Secondary nucleation overcomes seeding template in amyloid-like fibril formation

Katažyna Milto, Tomas Šneideris, Vytautas Smirnovas

Prions are infectious proteins where the same protein may express distinct strains. The strains are enciphered by different misfolded conformations. Strain-like phenomena have also been reported in a number of other amyloid-forming proteins. One of the features of amyloid strains is the ability to self-propagate, maintaining a constant set of physical properties despite being propagated under conditions different from those that allowed initial formation of the strain. Here we report a cross-seeding experiment using strains formed under different conditions. Using high concentrations of seeds results in rapid elongation and new fibrils preserve the properties of the seeding fibrils. At low seed concentrations secondary nucleation plays the major role and new fibrils gain properties predicted by the environment rather than the structure of the seeds. Our findings could explain conformational switching between amyloid strains observed in a wide variety of in vivo and in vitro experiments.

1 # Secondary nucleation overcomes seeding template in amyloid-like fibril

2 # formation

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8 **Abstract**

9 Prions are infectious proteins where the same protein may express distinct strains. The strains are

10 enciphered by different misfolded conformations. Strain-like phenomena have also been reported

11 in a number of other amyloid-forming proteins. One of the features of amyloid strains is the

12 ability to self-propagate, maintaining a constant set of physical properties despite being

13 propagated under conditions different from those that allowed initial formation of the strain.

14 Here we report a cross-seeding experiment using strains formed under different conditions.

15 Using high concentrations of seeds results in rapid elongation and new fibrils preserve the

16 properties of the seeding fibrils. At low seed concentrations secondary nucleation plays the major

17 role and new fibrils gain properties predicted by the environment rather than the structure of the

18 seeds. Our findings could explain conformational switching between amyloid strains observed in

19 a wide variety of *in vivo* and *in vitro* experiments.

20

Introduction

Prions are infectious particles which play the main role in a group of fatal neurodegenerative disorders, also known as the transmissible spongiform encephalopaties (TSE's). Prion diseases propagate by self-replication of a pathogenic prion isoform (PrP^{Sc}) using cellular prion protein (PrP^{C}) as a substrate (Prusiner, 1998; Collinge, 2001). Although structures of infectious forms of PrP are still only partially defined, it is known that PrP^{Sc} is rich in beta-sheet structure and demonstrates fibrillar morphology (Sim & Caughey, 2009; Colby & Prusiner, 2011). Different conformations of PrP^{Sc} are responsible for variations in prion disease phenotypes and are usually referred to as strains (Safar et al., 1998). For a long time prion protein was the only suspected infective protein in humans, however recently there is growing evidence that proteins in other amyloid-related diseases may spread via prion-like mechanisms (Lundmark et al., 2002; Soto, Estrada & Castilla, 2006; Frost & Diamond, 2010; Brundin, Melki & Kopito, 2010; Eisele et al., 2010; Angot et al., 2010; Westermark & Westermark, 2010; Masuda-Suzukake et al., 2013; Eisele, 2013; Goedert et al., 2014). Moreover, the most recent data suggest that variants of Alzheimer's disease are encoded by different strains (Stöhr et al., 2014; Watts et al., 2014; Aguzzi, 2014).

A lot of information on possible mechanisms of amyloid-like fibril formation comes from *in vitro* studies of the aggregation kinetics (Knowles et al., 2009; Arosio et al., 2014; Meisl et al., 2014). It is thought that four major steps are involved in fibril formation (Meisl et al., 2014). In the case of spontaneous aggregation, everything starts from primary nucleation. It takes time for a group of soluble protein molecules to get together and misfold into an amyloid-like structure, which serves as a nucleus for fibrillation. Once nuclei are formed, they start elongation into fibrils by attaching soluble protein at the ends and refolding it into an amyloid-like structure.

44   Although nucleation and elongation could be sufficient for describing fibrillation, in many cases

45   secondary processes, such as fibril fragmentation and secondary nucleation are extremely

46   important (Knowles et al., 2009; Meisl et al., 2014). Fibril fragmentation increases the number of

47   fibril ends, which leads to faster elongation. The presence of fibrils can induce formation of new

48   nuclei with much shorter lag times compared to primary nucleation; this is referred to as

49   secondary nucleation (Meisl et al., 2014).

