Tyrosine Residues Mediate Fibril Formation in a Dynamic Light Chain Dimer Interface

Received for publication, March 15, 2012, and in revised form, June 18, 2012. Published, JBC Papers in Press, June 27, 2012, DOI 10.1074/jbc.M112.362921

Ara Celi DiCostanzo, James R. Thompson, Francis C. Peterson, Brian F. Volkman, and Marina Ramirez-Alvarado

From the Departments of Biochemistry and Molecular Biology and Physiology and Biomedical Engineering, College of Medicine, Mayo Clinic, Rochester, Minnesota 55905 and Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Background: Light chain amyloidosis is a protein misfolding disease characterized by the deposition of immunoglobulin light chains in vital organs.

Results: Tyrosines are important in amyloidogenesis of light chain protein AL-09, but not AL-103.

Conclusion: AL-09 populates amyloidogenic dimeric structures where tyrosine residues may mediate fibril formation.

Significance: Tyrosine residues may be important targets for new therapeutic strategies for light chain amyloidosis.

Light chain amyloidosis is an incurable protein misfolding disease where monoclonal immunoglobulin light chains misfold and deposit as amyloid fibrils, causing organ failure and death. Previously, we determined that amyloidogenic light chains AL-09 and AL-103 do not form fibrils at pH 10 (tyrosine pKa). There are three tyrosine residues (32, 91, and 96) clustered in the dimer interface, interacting differently in the two light chain proteins due to their two different dimer conformations. These tyrosines may be ionized at pH 10, causing repulsion and inhibiting fibril formation. Here, we characterize single and double Tyr-to-Phe mutations in AL-09 and AL-103. All AL-09 Tyr-to-Phe mutants form fibrils at pH 10, whereas none of the AL-103 mutants form fibrils at pH 10. NMR studies suggest that although both AL-09 and AL-103 present conformational heterogeneity, only AL-09 favors dimer conformations where tyrosine residues mediate crucial interactions for amyloid formation.

Amyloidoses are a subset of protein misfolding diseases characterized by the presence of insoluble, extracellular amyloid fibrils. Amyloid fibrils have a distinct cross-β-sheet structure in which a series of β-strands stack together forming long, straight fibrils. Despite being formed by a diverse group of >20 human precursor proteins, amyloid fibrils show remarkable similarities in low resolution structure, suggesting common mechanisms of disease (1).

Light chain (AL) amyloidosis is caused by abnormal proliferation of plasma cells in the bone marrow that produce monoclonal immunoglobulin light chains. Normal immunoglobulin structure consists of a heterotetramer formed by two light chains and two heavy chains. This heterotetramer is normally secreted from the plasma cell into circulation. However, in AL amyloidosis, large amounts of free light chains are secreted; these light chains misfold, aggregate, and form extracellular amyloid fibrils in vital organs, leading to organ failure and death (2). AL amyloidosis patients have a median survival time of 12–40 months following diagnosis, although patients with advanced disease have a median survival of only 6 months (3, 4). Current treatments aim to suppress proliferation of the plasma cells, but they are poorly tolerated and noncurative. Recent reports suggest that soluble light chain species populated during the misfolding reaction could be one of the pathologic species (5–7); therefore, it is important to understand the etiology of the misfolding process of amyloidogenic proteins to target the toxic species that cause organ failure.

In this study, we investigate the role of tyrosine residues on in vitro fibril formation of two AL proteins, AL-09 and AL-103, which have been extensively studied by our laboratory (8–12). AL-09 and AL-103 are patient-derived proteins from the κ O18/O8 germ line that share greater than 90% sequence identity but exhibit vastly different clinical phenotypes. Patient AL-09 survived only a few months post diagnosis as a result of cardiac amyloidosis; patient AL-103 survived three years post diagnosis despite having amyloid deposition in the heart and tongue. Interestingly, neither protein forms fibrils at pH 10. We noted that tyrosine has a pKa of 10 and that there are three conserved tyrosine residues (32, 91 and 96) clustered and located in β-strands C, F, and G (Fig. 1). We hypothesized that mutating these tyrosine residues to phenylalanine (lacking the ionizable –OH group that may cause repulsion and amyloid inhibition when tyrosine residues are present) would allow these proteins to form fibrils at pH 10. If our hypothesis is correct, targeting of tyrosine residues in amyloidogenic light chains could become a potential therapeutic target for AL amyloidosis. Although studying fibril formation at pH 10 may not seem physiologically relevant, these studies will allow us to elucidate the structural changes that must occur for immunoglobulin light chains to form fibrils under physiological conditions.

Here, we characterized tyrosine to phenylalanine (Tyr to Phe) single mutant proteins in positions 32, 91, and 96 for...
AL-09 and AL-103 as well as the double mutants in positions 32 and 96 (herein called AL-09 2YF and AL-103 2YF). Our results show that all AL-09 mutants form fibrils at pH 10. We determined the crystal structures for AL-09 2YF and AL-103 2YF; both adopt the canonical dimer interface (analogous to the dimer structure normally formed by a light chain and a heavy chain within an immunoglobulin) with minor structural alterations. In previous work by our laboratory, we found that the light chain proteins AL-09 and kO18/O8 (its germ line) populate a promiscuous dimer interface (11). Solution nuclear magnetic resonance (NMR) two-dimensional 15N-1H heteronuclear single quantum coherence (HSQC) spectra of AL-09 and kO18/O8 (its germ line) were obtained; peak broadening and low peak counts indicated that multiple dimer structures were present. In this study, we attempted to solve the solution structure of AL-103; however, complete assignment of all residues was not possible because of missing and unassigned resonances located within the dimer interface. We obtained 15N-1H and 13C-1H HSQC spectra of AL-09 2YF, AL-103 2YF, and their parent proteins from pH 7.5–10.5 to assess how the Tyr-to-Phe mutations affect the dimer structure at high pH. Although all of the proteins possess dynamic dimer structures, AL-09 and AL-09 2YF appear to assume dimer conformations that are more amyloidogenic than AL-103 and AL-103 2YF. The amyloidogenic dimer conformations involve key tyrosine interactions that drive aggregation. The theoretical pKₐ for tyrosine is pH 10. Unfortunately, we were unable to determine the experimental pKₐ values for each of the tyrosine residues in these proteins with the 13C-1H aromatic HSQC experiments.

