Biochemical and Functional Analysis of the Assembly of Full-length Sup35p and Its Prion-forming Domain*

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2 The abbreviation used is: eRF, eukaryotic release factor.

The protein Sup35 has prion properties. Its aggregation is at the origin of the [PSI+] trait in Saccharomyces cerevisiae. In vitro, the N-terminal domain of Sup35p alone or with the middle domain assembles into fibrils that exhibit the characteristics of amyloids. The vast majority of in vitro studies on the assembly of Sup35p have been performed using Sup35pNM, as fibrils made of Sup35pNM assembled in vitro propagate [PSI+] when reintroduced into yeast cells. Little is known about the assembly of full-length Sup35p and the role of the functional C-terminal domain of the protein. Here we report a systematic comparison of the biochemical and assembly properties of full-length Sup35p and Sup35pNM. We show that the native structure of the C-terminal domain is retained within the fibrils. We determined the size of Sup35p nuclei and the critical concentration for assembly that both differ from that of Sup35pNM. We demonstrate that Sup35pNM co-assembles with the full-length protein and that fibrils made of Sup35p or Sup35pNM seed the assembly of soluble Sup35pNM and Sup35p with different efficiencies. Finally, we show that fibrils made of full-length Sup35p induce with higher efficiency [PSI+] appearance as compared with those made of Sup35pNM. Our findings reveal differences and similarities in the assembly of Sup35p and its NM fragment and validate the use of Sup35pNM in studying some aspects of Sup35p aggregation but also underline the importance of using full-length Sup35p in studying prion propagation both in vivo and in vitro.

The eukaryotic release factor (eRF3 or Sup35p) mediates together with eRF1 (Sup45p) translation termination of protein biosynthesis in the yeast Saccharomyces cerevisiae (1–3). In the [PSI+] prion state, insoluble aggregates of Sup35p lead to altered translation termination, as manifested by an increased tendency of ribosomes to read through nonsense ochre stop codons (4, 5). The self-propagating [PSI+] exhibits a range of phenotypic strain variants that possess different mitotic stabilities, stop codon suppression capacities, and susceptibility to cellular molecular chaperones, possibly as a consequence of different prion structures (6, 7).

Sup35p consists of a 685-amino acid polypeptide chain that can be divided into three distinct regions. The N-terminal domain, extending from residue 1 to 123 (Sup35pN), is enriched in uncharged polar residues and is indispensable for prion behavior. The conserved and highly charged middle region (Sup35pM) has probably a structural function. It modulates [PSI+] propagation and extends from amino acid residues 124 to 253 (8). The N and M domains are not required for translation termination or cell viability, whereas the C-terminal region is the functional domain of the protein, providing translation termination activity (2, 9). Fibrils formed from Sup35pN alone or together with the middle domain (Sup35pNM) exhibit the characteristics of amyloids in that they (i) are unbranched, (ii) have increased resistance to proteolysis, (iii) bind the dyes Congo red and thioflavin T, (iv) assemble in a cooperative manner following a nucleation growth process that can be greatly facilitated with preformed fibrils, and most important, (v) exhibit the 4.7 Å reflection in x-ray fiber diffraction images typical of amyloids (11–14). Essential for viability and for translation termination, the C-terminal region of Sup35p contains GTP- and eRF1-binding motifs (2, 3, 15–19). Sup35p possesses a low intrinsic GTPase activity that is strongly stimulated by the synergistic action of eRF1 and the ribosome and is essential for its association with Sup45p, recognition of translation termination signals by the latter, and efficient cleavage of the ester bond linking the polypeptide chain to the tRNA leading to polypeptide chain release from the ribosome (20–23).