50   How would such a mechanism of fibril formation work in the case of different amyloid strains?

51   Strain-like structural polymorphism was observed in a number of different amyloid-forming

52   proteins (Tanaka et al., 2004, 2005; Yamaguchi et al., 2004; Dzwolak et al., 2004; Petkova et al.,

53   2005; Jones & Surewicz, 2005; Heise et al., 2005; Paravastu et al., 2008; Makarava et al., 2009;

54   Colby et al., 2009; Dinkel et al., 2011; Jones et al., 2011; Chatani et al., 2012; Bousset et al.,

55   2013; Ghaemmaghami et al., 2013; Cobb et al., 2014; Tycko, 2014; Surmacz-Chwedoruk,

56   Babenko & Dzwolak, 2014). To form different amyloid strains *de novo* using the same protein,

57   different environmental conditions, such as temperature (Tanaka et al., 2005), shear forces

58   (Makarava et al., 2009), concentration of denaturants (Cobb et al., 2014) or co-solvents

59   (Dzwolak et al., 2004) are involved. Once nuclei are formed, they are able to carry strain-specific

60   properties even in unfavorable environments (Dzwolak et al., 2004; Petkova et al., 2005;

61   Makarava et al., 2009; Cobb et al., 2014; Surmacz-Chwedoruk, Babenko & Dzwolak, 2014).

62   This indicates that environment defines different strains during primary nucleation, but affects

63   only kinetics, not the structure, of fibrils formed via elongation. In the case of secondary

64   nucleation, formation of new nuclei is induced by existing fibrils, but there is no experimental

65   evidence if the structure of these nuclei is determined by the environment conditions, or by

66 structure of the fibrils. Or in other words, can secondary nucleation be responsible for

67 conformational switching in amyloid-like fibril strains?

Materials and Methods

69 Recombinant mouse prion protein fragment (rMoPrP(89-230)) used in this study was purified

70 and stored as described previously (Milto, Michailova & Smirnovas, 2014). Protein grade

71 guanidine hydrochloride (GuHCl) was purchased from Carl Roth GmbH, guanidine thiocyanate

72 (GuSCN) and other chemicals were purchased from Fisher Scientific UK.

73 To prepare different fibril strains, monomeric protein from a stock solution was diluted to a

74 concentration of 0.5 mg/ml in 50 mM phosphate buffer (pH 6) containing 2 M or 4 M GuHCl,

75 and incubated for one week at 37°C with 220 rpm shaking (in shaker incubator IKA KS 4000i).

76 For seeding experiments rPrP-A^{4M} fibrils were treated for 10 minutes using Bandelin Sonopuls

77 3100 ultrasonic homogenizer equipped with MS72 tip (using 20% power, cycles of 30 s/30 s

78 sonication/rest, total energy applied to the sample per cycle – 0.36 kJ). The sample was kept on

79 ice during the sonication. Right after the treatment, fibrils were mixed with 0.5 mg/ml of mouse

80 prion solution in 2 M GuHCl in 50 mM phosphate buffer, pH 6, containing 50 mM ThT.

81 Elongation kinetics at 60°C temperature was monitored by ThT fluorescence assay (excitation at

82 470 nm, emission at 510 nm) using Qiagen Rotor-Gene Q real-time analyzer (Milto, Michailova

83 & Smirnovas, 2014). ThT fluorescence curves were normalized by dividing each point by the

84 maximum intensity of the curve.

85 For denaturation assays, amyloid fibrils were resuspended to a concentration of 25 mM in 50

86 mM phosphate buffer, pH 6, containing 0.5 M GuSCN and homogenized by sonication. These

87 solutions were diluted 1:4 in a buffer containing varying concentrations of GuSCN, and

88   incubated for 60 min at 25°C. Samples were then mixed 1:20 with 50 mM ThT, and fluorescence

89   was measured at 480 nm using the excitation wavelength of 440 nm. Denaturation curves were

90   normalized by dividing each point by the average intensity of the points in the plateau region.

91

Results

93   Conformational stability of PrP^{Sc} as defined by resistance to chemical denaturation has been one

94   of the key parameters used to define differences between strains (Colby et al., 2009). Different

95   strains of recombinant mammalian prion protein amyloid-like fibrils made in 2 and 4 M

96   guanidine hydrochloride (rPrP-A^{2M} and rPrP-A^{4M}, respectively) were thoroughly characterized

97   by Surewicz group (Cobb et al., 2014). We used recombinant N-terminally truncated mouse

98   prion protein (rMoPrP(89-230)) to create rPrP-A^{2M} and rPrP-A^{4M} strains of amyloid-like fibrils.

99   Similar to recent data on recombinant human PrP (Cobb et al., 2014), rMoPrP fibrils formed in 2

100   and 4 M guanidine hydrochloride (GuHCl) have different conformational stability (Fig.1). Due

101   to the fact that rPrP-A^{4M} fibrils could not be fully denatured using even 7.5 M GuHCl(Cobb et

102   al., 2014), a denaturation assay using a more strongly chaotropic salt, guanidine thiocynate

103   (GuSCN) was performed. Midpoint of denaturation of rPrP-A^{2M} is at ~2 M GuSCN and rPrP-

104   A^{4M} is at ~2.5 M GuSCN, respectively. This difference served as a simple, unbiased marker of

105   different strains in further experiments.

