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Differences in protein concentration dependence for nucleation and elongation in light chain amyloid formation

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Abstract

Light chain (AL) amyloidosis is a lethal disease characterized by the deposition of the immunoglobulin light chain into amyloid fibrils, resulting in organ dysfunction and failure. Amyloid fibrils have the ability to self-propagate recruiting soluble protein into the fibril by a nucleation-polymerization mechanism, characteristic of auto-catalytic reactions. Experimental data suggest the existence of a critical concentration to initiate fibril formation. As such, the initial concentration of soluble amyloidogenic protein is expected to have a profound effect on the rate of fibril formation.

In this work, we present *in vitro* evidence that fibril formation rates for AL light chains are affected by the protein concentration in a differential manner. *De novo* reactions of the proteins with the fastest amyloid kinetics (AL-09, AL-T05 and AL-103) do not present protein concentration dependence. Seeded reactions however, presented small protein concentration dependence. For AL-12, seeded and protein concentration dependence data suggests a synergistic effect for recruitment and elongation at low protein concentrations, while reactions of κ I had a poor efficiency to nucleate and elongate preformed fibrils. Additionally, co-aggregation and cross seeding of κ I variable domain (V_L) and the κ I full length (FL) light chain indicate that the presence of the constant domain in κ I FL modulates fibril formation, facilitating the recruitment of κ I V_L . Together, these results indicate that the dominant process in the fibril formation varies among the AL proteins tested with a differential dependence of the protein concentration.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information. Supplementary table 1 with crystallographic data collection; thermodynamic characterization of AL proteins, *De novo* and seeded fibril formation kinetics at different concentration regime are presented in supplemental figures S1 to S8.

Author Contributions

L.M.B.-M. and M.R.-A. designed the study. L.M.B.-M. conducted the experiments; L.M.B.-M. and M.R.-A. analyzed the data. L.M.B.-M. and M.R.-A wrote and edited the manuscript.

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Light chain (AL) amyloidosis is a progressive and incurable disease characterized by the anomalous aggregation of monoclonal immunoglobulin light chains into amyloid fibrils and tissue deposition, which results in organ dysfunction.^{1, 2} *In vitro*, amyloid fibrils are formed following a nucleation polymerization mechanism.^{3–8} In this mechanism; well-known features have been described: *A*) a critical concentration, below which fibrils cannot form; *B*) a lag phase before fibrils form, where the monomer quickly reaches equilibrium with oligomers, and nucleation occurs at a rate dictated by this equilibrium; *C*) a strong dependence of the fibril formation rate on concentration.^{7, 9, 10}

The ability to self-propagate is a fundamental property of all amyloid fibrils and proceeds as a continuous auto-catalytic process via the recruitment of additional protein molecules from the surrounding solution.^{1, 8, 11} In fact, the sigmoidal nature of the amyloid fibril formation kinetics is characteristic of auto-catalytic reactions^{4, 7, 8, 12}, where the rate increases as the materials react. Autocatalytic reactions proceed slowly in the beginning because there is little catalyst present. As the reaction continues, the rate of reaction accelerates with an increased amount of catalyst to then slow down as the reactant is consumed. In the case of amyloid formation reactions, the reaction reaches equilibrium at the plateau region.

One of the hallmarks of nucleation-polymerization processes is a strong dependency on the precursor concentration. In the case of amyloid formation, this would be a strong dependency on soluble (monomeric in most cases) protein precursor concentration. The critical concentration is defined as the lowest concentration in which a fibril formation reaction will occur. Powers and Powers have reported on the numerical solutions to the rate equations for nucleated polymerization and the analytical solutions to some limiting protein concentration cases.⁹ They show that nucleated polymerization is caused by the concentration approaching and then exceeding the equilibrium constant for dissociation of monomers from species smaller than the nucleus, a quantity that they have named the supercritical concentration. When the concentration of the reaction exceeds the supercritical concentration, the monomer, not the nucleus, is the highest energy species on the fibril formation pathway, and the fibril formation reaction behaves initially like an irreversible polymerization.

