Formation of Amyloid Fibers by Monomeric Light Chain Variable Domains*

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Background: Amyloid fibers are protein aggregates associated with numerous pathologies.

Results: Mcg light chain variable domains form amyloid fibers through monomers.

Conclusion: This light chain variable domain monomer is the fundamental unit required to form amyloid fibers.

Significance: Understanding the molecular mechanism of Mcg light chain amyloid fiber formation has implications for treating systemic amyloidosis.

Systemic light chain amyloidosis is a lethal disease characterized by excess immunoglobulin light chains and light chain fragments composed of variable domains, which aggregate into amyloid fibers. These fibers accumulate and damage organs. Some light chains induce formation of amyloid fibers, whereas others do not, making it unclear what distinguishes amyloid formers from non-formers. One mechanism by which sequence variations may reduce propensity to form amyloid fibers is by shifting the equilibrium toward an amyloid-resistant quaternary structure. Here we identify the monomeric form of the Mcg immunoglobulin light chain variable domain as the quaternary unit required for amyloid fiber assembly. Dimers of Mcg variable domains remain stable and soluble, yet become prone to assemble into amyloid fibers upon dissociation into monomers.

Bence-Jones proteins were first reported over a century and a half ago as disease-associated substances and later shown to be immunoglobulin light chains involved in systemic light chain amyloidosis, amyloid formation frequently associated with multiple myeloma (1–5). Light chains (LC) are classified by amino acid sequence as either of two types, κ or λ, and consist of two domains, the constant (Cκ) and the variable (Vκ) connected by a joining (J) segment (6). In this disease, immunoglobulin light chains are overexpressed, increasing the ratio of light to heavy chains. This disturbance of the balance between light and heavy chains results in the formation of LC homodimers termed Bence-Jones proteins.

Amyloid fibers are insoluble protein deposits, often associated with disease, which are induced by particular peptide sequences and exhibit common structural and biochemical characteristics. Specifically, all amyloid fibers contain a β-sheet rich spine, which serves as a scaffold for fiber extension and gives rise to a distinctive cross-β x-ray diffraction pattern (7–9). The core of amyloid fibers consists of a pair of β-sheets, whose side chains interdigitate, forming a stable spine termed a steric zipper (10). Formation of a steric zipper requires the presence of a short peptide segment with an amino acid sequence capable of forming a self-complimentary propagating structure upon exposure to solvent (11). In systemic LC amyloidosis, both the full-length LCs (Vκ-J-Cκ) and their Vκ-s are found to be constituents of amyloid fibers, yet the ubiquitous presence of Vκ-s indicates that this domain may be essential for their assembly (12–17). The peptide segments within Vκ-s responsible for forming the steric zipper remain unknown. Due to somatic recombination of immunoglobulins, the LC amino acid sequence is different in every patient; however, the disease has a common progression and pathology leading to the secretion of excess monoclonal LCs through the renal tract (18). Despite renal clearance, full-length LCs or LC fragments that contain the variable domain still form amyloid deposits in the tissues of patients, thereby resulting in organ failure (19).

Although excess LCs and Vκ-s form amyloid fibers, most of their known molecular structures are homo-dimers with the two variable domains resembling a light chain and a heavy chain variable domain in a full antibody (20–22). The Vκ homo-dimer resembles the structure of the region of antibodies termed the antigen-binding fragment (Fab), which is composed of both light chains and heavy chains, each with constant (Cκ) and variable (Vκ) immunoglobulin domains. In the Fab, a disulfide bond covalently links the C termini of the LC and heavy chains, and noncovalent dimer interfaces form hydrophobic cavities between the Cκ and Cκ and between the Vκ and Vκ. Similarly to the Fab, a disulfide bond links full-length LC homo-dimers.
dimers at the C termini, and the two $V_L$s form a noncovalent dimer. The dimer interfaces of both the $V_\text{L1}$-$V_\text{L2}$ hetero-dimer of Fab and the pathologic $V_\text{L1}$-$V_\text{L1}$ homo-dimer are lined with apolar residues enclosing a cavity capable of accommodating hydrophobic molecules (23, 24).