In vitro, under physiologically relevant conditions, full-length Sup35p, Sup35pNM, and Sup35pN assemble into protein fibrils in a concentration-dependent manner (11, 12, 24). The self-assembly of Sup35p and Sup35pNM is a cooperative process where one can distinguish a lag phase where nucleation occurs followed by an elongation phase where assembly accelerates preceding the onset of a steady state. The lag phase is shortened significantly upon increasing the protein concentra-
tion or seeding the reaction with preformed fibrils, further demonstrating the cooperative character of the assembly reaction (11, 24). In the lag phase preceding assembly, heterogeneous oligomers containing 20 – 80 Sup35pNM molecules were observed and have been proposed to act as nucleating units in the polymerization pathway. These particles have been proposed to give rise to fibrils following a linear colloidal aggregation process (13, 25). However, later kinetic studies indicate that the smallest stable amyloid-forming species are made of no more than three Sup35pNM molecules and that the fibrils grow by addition of monomeric Sup35pNM to their ends (26).

The majority of in vitro studies on the assembly of Sup35p have been performed using its prion-determining region (Sup35pNM) as fibrils made of Sup35pNM assembled in vitro propagate [PSI+] when reintroduced in yeast cells (27, 28). Little is known about the assembly of full-length Sup35p. It is therefore not clear whether the functional C-terminal domain of the protein affects assembly. We set up a systematic comparison of the biochemical and assembly properties of full-length Sup35p, as Sup35p is not degraded in cells exhibiting the [PSI+] trait. Our findings validate the wide use of Sup35pNM as an adequate tool for studies of the nature of amyloids and some aspects of the Sup35p aggregation process. However, we present here differences in the assembly of Sup35p and its NM fragment underlining the usefulness of full-length Sup35p in studying prion propagation both in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Full-length Sup35p was cloned and purified as reported previously (24). A PCR product of the NM fragment was cloned as NdeI-BamHI fragment into pET15b vector. Sup35pNM was overexpressed in Escherichia coli strain BL21-CodonPlus, in Luria Bertani medium supplemented with chloramphenicol (34 μg/ml) and carbenicillin (100 μg/ml) at 30 °C. Protein expression was induced with 1 mM isopropyl-1-thio-D-galactopyranoside at A600 = 0.5–0.7. The bacterial pellet was resuspended in buffer A (8 M urea, 20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol) and disrupted by sonication. The lysate was clarified by centrifugation and loaded directly onto the chelating Sepharose column (Amersham Biosciences) equilibrated in buffer A. Bound proteins were eluted with linear gradients of imidazole (20–500 mM). Fractions containing Sup35pNM were concentrated (VivaSpin 10,000 concentrators) and loaded onto the Sephacryl S-100 column (Amersham Biosciences) equilibrated in buffer B (8 M urea, 20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 5 mM β-mercaptoethanol). Fractions containing Sup35pNM were further concentrated and stored at −80 °C.

Size Exclusion Chromatography—Sup35p was dialyzed against the assembly buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol, 5 mM β-mercaptoethanol, 10 mM MgCl2) and loaded onto Superose 12 HR 10/30 column (Amersham Biosciences) equilibrated in assembly buffer. Chromatography was carried out at a flow rate of 0.7 ml/min at 8 °C. Fractions (600 μl) were collected and analyzed by SDS-PAGE.

Sedimentation Velocity—Sedimentation velocity experiments were carried out in a Beckman Optima XL-A analytical ultracentrifuge equipped with a 60 Ti four-hole rotor and cells with two-channel 12-mm path length centerpieces. Samples were centrifuged at 40,000 and 60,000 rpm at 10 °C. Radial scans of absorbance at 276 nm were taken at 6-min intervals. Data analysis to provide the apparent distribution of sedimentation coefficients was performed using the software SVEDEBERG and DCDT+ (29).

GTPase Activity Measurements—GTP hydrolysis at 20 °C was measured in assembly buffer containing 1 mM [γ-32P]GTP by extraction of the [32P]phosphomolybdate complex formed in 1N HCl as described (30).