It is well established that the rate of fibril formation is accelerated by the addition of preformed fibrils (a phenomenon called “seeding” because of its analogy with the crystallization process). Recent experimental evidence from a number of laboratories,^{13, 14}

supports the idea that *in vitro*, the recruitment of the monomers to the nascent fibril can also occur via a different pathway, by lateral nucleation and fibril fragmentation, known as “secondary nucleation”.^{15, 16} which can happen following three different mechanisms: (a) Fragmentation breaks fibrils to produce new ends suitable for growth with a rate depending only upon the concentration of existing fibrils, and results in fibrils with a long and linear morphology.¹⁷ (b) Branching allows a new fibril to grow from within an existing fibril. (c) lateral interactions occur when addition of monomer facilitates side to side interactions with another fibril, with a rate dependent on both the concentration of monomers and that of existing fibrils.¹³

Within the context of the proteins selected for this study, immunoglobulin light chains exist as dimers. We have characterized the structure of κ I dimers and have proposed that an altered dimer is thermodynamically unstable and amyloidogenic.^{18, 19} In addition to the monomer concentration described by Powers and Powers⁹, an added equilibrium within AL amyloid formation is the dimer/monomer equilibrium and the dynamic nature of these dimers.

The germline κ I crystalized as a canonical dimer, whereas the amyloidogenic protein AL-09 adopted an altered dimer with a 90° rotation with respect to the canonical dimer structure¹⁹, characterized as weak homodimers (κ I $K_d = 217 \mu\text{M}$; AL-09 $K_d = 23 \mu\text{M}$). In addition, AL-103 and AL-12 retain the canonical germline dimer interface with subtle structural alterations in dynamic regions of both proteins.²⁰ AL-103 presents a strong kinetic protein folding control that may promote conformational changes that allow the protein to sample amyloidogenic conformations.²¹ AL-103 and AL-12 dimerization K_d values are 200–700 nM and 400 μM respectively (unpublished observations). AL-T05 is a λ 1b protein (48% sequence identity with respect to the κ I germline) that presents the fastest amyloid formation kinetics of the proteins characterized in our laboratory.²²

In this work, we present *in vitro* evidence that the overall rates of AL light chain *de novo* and self-seeded amyloid formation reactions are affected by protein concentration in a differential manner. *De novo* reactions of the proteins with the fastest amyloid kinetics (AL-09, AL-T05 and AL-103) do not present protein concentration dependence while we observe a minor protein concentration dependence on their seeded reactions. For AL-12, self-seeded and protein concentration dependence data suggests a synergistic effect for recruitment and elongation at low protein concentrations, while reactions of κ I had a poor efficiency to nucleate and elongate preformed fibrils at all concentrations. Co-aggregation and cross seeding of κ I V_L and the κ I full length (FL) light chain indicate that the presence of the constant domain in κ I FL modulates fibril formation, facilitating the recruitment of κ I V_L. Additionally, we present the crystal structure of λ 1 AL-T05 with an altered dimer rotated 180° from the canonical dimer, like the one reported for κ I Y87H.¹⁸

Together, these results suggests that protein stability and altered interfaces in amyloidogenic light chain dimers confer a synergistic effect for recruitment and elongation at low protein concentrations in the primary nucleation process, with secondary nucleation the most dominant process as the protein concentration increases. It is clear that minor disruptions in the quaternary structure could significantly increase the protein amyloidogenic propensity.

Experimental Procedures

Chemicals

Water was Milli-Q grade. Yeast extract and tryptone were from Difco. Other reagents were from Sigma-Aldrich.

Cloning, expression, extraction, and purification of light chain proteins

AL-09, AL-T05, AL-12, and AL-103 are patient-derived variable domain proteins. The κ I variable domain and the full length light chain κ I FL are derived from the germline gene product (also known as IGKV 1-33). We omitted V_L from the name of all proteins herein for simplicity, unless we are talking about co-aggregation and cross seeding between V_L and FL proteins.

Protein expression was performed as reported previously.^{19, 20, 22–26} Protein concentration was determined by UV absorption at 280 nm using an extinction coefficient calculated from the amino acid sequence as follows: $\epsilon=14,890 \text{ M}^{-1} \text{ cm}^{-1}$ for κ I and AL-103; $\epsilon=13,610 \text{ M}^{-1} \text{ cm}^{-1}$ for AL-09 and AL-12; $\epsilon=18,020 \text{ M}^{-1} \text{ cm}^{-1}$ for AL-T05; and $\epsilon=25,940 \text{ M}^{-1} \text{ cm}^{-1}$ for κ I FL. Pure proteins were frozen and stored at -80°C .

Circular Dichroism spectroscopy

As a quality control measurement, far UV circular dichroism (CD) spectroscopy was used to confirm that the proteins retained their native secondary structure at pH 7.4. 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 AL samples (20 μM) were prepared in 10 mM Tris-HCl pH 7.4. For κ I FL, experiments were carried out (2 μM) in PBS buffer, in a 1.0 cm cuvette under continuous stirring, as reported previously.²⁴ We have previously reported that at pH 2.0 in the presence of 150 mM NaCl, AL-09 shows stable β -sheet structure. AL-12, κ I, and AL-T05 also retain their secondary structure. AL-103 presented an increment in random coil content.²⁷ Thermal unfolding/refolding experiments were carried out following the ellipticity at 217 nm over a temperature range of $4\text{--}90^\circ\text{C}$ to determine the melting temperature (T_m), and to ensure the quality of the preparation, as reported previously.^{24, 28} Temperature was regulated within $\pm 0.002^\circ\text{C}$ using a Peltier system.

