Site-directed Mutagenesis Reveals Regions Implicated in the Stability and Fiber Formation of Human λ3r Light Chains*

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Background: λ6a and λ3r are the most implicated germ lines in light chain amyloidosis.

Results: Mutagenesis at N-terminal, loop 40–60, and CDR3 regions affected λ3r fibrillogenesis.

Conclusion: Changes at residues 7, 48, and 91 increased fibril formation while changes at residues 8 and 40 reverted fibrillogenesis.

Significance: Characterization of light chain germ lines helps to identify key regions implicated in amyloidosis.

Light chain amyloidosis (AL) is a disease that affects vital organs by the fibrillar aggregation of monoclonal light chains. λ3r germ line is significantly implicated in this disease. In this work, we contrasted the thermodynamic stability and aggregation propensity of 3mJL2 (nonamyloidogenic) and 3rJL2 (amyloidogenic) λ3r germ lines. Because of an inherent limitation (extremely low expression), Cys at position 34 of the 3r germ line was replaced by Tyr reaching a good expression yield. A second substitution (W91A) was introduced in 3r to obtain a better template to incorporate additional mutations. Although the single mutant (C34Y) was not fibrillogenic, the second mutation located at CDR3 (W91A) induced fibrillogenesis. We propose, for the first time, that CDR3 (position 91) affects the stability and fiber formation of human λ3r light chains. Using the double mutant (3rJL2/YA) as template, other variants were constructed to evaluate the importance of those substitutions into the stability and aggregation propensity of λ3 light chains. A change in position 7 (P7D) boosted 3rJL2/YA fibrillogenic properties. Modification of position 48 (I48M) partially reverted 3rJL2/YA fibril formation. Finally, changes at positions 8 (P8S) or 40 (P40S) completely reverted fibril formation. These results confirm the influential roles of N-terminal region (positions 7 and 8) and the loop 40–60 (positions 40 and 48) on AL. X-ray crystallography revealed that the three-dimensional topology of the single and double λ3r mutants was not significantly altered. This mutagenic approach helped to identify key regions implicated in A3 AL.

Amyloidosis refers to a group of diseases caused by the extracellular deposition of misfolded proteins as insoluble fibrillar aggregates, which show a periodic and ordered cross-β-spine structure (1–4). Light chain amyloidosis (AL)6 is a systemic and progressive disease caused by the deposition of large amounts of misfolded light chains. Approximately 70% of patients in an advanced phase of AL suffer from damage to tissues or organ function, mainly the kidney, heart, liver, lungs, and peripheral nervous system (5, 6). Light chains involved in amyloid fibril formation are of monoclonal origin, derived from a clone of plasma B cells that possess alterations in their regulation of Ig expression, resulting in an overproduction of its cognate light chain (3, 7). Each light chain consists of a variable (V) and a constant domain (C). Each of these domains is characterized by the topological organization of nine β-strands (A, B, C, C’, C”, D, E, F, and G) as a Greek key motif. Similar to other proteins rich in β-structures, V and C domains contain several anti-aggregation motifs (8). When these protective motifs are modified by mutations or environmental conditions, the β-edge strands of the light chain domains could potentially interact with β-edge strands from another monomer.

Because of the intrinsic diversity of the involved precursor proteins, it is difficult to understand the cause and mechanism of AL. Although a few germ line gene families have been implicated in the disease, hundreds of different sequences have been isolated from AL patients, each of which originates from a combinatorial arrangement of germ line gene segments that undergo a particular pattern of somatic mutations. The changes introduced into the V and somatic mutations can affect its stability and prevent normal association with its correspond-

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6 The abbreviations used are: AL, light chain amyloidosis; ThT, thioflavin T.
ing V_{14}, allowing its free secretion (9, 10). The loss of the Igs heterotetrameric structure may contribute to the amyloidogenicity of light chain variable domains because the fibrils of most AL patients predominantly comprise a single variable domain (11).

The comparison of amyloidogenic and nonamyloidogenic V_{L} sequences has allowed other researchers to identify certain amino acid changes that destabilize the V_{L} domain (12–16). The introduction of some of these mutations into nonamyloidogenic V_{L} domains decreases their stability and increases their tendency to aggregate and form amyloid fibers in vivo (17). Several studies have suggested that a less stable V_{L} domain is more likely to aggregate into amyloid fibers (17, 18).

The λ light chains are responsible for the majority of AL cases, with a 3:1 ratio over the κ isotype (19, 20). Several gene segments belonging to the λ3 and λ6 subgroups, particularly the 3r and 6a germ lines (6, 21), are significantly associated with AL. Despite the predominance of the λ isotype in the disease, only one λ germ line has been characterized for its propensity to form amyloids. Our group previously reported the characterization of the recombinant λ germ line 6aJL2, a protein encoded by the 6a and the j2 gene segments (22). 6aJL2 is thermodynamically more stable than other λ6 light chains derived from patients with multiple myeloma, although it is capable of forming fibers in vitro after long periods of incubation (22).

The λ3 light chain family comprises 21 genes, nine of which are polyclonal; only six of these nine genes have been associated with AL (23). Although several λ3 amyloidogenic light chains isolated from patients have been analyzed (6, 24–26), the role of germ line-encoded features (protein regions) in the amyloidogenic capability of the protein has not been determined.

