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Ion and seed dependent fibril assembly of a spidroin core domain

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ABSTRACT

Recombinant eADF4(C16) represents an engineered spider silk variant based on the sequence of the core domain of the natural dragline silk protein ADF4 of *Araneus diadematus*. Previously eADF4(C16) has been shown to self-assemble into cross-β fibrils in a two-step process of nucleus formation and fibril growth. Here, it is shown that structurally converted low molecular weight oligomers can act as nuclei. Further, it could be determined that specifically potassium and phosphate ions strongly influence both nucleus formation as well as fibril growth. Nucleation of fibril assembly could be surpassed by seeding soluble protein with pre-assembled fibrils but also, unexpectedly, with eADF4(C16) sub-micrometer particles. The latter finding reveals that spider silk fibril assembly seems to be rather dependent on the protein sequence than on the structural features, since cross-seeding with other proteins was not possible.

1. Introduction

Spider silk is a high-performance protein fiber with outstanding mechanical and biological properties (Kaplan et al., 1993; Lewis, 1996). Female orb web spiders can produce up to seven silk types in different glands, and five of these are used to build an orb web. The outer web frame, radial and lifeline fibers consist of silk proteins produced in the Major Ampullate (MA) gland, reflecting the best characterized spider silk fiber aka dragline (Gosline et al., 1999). The toughness of dragline silk exceeds that of most biological and engineered materials including steel or Kevlar (Gosline et al., 1999), with spider silk having a unique balance of strength, elasticity and viscoelastic characteristics.

Dragline silk comprises two different protein classes, mostly referred to as Major ampullate Spidroin 1 and 2 (MaSp 1 and 2) mainly differing in their proline content. All MaSp sequences are composed of highly repetitive sequence motifs comprising ~40 amino acids being repeated up to one hundred times within the core domain, which is intrinsically unstructured in solution (Ayoub et al., 2007; Hijirida et al., 1996; Humenik et al., 2011; Lefevre et al., 2008; Xu and Lewis, 1990). Non-repetitive (NR) domains are located at the carboxy- and amino termini of MaSps and both possess highly conserved α-helical structures (Askarieh et al., 2010; Hagn et al., 2010). Structure formation of the core domain is induced by several triggers including exchange of "salting in" ions like sodium and chloride for "salting-out" ions such as potassium and phosphate (Dicko et al., 2004a; Knight and Vollrath, 2001; Vollrath and Knight, 2001; Vollrath et al., 1998).

In order to analyze structure formation and self-assembly of a MaSp core domain in more detail, a previously established recombinant spider silk protein eADF4(C16), based on the consensus sequence of the core domain of *Araneus diadematus* Fibroin 4 (ADF4) was used as a model. Previously, it was shown that self-assembly of eADF4(C16) is triggered in the presence of phosphate ions (Pi). High concentrations of Pi (>400 mM) yield particles upon fast salting out and phase separation (Helfricht et al., 2013; Lammel et al., 2008; Slotta et al., 2008), while low Pi concentrations (<300 mM) trigger self-assembly into nano-fibrillar structures (Humenik and Scheibel, 2014; Humenik et al., 2014; Slotta et al., 2007, 2008) (Fig. 1 in Ref. (Humenik et al., 2015)). Assembly kinetics of eADF4(C16) fibrils (Humenik et al., 2014) could be explained by a nucleation dependent mechanism (Cohen et al., 2012; Eichner and Radford, 2011; Jarrett and Lansbury, 1993).

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Here, nucleus formation, seeded fibril assembly and the specific role of distinct ions on eADF4(C16) assembly were investigated in detail.

2. Materials and methods

2.1. Protein production and purification

Proteins eADF3(AQ)12, eADF4(C16) (Huemmerich et al., 2004) and Sup35p-NM (Scheibel and Lindquist, 2001) were produced in *Escherichia coli* and purified according to the published procedures.

eADF4(C16) sequence: GSSAAAAAAAASGPGGYGPENQGPSGPGG YGPGGP$_{16}$G.
eADF3(AQ)12 sequence: GPYGPGASAAAAAAGGYGPGSGQQGPG QQGPGQQGPGQQGPGQQ$_{12}$G; for detection purposes an amino terminal T7-tag was added to eADF4(C16) and eADF3(AQ)12 sequences.
Sup35p-NM sequence: MSDSNQGNNQQNYQQYSQNGNQQQGN NRYQGYQAYNAQAQPAGGYYQNYQGYSGYQQGGYQQYNPDAGY QQQYNPQGGYQQYNPQGGYQQQFNPQGGRGNYKNFNYNNNLQG YQAGFQPQSQGMSLNDFQKQQKQAAPKPKKTLKLVSSSGIKLANAT KKVGTKPAESDKKEEEKSAETKEPTKEPTKVEEPVKKEEKPVQTEEKT EEKSELPKVEDLKISESTHNTNNANVTSADALIKEQ EEEVDDEVVND.

