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Solid-state NMR chemical shift assignments for AL-09 V_L immunoglobulin light chain fibrils

Dennis W. Piehl^{a,e}, Luis M. Blancas-Mejía^{b,e}, Marina Ramirez-Alvarado^b, and Chad M. Rienstra^{a,c,d}

^aDepartment of Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^bDepartment of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN 55905, USA

^cDepartment of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

^dCenter for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA

Abstract

Light chain (AL) amyloidosis is a systemic disease characterized by the formation of immunoglobulin light-chain fibrils in critical organs of the body. The light-chain protein AL-09 presents one severe case of cardiac AL amyloidosis, which contains seven mutations in the variable domain (V_L) relative to its germline counterpart, κ I O18/O8 V_L. Three of these mutations are non-conservative—Y87H, N34I, and K42Q—and previous work has shown that they are responsible for significantly reducing the protein's thermodynamic stability, allowing fibril formation to occur with fast kinetics and across a wide-range of pH conditions. Currently, however, there is extremely limited structural information available which explicitly describes the residues that are involved in supporting the misfolded fibril structure. Here, we assign the site-specific ¹⁵N and ¹³C chemical shifts of the rigid core residues of AL-09 V_L fibrils by solid-state NMR, reporting on the regions of the protein involved in the fibril as well as the extent of secondary structure.

Keywords

AL amyloidosis; solid-state NMR; light chain fibrils; protein structure

BIOLOGICAL CONTEXT

Light chain (AL) amyloidosis is an acute systemic disease in humans that is described by the formation of immunoglobulin light-chain fibrils in the extracellular matrix of critical organs

Corresponding authors: Chad M. Rienstra, University of Illinois at Urbana-Champaign, Department of Chemistry, 600 S Matthews Ave, Urbana, IL 61801, (217)-244-4655, rienstra@illinois.edu. Marina Ramirez-Alvarado, Mayo Clinic, Biochemistry and Molecular Biology, 200 First St SW, Rochester, MN 55905, (507)-284-2706, ramirezalvarado.marina@mayo.edu.

^eThese authors contributed equally to this work.

ACCESSION NUMBERS

Chemical shift assignments for AL-09 V_L fibrils can be accessed on the BioMagResBank (BMRB) under entry number 26879. The GenBank accession number for the light chain cDNA of AL-09 V_L is AF490909.

of the body (Baden et al. 2009). AL patients typically die within two to three years of diagnosis, and this expectancy is reduced to under a year if the affected organ is the heart—which comprises approximately 50 % of all AL cases (Abraham et al. 2003; Levinson et al. 2013; McLaughlin et al. 2006). Currently, there are only a limited number of therapies that exist which can marginally extend this outlook (Baden et al. 2009; McLaughlin et al. 2006). The light chains that compose these fibrils originate from abnormally proliferative monoclonal plasma cells, which secrete a large amount of the amyloidogenic protein into the bloodstream (Ramirez-Alvarado 2012). Although these proteins are natively innocuous, they can become toxic in the blood upon misfolding, providing the nucleation event for fibril elongation and deposition to occur (Baden et al. 2009). Although the exact reason for the misfolding of these proteins is not completely understood, studies on the thermodynamic stability of patient-derived light chains reveal that the introduction of particular somatic (non-inherited) mutations within the V_L of the germline sequence can predispose them to forming amyloidogenic fibrils (Baden et al. 2009; Blancas-Mejia et al. 2014).