Because the λ3r subfamily might be intrinsically amyloidogenic, the aim of this work was to characterize the structural and biophysical properties of two germ lines of the λ3 subgroup. We are interested in the 3r subgroup because of its high prevalence in AL, whereas the 3m germ line, which has not been associated with AL, was used as a control (6). Each 3m and 3r gene segment was joined to the j2 segment to obtain the whole variable domains 3mJL2 and 3rJL2. Because of an inherent limitation (extremely low expression), Cys at position 34 of the 3r germ line was replaced by Tyr reaching a good expression yield. A second substitution at CDR3 (W91A) was introduced in 3r to obtain a better template to incorporate additional mutations. Taking the double mutant (3rJL2/YA) as template, other variants were constructed to evaluate the importance of those substitutions on the stability and aggregation propensity of λ3 light chains. Mutations were introduced into two regions thought to protect against light chain fibril formation: the switch joint (positions 7 and 8) (13) and the loop 40–60 region (positions 40 and 48) (14). X-ray crystallography revealed that the three-dimensional topology of the single and double λ3r mutants was not significantly different. The majority of λ3 variants evaluated in this study showed higher stability compared with other germ lines associated with AL, such as 6a6 and κO18/O8 (22, 27, 28). This mutagenic approach helped to confirm or identify other key regions implicated in λ3 AL.

### Materials and Methods

**Cloning, Expression, Extraction, and Purification**—Germ line sequences were obtained from the VBASE database. 3rJL2 and 3mJL2 contained the sequences of the corresponding human VA 3r and 3m germ lines. Both proteins contained the j2 segment. The j2 segment was used because it is frequently present in clonal plasma cells (6) 3r and 3m were synthesized by recursive PCR as described by Prodromou and Pearl (29). The DNA was cloned into the pET22b vector (Novagen, Darmstadt, Germany). Site-specific mutations in 3rJL2 were generated using a mutagenic mega-primer (30). All constructs were verified by nucleotide sequencing.

Vectors containing the light chain recombinant proteins were transformed into the Escherichia coli strain BL21(DE3). The cells were grown in 2XYT medium containing 100 μg/ml ampicillin at 37 °C and 150 rpm. When the culture reached an A_{600} of 1–1.4, protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.2 mM. The cultures were grown for 5 h under low agitation (115 rpm) at 22 °C.

The periplasmic proteins were extracted as described previously (22). The periplasmic extracts were precipitated twice with 30–90% saturated ammonium sulfate and stored at 4 °C. The precipitated proteins were removed by centrifugation (6000 rpm for 30 min), and the pellet was resuspended and dialyzed overnight in 50 mM NaCl and 40 mM Tris, pH 8.2 (buffer A). The proteins were separated by size exclusion chromatography using a Sephacryl S-100 HR column (Amersham Biosciences) connected to an Äkta FPLC system (GE Healthcare). The column was previously equilibrated with buffer A at a flow rate of 2 ml/min. The peak corresponding to the light chains was collected and precipitated with 70% saturated ammonium sulfate.

The precipitated protein was harvested by centrifugation (6000 rpm for 30 min), and the pellet was resuspended in buffer A. The soluble protein was extensively dialyzed at 4 °C in buffer A while gradually decreasing the NaCl concentration. The final dialysis was performed in 40 mM Tris, pH 8.2. The dialyzed protein was applied onto an anion exchange chromatography column (Bioscale mini macro prep High Q; Bio-Rad) that was precooled to 4 °C in buffer A. The proteins were separated by size exclusion chromatography using a Superdex 75 prep grade 10/30 prepacked column (Amersham Biosciences) that was precooled to 4 °C in buffer A. The proteins were separated by size exclusion chromatography using a Superdex 75 prep grade 10/30 prepacked column (Amersham Biosciences) that was precooled to 4 °C in buffer A. The fractions containing the light chain proteins were pooled and stored at 4 °C until needed. The purity of the protein was verified by SDS-PAGE. The protein concentration was determined by UV absorption using the extinction coefficient value calculated with the ProtParam Tool in the ExPASy Web site.

**Analytical Size Exclusion Chromatography**—The oligomeric state of the λ light chain was determined using a Superdex 75 (10/30) prepacked column (Amersham Biosciences). The column was previously equilibrated with buffer A. The molecular masses were calculated using linear interpolation from a calibration curve that included serum albumin (66.3 kDa), ovalbumin (43.5 kDa), carbonic anhydrase (28.8 kDa), myoglobin (17 kDa), and cytochrome c (11.7 kDa) as molecular mass markers.
**Unfolding Experiments: Data Analysis**—The changes in tryptophan fluorescence were analyzed after normalization of the transition curves to the apparent fraction of unfolded molecules, $F_{\text{app}}$:

$$F_{\text{app}} = \frac{(Y_{\text{obs}} - Y_i)}{(Y_u - Y_i)} \quad (\text{Eq. 1})$$

where $Y_{\text{obs}}$ is the observed fluorescence intensity at a given temperature or guanidine HCl concentration, and $Y_i$ and $Y_u$ are the fluorescence signals for the native and unfolded forms, respectively. A linear dependence of $Y_{\text{obs}}$ was observed in both the native and unfolded baseline regions; therefore, linear extrapolations from these baselines were made to obtain estimates of $Y_i$ and $Y_u$ in the transition region.