The pathway by which variable domains form amyloid fibers remains uncertain; however, there are several hypotheses. Early models of $V_L$ amyloid assembly proposed that continuous semi-crystalline fibers are formed by $V_L$ dimers that preserve their native or a native-like conformation with a conserved hydrophobic dimer interface (25). We term these dimers canonical dimers and term this model the canonical dimer model. This model suggests that canonical $V_L$ dimers, which resemble the structure of antigen-binding variable domains in a full antibody, stack to form noncovalent filamentous polymers with the width of a single $V_L$ dimer. The dimer interface of the two $V_L$ domains would need to form a continuous pair of $\beta$-sheets to give rise to the observed cross-$\beta$-x-ray diffraction pattern. Then, several of these filaments pack into stable amyloid fibers through intermolecular interactions between strands $G$-$F$-$C$-$C'$ of the Greek fold (26). This model proposes that semi-crystalline amyloid fibers contain globular $V_L$s in a conformation similar to that observed in crystals (Protein Data Bank [PDB] 1REI) (27). Because the canonical dimer model assumes the presence of globular $V_L$s with a conserved three-dimensional structure, it also assumes that amyloid formation is reversible.

Although most $V_L$s crystallize as canonical dimers, several $V_L$s, both $\kappa$ and $\lambda$ types, display conformations in which the orientation of domains relative to each other is altered. The most pronounced change includes a rotation up to 180° around an axis perpendicular to the plane of the dimer interface, whereas the hydrophobic environment of the cavity between the $V_L$s is conserved (28, 29). These structural alterations suggest the possibility of alternative amyloid formation pathways through which noncanonical dimers, lacking the stability of canonical dimers, assemble amyloid fibers in either the globular or the partially unfolded state. If the globular state is maintained in the fiber, the noncanonical dimers would need to stack as continuous $\beta$-sheets, similar to the model for amyloid formation by canonical dimers. Alternatively, the $V_L$s of a noncanonical dimer may partially unfold without disassociation to form steric zippers, whereas the hydrophobic interface between domains is maintained. Based on this model, which we term the noncanonical dimer model, destabilizing mutations would induce partial unfolding to an amyloid-prone tertiary conformation, whereas stabilizing mutations would inhibit amyloidosis (30–32).

In addition to forming canonical and noncanonical dimers, $V_L$s also exist as monomers in solution. Experiments conducted in denaturing conditions indicate that reducing the thermodynamic stability of the monomeric state promotes amyloid fiber formation (33–35). Specifically, the introduction of mutations that induce dimer disassociation or promote monomer unfolding increases the propensity to form amyloid fibers. These findings gave rise yet another model for $V_L$ amyloid assembly; partially unfolded monomers polymerize into amyloid fibers, whereas dimers protect against amyloidosis. We term this the monomer model.

Although many $V_L$s have been examined and different models for formation of their amyloid fibers have been proposed, it remains unclear which $V_L$ quaternary state promotes amyloid fiber assembly and whether fibers contain unfolded, partially folded, or globular $V_L$s (36). Such ambiguity limits our ability to identify peptide segments that form the amyloid spine. If amyloid fibers contain globular dimers with a preserved dimer interface, amyloidogenic segments can map only to solvent-accessible areas of $V_L$s. However, if unfolded $V_L$s monomers compose amyloid fibers, then amyloidogenic segments can map anywhere within the entire $V_L$ sequence.

We seek to identify the immunoglobulin $V_L$ quaternary state that is the precursor to formation of amyloid fibers and to identify which of the three models best describes the quaternary state most prone to forming amyloid fibers.

