Fibril Formation by the TIA1 Low Complexity Sequence Domain

Rachelle Stowell*

Mentor: Dr. Dylan T. Murray
Department of Chemistry at UC Davis

Abstract

Amyotrophic lateral sclerosis and frontotemporal dementia are devastating neurodegenerative diseases that are poorly understood with no effective treatments. Mutations in the T cell-restricted intracellular antigen-1 (TIA1) protein linked to these diseases correlate with an increased incidence of pathogenic fibrillar aggregates in patient tissue, similar to the buildup of amyloid plaques observed in Alzheimer's patients. TIA1 is an RNA-binding protein that is essential for the formation of RNA stress granules, which are micron sized structures that protect cells in disease conditions. Pathogenic fibrillar assemblies of TIA1 are thought to nucleate from the RNA granule structures. In this study, we produced the TIA1 protein using a recombinant bacterial expression system, obtained milligram quantities of the protein, and have purified it to greater than 95% purity. TIA1 fibrils were prepared and transmission electron microscopy was used to visually characterize their appearance. A protein denaturation assay is employed to measure the thermodynamic stability of TIA1 in fibril form and solid state nuclear magnetic resonance to characterize the fibril structure. The aim of this study is to quantitatively characterize the mechanisms by which RNA granule formation and fibrillization occur. Results will allow

for a better understanding of the TIA1 assembly in a biological context.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating disease that leaves patients with a short-lived future. Some patients with ALS also express symptoms of frontotemporal dementia (FTD). The death of motor neurons in ALS patients leads to symptoms such as weak limbs, respiratory failure, and impairment of speech. In FTD, neuronal degeneration occurs in the frontal and anterior temporal lobes and is identified through behavioral symptoms such as changes in mood, apathy, and repetitive behaviors or actions (Langenhove, Zee, & Broeckhoven, 2012). Approximately 50% of ALS patients exhibit impaired cognitive abilities (Ringholz et al., 2005). After onset and prognosis, the typical lifespan of an ALS patient with or without FTD is approximately 3-5 years (Rowland & Shneider, 2001). While ALS and FTD have been commonly distinguished as different diseases, there is evidence that these disease pathways may be linked (Ling, Polymenidou, & Cleveland, 2013). Current, clinically approved drugs only marginally increase the lifespan of a patient by 2-3 months (Renton, Chiò, & Traynor, 2014). There is currently no cure or concrete understanding of what causes these diseases.

With gene sequencing becoming more accessible, we have discerned underlying genetics of neurodegenerative diseases. Whole genome sequencing has identified six specific amino acid mutations in the T cell-restricted intracellular antigen-1 (TIA1) protein linked to ALS pathology (Mackenzie et al., 2017). TIA1 is an RNA-binding protein (RBP) that regulates alternative RNA splicing (Meyer et al., 2018). Mutations of TIA1 that are linked to ALS occur in the low complexity

sequence (LCS) domain: a domain that drives the protein-protein interactions of TIA1.

RBPs with LCS domains exists in at least three states: intrinsically disordered, dynamic phase separated, and rigid fibril. These proteins can be recruited into micron sized membraneless organelles, called RNA granules. This process is a normal and healthy function of the cell. A proposed purpose of an RNA granule is to help regulate translation (Anderson & Kedersha, 2002). Little is known about the mechanisms that create RNA granules, but the LCS domains in RBPs play a significant role.

An LCS domain is a protein amino acid sequence that is highly enriched in only a few amino acid types. Four amino acids comprise 52% of the TIA1-LCS domain, which is 116 residues long. LCS-domains typically do not adopt a rigid three-dimensional structure, but are important for cellular functions such as RNA granule formation (Banani, Lee, Hyman, & Rosen, 2017). The LCS domain is necessary and sufficient for phase separation (Gilks et al., 2004; Kedersha, Gupta, Li, Miller, & Anderson, 1999; Lin et al., 2015; Mackenzie et al., 2017). Experiments that investigated the ability of different domains of RBPs with LCS domains show that the LCS domain, but not the RNA-binding domain, will form phase separations (Gilks et al., 2004; Molliex et al., 2015).