**Guanidine HCl Denaturation Experiments**—The intrinsic fluorescence emission measurements were recorded on an LS50B spectrofluorimeter (PerkinElmer Life Sciences) using a 1-cm-path length quartz cuvette. Protein samples were prepared at concentrations of 20, 50, and 200 $\mu$g/ml in 20 mM sodium phosphate buffer, pH 7.5. Different concentrations of guanidine HCl from an 8 M stock solution were used to unfold the protein; the samples were then incubated for 12 h at 25 °C (equilibrium conditions). Samples incubated for 6 or 12 h showed similar results. The fluorescence emission was collected at 355 nm using an excitation wavelength of 295 nm. Thermodynamic parameters were calculated assuming the unfolding reaction followed a two-state model in which the native monomers (N) are in equilibrium with the unfolded monomers (U),

$$N \leftrightarrow U \quad (\text{Eq. 2})$$

where $K_{\text{app}} = F_{\text{app}}/(1 - F_{\text{app}})$. A linear dependence of $\Delta G^\circ$ on the concentration of guanidine HCl (GndHCl) was assumed (30),

$$\Delta G^\circ = \Delta G_{\text{HDO}} - m[\text{GndHCl}] \quad (\text{Eq. 3})$$

where $\Delta G^\circ$ represents the Gibbs free energy at a given concentration of guanidine HCl, $\Delta G_{\text{HDO}}$ represents the Gibbs free energy in the absence of guanidine HCl, $m$ is the gradient of the linear transition region and reflects the cooperativity of the process, and $C_m$ is the denaturant concentration at which $\Delta G^\circ = 0$ and was calculated as $C_m = -m/m$.

**Thermal Unfolding**—The samples were solubilized at 50 $\mu$g/ml in 3 ml of PBS solution and then placed into quartz cuvettes. A micro-stir bar was used at a low speed to maintain a uniform solution temperature. The fluorescence intensity was measured as described for the chemical denaturation experiments. The protein solution was heated using a water bath with a recirculation system connected to the cuvette holder of the spectrofluorimeter (LS50B: PerkinElmer Life Sciences). The temperature of the solution was measured using a thermistor thermometer. The thermal unfolding data were analyzed as described by Pace et al. (31) assuming a two-state process and the van’t Hoff equation,

$$\frac{d(\ln K_{\text{app}})}{d(1/T)} = -\frac{\Delta H_{\text{app}}}{R} \quad (\text{Eq. 4})$$

where $T$ is the temperature, $\Delta H$ is the enthalpy, and $R$ is the gas constant.

The value of $\Delta G$ at room temperature was determined using the following equation,

$$\Delta G(T) = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_p \left(T_m - T\right) + T \ln \left(\frac{T}{T_m}\right) \quad (\text{Eq. 5})$$

where $T$ is 298 K, and $C_p$ is the change in heat capacity associated with unfolding, calculated theoretically according to the method described by Milardi et al. (32).

**In Vitro Fibril Formation**—Protein samples at a concentration of 100 $\mu$g/ml were filtered through a 0.22-μm pore-size Millex GV membrane filter (Millipore) and poured into a polystyrene cuvette in PBS. The cuvette was incubated at 37 °C under agitation with a magnetic stir bar. Fiber formation was monitored at different time points by following the incorporation of thioflavin T (ThT) (Sigma Aldrich) (33). The fluorescence emission was recorded at 482 nm using an excitation wavelength of 340 nm. The fibril formation kinetics was analyzed by fitting the time-dependent changes in the ThT fluorescence intensity to the following equation,

$$F_{\text{ThT}} = A/(1 + \exp[-B(t - t_l)]) \quad (\text{Eq. 6})$$

where $F_{\text{ThT}}$ is the ThT fluorescence intensity, $A$ is the ThT fluorescence intensity in the post-transition plateau, $t_l$ is the midpoint of the transition region, $B$ is the fibril growth rate constant, and $t$ is time. An absolute value of the nucleation lag time, $t_{\text{lag}}$, was calculated by extrapolating the linear region of the hyperbolic phase back to the abscissa (34).

**Circular Dichroism Experiments**—CD spectra and thermal denaturation experiments were recorded on a JASCO J-715 spectropolarimeter (JASCO Inc., Easton, MD) equipped with a water-cooled peltier. Far-UV CD spectra were recorded using a 0.2-cm-path length quartz cuvette at a concentration of 200 $\mu$g/ml protein in 20 mM Na$_2$HPO$_4$, pH 7.5.

The raw data were converted to molar ellipticity using the formula,

$$[\theta] = 100\times\theta/(C*l) \quad (\text{Eq. 7})$$

where $C$ is molar concentration, and $l$ is the cell path length in cm.

