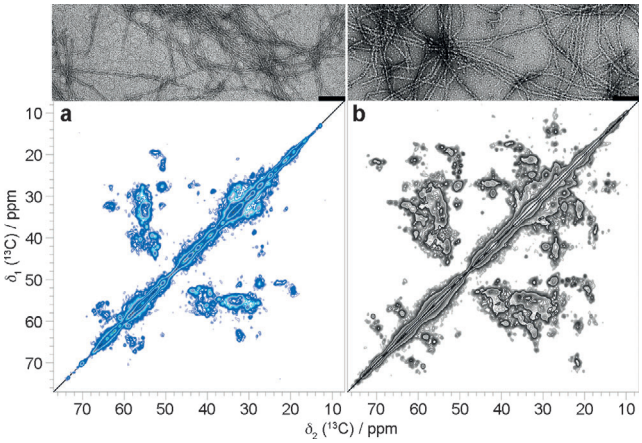

Figure 1. ^{13}C–^{13}C correlation spectra (DARR) with a mixing time τ_{mix} of 20 ms from fibrillar Sup35pNM (a) and Sup35p (b). All spectra were recorded at a magnetic field of 20 T and a MAS spinning frequency of 17.5 kHz. Negatively stained electron micrographs of fibrillar Sup35pNM and Sup35p are shown above the corresponding spectra (scale bar 0.2 μm).

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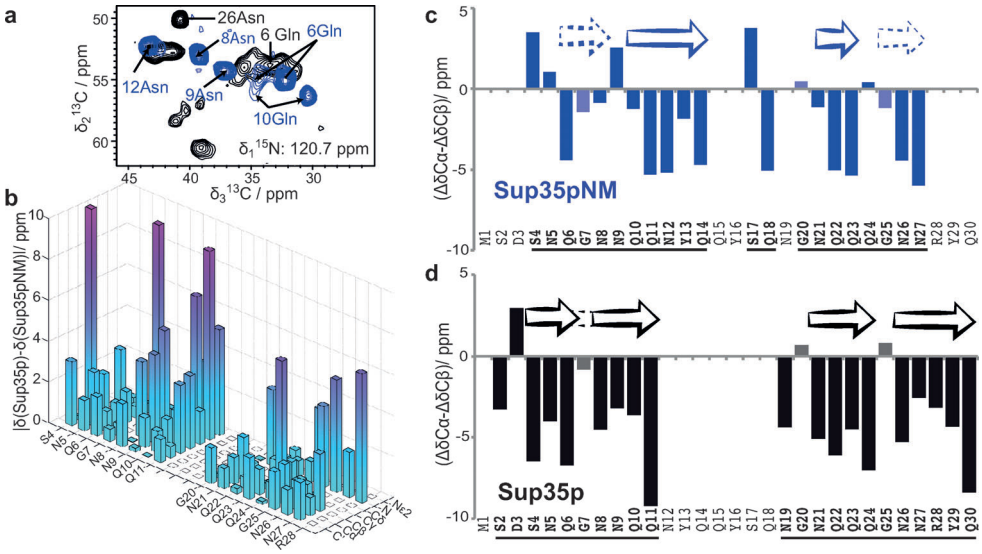

Figure 2. a) Overlay of a representative plane of the 3D NCACB spectra of Sup35pNM (blue) and Sup35p (black). Sequential resonance assignments are indicated. Experimental details are given in Table S3. b) Chemical-shift comparison between Sup35pNM and Sup35p. The absolute value of the chemical-shift difference for all ^{13}C and ^{15}N nuclei that are assigned in both constructs is plotted. c,d) Comparison of secondary chemical shifts[11] for Sup35pNM (c) and Sup35p (d). Light shades refer to $\Delta\delta C_\alpha$ of glycine. Solid arrows refer to β-strands, dashed arrows to possible extensions thereof, including Gly residues.

ψ.[10] We observe chemical-shift differences between Sup35p and Sup35pNM up to more than 10 ppm, thus spanning almost the entire chemical-shift range canonically observed for some of these spins. Thus, the conformation of the majority of the assigned residues must be significantly different in Sup35pNM and Sup35p. Moreover, the chemical shifts we observed do not correspond to those recorded for the fibril and crystal polymorphs of the peptide GNNQQNY from residues 7 to 13 of Sup35p (Supporting Information, Figure S3).

