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ARTICLE

Solid-state NMR sequential assignments of the amyloid core of Sup35pNM

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Abstract Sup35pNM represents the N-terminal and middle (M) domains of the yeast *Saccharomyces cerevisiae* prion Sup35p. This fragment is commonly used for structural and functional studies of Sup35p. We here present a solid-state NMR study of fibrils formed by this fragment and show that sequential assignments can be obtained for the rigid and well-ordered parts of the protein using 3D spectroscopy. We describe in detail the sequential assignment of the 22 residues yielding strong, narrow signals with chemical shifts that correspond mostly to β -sheet secondary-structured amino acids that form the fibril core.

Keywords Sup35pNM · Fibrils · Solid-state NMR · Assignments · Secondary structure

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Biological context

Sup35pNM is an N-terminal fragment of the 685 amino-acid protein Sup35 of *Saccharomyces cerevisiae* that has prion properties at the origin of the $[PSI^+]$ phenotype (Cox 1965; Wickner et al. 1995; Krzewska and Melki 2006; Krzewska et al. 2007). It spans residues 1–253 of Sup35p and comprises the N-terminal domain, essential for prion formation (DePace et al. 1998), as well as the middle domain (M-domain) whose function is yet unknown. While the N-terminal domain (N-domain) is rich in glutamine and asparagine residues, the M-domain consists mainly of charged residues. Sup35pNM forms self-seeding amyloid fibrils in vivo and in vitro under physiological pH and salt concentrations. Sup35pNM is the most extensively studied fragment of the Sup35p prion (Glover et al. 1997; Chien et al. 2005; Krishnan and Lindquist 2005; Toyama et al. 2007; Foo et al. 2011; Verges et al. 2011; Halfmann et al. 2011). Nevertheless, structural studies on the molecular level of Sup35pNM fibrils are scarce since they are not amenable to X-ray crystallography or solution NMR. We present here the solid-state NMR sequential assignments of this protein, as well as a secondary structure analysis, of the well-ordered, rigid parts of the protein. The assignment of the mother protein, namely the full-length prion Sup35p, is described in a companion paper (Schütz et al. 2013). A detailed comparison and a biophysical interpretation of the differences will be presented elsewhere (Luckgei et al. 2013).

Methods and experiments

Protein expression and purification; sample preparation

The protein was expressed and purified as shortly summarized here, and described in detail in (Krzewska and

Melki 2006; Krzewska et al. 2007). The protein has an N-terminal His-tag for purification purposes (for the full amino acid sequence see Fig. 1), and was labeled with ^{13}C and ^{15}N in M9 medium. For fibrillization, Sup35pNM (in 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 pelleted at 100,000g in a TL-100 tabletop centrifuge for 20 min at 4 °C. The fibrils were resuspended in distilled water, washed twice, and filled into 3.2 mm rotors by ultracentrifugation as described (Böckmann et al. 2009).

NMR spectroscopy

We used a suite of 2D and 3D experiments, namely 2D DARR and DREAM, and 3D NCACB, NCACO, NCACX, NCOCA, NCOCX, CANCO and CCC experiments to perform the assignment as described in detail in references (Schuetz et al. 2010; Habenstein et al. 2011). Each 3D spectrum took between 2 and 5 days to be recorded. The sequential walk was achieved by connecting resonances from NCACO/NCACX, CANCO and NCOCA/NCOCX spectra. Side-chain assignments were performed using NCACB, NCACX, NCOCX and CCC spectra. Full experimental details are given in Table 1. All assignment spectra were recorded on the same sample. However, 2D DARR spectra were recorded for two different preparations to verify reproducibility of the sample preparation.

All spectra were recorded on a Bruker Avance II+850MHz spectrometer operating at a static magnetic field of 20 T. A 3.2 mm Bruker triple-resonance MAS probe equipped with an LLC coil was used to reduce r.f. heating during the experiments. The spectra were recorded at sample temperatures between 5 and 10 °C. The pulse sequences were implemented as recently reported (Schuetz et al. 2010). All spectra were processed using TopSpin 2.0 (Bruker Biospin) by zero filling to the next power of two and the time domain signals were apodized with a squared

cosine function. Spectra were analyzed and annotated using the CcpNmr Analysis package (Fogh et al. 2002; Vranken et al. 2005; Stevens et al. 2011).