**Transmission Electron Microscopy**—A Formvar/carbon-coated copper G200 grid (Electron Microscopy Sciences, Hatfield, PA) was floated onto a 20-μl drop of protein sample for 2 min. The excess liquid was drained off with filter paper, and the specimen was negatively stained by floating onto a 4% (w/v) uranyl acetate for 2 min and blotted dry. The specimens were analyzed at 80 kV on a Zeiss EM900 Transmission electron microscope. The images were recorded with a CCD DualVision 300W camera (Gatan, Pleasanton, CA) at a resolution of 1030 × 1300 pixels. The image processing was performed using Adobe Photoshop version 7.0.
**Biophysical Characterization of λ3 Germ Lines**

**TABLE 1**

| Data set            | 3mJL2    | 3rJL2/Y  | 3rJL2/YA |
|---------------------|----------|----------|----------|
| Space group         | C 1 2 1  | P3,      | P3,      |
| Unit cell parameters (a, b, c (Å); α, β, γ (°)) | 89.4, 40.9, 106.7; 90, 90, 105, 90 | 106/4    | 106/4    |
| Residues per monomer/monomers per AU | 106/4    | 106/4    | 106/4    |
| Matthews coefficient (Å² Da⁻¹)/solvent content (%) | 2.1/41.3 | 1.9/33.9 | 1.8/33.6 |
| X-ray source        | NSLS, X6A| NSLS, X6A| NSLS, X6A|
| Detector            | ADSC Q270 CCD | ADSC Q270 CCD | ADSC Q270 CCD |
| Wavelength          | 0.9350   | 0.9720   | 0.9720   |
| Resolution range (Å) | 3.40–1.75 | 24.86–1.80 | 27.63–1.70 |
| Unicoherence (%)     | 31,323   | 31,131   | 36,831   |
| Multiplicity         | 6.8 (2.2) | 5.7 (5.7) | 5.7 (5.6) |
| Completeness (%)     | 79.4 (74.3) | 100 (100) | 99.9 (99.7) |
| Rmerge (%)           | 7 (37)   | 6 (32)   | 6 (43)   |
| Wilson plot B value (Å²)/mean B value from coordinates | 17.2/22.50 | 16.8/21.1 | 17.4/21.1 |
| Protein Data Bank code | 4AIZ      | 4AIX     | 4AJ0     |

**RESULTS**

3mJL2 and 3rJL2/YA as Nonamyloidogenic and Amyloidogenic λ3 Variable Domains—3mJL2 was expressed and purified efficiently. In contrast, 3rJL2 was scarcely expressed. λ3 germ line sequences were analyzed to identify single substitutions that would allow improve protein expression (Fig. 1). We found that 3r is the only germ line with a cysteine residue at position 34 (according to Kabat numbering), a solvent-exposed position at the N terminus of strand C. Although both amyloidogenic and nonamyloidogenic 3r-derived sequences bear a Cys in this position, other λ germ lines (such as 3m) contain Tyr or another polar residue (Fig. 1). These data suggested that Cys-34 should not substantially modify the fibrillogenic properties of the proteins derived from the 3r sub-family. Because the presence of a solvent-exposed cysteine residue may affect 3rJL2 expression, we constructed 3rJL2/Y, a mutant in which Cys-34 was mutated to tyrosine (3rJL2/C34Y), the equivalent residue found in the 3m germ line (Fig. 1). The expression, purification, and yield of 3rJL2/Y were comparable with those of 3mJL2. We assessed the thermodynamic stability of 3mJL2 and 3rJL2/Y by thermal and chemical unfolding experiments. Based on the tryptophan fluorescence measurements, monophasic and reversible transitions were observed; therefore, as a first approximation, we analyzed the data assuming a two-state process (Fig. 2). The unfolding kinetics of 3rJL2/Y and 3mJL2 were very similar, as deduced from the comparison of the midpoints of chemical or thermal unfolding (Cₘ and Tₘ, respectively; Table 2) and the values for the cooperativity of the unfolding process (m and ΔHᵥᵤ, respectively; Table 2). The ΔG values showed that 3rJL2/Y and 3mJL2 were more stable than 6aJL2 and xI018/08, the only amyloid germ lines previously evaluated (22, 27, 28). We found that 3rJL2/Y had refolding transitions that were not superimposable with the unfolding transitions (Fig. 2B). These results may reflect the presence of an irreversible refolding process.

**Crystallization**—Prior to the crystallization trials, the 3mJL2, 3rJL2/Y, and 3rJL2/YA proteins were maintained in 40 mM Tris, pH 8.2, containing 20 mM NaCl. Crystal screens 1 and 2 from the Molecular Biology Core (Lugano, Switzerland) were used to screen 10 different conditions. The 3mJL2, 3rJL2/Y, and 3rJL2/YA proteins were concentrated to 30% MPD; 3rJL2/Y, 2.3M ammonium sulfate and 40% trehalose. Because all of these conditions are suitable for cryocooling, crystals of 3rJL2/Y and 3rJL2/YA appeared using condition 5 from the micro-batch method under paraffin oil. Micro-crystals were obtained under several conditions, as follows: 3mJL2 crystals with the best shape appeared using condition 5 from Crystal Screen 1 (Hampton Research, Aliso Viejo, CA) containing 0.2M sodium citrate, 0.1M HEPES, pH 7.5, and 30% MPD, and crystals of 3rJL2/Y and 3rJL2/YA appeared using condition 32 from Crystal Screen 1 (Hampton Research) containing 2M ammonium sulfate. Upon optimization, 0.30×0.30×0.30-mm crystals were generated with the hanging drop method at 291 K after 15 days for each of the three proteins under the following conditions: 3mJL2, 0.2M sodium citrate, 0.1M HEPES, pH 7.5, and 40% MPD; 3rJL2/Y, 2.3M ammonium sulfate and 40% trehalose; and 3rJL2/YA, 1.6M ammonium sulfate and 40% trehalose. Because all of these conditions are suitable for cryocooling, the crystals were mounted in rayon cryoloops and flash-cooled in a 100 K nitrogen stream.