**EXPERIMENTAL PROCEDURES**

**Preparation of Mcg Mutant Proteins**—The peptide sequence for the Mcg protein was obtained from the PDB entry 3MCG. The DNA sequence was synthesized by GenScript and cloned into the pET26b+ expression vector. The N termini of the proteins contained a His$_6$ tag followed by a TEV protease cleavage site. Mcg-Y38E-F99A-F101E and Mcg-A45C-F101C were prepared by means of site-directed mutagenesis PCR. Plasmids with the appropriate sequences were cloned into BL21(DE3)Gold (Agilent Technologies) Escherichia coli expression cells.
Terrific Broth (Fisher Scientific) media were inoculated with expression cultures and allowed to reach $A_{600 \text{ nm}} = 1.0$. At this density, production of proteins was induced with 1 mM isopropyl-$\beta$-D-thiogalactopyranoside and performed overnight at 20 °C. Cells were harvested, resuspended in a 50 mM Tris, pH 8, 100 mM NaCl buffer, and sonicated, and an inclusion bodies pellet was separated from the supernatant by centrifugation. VLs were found in inclusion bodies of the pellet. Inclusion bodies were washed by dissolving the pellet in 50 mM Tris, pH 8, 100 mM NaCl buffer, centrifuged, and separated from the wash supernatant. To solubilize VLs from inclusion bodies, we used a solution of 7M urea, 50 mM Tris, pH 8, 100 mM NaCl, 0.05% Tween 20. VLs were purified by His tag capture on Ni-NTA columns and eluted with 7M urea, 50 mM Tris, pH 8, 100 mM NaCl, 0.05% Tween 20, 0.5 M imidazole. Following Ni-NTA capture, the proteins were dialyzed against a solution of 50 mM Tris, pH 8, 100 mM NaCl, 10% glycerol, 0.1 M l-arginine, 0.05% Tween 20. TEV protease digestion was performed by the addition of 1% w/w of TEV protease to the total protein mass and the addition of reduced/oxidized glutathione to 3 mM/0.3 mM, respectively. TEV protease digestion was conducted overnight at room temperature and monitored by SDS-PAGE. Urea was added to a final concentration of 7 M, and undigested protein, cleaved His tags, and His-tagged TEV protease were separated from VLs by means of Ni-NTA columns. The flow-through was collected and contained VLs denatured in urea buffer. Proteins were dialyzed against 50 mM Tris, pH 8, 100 mM NaCl, 10% glycerol, 0.1 M l-arginine, 0.05% Tween 20. To assist with proper refolding of the Mcg-A45C-F101C mutant, DTT was added to a final concentration of 5 mM in the dialysis buffer. After refolding, the proteins were concentrated in Centricon devices with a 10-kDa cutoff and washed three times with 25 mM Tris, pH 8, 50 mM NaCl.
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TABLE 1
X-ray data collection and refinement statistics
Each structure was derived from a single crystal.

| Data collection | Mcg PDB 4UNU | Mcg-A45C-F101C (covalent dimer) PDB 4UNV | Mcg-Y38E-F99A-F101E (monomer) PDB 4UNT |
|-----------------|--------------|------------------------------------------|----------------------------------------|
| Space group     | P12,1        | C222₁                                   | C121                                   |
| Cell dimensions | a, b, c (Å)  | 41.3, 34.1, 63.3                         | 37.4, 60.2, 79.4                       | 103.1, 90.3, 99.2 |
| a, b, γ (°)     | 90.0, 104.9, 90.0 | 90, 90, 90                             | 90, 118.8, 90                          | 65.2-7.27 |
| Resolution (Å)  | 40-0.95 (0.97) | 30-1.6 (1.64)                          | 65-2.7 (2.77)                         |
| Rmerge (%)      | 0.07 (0.7)  | 0.06 (0.4)                              | 0.09 (0.3)                             |
| Completeness (%)| 21 (1.9)     | 39 (4.2)                                | 13 (4.3)                               |
| Redundancy      | 88 (34)      | 98 (86)                                 | 98 (99)                                |
| Refinement      | 12 (32)      | 7 (3.6)                                 | 4 (3.8)                                |

Crystallization conditions
- 2 M NaCl, 2 M (NH₄)₂SO₄, 0.1 M CH₃COONa pH 4.6, 2 M (NH₄)₂SO₄, 0.5 M LiCl, 1.65 M (NH₄)₂SO₄

Amyloid Fiber Formation Assays—Thioflavin T (ThT) assays were performed by diluting the protein with 50 mM acetic acid/sodium acetate, pH 4, 150 mM NaCl buffer, and 0.05 mM ThT to a final concentration of 0.5 mg/ml. In reducing conditions, the proteins were first treated with 1 mM DTT for 5 min and then diluted to 0.5 mg/ml with 50 mM acetic acid/sodium acetate, pH 4, 150 mM NaCl buffer, 0.05 mM ThT, and 1 mM DTT. Formation of amyloid fibers was performed at 37 °C with constant shaking in black NUNC micro plates with Teflon balls with radii of 0.125 inches as stirrers. Fluorescent measurements of ThT were acquired with a Varioskan spectrophotometer at 444/482 nm excitation/emission wavelengths.