EXPERIMENTAL PROCEDURES

Cloning, Expression, Extraction, and Purification—Variable domain (V₄) recombinant proteins were expressed as reported previously (8, 12, 13). Briefly, the proteins were expressed in BL21 cells, harvested by centrifugation, refolded from inclusion bodies, and purified using size exclusion chromatography (HiLoad 16/60 Superdex 75 column) on an AKTA FPLC (GE Healthcare). SDS-PAGE and Coomassie Blue staining were used to identify pure protein fractions. Western blot analysis and/or mass spectroscopy were used to confirm the identity of each protein. The proteins were flash frozen and stored at −80 °C.

Isotopically labeled proteins for NMR experiments were produced using M9 media supplemented with Isogro containing [15N]ammonium chloride and [13C]glucose. 15N, 13C AL-09, AL-103, AL-09 2YF, and AL-103 2YF were harvested, and purified as discussed above. Proteins for NMR were dialyzed into 10 mM Na₂HPO₄ (pH 7.4), flash frozen, and stored at −80 °C.

CD Spectroscopy—Circular dichroism (CD) spectroscopy was used to determine the secondary structure and the thermal stability of all Tyr-to-Phe mutant proteins on a Jasco Spectropolarimeter 810 (JASCO, Inc., Easton, MD) as described previously (14). Briefly, far UV-CD spectra from 260–200 nm were acquired to determine secondary structure. The minimum wavelength between 215–218 nm, indicative of β-sheet structure, was chosen to follow the unfolding of the protein from 4–90 °C to determine the melting temperature (Tₘ). This CD analysis was performed as a quality control measure to ensure
that all batches of protein displayed similar secondary structure and thermal stability.

**Fibril Formation Assays**—Before fibril formation assays, preformed aggregates were removed from soluble protein by ultracentrifugation to a sedimentation coefficient of 3.0 S (the size of a dimer or 24 kDa) determined by the following equation,

\[ s = k \times M^{2/3} \]  

(Eq. 1)

where \( s \) is the sedimentation coefficient, \( M \) is the molecular weight, and \( k \) is 0.00363 (based on the approximation that the particles are spheres) (15). This corresponds to 3.3 h at a speed of 90,000 rpm in a NVT-90 rotor on an Optima L-100 XP centrifuge according to centrifuge specifications.

All fibril formation assays were performed in triplicate using black 96-well polystyrene plates and shaken continuously at 300 rpm at 37 °C in a New Brunswick Scientific Innova40 incubator shaker. Each well contained 260 μL of 20 μM protein, 150 mM NaCl, 10 μM thioflavin T, 0.02% NaN₃ in 10 mM sodium acetate, boric acid, and sodium citrate (ABC) buffer at the appropriate pH value. Fibril formation was monitored daily for 1 month (~750 h) by thioflavin T fluorescence on a plate reader (Analyst AD, Molecular Devices) with an excitation wavelength of 440 nm and an emission wavelength of 480 nm. A fibril formation reaction was considered positive when thioflavin T fluorescence readings reached at least four times the lowest fibril sample reading of that particular reaction (usually ~200,000 counts/s). The \( t_{50} \) value, or the time at which the fibril formation reaction is 50% complete, was calculated by linear regression of the first exponential phase (from base line to 4-fold fluorescence) of each fibril formation reaction. These \( t_{50} \) values may seem counterintuitive; a larger bar indicates a longer time required to form fibrils.

**Electron Microscopy**—A 3-μl fibril sample was placed on a 300 mesh copper formvar/carbon grid, 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).

**X-ray Crystallography**—Purified AL-09 2YF and AL-103 2YF were concentrated to 13 mg/ml and 50 mg/ml, respectively. AL-09 2YF crystals were obtained in hanging drops using vapor diffusion against 20% (w/v) polyethylene glycol monomethyl ether and 0.2 M zinc acetate in 0.1 M Tris-HCl buffer (pH 7.0) at 22 °C. AL-103 2YF crystals were obtained in hanging drops using vapor diffusion against 20% (w/v) polyethylene glycol monomethyl ether and 0.2 M cattle acetate in 0.1 M MES buffer (pH 8.2) at 4 °C. Crystals were cryoprotected with a 10% glycerol solution in liquid N₂.

Diffraction data were collected using a Rigaku/MS 007 microfocus x-ray diffractometer with Osmic Varimax optics, Xstream cryostream and an R-axis IV2⁺ detector. All data were collected at 100 K.