One particularly severe case of AL arises from the protein AL-09 (from patient AL-09) (Martin and Ramirez-Alvarado 2010). The AL-09 V_L domain is a 108 amino acid residue protein composed of primarily β -sheet secondary structure that contains seven mutations relative to its non-amyloidogenic germline counterpart, κ I O18/O8 (Fig. 1a–b) (Baden et al. 2008; Baden et al. 2009; Blancas-Mejia et al. 2014). Notably, AL-09 is observed to form fibrils spontaneously and with fast kinetics at a physiological pH of 7.4, whereas κ I O18/O8 requires the addition of a pre-formed fibril to initiate the aggregation reaction (yet, both proteins spontaneously form fibrils at a pH of 2.0) (Fig. 1c) (Blancas-Mejia et al. 2014; Martin and Ramirez-Alvarado 2010; Ramirez-Alvarado 2012). Interestingly, restorative mutational analyses for AL-09 have revealed that the three nonconservative mutations are responsible for a significant amount of the destabilizing effect—Y87H, N34I, and K42Q (in decreasing order of influence) (Baden et al. 2008; Baden et al. 2009; Ramirez-Alvarado 2012). However, due to a lack of high-resolution structural or chemical shift information of the fibrillar species, the exact influence that these mutations are suspected to have on increasing the fibril-forming propensity of AL-09 relative to κ I O18/O8 remains unclear (Baden et al. 2009; Ramirez-Alvarado 2012). Accordingly, in this study, the sequence composition and secondary structure of the rigid regions for AL-09 V_L fibrils were investigated by magic-angle spinning (MAS) SSNMR spectroscopy, revealing an extensive and highly ordered fibril core that involves residues of both the N- and C-terminus.

METHODS AND EXPERIMENTS

Protein Expression and Purification

AL-09 V_L is a patient-derived variable domain proteins belonging to the I O18/O8 germline gene product (also known as IGKV 1-33) (Abraham et al. 2003). Uniformly [¹³C, ¹⁵N]-labeled AL-09 V_L protein was expressed using a modified version of the protocol described by Marley et al. 2001. Briefly, *Escherichia coli* BL21 (DE3) Gold competent cells (Stratagene, La Jolla, CA) were transformed with pET12a-AL-09 V_L and grown in 1 L of 2XYT media at 37 °C and 250 rpm. Upon reaching an A_{600nm} of 0.6, cells were pelleted for 20 min at 4,000 g, and subsequently washed and re-pelleted (20 min, 4,000 g) using an M9

salt solution (Sambrook et al. 2001) that excluded all nitrogen and carbon sources. The cell pellet was resuspended in 250 mL of isotopically labeled M9 minimal medium containing [^{13}C]-glucose and [^{15}N]-ammonium chloride (Cambridge Isotope Laboratories, Tewksbury, MA) as the sole carbon and nitrogen sources, and incubated for 1 h at 37 °C and 250 rpm to allow for recovery of growth. Cells were induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) for a concentration of 0.8 mM and then grown at 25 °C and 100 rpm for 12 h before harvesting by centrifugation at 8,000 rpm for 20 min.

Extraction of uniformly [^{13}C , ^{15}N]-labeled AL-09 V_L protein was accomplished by osmotic shock, as follows. Harvested cell pellets were resuspended in 100 mL (each) of cold phosphate-buffered saline (PBS), incubated on ice for 10 min, and pelleted for 20 min at 4,000 *g*. Subsequently, cell pellets were resuspended in 100 mL of 20% sucrose solution, incubated on ice for 10 min, and pelleted for 20 min at 8,000 *g*. Upon resuspension in 100 mL of 10 mM Tris–HCl buffer, pH 7.4, each pellet was sonicated three times for 10 s at 35% amplitude (with 1 min intervals of incubation on ice), using a Misonix S-400 sonicator (Osonica, Newtown, CT). Sonicated cells were pelleted for 20 min at 8,000 *g*, followed by resuspension in 100 mL of 5 M urea, incubation on ice for 30 min, and re-centrifugation for 1 h at 20,000 *g*. The resulting pellets were discarded and the urea supernatant was dialyzed overnight against 4 L (per pellet) of 10 mM Tris–HCl buffer, pH 7.4. Uniformly [^{13}C , ^{15}N]-labeled AL-09 V_L monomer was purified under the same buffer conditions by size exclusion chromatography with a HiLoad 16/60 Superdex 75 column on an AKTA FPLC system (GE Healthcare, Piscataway, NJ).