Electron Microscopy—Following the completion of ThT assays, samples were collected, diluted with water to 10% v/v, and applied onto electron microscopy copper grids. The grids were stained with 2% uranyl acetate, and images were collected by means of a Tecnai T12 electron microscope at 120 kV with a Gatan CCD camera.

Crystal Structure Determination—Crystallization trials were set up in hanging drop plates with a Mosquito micro-crystallization robot. Crystals were cryo-protected with 25% glycerol, and data were collected with a Rigaku HTC or at the Advanced Photon Source (APS), Argonne, IL, at the 24-ID-C and 24-ID-E beamlines. Data were processed with Denzo and scaled with Scalepack or with the XDS package. Phases were determined by molecular replacement with Phaser, using a variable domain fragment from chain A of PDB 3MCG as the initial model. Protein models were refined with Refmac5. Graphics were rendered with PyMOL. PDB accession codes and x-ray data collection and refinement statistics are in Table 1.

Native State Mass Spectrometry, Quadrupole Time-of-flight—For native mass spectrometry, protein samples were dialyzed into 50 mM ammonium bicarbonate immediately prior to analysis under native conditions. The sample solution was infused by a syringe pump (20 μl/min) to the electrospray source of a quadrupole time-of-flight mass spectrometer operating in positive ion mode (Agilent Technologies 6550 with dual Agilent JetStream source). The instrument was operated in the “extended mass range” mode at 2 GHz for scanning to 10,000 m/z. Quadrupole transmission was set to 1,000 m/z, and various parameters were adjusted to lower the energy delivered to the analyte, including the sheath gas temperature of 125 °C and flow of 3 liters/min with a fragmentor voltage of 200 V. Mass spectra were analyzed with MassHunter Qualitative Analysis software (Agilent Technologies).

Analytical Ultracentrifugation—Sedimentation equilibrium runs were performed on the native Mcg, the covalent Mcg dimer, and the V₁ monomer at 4 °C in a Beckman Optima XL-A analytical ultracentrifuge using absorption optics at 280 nm. Samples were at concentrations of 0.094, 0.28, and 0.44 mg/ml. Additional concentrations of 0.38 and 1.5 mg/ml were also run for the native Mcg. A 12-mm path length six-sector cell was used for all samples, except for the sample at 1.5 mg/ml, for which a 3-mm path length double sector cell was used. Samples were in 50 mM NaCl, 25 mM Tris, pH 8. Sedimentation equilibrium profiles were measured at speeds of 14,000, 17,000, and 22,000 rpm. The data were initially fitted with a nonlinear least-squares exponential fit for a single ideal species using the Beckman Origin-based software (Version 3.01). The Beckman global analysis software (the “multifit” option of the above mentioned soft-
**FIGURE 3.** Similarity of the Mcg VL homo-dimer to the VL-VH variable domain hetero-dimer of a physiological Fab (PDB 3TNM). 

A, the crystal structure of the Fab (PDB 3TNM) reveals a hydrophobic cavity in between the variable domains of its light and heavy chains. The cavity between the VL-VH is lined with side chains of hydrophobic residues. VL is shown in blue, and VH is in green. 

B, overlay of Fab with Mcg (yellow). Despite differences in residues, the overall structures have identical orientations of domains. 

C, crystal structure of the Mcg VL-VL dimer. 

D, sequence alignment of 3TNM and Mcg. Yellow indicates residues that form the hydrophobic interface between variable domains. Notice the similarity of the interface-forming residues of the Mcg VL-VL homo-dimer and the VL-VH hetero-dimer.

