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Incomplete Refolding of Antibody Light Chains to Non-native, Protease-sensitive Conformations Leads to Aggregation—A Mechanism of Amyloidogenesis in Patients?

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Abstract

Genetic, biochemical and pharmacologic evidence supports the hypothesis that conformationally altered or misfolded protein states enable aggregation and cytotoxicity in the systemic amyloid diseases. Reversible structural fluctuations of natively folded proteins are involved in the aggregation of many degenerative disease associated proteins. Herein, we use antibody light chains (LCs) that form amyloid fibrils in AL amyloidosis to consider an alternative hypothesis of amyloidogenesis: that transient unfolding and incomplete extracellular refolding of secreted proteins can lead to metastable, alternatively folded states that are more susceptible to aggregation or to endoproteolysis that can release aggregation-prone fragments. Refolding of full-length λ 6a LC dimers comprising an inter-chain disulfide bond from heat- or chaotrope-denatured ensembles in buffers yields the native dimeric state as well as alternatively folded dimers and aggregates. LC variants lacking an inter-chain disulfide bond appear to refold fully reversibly to the native state. The conformation of a backbone peptidyl-proline amide in the LC constant domain, which is *cis* in the native state, may determine whether the LC refolds back to the native state. A proline to alanine (P147A) LC variant, which cannot form the native *cis*-amide conformation, forms amyloid fibrils from the alternatively folded ensemble, whereas all the full-length λ 6a LCs we have studied to date do not form amyloid under analogous conditions. P147A LC variants are susceptible to endoproteolysis by thrombin, enabling amyloidogenesis of the fragments released. Thus, non-native LC structural ensembles containing a tyrosine 146-proline 147 *trans*-amide bond can initiate and propagate amyloid formation, either directly or after aberrant endoproteolysis.

Introduction

Misfolding and/or aggregation of antibody light chains (LCs) into various structures, including low molecular weight (MW) non-native oligomeric conformers and high MW cross- β -sheet amyloid fibril aggregates, appears to drive organ damage in the disease light chain (or AL) amyloidosis.^{1–3} This relentlessly progressive and often fatal disease affects 1

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individual per 100,000 per year. The underlying cause of AL amyloidosis is an expanded, or in some cases cancerous, population of monoclonal plasma cells, which are specialized antibody-secreting cells. LCs may be secreted both as “free” LCs and as complete antibodies, although clonal plasma cells from approximately half of AL amyloidosis patients have a chromosomal translocation that prevents antibody heavy chain expression.^{3–4} Each patient has a unique LC sequence.⁵ Most LCs are excreted by the kidneys,⁶ but in AL amyloidosis LCs misfold and aggregate leading to deposition of amyloid fibrils. AL amyloidosis is generally diagnosed after the onset of heart, liver and kidney damage. Patients with significant cardiac involvement are often too sick to tolerate disease-modifying chemotherapy regimens (typically a combination of a DNA alkylating agent and a proteasome inhibitor) that kill the underlying plasma cell clone and prevent further LC amyloidogenesis-mediated organ toxicity.⁷ Additional early diagnostic methodology and mechanistically diverse, low toxicity, LC amyloid disease-modifying treatments are urgently needed, especially for those patients presenting with severe cardiomyopathy.^{7–9}

LCs are generally secreted as folded monomers or homodimers from AL-associated plasma cells. There are two classes of LCs, λ and κ , with similar structures, although λ LCs are more likely than κ LCs to form dimers.^{10–11} Each LC protomer consists of two immunoglobulin (Ig) domains: An N-terminal variable domain (V-domain) and C-terminal constant domain (C-domain) (Figure 1A). Dimers may be stabilized by an inter-chain disulfide bond. LC genes are formed by germline recombination of either the λ or κ locus, each consisting of arrays of “variable”, “joining” and “constant” gene segments.¹² The κ locus has a single constant segment, whereas the λ locus encodes five constant segments with >90% protein sequence identity. During the process of B cell development, one of each type of segment is joined together to produce a precursor gene, which is then subject to additional mutagenesis to select for optimal antigen binding. Amyloid deposits isolated from AL amyloidosis patients typically contain full-length LCs, individual Ig domains and peptide fragments thereof, which are apparently produced by aberrant endoproteolysis.^{13–17} However, the role of these different LC species in proteotoxicity^{18–20} and the influence of proteolysis on the pathogenesis of the disease are not well understood. Identifying the molecular mechanisms by which LC misfolding and/or aggregation lead to dysfunction and loss of post-mitotic tissue in AL amyloidosis should lead to new therapeutic strategies.

We recently reported that natively folded full-length λ 6a LCs from AL amyloidosis patients are less kinetically stable than a non-amyloid-associated LC from a multiple myeloma patient and the λ 6a LC germ line sequence from which the other LCs are derived.²² λ 6a LCs (containing the IGLV6-57 variable gene segment) are over-represented in AL amyloidosis patients, hence our focus on them here.^{23–24} Kinetic stability is quantified by the magnitude of the free energy activation barrier associated with unfolding, which determines the frequency at which a protein visits non-native conformations at equilibrium.²² Low kinetic stability translates to fast unfolding from the native state. Faster unfolding makes full length LCs more susceptible to aberrant processing by endoproteases, which preferentially cleave extended protein conformations. Formation of amyloid fibrils from patient-derived LCs was originally observed following treatment with proteases.¹⁴ We demonstrated that specific endoproteolysis between the LC V- and C-domains (Figure 1A) is required to release amyloidogenic fragments, and we showed that not all cleavage sites

between the V- and C-domains afford variable domains that can aggregate.²² We and others have reported that full-length LCs do not readily form amyloid under laboratory conditions where their variable domains do so—the presence of the constant domain seems to be protective *in cis*.^{22, 25–26} However, full-length LCs have been shown to aggregate under some conditions and do so in patients.^{16, 25} During our attempts to quantify LC thermodynamic stability, we observed that full-length, disulfide-tethered λ 6a LC dimers do not fully refold from denaturant but instead form a conformational ensemble exhibiting a non-native intrinsic tryptophan fluorescence spectrum.^{22, 27} Unfolding of full-length LCs by heat is predominantly irreversible^{25–26}. These results suggest that attempted extracellular refolding, i.e., in the absence of the proteostasis network components that are present in the endoplasmic reticulum of plasma cells,²⁸ is not completely efficient. Hence, transient unfolding of native LCs after secretion could afford a population of circulating LCs that adopts a non-native conformational ensemble, as well as a fraction that refold to the native state. We hypothesize that the non-native conformational ensemble resulting from unsuccessful refolding makes certain LCs susceptible to amyloidogenesis directly or to aberrant endoproteolysis enabling LC fragment aggregation. This is distinct from the widely accepted conformational change hypothesis, wherein higher energy, aggregation-competent conformations are accessed by transient, reversible fluctuations from the native state.²⁹

Here, we investigate the properties of the misfolded ensemble of λ 6a LCs. Triggering refolding by dilution of urea or upon cooling after thermal denaturation results in a mixed population of species, including the native LC dimer. Another species afforded is a non-native, but collapsed conformational ensemble that appears to harbor a non-native tyrosine 146-proline 147 *trans* backbone amide bond. We show that the misfolded ensemble is kinetically destabilized compared to the LC native state, and accordingly is rapidly degraded by endoproteolysis. The misfolded ensemble is also more prone to aggregation than the native state. Population of this misfolded conformational ensemble in the extracellular space could lead to aggregation directly, or alternatively the misfolded ensemble could undergo aberrant endoproteolysis enabling the resulting LC fragments to aggregate efficiently. Both of these processes could contribute to AL amyloidosis pathology in humans.