**Structure Refinement**—Diffraction patterns were processed with Crystal Clear (AL-09 2YF) and XDS (AL-103 2YF). All structures were solved by molecular replacement with PHASER as implemented by the Phenix software package using the αL O18/O8 structure (Protein Data Bank code 2Q20) as a probe molecule. Phenix and COOT were used for structure refinement and model building.

**Results**

Tyrosine Role in Amyloid Formation

**Accession Numbers**—Structures were deposited into the Protein Data Bank with the following codes: 3U7A (AL-09 Y32F Y96F) and 3U79 (AL-103 Y32F Y96F).

**NMR Spectroscopy**—NMR experiments were performed at 25 °C on the AL-103 and AL-09 parent proteins and 2YF mutants using a Bruker Avance 600 MHz and an Avance 500 MHz spectrometer, respectively. All NMR samples contained 0.8–1.2 mM protein, 10 mM sodium phosphate, 90% H₂O, 10% D₂O, and 0.02% sodium azide. The initial pH of each sample was 7.5, and the pH was incrementally adjusted to 8.5, 9.5, and 10.5 using 0.1 N sodium hydroxide. All pH values were measured to within ± 0.1 pH units. Standard 11N-1H HSQC (16), 13C-1H aromatic HSQC (16), and 13C-1H constant time aromatic HSQC (17) spectra were collected at each discrete pH value. Diffusion coefficients were also measured at each pH value using a pulse field gradient water-suppressed longitudinal encoded-decoded experiment (18). The gradient strength was varied from 10 to 75% in 1% increments. The maximum gradient strength was calibrated using a 1% (w/v) β-cyclodextrin solution in 90% H₂O and 10% D₂O with a diffusion coefficient of 3.239 × 10⁻⁶ cm² s⁻¹ at 25 °C (19). Nonlinear least-squares fitting was used to obtain self-diffusion coefficients (\( D_s \)) from the following equation,

\[ A(2\tau) = A(0)e^{(-\gamma G^2 I(\Delta - \delta^2/3))D_s} \]  

(Eq. 2)

where \( \gamma \) is the gyromagnetic ratio of \(^1\)H, \( \delta \) is the gradient pulse length, \( G \) is the gradient intensity, and \( \Delta \) is the diffusion delay.

**Results**

**Tyro-Pho Mutants Retain Secondary Structure and Thermodynamic Stability at pH 7.4**—AL-09 Tyro-Pho mutants and AL-103 Tyr-to-Phe mutants present the typical β-sheet secondary structure we have observed with other V₃ light chains by far UV-CD spectra (Fig. 2, A and B). The spectra have two minima; one at ~216 nm (typical for β-sheets), and a second at ~235 nm (aromatic residues that are optically active in the far UV region (20)).

Thermal denaturation experiments were performed to compare the thermodynamic stability between Tyr-to-Pho mutants (Fig. 2, C and D). All proteins refold reversibly with \( T_m \) values ranging from 35.0 °C (AL-09 Y91F) to 41.9 °C (AL-103 Y96F). Most of the Tyr-to-Pho mutants have similar \( T_m \) values to their parent proteins: AL-09 (41.1 °C) and AL-103 (41.6 °C) (supplemental Table S1).

**AL-09 Tyr-to-Pho Mutants Form Fibrils at pH 10**—AL-09 forms fibrils from pH 2–9; fibril formation is more stochastic and delayed from pH 6–9. AL-09 Tyr-to-Pho mutants form fibrils from pH 2–9; fibril formation is more stochastic and delayed from pH 6–9. AL-09 Tyr-to-Pho mutants form fibrils at pH 10—AL-09 forms fibrils from pH 2–9; fibril formation is more stochastic and delayed from pH 6–9.
(herein whenever we mention “kinetics,” we are referring to the kinetic parameter $t_{50}$ values in the context of our study). AL-09 Y32F consistently displays the lowest $t_{50}$ values of all of the mutants. From pH 3–8, AL-09 2YF has similar $t_{50}$ values to both AL-09 Y32F and AL-09 Y96F; however, at pH 9 and 10, AL-09 Y32F and AL-09 2YF have smaller $t_{50}$ values than AL-09 Y96F. At pH 10, its $t_{50}$ value is delayed 150 h for Y96F with respect to Y32F and 2YF. These results suggest that tyrosine 32 is an important mediator of fibril formation for AL-09.

AL-09 Tyr-to-Phe Mutants and AL-09 Have Similar $t_{50}$ Values—AL-09 and AL-09 Tyr-to-Phe mutants form fibrils from pH 2–9 with similar $t_{50}$ values (Fig. 3B); none of the proteins form fibrils at pH 10 (confirmed by electron microscopy (EM), data not shown). AL-103 Tyr-to-Phe mutants display more stochastic fibril formation around pH 4–5, which is near the calculated isoelectric point (pl) of 5.5. This behavior was also observed to a certain extent for the AL-09 mutants (Fig. 3A).

AL-09 Tyr-to-Phe mutant fibrils formed at the pH 10 cluster as short rods laterally stacked together as shown by EM (Fig. 3, C–F). These Tyr-to-Phe mutant fibrils cluster in large fibrillar networks in which the individual fibrils are sometimes difficult to discern, similar to other AL protein fibrils previously characterized in our laboratory and others (9, 10, 21–25). Images of fibrils at pH 2 for AL-09, AL-103, and their respective Tyr-to-Phe mutants are shown for comparison (supplemental Fig. S3).