106

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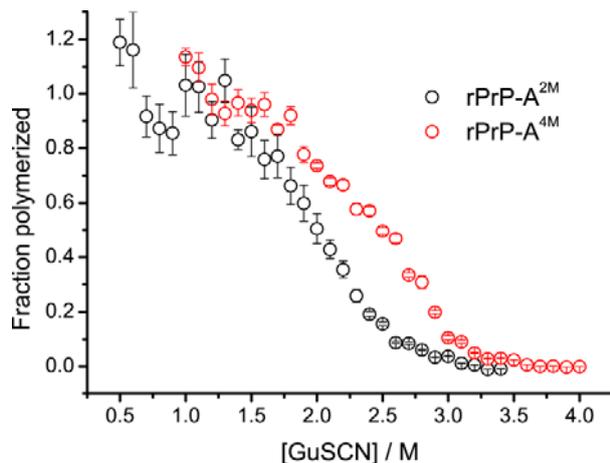
Figure 1. Denaturation profiles of rPrP-A²ᴹ and rPrP-A⁴ᴹ fibrils in GuSCN reveal different conformational

stabilities. Standard errors calculated from 12 measurements using Student's t-distribution at P=0.05.

110

111 In our previous work we have described elongation kinetics at different temperatures and

112 guanidine hydrochloride (GuHCl) concentrations, using rPrP-A²ᴹ as a seed (Milto, Michailova

113 & Smirnovas, 2014). It was not possible to get reliable data above 2.5 M GuHCl due to

114 depolymerization of rPrP-A²ᴹ. Thus only one way cross-seeding is possible for rPrP-A²ᴹ and

115 rPrP-A⁴ᴹ strains. We followed cross-seeding kinetics using different concentrations of seeds. As

116 seen in figure 2, five percent seeds led to fast growth of amyloid-like fibrils from the very

117 beginning, suggesting fast fibril elongation. At 1% seed volume elongation is slower, but after

118 some time the rate of aggregation explodes. At a lower concentration of seeds elongation is very

119 slow and the curve looks sigmoidal, as usually seen in case of spontaneous fibrillation. However

120 in absence of seeds no aggregation was detected within the experimental timeframe, which

121 means the observed process, is fibril-induced secondary nucleation (see Supplementary

122 information for the fitting data).

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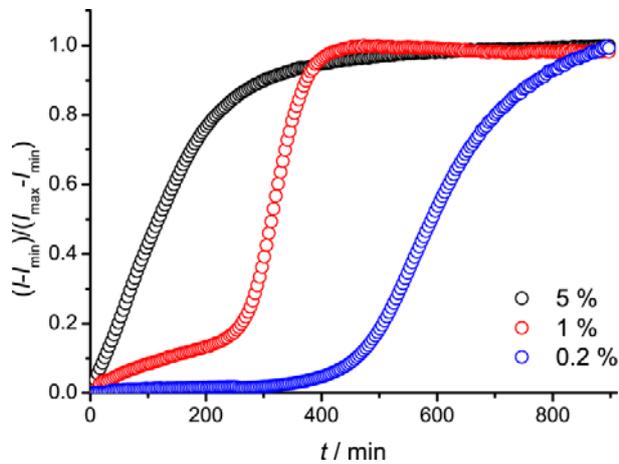
Figure 2. Concentration of seeds determines the mechanism of aggregation. Different amounts of rPrP-A^{4M} fibrils
(sonicated for 300 s) were added to the solution of rMoPrP, prepared in 2 M GuHCl, 50 mM phosphate buffer, pH6.
The kinetics was followed at 60°C using Thioflavin T (ThT) fluorescence assay. No change of ThT fluorescence
was observed in samples without seeds.

129

Similar change from elongation-driven to secondary nucleation-driven processes can be observed

using sonicated versus unsonicated fibrils as seeds (Fig. 3A). The fibril denaturation assay (Fig.

