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ScienceDirect
Biochemical and Biophysical Research Communications 353 (2007) 139–146
BBRC
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Effects of randomizing the Sup35NM prion domain sequence on formation of amyloid fibrils *in vitro*

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Received 18 November 2006
Available online 6 December 2006

Abstract

The mechanism by which proteins aggregate and form amyloid fibrils is still elusive. In order to preclude interference by cellular factors and to clarify the role of the primary sequence of Sup35p prion domain in formation of amyloid fibrils, we generated five Sup35NM variants by randomizing amino acid sequences in PrDs without altering the amino acid composition and analyzed the *in vitro* process of amyloid fibril formation. The results showed that each of the five Sup35NM variants polymerized into amyloid fibrils *in vitro* under native conditions. Furthermore, the Sup35NM variants showed differences in their aggregation time courses. These findings indicate that specific amino acid sequence features in PrD can modify the rate of conversion of Sup35p into amyloid fibrils *in vitro*.

Keywords: Fibril formation; Sup35NM; Yeast prion; Prion domain

Amyloid fibrils are highly ordered protein aggregates characterized by high β-sheet content, protease-resistance, and apple green birefringence upon staining with Congo red [1]. Such fibrils have been found in about 20 human diseases [2]. However, little is known about what makes a protein amyloidogenic and there is no effective treatment for amyloid diseases.

The nonchromosomal genetic element [PSI^+] is the prion form of the *Saccharomyces cerevisiae* protein Sup35p [3]. It is called a ''yeast prion'' because of the similarity between its proposed mechanism of propagation and that of the TSEs [4]. The [PSI^+] formation results from the conversion of the active Sup35p into an insoluble and inactive, self-propagating, and infectious amyloid form. Therefore, [PSI^+] offers a useful model for studying the prion-like transmission of protein conformation as well as the formation of amyloid.

Sup35p is composed of three distinct regions: N, M, and C [5]. The N region constitutes the prion determining domain (PrD) that is required for the induction and maintenance of [PSI^+]. The M region is highly charged and its function remains unclear. The C region provides the essential translation termination function in yeast. The N domain is extremely rich in glutamine (Q) and asparagine (N), and contains five imperfect nine-residue repeats (PQGGYQQYN) [6], which is similar to the mammalian prion protein repeats [7].

Previous study showed variants of Sup35p with randomized amino acid sequences in PrDs form prions *in vivo* [8]. However, cellular expression level, localization of proteins,

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.11.143

and other cellular factors may influence prion formation *in vivo*. A number of cellular factors are likely to play an important role in the maintenance and propagation of the Sup35p prion state. For example, several lines of evidence have indicated that an increase or deletion of *HSP104* in [PSI$^+$] leads to the loss of the [PSI$^+$] [9,10]. Furthermore, the structural change resulting from randomized amino acid sequences would affect Sup35p's interaction with Hsp104, and this might also affect the stability of [PSI$^+$]. Thus, prion formation and maintenance *in vivo* could be linked to various factors. The aggregating dynamics of the variants have not been characterized *in vitro*.

In the study, we assay randomized sequence variants of Sup35p *in vitro*, providing uniform protein concentration in simple aggregating buffer. We evaluate the effect of randomized amino acid sequences on amyloid fibril formation to see whether there are intrinsic sequence features of Sup35p PrD that affect protein aggregation.

Materials and methods

Sequence randomization of Sup35p PrD and gene synthesis. Sup35p amino acids 2–123 were randomized as described previously [8]. Five cDNAs encoding sequence-scrambled Sup35p PrD variants, named Sup35N-1 to -5, were synthesized by Shanghai Sangon Biotechnology Co. Ltd. (Shanghai, China). Each of the synthesized genes was inserted into the pUC57 vector, respectively.