2YF Mutants Crystallize as Canonical Dimers—We wanted to compare the structures of the Tyr-to-Phe mutants with their corresponding parent proteins to identify the structural basis for their fibril formation occurring at pH 10. First, we obtained x-ray crystal structures of AL-09 2YF and AL-103 2YF structures that were refined to 2.0 and 1.6 Å resolution, respectively (Table 1). Both 2YF mutants crystallized as canonical dimers at pH 7.0 (AL-09 2YF) and pH 8.2 (AL-103 2YF) with minor structural alterations. Comparison of AL-09 and AL-09 2YF dimer structures show significant structural differences due to the altered dimer interface of AL-09 (Fig. 4) and a decrease in the distance between the Y96F pair from 9.03 to 5.43 Å; yet, the YF mutations did not have a profound effect on the position of residues 32 and 96. AL-103 2YF displays the canonical dimer interface and shows a 90° rotation of the Phenylalanine aromatic ring at position 32 compared...
with AL-103 (Fig. 5). We were unable to obtain crystals of AL-09 2YF or AL-103 2YF at pH 10 or above.

In Solution, AL-103 Assumes Heterogeneous Dimer Conformations That Appear to Differ from AL-09—In an effort to assess possible dynamic behavior of AL-09 2YF and AL-103 2YF, we first needed to characterize AL-103 by NMR because we had previously only studied the conformational heterogeneity of AL-09 in solution (11). The $^{15}$N-$^1$H HSQC spectrum of AL-103 shows a pattern of well resolved peaks (Fig. 6A) containing nearly all of the 127 expected resonances (including both backbone and side chain NH groups). Close inspection of the data revealed that the missing resonances were mainly backbone amides. Based on the amino acid sequence, AL-103 should yield a total of 103 observable backbone amide resonances in the $^{15}$N-$^1$H HSQC spectrum, but only 93 were detected. Assignments for 73 of these peaks were completed using the three-dimensional triple-resonance NMR data; the other 20 HSQC peaks could not be assigned due to weak or missing signals in the three-dimensional spectra (Fig. 6A). The undetectable and unassigned residues in AL-103 are localized to three regions: residues 27–35 ($\beta$-strand B, loop B-C, $\beta$-strand C), residues 41–50 (loop C-C), and residues 86–99 ($\beta$-strand F, loop F-G, $\beta$-strand G). Mapping of the missing assignments onto the AL-103 structure (Protein Data Bank code 3DVI) showed that all of the unassigned resonances were contained within the dimer interface (Fig. 6, B and C).

Comparison of the $^{15}$N-$^1$H HSQC spectra of AL-09 and AL-09 2YF from pH 7.5 to 10.5 (supplemental Fig. S4, A and B) suggest that although both proteins remain folded at pH 10.5, AL-09 displays decreased peak counts and peak intensity at pH 10.5 compared with AL-09 2YF. Peak counts and intensity are expected to decrease due to high amide exchange rates at high pH, although it can also indicate changes in tertiary and quaternary structure or aggregation state of the protein.

As with AL-09 and AL-09 2YF, comparison of the $^{15}$N-$^1$H HSQC spectra of AL-103 and AL-103 2YF at different pH values (from pH 7.5 to 10.5) to determine whether there were structural differences between the parent proteins and their corresponding 2YF mutants.

The $^{15}$N-$^1$H HSQC spectra of AL-09 2YF from pH 7.5 to 10.5 show a subset of peaks (corresponding to residues located at the dimer interface as determined previously) that are broad, suggesting conformational heterogeneity due to a dynamic dimer interface (supplemental Fig. S2B), in agreement with what has been observed for AL-09 and germline kO18/O8 (11). Comparison of the $^{15}$N-$^1$H HSQC spectra of AL-09 and AL-09 2YF from pH 7.5 to 10.5 (supplemental Fig. S4, A and B) suggest that although both proteins remain folded at pH 10.5, AL-09 displays decreased peak counts and peak intensity at pH 10.5 compared with AL-09 2YF. Peak counts and intensity are expected to decrease due to high amide exchange rates at high pH, although it can also indicate changes in tertiary and quaternary structure or aggregation state of the protein.

As with AL-09 and AL-09 2YF, comparison of the $^{15}$N-$^1$H HSQC spectra of AL-103 and AL-103 2YF at different pH values from pH 7.5 to 10.5 to determine whether there were structural differences between the parent proteins and their corresponding 2YF mutants.
Tyrosine Role in Amyloid Formation

FIGURE 4. A, superposition of AL-09 2YF (orange) crystal structure onto one monomer of AL-09 (green); significant structural alterations in the left side monomers are the result of the altered dimer interface of AL-09 B (red box). Shown is a detailed view of the Tyr-to-Phe mutations (from residues 30–36 and 91–97). C (blue box), close up of alterations in loop of one monomer between β-strands C and C' (residues 39–44).

FIGURE 5. A, superposition of AL-103 2YF (teal) and AL-103 (purple) crystal structures shows few structural differences. B (yellow box), detailed view of the Tyr-to-Phe mutations shows rotation of Phe-32 (from residues 30–36 and 91–96). C (blue box), close up of alterations in loops between β-strands C and C' (residues 39–44) show minor differences.

Tensile differences at high pH (supplemental Fig. S5, A and B). AL-103 displays decreased peak counts and peak intensity compared with AL-103 2YF.