Assignment and data deposition

Figure 2 shows the 2D NCA and NCO spectra of Sup35pNM. The most intense correlations could be sequentially assigned and are labeled. A sequential assignment was possible for those signals that lead to sufficiently strong signals in the 3D spectra, which indeed reveal only a small subset of the resonances of the residues of the NM domains, due to static and/or dynamic disorder for the remaining parts of the protein. The line widths of the resonances detected in the 3D spectra are mostly between 0.5 and 1 ppm, and the spectra show a good resolution and dispersion (Fig. 3). Assignments were established for all residues with corresponding signals present in the NCACO/CX, CANCO and NCOCA/CX spectra (Schuetz et al. 2010; Habenstein et al. 2011).

To illustrate sequential assignments, a representative plane of the 3D NCACB, NCACO, CANCO and NCOCA experiments is shown in Fig. 3. Shifts for 22 residues could be assigned, which are all located in the N-terminal prion domain. The chemical shifts have been deposited in the BMRB under the accession number 18406.

As a probe for flexibility, highly dynamic residues generally can be detected in INEPT spectra without homonuclear proton decoupling during polarization transfer. For Sup35pNM, all amino-acid types with the exception of Arg, Phe and Tyr could be observed in this scalar-coupling based spectrum, as shown in Fig. 4. The absence of Arg in the spectra coincides with the absence of Arg residues in the M-domain. Also, all residue-types present in the M-domain show signals in the INEPT spectrum.

Assigned resonances are shown in the assignment graph in Fig. 5. As shown in Fig. 2, most strong cross peaks in the 2D NCA spectrum can be explained based on this assignment.

Fig. 1 Amino-acid sequence of the Sup35pNM construct used. The tag is shown in grey, the N domain in light blue, and the M domain in deep blue

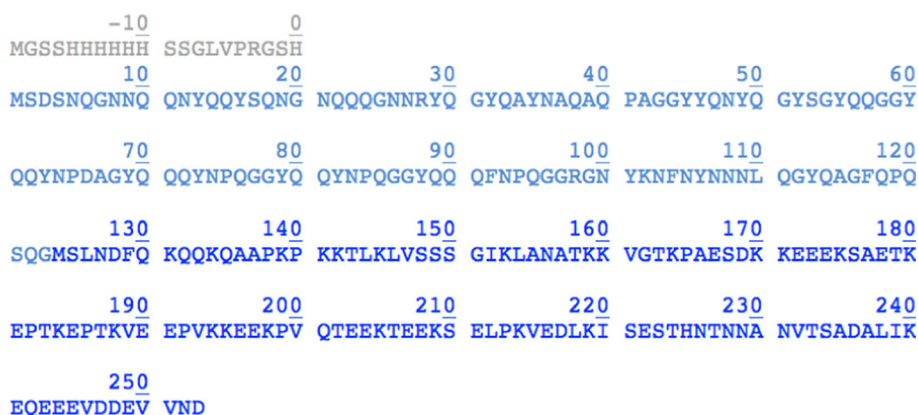


Table 1 Experimental parameters

Experiment	DARR 20 ms	DARR 100 ms	NCA	NCO	INEPT
MAS frequency [kHz]	18	17.5	17.5	17.5	18
Transfer 1	HC-CP	HC-CP	HN-CP	HN-CP	INEPT
Field [kHz]- ¹ H	64.7	72.6	55.0	64.7	62.5
Field [kHz]- ¹³ C/ ¹⁵ N	55.5	63.7	41.6	41.6	50.0
Shape	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Hard pulses
¹³ C carrier [ppm]	55.6	55.6	–	–	–
Time [ms]	0.5	0.5	0.8	1	–
Transfer 2	MIRROR	MIRROR	NC-CP	NC-CP	–
Field [kHz]- ¹ H	5.4	6.1	101.5	101.5	–
Field [kHz]- ¹³ C	–	–	10.9	10.9	–
Field [kHz]- ¹⁵ N	–	–	6.6	8.2	–
Shape	–	–	Tangent ¹³ C	Tangent ¹³ C	–
¹³ C carrier [ppm]	–	–	61.2	178.6	–
Time [ms]	20	100	6	6	–
t ₁ increments	1,024	1,216	512	256	192
Sweep width (t ₁) [kHz]	66.667	80	20	10	10
Max. acq time (t ₁) [ms]	7.68	7.60	12.80	12.80	9.60
t ₂ increments	2,048	2,048	2,048	2,560	2,048
Sweep width (t ₂) [kHz]	100	100	100	100	100
Max. acq time (t ₂) [ms]	10.24	10.24	10.24	12.80	10.24
t ₃ increments	–	–	–	–	–
Sweep width (t ₃) [kHz]	–	–	–	–	–
Max. acq time (t ₃) [ms]	–	–	–	–	–
¹ H Spinal64 Decoupling power [kHz]	100	100	100	100	76.9
Inter-scan delay [s]	2.5	2.5	2	2	2
Number of scans	8	24	8	8	8
Total measurement time [h]	5.8	21.2	2.3	1.1	0.9