RNA granules are transient structures composed of molecules with highly fluid and dynamic properties (Buchan & Parker, 2009). A cell regulates RNA granule formation and dissociation based on extracellular conditions. RNA-binding proteins with mutations in the LCS-domain can cause RNA granules to mature, and result in proteinaceous aggregates in the cell (Murakami et al., 2015). Protein-protein phase separations of purified proteins *in vitro* reproduce the

macroscopic behaviors of RNA granules (Riback et al., 2017). TIA1 phase separations mature and become less dynamic over time and mutations in the TIA-LCS domain increase the likelihood in which this maturation occurs (Mackenzie et al., 2017). These physical changes are due to the formation of amyloid-like protein fibrils (Lin et al., 2015). These fibrils have a well-defined structure that can be studied to gain a greater understanding of the biological behaviors of the RBPs, such as TIA1.

There are few molecular studies that investigate TIA1-LCS in fibril form and how this relates to the distinct thermodynamic states in living cells. Here, we show that the TIA1-LCS can be expressed and purified using a recombinant *E. coli* expression system and that fibrils of TIA1-LCS can be reliably produced from this material. Our work lays a foundation for future structural and thermodynamic studies of TIA1-LCS in the phase separated and fibril states.

Methods

E. coli Transformation and Expression

An expression plasmid for 6x His-tagged TIA1-LCS was transformed into chemically competent BL21(DE3) *E. coli* cells using standard methods. An aliquot of the BL21(DE3) *E. coli* was thawed on ice. 2 µl of the plasmid DNA was added to the bacteria, was gently mixed, and placed on ice for 15 min. The mixture was then heat shocked at 42 °C for 90 s. 500 µl of Luria Broth (LB) media was added and the mixture was placed in a shaker-incubator at 37 °C, 220 rpm, for 20 min. The transformed *E. coli* were streaked onto an LB agar plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight.

For large-scale expression in LB media, a single colony was picked from the plate and was used to inoculate 50 ml of liquid LB media containing 100 µl/ml ampicillin. The culture was placed in an incubator at 37 °C overnight with shaking at 220 RPM. The following morning, 20 ml of this culture was transferred into 1 l of LB media with 100 µg/ml ampicillin in a 4 l baffled flask. The culture was grown at 37 °C with shaking at 220 RPM until the OD_{600} reached 3.32. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture grown at 37 °C with 220 RPM shaking for 3 hr. The cells were harvested by centrifugation at 6,000 g, flash frozen in liquid nitrogen, and stored at −80 °C.

For the expression of TIA1-LCS in minimal media (45 mM sodium phosphate dibasic heptahydrate, 8 mM sodium chloride, 20 mM potassium phosphate monobasic, 19 mM ammonium chloride, 5% w/v glucose, 2 mM magnesium chloride, 100 µM calcium chloride), an LB agar plate containing 100 µg/ml ampicillin was streaked from a −80 °C stock of the previously transformed BL21(DE3) *E. coli* using a pipette tip and incubated at 37 °C overnight. Two 50 ml LB cultures were inoculated with the bacteria from the agar plate using a pipette tip. 100 µg/ml of ampicillin was added to each culture and incubated overnight at 37 °C and 225 RPM. The next morning, the cultures were removed from the shaker and 20 ml of each culture was transferred into a separate 1 l of LB media. 100 µg/ml of ampicillin was added to each 1 l flask. The flasks were incubated at 37 °C with 225 RPM shaking. After approximately three hours, the cultures were harvested at 6,000 g for 10 min. The supernatant was discarded, the cell pellets were resuspended with approximately 10 ml of minimal media each and transferred together into 1 l of minimal media. 100 µg/ml of ampicillin was added to the minimal media and the solution was incubated at 37 °C, 225 RPM, for 30 min. The

OD_{600} was 1.626 a.u. measured on a 1 cm pathlength. 0.5 mM IPTG was added to the culture to induce protein expression and the culture was incubated for approximately 3 hr at 37 °C, 225 RPM. The OD_{600} after three hours was 2.12 a.u. measured on a 1 cm pathlength. The culture was harvested at 6,000 g for 15 min and the cell pellet was flash frozen in liquid nitrogen and stored at −80 °C until purification. The wet cell pellet weight was 2.8 g.