Sample preparation for *in vitro* fibril formation assay

Proteins were thawed at 4°C , filtered using $0.45 \mu\text{m}$ membranes, and equilibrated for 24 hours at 4°C . Filtered, equilibrated proteins were ultracentrifuged at a speed of 90,000 rpm ($645,000 \times g$) for 3.3 h in a NVT-90 rotor on an Optima L-100 XP centrifuge (Beckman Coulter). This step was carried out to remove any preformed aggregates formed during the thawing process of the soluble protein, as reported previously.²⁸ After ultracentrifugation, and prior to initiation of the fibril formation reaction, the global structure and oligomeric state (monomer/dimer equilibrium) of the proteins were confirmed via Far UV-CD spectra, thermal unfolding, and analytical size-exclusion chromatography to ensure the integrity and homogeneity of the proteins before the initiation of the fibril formation reaction.

Size exclusion chromatography

Analytical size-exclusion chromatography was carried out at 4°C using a BioSil 125-5 HPLC (Bio-Rad) size exclusion column on an AKTA FPLC system (GE Healthcare). The column was equilibrated with 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 150 mM NaCl, and 0.02% NaN₃ at pH 6.8, the recommended buffer system for this column and the molecular weight standards we use (thyroglobulin, bovine gamma-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12) were used to calibrate the column (BioRad, Hercules, CA). The same molecular weight standards have been used with PBS, pH 7.4 and 10 mM Acetate Borate Citrate, 150 mM NaCl at pH 2.0. Chromatographic analyses were carried out at 0.2 mL/min. κ light chain variable domains have been previously characterized as weak homodimers at relatively high concentrations (~700 μ M), with dissociation constants that range between 300 μ M to 0.2 μ M as calculated by NMR diffusion experiments.¹⁸ In order to re-establish the dimer-monomer equilibrium, protein samples (200 μ L of pure proteins diluted to 20 μ M) were incubated 24 hours at 4°C prior to injection. Chromatographic peaks were detected by UV absorbance at 280 nm. Molecular weight and oligomeric states were estimated from elution volumes using a molecular weight calibration curve as reported before.²⁰ All proteins studied under these experimental conditions were monomeric when injected at 20 μ M at pH 6.8, demonstrating the monodispersity of the protein before the beginning of the reaction. Our control experiments have also shown monodispersity and monomeric protein at pH 7.4 and pH 2.0.

In vitro fibril formation assay

***De novo*, nucleation (non-seeded) experiments**—Samples of each filtered and ultracentrifuged protein 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), 0.02% NaN₃, in 1.5 mL low binding microcentrifuge tubes (1.0 mL total volume).

In vitro fibril formation reactions were carried out by monitoring the fluorescence emission enhancement of Thioflavin T (ThT) that occurs when ThT binds to amyloid fibrils. All fibril formation assays were performed in triplicate (260 μ L per well) using black 96-well polystyrene plates (Greiner, Monroe, NC) sealed with plate sealers (Nunc; Roskilde, Denmark), covered with a black polystyrene cover, and sealed with tape to reduce evaporation. Samples were incubated at 37°C with continuous orbital shaking (300 rpm) in a New Brunswick Scientific Innova40 incubator shaker. Fibril formation was monitored daily for 1 month (~750 h) following fluorescence on a plate reader (Analyst AD, Molecular Devices; Sunnyvale, CA). The excitation wavelength used was 440 nm and the emission wavelength was 480 nm. The plate sealer and the tape sealing the cover were replaced daily.

We considered that a fibril formation reaction had occurred when we observe at least a four-fold ThT-fluorescence enhancement (~200,000 A.U. in our system). However, we are aware of the drawbacks associated with measuring ThT fluorescence enhancement as the only measure of amyloid formation. This includes but is not limited to: non-specific ThT binding to non-cross beta structures, inner filter effects, loss of ThT binding sites due to excessive fibril clustering, and inadequate amount of available ThT to bind to amyloid fibrils.

present.^{29, 30} For that reason, the presence of amyloid fibrils was confirmed by electron microscopy.