**FIGURE 4.** Deconvoluted protein masses from native state mass spectrometry measurements. 

A, detected masses for Mcg. The two major peaks show that the protein is found as both a monomer (11557.6 Da) and a dimer (23113.19 Da) in solution. amu, atomic mass units. 

B, detected mass for the covalent Mcg dimer (Mcg-A45C-F101C, 23150.53 Da). 

C, detected mass for the induced monomer (Mcg-Y38E-F99A-F101E, 11427.35 Da). Thus, mass spectrometry shows that Mcg is in equilibrium between monomers and dimers.
ware) was then used to analyze multiple scans, corresponding to different input concentrations and speeds, simultaneously for various models. Partial specific volumes of 0.709 (native Mcg) and 0.707 (Vₐ monomer and covalent Mcg dimer) were used. These were calculated from the amino acid composition and corrected to 4 °C (38, 39).

**Chemical Denaturation Analysis**—The stability of the proteins with and without 1 mM DTT was assessed by titrating the samples with urea and monitoring tryptophan fluorescence (40). The excitation wavelength was set at 295 nm, and the emission spectra were recorded from 325 to 400 nm. The maximal emission change along the titration occurred at 355 nm. The experiment...
was performed with a Molecular Devices SpectraMax fluorometer and quartz cuvette with a 1-cm path length. The concentration of protein in all samples was 0.1 mg/ml.

Fluorescence data points indicating cooperative denaturation through logarithmic analysis were fit to the sigmoidal equation (\(Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - X) \times \text{Hill}}})\) (where the \(^\wedge\) symbol indicates to the mathematical power of) using GraphPad Prism version 6 for Windows (GraphPad Software, La Jolla, CA). The urea concentration corresponding to denaturation of half of the protein (IC\(_{50}\)) was used to assess the stability of the proteins.

RESULTS

Design of a Covalently Linked \(V_L\) Dimer and an Induced \(V_L\) Monomer—The pathologic Mcg identified by Edmundson and co-workers (21, 41) is a \(\lambda\) LC homo-dimer that was isolated from a patient and served as a model in many previous experiments, making it a particularly informative variant for studying the amyloidogenic behavior of \(V_L\)s in our research. The crystal structure of Mcg \(V_L\)s appears as a homo-dimer (PDB 3MC-G) with an overall conformation similar to the canonical \(V_H\)-\(V_L\) hetero-dimer of Fab (42, 43). The structural integrity of the Fab is mimicked in Mcg because the dimer interface between the \(V_L\)s, which contains strands G-F-C-C of the immunoglobulin Greek key fold, is preserved (26).

To verify whether pure \(V_L\) monomer or a dimer forms amyloid fibers and to examine in physiological conditions findings previously found in denaturing conditions (33, 44, 45), we generated distinct, soluble, quaternary forms of the model \(V_L\) domains: the native Mcg dimer consisting of two individual \(V_L\)s bound by noncovalent interactions, a covalently linked Mcg dimer with disulfide bonds welding the two individual \(V_L\)s as a dimer, and an induced \(V_L\) monomer. In near physiological conditions, we examined which of the three states of Mcg can assemble into amyloid fibers.

To produce the covalent Mcg dimer, residues appropriately located for site-directed mutagenesis to cysteine were identified based on the crystal structure of the native Mcg \(V_L\) dimer (Fig. 2, Table 1). The Mcg crystal structure shows the proximity of residues Ala-45 and Phe-101 at the interface of the identical \(V_L\)s of the dimer. Therefore, we created a covalent Mcg dimer by mutagenesis of both of these residues to cysteine (Mcg-A45C-F101C). When purified, a nonreducing SDS-PAGE shows a molecular mass of \(\sim 30\) kDa as expected for a Mcg dimer. In addition, the crystal structure of Mcg-A45C-F101C clearly shows the presence of the two designed disulfide bonds between the adjacent \(V_L\)s (Fig. 2). Upon reduction of the disulfide bonds, the SDS-PAGE shows a molecular mass that matches that of the native Mcg, \(\sim 15\) kDa.