Construction of expression vectors for Sup35NM and its variants in Escherichia coli. The DNA sequence encoding the Sup35 M region was amplified and a 8-fold-histamine (His_8)-tag was integrated by PCR with the N-terminal primer containing *Bam*HI and *Eco*RI sites, 5′-CGACATA TGCACCACCACCATCATCATCATCATTCAGGCGGATCCCCGC GGGAATTCATGTCTTTGAACGACTTTC-3′ and C-terminal primer, 5′-CGGCTCGAGTTAATCGTTAACAACTTCGTCATC-3′, using p2HGNMsGFP (kindly provided by Dr. Susan L. Lindquist of Whitehead Institute, Massachusetts Institute of Technology) as a template and inserted into pET30a (Novagen) vector at the *Nde*I/*Xho*I sites. The construct was named pET30a-Sup35M. The synthesized Sup35N-1 to -5 sequences were then inserted into the plasmid pET30a-sup35M at the *Bam*HI/*Eco*RI sites. The five constructs were designated Sup35NM-1 to -5. Wild type (wt) Sup35NM was amplified by PCR and inserted into the pET30a vector at *Nde*I/*Eco*RI sites. All constructs contained N-terminal His_8-tags.

Expression and purification of protein. Versions of Sup35NM-1 to -5 and wt Sup35NM were prepared following the method by Glover et al. [5] with minor modifications. The details were available elsewhere (see Supplemental material).

Aggregation of wt Sup35NM and its five variants. Concentrated protein solutions were diluted into phosphate buffered saline (PBS) (pH 7.4) to a final concentration of 16 μM. The fresh protein can aggregate into fibrils by incubating at room temperature. These fibrils can be used as seeding materials.

Electron microscopy. For negative staining [11], 10 μl of protein solution was applied to a glow-discharged 200 mesh carbon-coated copper grid for 1 min and stained with several drops of 2% (w/v) aqueous uranyl acetate, then air-dried. The specimens were examined with a Philips Tecnai 12 at an accelerating voltage of 80 kV.

Secondary structure analysis. Circular dichroism (CD) spectra were recorded on a Jasco 715 spectropolarimeter equipped with a 0.1 cm pathlength Supracil cuvette at ambient temperature. All samples were briefly sonicated before measurement. Samples were scanned at 100 nm/min, response time = 4 s, bandwidth = 1 nm, accumulations = 4.

Protease K resistance assay. The method was modified from a Sup35pN proteinase K resistance assay [12]. About 50 μg of monomeric or amyloid fibril samples were suspended in 90 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). Ten microliters of protease K (Sigma) solution in TE buffer at concentrations of 1 or 4 μg/ml were added and the mixture was incubated for various periods of time at 37 °C. Reactions were terminated by adding phenylmethylsulfonyl fluoride to a final concentration of 5 mM. SDS gel loading buffer was added at 1× and boiled for 10 min. Samples were run on a 12% SDS–PAGE and detected with Coomassie blue staining.

Thioflavin T (ThT) binding assay. ThT assay was carried out as described elsewhere [13]. At time intervals, 10 μl protein solutions were taken for measurement of ThT fluorescence spectrum. Proteins were diluted to 0.3 μM in the presence of 20 μM ThT solution (50 mM Tris–HCl and 0.2 M NaCl, pH 7.4). ThT fluorescence was monitored using a Shimadzu RF5301PC spectrofluorometer, with excitation at 450 nm and emission at 482 nm (excitation slit, 10 nm; emission slit, 10 nm).

Western blot. Sup35NM variants were analyzed by 12% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). The blots were blocked in 5% skim silk at room temperature. Then anti-His_6 monoclonal antibody (Sigma) was added. Following PBS washing, secondary anti-mouse antibody conjugated to horseradish peroxidase was added. The proteins were visualized with 3,3′-diaminobenzidine (Sigma).

Seeding assay. Preformed fibrils (1% w/w) were briefly sonicated and then added to 10 μM solutions of freshly prepared proteins. Conversion occurred at room temperature without agitation and was monitored by fluorescence emission of ThT.

Results

Randomization of Sup35 PrD sequences and expression of Sup35NM variants

Amino acids of 1–123 of the Sup35 PrD are rich in Q/N (44%), whereas amino acids 41–114 comprise five imperfect oligopeptide repeats that are thought to be important for prion maintenance [14]. We created five variants of Sup35NM PrD, in which the order of amino acids 2–123 was randomized with the amino acid composition kept constant. The variants sequences are shown in Table 1. Randomization disrupts the oligopeptide repeats of Sup35NM PrD (Table 2). In addition, the Sup35NM-1, -2, and -3 variants have glutamine-runs of at least four amino acids in length (Tables 1 and 2). The proteins were purified from *E. coli* by chromatography to levels as high as >90% (see Supplemental material).