Materials and Methods

Protein sequences, expression and purification

LC sequences were cloned as previously described.²² Mutagenesis was carried out by the QuikChange method (Agilent). Numbering here refers to the order of residues in the 6aJL2-FL construct;²² Kabat residue numbers³⁰ are provided for reference. A sequence alignment is shown in Figure 1B. Each construct comprises the human CL3 constant domain sequence (Figure 1B). Full-length LCs were expressed as inclusion bodies in *E. coli* BL21 (DE3) as described.²² Cells were lysed by sonication, and inclusion bodies were washed by cycles of centrifugation, resuspension and sonication. Inclusion bodies were dissolved in 10–20 ml 4 M guanidine hydrochloride containing 5 mM dithiothreitol. LCs were refolded by 20-fold dropwise dilution into 50 mM Tris-Cl containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione on ice. After partial refolding overnight, ammonium sulfate, (NH₄)₂SO₄, was added to 25% saturation at 4 °C and insoluble material was pelleted by

centrifugation. Additional ammonium sulfate was added to bring the supernatant to 75% saturation and the resulting insoluble material was pelleted by centrifugation and then redissolved in 25 mM Tris-Cl at 4 °C, pH 8.5. LCs were dialyzed against 25 mM Tris-Cl, centrifuged and filtered to remove insoluble material. LCs were purified on a Source 15Q column (GE) at 4 °C in 25 mM Tris-Cl, pH 8.5, eluting with a 0–300 mM NaCl gradient. LC-containing fractions were concentrated and further purified by size exclusion chromatography, using a Superdex 75 column (GE) equilibrated in phosphate buffered saline solution (PBS, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl, pH 7.4). We refer to this LC as “folded and purified.”

P147A LCs were purified as above, except that the LC inclusion bodies dissolved in guanidine hydrochloride were refolded into 0.5 M arginine, pH 9, containing 5 mM reduced glutathione and 0.5 mM oxidized glutathione. The resulting material was centrifuged to remove insoluble material, dialyzed against 25 mM Tris-Cl, pH 8.5, and purified using column chromatography as above. We refer to this LC as “misfolded and purified.”

The λ C domain and LC V-domains were expressed as fusions with the *pelB* leader sequence from the plasmid pET-22. Expression was induced overnight at 18 °C with isopropyl β -D-1-thiogalactopyranoside. LC domains were harvested by periplasmic shock as previously described.²² LC domains were purified by ammonium sulfate precipitation and column chromatography as described above.

Unfolding and refolding experiments

Folded and purified recombinant LCs (150 μ M) were unfolded by dilution with 2 volumes of 9 M urea in 50 mM sodium phosphate buffer at 25 °C, pH 7. The final urea concentration was 6 M, sufficient to completely unfold all LCs.²² Refolding was initiated after a 1 h incubation in 6 M urea unless otherwise indicated, by diluting the LC into 9 volumes of 50 mM sodium phosphate buffer at 25 °C, pH 7 to give a final LC concentration of 5 μ M. Refolding was allowed to proceed for at least 1 h after dilution of urea. Folded and purified LC samples in low concentrations of urea were prepared for comparison to the refolded LCs in the same urea concentration. Dilution of the folded and purified LC with 29 volumes of 0.621 M urea in 50 mM sodium phosphate buffer at 25 °C, pH 7 to a final urea concentration of 0.6 M enables comparison with the refolded LCs in 0.6 M urea. Total LC (5 μ M) and urea concentrations in “folded and purified” and “refolded” samples were therefore identical for each comparison. For circular dichroism (CD) experiments, the unfolded LC in 6 M urea was concentrated 5-fold using a 10 kDa-cutoff centrifugal concentrator, and then refolded by 50-fold dilution to a final urea concentration of 0.12 M. Refolding did not result in the formation of visible aggregates. Two populations of refolded JTO-FL could be partially separated on a MonoQ column (GE) at 4 °C in 25 mM Tris-Cl, pH 8.5, eluting with a 0–250 mM NaCl gradient. Fractions were collected for analysis by fluorescence spectroscopy.

Spectroscopy

Spectra were recorded at 25 °C in 50 mM sodium phosphate buffer, pH 7 unless otherwise indicated. CD spectra were measured using an Aviv 420SF instrument as previously described.²² The intensity of the CD spectra and the fluorescence spectra of JTO-FL

following ultracentrifugation were corrected for the relative concentration of LC by unfolding each sample in 6 M urea and measuring the intrinsic tryptophan fluorescence. Steady state and kinetic fluorescence measurements were carried out on a Jasco FP-8500 or Aviv ATF 105 fluorimeter equipped with a stopped flow apparatus as previously described.²² The excitation wavelength (λ_{ex}) was 280 nm for all experiments and the emission wavelengths (λ_{em}) were 350 nm for stopped flow experiments and 300–420 nm for the fluorescence spectra. For comparison of emission spectra, the average wavelength ($\langle\lambda\rangle$ or center of emission spectral mass)³¹ was calculated:

$$\langle\lambda\rangle = \frac{\sum_i \lambda_i I_i}{\sum_i I_i}$$

where λ_i and I_i are the wavelength and intensity at data point i , respectively. All fluorescence spectra are normalized and scaled so that the maximum intensity of one spectrum (defined in each figure legend) is set to 1, to facilitate comparisons between spectra.

Molecular size determination

Analytical ultracentrifugation sedimentation velocity experiments and analytical size exclusion chromatography (SEC) were carried out as previously described.²² Analytical ultracentrifugation data were processed using SEDFIT software³² to produce a continuous distribution of sedimentation coefficients. Analytical SEC was monitored by absorbance at 280 nm and fluorescence emission (λ_{ex} 280 nm) at 325 nm and 355 nm. Dynamic light scattering (DLS) was measured after incubation of refolded JTO-FL for 2 h at 25 °C in 50 mM sodium phosphate buffer containing 0.6 M urea, pH 7, using a DynaPro Nanostar instrument (Wyatt) in a 4 × 1 mm cuvette. Samples were not centrifuged or agitated during the refolding process. 10 scans of 10 s each were measured and combined using Dynamics software (Wyatt). Mass distributions are shown using the “optimal resolution” setting of the software.

Proteolysis

Endoproteolysis was carried out at 37 °C in PBS buffer as described previously.²² For pulse proteolysis, equimolar proteinase K (Fermentas) was incubated for 1 min with LC. For proteolysis kinetics, 1% mole fraction of proteinase K was used. Samples were quenched with phenyl methyl sulfonyl fluoride (PMSF) and quantitated by analytical SEC as above or by SDS-PAGE electrophoresis. Gels were imaged by reaction of LC tryptophan residues with 2,2,2-trichloroethanol³³ using a BioRad ChemiDoc. Quantitation by densitometry was carried out using BioRad ImageLab software.

Amyloidogenesis

Filtered LC solutions (200 μ l of 10 μ M LC in PBS buffer containing 5 μ M thioflavin T) were incubated in 96-well, black, clear-bottomed microplates (Corning #3631). Plates were sealed with Nunc polyester film (ThermoFisher) and a black lid was secured to the plate with tape. After equilibration to 37 °C, aggregation was initiated by shaking at 1000 rpm in a plate

shaker at 37 °C. Plates were read ($\lambda_{\text{ex}} = 440 \text{ nm}$, $\lambda_{\text{em}} = 480 \text{ nm}$) in a Spectramax Gemini EM plate reader (Molecular Devices).

Differential scanning calorimetry (DSC)

LCs (30 μM) were dialyzed overnight at room temperature (22 °C) against PBS. Thermal transitions were measured on an automated Microcal VP-Capillary DSC instrument (Malvern Instruments). The dialysis buffer was filtered and used as a blank for DSC measurements. LCs were heated from 25–90 °C at a rate of 90 °C per h, then cooled to 20 °C and then heated again. Data were recorded at 1 °C resolution with an 8 s filter period and using passive feedback mode. Data were recorded and processed using Origin software.

Electron microscopy

Electron micrographs were recorded by the TSRI microscopy core facility as described previously.²² Samples were stained with phosphotungstic acid or uranyl acetate.