The secondary chemical shifts (Figure 2c,d) are a good proxy for the backbone conformation of a protein[11] and clearly point to high β-strand content for residues 2 to 30. However, the distribution of the β-strands is distinct for the two proteins. Dashed arrows are shown for β-strands containing Gly residues, which might not be part of the β-strands, because in the few existing fibril structures, they are all located in turns.[12]

The majority of residues in both Sup35p and Sup35pNM do not yield detectable correlation peaks in the 3D solid-state NMR spectra, which is either due to dynamic or to static disorder. In INEPT spectra[13] we could detect signals corresponding to most amino acid residue types. The spectra appear to be similar for Sup35p and Sup35pNM.[9] Dynamic resonances can also be detected selectively in directly pulsed ^{13}C 2D correlation spectra recorded with a short interscan relaxation delay. An extract of this spectrum is shown for Sup35pNM in Figure 3a in blue (for full aliphatic regions, see the Supporting Information, Figure S4). The spectrum is clearly dominated by signals at the predicted random-coil chemical shifts.[14] The random-coil shifts of a given amino acid type overlap, except for the residues preceding a proline, which are clearly shifted. We find strong indications that the vast majority of these flexible residues are located within the M-domain: 1) all residue-types present in the M-domain show signals in the spectrum, and residues that are present only within Sup35p N- and C-terminal domains are absent from the spectra; 2) we observe Ala-Pro and Lys-Pro pairs, which are uniquely present in the M domain; 3) the peak intensities correspond well to the occurrence of the residues in the M domain for both Sup35pNM and Sup35p (Figure 3e); 4) Thr signals are observed in these spectra, whereas none are observable in cross-polarization (CP)-based 2D DARR spectra with short mixing times (Supporting Information, Figure S5). Indeed, in Sup35pNM, all 11 Thr residues are found in the M domain. The polarization buildup of the Thr signal under cross-polarization is slow (Figure 3c), underlining the flexible behavior of the M-domain residues. Signals arising from a random-coil AP pair, as well as from random-coil Thr, are observable in directly pulsed Sup35p spectra as well; however, potential overlap with signals from the C-terminal domain does impede a similar analysis of the behavior of these peaks. An analysis of the signal intensities for Sup35pNM (Figure 3f) suggests that the M-domain is at the origin of these signals in Sup35p as well. Thus, the M domain is flexible and adopts a random-coil conformation in both Sup35pNM and Sup35p, which explains the missing of the corresponding peaks in the 3D spectra.

To characterize the remainder of the N domain, Tyr resonances are particularly well suited because they appear only in this segment of the protein. In 2D experiments, they exhibit featureless correlations resonating over the entire range of β-strand-typical chemical shifts (Supporting Information, Figure S6). The T_2 relaxation times of the C_α resonances are long (Supporting Information, Table S2), and the CP buildup is fast (Figure 3c). We therefore exclude significant dynamical contribution to the linewidth and conclude that the Tyr residues are mainly in β-strand conformation, in accordance with earlier findings,[15] but in contrast to the core amyloid residues, they are in a statically disordered environment. Besides Tyr, the 2D DARR spectra of Sup35pNM also additional broad resonances (Figure 3a), which can be identified for the Pro, Ser, and Ala residues. Ala residue chemical shifts cover the entire range of α-helical/turn and β-strand typical resonance positions. Their dynamical behavior (Figure 3c; Supporting Information, Table S1) is the same as for the Tyr. We therefore strongly suspect that these residues are also located within the remainder of the N