Experiment	NCACB	CANCO	NCOCA	CCC	NCACO	NCACX	NCOCX
MAS frequency [kHz]	17.5	17.5	17.5	17.5	17.5	17.5	17.5
Transfer 1	HN-CP	HC-CP	HN-CP	HC-CP	HN-CP	HN-CP	HN-CP
Field [kHz]- ¹ H	55.0	69.3	67.8	72.6	59.7	67.8	67.8
Field [kHz]- ¹³ C/ ¹⁵ N	41.6	59.5	43.5	63.7	46.7	44.0	43.5
Shape	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H	Tangent ¹ H
Carrier [ppm]	–	48.5	–	–	–	–	–
Time [ms]	0.8	0.6	1.2	0.5	0.8	1.2	1.2
Transfer 2	NC-CP	CN-CP	NC-CP	DREAM	NC-CP	NC-CP	NC-CP
Field [kHz]- ¹ H	101.5	116.6	82.5	101.5	101.5	102.7	82.5
Field [kHz]- ¹³ C	10.9	11.0	11.0	8.3	10.5	11.0	11.0
Field [kHz]- ¹⁵ N	6.6	6.8	8.1	–	8.3	7.2	8.1
Shape	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	Tangent ¹³ C	tangent ¹³ C	tangent ¹³ C	tangent ¹³ C
Carrier [ppm]	61.2	61.6	178.6	55.0	57.9	57.9	178.6
Time [ms]	6	8	5	4	5.5	6.5	5
Transfer 3	DREAM	NC-CP	MIRROR	MIRROR	MIRROR	MIRROR	MIRROR
Field [kHz]- ¹ H	–	–	21.8	3.4	21.3	3.2	21.8
Field [kHz]- ¹³ C	8.0	11.0	–	–	–	–	–
Field [kHz]- ¹⁵ N	–	8.2	–	–	–	–	–
Shape	tangent ¹³ C	tangent ¹³ C	–	–	–	–	–
Carrier [ppm]	59.0	178.6	–	–	–	–	–

Table 1 continued

Experiment	NCACB	CANCO	NCOCA	CCC	NCACO	NCACX	NCOCX
Time [ms]	4	6	80	200	20	80	80
T ₁ increments	128	128	80	216	80	80	80
Sweep width (t ₁) [kHz]	6.8	10	4	20	4	4	4
Max. acq time (t ₁) [ms]	9.4	6.4	10	5.4	10	10	10.00
t ₂ increments	96	96	96	216	96	128	96
Sweep width (t ₂) [kHz]	9.6	4.8	8	20	8	10	8
Max. acq time (t ₂) [ms]	5	10	6	5.4	6	6.4	6.00
t ₃ increments	1,536	2,048	2,048	2,048	2,048	2,048	2,048
Sweep width (t ₃) [kHz]	100	100	100	100	100	100	100
Max. acq time (t ₃) [ms]	7.3	10.3	10.3	10.3	10.3	10.3	10.0
¹ H Spinal64 Decoupling power [kHz]	100	100	100	100	100	100	100
Interscan delay [s]	2.22	2.45	2.3	1.9	2.3	1.94	2.3
Number of scans	8	8	16	4	16	8	16
Total measurement time [h]	61.2	67.7	81.9	109.6	79.9	46.4	81.7

All spectra were recorded with 850 MHz WB Bruker spectrometer between 5 and 10 °C

Fig. 2 2D NCA and NCO spectra of Sup35pNM with the sequentially assigned residues labeled. The spectra were acquired at 850 MHz with MAS at 17.5 kHz