Assembly of Sup35 into Protein Fibrils—Sup35p was dialyzed against the assembly buffer. To remove urea, Sup35pNM was loaded onto NAP-10 desalting column (Amersham Biosciences) equilibrated in the assay buffer. Sup35p was supplemented with GTP and an ATP/GTP-regenerating system (10 mM creatine phosphate, 100 μg/ml creatine kinase). The reaction mixtures were incubated at 7 °C with gentle agitation. At regular time intervals, aliquots were removed and the assembly reaction was monitored using a Thioflavin T binding assay (31). Thioflavin T binding was measured by averaging the emission signal over 60 s using a Quantamaster QM 2000–4 spectrofluorometer (Photon Technology International, Inc., Lawrenceville, NJ). The lag time was estimated from the first derivative of the assembly kinetics.

Preparation of Spheroplasts for Transformation and Infectivity Assays—Spheroplasts of [psi−] yeast strain were prepared and transformed as described previously (27). Sup35 and Sup35pNM were assembled as described above. The fibrils were collected by centrifugation at 20,000 × g for 20 min at the early stages of steady state, resuspended with 10 mM Tris buffer (pH 7.5) containing 1 M sorbitol and 10 mM CaCl2, and sonicated. The final concentration of fibril particles in infection experiments was 3 μM.

Electron Microscopy—Samples of fibrillar Sup35p and Sup35pNM were negatively stained on carbon-coated grids (200 mesh) with 1% uranyl acetate and examined in a Philips EM 410 electron microscope.

Circular Dichroism Measurements—Far-UV CD spectra were recorded on a J810 (Jasco) spectropolarimeter using a 0.2-mm path length cuvette. Ten independent scans were recorded at 20 °C in the assembly buffer at a protein concentration of 0.8 mg/ml. Spectra were then overlaid and averaged.

SDS-PAGE—SDS-PAGE was performed on 10% polyacrylamide gels (14 × 15 × 0.15 cm) following the standard method of Laemmli (32). The gels were stained with Coomassie Blue, destained, imaged using a Sony CCD camera, and further analyzed on a Macintosh computer (Apple Computer, Inc., Cupertino, CA) using NIH image software (available at rsb.info.nih.gov/nih-image).

RESULTS

Secondary and Quaternary Structures of Soluble Full-length Sup35p—Recombinant full-length Sup35p has a molecular mass of 74 kDa upon analysis by SDS-PAGE (Fig. 1A), consistent with the calculated mass of full-length Sup35p (76,551 Da), and elutes from Superose 12 gel filtration columns in a single peak with apparent mass of 130 kDa (Fig. 1B). To investigate whether the elution behavior of Sup35p from the size exclusion...
column is due to its oligomerization or to its behavior as a non-globular protein, we carried out sedimentation velocity experiments and analyzed them to yield the apparent distribution of sedimentation coefficients, $g(s)$. Measurements were done at 8 and 5 $\mu$M of Sup35p and Sup35pNM. The raw distribution of sedimentation coefficients (symbols in Fig. 1C) were fitted by nonlinear least squares procedures (solid lines) as described by Philo (29). The data fit very well to a single-component system with sedimentation coefficients of 2.85 and 1.8 $s$ for Sup35p and Sup35pNM, respectively. No concentration-dependent oligomerization was observed. The sedimentation coefficient of 1.9 $s$ previously reported for Sup35pNM (26) is consistent with that that we measured. The sedimentation coefficient of Sup35p is low and suggests that the protein has an elongated shape with a calculated friction coefficient 1.807.

The overall secondary structure of Sup35p and Sup35pNM were probed by circular dichroism (CD) spectroscopy (Fig. 1D). Sup35pNM displays a far-UV CD signal representative of a molecule rich in random coils, in agreement with previous observations (33), whereas Sup35p is $\alpha$-helical. The CD difference spectrum (not shown) of Sup35p and Sup35pNM confirms the high $\alpha$-helical content of the C-terminal domain of Sup35p.