Scrambled PrDs did not prevent Sup35NM variants from polymerizing into amyloid fibrils in vitro

In order to examine whether the scrambled PrD of Sup35NM would abolish the formation of amyloid, Sup35NM variants were incubated at room temperature. After 5 days, all the solutions became viscous. The scrambled variants were seen by TEM to be polymerized into ordered filaments. In all cases, the fibrils were extremely long and showed no remarkable difference in morphological character from wt Sup35NM (Fig. 1A). They had a smooth appearance with an average diameter of 8–14 nm. Further probing the properties of these ordered filaments by CD spectra, ThT binding assay, and Protease K resistance showed that they met the classification criteria for amyloid fibrils.

Table 1
Amino acid sequences of scrambled Sup35p prion domains

Variants	Amino acid sequences of scrambled Sup35 prion domains
Wt Sup35NM	MSDSNQGNNQQNYQQYSQNGNQQQGNNRYQGYQAYNAQAQ PAGGYYQNYQGYSGYQQGGYQQYNPDAGYQQQYNPQGGYQQYN PQGGYQQQFNPQGGRGNYKNFNYNNNLQG YQAGFQPQSQG…
Sup35NM-1	MYSGKNQAAQPSAGQQYQYQYYDNQGGGGAQNQGSNQRPFYNS QPNAQGLPNYYQGQNYYDNGQRNQYQYNGAGQQNYYNNGFYQQ QGQQGNPPYNYYYGYNQQGQQQQGGNGNGFSNQQQQQ…
Sup35NM-2	MQQANAQNSQQQGQGNGNNQSYNYSLANGQGNQYQNYGGFQQY GQYDYRNPPQQQQAQQYYYFQQQNQPYNYGYGFNGQNQYQNSQ YQYGAYGNQGSDPGAGGGPKYQNQRGQNYNGNPGYQQ…
Sup35NM-3	MQYRQGSGGYGSQGNQGANNQAGQGSQYQNNQYNQGPNNQDQQ QGPQQQQNGRYQQQQYKSYQAGGQGQNQGNPFYQAQNYFQPYG GAQYNNYYYYGGLQYGYPQYQNNPNYGNQFQSNNADY…
Sup35NM-4	MPYGAYANGPGNNYQDQQGGNQQGQSQNGQGQQQPYQQNQRGQ NQANNQGGPNYYYGYNGQGYPYQYGQNQSSYFYNQNANFGQRQ ANNQLNYQYYQQSGQQQGYGKNYGFNSAPQQDYQYQG…
Sup35NM-5	MGYNGGQGYSQGYQQQFPNNFQQPQQYGQPGYNYGGDQNQNQG QQQYGQANNSNYGYSNQGRFYNGPYGNDYQQSNQQYAYYQNLA QQYQANYNQGNQNQKQQRGNPGYGQPQYAGYAQNQGS…

The five imperfect oligopeptide repeats are shaded in gray and the runs of glutamine longer than three amino acids are underlined.

Table 2
Aggregating tendencies of Sup35NM variants

Variants	Time for reaching ThT binding plateau (h)	Aggregation tendency[a]	Quantity of oligopeptide repeats	Quantity of Q-runs of at least four amino acids
wt Sup35NM	6	++++	5	0
Sup35NM-1	8	+++	0	2
Sup35NM-2	8	+++	0	1
Sup35NM-3	8	+++	0	2
Sup35NM-4	10	++	0	0
Sup35NM-5	12	++	0	0

[a] Moderate (++), strong (+++), very strong (++++) aggregating tendency of Sup35NM variants.

A diagnostic feature of amyloid proteins is fibrils formation with a high β-sheet content. The CD spectra of amyloid fibrils formed by wt Sup35NM and variants exhibited a minimum near 216 nm, which is characteristic of structures rich in β-sheet. As a control, the CD spectrum of a freshly prepared solution of wt Sup35NM presented α-helical characteristics with two negative peaks at 222 and 208 nm (Fig. 1B). The results demonstrated that the five variants and wt Sup35NM underwent extensive changes in secondary structure on aging. Another distinctive property of amyloid fibrils is their ability to bind ThT [15]. As shown in Fig. 1C, after 10 h, the ThT fluorescence intensity of each sample increased significantly relative to the 0-h solution.