Preparation of amyloid seeds

For the seeding experiments, freshly amyloid seeds were prepared as follows: 800 µL of amyloid fibril solution (20 µM of soluble monomer at the beginning of the reaction) from *de novo* experiments were collected and transferred into low-bind microcentrifuge tubes. Samples were sonicated at room temperature for 10 seconds using a water bath sonicator Branson 8510 (Branson Ultrasonics Corp, Danbury, CT).

Fibril elongation (self-/homologous and cross-/heterologous seeding) experiments

Fresh solutions of 20 µM of each AL protein were prepared as we prepared them for *de novo* reactions. 10 µL or 100 µL freshly prepared seeds, (1%, 10% V/V, respectively) were sonicated, and added to the reaction mixture. ThT fluorescence was monitored in the plate reader as previously described for the *de novo* experiments. In all cases, the same batch of freshly prepared seeds was used to avoid variability within the seeded reactions. Samples were kept on ice until the beginning of the reaction.

De novo control reactions, as well as control wells containing only ThT without protein were carried out in parallel, to ensure that any differences in fibril formation kinetics (measured as t_{50} values or the time it takes to complete 50% of the fibril formation reaction as well as elongation rates) could be attributed directly to the presence of the specific seed, while ensuring the reproducibility of the observations.

Analysis of Experimental Kinetic Data

The t_{50} value was obtained by fitting each independent kinetic trace to a sigmoidal function (defined as a Boltzmann function by the Origin software package <http://www.originlab.com/www/help/online/Origin/en/UserGuide/Boltzmann.html>) as previously reported.^{21, 31, 32}

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2 \quad (\text{Eq. 1})$$

Where A_1 is the initial fluorescence value, A_2 is the final fluorescence value, x_0 is the midpoint (or t_{50} value) and dx is defined as the time constant.

A shorter t_{50} value indicates a faster reaction to form fibrils. The rates of fibril elongation were calculated from the slope of the linear fitting of the growth phase. The uncertainties measured are independent triplicates for each protein concentration tested. Triplicates were measured in the same plate during each experimental run. However each kinetic trace was individually analyzed. The kinetic parameters reported are the average of the t_{50} values determined for each kinetic trace. The comparison and statistical analysis of the effects of homologous and heterologous seeding over the fibril formation, was based on paired Student's t-test. Significance was reported at the 95% ($p < 0.05$) confidence level.

Electron Microscopy

3 μL 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 H_2O , and air-dried. Grids were analyzed on a Philips Tecnai T12 transmission electron microscope at 80 kV (FEI, Hillsboro, OR).

Crystallization/X-ray Data Collection

Purified AL-T05 was concentrated to 29.3 mg/mL. AL-T05 crystals were obtained in hanging drops using vapor diffusion against 30% (w/v) polyethylene glycol 8000 and 100 mM sodium cacodylate in 100 mM sodium acetate buffer (pH 6.5) at 25 °C. Crystals were cryoprotected with a 10% glycerol solution in liquid N_2 .

Diffraction data were collected at 1.5241 nm on a home X-ray source, Rigaku/MSO 007 microfocus generator, with Osmic VariMax optics, Xstream2000 cryostream, and an R-axis IV++ detector. The data sets were collected at 100 K. Diffraction patterns were processed with Crystal Clear and XDS. All structures were solved by molecular replacement with PHASER as implemented by the Phenix software package using the $\kappa\text{I O18/O8}$ structure (Protein Data Bank code 2Q20) as a probe molecule. Phenix and Coot were used for structure refinement and model building. Table 1 summarizes the statistics for the crystallographic diffraction data collection and structural refinement. Structure was deposited into the Protein Data Bank with the code 5T93.

Results

Concentration dependence of fibril formation reaction rates

To ensure the reproducibility of the fibril formation reactions, we initiate all reactions with proteins in the native conformation. Far-UV CD spectra and thermal unfolding experiments were carried out (Figure S1). All proteins presented the characteristic Far UV-CD spectrum at pH 7.4 (Figure S1), for V_L proteins, as previously reported.^{21, 24, 27, 28, 31–36}

ThT fluorescence was chosen to monitor the fibril formation because of its high sensitivity and signal to noise ratio compared to other common techniques, like dynamic light scattering, particularly important at low protein concentrations. Figure 1 shows representative parameters derived from *de novo* fibril formation at different protein concentrations (0.1 μM –90 μM). Raw kinetic data for all proteins and concentrations can be found on figure S2–S4.