Purification of TIA1-LC

For the purification of TIA1-LCS expressed in LB media, the cell pellet was removed from the −80 °C freezer and thawed on ice. 20 ml of lysis buffer (6 M urea, 20 mM Tris pH 7.5, 200 mM sodium chloride, 200 mM imidazole, 1% v/v Triton X-100) containing 0.028 g of powdered hen egg white lysozyme were used to resuspend the cell pellet. The cell suspension was lysed using a Branson Sonifier 250 with a 1/4" tip with the settings 1 s on, 3 s off, 30% output until the solution was no longer viscous. The solution was transferred to a 50 ml centrifuge tube, 1 ml of lysis buffer was added, and spun down at 75,600 g for 30 min at 4 °C. The supernatant was harvested and loaded onto a 5 ml Bio-Rad Mini Nuvia IMAC Ni^{2+}-Charged column equilibrated in equilibration buffer (6 M urea, 20 mM Tris pH 7.5, 200 mM sodium chloride). The column was washed with equilibration buffer and then equilibration buffer with 20 mM imidazole, each time until the A_{280} absorbance returned to baseline. The protein was eluted with 200 mM imidazole. Fractions were collected from all steps of the purification and aliquots from each fraction were saved for SDS-PAGE analysis.

For the purification of TIA1-LCS expressed in minimal media, the cell pellet was removed from the −80 °C freezer and was thawed on ice. 25 ml of lysis buffer containing 625 µl of hen egg white lysozyme was used to resuspend the cells using a

serological pipette. Phenylmethylsulfonyl fluoride was added to the resuspension for a final concentration of 1 mM. The pellet was placed into a container of ice and water and was sonified with a Branson Sonifier 250 and 1/4" tip for 1 min using the following settings: 0.3 s on, 3 s off, 30% output. The sample was then centrifuged for 30 min at 75,600 g, 4 °C. The supernatant was loaded onto a 5 ml Bio-Rad Mini Nuvia IMAC Ni^{2+}-Charged column equilibrated with equilibration buffer and washed with equilibration buffer and equilibration buffer with 20 mM imidazole until the A_{280} absorbance returned to baseline. The protein was eluted using a gradient from 20 mM imidazole to 200 mM imidazole over 40 ml. Fractions were collected from all steps of the purification and aliquots from each fraction were saved for SDS-PAGE analysis.

SDS-PAGE of TIA1-LCS

An SDS-PAGE was run with aliquots from the fractions of all steps of each purification process. A BIO-RAD TGX Stain-free Fast Cast kit was used to make a 10% gel for the protein expressed in LB media. A 12% resolving, 4% stacking, gel was casted using a recipe adapted from Cold Springs Harbor Press for the protein purified from minimal media. All gel samples used 4x NuPAGE LDS loading buffer. The final samples contained 1x loading buffer containing a volume of each fraction normalized to the volume of the 20 mM wash. Samples were heated to 70 °C and then spun down at 20,000 g for 10 min and loaded into the gel. The molecular weight ladder used for both gels was the Fisher BioReagents EZ-Run *Rec* Protein Ladder.

Fibrillization of TIA1-LCS

The concentrations of protein solutions were determined using Beer's Law, a molecular weight of 15,516.97 g/mol, and an extinction coefficient of 53,860 $M^{-1}cm^{-1}$. The molecular weight and extinction coefficients were calculated from the protein

primary sequence with the 6x His tag using the online tool ProtParam (https://web.expasy.org/protparam/). 200 µl of an elution fraction containing 58.86 µM TIA1-LCS purified from the LB media expression was put into a 0.5 ml Amicon Ultra 3K MWCO spin filter and centrifuged at 14,000 g for 30 min, twice, until the final volume remaining was approximately 50 µl. The spin filter was then inverted into a new tube and spun down for 2 min at 1,000 g to collect the concentrated protein. The concentrated protein solution was mixed with 150 µl of fibril buffer in an Eppendorf tube. The tube was rotated for 11 d. 100 µl of the fibrillized solution was added to 300 µl of HEPES and was sonicated using a Branson Sonifier 250 with an 1/8" tip for 5 min total using the settings 0.2 s on, 1 s off, 15% output. The fibrillized solution was sonicated to break up the fibrils and create small fibril fragments, or fibril seeds.
A 65 µM protein elution from the minimal media expression was dialyzed in 20 mM HEPES pH 7.4 buffer. 200 µl of sample was spun down for 1 hr at 80,000 rpm and 8 °C to precipitate any aggregates. The supernatant was harvested and was combined with 75 µl of the fibril seeds. The sample was left on the benchtop to fibrillize for 10 d.