The aggregates of the variants showed greater protease K resistance than native proteins. Monomeric and amyloid variants were treated with 1 μg/ml or 4 μg/ml protease K at 37 °C for three durations: 15, 30, and 60 min. At the lower enzyme level, the amyloid form was still detectable at 60 min after the addition of protease (Fig. 1D), while monomers of wt Sup35NM and the variants were readily degraded. Digestion at the higher enzyme level obtained similar results.

Collectively, these findings showed that the randomized Sup35NM could polymerize into fibrils *in vitro* under native conditions, indicating that the fibrils formation of Sup35NM is primary sequence independent. These results are identical to those obtained *in vivo* experiments previously [8].

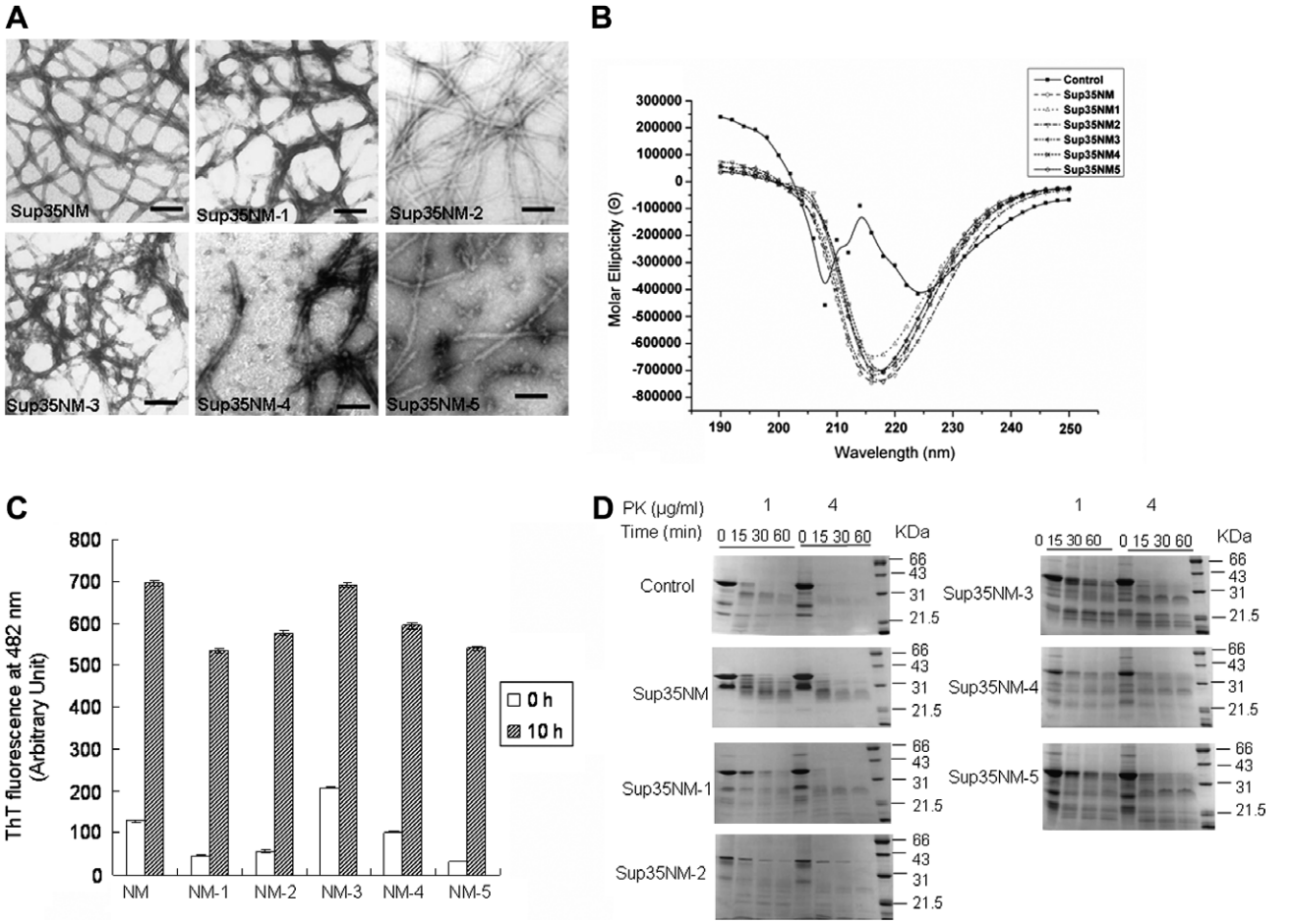

Fig. 1. Sup35NM variants polymerized into amyloid fibrils *in vitro*. (A) Electron micrographs of Sup35NM variants after incubation at room temperature for 18 h. Bar = 100 nm. (B) Circular dichroism spectra of fibrils formed by Sup35NM variants. Freshly prepared Sup35NM solution was measured as a control. (C) Thioflavin T binding assays of fibrils formed by Sup35pNM variants. The fluorescence emission intensity at 482 nm ($\lambda_{Ex} = 450$ nm) of each sample was measured after incubation for 0 and 10 h. Data were expressed as means ± standard deviation (SD) and obtained from triplet measurements. (D) Protease K resistance of filamentous aggregates of Sup35NM variants as determined by SDS–PAGE. Monomeric Sup35NM was used as a control.

Conversion to amyloid fibrils of Sup35NM variants can be promoted by seeding