Figure 1 A–E shows that for all proteins except AL-T05, the critical concentration seems to be the same (0.02 μM), the lowest protein concentration we were able to follow by ThT fluorescence. For AL-T05, 0.7 μM was the lowest concentration where fibril formation was detected (Figure 1E). It is worth to note that the experiments performed at the low range of protein concentration (0.1 μM –0.9 μM) present, as expected, the lowest signal to noise ratio for most proteins (except AL-103, see Figure S2–S4) and the variability between them could be larger than the error as has been reported previously for $\beta 2$ -microglobulin.³⁷ The only

protein that presents concentration dependence is AL-12. As AL-12 concentration increases, the t_{50} values increase as well. This behavior has been reported previously for another AL light chain protein (κ IV SMA).³⁸ The inverse concentration dependence on the kinetics of amyloid formation was attributed to the protective effect of the SMA dimer. This is a possibility for AL-12, as the K_d for dimerization has been calculated around 400 μ M. However, the delay in amyloid formation as a function of increased protein concentration can also be attributed to off-pathway species formation.³⁹

As we mentioned before, AL proteins presented a broad range of dimer dissociation constants. At 20 μ M, the fraction of dimers calculated is around 96.9 % for AL-103 ($K_d \sim 700$ nM); 46.5% for AL-09 ($K_d = 23$ μ M); 8.4% for κ I ($K_d = 217$ μ M); and 4.8% for AL-12 ($K_d = 400$ μ M), while at 90 μ M is 99.8% for AL-103, 80.0% for AL-09, 29.2% for κ I, and 18.4% for AL-12, respectively. No dissociation constant information has been obtained for AL-T05 although its behavior by analytical size exclusion chromatography leads us to believe that its dimerization K_d is within the range found for the other proteins (700 nM-400 μ M).

Log-log plots of t_{50} values versus protein concentration are used to elucidate the dominant molecular mechanisms, because the behavior of t_{50} with varying monomer concentration give us information about whether two or more processes are in competition and which aggregation mechanisms are dominant.^{15, 16, 40, 41}

In this regard, analysis of the t_{50} vs. protein concentration log-log plots in Figure 1 shows a linear dependence for most proteins. Deviations from the linear relationship reflect changes in the dominant molecular mechanism of the fibril formation reaction. Kinks in the log-log plot curves were observed for AL-103 and AL-12, with a linear dependence at low protein concentrations and a kink at the medium-concentration regime.

The behavior at the medium concentration regime indicates that the classic nucleation-condensation mechanism at the medium-concentration regime is not dominant, suggesting a competition between nucleation condensation and secondary nucleation.^{9, 42}

We observe two general behaviors when we plot the maximum ThT fluorescence as a function of protein concentration:

- a. For AL-09 and AL-103 (Figure 1K and L), present an exponential behavior
- b. For AL-12 (Figure 1M) and κ I (Figure 1N) show a pseudo linear increase in the ThT fluorescence as the protein concentration of the reaction increases. In the case of κ I, the fluorescence values reach a plateau at the highest concentrations. AL-T05 presents a pseudo linear increase in the fluorescence at low and medium protein concentration, with outlier data at high protein concentration, due probably to inner filter effect.

The log-log plots and the ThT maximum fluorescence of the reactions show that for AL-09 and AL-103, the concentration dependence affects the amount of fibrils it makes (interpreted as the maximum ThT fluorescence, and taking into account all the drawbacks interpreting ThT fluorescence may entailed, see methods section for a description of these drawbacks) up

to the point where the ThT molecules are all bound and therefore, there is no increase in the ThT fluorescence for higher concentrations. For AL-12 and κ I, the increase of ThT maximum fluorescence is linear, suggesting that the amount of ThT-binding regions formed in the fibrils are increasing within the concentration range tested. Interestingly, this is also the case for our fastest amyloid former, AL-T05 but with a larger slope for the linear dependence compared to the proteins with slow amyloid formation.

Figure 2 shows kinetic parameters of reactions with 1% (v/v) of homologous seeds. Panels A–D present a comparison between the t_{50} values of seeded and unseeded reactions. Log-log plot of AL-09 (Figure 2E) presents very small changes in the kinetic parameters between the *de novo* and elongation reactions. The only observable change is the ThT maximum fluorescence values that plateau at a lower concentration for the reactions with seeds (Figure 2J). For AL-103 (Figure 2F) most of the low concentration reactions are accelerated in the presence of seeds, while the reactions slow down at 20 μ M. For AL-12 (Figure 2G), the behavior is just the opposite, the seeded reactions are accelerated starting at 40 μ M, while κ I presents a similar behavior but with reduced t_{50} values over the whole protein concentration range (Figure 2H).