Results

Full-length, disulfide-bonded LC dimers misfold upon dilution from chaotrope

Unfolding of full-length $\lambda 6a$ LCs in urea, followed by attempted refolding by dilution of urea results in a conformational ensemble exhibiting a non-native tryptophan fluorescence emission spectrum,^{22, 27} similar to what is seen after LC heat denaturation followed by cooling-associated reconstitution.²⁵ Many single domain proteins refold efficiently upon denaturant dilution, but multi-domain proteins often misfold and/or aggregate when refolded in the absence of proteostasis network assistance.²⁹ We hypothesize that refolding affords a mixture of native and non-native LC dimers, and possibly also some aggregates. A sequence alignment of the LCs used in this study is shown in Figure 1B—all LCs have the same $\lambda C3$ constant domain. Recombinant LCs were purified from the cytoplasm of BL21 *E. coli* as inclusion bodies.^{22, 34} The inclusion bodies were unfolded in guanidine hydrochloride (GuHCl) and then refolded by dilution, before purification by an ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$ precipitation, followed by column chromatography steps (Figure 2A; see Materials and Methods). Refolding of the dissolved inclusion bodies results in >90% pure LCs, which appear to be partially misfolded as evidenced by their red-shifted fluorescence emission spectrum (Figure 2B, dotted purple line). $(\text{NH}_4)_2\text{SO}_4$ fractionation (25%–75% insoluble fraction) and redissolution affords LCs with a native-like fluorescence spectrum (Figure 2B, red line). Only 45% of the LC is recovered upon redissolution of the $(\text{NH}_4)_2\text{SO}_4$ -insoluble material (74 mg from 166 mg of LC in the inclusion bodies). Some of this loss may be LC that did not correctly form all five disulfide bonds. In support of this hypothesis, $(\text{NH}_4)_2\text{SO}_4$ precipitation of folded and purified LCs resulted in apparently complete recovery of the LC. Therefore, we conclude that this denaturation, refolding and purification procedure removes misfolded and/or aggregated LCs, producing “folded and purified” recombinant LCs that are apparently structurally homogeneous. Each full-length $\lambda 6a$ LC protomer contains three tryptophan (Trp) residues. Trp 37 (residue 35 in the Kabat numbering system³⁰) packs against the intra-domain disulfide bond in the V-domain and Trp 154 (Kabat residue 158) packs against the intra-domain disulfide bond in the C-domain (Figure 1A). The proximity of the disulfide quenches the fluorescence of these Trp residues

in the native state. Trp 191 (Kabat residue 186) is buried in the C-domain,^{21, 35} and is likely largely responsible for the native state fluorescence spectrum. The fluorescence spectral change upon refolding indicates that the local environment of at least one of the six Trp residues comprising each LC dimer differs between the folded and purified LCs and the improperly refolded LC dimer conformational ensemble.

Reasoning that any soluble misfolded LC conformational ensemble is likely to be less stable than natively folded LCs, we initially focused on JTO, a non-amyloid-associated LC from a multiple myeloma patient.³⁶ JTO is the most kinetically stable of the LCs that we have investigated,²² which might make distinguishing folded and misfolded states easier. We initially employed fluorescence and circular dichroism (CD) spectroscopy to investigate the natively folded conformation, and to study chaotrope unfolding, as well as to assess the reversibility of JTO refolding. We refer to full-length, dimeric JTO as JTO-FL. We also studied the isolated variable domain (JTO-V) and the isolated constant domain (λ C). This constant domain sequence comprises all the LCs previously studied by us (Figure 1B).²² The intrinsic fluorescence spectra of folded and purified recombinant JTO-FL and variants thereof ($\lambda_{\text{ex}} = 280$ nm) are shown in Figures 2C and 2D. Recombinant, folded and purified LCs were denatured by dilution into 6 M urea at 25 °C for 1 h then refolded by dilution into 50 mM sodium phosphate buffer at 25 °C, pH 7 to a final urea concentration of 0.6 M. Disulfide bonds were not reduced during this process. JTO-FL, with its intact inter-chain disulfide bond between cysteine 217 residues (Kabat residue 214), refolds to a conformational ensemble exhibiting a red-shifted tryptophan fluorescence emission spectrum after a 1 h refolding period at 25 °C, pH 7 (Figure 2C, dotted blue line). The fluorescence spectrum of JTO-FL after a 16 h refolding period at 25 °C exhibits a decreased intensity and an emission maximum between that of the folded and purified LC and the LC allowed to refold for 1 h (Figure 2C, dashed blue line). Mutation of cysteine 217 to serine (C217S) kinetically destabilized all LCs evaluated to date.²² Yet, notably, the JTO-FL C217S variant refolds fully reversibly from 6 M urea upon dilution into 50 mM sodium phosphate buffer to a conformation exhibiting an indistinguishable emission spectrum from that of folded and purified JTO-FL C217S (Figure 2D; top right panel). Similar fully reversible refolding occurs with other LC C217S variants (data not shown). Ammonium sulfate precipitation and redissolution of the pellet of JTO-FL C217S during purification from inclusion bodies yields 60% recovery of LC (61 mg from a 101 mg of LC in the inclusion bodies), more than the 45% of JTO-FL that was recovered from inclusion bodies. The JTO V-domain and the λ C domain both refold reversibly (Figure 2D), as has been reported for other variable domains^{22, 37–39} and the κ C domain.⁴⁰

After unfolding for 1 h in 6 M urea, we followed the kinetics of JTO-FL refolding initiated by dilution to 0.6 M urea. Fluorescence spectra of JTO-FL were recorded as a function of refolding time (Figure 2E). The average wavelength of the spectra (also known as the center of spectral mass; see Materials and Methods) changes with multiphasic kinetics, but does not reach the level of the initially folded and purified ensemble even after a 48 h refolding period. The data are consistent with slow reorganization of a fraction of the misfolded LC formed during refolding proceeding to the native state. The far-UV CD spectrum of recombinant, folded and purified JTO-FL is characteristic of a β -sheet-rich protein (Figure 2F, solid black line), whereas the spectrum of refolded JTO-FL exhibits reduced ellipticity,

consistent with refolding to mostly the native state, but refolded LC clearly also populates a misfolded ensemble (Figure 2F, dotted blue line). The far-UV CD spectra of both folded and purified JTO-FL C217S, and refolded JTO-FL C217S are very similar (Figure 2F, right panel), consistent with this variant's ability to refold quantitatively from denaturant. Collectively, the data described thus far suggest that partial misfolding of the full-length, inter-chain disulfide bonded LC dimers upon attempted refolding results from trapping an otherwise transient conformational state by the restraints imposed by the inter-chain disulfide bond.

Many proteins aggregate upon refolding by dilution of chaotrope. To determine whether this is the case for the full length LCs, we measured the apparent molecular weight of the refolded JTO-FL by size exclusion chromatography (SEC), employing parallel absorbance and fluorescence emission detection. Folded and purified JTO-FL, and both folded and purified, as well as refolded JTO-FL C217S elute from the column as a single major peak at similar elution volumes, consistent with a dimeric full-length quaternary structure (Figure 3A). However, refolded JTO-FL had a more complex elution profile, exhibiting a reduced intensity dimer peak and the presence of a very broad peak eluting earlier, consistent with an expanded misfolded ensemble or, more likely, an aggregated LC population (Figure 3A, dotted blue lines in upper panel). The total absorbance intensity of refolded JTO-FL, including the dimer-like and larger species, is lower than that of the folded and purified recombinant JTO-FL, suggesting that approximately 15% of LC is aggregated and does not enter the column, although we have not been able to identify such aggregates directly. The ratio of the fluorescence emission intensity ($\lambda_{\text{ex}} = 280 \text{ nm}$) at 325 nm and 355 nm of the dimer SEC peak differs between folded and purified JTO-FL and refolded JTO-FL (Figure 3B). This is not the case for folded and purified JTO-FL C217S and refolded JTO-FL C217S, which exhibit indistinguishable fluorescence ratios (Figure 3B, right panel). That the altered 325 nm/355 nm fluorescence emission ratio of the refolded dimeric JTO-FL is red-shifted compared to that of the folded and purified JTO-FL is consistent with the hypothesis that the dimer peak comprises both the alternatively folded ensemble and natively folded JTO-FL. To ask whether the spectroscopic differences between folded and purified JTO-FL and refolded JTO-FL are due to the presence of aggregates, we subjected the refolded material to ultracentrifugation for 150 min at 355,000 *g*, which clears species larger than dimers from solution. This treatment results in loss of 7% of the refolded JTO-FL (possibly the fraction aggregated), but ultracentrifugation does not alter the fluorescence emission spectrum of the remaining soluble material (Figure 3C), indicating that a soluble, non-native dimer is formed upon refolding. Dynamic light scattering measurements, which are generally a sensitive method to detect small amounts of aggregates, do not reveal differences in apparent molecular size between folded and purified versus refolded JTO-FL (Figure 3D). Overall, these data suggest that refolded JTO-FL predominantly forms dimer-sized structures, and these structures are responsible for the spectral changes observed upon refolding. However, some material (7–15%) appears to be lost upon refolding, which we attribute to aggregation, although we are unable to characterize these aggregates in detail.