It is one of the most important properties of prion-like proteins that the formation of amyloid fibrils can be induced by seeding [5]. The key experiment showed that seeding with fibrils of Sup35NM variants promote the conversion of monomers into fibrils. After adding 1% preformed filaments to freshly prepared solutions of Sup35NM variants, the fluorescence intensity significantly increased in contrast to reactions without seeding, suggesting a marked increase of conversion rate after seeding. However, the amount that seeding increased the conversion rate differed among the variants (Fig. 2).

Specific amino acid sequence features influence the conversion rate of Sup35p into amyloid fibrils in vitro

To evaluate the impact of amino acid sequence on the dynamic process of amyloid formation *in vitro*, we analyzed the time course of variant aggregation. Samples captured from different stages during polymerization were firstly analyzed with TEM. Two hours after the polymerizing initiation, the variants Sup35NM-1, -2, -3 and wt Sup35NM showed all kinds of forming fibrils with oligomers attached (Fig. 3A). In contrast, Sup35NM-4 and -5 mainly displayed oligomers of small beads, short rods and longer linear strings of beads. Later, mature fibrils (described in Fig. 1A) appeared and increased with the intermediates diminishing. Eighteen hours later, only fibrils were observed in samples of the variants Sup35NM-1, -2, -3 and wt Sup35NM. However, the variants Sup35NM-4 and -5 exhibited a mixture of structures including fibrils and oligomers. Sup35NM-4 and -5 aggregated much more slowly than the others.

To show the differences among the conversion rates of Sup35NM variants quantificationally, ThT binding assay was performed. Time course of fibrils formation of Sup35NM variants measured by ThT fluorescence displayed an approximately sigmoidal growth curve, which was composed of an initial lag phase, a subsequent rapid growth phase, and a final equilibrium phase (Fig. 3B).