**X-ray Data Collection**—The data collection was performed at National Synchrotron Light Source Beamline X6A at two x-ray wavelengths: 0.9720 Å (for both 3r mutant crystals) and 0.9330 Å (for 3mJL2), using an Oxford Cryosystems 700 series Cryostream and an ADSC Q270 CCD detector (ADSC, Poway, CA).

**Data Processing and Model Refinement**—Indexing and integration were performed using MOSFLM for 3rJL2/Y (35) and XDS (36) for 3mJL2 and 3rJL2/YA. The integrated reflections were sorted, scaled, and truncated with SORTMTZ, SCALA, and TRUNCATE (37) from the CCP4 suite, respectively. Molecular replacement was carried out in PHASER (38) using the edited Protein Data Bank code 1LIL atomic coordinates, corresponding to the λ3 immunoglobulin Cle, as the starting model (24). The resulting model for each protein was subjected to rigid body refinement followed by restrained refinement in REFMAC5 (39). Once convergence was reached, the refinement was continued in PHENIX 1.5 (40). The refinement was alternated with manual building in COOT (41). The refinement cycles ended when Rwork and Rfree values were lower than 0.18 and 0.22, respectively. Validation of the final models was performed using PROCHECK (42). The coordinates and structural factors of the final models were deposited in the Protein Data Bank (43) with the following codes: 4AI2 (3mJL2), 4AIX (3r JL2/Y), and 4AJ0 (3rJL2/YA). The crystallographic data and refinement statistics for the three structures are shown in Table 1.
and/or photo-physical damage of a Trp residue. Fluorescence spectra of samples kept in the dark or illuminated during the thermal melt were very similar, indicating that photo-oxidation is not responsible for these differences. Size exclusion chromatography and electron microscopy analyses were performed to know whether some type of aggregation would explain irreversibility. No detectable aggregates were found using both alternatives (data not shown). The far UV CD spectra of 3rJL2/Y before

FIGURE 1. Sequence alignment of λ3 germ lines, λ3 protein obtained from patients and healthy individuals, and 6aJL2 germ line. The amino acid residues are colored by relative conservation using the ALIGN program (54). The dots indicate gaps inserted to maximize the alignment. The top group shows the λ3 germ line sequences, including the 6aJL2 germ line sequence. Green, slate, and purple triangles above the alignment mark the sites that were mutated in 3rJL2, 3rJL2/Y, and 3rJL2/YA, respectively. Black rectangles above the alignment mark the potential aggregation regions predicted by TANGO, PASTA, and Aggrescan servers. Framework (FR), complementarity determining regions (CDR), and joining (JL2) segments are indicated below the alignment with pink, indigo, and gray stripes, respectively. The secondary structure elements of 3rJL2/Y are marked below the alignment. The middle and lower groups comprise the λ3 sequences derived from patients with AL amyloidosis and healthy individuals, respectively. The germ line sequences were obtained from the VBASE database, and the λ3 sequences derived from patients and healthy individuals were obtained from the National Center for Biotechnology Information server.
and after the thermal melt are not the same (Fig. 3), suggesting that some sort of irreversibility not linked to aggregation is present in this protein.

All of the recombinant λ chain proteins previously analyzed have shown reversible temperature-induced transitions. In addition to the buried Trp-35 in the hydrophobic core of all λ light chains, 3rJL2 contains a second tryptophan residue at position 91, which is also present in most λ3 light chain sequences (Fig. 1). To simplify the interpretation of unfolding transition reversibility followed by fluorescence, Trp-91 was

FIGURE 2. Denaturation curves of λ3 proteins. A and C, fraction of unfolded protein as a function of guanidine HCl concentration. The solid lines represent a two-state fit of the data from 0 to 5 M of the denaturant. B and D, temperature-induced unfolding. The inset in B shows the fluorescence spectra of 3rJL2/Y and 3rJL2/YA. The filled symbols indicate denaturation, and the open symbols indicate renaturation. The 6aJL2 protein is shown for comparison purposes. The calculated thermodynamic parameters are shown in Table 2.

### TABLE 2

**Thermodynamic and fibrillogenesis parameters of λ3 proteins**

The error shown is the standard deviations from three independent experiments. NA, not applicable, i.e. fibril formation was not observed after 70 h.