Analytical ultracentrifugation data for the covalent Mcg dimer and \(V_L\) monomer gave concentration-independent molecular weights consistent with a dimer and monomer respectively. The native Mcg, however, gave molecular weights that were both speed-dependent (indicating molecular weight heterogeneity) and concentration-dependent (indicating association). A group analysis of 12 files (four concentrations at three speeds) of Mcg found the best fit to be a monomer-dimer equilibrium with a dissociation constant (\(K_D\)) of \(0.2 \pm 0.01\) mM (Fig. 5).

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To induce a soluble \(V_L\) monomer, residues at the hydrophobic interface between the two \(V_L\) domains of the native Mcg were mutated to decrease interdomain van der Waals forces and increase solubility. These residues were identified based on the sequence and structural similarity between the native Mcg and its analogous Fab (Fig. 3) and according to their effect on dimerization constants as noted by Stevens et al. (46). The crystal structure of the most similar full-length antigen-binding fragment (Fab) (PDB 3TNM) was determined by means of amino acid sequence alignment of the native Mcg with proteins deposited in the Protein Data Bank. Comparison of the three-dimensional \(V_L\) models indicates that not only do the native Mcg and the corresponding Fab overlay closely, but apolar residues at the hydrophobic interface overlay as well. Upon the alignment of crystal structures, Mcg residues Tyr-38, Phe-99, and Phe-101 were identified among several conserved key residues that form the hydrophobic interface of the \(V_L\) dimer. The residues Tyr-38 and Phe-101 were also identified previously by Stevens et al. (46) to affect dissociation constants of \(V_L\) dimers in \(\kappa\) variable domains. The triple mutant of Y38E, F99A, F101E removed hydrophobic contacts and introduced two additional charges per monomer, resulting in a soluble \(V_L\) monomer (Mcg-Y38E-F99A-F101E).

**Quaternary States of the Proteins in Solution**—The distribution of quaternary states of the native Mcg and two mutants in solution was verified by two methods: quadrupole time-of-flight native mass spectrometry and analytical ultracentrifugation. Native state mass spectrometry identified molecular masses for the three proteins (Fig. 4). The \(V_L\)s of the native Mcg were found to be both dimeric and monomeric, 23.1 and 11.5 kDa respectively, indicating that equilibrium exists between the two quaternary states. The molecular mass of the covalent Mcg dimer was 23.1 kDa, and that of the \(V_L\) monomer was 11.4 kDa. These results verified the intended effects of the designed mutations on the Mcg quaternary states in solution and confirmed that the native Mcg exists in equilibrium between a \(V_L\) monomer and dimer.

Analytical ultracentrifugation data for the covalent Mcg dimer and \(V_L\) monomer gave concentration-dependent molecular weights consistent with a dimer and monomer respectively. The native Mcg, however, gave molecular weights that were both speed-dependent (indicating molecular weight heterogeneity) and concentration-dependent (indicating association). A group analysis of 12 files (four concentrations at three speeds) of Mcg found the best fit to be a monomer-dimer equilibrium with a dissociation constant (\(K_D\)) of \(0.2 \pm 0.01\) mM (Fig. 5).
Stability of the Native Mcg, Covalent Mcg Dimer, and VL Monomer—The stability of the three proteins was examined by titrating with urea and monitoring fluorescence. Logarithmic analysis of the fluorescence data indicates that all the proteins proceed through cooperative unfolding at lower concentrations of urea followed by noncooperative unfolding at higher concentrations (Fig. 6). The urea concentration corresponding to denaturation of half of the protein (IC50) is

![Graphs showing denaturation of proteins](image-url)
highest for the covalent Mcg dimer, followed by the native Mcg, and then the induced VL monomer, indicating that the monomer is the least stable. In the presence of DTT, the IC\textsubscript{50} of each protein decreases, indicating that the proteins are less stable under reducing conditions. This shows that in addition to reducing the disulfide bond that links the Mcg covalent dimer, the intrachain disulfide bond between residues 22 and 90 of each variable domain is also reduced despite its location in the core of the domain.