Protein purity and homogeneity were determined by SDS-PAGE. Protein concentration was determined by UV absorption at 280 nm using an extinction coefficient of $\epsilon = 13,610 \text{ M}^{-1} \text{ cm}^{-1}$ for AL-09 V_L, calculated from the amino acid sequence. Pure protein was flash frozen and stored at –80 °C. As a quality control measurement, far UV circular dichroism (CD) spectroscopy was used to confirm that the protein retained the native secondary structure previously reported at the beginning of the fibril formation reaction. Far UV-CD spectra from 260–200 nm (1 nm bandwidth) were acquired at 4 °C, on a Jasco Spectropolarimeter 810 (JASCO Inc., Easton, MD) using a 0.2 cm path-length quartz cuvette. All samples were prepared with 20 μM protein in 10 mM Tris–HCl pH 7.4.

Mass Spectrometry

The molecular mass of uniformly [^{13}C , ^{15}N]-labeled AL-09 V_L protein was determined using MALDI-TOF. The average molecular weight was measured to be 12776 Da, indicating ~99 % [^{13}C , ^{15}N] incorporation relative to the calculated masses for unlabeled and 100 % [^{13}C , ^{15}N]-labeled protein (11933 Da and 12789 Da, respectively).

In vitro Fibril Formation

Uniformly [^{13}C , ^{15}N]-labeled AL-09 V_L fibril samples were prepared from protein monomer solutions, according to previously reported protocols (Blancas-Mejia et al. 2014). Briefly, purified protein was thawed at 4 °C, filtered using 0.45 μm membranes and subjected to ultracentrifugation at a speed of 90,000 rpm (645,000 *g*) for 3.3 h in a NVT-90

rotor on an Optima L-100 XP centrifuge (Beckman Coulter, Brea, CA), to remove any pre-formed aggregates formed during the thawing of the soluble protein.

Ultracentrifuged protein samples were prepared on ice at a final concentration of 20 μ M in 10 mM Acetate Borate Citrate (ABC) buffer pH 2.0, containing 150 mM NaCl, 10 μ M Thioflavin T (ThT), and 0.02 % NaN₃ in 1.5 mL low binding microcentrifuge tubes (1.0 mL total volume). Fibril formation reactions were conducted in triplicate using sealed, black 96-well polystyrene plates (Nunc, Roskilde, Denmark; Greiner, Monroe, NC) with 260 μ L reaction mixture per well and incubating at 37 °C with continuous orbital shaking (300 rpm) in a New Brunswick Scientific Innova40 incubator shaker until fibril formation was complete. Fibrillation progress was monitored by the change in enhanced fluorescence intensity of ThT, and the presence of fibrils was subsequently confirmed by transmission electron microscopy (TEM). On average, the time required for fibrillation to conclude for AL-09 V_L fibrils is ~2 days (50 hours).

Transmission Electron Microscopy (TEM)

To confirm the presence of fibrils, a 3 μ L fibril sample was placed on a 300 mesh copper formvar/carbon grid (Electron Microscopy Science, Hatfield, PA), and excess liquid was removed. The samples were negatively stained with 2 % uranyl acetate, washed twice with H₂O, and air-dried. Grids were analyzed on a Philips Tecnai T12 transmission electron microscope at 80 kV (FEI, Hillsboro, OR).

Preparation of Fibrils for SSNMR Studies

Fibril samples were transferred to polypropylene microfuge tubes (Item #: 357448) and pelleted in a table-top ultracentrifuge with a TLA-100.3 (Beckman Coulter) rotor and microfuge tube adapters (Item #: 355919), for 1 hr at 4 °C and 55,000 rpm (or ~100,000 *g*, accounting for the shorter radius of the microfuge tubes). Following the removal of the fibrillation buffer, fibril pellets were washed twice with deionized water using homogenization and subsequent ultracentrifugation. Washed fibril pellets were dried completely under nitrogen gas by monitoring the mass of the sample until it remained unchanged. Dried AL-09 V_L fibril samples were packed into either 3.2 mm (outer-diameter) standard-wall rotor or 3.2 mm thin-wall rotor (Agilent Technologies, Santa Clara, CA), and hydrated to a level of 50 % water by mass. Kel-F and rubber spacers were used to retain hydration level of sample.