$^{13}$C-$^1$H aromatic HSQC spectra of AL-09, AL-103, AL-09 2YF, and AL-103 2YF were acquired in an attempt to titrate the individual tyrosine residues and determine their $pK_a$ values. Unfortunately, we were unable to resolve the individual tyrosine residues, and the peak counts were too low due to conformational exchange of the aromatic residues at the dimer interface. From pH 7.5 to 9.5, the AL-09 spectra have only a few peaks with significant peak loss, peak shifts, and the appearance of new peaks at pH 10.5, whereas the AL-09 2YF spectra from pH 7.5 to 10.5 are similar to one another with better resolved peaks (supplemental Fig. S6). Likewise, AL-103 has only a few peaks from pH 7.5 to 9.5 with significant changes at pH 10.5, whereas AL-103 2YF has more peaks from pH 7.5 to 9.5 (for AL-103 2YF, most peaks disappear at pH 11) (supplemental Fig. S7).

Translational self-diffusion ($D_s$) values were obtained from pH 7.5 to 10.5 (at 25 °C) for AL-09, AL-103, AL-09 2YF, and AL-103 2YF. We observed that the $D_s$ values for all the proteins studied decreased at pH 10.5, indicating an increase...
Tyrosine Role in Amyloid Formation

We hypothesized that at pH 7.4 (under physiological conditions), tyrosine residues are neutral and capable of mediating crucial interactions to initiate amyloid formation. Tyrosine residues may be ionized at pH 10, causing electrostatic repulsion that is sufficient to inhibit the aberrant self-association that leads to fibril formation in AL-09 and AL-103. We mutated three clustered, conserved tyrosine residues to phenylalanine in AL-09 and AL-103 and found that all AL-09 Tyr-to-Phe mutants form amyloid fibrils at pH 10. In particular, tyrosine 32 appears to play a dominant role in the initiation of fibril formation in AL-09. In addition, we were able to demonstrate that although both AL-09 and AL-103 have dynamic dimer structures, AL-09 populates amyloidogenic dimeric structures more easily than AL-103. Although these three tyrosine residues are conserved in both proteins, their orientations within the dimer interface vary due to the differences in dimer structures adopted by AL-09 and AL-103.

Patient AL-09 suffered from an aggressive form of the disease (patient died within 1 year after diagnosis), whereas patient AL-103 died 3 years after diagnosis. In one of our previous studies (10), we compared the amyloid formation kinetics of AL-09 and AL-103. We utilized ultracentrifugation to remove preformed aggregates prior to the initiation of fibril formation reactions. In our previous study, our preformed aggregate protocol enriched the solution with monomeric species. The differences we observed between AL-09 (fast fibril formation kinetics as measured with \( t_{50} \) values) and AL-103 (slow, very stringent conditions that allow fibril formation kinetics) correlated very well with the relative aggressiveness of the two proteins in vivo. In the present study, we have removed preformed aggregates and enriched our solutions with dimeric species because the tyrosine residues are clustered within the dimeric structures of these proteins. Our results show that the differences in \( t_{50} \) values of AL-09 and AL-103 are minimal and not statistically significant. The results of this study and our previous report suggest that the initial species present in the beginning of the reaction play a major role in the kinetics of \( V_L \) light chain amyloid formation.

In general, the AL-09 and AL-103 Tyr-to-Phe mutants display similar structure and thermal stability to their parent proteins at pH 7.4 (Fig. 2). The Y91F mutations are the most destabilizing; the melting temperatures (\( T_m \)) for AL-09 Y91F and AL-103 Y91F are 35.0 and 35.6 °C, respectively, compared with 41.1 and 41.6 °C for AL-09 and AL-103 (supplemental Table S1). Interestingly, the Y91F mutants display \( t_{50} \) values that are more stochastic (at some pH values) and are longer compared with other Tyr-to-Phe mutants (Fig. 3, A and B, and supplemental Fig. S1). The remaining Tyr-to-Phe mutants have melting temperatures ranging from 38.2–41.9 °C and display similar \( t_{50} \) values between each other at pH 2–10 (with the exception of the AL-09 Tyr-to-Phe mutants at high pH). At 37 °C, all of the proteins are within the unfolding transition (Fig. 2, C and D). However, the Y91F mutants populate a higher percentage of unfolded protein (~70% unfolded) than the rest of the Tyr-to-Phe mutants (~30% unfolded) at 37 °C. This difference may explain the stochastic amyloid formation behavior of the Y91F mutants.

From the \( t_{50} \) value data in Fig. 3A, we can conclude that substitution from Tyr to Phe in AL-09 appears to be favorable because the reactions are significantly accelerated for most mutants under many pH values tested, particularly at pH 7. The most notable example is pH 10 where AL-09 Y91F (one of three wells) and AL-09 Y96F present \( t_{50} \) values of 161 and 199 h respectively, whereas AL-09 Y32F and AL-09 Y2F have \( t_{50} \) values of 40 h. The 2YF mutants were designed in residues Tyr-32 and Tyr-96 because the side chains of these two tyrosine residues are located in a stacking mode along the dimer interface with their phenol groups oriented across the dimer (Fig. 1B); Tyr-91 side chain points away from these interactions. Our data

![Image](image-url)
suggests that Tyr-32 may contribute with the dominant tyrosine interactions mediating amyloid formation followed by Tyr-96 and to a lesser extent Tyr-91. In patients, natural somatic mutations from Tyr to Phe in these positions may further enhance the amyloidogenic potential of these proteins.