In summary, Figures 1–2 show that *de novo* reactions of the proteins with the fastest amyloid kinetics do not present protein concentration dependence. Seeded reactions presented small protein concentration dependence. For AL-12, seeded and protein concentration dependence data suggests a synergistic effect for recruitment and elongation at low protein concentrations, while reactions of κ I had a poor efficiency to nucleate and elongate preformed fibrils. Due to its fast fibril formation kinetics (the fastest of the proteins we have characterized so far) in *de novo* reactions and in the presence of seeds²⁷, we are not including AL-T05 self-seeding at different concentrations as *de novo* reactions present no protein concentration dependence (Figures 1E, 1J, 1O).

Figure 3 shows the concentration dependence of mixtures of V_L and FL soluble κ I in both *de novo* and seeded reactions with either 1% (v/v) V_L seeds or FL seeds. The reactions were conducted under the only solution condition in which κ I V_L forms fibrils (pH 2.0). Only reactions that have an excess of soluble κ I V_L (2:1 to 10:1 V_L :FL) form amyloid fibrils in agreement with what we have reported previously regarding the role of the constant domain within κ I FL modulating amyloid formation.²⁴ For *de novo* reactions (Figure 3A), the fastest reaction with statistical significance occurs with 2:1 V_L :FL. Surprisingly, we observe statistically significant differences between the reactions with V_L and FL seeds as the latter proceed faster than the reactions with V_L seeds. Reactions with V_L seeds (Figure 3B) present a 2nd phase in the amyloid formation reaction, enhancing the final ThT fluorescence. Biphasic reactions have been observed for κ I V_L before, although their appearance has been quite stochastic.²⁷ In the presence of FL seeds (Figure 3C), the reactions show statistical differences between 2:1 and higher ratios (Figure 3D). These results suggest that the constant domain present in κ I FL can accelerate the reaction, possibly because κ I FL is a better fibril nucleator than κ I V_L .

Transmission electron microscopy was employed to confirm and determine the morphology of protein aggregates formed in the mixtures of V_L and FL proteins. As illustrated in Figure

4, fibrils from mixtures present the classical morphology of long and straight amyloid fibrils, but slightly different degrees of clustering, consistent with the acceleration of the reaction observed in presence of seeds. In the case of *de novo* and seeded reactions with κ I V_L, aggregates look like bundled long fibrils, while in the presence of κ I FL seeds, aggregates appear like a dense network of clustered fibrils. However, individual amyloid fibrils did not present any significant difference in the morphology compared to the results of previous reports.²⁴ Our transmission electron microscopy results are consistent with the acceleration of the seeded reactions, as well as the idea that κ I FL may be a better fibril nucleator while κ I V_L seems to be a better fibril elongator, as we reported previously.^{24, 27}

The crystal structure of AL-T05 (Figure 5) reveals a dimer interface rotated $\sim 180^\circ$ with respect to the canonical dimer interface of κ I, and $\sim 90^\circ$ from the AL-09 dimer interface. This non-canonical dimer interface is also found in κ I Y87H NMR structure.¹⁸ As in the case of κ I Y87H, the intermolecular contacts are significantly reorganized, the β -sheets at the interface are now roughly orthogonal to the two-fold axis, and symmetry-related β -strands in the opposing monomers are oriented antiparallel relative to each other. The existence of an altered dimer interface does not seem to be dependent on the crystallographic/solution conditions as we have obtained altered dimer interface crystals at pH 7.9–8.9 for AL-09¹⁹ and NMR structures for κ I Y87H at pH 6.8¹⁸. NMR studies of AL-T05 are ongoing. Interestingly, fibril formation kinetics of κ I Y87H are twice as fast as κ I V_L³³, suggesting that the altered dimer interface found in AL-T05 may allow access and populate amyloidogenic dimer conformations just like we found with AL-09.

Discussion

In this study we have evaluated amyloid formation as a function of protein concentration for five immunoglobulin light chains. We see a differential effect of protein concentrations among the proteins studied.

The most amyloidogenic proteins AL-09, AL-T05 and AL-103 —defined by their fast kinetics of amyloid formation— do not present a concentration dependence on t_{50} values for *de novo* reactions. In contrast, the mildly amyloidogenic protein AL-12 presents an inverse concentration dependence on t_{50} values (reflected in the log-log plot as well) where the reaction takes longer time with increasing protein concentration.

