Biophysical Chemistry 237 (2018) 1–8

Contents lists available at ScienceDirect

Biophysical Chemistry

journal homepage: www.elsevier.com/locate/biophyschem

Amyloid fibrillation of an intrinsically disordered plant phloem protein *At*PP16-1 under acidic condition

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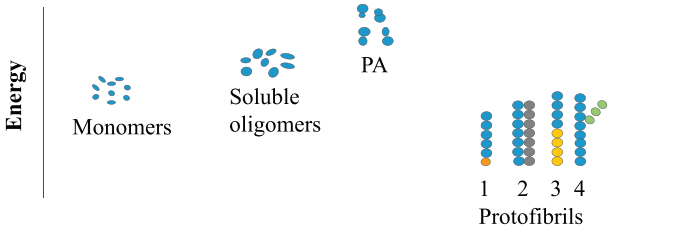
HIGHLIGHTS

- *Arabidopsis* phloem protein AtPP16-1 is an intrinsically disordered RNA transporter.
- The protein readily enters the amyloidogenic state at acid pH and high temperature.
- Fibrillation kinetics and structural changes are typical of amyloid fibrillation process.
- The fibrils are simple and occasionally branched; their dimensions have been scaled.

GRAPHICAL ABSTRACT



1 oligomer condensing at the end
2 lateral association of protofibrils
3 end-to-end joining of protofibrils
4 branching of protofibril

ARTICLE INFO

Keywords:
Arabidopsis thaliana
Intrinsically disordered phloem protein
Amyloid fibrillation of phloem protein

ABSTRACT

Arabidopsis thaliana Phloem Protein 16-1 (*At*PP16-1) is a 156-residue intrinsically disordered nucleic acid binding protein which is putatively involved in long-distance systemic transport of RNA to budding regions in plants. Dimerization or oligomerization of the protein at pH higher than about 4.1 leaves no apolar surface exposed for interaction with the dye 8-Anilinonaphthalene-1-sulfonate (ANS). The most stable monomeric state is found near pH 4 where the structure of the protein is determined to have three short β-strands and a single α-helix. By surveying the pH-dependent propensity of fibrillation we find the protein enters the amyloidogenic state at pH 2, 60 °C. The reaction product is not amorphous aggregate, but simple amyloid fibrils with sparse or no branching. The mean diameters of the fibril population scaled from AFM images are 13.2 and 21.2 nm for precursor aggregates (PA) and proto- or elongated fibrils, respectively. These values are somewhat larger than the fibril diameters generally cited, and the reason could be larger lateral association for both PA and protofibrils. The protein *At*PP16-1 is strictly pH-selective in terms of its structure and stability, and the solution structure is known at pH 4. Under the conditions of pH 2 used here for fibrillation, the protein retains substantial secondary structure. Even if the pH and temperature conditions used for fibrillation are hardly physiological, there is a finite possibility that some aggregation of *At*PP16-1 would occur in vivo, as the case of transglutaminase aggregates in the chloroplast of transplastomic plants, for example. The pH related problem has been discussed in detail, but the questions emanating are: do phloem proteins fibrillate in vivo, and if so what implication fibrillation has for plant physiology?

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https://doi.org/10.1016/j.bpc.2018.03.004
Received 11 January 2018; Received in revised form 22 March 2018; Accepted 22 March 2018
Available online 26 March 2018
0301-4622/

1. Introduction

The association of unbranched elongated fibrils with a variety of amyloid diseases, including prion and Alzheimer [1,2] has been known for a long time. But numerous globular proteins have been shown to form fibrils or forced to enter the amyloidogenic state under a variety of conditions [3–6] that may not even be physiologic. In general, the proteins are partly denatured in acid or alkali, or by adding solvent additives so as to convert them to branched or unbranched fibrillar states. Fibrillation observed for overwhelmingly large number of proteins establish that the fibrillar state is the fourth state of proteins − the native, unfolded, and molten globule states are the other three. In fact it is the premolten globule-like state that is often found to enter the amyloidogenic state [7].

Structurally, the fibrillar-state model of Astbury is characterized by a cross-β-sheet fibril [8]. Recent high-resolution NMR has shown extensive rearrangement of the native-state β-structures to produce a β-strand conformation in the fibril core [9]. Since not only β-sheet proteins but also those having both α-helix and β-sheet structures fibrillate, it is thought that the α-helices may unfold and then refold to β-strands in the course of fibrillation [10].