Solid-State NMR Spectroscopy

MAS SSNMR spectra of AL-09 V_L fibrils were acquired at 11.7 T (500 MHz ¹H frequency) and 17.6 T (750 MHz) on VNMRS (Agilent Technologies) spectrometers with a 3.2 mm Balun or BioMAS ¹H-¹³C-¹⁵N probe as well as at 14.1 T (600 MHz) on an InfinityPlus spectrometer using a 3.2 mm Balun or T3 probe. For all multidimensional experiments, the MAS rate was set to 11.111 kHz (500 MHz), 12.5 kHz (750 MHz), or 13.333 kHz (600 MHz), and a variable-temperature (VT) stack was used to provide a constant flow of -10 °C, 0 °C, or 10 °C air, for a sample temperature of -5 to 15 °C (due to heating caused by MAS and 65–80 kHz ¹H SPINAL-64 (Comellas et al. 2011) decoupling power). Multidimensional SSNMR experiments performed on AL-09 V_L fibril samples include: ¹³C-¹³C (CC)

2D, ^{15}N - $^{13}\text{C}\alpha$ (NCA) 2D, ^{15}N - $^{13}\text{C}'$ (NCO) 2D, ^{15}N -($^{13}\text{C}\alpha$)- $^{13}\text{C}\chi$ (N(CA)CX) 2D, ^{15}N -($^{13}\text{C}'$)- $^{13}\text{C}\chi$ (N(CO)CX) 2D, ^{15}N - $^{13}\text{C}\alpha$ - $^{13}\text{C}\chi$ (NCACX) 3D, ^{15}N - $^{13}\text{C}'$ - $^{13}\text{C}\chi$ (NCOCX) 3D, $^{13}\text{C}\alpha$ - ^{15}N - $^{13}\text{C}'$ (CANCO) 3D, $^{13}\text{C}\alpha$ - ^{15}N -($^{13}\text{C}'$)- $^{13}\text{C}\chi$ (CAN(CO)CX) 3D, and ^{13}C - ^{13}C - ^{13}C (CCC) 3D. One-bond ^{13}C - ^{13}C correlations were achieved using either dipolar-assisted rotational resonance (DARR; $\tau_{\text{mix}} = 25\text{--}90$ ms) (Takegoshi et al. 2001) or supercycled POST-C7 (SPC-7; $\tau_{\text{mix}} = 0.9$ ms or 1.2 ms) (Hohwy et al. 1999) for magnetization transfer, and one-bond ^{15}N - ^{13}C or ^{13}C - ^{15}N correlations were accomplished using SPECIFIC-CP (Baldus et al. 1998). Further experimental details are summarized in Supplementary Table 1.

Solid-State NMR Data Processing and Analysis

All SSNMR data were processed with forward or backward linear prediction, zero filling, and Lorentzian-to-Gaussian apodization using NMRPipe (Delaglio et al. 1995). Peak assignments, signal-to-noise ratio (SNR) measurements, and linewidth measurements were performed using Sparky (Goddard and Kneller). Secondary structure analysis of site-specifically resolved residues was performed using TALOS-N (Shen and Bax 2013).

ASSIGNMENTS AND DATA DEPOSITION

The number of residues involved in the structured region of uniformly [^{13}C , ^{15}N]-labeled AL-09 V_L fibrils was assessed by the acquisition and analysis of multidimensional MAS SSNMR spectra (Fig. 2). Specifically, a count of the number of peaks observed in initial 2D ^{13}C - ^{13}C (CC) spectra allowed for an approximation of >50 spin-systems at a signal threshold of 10 times the noise floor, σ . This process was facilitated by the focus on Ala and Thr spin-systems, which exhibit highly resolved side-chain peaks in ^{13}C - ^{13}C 2D spectra (Fig. 2b–c). This estimate was further improved by repeating the process for higher resolution 3D data sets—specifically, NCACX, NCOCX, and CAN(CO)CX spectra (at a signal threshold of 8σ)—which consistently revealed the presence of 70 ± 15 spin-systems. Notably, as there are five alanine residues in the AL-09 V_L sequence, the observation of no more than five alanine $^{13}\text{C}\alpha$ - $^{13}\text{C}\beta$ peaks in the CC 2D (Fig. 2b) and 3D spectra provided initial support for the existence of a homogenous fibril conformation. Furthermore, the majority of the resolved cross-peaks between the α - and β -carbons in each residue are indicative of a primarily β -sheet secondary structure (Fig. 2a–c). Together, these data reveal that approximately 70 residues of the 110 total in the AL-09 sequence are observed in the rigid fibril structure.