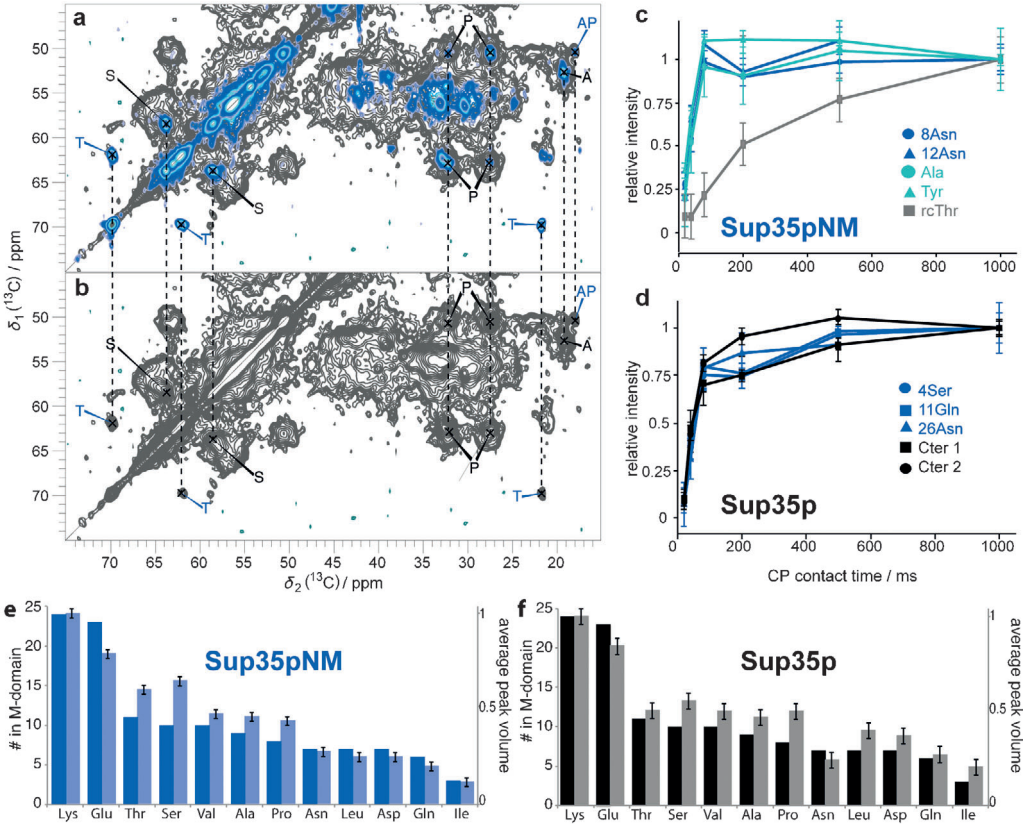

Figure 3. a) Overlay of Sup35pNM 100 ms DARR spectrum (gray) and directly pulsed 100 ms DARR spectrum (blue) with a T_1-filter on ^{13}C by use of a short relaxation delay (4 s). Isolated signals of amino acids from both N- and M-domain are labeled in black, those appearing only in the M- domain in blue. b) only the 100 ms DARR spectrum. c,d) Cross-polarization buildup dynamics for selected residues (of C_α–C_β cross-peak). Residues from the core amyloid are colored dark blue, residues from the remainder of the N domain light blue, from the M domain in gray, and from the C domain in black. e,f) Comparison of the C_α–C_β cross-peak volume from the directly pulsed PDSD spectra (Supporting Information, Figure S3), with the amino acid distribution in the M domain, normalized on Lys residues. The error is the standard deviation of the noise in direct-pulsed PDSD spectra.

domain. For the full-length protein, the unequivocal assignment of isolated peaks to the remaining N domain is more difficult, but the spectra are compatible with the assumption that the parts of the N domain, which do not form part of the ordered core amyloid, are statically disordered in the sense that the conformation of each residue in this part is variable. Nevertheless, these residues are organized in β-sheets. Whether the disorder is within one fibril, which we suspect, or between fibrils in a way that may be described as an extensive polymorphism in this part cannot be decided by an ensemble method like NMR spectroscopy.

The majority of Sup35p residues belong to the C-terminal domain, which remains biochemically functional in fibrils,[5b] suggesting that it retains its fold. We created a homology model from the X-ray structure of the homologous protein from *S. pombe*[16] and predicted chemical shifts using Sparta[17] (Supporting Information, Figure S7), which show a similar dispersion and distribution of signals. The resolved signals that do not have a counterpart in the Sup35pNM spectra can thus tentatively be assigned to the C-terminal domain. They show narrow line width and many resolved signals, which confirms that the C-terminal domain is ordered and well-structured. As shown in Figure 3 d (in black), the CP buildup of the signals from the C-terminal domain is very similar to one of the core amyloid residues, indicating that no large-scale amplitude motions take place. Nevertheless, these residues are not observed in the 3D spectra, indicating that subtle dynamic effects, probably a not yet fully characterized overall motion of the globular domain, are at the origin of signal attenuation in the 3D spectra.