Misfolded LCs are prone to endoproteolysis

Folded and purified JTO-FL is resistant to endoproteolysis by proteinase K, consistent with its high kinetic stability and consequent slow rate of unfolding.²² We took advantage of this property to ask whether refolded, dimeric JTO-FL adopts a non-native conformation exclusively, reasoning that a non-native, misfolded JTO-FL population would be more susceptible to endoproteolysis. We used a short pulse of a high concentration of proteinase K followed by quenching with PMSF, since this allows us to partially differentiate between a population of misfolded LCs having a relatively long lifetime versus folded LCs that transiently populate an unfolded state.⁴¹ We measured protease sensitivity after a 1 h and a 24 h refolding period. The products of proteinase K treatment were quantified by SEC in order to measure endoproteolysis of both the dimeric LC and the apparent higher order oligomeric species preceding the 1.5 min peak (Figure 4A, quantified in Figure 4B). Pulse proteolysis of folded and purified JTO-FL resulted in a <5% reduction in the absorbance of the dimer peak at 1.5 ml (Figure 4A, left panel). Pulse proteolysis of refolded JTO-FL after a 1 h refolding period at 25 °C results in loss of absorbance from both the dimer and oligomer regions of the chromatograms (15% reduction in absorbance of the dimer peak at 1.5ml), and accumulation of species eluting more slowly, consistent with cleavage of LC dimers into fragments (Figure 4A, center panel). After 24 h of refolding at 25 °C, the dimer peak was reduced to a lesser extent by proteolysis, consistent with a misfolded-to-folded transition (Figure 4A, right panel). Both folded and purified JTO-FL C217S and refolded JTO-FL C217S are cleaved to a similar extent by pulse proteolysis (Figure 4B), consistent with the decreased kinetic stability of this variant²² and its apparent reversible refolding (Figure 2D). These data suggest that refolding of JTO-FL from 6 M urea triggered by dilution results in the formation of at least three species: a native kinetically stable dimer; an alternatively-folded dimer; and larger, partially unstructured aggregates. Both the alternatively-folded dimer and the aggregates are more susceptible to proteolysis than the native folded dimer, likely due to reduced kinetic stability (faster unfolding) or accessible protease cleavage sites in their relatively stable misfolded ensembles. The alternatively-folded dimer appears to slowly convert into the more protease-resistant natively folded dimer, consistent with the changes in its fluorescence spectrum over a similar time course (Figure 2E). These results support the hypothesis that the red-shifted fluorescence emission observed for refolded JTO-FL in Figure 2 originates from formation of a misfolded dimer population, in addition to the native-like dimer. The resistance of the folded and purified recombinant JTO-FL to endoproteolysis is consistent with the misfolded JTO-FL having been removed during LC purification, as described above (Figure 2B). These data also suggest that the LC refolding process itself leads to about 10% aggregation, at least *in vitro*, and possibly in AL amyloidosis patients.

To ask whether this behavior is unique to JTO-FL or is a feature of other λ 6a LCs, we compared folded and purified recombinant samples to refolded samples of five other LCs that we have previously investigated. These are the germline sequence 6aJL2-FL and its amyloid-associated variant 6aJL2-FL R25G⁴² and three AL amyloidosis patient-derived sequences named WIL-FL³⁶, ALLC-FL⁴³ and 6aJL7AL-FL.²² In each case, refolding results in a reduction in the intensity of the SEC peak corresponding to dimeric LC, suggesting that under these conditions they misfold and/or aggregate to similar extents

(Figure 4C). The dimer peak in the SEC chromatograms of folded and purified recombinant ALLC-FL and WIL-FL decreases in intensity following pulse proteolysis treatment by more than that of the other LCs (Figure 4C). This is likely due to their reduced kinetic stabilities²² resulting in a greater population of the initially natively-folded LCs transiently unfolding during the pulse proteolysis period. Another interpretation is that folded and purified recombinant ALLC-FL and WIL-FL, as defined above, could include a population of misfolded LC.

To distinguish between these scenarios, we measured the kinetics of endoproteolysis of four folded and purified recombinant LCs (ALLC-FL, WIL-FL, JTO-FL and 6aJL2-FL) versus their refolded ensembles 1 h after initiation of refolding by dilution of urea (Figure 5A). We used a low proteinase K concentration (50 nM proteinase K to 5 μ M LC) and a longer timecourse in order to compare the rates at which these LCs visit unfolded states under these conditions. Since the misfolded state is more sensitive to endoproteolysis than the native state, a mixture of natively folded and misfolded LC would result in biphasic (or higher order) kinetics, whereas a single LC species would likely be cleaved with first order kinetics (see the model in Figure 5B). In each case, the folded and purified recombinant LC is degraded with apparent first order kinetics (circles and solid lines in Figure 5A). In contrast, the refolded LCs exhibit biphasic kinetics (triangles and dashed lines in Figure 5A), with rapid degradation of the misfolded dimeric and aggregated LC within the dead time of the experiment, followed by a slower phase corresponding to endoproteolysis of LCs that are properly refolded and transiently populating partially folded states. All data could be fitted to a double exponential function (lines in Figure 5A). That the amplitude of the fast phase corresponds to the proportion of misfolded LC in the refolded sample is supported by the enhanced rate of proteolysis of the refolded dimeric LCs observed during the pulse experiments shown in Figure 5C. The slow kinetic phase represents the rate of endoproteolysis derived from transient unfolding of the native dimeric state. In the case of JTO-FL, the rate of the slow phase is too low to measure, consistent with its high kinetic stability and associated resistance to endoproteolysis.²² For the two amyloid-associated LCs, ALLC-FL and WIL-FL, where more rapid endoproteolysis from the native state leads to a large slow phase amplitude, the slow phase rate is indistinguishable between the folded and purified data and the refolded data (Figure 5C). This is expected since the dimeric native state is partially formed in the refolding experiment.

The ion exchange chromatography elution profile of refolded JTO-FL (Figure 6, dotted blue line) shows two main peaks at elution volumes of 19.5 ml and 20 ml. The tryptophan fluorescence emission spectrum of the collected peak eluting at 20 ml (right inset, dotted blue line) is red-shifted compared to that of the peak collected at 19.5 ml (left inset, dotted blue line), consistent with the 20 ml peak having a higher proportion of the misfolded LC than the peak at 19.5 ml, which represents LC that has refolded to the native state. To further scrutinize the hypothesis that native-like LCs are formed upon attempted refolding, refolded JTO-FL (3 μ M) was treated with 1 μ M proteinase K for 30 min at 25 °C, the reaction was quenched with PMSF, then JTO-FL was repurified by ion exchange chromatography. Protease treatment results in an elution profile where the peak at 20 ml is reduced in size (Figure 6, solid purple line). The tryptophan fluorescence emission spectrum of the collected peak at 20 ml (right inset, solid purple line) is indistinguishable from that of the collected

peak at 19.5 ml, consistent with the material in this fraction consisting of the tail of the 19.5 ml peak, and with the misfolded material having been removed by proteolysis. The characteristics of the peak at 20 ml are consistent with that of the misfolded LC fraction. This result is fully consistent with a population of LC that refolds to a native-like state that is protected from proteinase K proteolysis under these conditions.

Full-length LC misfolding may be due to *cis-trans* prolyl isomerization

The λ LC constant domain native state contains a *cis* tyrosine 146-proline 147 backbone amide bond (Figure 1, residue 140–141 in the Kabat numbering system) in the reverse turn between β -strands B and C, which forms part of the interface between the C- and V-domains. The Pro 147 residue is conserved in all LCs. The presence of this *cis* backbone amide bond affects the folding of two single-domain proteins: the isolated mouse κ LC C-domain⁴⁰ (C κ), which has 62% sequence identity with the human C κ domain and 40% sequence identity with the human C λ domain; and human β_2 -microglobulin⁴⁴ (β_2 m), which has 15% sequence identity with the human C λ domain. The equivalent amide bonds are Tyr 34-Pro 35 in C κ and His 31-Pro 32 in β_2 m. In the unfolded state of these proteins, the *trans*-amide bond conformation is favored, which delays folding and causes an intermediate species harboring a *trans*-amide bond, referred to as I_T, to accumulate.^{40, 44–47}