We hypothesized that the introduction of phenylalanine residues in the Tyr-to-Phe mutants could allow stacking $\pi-\pi$ interactions to occur more easily and these interactions may drive amyloid formation. This appears to be the case for AL-09 where the Tyr-to-Phe mutants present faster amyloid fibril formation (low $t_{50}$ values) but not AL-103, which is likely a result of their differences in dimer conformations (see below). Assembly of partially unfolded proteins exposing hydrophobic stretches has been implicated in amyloid fibril formation (26, 27). When these tyrosine residues are mutated to phenylalanine, their propensity for fibril formation may be enhanced due to increased local hydrophobicity. Marshall et al. (28) found that maintaining four phenylalanines is necessary for fibril formation of the peptide KFFEAAAKKFFE; furthermore, their results suggest that hydrophobicity, not aromaticity of the phenylalanines, causes increased amyloidogenicity. In another study, Marek et al. mutated the three aromatic residues (Phe-15, Phe-23, and Tyr-37) in the islet amyloid polypeptide (IAPP) to leucine; they found that although the triple mutant (IAPP-3XL) could form fibrils, fibril formation was significantly slower at pH 7.4, and fibril morphology was less ordered when observed by atomic force microscopy (AFM) (29). Our experiments with AL-09 Tyr-to-Phe mutants concur; they demonstrate generally faster fibril formation (low $t_{50}$ values) (Fig. 3A) at high pH where the lack of negative charge repulsion may be of importance in comparison with AL-09.

Both AL-09 2YF and AL-103 2YF crystallized as canonical dimers, and we pursued further characterization by solution NMR to explore the nature of their dimer interactions at high pH. Previously, the AL-09 H87Y restorative and $\kappa$Y87H reciprocal mutants were characterized by two-dimensional HSQC in addition to AL-09 and $\kappa$O18/O8 (the germ line) (11). Their HSQC spectra were disparate, yet they had both crystallized as canonical dimers (8). Their solution structures were solved; the NMR structure of $\kappa$Y87H revealed that one monomer is rotated 180° compared with the canonical dimer. Comparison of unassigned peaks for AL-09 and $\kappa$O18/O8 and the positions of the different dimers (AL-09 H87Y and $\kappa$Y87H) indicated that AL-09 and $\kappa$O18/O8 have a promiscuous dimer interface and can switch between multiple dimeric structures. We chose to undertake similar studies with AL-09 2YF and AL-103 2YF because their crystal structures were similar to $\kappa$Y87H.

Analysis of the NMR spectrum of AL-103 revealed a pattern of broadening in the dimer interface that is distinct from that observed previously for AL-09 and $\kappa$O18/O8. The dimer interface exhibits conformational exchange broadening in all three proteins; however, the AL-103 HSQC contains 93 of the 103 possible backbone amide signals, whereas the $\kappa$O18/O8 and AL-09 spectra were much less complete, with 82/104 and 85/104 peaks detected, respectively. Based on our previous structural studies, it is likely that the $\kappa$O18/O8 and AL-09 proteins interconvert between three distinct dimer arrangements. Two-site conformational exchange on micro- to milli-

second time scales is well known to enhance apparent NMR relaxation rates (30); however, three-site exchange or more complex equilibrium processes can lead to extreme broadening and the total loss of NMR signals, as observed for AL-09 and $\kappa$O18/O8 (11).

In contrast, the pattern of broadened but mostly observable resonances in the AL-103 dimer interface is more consistent with simple two-state conformational exchange. Thus, in addition to the stable, non-exchanging dimers and the highly promiscuous dimers, which interconvert between three different interfaces, we hypothesize that AL-103 represents a third distinct category of a light chain dimer that undergoes simple two-state conformational exchange at the dimer interface. Given the slow/stochastic fibril formation behavior exhibited by AL-103 previously (10), a further implication of this model is that promiscuity in the light chain dimer interface is necessary but not sufficient to promote amyloidogenicity. We propose that the highly amyloidogenic AL-09 frequently samples a specific dimer arrangement that promotes fibril formation; in contrast, AL-103 samples different dimer arrangements that are less amyloidogenic than those populated by AL-09. The Tyr-to-Phe point mutations studied were not sufficient to force AL-103 to assume amyloidogenic dimeric structures at pH 10.

Given the >90% sequence identity between AL-09 and AL-103, what could make their Tyr-to-Phe mutants behave differently, most prominently at pH 10? One of the main differences between AL-09 and AL-103 is the insertion of proline 95a in AL-103. This insertion creates a di-proline motif with proline 95 in trans conformation and proline 95a in cis conformation. This insertion causes a major backbone rearrangement at the end of the CDR3 and the beginning of $\beta$-strand G in AL-103. In contrast, AL-09 has proline 95 in cis conformation. It is possible that proline isomerization plays a role in the different rates of fibrillogenesis of AL-09, AL-103, and their Tyr-to-Phe mutants.

To explore whether other residues might be involved in the interactions that allow fibril formation at pH 10 for Tyr-to-Phe mutants, we measured the distances between charged residues (Lys, Arg, His, Asp, and Glu) and positions 32, 91, and 96 in the crystal structures of AL-09, AL-103, AL-09 2YF, and AL-103 2YF. We noted that Asp-50 is the only charged residue that appears to be close to any of the tyrosine residues, at a distance of <3 Å from Tyr-91 in all of the structures. Additionally, we identified polar contacts between positions 32, 91, and 96 and water molecules in the crystal structures of AL-09, AL-09 2YF, AL-103, and AL-103 2YF. In the AL-103 structure, Tyr-32 appears to form a hydrogen bond with a water molecule located 2.6 Å away. Taken together, although we identified some interactions, we have been unable to identify key interactions between tyrosine residues and charged side chains and/or water molecules that may explain the observed differences between AL-09, AL-103, and their Tyr-to-Phe mutants.