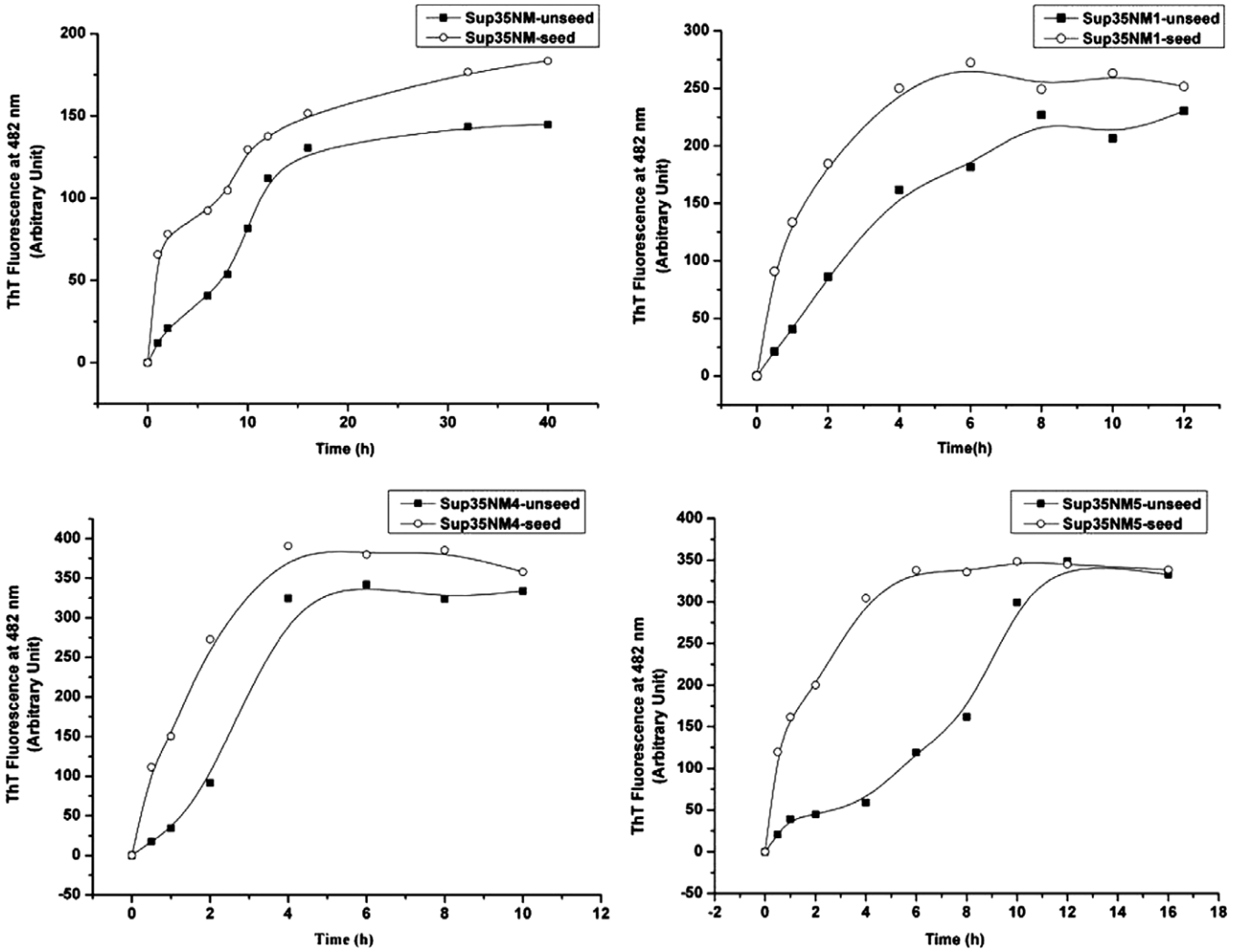

Fig. 2. Effects of seeding on fibril formation of Sup35NM variants. In the seeded reactions, 1% (w/w) preformed fibrils were added to 10 μM solutions of freshly prepared proteins, respectively.

Because increased protein concentration shortens the lag time [5], here at a fairly high concentration of 16 μM, we did not detect the typical lag phase for Sup35NM and variants Sup35NM-1, -2, and -3. However, the ThT fluorescence curve of Sup35NM-4 and -5 revealed a prolonged lag period of nearly 3 and 5 h, respectively. ThT binding reached a plateau in 6 h for wt Sup35NM, in 8 h for Sup35NM-1, -2, and -3, but took 10–12 h to plateau for Sup35NM-4 and -5 (Table 2).

It has been confirmed that insoluble aggregates stick in the loading wells of gels and are not stained well by Coomassie blue, but they can be transferred to nitrocellulose membranes and detected by Western blot [16]. On a time course from 0 to 18 h, samples of aggregating Sup35NM variants were analyzed by Western blot. Monomeric Sup35pNM variants decreased while insoluble aggregates trapped in the loading wells increased gradually (Fig. 3C). Consistent with the results of ThT binding assays, monomeric Sup35NM-4 and -5 disappeared much more slowly than the other variants and wt, with soluble monomers still detectable after 18 h.

Taken together, the findings showed that aggregating dynamics and tendency of the five variants varied with the primary amino acid sequences, indicating that specific amino acid sequence might be an influence element during the amyloid fibril formation.