Our study provides insight into the global structure and the dynamics of Sup35p N, M, and C domains in fibrils and demonstrates that the different domains feature different behavior. The ordered core amyloid of the fibrils is located within the first thirty residues and shows β-strand secondary structure in agreement with the slow H/D exchange rates observed for residues 4-37 in Sup35pNM fibrils assembled at 4 °C.[4] The faster proton–deuterium exchange rates recorded for the remainder of Sup35pNM[4] also agree with the absence of regular, well-ordered secondary structure we observe, although the observation by Toyama et al.[4] of moderate protection in some parts may hint at residues forming more stable hydrogen-bonded interactions. The disordered but static nature of a significant fraction of Sup35pNM suggests that non-residue-specific interactions between aromatic residues located outside the core amyloid may play an important role in the initial steps of aggregation.[18] Also, the presence of this possibly polymorphic region might add to the observation of various strains in Sup35p, because although the core amyloid shows clearly a well-defined structure, these immobilized segments of the N domain may have different conformations in different strains.

These findings shed new light on the surprisingly diverse world of prion assemblies, where conformational variability plays a staggering and confusing role. It supports the emerging picture that prions are complex structural units. Indeed, even if displaying a highly defined structure, a given

domain can adopt different conformations dependent on the setting (in isolation, in the context of a larger fragment, or the full-length protein) or the environment (buffer conditions, chaperones). For functional prions, such as the HET-s/HET-S systems, these properties can give rise to a functional switch that triggers apoptosis.[19] Our findings give a molecular-level explanation for the contrasting assembly propensity and infectivity of Sup35pNM and Sup35p, and stress the paramount importance of a molecular-level structural characterization of the aggregates employed in functional studies.

Experimental Section

The proteins were expressed and purified as described previously, using M9 medium with ^{15}N and ^{13}C labeling.[5a,b] In detail, Sup35p in buffer solution (20 mM Tris·HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$) was incubated at 10°C under orbital shaking at 30 rpm, 0.5 cm amplitude, for 3 weeks. The fibrils were spun at 100000 g in a TL-100 tabletop centrifuge for 20 min at 4°C. The pellets were resuspended in distilled water, washed twice, and filled into 3.2 mm rotors by ultracentrifugation as described.[20]

All spectra were recorded on a Bruker Avance II + 850 MHz spectrometer operating at a static field of 20 T. A 3.2 mm Bruker triple-resonance MAS probe equipped with an LLC coil was used. The sample temperature was about 7°C. The pulse sequences were implemented as recently reported,[8a] and the experimental parameters are given in the Supporting Information, Table S3. All spectra were processed using TopSpin 2.0 (Bruker Biospin) with zero filling and apodization by a squared cosine function. Spectra were analyzed and annotated using the CCPNmr Analysis package.[21]