The Sup35p Fibrils Retain a Native C-terminal Domain—Sup35p assembles into long straight fibrils that are 25 nm wide and over 1 $\mu$m long (24). Functional Sup35p binds and hydrolyzes GTP (3, 9, 21, 22). To determine whether the native structure of Sup35p is retained within the fibrils, the ability of Sup35p to bind and hydrolyze GTP was assessed during assembly (Fig. 2A). The data clearly show that GTP hydrolysis occurs during the assembly of Sup35p at steady state and at a steady rate. In contrast, no GTPase activity is detected during the assembly of Sup35pNM. To further compare the GTPase activities of soluble and fibrillar Sup35p, we monitored GTP hydrolysis of soluble and preassembled Sup35p at identical protein concentrations. The data presented in Fig. 2B show that nucleotide exchange occurs at the same rate on the two forms of the protein. Following exchange, GTP is hydrolyzed at very similar rates by soluble and fibrillar Sup35p. The specific activities, calculated from the slopes of the linear rates of hydrolysis, for Sup35p during the early and late stages of assembly, are 6.5 and 7.8 min$^{-1}$, respectively. These data suggest that fibrils of Sup35p contain native-like catalytic/functional domains.

It is worth noting that the assembly kinetics of Sup35p into fibrils, monitored using thioflavin T binding, in the absence and the presence of GTP are identical. Furthermore, the fibrils appear morphologically indistinguishable in the electron microscope (data not shown).

The Critical Concentrations of Sup35p and Sup35pNM Assemblies Are of the Same Order of Magnitude—To further characterize the assembly process and its dynamic aspect, we determined the critical concentrations of Sup35p assembly into fibrils and compared this constant with that of Sup35pNM under the same experimental conditions. The kinetic parameters for elongation of Sup35p and Sup35pNM fibrils can be determined using Oosawa’s plot (34) of the monomer concentration dependence of the rate of growth, $J(c)$. The following equation describes polymer growth: $J(c) = dc/dt = k_+ [P]c - k_- [P]$ in the case of a reversible polymerization. $c$ and $P$ represent the concentrations of monomer and polymer elongating sites, respectively; $k_+$ and $k_-$ are the rate constants for monomer association to and dissociation from polymer ends. According to this equation, $k_-$ is easily derived from the slope of the plot, while $k_+$ is given by the extrapolation of $J(c)$ at $c = 0$. The critical concentration is given by solving $J = 0$ (intercept on the $c$ axis). In the case of an irreversible polymerization, which is the case for Sup35p assembly as fibrillar Sup35p does not disassemble upon dilution, the above equation becomes $J(c) = k_+ [P]c$. Fig. 3 shows the initial growth rates of preformed Sup35p and Sup35pNM fibrils (1 $\mu$m) followed by thioflavin T binding in the presence of increasing concentrations of soluble Sup35p and Sup35pNM (2–6 $\mu$m). Control reactions, where both soluble proteins (6 $\mu$m) were incubated under the same experimental conditions with no added nuclei, demonstrate the absence of de novo nucleation and assembly throughout the measurements. The $J(c)$ plots derived from the data in Fig. 3, A and B, are presented in Fig. 3C. The critical concentrations of Sup35p and Sup35pNM are 2.5 and 0.8 $\mu$m, respectively.

3 J. Krzewska and R. Melki, unpublished observations.
Size of Sup35p Nuclei—The nucleus is a stable oligomer that has equal probabilities of lateral or longitudinal interaction with an additional molecule (34). The formation of nuclei occurs during the lag phase preceding Sup35p assembly. The size of the nucleus is equal to $2^m/H_{11002}$, where $m$ is the value of the slope of the log/log plot of the lag time versus the concentration of assembling proteins. The lag time that precedes Sup35p assembly is highly dependent on Sup35p concentration.

FIGURE 2. GTPase activity of soluble and fibrillar Sup35p. A, GTP hydrolysis was monitored for 80 h during the time course of Sup35p assembly into protein fibrils. The hydrolysis of $[\gamma-^{32}P]$GTP in the presence of $5 \mu M$ Sup35p (filled circles) and Sup35pNM (filled squares) was assayed as described under “Experimental Procedures” at 7 °C in assembly buffer during Sup35p and Sup35pNM polymerization. The assembly kinetics of Sup35p (filled circles) and Sup35pNM (filled squares), monitored using thioflavin T binding, are shown in the inset. A.U., arbitrary units. The error bars indicate the S.D. in three independent measurements. B, GTPase activity of soluble (filled circles) and preassembled (filled squares) Sup35p at 20 °C in assembly buffer following addition of $[\gamma-^{32}P]$GTP at time zero to soluble and fibrillar Sup35p.