2.2. Preparation of protein solutions

Stock solutions of eADF4(C16) and eADF3(AQ)12 were prepared by dissolving 3 mg/mL of protein in 6 M guanidinium thiocyanate (GdmSCN) followed by dialysis against 10 mM Tris/HCl, pH 7.5, with three buffer changes, twice after 2 h and once after 16 h. The protein concentration was determined using a Varian Cary 50 Bio UV–Vis spectrometer after centrifugation in a Beckman Optima ultracentrifuge at 55,000 rpm for 20 min at 4 °C. Before starting kinetic measurements, the monomeric state of eADF4(C16) was confirmed by size exclusion chromatography connected to a multiangle light scattering detector (Humenik and Scheibel, 2014) (SEC-MALS, Fig. 2 in Ref. (Humenik et al., 2015)).

Sup35p-NM was solubilized and assembled as described previously (Suhre et al., 2009).

2.3. Fluorescence labeling of eADF4(C16)

Either 5-(and 6-)carboxyfluorescein, succinimidyl ester (NHS-fluorescein) or 5-(and 6)-carboxytetramethylrhodamine, succinimidyl ester (NHS-TMR) were coupled to eADF4(C16). 15 times molar excess of each fluorophore was incubated with eADF4(C16) in 50 mM HEPES-Na, pH 7.1 for 2.5 h at room temperature. Upon addition of an equal volume of 2 M potassium phosphate, pH 8, the solutions were incubated for 30 min and then centrifuged for 15 min at 13,000 rpm. Precipitated fluorescein-eADF4(C16) and TMR-eADF4(C16) were washed 3 times using MQ water by successive centrifugation and resuspension.

2.4. Turbidity measurements

Turbidity of eADF4(C16) solutions was recorded at 340 nm in the presence of different salts in a Varian Cary 50 Bio UV–Vis spectrometer at 22 °C. The data were fitted using a logistic function to calculate lag times and apparent growth constants k_{app} (Table 1) as described previously (Humenik et al., 2014).

2.5. Thioflavin-T measurements

eADF4(C16) (5 μM), potassium phosphate buffer, pH 7.5, (200 mM) and Thioflavin-T (15 μM) were mixed with water to adjust the required concentrations. Assembly was investigated in a Jasco FP-6500 fluorescence spectrometer at an excitation wavelength of 450 nm and an emission wavelength of 480 nm at 22 °C.

Table 1
Kinetic parameters calculated from the turbidity measurements applying a logistic function (Humenik et al., 2014).

	k_{app} (min^{-1})	Lag time (min)
K-Pi	0.00435 ± 3.62E−5	856 ± 8.0
Na-Pi	0.00431 ± 1.74E−5	946 ± 3.6
K_2SO_4	0.00367 ± 1.76E−5	1200 ± 4.5
Na_2SO_4	0.00344 ± 1.94E−5	1403 ± 6.8
$(NH_4)_2SO_4$	0.00317 ± 2.69E−5	1255 ± 11.1

2.6. Tetramethylrhodamine measurements

Fluorescence emission of TMR-eADF4(C16) (5 μM) in K-Pi (200 mM), pH 7.5, was investigated in a Jasco FP-6500 fluorescence spectrometer using an excitation wavelength of 555 nm and an emission wavelength of 576 nm at 22 °C.

2.7. Sedimentation measurements

The supernatant of 120 μL samples was analyzed at specific time points using UV–Vis spectroscopy at 280 nm after centrifugation at 55,000 rpm in a Beckmann optima centrifuge for 20 min at 4 °C.

2.8. Seeding experiments using sonicated fibrils

Pre-assembled mature fibrils (Fig. 1 in Ref. (Humenik et al., 2015)) (assembled from 1 mg/mL eADF4(C16) in 100 mM K-Pi) were used either directly as seeds or after sonication using a MS73 tip set to 10% amplitude for 15 s with overall six repetitions while keeping the samples on ice. Seeds were added to an eADF4(C16) solution in the presence of 100 mM potassium phosphate at distinct percentages (w/w, seed/soluble protein). Seeds obtained upon sonication were visualized using atomic force microscopy (Fig. 3 in Ref. (Humenik et al., 2015)) as described previously (Humenik and Scheibel, 2014).

2.9. Seeding experiments using particles

Particles were prepared as described previously (Lammel et al., 2008). Briefly, recombinant proteins (1 mg/ml), were incubated in 10 mM Tris/HCl, 1 M K-Pi, pH 8, in a head-over-head mixer for 15 min. Particles were washed 3 times using MQ water using successive centrifugation and resuspension. A 100 μL reaction solution containing 0.05 mg/mL TMR-eADF4(C16) particles (1 μM), 1.0 mg/mL fluorescein-eADF4(C16) (20 μM) and 10 mM potassium phosphate, pH 8, was incubated at RT, and samples were taken at various time points for TEM and fluorescence microscopy.

2.10. Crosslinking experiments

eADF4(C16) (3 mg/mL) in GdmSCN was dialyzed against 10 mM HEPES-Na, pH 8, and ultracentrifuged as described above. eADF4(C16) assembly (2 mg/mL, 40 μM) was initiated in 150 mM K-Pi buffer, pH 7.5. Tris(2,2-bipyridyl)dichlororutheniu m(II) (Ru(Bpy)) (1 mM) and ammonium persulfate (APS) (20 mM) were added after 42 min (1/3 of the lag time), and the mixture was exposed to a tungsten lamp for 10 s. The mixture was further incubated with NHS-fluorescein (4 mM) for 5 min and quenched using β-mercaptoethanol (10 mM). Proteins were separated using