TEM Imaging

5 µl of the fibril solution was pipetted onto a glow discharged formvar-coated copper grid and sat for 2 min. The grid was quickly blotted with a laboratory wipe and washed twice with 5 ml of water and then negatively stained with 3% uranyl acetate for 10 s. Grids were then imaged with a JEOL 1230 electron microscope operating at 100kV with a 2k X 2k Tietz CCD camera.

Results

His-tagged TIA1-LCS can be expressed in E. coli and purified

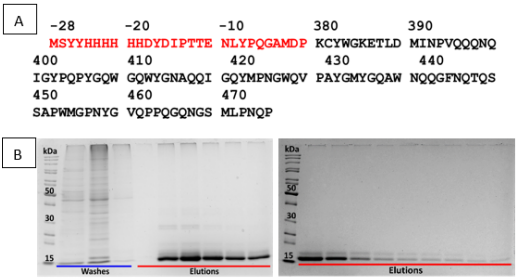

Figure 1: His-tagged TIA1-LCS preparation. (A) The single letter amino acid sequence of TIA1-LCS, with the N-terminal His-tag indicated in red. (B) SDS-PAGE of fractions from the stepwise purification procedure. The His-tagged TIA1-LCS has a calculated molecular weight of 15517 kDa.

The His-tagged TIA1 low complexity sequence domain (TIA1-LCS), with the amino acid sequence shown in Figure 1A was expressed in *E. coli* and purified using Ni^{2+} affinity chromatography with stepwise washing and elution from 20 mM to 200 mM imidazole. The purity of the TIA1-LCS protein obtained from the procedure was analyzed using the SDS-PAGE gel shown in Figure 1B. The dark bands near the bottom of the gel are consistent with the TIA1-LCS calculated molecular weight of 15517 kDa. Expression of TIA1-LCS in M9 minimal media yielded 18.22 mg from 1 l culture. Figure 2 shows that the TIA1-LCS protein obtained from the M9 minimal media is greater than 95% pure based on SDS-PAGE. Minor impurities observed in the elutions from the stepwise purification were removed using a gradient elution from 20 mM to 200 mM imidazole. Faint bands are present at approximately 16 kDa in Figure 2. Very faint bands can also be seen on some elutions in Figure 2 with a molecular weight of approximately 27 kDa.

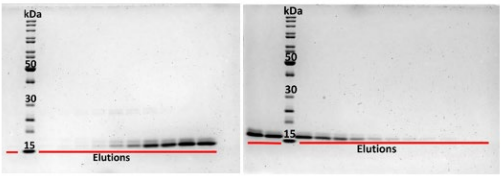

Figure 2: Preparation of TIA1-LCS in Minimal Media. SDS-PAGE of TIA1-LCS purified from M9 minimal media using a gradient elution.

Fibril Formation by His-tagged TIA1-LCS

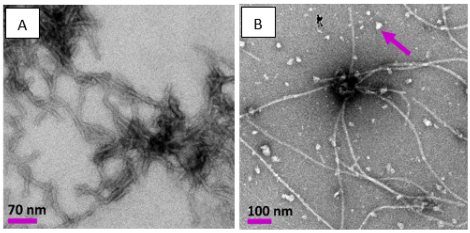

Figure 3: TEM Images of TIA1-LCS Fibrils
A. TEM images of negatively stained TIA1-LCS fibrils from initial fibril formation taken at 30,000x magnification.
B. TEM images of negatively stained TIA1-LCS fibrils formed through repetitive seeding taken at 25,000x magnification.