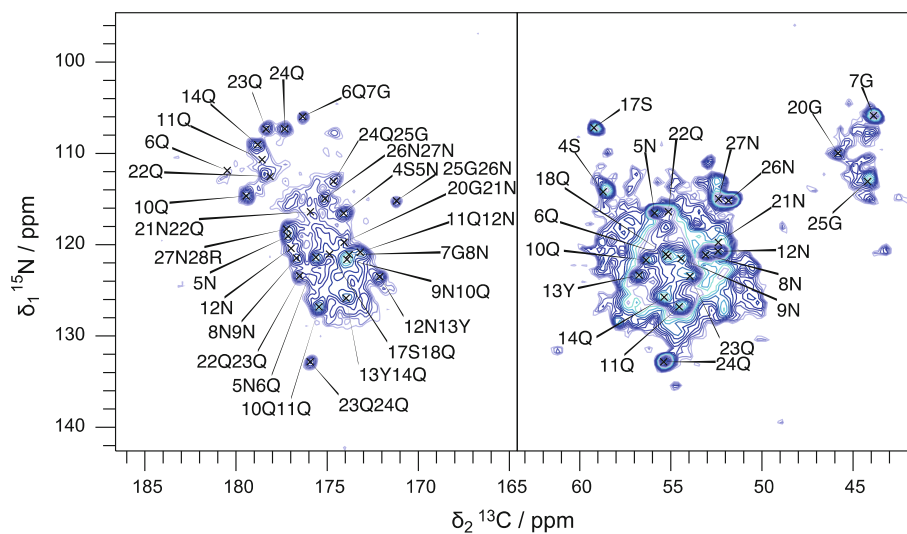
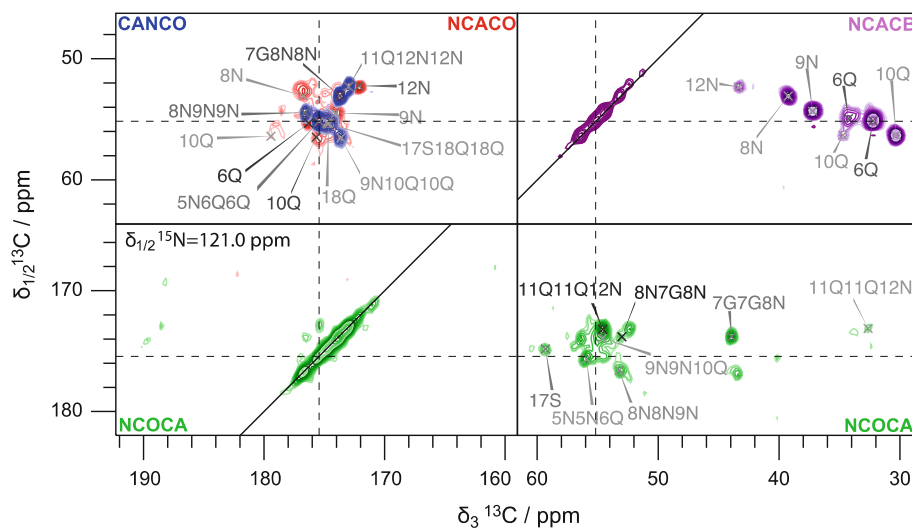


Fig. 3 Representative planes of 3D NCACB, NCACO, CANCO and NCOCA spectra of Sup35pNM with the sequentially assigned residues labeled. Residues labeled in grey have the center of the peak in another plane. Dotted lines highlight the connection between Q6 and N5