3B) revealed that stability of fibrils formed in elongation-driven process is the same as of the

rPrP-A^{4M} strain, which was used as a seed. However for the secondary nucleation-driven

process, stability of fibrils is the same as the rPrP-A^{2M} strain, which is favored by the

environment. This leads to the conclusion that fibril formation from secondary nucleation does

not follow the seeding template, despite using template fibrils as nucleation sites.
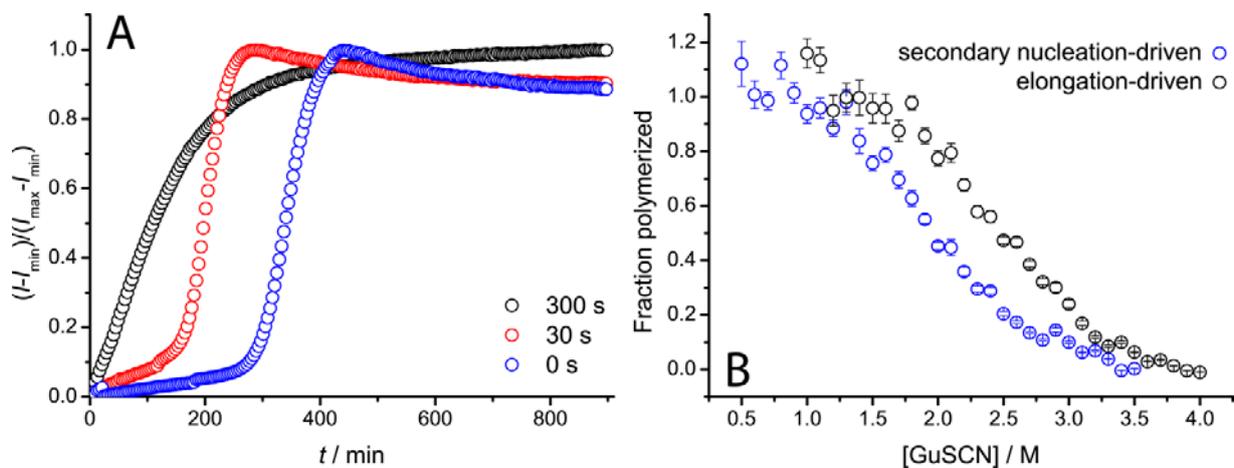
Figure 3. Amount of fibril ends determines the mechanism of aggregation and conformation of the final strain. (A) Different times of sonication were used to prepare rPrP-A^{4M} seeds. Sonication breaks fibrils into shorter pieces increasing number of fibril ends. The same amount of seeds (5%) was used in all experiments. (B) Denaturation profiles of fibrils obtained using unsonicated (secondary nucleation pathway) or highly sonicated (elongation pathway) rPrP-A^{4M} fibrils as seeds.

Discussion

Amyloid strain switching has been observed in animal studies (Bartz et al., 2000; Asante et al., 2002; Lloyd et al., 2004; Ghaemmaghami et al., 2013), cell culture (Li et al., 2010), and experiments in vitro (Castilla et al., 2008; Makarava et al., 2009; Surmacz-Chwedoruk, Babenko & Dzwolak, 2014). Two possibilities are suggested to explain this phenomenon (Collinge & Clarke, 2007; Cobb & Surewicz, 2009). The first one describes coexistence of multiple structures in the infective material, when only the dominant type would be recognized experimentally; however upon transmission to different host, the minor population may self-propagate much better and become dominant, reflected in the change of strain properties. Recently this way of amyloid strain switching was demonstrated for insulin fibrils *in vitro* (Surmacz-Chwedoruk,

154   Babenko & Dzwolak, 2014). The second possibility suggested that sometimes host protein can

155   adopt amyloid conformations distinct from the heterologous template. The Baskakov group

156   demonstrated adaptive conformational switching within individual fibrils as a possible

157   mechanism for such change (Makarava et al., 2009). Our data suggests a possibility of strain

158   switching via secondary nucleation pathways. Moreover, secondary nucleation can explain

159   switching of strains in absence of species barrier, for example in case of recently described

160   Darwinian evolution of prions in cell culture, which showed strain mutations within a single host

161   protein (Li et al., 2010) or in case of protein misfolding cyclic amplification (PMCA) of

162   recombinant PrP (Smirnovas et al., 2009). In summary, continuous propagation or switching

163   between amyloid strains may be determined by the mechanism of replication in addition to the

164   environment. In cases when a species barrier or environmental barrier stops or slows down fibril

165   elongation, there is the possibility of secondary nucleation events to seed the formation of

166   different strains. The mechanism is dependent on the concentration of fibril ends, which opens

167   up a new dimension in cross-species and cross-environment seeding/infection experiments.

168   Assuming the same mechanisms of prion propagation *in vivo*, there is a possibility of one strain

169   of PrP^{Sc} causing different disease variants. For example a hypothesis of both variant Creutzfeldt-

170   Jakob disease (CJD) and sporadic CJD to be caused by different amounts of the same PrP^{Sc} could

171   be valid.

172

173   **Acknowledgments**

174   The authors thank Prof. Witold Surewicz for sharing MoPrP(89-230) plasmid and Dr. Jonathan

175   G. Cannon for critical reading of the manuscript.

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