FIGURE 3. Kinetic determination of the critical concentration of Sup35p and Sup35pNM assembly into fibrils. A, time course of seeded assembly of soluble Sup35p, $1 \mu M$ (filled circles), $3 \mu M$ (filled squares), $4 \mu M$ (filled diamonds), $5 \mu M$ (filled triangles), and $6 \mu M$ (half-filled squares), using preformed Sup35p fibrils ($1 \mu M$). B, time course of seeded assembly of soluble Sup35pNM, $1 \mu M$ (filled circles), $3 \mu M$ (filled squares), $4 \mu M$ (filled diamonds), and $5 \mu M$ (filled triangles), using preformed Sup35pNM fibrils ($1 \mu M$). The elongation reactions were carried out at 7 °C in assembly buffer and were monitored using thioflavin T binding, A.U., arbitrary units. The error bars in panels A and B indicate the S.D. in two independent measurements. C, $J(c)$ plot for Sup35p and Sup35pNM assemblies showing the linear dependence of the rate of fibril elongation on Sup35p (filled squares) or Sup35pNM (filled circles) concentrations. The intercepts of the plots on the abscissa are the critical concentrations.
Sup35p Fibril Formation

Figure 4. Estimation of the size of Sup35p and Sup35pNM nuclei in the early stages of assembly. A and B, dependence of the lag time preceding assembly on the concentration of Sup35p and Sup35pNM, respectively. A, soluble Sup35p, 5 μM (filled circles), 6 μM (filled squares), 8 μM (filled diamonds), 9 μM (filled triangles), and 10 μM (half-filled squares). B, soluble Sup35pNM, 6 μM (filled squares), 8 μM (filled diamonds), 9 μM (filled triangles), and 10 μM (half-filled squares), 11 μM (filled circles), and 12 μM (filled reversed triangles). Samples were incubated at 7 °C in assembly buffer, and polymerization was monitored using thioflavin T binding. A.U., arbitrary units. The error bars in panels A and B indicate the S.D. in two independent measurements. C, dependence of the lag time, determined from the assembly reactions presented in panels A and B, on the concentration of Sup35p (filled squares) or Sup35pNM (filled circles). The log of lag time is plotted as a function of the log protein concentration.

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centration and decreases from 26 to 3 h upon increasing the protein concentration from 5 to 12 μM (Fig. 4A). The value of \( m \) derived from the kinetics presented in Fig. 4A is 3.4 (Fig. 4C); thus, Sup35p nuclei are made of six molecules. In comparison, the value of \( m \) derived from the data obtained under the same experimental conditions for Sup35pNM (Fig. 4B) is 2 (Fig. 4C), indicating that Sup35pNM nuclei are made of three molecules. The latter observation is fully consistent with results obtained under slightly different experimental conditions (26). The finding that full-length Sup35p and Sup35pNM nuclei differ in size might either reflect structural differences or differences in their intrinsic stabilities.

The Elongation Rate of Sup35pNM Fibrils Is Significantly Greater than That of Full-length Sup35p Fibrils—To determine whether the difference in the size of Sup35p and Sup35pNM nuclei reflects a difference in their intrinsic stability or structural features, the elongation rates of increasing concentrations of preformed Sup35p and Sup35pNM fibrils in the presence of a constant amount of full-length Sup35p (Fig. 5A) and Sup35pNM (Fig. 5B) were compared. As expected, the elongation rates of the different fibrils vary linearly with the amount of added seeds in the range 0.1–2 μM (Fig. 5C). However, when the rates for Sup35p and Sup35pNM are compared a 4.5-fold greater seeding activity of Sup35pNM is observed as compared with that of full-length Sup35p (Fig. 5C).