Accumulation of this intermediate state has been shown to promote aggregation of β_2 m.⁴⁴ Peptidyl-prolyl backbone amide bond isomerization typically occurs in the unstructured ensemble, wherein the half-time for isomerization is several seconds, but is restricted by steric constraints imposed by secondary and tertiary structure. We hypothesize that isomerization can be inhibited by the structure adopted in the misfolded LC conformational ensemble, reasoning that the constant domains within the full-length LCs may fractionally populate an ensemble containing the *trans*-amide bond in the unfolded state, and be trapped in an I_T-like misfolded state upon attempted refolding. Steric constraints imposed by the variable domain dimer interface and/or the inter-chain disulfide bond may considerably slow peptidyl-prolyl amide bond isomerization, retarding the rate at which the misfolded ensemble reforms the native state. Such a misfolded ensemble containing the *trans* Tyr 146-Pro 147 amide bond might also be able to aggregate efficiently, analogous to human β_2 m⁴⁴ and the isolated mouse C κ domain.⁴⁰ If isomerization of the peptidyl-prolyl amide bond to the *trans* form is involved in the LC misfolding process, the extent of misfolding would be expected to increase with time spent in the unfolded state. To test this hypothesis, we carried out refolding experiments wherein ALLC-FL and JTO-FL were unfolded by dilution into 6 M urea for different time periods, before the LCs were refolded by dilution from chaotrope with buffer (50 mM sodium phosphate, pH 7) to a final concentration of 0.6 M urea. After a refolding period of 16 h, tryptophan fluorescence emission spectra were recorded (Figure 7A). This experiment reveals that the red shift in the average wavelength of the emission spectrum increases as a function of time that the LC spent in 6 M urea. The misfolded state of both ALLC-FL and JTO-FL accumulates with very similar kinetics. The accumulation of the misfolded state shows an initial burst phase followed by a phase that can be described by a double-exponential model (Figure 7A) with rate constants of $\approx 10^{-4}$ and 10^{-3} s^{-1} .

Peptidyl prolyl isomerases (PPIases) are enzymes that catalyze peptidyl-prolyl amide bond isomerization. They can accelerate folding of proteins by reducing the energy barrier for

peptidyl-prolyl amide bond isomerization, thus PPIases can be added to determine whether proline isomerization is rate limiting in the folding kinetics of a protein. PPIases do not alter the relative populations of proline isomers, but rather accelerate their interconversion, and preferentially interact with unstructured polypeptide conformations. Refolding in the presence of the PPIase SlyD from *E. coli*⁴⁸ (which has no tryptophan residues and therefore does not contribute significantly to the LC's fluorescence signal) did not result in the recovery of a native-like fluorescence spectrum (Figure 7B). It may be that the misfolded state is collapsed enough to prevent access of SlyD to the peptidyl-prolyl amide bond in the LCs.

Mutation of proline 147 to alanine traps LCs in a misfolded ensemble

To further probe whether the misfolding of LCs is due to populating a non-native *trans* 146–147 peptidyl-prolyl amide bond conformation, and to investigate this species in the absence of aggregates induced by refolding, we mutated the proline at position 147 to alanine in both ALLC-FL and JTO-FL. A similar strategy has been used to trap the folding intermediates of the isolated mouse C κ domain⁴⁰ and human β_2m .^{44–45, 49} We expressed the proteins as inclusion bodies in *E. coli*, as for the other LCs studied above. Notably, refolding of both P147A variants from reduced, denatured states resulted in substantial aggregation of the P147A LCs. We were able to refold the LCs by dilution into 0.5 M arginine at pH 9, on ice, which has been shown to reduce the aggregation of other proteins.⁵⁰ We did not use ammonium sulfate precipitation in the purification process, because it led to the loss of misfolded LC states (Figure 2B). Subsequent purification by ion exchange chromatography and SEC resulted in the generation of pure, homogeneous P147A variants, which we refer to as “misfolded and purified” (Figure 8A). Analytical ultracentrifugation sedimentation velocity measurements of the P147A variants (Figure 8B) indicate that ALLC-FL P147A sediments as a monomer, whereas JTO-FL P147A appears to sediment as a mixture of monomer and dimer. SDS-PAGE under reducing and non-reducing conditions (Figure 8C) shows that the majority of the samples of the P147A LC variants migrates at a similar size to the C217S LC variants, indicating the absence of an inter-chain disulfide bond. This suggests that the conformation of the C-domains in these variants restricts the formation of the inter-chain disulfide bond during the refolding process. This interplay between interchain disulfide bond formation and the folding of the constant domain may be related to the slow rate of peptidyl-prolyl backbone amide bond isomerization in the LC misfolded state.

The fluorescence emission spectrum of misfolded and purified (*trans* 146–147 amide bond) JTO-FL P147A is red shifted compared to that of JTO-FL (Figure 8D), indicating solvent exposure of one or more tryptophan residues. The far-UV CD spectrum of misfolded and purified JTO-FL P147A shows reduced ellipticity in the β -sheet region compared to folded and purified JTO-FL (Figure 8E). Consistent with the lack of the inter-chain disulfide bond, refolded JTO-FL P147A reversibly regains its misfolded conformation, affording a similar fluorescence emission spectrum and CD spectrum to the misfolded and purified JTO-FL P147A LC. ALLC-FL P147A displays similar spectra (data not shown). Using stopped-flow kinetics to measure the unfolding rates of these variants, we observed that ALLC-FL P147A (Figure 8F) and JTO-FL P147A (Figure 8G) unfold with apparent single-exponential kinetics, with an unfolding rate and denaturant dependence (*m*-value, the slope of the plots

in Figures 8F and 8G) similar to that of their respective variable domains. Together, these data suggest that the C-domain of the P147A variants adopts a misfolded state. Kinetic data are shown in Table 1.

To further investigate the misfolded and purified state, we measured the kinetics of proteinase K digestion of ALLC-FL P147A and JTO-FL P147A. Both LCs are rapidly degraded by proteinase K with single-exponential kinetics (Figure 9A, filled circles), much faster than the endoproteolysis of their Pro 147-containing counterparts (Figure 9A, unfilled circles). These rates of degradation are fast enough to account for the burst phase seen in the proteolysis of the refolded LCs (Figure 5), suggesting that the burst phase arises from proteolysis of the misfolded dimeric LC ensemble.

The λ LC sequence at the N-terminus of the constant Ig domain, ¹¹³GQPKAA¹¹⁸, resembles the consensus cleavage sequence for the blood plasma protease thrombin, LVPRGS. We previously showed that mutation of the LC linker region to a thrombin consensus sequence resulted in LCs (referred to as ALLC-FL-Th and JTO-FL-Th) that could be cleaved by thrombin to yield amyloidogenic fragments.²² Insertion of the thrombin site destabilized both ALLC-FL and JTO-FL.²² Although thrombin is regarded as a highly specific protease, it has some promiscuity in its recognition sequence.⁵¹ The susceptibility of the P147A LC variants to proteinase K suggested that the linker region may be accessible to thrombin. We therefore tested whether thrombin could cleave the misfolded and purified P147A variants of ALLC-FL and JTO-FL. Treatment of both ALLC-FL P147A and JTO-FL P147A with thrombin resulted in endoproteolysis and thus production of LC fragments (Figure 9B). Therefore, we conclude that thrombin is able to cleave the λ LC linker region, likely due to facile conformational excursions from the misfolded state.

Thermal and chaotrope denaturation result in similar misfolding

Patient-derived and recombinant full-length LCs are established to misfold after thermal denaturation.^{25–26, 52} We therefore asked whether heating and cooling causes folded and purified recombinant LCs to fractionally misfold to a conformational ensemble similar to that formed following partial refolding from 6 M urea by dilution. We measured the apparent melting temperature and assessed folding reversibility by differential scanning calorimetry (DSC, Figure 10A and 10B). Initial heating of all folded and purified LC proteins resulted in an apparently cooperative melting transition. After cooling, each sample was heated again to assess whether reversible refolding had occurred. Both ALLC-FL and JTO-FL exhibited no thermal DSC transition during the second round of heating, suggesting that the proteins had aggregated substantially. In agreement with their reversible unfolding-refolding from urea, however, the V-domains, the full-length C217S variants and the full-length P147A variants of both ALLC and JTO had indistinguishable melting temperatures in both DSC rounds, although the amplitude of the unfolding transition was lower in each case for the second round of heating. Apparent aggregation in the DSC experiments may be due to extended incubation periods at temperatures above the melting temperature. We used a shorter heating protocol to further investigate refolding after thermal denaturation. We incubated JTO-FL at 65 °C for 120 s followed by attempted refolding at 25 °C for 1 h and analyzed the refolded LC by SEC and fluorescence spectroscopy. Chromatograms of JTO-FL following heat

treatment show a loss of the dimer peak and accumulation of larger species (Figure 10C). Identical treatment of JTO-FL C217S resulted in complete recovery of the dimer SEC peak (Figure 10C). The intrinsic tryptophan fluorescence spectrum of heated and cooled JTO-FL is red-shifted compared to the spectrum of folded and purified JTO-FL and shows evidence of light scattering, indicative of a misfolded and/or aggregated ensemble (Figure 10D). The increased fluorescence emission intensity compared to the spectra of LCs refolded from chaotrope (Figure 2) indicates a larger population of misfolded or aggregated LC relative to that produced by refolding from chaotrope dilution. The fluorescence spectra of JTO-FL C217S (Figure 10D) are similar before and after heating. Collectively, these data suggest that LC misfolding occurs upon attempted refolding after either heat or urea denaturation and that the presence of the inter-chain disulfide bond is required to trap the misfolded state.