In the case of AL-T05, AL-09 and AL-103, the lack of concentration dependence on t_{50} values indicates a nucleation-elongation process, where secondary nucleation is negligible, if we assume that the lack of concentration dependence is not influenced by other factors.⁴³ If we consider other factors, it is possible that the partial unfolding occurs at any concentration and is driving the reaction. For AL-12 the inverse linear concentration dependence observed on t_{50} values, suggest not only that the dominant mechanism does not change within the range of protein concentration, but also there is a delaying effect at high protein concentration, similar to the effect described for the κ 4 protein SMA.³⁸

We cannot discard the presence of off-pathway processes such as the formation of irreversible amorphous (non-fibrillar) aggregates that could substantially slow down the

fibril formation reaction, and can cause the fibril formation to have an inverted concentration dependence; that is, fibril formation can become slower as the protein concentration increases.³⁹

Fibril formation reactions are expected to be more complex for globular proteins like immunoglobulin light chains compared to the reactions for peptides and intrinsically disordered proteins.⁴⁴ Global or partial unfolding is required prior to the aggregation of a globular, folded protein favored by the solution conditions or by the transient population of these states from the native state.^{35, 38, 45}

It is widely accepted that thermodynamic stability is one of the major modulators of the amyloidogenicity of AL proteins, although there are notable exceptions as we have reported two AL proteins with similar protein stability but different fibril formation tendency.^{27, 31} AL proteins are weak homodimers with different affinities. AL-09 dimer interface is rotated 90°, while AL-T05 is rotated 180° relative to canonical dimers populated by κ I V_L, AL-103 and AL-12 (Figure 6).^{18, 19} Because quaternary structure confers stability, protein concentration dependence in the monomer-dimer equilibrium is expected. In this sense Qin et al.,³⁸ attributed the inverse concentration dependence on the kinetics of amyloid formation to a protective effect of the canonical dimer for κ 4 protein SMA. The unusual dimeric structure of AL-09 and AL-T05 could facilitate sampling altered conformational states that may be amyloidogenic.

Comparison of the crystallographic structures of AL-09 and AL-T05 with respect to the κ I NMR structure indicates that an altered dimer interface may allow AL proteins to populate amyloidogenic dimer conformations. We speculate that non-canonical dimer interfaces play a major role in the initiation of fibril formation.

Our experiments with mixtures of κ I V_L and FL informed us of the complexity of the fibril formation reactions that may occur in patients with AL amyloidosis with amyloid deposits that consist of mixtures of full length light chains and fragments consisting primarily of V_L.

The reactions with a small amount or equal amount of V_L protein did not form any amyloid fibrils detected via ThT fluorescence. It was not until we reached a ratio of 2:1 (V_L:FL) that the reactions proceeded quite rapidly ($t_{50}=75.9\pm 12$ h) compared to 20 μ M V_L (266.2 $\pm$ 11 h) and 20 μ M FL (33.5 $\pm$ 22 h). The t_{50} values for the 2:1 (V_L:FL) mixture is in between the values for the two κ I proteins. It is worth mentioning that κ I FL forms fibrils more readily than its V_L counterpart. Interestingly, having more κ I V_L in the reaction does not necessarily accelerate the reaction, suggesting that 2:1 V_L:FL is the optimal ratio to initiate the reaction and recruit κ I V_L. This suggests that the constant domain may allow κ I FL to be a better nucleator while κ I V_L acts as a better elongator. It is also tempting to speculate that having two V_L molecules per one FL (V_L+CL) may offer the appropriate surface ratio for aggregation to occur, which is compatible with a secondary nucleation-elongation mechanism. We suggest that κ I V_L monomers bind to the lateral sides of preformed fibrils during the seed-dependent elongation.

Further studies of the growing fibrils by real-time observations can give us a more detailed outlook of the molecular basis for the recruitment of soluble protein in AL amyloidosis, and

determine if secondary nucleation plays an important role in the recruitment of soluble protein into fibrils.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations and Textual Footnotes

AL	(amyloidogenic) Light Chain
ThT	Thioflavin T
CD	Circular Dichroism
V_L	variable domain of immunoglobulin light chain
FL	Immunoglobulin light chain
t₅₀	time where 50% of the fibril formation reaction is complete

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Figure 1.

Protein concentration dependence of fibril formation kinetics. t_{50} values of *de novo* reactions as a function of the total protein concentration for (A) AL-09; (B) AL-103; (C) AL-12; (D) κ I; and (E) AL-T05. Stripped pattern on bars indicates reactions with less than 4-fold ThT fluorescence ($\ll 200,000$ A.U.). Crossed pattern on bars represent positive reactions with really low ThT fluorescence, due to inner filter effects caused by a large amount of fibrils. Reactions were conducted using concentrations of monomer ranging from $0.1 \mu\text{M}$ to $90 \mu\text{M}$. Log-log plots of the rescaled t_{50} against protein concentration (F) AL-09; (G) AL-103; (H)

AL-12; (*I*) κI; and (*J*) AL-T05. Dependence of the fluorescence intensity of ThT at the final point of the reaction as a function of total protein concentration (*K*) AL-09; (*L*) AL-103; (*M*) AL-12; (*N*) κI; and (*O*) AL-T05. Average values from triplicates.