Effect of Quaternary Structure on Amyloid Fiber Formation—The ability of the native Mcg, covalent Mcg dimer, and VL monomer to form fibers was monitored by an increase in ThT fluorescence and by analysis of transmission electron micrographs for the presence of fibers after the ThT assays. When amyloid fibers formed, catalyzed by an acidic environment and temperature of 37 °C, the ThT assay indicated an increase in fluorescence, and electron micrographs confirmed the presence of fibers (Fig. 7). Although the increase in ThT fluorescence correlates with formation of amyloid fibers, the experiments indicated a variation in intensity and lag times. This variation probably occurs due to differences in nucleation heterogeneity, and therefore, we qualitatively examined ThT fluorescence.

The native Mcg and VL monomer readily formed fibers in oxidizing and reducing conditions. However, the covalent Mcg dimer did not form fibers in oxidizing conditions. Upon reduction of the disulfides with DTT, the dimer formed amyloid fibers resembling those of the native Mcg and the VL monomer (Fig. 7). Confinement of the covalent Mcg to its canonical dimer conformation abolishes its amyloidogenic property, whereas both the native Mcg, existing in equilibrium between dimers and monomers, and the pure VL monomer are able to form amyloid fibers.

Structures of Designed VL Domains—The structures of the covalent Mcg dimer and of the VL monomer were determined by means of x-ray crystallography, and their conformations remained similar to the native Mcg dimer. The covalent Mcg dimer showed two disulfide bonds between residues 45 and 101 of adjacent VLs, which remained in the same orientation relative to each other as in the native Mcg (Fig. 2). Despite mutations that induced the VL monomer in aqueous solution, it also crystallized in a conformation similar to the native Mcg. However, its crystal structure displayed a phenomenon new to immunoglobulin variable domains; G-strands were swapped among crystallographic
FIGURE 7. Thioflavin T fiber formation assays and electron micrographs of the native Mcg, covalent Mcg dimer, and induced V\textsubscript{L} monomer. A and B, averaged fluorescence readings for each protein based on three repeated thioflavin T assays in oxidized and reduced conditions. Error bars are not shown for clarity. C and D, electron micrographs. The only sample that does not form amyloid fibers within 5 days is the covalent Mcg dimer. However, when the covalent cysteine linkages are reduced by DTT, it forms amyloid fibers as readily as the native Mcg and the induced V\textsubscript{L} monomer. E, raw ThT fluorescence (F\textsubscript{T}) data for the three replicated experiments, which are shown averaged in columns A and B.
dimers, yet the overall immunoglobulin fold remained preserved (Figs. 2 and 8).

DISCUSSION

Identification of pathways of assembly from globular proteins into amyloid fibers is essential for understanding amyloid associated diseases. Until now, it has not been clear whether a monomer, dimer, or some other multimeric state of immunoglobulin LCs is the precursor to assembly of amyloid fibers. Three models for the assembly pathway are presented in Fig. 1. Our experiments show that the covalent Mcg dimer is unable to form fibers due to disulfide bonds that bind pairs of VLs and prevent transformation into monomers or a noncanonical dimer. However, upon reduction of the disulfide bonds, the dimer regains the ability to form fibers as readily as the native Mcg and the VL monomer. Therefore, this demonstrates that a canonical dimer of Mcg is protective against amyloid formation, whereas a VL monomer is prone to assemble into amyloid fibers (29, 32, 33). In other words, the monomer model is the most consistent with our data.