More recent amyloid fibrillation studies endeavor to understand the details of the mechanism of fibrillation, often at residue level [11–14]. Another important subject involves studies on plant protein fibrillation. Amyloidosis of plant proteins should never be a surprise because the fibrous state is a generic state of proteins as speculated by Astbury. However, reports of plant protein amyloid fibrillation have been relatively scarce, because of either sparse study or a general lack of propensity for fibrillation or both. Plant protein fibrillation most often reported are those of the well-studied monellin [15–17]. Amyloid fibrillation and amorphous aggregation of concanavalin A that belongs to the legume lectins family have been studied in fair detail [14,18]. Very recently concanavalin A has been induced to fibrillate by the use of cationic Gemini surfactant [19]. Amyloid-like inclusions of maize transglutaminase that share structural features with Alzheimer's and Perkinson's-associated aggregates have been located in the chloroplasts of tobacco transplastomic plants [20]. Even though in vivo fibril-like aggregate formation in plant cells might be different from the well-studied amyloid formation in mammalian cells, designed protein aggregation has been used for selective protein knockdowns in plants [13]. Recently, we solved the solution NMR structure of *At*PP16-1 [21], a 17,316 Da protein that presumably serves as a RNA transporter in the phloem conduit that runs into shoot or bud regions. The protein is selective about the working pH that we set to 4.1 for structure determination. The secondary structure consists of three β-strands, β1, β2 (56 − 62), and β3 (133 − 135), and an α-helix, and are formed of residue stretches 3 − 9, 56 − 62, 133 − 135, and 96 − 110, respectively (Fig. 1A). The remainder of the chain segments, the N-terminal residues 10 − 55 in particular, are largely disordered with surface overhangs of side chains. As the space-filled structural model shows, the surface ledges arising out of some disordered stretches produce irregular depressions with a conspicuous cleft (Fig. 1B). These structural features have already been incorporated in the description of *At*PP16-1 as a prolate-shaped intrinsically disordered protein (IDP) whose global correlation time is 8.4(± 0.7) ns [21]. This identification makes the protein even more attractive for fibrillation studies, because IDPs are thought to undergo some refolding at the earliest stage of fibrillation. This work shows growth of unbranched or less branched amyloid fibrils under acid destabilized conditions where most of the secondary structure but little tertiary structure is retained.

2. Materials and methods

2.1. Protein preparation

Cloning and purification of *At*pp16-1 has already been described [21]. Briefly, the cDNA is initially cloned into pTZ57R/T vector, subcloned into pET28a(+) vector, and transformed into BL21(DE3)RIL cells for protein expression. Cells are grown in Luria Bertani broth containing kanamycin (50 μg/mL) at 37 °C up to o.6 optical density at 600 nm before allowing protein expression at 28 °C by the addition of IPTG (0.5 mM). After 5 h of growth cells are harvested and suspended in phosphate buffer saline. All purification steps are carried out at 4 °C. Cell lysis is achieved by a '20 s on and 40 s off' sonication cycle for 5 min in 20 mM Tris-HCl, 50 mM NaCl, 5 mM imidazole, pH 8. The supernatant of the sonicated cell suspension is collected by a 20 min centrifugation at 23447*g*, passed through a Ni-NTA column equilibrated with lysis buffer, washed by flowing the wash buffer (20 mM Tris-HCl, pH 8, 50 mM NaCl, 40 mM Imidazole), and eluted with the elution buffer (20 mM Tris-HCl, 50 mM NaCl, 150 mM imidazole, pH 8). The eluted fractions are dialyzed in 50 mM sodium acetate at pH 4, the part of the protein precipitated during dialysis is removed by centrifugation, and the purity is checked by SDS-PAGE. For production of ^{15}N-labeled protein, all steps are identical except that the cells are grown in M9 medium containing $^{15}NH_4Cl$.

2.2. pH titration and ANS binding

The buffer mixture consisted of 10 mM glycine, 4 mM each of sodium acetate, HEPES and PIPES, 8 mM Tris, and 2 mM CAPS. The protein concentration was ~8 μM. Fractions of the stock solution were set to different pH values in the range 1.6 − 10.75 using minimal volumes of HCl, acetic acid, and NaOH. Following equilibration at 25°C for 2 h 218-nm CD and fluorescence spectra (280-nm excitation) were

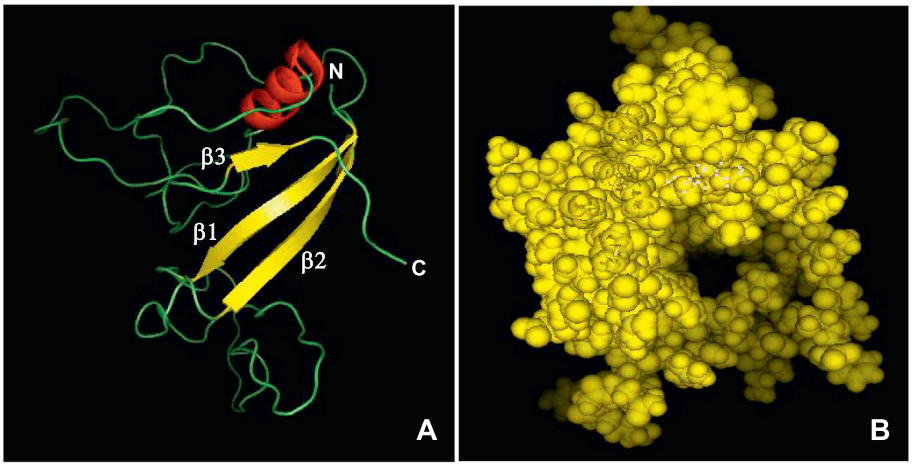

Fig. 1. Solution structure of *At*PP16-1 (PDB ID 5YQ3). (A) The secondary structure includes three antiparallel *β*-strands (βI, βII, and βIII corresponding to residues 3 − 9, 56 − 62, and 133 − 135, respectively, and an *α*-helix (residues 96 − 105). The disordered tertiary fold is noticeable. The stretch of residues 10 − 55 that connects strands βI and βII form a small lobe. (B) The space-filled model showing surface overhangs, a sign of disordered structure. A deep cleft that is supported laterally by the βII strand runs through the molecule and is thought to be important for DNA and RNA binding.