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[1] R. B. Wickner, *Science* **1994**, *264*, 566.
[2] J. R. Glover, A. S. Kowal, E. C. Schirmer, M. M. Patino, J. J. Liu, S. Lindquist, *Cell* **1997**, *89*, 811.
[3] B. S. Cox, *Heredity* **1965**, *20*, 505.
[4] B. Toyama, M. Kelly, J. Gross, J. Weissman, *Nature* **2007**, *449*, 233.
[5] a) J. Krzewska, R. Melki, *EMBO J.* **2006**, *25*, 822; b) J. Krzewska, M. Tanaka, S. G. Burston, R. Melki, *J. Biol. Chem.* **2007**, *282*, 1679; c) M. Kabani, B. Cosnier, L. Bousset, J.-P. Rousset, R. Melki, C. Fabret, *Mol. Microbiol.* **2011**, *81*, 640.
[6] a) G. Zhouravleva, L. Frolova, X. Legoff, R. Leguellec, S. Ingevechtomov, L. Kisselev, M. Philippe, *EMBO J.* **1995**, *14*, 4065; b) I. Stansfield, K. Jones, V. Kushnirov, A. Dagkesamanskaya, A. Poznyakovski, S. Paushkin, C. Nierras, B. Cox, M. Teravanesyan, M. Tuite, *EMBO J.* **1995**, *14*, 4365.
[7] a) C. King, R. Diaz-Avalos, *Nature* **2004**, *428*, 319; b) M. Tanaka, P. Chien, N. Naber, R. Cooke, J. Weissman, *Nature* **2004**, *428*, 323; c) F. Shewmaker, D. Kryndushkin, B. Chen, R. Tycko, R. B. Wickner, *Biochemistry* **2009**, *48*, 5074.
[8] a) A. Schuetz, C. Wasmer, B. Habenstein, R. Verel, J. Greenwald, R. Riek, A. Böckmann, B. H. Meier, *ChemBioChem* **2010**, *11*, 1543; b) B. Habenstein, C. Wasmer, L. Bousset, Y. Sourigues, A. Schütz, A. Loquet, B. H. Meier, R. Melki, A. Böckmann, *J. Biomol. NMR* **2011**, *51*, 235.
[9] a) A. Schütz, B. Habenstein, N. Luckgei, L. Bousset, Y. Sourigues, A. B. Nielsen, R. Melki, A. Böckmann, B. H. Meier, *Biomol. NMR Assignments* **2013**, DOI: 10.1007/s12104-013-9515-1; b) N. Luckgei, A. Schütz, B. Habenstein, L. Bousset, Y. Sourigues, R. Melki, B. H. Meier, A. Böckmann, *Biomol. NMR Assignments* **2013**, DOI: 10.1007/s12104-013-9518-y.
[10] D. S. Wishart, B. D. Sykes, F. M. Richards, *J. Mol. Biol.* **1991**, *222*, 311.
[11] Y. Wang, O. Jardetzky, *Protein Sci.* **2002**, *11*, 852.
[12] a) H. van Melckebeke, C. Wasmer, A. Lange, E. Ab, A. Loquet, A. Böckmann, B. H. Meier, *J. Am. Chem. Soc.* **2010**, *132*, 13765; b) C. Wasmer, A. Lange, H. van Melckebeke, A. B. Siemer, R. Riek, B. H. Meier, *Science* **2008**, *319*, 1523.
[13] A. Siemer, A. Arnold, C. Ritter, T. Westfeld, M. Ernst, R. Riek, B. H. Meier, *J. Am. Chem. Soc.* **2006**, *128*, 13224.
[14] K. Tamiola, B. Acar, F. A. A. Mulder, *J. Am. Chem. Soc.* **2010**, *132*, 18000.
[15] F. Shewmaker, R. B. Wickner, R. Tycko, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 19754.
[16] C. Kong, K. Ito, M. A. Walsh, M. Wada, Y. Liu, S. Kumar, D. Barford, Y. Nakamura, H. Song, *Mol. Cell* **2004**, *14*, 233.
[17] Y. Shen, A. Bax, *J. Biomol. NMR* **2007**, *38*, 289.
[18] Y. Ohhashi, K. Ito, B. H. Toyama, J. S. Weissman, M. Tanaka, *Nat. Chem. Biol.* **2010**, *6*, 225.
[19] C. Seuring, J. Greenwald, C. Wasmer, R. Wepf, S. J. Saupe, B. H. Meier, R. Riek, *PLoS Biol.* **2012**, *10*, e1001451.
[20] A. Böckmann, C. Gardiennet, R. Verel, A. Hunkeler, A. Loquet, G. Pintacuda, L. Emsley, B. H. Meier, A. Lesage, *J. Biomol. NMR* **2009**, *45*, 319.
[21] a) W. Vranken, W. Boucher, T. Stevens, R. Fogh, A. Pajon, P. Llinas, E. Ulrich, J. Markley, J. Ionides, E. Laue, *Proteins Struct. Funct. Bioinf.* **2005**, *59*, 687; b) T. J. Stevens, R. H. Fogh, W. Boucher, V. A. Higman, F. Eisenmenger, B. Bardiaux, B.-J. van Rossum, H. Oschkinat, E. D. Laue, *J. Biomol. NMR* **2011**, *51*, 437.