| Parameter | 3mJL2 | 3rJL2/Y (CDR1) | 3rJL2/YA (CDR1/CDR3) | 3rJL2/YA/P7D (FR1) | 3rJL2/YA/P8S (FR1) | 3rJL2/YA/P40S (FR2) | 3rJL2/YA/I48M (FR2) | 6aJL2 |
|-----------|-------|----------------|----------------------|-------------------|-------------------|---------------------|---------------------|-------|
| \( C_m \) (m) | 1.6 ± 0.1 | 1.8 ± 0 | 1.4 ± 0.1 | 0.9 ± 0 | 1.3 ± 0 | 1.3 ± 0 | 1 ± 0 | 1.6 ± 0 |
| \( m \) | -12 ± 1.2 | -13 ± 1.3 | -16 ± 1.0 | -17 ± 1.8 | -14 ± 1.2 | -17 ± 1.5 | -21 ± 1.2 | -14 ± 1.2 |
| \( T_m \) (°C) | 60 ± 0.8 | 63 ± 0.1 | 56 ± 0.5 | 50 ± 0.2 | 54 ± 1.4 | 55 ± 0.6 | 51 ± 0.7 | 49 ± 0.5 |
| \( \Delta G_m \) (KJ/mol) | 33 ± 2 | 37 ± 2.9 | 31.3 ± 0.2 | 22.1 ± 0.2 | 28 ± 2.4 | 28.4 ± 1.5 | 25.5 ± 1.1 | 27 ± 1.3 |
| \( \Delta H_m \) (KJ/mol) | 317 ± 2 | 331.3 ± 26 | 332 ± 5 | 291 ± 5.3 | 313.3 ± 29.4 | 312.7 ± 12 | 316.3 ± 15.6 | 359.5 ± 19.4 |
| Growth rate (h⁻¹) | NA | NA | 0.08 ± 0.01 | 0.84 ± 0.28 | NA | NA | 0.07 ± 0.01 | 1.20 ± 0.24 |
| \( t_{lag} \) (h) | NA | NA | 11.25 ± 5.22 | 9.24 ± 0.41 | NA | NA | 11.74 ± 0.87 | 5.17 ± 0.84 |
FIGURE 3. Far UV CD spectra at different temperatures of λ light chains. Unfolding and refolding traces correspond are shown as dashed lines and open circles, respectively. CD spectra were recorded during heating and cooling of the same sample. The continuous lines represent the native protein. The refolding of 3rJL2/Y, 3rJL2/YA, 3rJL2/YA/P7D, 3rJL2/YA/P40S, and 3rJL2/YA/I48M shows some changes in secondary structure as evidenced by the displacement of the refolding trace beyond the minimum (215 nm) or approximately at the maximum at 225 nm. The far UV CD recordings were carried out using a 0.2-cm-path length cuvette (200 μg/ml protein in 20 mM Na₂HPO₄, pH 7.5).
Biophysical Characterization of $\lambda$3 Germ Lines

changed to alanine (the equivalent residue present in the sequence of 3m). This double mutant named 3rJL2/YA (3rJL2/C34Y/W91A) showed superimposable unfolding and refolding transitions. Far UV CD results indicate that the double mutant recovered its native structure more significantly as compared with the single mutant. The better recovering of native properties after thermal unfolding encouraged us to use this double mutant as a template for the evaluation of the fibril formation propensity of additional mutants generated by site-directed mutagenesis.

It should be noted, however, that the thermodynamic analysis of the double mutant showed that W91A change in 3rJL2/YA caused a significant destabilization, as demonstrated by the $\Delta G$ decrement compared with that of 3rJL2/Y (Table 2). The $C_m$ and $T_m$ values decreased by 0.4 M and 7 °C, respectively (Table 2). The unfolding of 6aJL2 was evaluated to assess the thermodynamic stability difference between the A3 and A6 families. The results for 6aJL2 were similar to those previously reported (22). Far UV CD data showed that 6aJL2 germ line is completely reversible (Fig. 3). 3mJL2 and 3rJL2/Y had higher $T_m$ values than 6aJL2, indicating that the $\lambda3$ variable domains are more stable (Table 2).

Structural Characterization—The asymmetric units of the 3mJL2, 3rJL2/Y, and 3rJL2/YA crystal structures each contain four monomers, and each monomer comprises residues 2–107 of the light chain variable domain. The crystallographic data and refinement statistics are presented in Table 1. The superposition of monomers from each of the structures showed a backbone root mean square deviation between 0.4 and 0.9 Å, indicating that the global topology of the three models is similar (Fig. 4). However, there are subtle backbone deviations between the 3mJL2, 3rJL2/Y, and 3rJL2/YA structures at CDR1, CDR3, and the region around residue Pro-40, and the loop connecting strands C' and D (Fig. 4C).

The conformation of the N-terminal region in 3mJL2, 3rJL2/Y, and 3rJL2/YA was similar to that of 6aJL2 (13), including the proline $\beta$-bulge between the two segments of strand A. However, strand A in the $\lambda3$ structures was shorter and only comprised the segment after the $\beta$-bulge (Fig. 4B). The same hydrogen bonds are formed between the carbonyls of Pro-7 and Pro-8 in strand A and the carbonyls of Thr-103 and Lys-104 in strand G in the $\lambda3$ and 6aJL2 structures (Fig. 4B). 3mJL2 has an insertion (Y95b) at CDR3 compared with the other $\lambda3$ germ lines (Fig. 1). The OH of residue Y95b formed a hydrogen bond with the OE2 of residue E3, tightening the N terminus to the core of the domain (Fig. 4B and Table 3).

We compared the $\lambda3$ structures and 6aJL2 and found deviations of $\approx 2$ Å in the Ca position in the loop connecting $\beta$-strands C and C' (K39 to Q42) (Fig. 4A). This difference has been previously reported in AL proteins and may reflect the high mobility of this region (16).