Trapped misfolded LC variants are prone to amyloidogenesis

Protein variants of both mouse C κ and human β_2m with increased equilibrium populations of the *trans*-peptidyl-prolyl misfolded intermediates are more aggregation prone than their respective wild type sequences.^{40, 44–45, 53} We therefore asked whether the misfolded and purified P147A variants of ALLC-FL and JTO-FL would aggregate *in vitro*. We had previously shown that full-length LCs are very resistant to aggregation under conditions where their variable domains readily aggregate. However, cleavage between domains can generate amyloidogenic fragments, depending on the cleavage site. The fragments produced by endoproteolysis of ALLC-FL-Th by thrombin did not generate aggregates in PBS buffer at pH 7.4 after 24 h.²² We incubated 10 μ M ALLC and JTO variants with or without thrombin for 16 h in PBS, pH 7.4 at 37 °C, then initiated amyloidogenesis by shaking under the same conditions. Aggregation kinetics followed by thioflavin T fluorescence are shown in Figure 11A. Neither full-length LC aggregated over two weeks of shaking, whereas both V-domains did so, independently of the presence of thrombin. In contrast to our previous data, cleavage of ALLC-FL-Th by thrombin was sufficient to cause amyloidogenesis at pH 7.4, (Figure 11A), which we attribute to differences in the aggregation protocol. We employed shaking on plates herein versus shaking in microfuge tubes in previous experiments. We also increased the concentration of LC in the current experiments: 10 μ M here versus 5 μ M used previously.

The striking result is that both ALLC-FL P147A and JTO-FL P147A aggregated after several days of incubation, even though they comprise a C-domain which seems to inhibit full-length LC aggregation.²² Endoproteolysis of ALLC-FL P147A by thrombin resulted in a substantially reduced lag time for aggregation. Thrombin treatment resulted in a smaller reduction in lag time for JTO-FL P147A, which agrees with the reduced sensitivity to endoproteolysis of JTO-FL P147A compared to ALLC-FL P147A (Figure 9B).

Electron micrographs of the aggregates formed by ALLC-FL P147A and JTO-FL P147A at pH 7.4 in the absence of thrombin reveal that amyloid fibril-like structures are formed (Figure 11B). The aggregates formed after thrombin endoproteolysis have a distinct appearance, consisting of short, curvilinear structures (Figure 11C).

Discussion

Many amyloid disease-associated proteins transiently populate partially folded conformational states that aggregate. These states are generally depicted as having a higher free energy than the native state, but as being accessible from the native state at equilibrium.²⁹ Many such states have been characterized by altering solution conditions (e.g., temperature or pH) so that populations of the alternatively folded states are increased at equilibrium. We propose a distinct mechanism for LC aggregation herein. Following folding to the native state and secretion from plasma cells, full-length immunoglobulin LCs can unfold in the extracellular space. Upon spontaneous refolding, a fraction of the LC could form a misfolded structural ensemble in which the Tyr 146-Pro 147 amide bond adopts a *trans* conformation. We hypothesize that, outside the endoplasmic reticulum, in the absence of most proteostasis network components, including disulfide isomerases, such states could be kinetically trapped by the inter-chain disulfide bond as well as inter-domain interactions, and therefore be long-lived. Utilizing *in vitro* experiments, we have shown that these misfolded states can aggregate directly or be cleaved by proteases to generate fragments that aggregate (Figure 11).

This aberrant endoproteolysis could be due to locally unstructured regions in the misfolded ensemble and/or the more rapid unfolding of the misfolded state relative to the natively folded state. Since the rate at which the misfolded state accumulates is similar for ALLC-FL and JTO-FL (Figure 7), access to the misfolded state may be limited by unfolding rate in the absence of denaturant. Therefore, the kinetic stability of a particular natively folded LC will determine how often it has the opportunity to misfold or be aberrantly processed by proteases. This means that kinetically more stable light chains are less likely to populate the misfolded conformational ensemble, and are therefore less likely to be associated with amyloidosis, as we and others have observed.^{2, 22, 39} A kinetically-unstable LC dimer can therefore be cleaved into amyloidogenic fragments either via transient unfolding from the native state, or more readily after forming a longer lived misfolded conformational ensemble, as shown herein.

Refolding kinetics measured by fluorescence (Figure 2E) and protease sensitivity (Figure 4A) suggest that a population of misfolded LC is initially formed upon removal of denaturant by dilution to initiate refolding. This conformational ensemble then spontaneously converts to a more native-like ensemble over several hours (Figure 2E). Minimal aggregation (Figure 3) upon refolding indicates that the majority of the LC refolds to a dimer-like state. The misfolded state observed here requires that the inter-chain disulfide bond between cysteine residues at position 217 be intact to kinetically trap this conformational ensemble. Mutation of this cysteine residue to serine results in significant kinetic destabilization of LCs, leading to faster unfolding and endoproteolysis. Notably, in the absence of the inter-chain disulfide bond, the LCs appear to refold completely to their native state, suggesting that this disulfide bond, in combination with the inter-domain molecular interactions, restricts the motion of the C-domain, trapping the refolding intermediate. Several lines of evidence support the hypothesis that dimerization of LCs is protective against amyloidogenesis. Patient-derived LCs can be induced to form amyloid by addition of reducing agent,⁵⁴ and multiple studies suggest that dimerization of V-domains

reduces the rate of amyloid formation.^{55–57} The functional role of Cys 217 is to stabilize the LC's interface with the antibody heavy chain in mature antibodies. Even though the inter-chain disulfide bond may increase the risk of misfolding, overall, its stabilization of LC dimers may reduce the likelihood of amyloidosis.

The P147A variants recapitulate some aspects of the misfolded state. Mutation of proline 147 to alanine, whose dipeptide amide bond can only adopt the *trans* conformation, traps the LC in a misfolded ensemble, enabling aggregation. However, the inability of P147A LC to form the inter-chain disulfide bond means that this LC is an imperfect model of the misfolded state. The slow disappearance of the misfolded ensemble, due to conversion to the native state and possibly some aggregates, has thus far prevented us from characterizing its structure in atomic detail, e.g., by nuclear magnetic resonance. We therefore cannot definitively state that population of the *trans* peptidyl-proline amide bond is responsible for full-length LC misfolding; however, evidence from related proteins supports this hypothesis.

The refolding of both mouse C κ and human β_2m from denaturant is rate limited by isomerization of the native *cis* amide bond (Tyr 34-Pro 35 in C κ and His 31-Pro 32 in β_2m).^{40, 44} A compact, misfolded intermediate in which the peptidyl-proline amide bond is in the *trans* conformation (I_T) has been identified and characterized in both proteins.^{40, 44–45, 49} In both cases, the I_T state is only transiently populated by the wild-type protein. Population of an I_T -like state has been linked to β_2m amyloidosis (also known as A β_2M amyloidosis or dialysis-related amyloidosis¹). In the case of β_2m , deletion of 6 residues from the N-terminus, referred to as N6 β_2m , also traps the protein in an I_T -like state.⁵³ This isoform of β_2m has been observed in amyloid deposits from patients with A β_2M amyloidosis, but the protease responsible has not been identified.⁵⁸ The predicted site at which thrombin cleaves the P147A LCs, Lys¹¹⁶, aligns with Lys⁶ in β_2m . Cleavage of β_2m at this site would afford N6 β_2m , suggesting that thrombin may be responsible for cleavage of β_2m in patients. The structures of I_T -like states of β_2m have been solved.^{45–46, 49} The solution structure of N6 β_2m shows a rearrangement of the loops at one end of the β -sandwich, where the N-terminal residues would be. β_2m is a chaperone for the class I major histocompatibility complex (MHC I), and this face of the molecule interacts with the MHC I heavy chain.⁵⁹ Unlike the single-domain β_2m , the LC C-domain is linked at its N-terminus to the V-domain. Structural changes analogous to those between native and I_T β_2m would disrupt the linker peptide, possibly breaking the backbone hydrogen bond between Ala 117 and Tyr 146, and the interfaces between the C- and V-domains and between LC protomers, as we observed (Figures 8B and 8C). The misfolded LC conformational ensemble persists for much longer than the I_T states of C κ and β_2m , which may allow increased self-association and/or proteolysis.