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Figure 2.

Protein concentration dependence of fibril formation kinetics. t_{50} values of seeded reactions as a function of the total protein concentration, for (A) AL-09; (B) AL-103; (C) AL-12; and (D) κ I. Reactions were conducted using concentrations of monomer ranging from 0.1 μ M to 90 μ M. 1% v/v of seeds were employed. Log-log plots of the rescaled t_{50} against protein concentration, for (E) AL-09; (F) AL-103; (G) AL-12; and (H) κ I. Data for AL-12 in presence of 10% of seeds are presented in blue. Dependence of final fluorescence intensity

of ThT at the final point of the reaction of (*J*) AL-09; (*J*) AL-103; (*K*) AL-12; and (*L*) κI.
Average values from triplicates.

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Figure 3.

Differential effect of co-aggregation and cross-seeding of κ I V_L and κ I FL. Kinetics of fibril co-aggregation for (A) *de novo* reactions, (B) seeded reactions in the presence of 1% v/v κ I V_L seeds, or (C) in presence of 1% v/v of κ I FL seeds. Fibril formation reactions were carried out at different ratios of monomeric V_L:FL κ I proteins (■) 1:10, (●) 1:5, (▲) 1:2, (▼) 1:1, (◆) 2:1, (◀) 5:1, and (▶) 10:1. Total protein concentration was 20 μ M. (D) Comparison of the t_{50} values as a function of the seeds employed, at different κ I V_L: κ I FL ratio. Reaction was considered positive when ThT fluorescence increased four-fold ($>200,000$ A.U). Not significant (ns)= $P > 0.05$, *= $P = 0.05$, **= $P = 0.01$, ***= $P = 0.001$.

Figure 4.

Transmission electron microscopy images of κ I V_L + κ I FL amyloid fibrils at the endpoint of the reaction. (A) Fibrils from 10:1 κ I V_L + κ I FL protein, *de novo reaction*; (B) fibrils from 10:1 κ I V_L + κ I FL protein, reaction seeded with 1% κ I V_L seeds; and (C) reaction seeded with 1% κ I FL seeds. In all cases, fibrillar species were observed. Scale bar represent 200 nm.

Figure 5.
Electron density for AL-T05. Electron density map ($2F_o - F_c$ at 1σ contouring) the dimer.
One monomer is colored in teal, the other in orange.

Figure 6.

Comparison of the different dimer interfaces adopted by the AL proteins. X-ray crystal structures of (A) κ I (gold), (B) AL-12 (blue), (C) AL-103 (red), (D) AL-09 (black), and (E) AL-T05 (orange) (PDB codes 2Q20, 3DVF, 3DVI, 2Q1E, and 5T93, respectively).

Structures were superimposed using one monomer (surface, cyan) of the homodimer. κ I, AL-12 and AL-103 present the canonical dimer interface. AL-09 adopts an altered dimer interface that is rotated $\sim 90^\circ$ with respect to canonical dimer present in κ I, AL-12 and AL-103. The crystallographic structure of AL-T05 is rotated $\sim 180^\circ$ with respect to the canonical dimer interface and $\sim 90^\circ$ from the AL-09 dimer interface shown in (D).

Table 1

Data collection and refinement statistics (molecular replacement).

Data collection^a	
Space group	P 1
Cell dimensions	
a,b,c	38.46, 42.12, 65
α,β,γ	80.66, 86.02, 63.02
Resolution (Å)	37.10–1.90 (37.11–1.90) ^b
Unique reflections	24,891 (1,477)
Wilson B-factor	23.7
Rmerge (%)	6.0 (15.4)
I/sI	8.13 (1.90)
Completeness (%)	88.1 (37.10–1.90)
Redundancy	90.7
Refinement	
Reflections used	24,891 (1,447)
Rwork/Rfree (%)	21.1/23.5 (28.4/29.6)
Atom number	
Number of Protein atoms	9351
Number of solvent atoms	847
Zn	7
r.m.s.^c deviations	
Average B-factors	26
Bond length(Å)	0.013
Bond angles(°)	1.49
Bond lengths	0.006
Bond angles	1.236
MolProbity clashscore	12.11
%Ramachandran outliers	0
%Rotamer outliers	0

^aOne crystal was used to determine each structure.^bValues in parentheses are for highest-resolution shell.^cr.m.s. root mean square.