Discussion

Previous work has elucidated that randomized Sup35p PrDs form prions *in vivo* [8]. Here we report the effects of PrD randomization on fibril formation *in vitro*, more quantitatively describing the time course in the absence of *in vivo* cellular factors. We generated five Sup35NM variants bearing randomized PrDs that polymerized into amyloid *in vitro* under native conditions. The ordered filaments retained the morphological and biochemical properties reported for

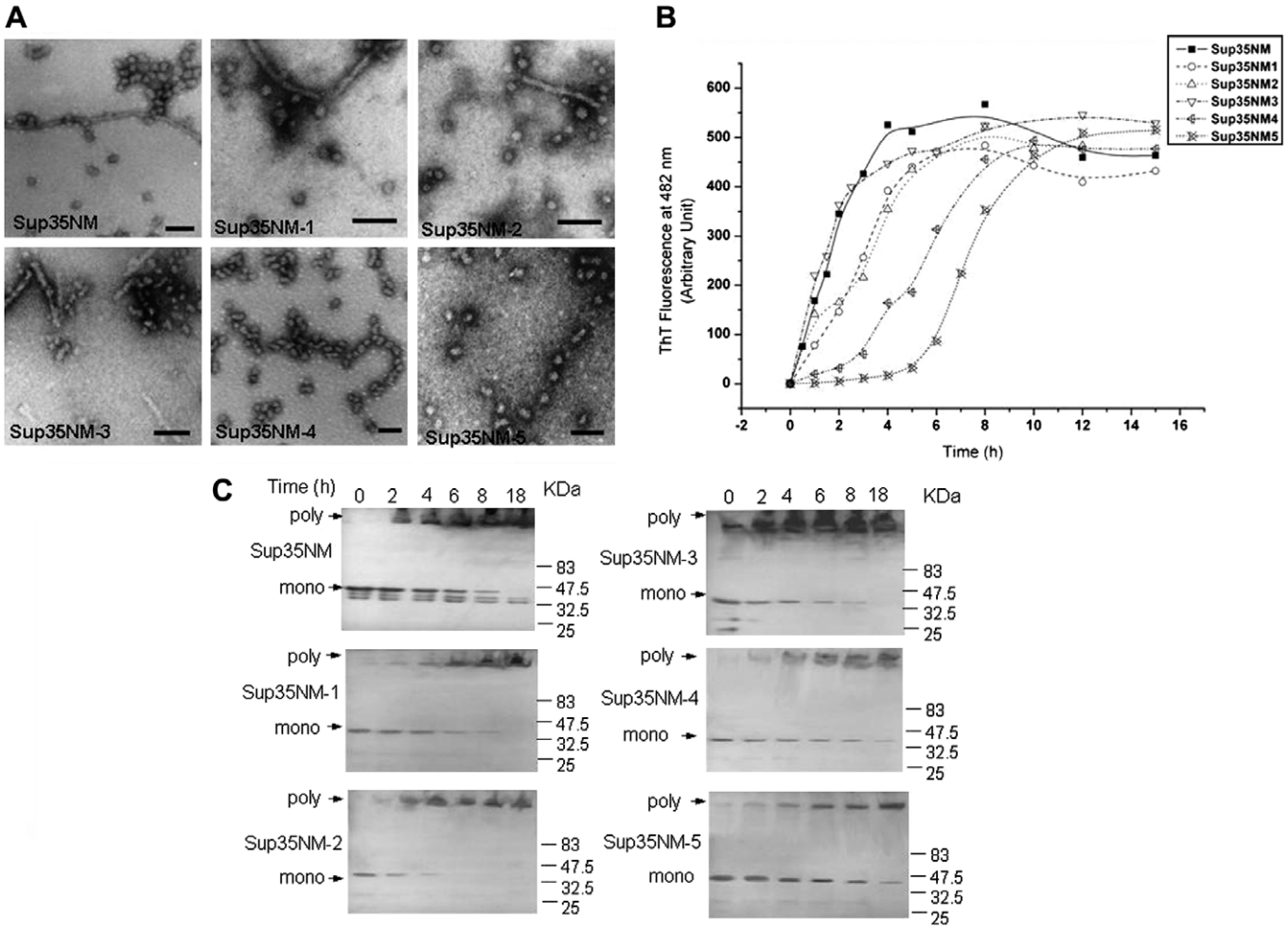

Fig. 3. Time courses of fibril formation by Sup35NM variants. (A) Electron micrographs of Sup35NM variants after incubation for 2 h. Bar = 100 nm. (B) ThT fluorescence intensities of each sample at the same protein concentration of 16 μM were determined at multiple time points. (C) Western blot assay of the samples with anti-His_6 monoclonal antibody. Aliquots from the reactions at multiple time points were mixed with sample buffer and loaded without heating.

in vitro Sup35p aggregates (Fig. 1). However, aggregation speed of these proteins depends on their sequence (Fig. 3). The results indicate that the ability of Sup35p to form amyloid fibrils is determined primarily by its amino acid composition, while specific sequence features modulate the rate of conversion into amyloid *in vitro*.