The rate at which the misfolded state accumulates (Figure 7) is slower than would be expected from peptidyl-prolyl amide bond isomerization alone.⁴⁴ However, proline residues preceded by aromatic residues, such as the Tyr 146-Pro 147 amide bond in LCs, have a higher barrier to peptidyl-prolyl amide bond isomerization than other proline residues, which may account for some of the effect observed. It is also possible that residual structure in the unfolded state in 6 M urea—perhaps influenced by the presence of the disulfide bond between residues Cys 140 and Cys 199—restricts the conformational mobility of the Tyr

146-Pro 147 peptide bond. Alternatively, the proline 147 residues in both LC protomers may need to be in the *trans* form in order for the protein to misfold. The initial, rapid phase in Figure 7 is of a comparable rate to the isomerization of His 31-Pro 32 in β_2m . The slower phases are however compatible with other published rates of proline isomerization.⁶⁰ The rate of accumulation of the misfolded state is also faster than the typical rates observed for carbamoylation of proteins in urea,⁶¹ indicating that covalent modification of the LCs is unlikely.

Since free LCs are secreted in large quantities from clonally expanded plasma cells, the sheer mass of protein produced implies that even a small population of a misfolded LC ensemble could contribute to disease. Systemic AL amyloidosis is a slow, chronic disease characterized by overproduction of a precursor LC over many years, so relatively rare events may lead to accumulation of amyloid-competent species. Misfolded LC may be secreted by plasma cells, or its formation may result from incomplete extracellular refolding. Once formed, our data suggest that misfolded LC ensembles can persist long enough to aggregate or be proteolyzed, contributing to amyloidosis. Importantly, kinetically and/or thermodynamically unstable LCs will have a higher steady-state population of misfolded LC, which would promote the aggregation of these LCs and the progression of AL amyloidosis. This suggests that pharmacological stabilization of LC dimers could be a useful therapeutic strategy, as we²² and others^{9, 62–63} have previously suggested.

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Figure 1.

Antibody λ LCs used in this study. (A) Structural diagram of human λ LCs, from the structure of the λ LC CLE, PDB code 1LIL (reference ²¹). One chain of the homodimer is depicted as a surface representation, the other as a backbone ribbon with the V-domain shown in blue and the C-domain in red. Disulfide bonds are shown between cysteine residues as yellow spheres. Tryptophan residues and the native *cis*-proline residue (position 147) are shown in stick format. Numbering is based on the sequence of the 6aJL2 germline construct used.²² (B) Sequence alignment of the λ 6a LC constructs used in this study: ALLC-FL, JTO-FL and the germline sequence, 6aJL2-FL. Each has an N-terminal methionine residue for expression in *E. coli*. Residues conserved in all three sequences are highlighted in blue. The C-domain variants P147A and C217S are indicated at their positions in the C-domain sequence.

Figure 2.

Refolding of LCs to an alternative state. (A) Experimental workflow for purification of LCs from inclusion bodies, and refolding experiments. “Folded and purified” LCs were obtained by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatographic purification from inclusion bodies. Dilution of folded and purified LCs into 6 M urea results in “unfolded” LCs. Dilution to a final urea concentration of 0.6 M results in “refolded” LCs. (B) SDS-PAGE gel demonstrating purity of folded JTO-FL (positions of molecular weight markers (kDa) are shown) and intrinsic tryptophan fluorescence emission spectra of folded JTO-FL before

(dotted purple line) and after (solid red line) purification by a 25–75% $(\text{NH}_4)_2\text{SO}_4$ precipitation. Spectra are individually normalized for comparison. (C) Intrinsic tryptophan fluorescence emission spectra of initially folded and purified JTO-FL under different conditions. Spectra are normalized to the intensity of the unfolded state in 6 M urea. (D) Fluorescence spectra of folded and purified (solid black lines) and refolded (dotted blue lines) LCs in 0.6 M urea, 50 mM sodium phosphate, pH 7. Spectra are scaled to the intensity of the folded and purified spectrum. (E) Folded and purified JTO-FL was unfolded in 6 M urea for 1 h, then refolded by dilution to 0.6 M urea. Intrinsic tryptophan fluorescence spectra were recorded to follow the refolding process. Average wavelength is shown by hollow symbols with colors from dark blue to cyan corresponding to refolding time. A fit to a single exponential model with an apparent rate of 0.76 h^{-1} is shown in grey. The average wavelength of the folded and purified JTO-FL is shown by the red dotted line. Spectra, scaled to the intensity of the spectrum recorded immediately after initiation of refolding and colored from dark blue to cyan according to refolding time, are shown in the inset. The spectrum of folded and purified JTO-FL is shown in red. All fluorescence spectra were recorded at 25 °C using an excitation wavelength of 280 nm. (F) Far-UV CD spectra of folded and purified JTO-FL and JTO-FL C217S diluted into 0.12 M urea (solid black lines), and refolded from 6 M urea to 0.12 M urea (dotted blue lines) at 25 °C, pH 7. Spectra are normalized based on the fluorescence intensity of the unfolded state in 6 M urea, so are not converted to molar ellipticity. The noise below 205 nm in the refolded spectrum is due to absorbance of residual urea, as shown by the absorbance signal.

Figure 3.

Refolding of full-length, disulfide bonded LCs results in a mixture of species. (A) Size exclusion chromatograms, $n=3$, of folded and purified (solid black lines) and refolded (dotted blue lines) JTO-FL (top) and JTO-FL C217S (bottom). Unfolding and refolding were carried out at 25 °C to a final concentration of 0.6 M urea in 50 mM sodium phosphate, pH 7. Inset: Expanded view of the region of the chromatograms where the native dimer-like species and soluble aggregates elute from the column. (B) Size exclusion chromatograms, $n=3$, of folded and purified, and refolded JTO-FL quantified by fluorescence emission, $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 325$ (blue peak) and 355 nm (red peak). The ratio of integrated peak areas are shown at right. Solid symbols represent folded and purified LC and hollow symbols represent refolded LC. (C) JTO-FL was unfolded and refolded as above, then an aliquot was subjected to ultracentrifugation to remove aggregates larger than dimers. Fluorescence emission spectra, as in Figure 2, of folded and purified (solid black line), refolded (dotted blue line), and refolded then centrifuged JTO-FL (dashed purple line) are shown. Spectra were corrected for total protein concentration, which showed that 7% of the refolded JTO-FL was lost after centrifugation. Spectra are normalized to the intensity of the spectrum of refolded JTO-FL. (D) Dynamic light scattering of folded and purified JTO-FL and refolded JTO-FL, $n=3$. The mass fraction of the sample attributed to species of a given size is shown.

Figure 4.

Refolded LCs are susceptible to proteolysis. Folded and purified LCs were either incubated in 0.6 M urea, or unfolded and refolded, for 1 h or 24 h, then were subjected to 60 s of proteolysis with equimolar (5 μ M) proteinase K before quenching with PMSF, $n=3$. (A) SEC of folded and purified JTO-FL or refolded JTO-FL at 1 h or 24 h after initiation of refolding before (black lines) and after (dotted red lines) proteinase K treatment. (B) Quantitation of the apparent native LC peak (eluting at 1.5 ml) before (black circles) and after (red triangles) proteinase K treatment. Solid symbols represent folded and purified LCs and hollow symbols represent LCs that have been unfolded and refolded. Data are normalized to the average area of the non-proteinase K treated, folded and purified LCs. (C) Quantitation of the remaining apparent dimer SEC peak of folded and purified LCs versus LCs refolded for 1 h, before (black circles) or after (red triangles) proteinase K treatment.

Figure 5.