To further reveal potential structural differences in Sup35p and Sup35pNM fibrils, cross-seeding experiments where fibrils of full-length Sup35p were used to induce the assembly of soluble Sup35pNM, and vice versa, were carried out (Fig. 6). A seeding activity is observed in all cases; however, the seeding capacity of fibrillar Sup35pNM in the presence of soluble Sup35pNM is 2.5-fold higher than that in the presence of full-length Sup35p. Similarly, the seeding capacity of fibrillar Sup35p in the presence of soluble Sup35pNM is 2.5 lower than that of fibrillar Sup35pNM. These results suggest either that fibrillar Sup35pNM and Sup35p differ structurally or that the rearrangements of the C-terminal domain or local rearrangements within this domain in full-length Sup35p constitute a limiting reaction in the assembly process. To further document the structural differences in the assembly of Sup35p and Sup35pNM, the soluble forms of the two polypeptides (5 μM) were mixed and their assembly compared with that of Sup35p and Sup35pNM (5 and 10 μM). The data presented in Fig. 7A clearly show that the assembly kinetic of mixed Sup35p and Sup35pNM is identical to that of full-length Sup35p (10 μM) and differs significantly from that of Sup35pNM (10 μM). When the fibrils obtained upon mixing soluble Sup35p and Sup35pNM are examined in the electron microscope, they are indistinguishable from that made of full-length Sup35p (Fig. 7, B–D). We conclude from these observations that soluble Sup35p and Sup35pNM co-assemble efficiently. We further conclude that the incorporation of Sup35pNM into mixed fibrils yields polymers that possess overall the shape of fibrils made of full-length Sup35p.

Fibrillar Sup35p Induces [PSI⁺] Appearance—The overexpression of Sup35p in vivo leads to induction of a range of [PSI⁺] prion strains that are distinguishable based on (i) the efficiency of suppression of the stop codon in ADE1 gene
that shows when the cells are grown on a rich medium through the colony color (red or various shades of pink) and (ii) their stability (homogeneous versus sectored colonies) (5). To establish whether Sup35p fibrils induce \( \text{PSI}/H11001 \) appearance in a manner similar to Sup35pNM, we carried out spheroplast transformation experiments of \( \text{psi}/H11002 \) cells containing a nonsense mutation in the \( \text{ade1} \) gene by preformed Sup35p and Sup35pNM fibrils (26). Co-transformation with \( \text{URA3} \) plasmid allowed selection of the fraction of yeast that had taken up material from solution. Transformants were selected on plates that were devoid of uracil. Ura\(^+\) colonies were streaked on modified YEPD plates where \( \text{psi}/H11002 \) and \( \text{PSI}/H11001 \) yeast show red and white (strong) or pink (weak) color phenotypes, respectively. The infection efficiencies of full-length Sup35p and Sup35pNM fibrils (3\% M) determined from counting the white and red/pink colonies are 27\% and 11\%\(^2\), respectively (Fig. 8). Preformed Sup35p fibrils not only display higher infectivity as compared with Sup35pNM but also induce strong \( \text{PSI}/H11001 \) with a higher frequency. Indeed, 64\% of the colonies exhibited a strong \( \text{PSI}/H11001 \) phenotype when transformed by fibrils made of full-length Sup35p in contrast with only 10\% for Sup35pNM (not shown).