In the 3rJL2/Y mutant, Tyr-34 (at $\beta$-strand C) forms part of a hydrophobic cluster with residues Tyr-32, Tyr-49, Leu-46, and Trp-91 (Fig. 4D). Trp-91 forms an additional hydrophobic interaction with Val-96 (Fig. 4D). The 3rJL2/YA mutant maintains the same hydrophobic interactions, with the exception of position 91. The change from cysteine to tyrosine at position 34 increases this hydrophobic patch in 3r structures. Because the backbone conformations of 3rJL2/Y and 3rJL2/YA are similar, we hypothesize that the W91A mutation ($\beta$-strand G) loosens the contacts at $\beta$-strands C, F, and G, explaining the slightly higher stability of 3rJL2/Y (Table 2) and the fibril formation capacity of 3rJL2/YA (see below and Table 3). Our structural analysis suggests that each germ line has a particular set of contacts that stabilizes its structure.

Many variable domains and complete light chains form dimers at physiological conditions (44, 45). Because the dimers do not seem to be involved in fiber elongation, they could modulate the aggregation of pathological light chain monomers (45). Therefore, variable domain dimerization could be a security lock to prevent fiber formation. We analyzed the $V_{H1}-V_{L1}$ interactions in the crystal lattices of the $\lambda3$ variants. In the asymmetric unit of 3mJL2, each monomer interacted with another monomer to form the equivalent of a Bence-Jones dimer (Fig. 4E), whereas the 3rJL2/Y and 3rJL2/YA monomers formed crystallographic dimers in which the second monomer was rotated 180° with respect to the corresponding 3mJL2 monomer (Fig. 4F). The $\lambda3$ monomers would only form the equivalent of a Bence-Jones dimer with their symmetric partners. At the center of the 3mJL2 B strand, the Arg-20 side chain pointed toward the solvent, preventing the formation of the alternate interface found in the 3rJL2/Y and 3rJL2/YA mutants, whereas the Ser-20 side chain is buried in the crystal dimer interface (Fig. 4F). It was previously proposed that the presence of a charged amino acid at the center of an edge strand may prevent the aggregation of proteins that contain a $\beta$-sandwich structure (8). The majority of $\lambda3$ germ lines and $\lambda3$ variants isolated from patients presented an arginine residue at position 20 (Fig. 1). The germ line 3r contains a Ser at this position, and this is an important difference between 3m and 3r. Presumably, Arg-20 may be one of the security locks that prevent fibril formation in the 3m germ line.

PISA (Protein Interfaces, Surfaces, and Assemblies) (46) analysis indicated that the 3mJL2 dimer interface contains 1,820 Å$^2$ of buried accessible interface area. This value is significantly higher than the canonical crystallographic dimer interfaces formed by 3rJL2/Y, 3rJL2/YA, and 6aJL2 (1,546, 1,361, and 1,227 Å$^2$, respectively) and other dimer interfaces in antibody fragments ($V_{H1}-V_{L1}$) and light chains ($V_{H1}-V_{L1}$) (Table 4). The size of the canonical interface of the 3mJL2 dimer would block conformations prone to aggregation.

To rule out those possibilities, we assessed the oligomerization state of the variable domains of 3mJL2, 3rJL2/Y, 3rJL2/YA, and 6aJL2, previously analyzed by SDS-PAGE (Fig. 5). Different protein concentrations (20, 50, and 200 μg/ml) were examined through analytical size exclusion chromatography (Fig. 5). The purified variable domains eluted as a single peak with an elution volume corresponding to the molecular mass of a monomer (Fig. 5). We did not observe any oligomeric species in the elution profile. We next performed guanidine HCl and temperature-induced unfolding experiments using the same protein concentrations (20, 50, and 200 μg/ml) (Fig. 6). These results, in addition to the hydrodynamic and thermodynamic data (Table 5), allowed the conclusion that the variable domains were mainly monomeric at the protein concentrations evaluated.
Site-directed Mutagenesis of the Protective Edges Identifies a Putative Fibrillogenic Region in 3r-derived Proteins—Proteins rich in β-structures have structural features that prevent their aggregation under harsh conditions or as a consequence of mutations. Light chains have two such anti-aggregation motifs that have been proposed to prevent intermolecular associations of edge strands that could lead to aggregation and fibril formation (8, 47). The N-terminal region of λ3 (residues 1–14) contains a “sheet switch” motif, a structural feature in light chains that is proposed to act as an anti-aggregation domain for β-strands B and G. Prolines at positions 7 and 8 are highly conserved in light chains (Fig. 1); these residues have been proposed to stabilize the sheet switch (13) (Fig. 4B). The functions of these residues were assessed using the 3rJL2/YA mutant.