LCs refold to form at least two species with differing protease sensitivities. Folded and purified or refolded LCs after refolding for 1 h at 25 °C (final urea concentration 0.6 M) were incubated with 1% molar equivalent proteinase K at 37 °C in PBS. Reactions were quenched with PMSF at the indicated time points and the remaining LC was quantified by SDS-PAGE and densitometry. (A) Endoproteolysis kinetics of folded and purified (circles) and refolded (triangles) LCs. Example SDS-PAGE gels (n=3) are shown above the graphs; F = folded and purified, R = refolded. A fraction of the refolded LCs is degraded within the 5

min dead time of the experiment. To account for this, a double exponential equation (shown in B) was used to fit to the data. Fits are indicated by solid lines for folded and purified LCs and dashed lines for refolded LCs. (B) Model for differential endoproteolysis of native and misfolded LCs. N, native LC; M, misfolded LC; P, proteolyzed LC. (C) Slow phase rate constants derived from the double exponential fits in (A). Circles represent data for folded and purified LCs and triangles represent data for refolded LCs.

Figure 6.

Native-like LCs can be recovered from refolded material by proteolysis. Folded and purified JTO-FL was unfolded in 6 M urea for 2 h, then refolded by 10-fold dilution into 25 mM sodium phosphate, pH 7, at 25 °C. A fraction of the refolded protein (3 μ M) was subjected to pulse proteolysis by 1 mM proteinase K for 30 min at 25 °C and then quenched with PMSF. The refolded JTO-FL, not proteolyzed or after proteolysis, was then purified by ion exchange chromatography. Chromatograms of refolded JTO-FL not proteolyzed (dotted blue lines) and after proteolysis (solid purple lines) are shown. The linear NaCl gradient used to elute the LCs is indicated by arrows. Fluorescence spectra (in 25 mM Tris-Cl at 25 °C, pH 8, $\lambda_{\text{ex}} = 280$ nm) of collected fractions corresponding to the major (19.5 ml elution volume) and minor (20 ml elution volume, dotted blue peak) peaks are shown in the insets to the left and right, respectively. Spectra are individually normalized to correct for differences in protein concentration. Proteolysis preferentially depletes the minor peak, removing the misfolded LC, which has a red-shifted fluorescence emission spectrum, to leave the native-like LC.

Figure 7.

Misfolding seems to be linked to proline isomerization. (A) Folded and purified ALLC-FL (red) and JTO-FL (blue) were unfolded in 6 M urea, incubated at 25 °C for the time period indicated, then refolded by dilution to a final urea concentration of 0.6 M. The extent of misfolding was quantified by the average wavelength of the intrinsic tryptophan fluorescence emission spectrum ($\lambda_{\text{ex}} = 280 \text{ nm}$). Solid lines indicate fits to a double exponential function, excluding the first point. The calculated rates are similar between the two LCs. (B) Refolding of JTO-FL from 6 M urea in the presence or absence of the PPIase SlyD results in states with indistinguishable tryptophan fluorescence emission spectra, indicating that SlyD does not alter the endpoint conformation of the LC. The intensity of each spectrum is shown relative to that of folded and purified JTO-FL. The fluorescence emission spectrum of SlyD (gray dashed line) was subtracted from that of JTO-FL refolded in the presence of SlyD.

Figure 8.

Mutation of proline 147 to alanine recapitulates features of the misfolded state. (A) Experimental workflow for purification of P147A LCs from inclusion bodies, and refolding experiments. “Misfolded and purified” P147A LCs were obtained by chromatographic purification from inclusion bodies. Dilution of folded and purified LCs into 6 M urea results in “unfolded” LCs. Dilution to a final urea concentration of 0.6 M results in “refolded” LCs. (B) Analytical ultracentrifugation (n=2) of LC variants (14 μ M) at 20 $^{\circ}$ C in PBS. Sedimentation velocity analysis of ALLC-FL P147A (red) indicates a monomeric species in

solution. Sedimentation velocity analysis of JTO-FL P147A (blue) is consistent with an equilibrium between monomer and dimer. Data for ALLC-FL²² is shown in gray for comparison. (C) SDS-PAGE gel of LCs run in the presence (left) or absence (right) of dithiothreitol (DTT). (D) Intrinsic tryptophan emission spectra of misfolded and purified (*trans* 146–147 amide bond) JTO-FL P147A (solid purple line), unfolded JTO-FL P147A (dotted red line) and refolded JTO-FL P147A (dashed blue line). Equivalent spectra for JTO-FL (from Figure 2C) are shown in gray. Data are normalized to the intensity of the unfolded state to correct for differences in concentration between the samples. (E) Far-UV CD spectra of misfolded and purified (solid purple line) and refolded (dashed blue line) JTO-FL P147A. The spectrum of folded and purified recombinant JTO-FL (solid gray line) from Figure 2F is shown for comparison. (F) Unfolding rates of ALLC-FL P147A (purple diamonds), extracted from fits of stopped flow unfolding data to a single exponential equation. Data for wild-type ALLC-FL (gray circles) and ALLC-V (gray triangles) are shown for comparison²². (G) Unfolding rates of JTO-FL P147A (purple diamonds), extracted from fits of stopped flow unfolding data to a single exponential equation. Data for wild-type JTO-FL (gray circles) and JTO-V (gray triangles) are shown for comparison.²² Fluorescence and CD data were recorded at 25 °C in 50 mM sodium phosphate, pH 7.

Figure 9.

Proteolysis of misfolded and purified P147A LCs by proteinase K and thrombin. (A) LCs (5 μ M) were digested with 50 nM proteinase K at 37 °C in PBS then quenched with PMSF. Residual LC was quantified by SDS-PAGE. Example gels (n=3), quantitation and fits to a single exponential model are shown. (B) LC variants were treated with 1% mole fraction thrombin (1 U in 20 μ l) for 16 h at 37 °C in PBS then quenched with PMSF. Residual LC was quantified by SDS-PAGE. An example gel (n=3) is shown. Positions of molecular weight markers (kDa) are indicated.

Figure 10.

Thermal denaturation and refolding leads to misfolding of LCs. (A) Differential scanning calorimetry (DSC) of JTO variants. Colors indicate LC variants. Solid lines indicate the first round of heating; dashed lines indicate the second round of heating. Representative traces (n=2) are shown. Similar data (n=2) were observed for ALLC variants (not shown). (B) Melting temperatures calculated from DSC data. Circles indicate the first round of heating and diamonds indicate the second round of heating (n=2). No transition was observed for folded and purified ALLC-FL or JTO-FL upon the second round of heating. (C) LCs were incubated at either 25 °C or 65 °C for 2 min, then at 25 °C for 60 min to refold. Residual soluble protein was quantified by SEC. (D) The intrinsic fluorescence spectra ($\lambda_{\text{ex}} = 280$ nm, 25 °C) of the samples from (C). Black lines indicate LCs incubated at 25 °C, dotted blue lines indicate LCs incubated at 65 °C for 2 min. Spectra are normalized to the maximum intensity of the folded and purified spectrum. The similarity between the spectra of folded and purified JTO-FL C217S and thermally unfolded and refolded JTO-FL C217S indicates that minimal concentration changes occurred due to evaporation of H₂O and that no protein was lost due to aggregation.

Figure 11.

Aggregation of misfolded and purified P147A LCs is accelerated by thrombin endoproteolysis. (A) Aggregation kinetics of LC variants measured by fluorescence of Thioflavin-T (ThT). LCs (10 μ M) were incubated at 37 $^{\circ}$ C overnight in 96-well plates in the absence (black lines) or presence (dashed red lines) of thrombin, after which aggregation was initiated by shaking at 1000 rpm, 37 $^{\circ}$ C on a plate shaker. ThT fluorescence (λ_{ex} = 440 nm, λ_{em} = 480 nm) was measured daily after the first 12 h. (B) Electron micrographs of fibrillary aggregates formed by ALLC-FL P147A and JTO-FL P147A without thrombin

digestion. (C) Electron micrographs of fibrillary aggregates formed by ALLC-FL P147A and JTO-FL P147A after overnight thrombin digestion. Scale bars represent 100 nm.

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Table 1

Unfolding rates and denaturant dependencies for P147A LC variants. k_{uH_2O} , extrapolated unfolding rate in the absence of urea; m_u , urea dependence of the unfolding rate (m-value); t_{50} , half-life of the folded state, $1/k_{uH_2O}$. Errors indicate goodness-of-fit.

Light chain	k_{uH_2O} ($\times 10^{-3} \text{ s}^{-1}$)	m_u ($\text{kJ mol}^{-1} \text{ M}^{-1}$)	t_{50} (s)
ALLC-FL P147A	21.6 ± 0.81	1.88 ± 0.016	46.4
JTO-FL P147A	22.1 ± 0.57	1.45 ± 0.011	45.2