**DISCUSSION**

The vast majority of *in vitro* studies on the assembly of Sup35p have been carried on so far using Sup35pNM fragment. The prion-determining region of Sup35pNM is certainly an important tool for studies of the nature of amyloids, measurements. C, linear dependence between the rates of elongation, calculated from the slopes of the elongation reaction shown in panels A and B, and the amounts of fibrillar Sup35p (filled circles) and Sup35pNM fibrils (filled squares).
Sup35p fibril formation. The finding that Sup35p nuclei are composed of six molecules whereas that of Sup35pNM are made of three molecules indicates that the two kinds of nuclei have different geometries. This might reflect a different packing of Sup35pNM domains, either free or when attached covalently to the C-terminal domain of the protein. Alternatively, this could also be due to a direct contribution of the C-terminal domain to Sup35p oligomerization. We also showed that the elongation rate of preformed Sup35pNM seeds is significantly greater than that of full-length Sup35p. When fibrillar Sup35pNM is used to seed the assembly of soluble Sup35pNM and full-length Sup35p, a higher cross-seeding capacity is observed in a homologous, as compared with a heterologous, system. This suggests either that fibrillar Sup35pNM and Sup35p differ structurally or that local rearrangements within the C-terminal domain in full-length Sup35p constitute a limiting reaction in the assembly process. Furthermore, it is worth noting that whereas soluble Sup35p is purified under native conditions, recombinant Sup35pNM is recovered from inclusion bodies and refolded from denaturant. Although the overall secondary structure and assembly kinetics of Sup35pNM purified under denaturing and non-denaturing conditions are similar (33), it is possible that the ensemble of conformational states adopted by Sup35pNM following its renaturation differs to some extent from that of the NM domain in full-length Sup35p. In that case, the observed differences in cross-seeding capacities would reflect either a poorly populated, assembly-competent conformation of the NM domain in full-length Sup35p, a limiting step upon displacement of the equilibrium between the assembly-incompetent and assembly-competent conformations toward the latter form, or both. Overall, the data presented here suggest that the differences in the assembly process of Sup35p and Sup35pNM are worth being taken into account when drawing general conclusions on Sup35p assembly when Sup35p fragments or short oligopeptides are used.

Similar to Sup35pNM, fibrils made in vitro of full-length Sup35p are infectious. This infectivity is either the consequence of prion induction or conversion. Indeed, upon induction of a prion phenotype different prion strains may be generated. These strains are defined from their capacity to reproduce pre-existing strains, i.e. the efficiency in stop codon read-through. As we do not present hereinafter a characterization of the strains generated upon transformation of yeast cells, we will refer to [PSI'] trait appearance upon treatment of the cells with fibrillar Sup35p and Sup35pNM as an induction of [PSI'].

The observed difference in the induction of [PSI'] trait appearance by fibrillar Sup35p and Sup35pNM suggests that the two macromolecular assemblies either somehow differ structurally or penetrate the cells with different efficiencies. It is also possible that the increased ability of Sup35p fibrils to induce [PSI'] is the consequence of differences in the interac-

because many kinetic features of Sup35pNM assembly are common for other amyloidogenic proteins (35–37) and fibrils made of Sup35pNM assembled in vitro propagate [PSI'] when reintroduced in yeast cells (27, 28). However, as the occurrence of the [PSI'] is not accompanied by Sup35p degradation, for a better understanding of the conversion of soluble Sup35p into prion particles it is important to investigate the assembly properties of the full-length protein. Such studies have only been initiated recently (24, 38), supposedly because of difficulties in purification and handling full-length Sup35p, a highly aggregation-prone polypeptide.