Differences in amino acid sequence between various VLs are likely to affect the stability of the dimer and the monomer, which can account for why some VLs are more likely than others to disassociate into monomers and form amyloid fibers (31). In the vast number of possible immunoglobulin variants, each differs from others by only several residues within each of the λ or κ families, and these differences may affect VL dimer–monomer disassociation constants (31, 47). Unstable dimers are more likely to transform between alternative conformations, which protect against amyloid formation to various degrees according to their thermodynamic stability. This transformation is likely to occur by disassociation of the dimer into amyloid-prone monomers, which then can partially unfold and expose a segment that forms the steric zipper spine of the fiber. Our results are consistent with previous findings that destabilization of VL dimers and unfolding of VL monomers are consecutive yet separate processes (29, 32, 44). Experiments with κ1 O18/O8, AL-09, and SMA VLs show that disassociation of dimers and unfolding of monomers promote amyloid assembly, and in denaturing conditions, mutations that destabilize VL monomers induce formation of amyloid fibers. Similarly, our chemical denaturation experiments suggest that the monomer is the quaternary state that is more prone to unfold, whereas the dimer is protective. Because formation of amyloid fibers depends on a soluble yet unstable monomer, both a stable dimer and a stable monomer would reduce propensity to form amyloid fibers. However, reduction of amyloid formation occurs by two different mechanisms; stable dimers reduce the concentration of soluble monomers, and stable monomers are less likely to unfold and form amyloid fibers. This is similar to pathways by which other amyloidogenic proteins form amyloid fibers; for example, transthyretin disassociates into monomers, and lysozyme must transition through a partially unfolded state to form amyloid fibers (33, 48–51).

Many variants of VLs have been found in patients with systemic light chain amyloidosis, and these variants only differ by a few amino acids (14). Although our experiments were performed with only one variant and a single conformation was imposed on the covalently linked dimer, the fact that a pure, soluble monomer forms amyloid fibers indicates that the monomeric state of the VL is the precursor to amyloid fibers. Due to the similarity in sequence, structure, and biochemical behavior of all light chain variable domains, we infer that our finding for Mcg may well be applied to other light chains and explain the differences in propensity to form amyloid; a stable dimer is less likely to disassociate into monomers, and therefore, less likely to form amyloid.
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Although VL dimers must dissociate into monomers to form fibers, structures of full-length LCs indicate that this conformational change can occur regardless of a connection to a constant domain (42). In full-length LCs, VLs are connected to CLs through a joining (J) segment, which provides the domains with the flexibility to acquire conformations independent of each other. Such an architecture of the protein does not impose a particular orientation on VLs relative to connected CLs, as affirmed by the existence of several crystal structures (42, 52, 53). Therefore, a dimer of VLs maintains the ability to dissociate into amyloid-prone VL monomers, while still covalently attached to CLs. This flexibility explains why amyloid fibers of immunoglobulin can consist of full-length LCs, just their VLs, or both.

The crystal structure of our induced VL monomer displays an overall conformation similar to the dimer formed by the native Mcg, yet it also shows a phenomenon new to immunoglobulin variable domains where strands are swapped among molecules of the asymmetric unit (Fig. 8). Swapping between segments of protein domains is known to be concentration-dependent and was previously observed in other proteins, such as diphtheria toxin, human prion protein, cystatin C, α2-microglobulin, T7 endonuclease, and helicase (54–58). In closed swapping, protein segments are exchanged between a discrete number of molecules, whereas in run-away swapping, the exchange occurs between an unlimited number, resulting in a continuous polymer (55). In the crystal structure of VL monomers, closed swapping of G-strands occurs between four VL dimers, with both domains of each dimer being swapped. In addition, canonical dimer interfaces link the pairs of swapped dimers in a noncrystallographic octamer with distorted 223 symmetry. Although swapping between domains of VL monomers in solution may play a role in linking molecules in a fiber, the closed swapping in the crystal structure does not resemble a steric zipper spine that would account for the cross-β x-ray diffraction pattern. Although the domain swapping cannot be dismissed as unrelated, its existence in immunoglobulins and relation to their amyloidogenic property is yet unclear.

A practical insight emerging from both past studies and the present study is the possible binding of molecules to the hydrophobic cavity between the variable domains to stabilize the dimer (59). Structures of VL dimers show that a variety of ligands bind to the hydrophobic cavity between the domains (24). Crystallographic models of Mcg indicate that the protein maintains its structure as a canonical dimer while bound to a ligand. The binding of molecules yields an energetically favorable complex, and the equilibrium between different conformations might shift toward a stable dimer rather than an amyloid-prone monomer. Such complexes may therefore inhibit the transition of LC or VL dimers to amyloid fibers, resulting in alleviated symptoms in systemic amyloidosis patients.

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