An increasing number of natural or designed sequences of proteins have been shown aggregate *in vitro* into fibrils. However, in most cases, nonphysiological conditions such as low pH are required [17]. In this study, the Sup35NM variants polymerized into amyloid *in vitro* under native conditions, suggesting that the Sup35NM variants with randomized PrD still retain the ability to form fibrils. This may be attributed to their high Q/N content (44%). Numerous evidences indicate that high Q/N content is an important factor in prion formation by Sup35 [18]. Besides, Q/N-rich domains are found in other yeast prion proteins, Ure2p and Rnq1p [18,19]. The Sup35p PrD is at one extreme end of the protein sequence and relatively unstructured [20], making them easily accessible for prion formation. Likewise, the PrDs of Sup35NM variants is at one extreme end. Moreover, the order of the amino acids in the PrDs of the Sup35NM variants have been shuffled, these domains are unlikely to contain stably folded structures.

Although the Sup35NM variants formed fibrils, they displayed different aggregation rates. The rates of fibrils formation by the variants Sup35NM-1, -2 and -3 were somewhat slower than that of the wt Sup35NM; Sup35NM-4, and -5 were much slower. Thus, the primary sequence plays an important role in aggregation even though amino acid composition is sufficient to support aggregation.

A number of studies have shown that an expansion of the oligopeptide repeats induces the spontaneous appearance of [PSI^+] and that deletion of the repeats eliminates it [14,21]. These data suggested that the number of oligopeptide repeats in the Sup35-PrD could modify the rate of conversion of Sup35p. Most probably, the oligopeptide

repeats act to stabilize the Sup35p–Sup35p interactions required for the formation of oligomeric intermediates [22]. In this study, the oligopeptide repeats were disrupted by randomizing the Sup35p PrD. Disruption of the repeats may affect the strength of Sup35p–Sup35p interactions and reduce the rate of oligomerization. While the repeats were disrupted in five variants, the aggregation rates were not uniformly slowed. These data indicated that oligopeptide repeats might not be the only sequence characteristic that impacts the complex dynamics of protein conversion and polymerization.

Expansion of the poly-glutamine repeats in certain proteins accounts for the pathogenesis of several inherited neurodegenerative diseases, e.g., Huntington's disease. These diseases are characterized by the accumulation of protein aggregates in neurons [23]. PolyQ aggregate with amyloid-like appearance has also been observed *in vitro* [24]. Moreover, it has been demonstrated that aggregating variants of the polyQ-containing domain of the huntingtin protein promote the *de novo* conversion of Sup35 into [PSI^+] and facilitate Sup35NM aggregation *in vitro* [25]. This suggests that amyloidogenic proteins containing polyQ have an intrinsic propensity to promote amyloid formation. Here the variants Sup35NM-1, -2, and -3, which formed fibrils at a rate comparable to wt Sup35NM, contain at least one run of four sequential glutamines (Tables 1 and 2). This suggests that Q-runs may be one of the important factors that determine aggregation rate. A reasonable explanation is that the many potential hydrogen bonded chemical groups in the Gln and Asn residues of the PrDs could form "polar zippers," facilitating the association of prion monomers into fibrils [26].

In summary, our study showed that specific sequence features in PrD can modulate the rate of conversion of Sup35p into amyloid fibrils *in vitro*, though it is dispensable to the ability of amyloid formation, which is determined primarily by its unusual amino acid composition. The findings may provide some clues for a strategy to prevent the amyloid fibril formation by directly targeting the oligopeptide repeats and Q/N-rich domains.

Acknowledgments

We are grateful to Dr. S. Lindquist (Whitehead Institute, MIT) for the gift of p2HG-NMsGFP, Dr. Chin-chen Wang and Chunjuan Huang (Institute of Biophysics, Chinese Academy of Sciences) for their assistance with ThT binding assay. This research was supported by a grant from the Major Program of National Science Foundation of China (No. 30291000).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.11.143.

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