To pursue the site-specific identification of the residues involved in the highly structured fibril region, chemical shift assignments were made via a backbone-walk using a set of NCACX, NCOCX, and CAN(CO)CX 3D spectra (experimental details for each are described in Supplementary Table 1). This analysis enabled the complete backbone assignments of 48 residues site-specifically within the AL-09 sequence, as well as partial assignments for an additional 8 residues (Fig. 3 and S1). Furthermore, we have also made tentative assignments for residues I34, R61–G66, and S76–S77 (9 total); however, because the sensitivity of these spin-systems was relatively limited in the 3D data sets we could not confidently confirm the connectivity between these residues and their neighbors, and thus

opted to exclude these assignments here. Interestingly, the majority of the assigned resonances are located within the first 35 and last 20 amino acids of the N- and C-termini, respectively. In addition, TALOS-N predictions of the secondary structure and order parameters (based on the chemical shift values of the assignments) support the existence of a highly ordered and β -sheet rich fibril structure (Fig. 3) (Shen and Bax 2013).

Overall, our results reveal that approximately 70 of the 110 residues in the AL-09 V_L sequence are involved in the highly ordered fibril with β -strand secondary structure. Moreover, we observed that the majority of these rigid residues are located in the N- and C-termini of the AL-09 V_L sequence and generally demonstrate strong and well-resolved backbone signals. By contrast, the residues located between the two termini (i.e., those immediately following N30 and preceding L94) exhibit relatively weaker signal intensities and/or chemical shift degeneracy, consequently leading to ambiguity. Nevertheless, the majority of these spin-systems were able to be assigned by amino acid type based on comparison with empirical chemical shift statistics. Further, the cross-peak intensities exhibited by these spin-systems were observed in most cases to range from 60–130% relative to the average peak intensity of site-specifically assigned residues. Together these observations provide support for the existence of a single conformation of the AL-09 V_L fibrils, as the distribution of total intensity between several different conformations of a single spin-system would lead to a substantial reduction in the intensity of each cross-peak. Finally, one interesting correlation underlying the extent of chemical shift assignments reported here is that all seven mutations are positioned in the central portion of the AL-09 V_L sequence, and only two of which have assignments for all backbone atoms (N30 and L83). Although a detailed understanding of the interactions engaged by the non-conservative mutations within the fibril structure will require completion of all chemical shift assignments, it is nonetheless noteworthy that the mutations are not the sole constituents of the ordered fibril core.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

a. Sequence alignment for AL-09 and κ I O18/O8 V_L (residues in red indicate mutations; those in blue are purification artifacts). **b.** X-ray structures of AL-09 and κ I V_L dimers in their native forms (PDBs: 2Q1E and 2Q20, respectively) (Baden et al. 2008). **c.** TEM images of [¹³C, ¹⁵N]-labeled AL-09 fibrils used for SSNMR studies

Fig. 2.

a. ^{13}C - ^{13}C 2D SSNMR spectra of $[^{13}\text{C}, ^{15}\text{N}]$ -labeled AL-09 V_L fibrils collected at 750 MHz and 0 °C using 40 ms ^{13}C - ^{13}C DARR (dipolar) mixing and 12.5 kHz MAS. Labels indicate a subset of site-specific residue assignments and spectral regions unique to specific amino acid types. **b.** Expansions of the Ala ^{13}Ca - $^{13}\text{C}\beta$ region and **c.** Ser/Thr $^{13}\text{C}\beta$ - ^{13}Ca region illustrate the extent of assignments and predominantly β -sheet secondary chemical shift trends

Fig. 3.

Secondary structure and extent of chemical shift assignments for AL-09 V_L fibrils. TALOS-N predicted backbone dihedral angles psi (closed squares) and phi (open circles) are plotted a function of residue number; error bars correspond to the standard deviation of the averaged TALOS-N matches. AL-09 residues for which chemical shifts have been assigned are labeled in blue; those in bold are unambiguous and have resonances assigned to all three backbone atoms. Highlighted residues indicate the location at which mutations occur; those that are underlined are non-conservative