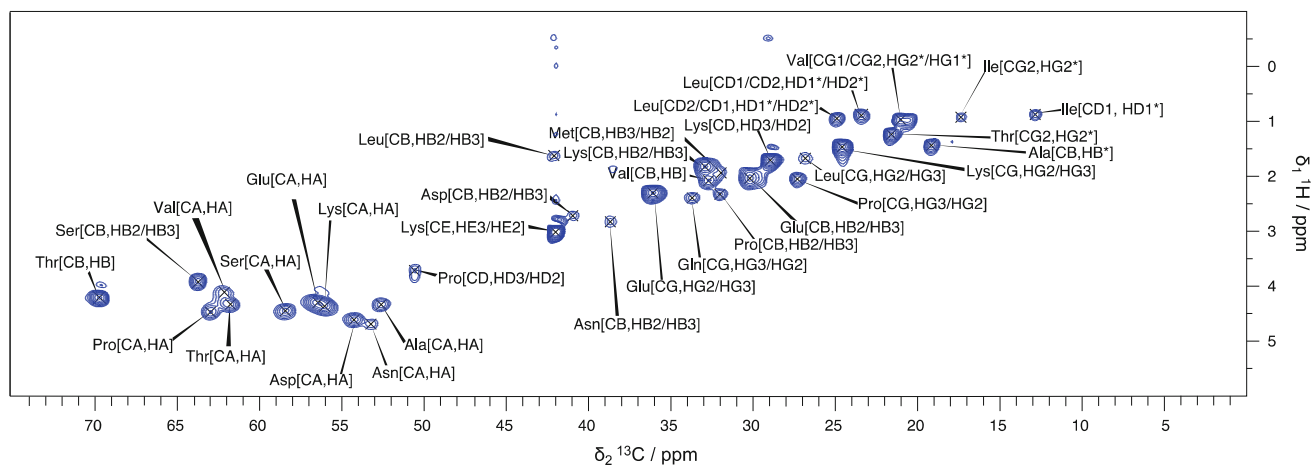


Fig. 4 INEPT spectrum of Sup35pNM. Signals are assigned to residue types according to the random coil chemical shifts (Wang and Jardetzky 2002)

Fig. 5 Assignment graph (created using the CcpNmr software (Fogh et al. 2002; Vranken et al. 2005; Stevens et al. 2011)) of the N-terminal amino acids of Sup35pNM

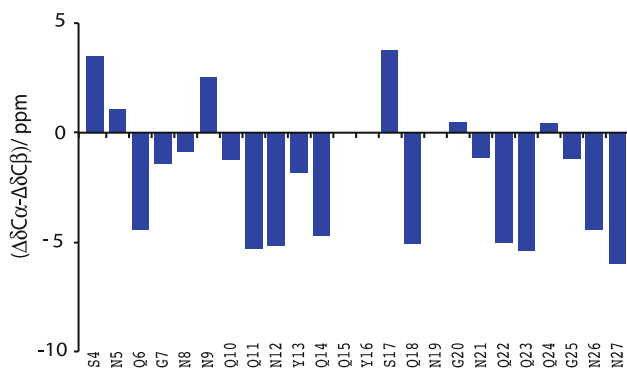
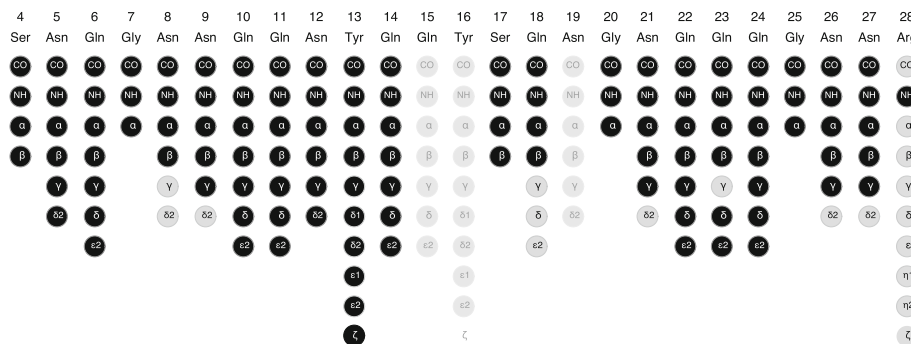


Fig. 6 Secondary chemical shifts for Sup35pNM. Secondary chemical shifts have been calculated as given in (Wishart and Sykes 1994) and we report the difference between C α and C β secondary shifts (Luca et al. 2001). Three or more negative values in a row suggest β -strand conformation. Isolated positive or negative values for a given residue suggest turn or random-coil conformation. For Gly, the secondary shift of C α is plotted

The secondary chemical shifts for the assigned residues are given in Fig. 6. The assigned residues mostly display secondary chemical shifts typical for β -strand conformation. For glycines, only $\Delta\delta C\alpha$ is plotted.

The sequential assignments of the rigid and well-ordered amino acids of Sup35pNM represent a first step towards the structural analysis of this prion.

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