AL-09 and AL-103 have other tyrosine residues that were not included in this study because we had characterized them already (9, 11); they point toward the hydrophobic core or their location is far away from the dimer interface (Tyr-36, Tyr-49, Tyr-86, and Tyr-87). AL-09 has a histidine mutation at position 87 that is highly amyloidogenic. Tyr-49, located $\pm$8 Å from Tyr-32, Tyr-91, and Tyr-96, has its phenol group oriented away
in both AL-09 and AL-103, so interaction with Tyr-49 is unlikely. Likewise, Tyr-36, Tyr-86, and Tyr-87 are located near each other on β-strands C and F, respectively, but they are on the opposite end of the strands from Tyr-32, Tyr-91, and Tyr-96. The phenol groups of Tyr-36 and Tyr-86 face the hydrophobic core at orthogonal angles, whereas Tyr-87 is oriented in the opposite end of the strands from Tyr-32, Tyr-91, and Tyr-96.

The sequences of complementarity-determining regions favor certain amino acid residues, including tyrosine and serine (31). Tyrosine has been found to play a role in antigen binding, making ~25% of all contacts to the antigen. Fellouse et al. (32, 33) proposed that the abundance of tyrosine residues in antibodies could be due to the unique character of tyrosine; it is able to interact with many different types of surfaces and reduces nonspecific binding in the unbound state.

In conclusion, we have found that tyrosine residues, especially position 32, mediate fibril formation within the dynamic dimeric structures in AL-09, but not AL-103. This can be explained by the differences in dimer structures between AL-09 and AL-103. Although AL-09 and AL-103 both have dynamic dimer structures, we propose that AL-103 interconverts between dimer interfaces that are less amyloidogenic than the highly promiscuous AL-09 that interconverts between three different dimer interfaces (at least one of which is highly amyloidogenic). This implies that promiscuity of dimer structures is necessary, but not sufficient for amyloid formation. Understanding how dimer structure dynamics translates into amyloidogenicity is critical to elucidating the exact mechanisms of light chain amyloid formation and discovering new therapeutic targets for AL amyloidosis.

REFERENCES

1. Martin, D. J., Randles, E. G., and Ramirez-Alvarado, M. (2010) in Amyloidosis: Diagnosis and Treatment (Gertz, M. A., and Rajkumar, S. V., eds), pp. 1–14, Springer Science and Business Media, LLC, New York

2. Baden, E. M., Sikkink, L. A., and Ramirez-Alvarado, M. (2009) Light chain amyloidosis: Current findings and future prospects. Curr. Protein Pept. Sci. 10, 500–508

3. Kumar, S. K., Gertz, M. A., Lacy, M. Q., Dingli, D., Hayman, S. R., Buadi, F. K., Short-Detweiler, K., Zeldenrust, S. R., Leung, N., Greipp, P. R., Lust, J. A., Russell, S. J., Kyle, R. A., Rajkumar, S. V., and Dispenzieri, A. (2011) Recent improvements in survival in primary systemic amyloidosis and the importance of an early mortality risk score. Mayo Clin. Proc. 86, 12–18

4. Wechalekar, A. D., Hawkins, P. N., and Gillmore, J. D. (2008) Perspectives in treatment of AL amyloidosis. Br. J. Haematol. 140, 365–377

5. Brenner, D. A., Jain, M., Pimentel, D. R., Wang, B., Connors, L. H., Skinner, M., Apstein, C. S., and Liao, R. (2004) Human amyloidogenic light chains directly impair cardiomyocyte function through an increase in cellular oxidant stress. Circ. Res. 94, 1008–1010

6. Shi, J., Guan, J., Jiang, B., Brenner, D. A., Del Monte, F., Ward, J. E., Connors, L. H., Sawyer, D. B., Semigran, M. J., Macgillivray, T. E., Seldin, D. C., Falk, R., and Liao, R. (2010) Amyloidogenic light chains induce cardiomyocyte contractile dysfunction and apoptosis via a non-canonical p38MAPK pathway. Proc. Natl. Acad. Sci. U.S.A. 107, 4188–4193

7. Sikkink, L. A., and Ramirez-Alvarado, M. (2010) Cytotoxicity of amyloidogenic immunoglobulin light chains in cell culture. Cell Death Dis. 1, e98

8. Baden, E. M., Owen, B. A., Peterson, F. C., Volkman, B. F., Ramirez-Alvarado, M., and Thompson, J. R. (2008) Altered dimer interface decreases stability in an amyloidogenic protein. J. Biol. Chem. 283, 15853–15860

9. Baden, E. M., Randles, E. G., Aboagye, A. K., Thompson, J. R., and Ramirez-Alvarado, M. (2008) Structural insights into the role of mutations in amyloidogenesis. J. Biol. Chem. 283, 30950–30956

10. Martin, D. J., and Ramirez-Alvarado, M. (2010) Comparison of amyloid fibril formation by two closely related immunoglobulin light chain variable domains. Amyloid 17, 129–136

11. Peterson, F. C., Baden, E. M., Owen, B. A., Volkman, B. F., and Ramirez-Alvarado, M. (2010) A single mutation promotes amyloidogenicity through a highly promiscuous dimer interface. Structure 18, 563–570

