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Effects of randomizing the Sup35NM prion domain sequence on formation of amyloid fibrils *in vitro*

Yingxia Liu [a,d,1], Haiyan Wei [b,d,e,1], Jianwei Wang [c,d,*], Jianguo Qu [d], Weiming Zhao [b], Hung Tao [a,b,d]

[a] *Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, School of Basic Medicine, Peking Union Medical College, Beijing 100005, China*
[b] *School of Medicine, Shandong University, Jinan 250012, China*
[c] *State Key Laboratory of Molecular Virology and Genetic Engineering, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing 100730, China*
[d] *National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 100052, China*
[e] *Technical Center of Beijing Entry–Exit Inspection and Quarantine Bureau, Beijing 100026, China*

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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,

* Corresponding author. Fax: +86 10 65105188.
E-mail address: wangjw28@163.com (J. Wang).
[1] These authors contributed equally to this work.

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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.