Figure 3 shows micrographs of TIA1-LCS fibrils from the initial fibrillization of fibrils produced through seeded growth. The initial fibrils were bundled together and varied in length. The fibrils produced through the seeding protocol are more separated and longer than the first preparation of fibrils, but the individual fibrils from each preparation were similar in diameter and lack of a distinct twist. In Figure 3B, there appears to be amorphous aggregates, indicated with an arrow.

Discussion

In this paper we show that His-tagged TIA1-LCS can be expressed in both LB media and minimal media and purified to 95% purity using immobilized metal affinity chromatography (Figures 1B and 2). Additionally, we show that TIA1-LCS readily forms protein fibrils when at concentrations above 58 µm in the absence of a denaturing agent or through a seeding procedure (Figure 3).

An initial concern was that the *E. coli* BL21(DE3) strain would not be able to express substantial amounts of TIA1-LCS due to toxicity of the human protein in *E. coli.* The results from the SDS-PAGE in Figure 1B confirm that we can successfully express and isolate pure TIA1-LCS using our recombinant bacterial expression system.

Once we confirmed the protein could be expressed in rich (LB) media, we investigated the expression of TIA1-LCS in minimal media (M9). Future solid state NMR measurements aimed at obtaining an atomic resolution structural model for the TIA1-LCS fibrils will require milligram quantities of isotopically labeled protein. We were able to confirm that adequate quantities of TIA1-LCS, greater than 10 mg per 1 l of culture, are obtainable in minimal media appropriate for isotopic labeling. The SDS-PAGE analysis of the protein obtained from our procedure is shown in Figure 2 and confirms that the protein is at least 95% pure.

Stepwise washing and elution of the protein was not sufficient to obtain the highly pure protein needed for structural studies. We altered our initial protocol to eliminate the impurities present in the elution from our stepwise purification protocol and found that an imidazole gradient was sufficient to remove almost all impurities. The gradient elution improved the purity of the elution, as determined by a decrease in bands on the SDS-PAGE gel that were not consistent with the molecular weight of TIA1-LCS (Figure 2).

In both gels, Figures 1B and 2, there are bands that are not consistent with His-tagged TIA1-LCS. There are faint bands right above the TIA1-LCS bands, indicating they are close in molecular weight. It is possible that it could be due to a cleavage of the His-tag. However, this cleavage would have to take place either during or after the elution step of the

purification, otherwise, the protein would not be able to bind to the column and would have been washed out before the elution. Another proposed explanation for the double band is TIA1-LCS adopting two conformations in the gel, resulting in varying amounts of interactions with SDS, altering its velocity down the gel. Part of the C-terminus of the protein may also have been cleaved during the expression of the protein, creating two different length proteins with the His-tag. The other band that is of interest is the one seen in both gels around 27 kDa. Based on the molecular weight of the monomeric TIA1-LCS, we propose that this could be a dimer of the TIA1-LCS. The formation of a dimer is unusual due to the sample containing 6 M urea and being run in SDS-PAGE, which contains a substantial amount of detergent. This proposal was made based on previous work with FUS, another protein with a LCS-domain, which runs on a gel with an apparent molecular weight of a trimer in the SDS-PAGE, despite also being solubilized in 6 M urea (Murray et al., 2017).

The TEM images, Figure 3, confirm that TIA1-LCS fibrils can be formed through seeded growth. Further optimization for fibrillization is needed to reduce the amorphous aggregates that are present in the sample. More rounds of fibrillization via seeded growth can be performed to create a more homogeneous preparation of fibrils. Amorphous aggregates could cause issues during a solid-state NMR experiment, such as inconclusive results due to signals that did not arise from the fibrils under investigation.

The data presented in this paper demonstrates that sufficient protein can be obtained for a structural investigation of TIA1-LCS. We have determined how to reliably prepare visually homogeneous fibrillar assemblies of the TIA1-LCS protein. Future work will be to record solid-state NMR measurements on the fibrils to determine the molecular conformation of the

protein in fibril form. Calorimetric, fluorescence, and denaturation assays will quantify the thermodynamics and kinetics of the fibrils. By investigating TIA1-LCS fibril assembly, we hope to understand the functional and pathogenic roles of TIA1-LCS at the molecular level.

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