12. Randles, E. G., Thompson, J. R., Martin, D. J., and Ramirez-Alvarado, M. (2009) Structural alterations within native amyloidogenic immunoglobulin light chains. J. Mol. Biol. 389, 199–210

13. McLaughlin, R. W., De Stigter, J. K., Sikkink, L. A., Baden, E. M., and Ramirez-Alvarado, M. (2006) The effects of sodium sulfate, glycosaminoglycans, and Congo red on the structure, stability, and amyloid formation of an immunoglobulin light-chain protein. Protein Sci. 15, 1710–1722

14. Sikkink, L. A., and Ramirez-Alvarado, M. (2008) Biochemical and aggregation analysis of Bence Jones proteins from different light chain diseases. Amyloid 15, 29–39

15. Haisall, H. B., and Wermeling, J. R. (1982) Sedimentation coefficient, frictional coefficient, and molecular weight: A preparative ultracentrifuge experiment for the advanced undergraduate laboratory. J. Chem. Ed. 59, 1076–1078

16. Talluri, S., and Wagner, G. (1996) An optimized 3D NOESY-HSQC. J. Magn. Reson. B 112, 200–205

17. Santoro, J., and King, G. C. (1992) A constant-time two-dimensional over-bodenhausen experiment for inverse correlation isotopically enriched species. J. Magn. Reson. 97, 202–207

18. Altieri, A. S., Hinton, D. P., and Byrd, R. A. (1995) Association of biomolecular systems via pulsed field gradient NMR self-diffusion measurements. J. Am. Chem. Soc. 117, 7566–7567

19. Uedaia, H., and Uedaia, H. (1970) Translational frictional coefficients of molecules in aqueous solution. J. Phys. Chem. 74, 2211–2214

20. Albinsson, B., and Norden, B. (1992) Excited-state properties of the indole chromophore: Electronic transition moment directions from linear dichroism measurements: Effect of methyl and methoxy substituents. J. Phys. Chem. 96, 6204–6212

21. Hu, D., Qin, Z., Xue, B., Fink, A. L., and Uversky, V. N. (2008) Effect of methionine oxidation on the structural properties, conformational stability, and aggregation of immunoglobulin light chain LEN. Biochemistry 47, 8665–8677

22. Meng, X., Fink, A. L., and Uversky, V. N. (2008) The effect of membranes on the in vitro fibrillation of an amyloidogenic light chain variable domain SMA. J. Mol. Biol. 381, 989–999

23. Qin, Z., Hu, D., Han, S., Hong, D. P., and Fink, A. L. (2007) Role of different regions of α-synuclein in the assembly of fibrils. Biochemistry 46, 13322–13330

24. Wall, J., Schell, M., Murphy, C., Hrnric, R., Stevens, F. J., and Solomon, A. (1999) Thermodynamic instability of human α6 light chains: Correlation with fibrillogenicity. Biochemistry 38, 14101–14108

25. Wall, J. S., Gupta, V., Wilkerson, M., Schell, M., Loris, R., Adams, P., Solomon, A., Stevens, F., and DeAlwis, C. (2004) Structural basis of light chain amyloidogenicity: Comparison of the thermodynamic properties, fibrillogenic potential, and tertiary structural features of four VA6 proteins. J. Mol. Recognit. 17, 323–331

26. Chiti, F., Webster, P., Taddei, N., Clark, A., Stefani, M., Ramponi, G., and Dobson, C. M. (1999) Designing conditions for in vitro formation of amyloid protofibrils and fibrils. Proc. Natl. Acad. Sci. U.S.A. 96, 3590–3594

27. Khurana, R., Gillespie, J. R., Talapatra, A., Minert, L. J., Ionescu-Zanetti, C., Millett, L., and Fink, A. L. (2001) Partially folded intermediates as critical precursors of light chain amyloid fibrils and amorphous aggregates. Biochemistry 40, 3525–3535

28. Marshall, K. E., Morris, K. L., Charlton, D., O’Reilly, N., Lewis, L., Walden, H., and Serpell, L. C. (2011) Hydrophobic, aromatic, and electrostatic interactions play a central role in amyloid fibril formation and stability. Biochemistry 50, 2061–2071

29. Marek, P., Abedini, A., Song, B., Kanungo, M., Johnson, M. E., Gupta, R., Zaman, W., Wong, S. S., and Raleigh, D. P. (2007) Aromatic interactions are not required for amyloid fibril formation by islet amyloid polypeptide
but do influence the rate of fibril formation and fibril morphology. *Biochemistry* 46, 3255–3261
30. Torchia, D. A. (2011) Dynamics of biomolecules from picoseconds to seconds at atomic resolution. *J. Magn. Reson.* 212, 1–10
31. Kossiakoff, A. A., and Koide, S. (2008) Understanding mechanisms governing protein-protein interactions from synthetic binding interfaces. *Curr. Opin. Struct. Biol.* 18, 499–506
32. Birtalan, S., Zhang, Y., Fellouse, F. A., Shao, L., Schaefer, G., and Sidhu, S. S. (2008) The intrinsic contributions of tyrosine, serine, glycine, and arginine to the affinity and specificity of antibodies. *J. Mol. Biol.* 377, 1518–1528
33. Fellouse, F. A., Barthelemy, P. A., Kelley, R. F., and Sidhu, S. S. (2006) Tyrosine plays a dominant functional role in the paratope of a synthetic antibody derived from a four amino acid code. *J. Mol. Biol.* 357, 100–114