Here we have compared the assembly properties of Sup35pNM and full-length Sup35p as the functional C-terminal domain of the protein may affect the reaction. We have shown that full-length Sup35p assembles in a manner similar to its NM fragment. Both polypeptides exhibit cooperative assembly and concentration dependence. However, the lag phases and extent of assembly as measured by thioflavin T binding show significant differences. Although the critical concentrations for assembly of both polypeptides are in the μM range, that of Sup35p is higher than that of Sup35pNM. The finding that Sup35p nuclei are composed of six molecules whereas that of Sup35pNM are made of three molecules indicates that the two kinds of nuclei have different geometries. This might reflect a different packing of Sup35pNM domains, either free or when attached covalently to the C-terminal domain of the protein. Alternatively, this could also be due to a direct contribution of the C-terminal domain to Sup35p oligomerization. We also showed that the elongation rate of preformed Sup35pNM seeds is significantly greater than that of full-length Sup35p. When fibrillar Sup35pNM is used to seed the assembly of soluble Sup35pNM and full-length Sup35p, a higher cross-seeding capacity is observed in a homologous, as compared with a heterologous, system. This suggests either that fibrillar Sup35pNM and Sup35p differ structurally or that local rearrangements within the C-terminal domain in full-length Sup35p constitute a limiting reaction in the assembly process. Furthermore, it is worth noting that whereas soluble Sup35p is purified under native conditions, recombinant Sup35pNM is recovered from inclusion bodies and refolded from denaturant. Although the overall secondary structure and assembly kinetics of Sup35pNM purified under denaturing and non-denaturing conditions are similar (33), it is possible that the ensemble of conformational states adopted by Sup35pNM following its renaturation differs to some extent from that of the NM domain in full-length Sup35p. In that case, the observed differences in cross-seeding capacities would reflect either a poorly populated, assembly-competent conformation of the NM domain in full-length Sup35p, a limiting step upon displacement of the equilibrium between the assembly-incompetent and assembly-competent conformations toward the latter form, or both. Overall, the data presented here suggest that the differences in the assembly process of Sup35p and Sup35pNM are worth being taken into account when drawing general conclusions on Sup35p assembly when Sup35p fragments or short oligopeptides are used.

Similar to Sup35pNM, fibrils made in vitro of full-length Sup35p are infectious. This infectivity is either the consequence of prion induction or conversion. Indeed, upon induction of a prion phenotype different prion strains may be generated. These strains are defined from their capacity to reproduce pre-existing strains, i.e. the efficiency in stop codon read-through. As we do not present hereinafter a characterization of the strains generated upon transformation of yeast cells, we will refer to [PSI'] trait appearance upon treatment of the cells with fibrillar Sup35p and Sup35pNM as an induction of [PSI'].

The observed difference in the induction of [PSI'] trait appearance by fibrillar Sup35p and Sup35pNM suggests that the two macromolecular assemblies either somehow differ structurally or penetrate the cells with different efficiencies. It is also possible that the increased ability of Sup35p fibrils to induce [PSI'] is the consequence of differences in the interac-
tion of the two kinds of polymers with the cellular pool of molecular chaperones. Indeed, fibrillar Sup35p and Sup35pNM most likely expose to the solvent surface areas with different properties. The reintroduction of full-length Sup35p fibrils in the cells should not therefore affect the molecular chaperone homeostasis to the same extent as that of Sup35pNM fibrils that are expected to bind molecular chaperones in a manner similar to other glutamine-rich polypeptides (39). A differential interaction between fibrillar Sup35p or Sup35pNM and molecular chaperones could lead to the depletion of specific molecular chaperones from the cellular pool and perturbed homeostasis. Such changes might (i) impact [PSI⁺] propagation as it is highly dependent on the expression levels of a number of molecular chaperones (40) and/or (ii) have major effects, in particular in cells where the folding capacities are limited, as the expression of polyglutamine expansions disrupts the global balance of protein-folding quality control, resulting in the loss of function of diverse metastable proteins (10). Our recent in vitro studies revealed that the assembly of Sup35p can be finely tuned by the amount of available molecular chaperones (24). Thus the finding that the efficiency of [PSI⁺] induction by Sup35p fibrils is higher than that of Sup35pNM fibrils certainly requires further investigation. The depletion of the functional pool of molecular chaperones within the cells by one kind of fibril might also allow the de novo formation of Sup35p propagons, thus leading to the observed increased efficiency of [PSI⁺] occurrence. Further identification of the molecular chaperones that bind to fibrillar Sup35p and Sup35pNM upon addition of the fibrils into yeast cell extracts by proteomic, biochemical, and structural approaches should allow a better understanding of the differences in the efficiency of [PSI⁺] induction. A quantitative analysis of [PSI⁺] induction by Sup35p and Sup35pNM fibrils pre-incubated in the presence of the molecular chaperones identified by the approach described above should allow assessment of the specific effect of each molecular chaperone, which in turn will contribute to the identification of the most infectious Sup35p particles and the characterization of [PSI⁺] propagation process.

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