Pro-7 was mutated to aspartic acid, which is normally present in the 3l germ line. We named the resulting protein (3rJL2/YA/P7D) 3rJL2/YA/P7D. We also mutated Pro-8 to serine, the residue present at the equivalent position in the 3l germ line. The resulting protein was named 3rJL2/YA/P8S (3rJL2/C34Y/W91A/P8S). The thermodynamic data for these mutants are shown in Table 2 and Fig. 2. The thermodynamic stability of 3rJL2/YA/P7D was significantly affected, whereas the stability of 3rJL2/YA/P8S was unaffected as compared with the double mutant (Table 2 and Fig. 2). Far UV CD data showed that mutation at position 7 exerted a subtle influence on the reversibility to the native structure. In the case of position 8, the effect on the reversibility of thermal unfolding was minimal (Fig. 3).
Residues 40–60 formed the other protective edge of light chains and shield strands C and C' (Fig. 1). Residue Pro-40 was of particular interest because it is highly conserved in both κ and λ isotypes. Furthermore, residue Pro-40 of the multiple myeloma LEN protein has been shown to confer stability onto several amyloid-related light chains (14). We evaluated the role of Pro-40 using the 3rJL2/YA/I48M mutant. Because Ile-48 is buried in the hydrophobic core of the protein (Fig. 4), mutation at position 48 exerted a moderate influence on the reversibility of the mutant so severely that it was not expressed. The thermodynamic results of 3rJL2/YA/I48M indicated that this mutant showed a decrease in its thermodynamic stability (Table 2 and Fig. 2). Far UV CD data showed that mutations at position 8 and 40 exerted a minimal influence on the reversibility to the native structure (Fig. 3).

We examined protein aggregation because it promotes amyloid fibril formation. The TANGO, PASTA, and Aggrescan servers are useful bioinformatics tools to evaluate the propensity of residues within a β-sheet to be facing one another on neighboring strands (48–50). Following these criteria, we identified four regions in A3 sequences (designated a–d) with different aggregation propensities (Fig. 7). Region b (residues 44–50) obtained the highest scores. 3rJL2/YA was subjected to in silico amino acid scanning around this region to find key residues that could modify its aggregation profile. When we mutated the conserved residue Ile-48 to methionine or proline, the servers predicted a decrease in the aggregation profile of this region in 3rJL2/YA (Fig. 7). Several light chain sequences contain a methionine residue at this position (Fig. 1). These results motivated us to generate the corresponding mutants; however, we were only successful on expressing the 3rJL2/YA/I48M (3rJL2/C34Y/W91A/I48M) mutant. Because Ile-48 is buried in the hydrophobic core of the protein (Fig. 4C), the mutation to proline presumably destabilized the mutant so severely that it was not expressed. The thermodynamic results of 3rJL2/YA/I48M indicated that this mutant showed a decrease in its thermodynamic stability (Table 2 and Fig. 2). Far UV CD data showed that mutation at position 48 exerted a moderate influence on the reversibility to the native structure (Fig. 3).

According to their thermodynamic parameters, the 3rJL2/YA/P8S and 3rJL2/YA/P40S mutants were considered stable. In fact, the Tm and Cm values of 3rJL2/YA/P7D and 3rJL2/YA/I48M are characteristic of relatively unstable proteins (22, 51). Based on the Cm and Tm values, the stability of the

**TABLE 3**

Comparison of interactions among specific residues of A3 and 6aJL2 proteins

| Hydrogen bonds | 3mJL2 | 3rJL2/Y | 3rJL2/YA | 6aJL2 |
|---------------|-------|---------|----------|-------|
| Glu-3 (OE2)   | Tyr-95b (OH) | —        | —        | —     |
| Pro-7 (O)     | Thr-102 (OG1) | Pro-7 (O) | Thr-102 (OG1) | Pro-7 (O) |
| Pro-8 (O)     | Lys-103 (N) | Pro-8 (O) | Lys-103 (N) | Pro-8 (O) |
| Tyr-34 (N)    | Glu-89 (O) | Tyr-34 (N) | Glu-89 (O) | Tyr-34 (N) |
| Tyr-34 (O)    | Q89 (N) | —        | —        | —     |
| —             | —        | —        | —        | —     |

**TABLE 4**

Interface areas of different antibody formats

| Protein         | Interaction | Format    | Area  | Protein Data Bank code |
|-----------------|-------------|-----------|-------|------------------------|
| Human myeloma IgG H2L | V3-V3     | Fab       | 1,551 | 8FAB                  |
| IgM rheumatoid factor | V3-V3    | Fab       | 1,726 | 1ADQ                  |
| 900G in complex with Cn2 | V3-V3 | SCFv      | 1,176 | 2YC1                  |
| LEN             | V3-V3      | Light chain dimer (L3) | 1,422 | 1J1L                  |
| AL-09           | V3-V3      | Light chain dimer (L3) | 1,080 | 2LYE                  |
| 8O18/08         | V3-V3      | Bence-Jones proteins (f) | 1,348 | 2QIE                  |
| 3mJL2          | V3-V3      | Light chain homodimer (germ line xL) | 1,237 | 2Q20                  |
| 3rJL2/Y        | V3-V3      | Light chain (germ line a) | 1,820 | 4AIZ                  |
| 3rJL2/YA       | V3-V3      | Light chain (germ line a) | 1,546 | 4AIK                  |
| 3rJL2/YA       | V3-V3      | Light chain (germ line a) | 1,361 | 4AJ0                  |

Biophysical Characterization of λ3 Germ Lines

Hydrogen bonds and hydrophobic interactions were determined between residues of 3mJL2, 3rJL2/Y, 3rJL2/YA, and 6aJL2 (according to Kabat numbering). The hydrogen bonds and hydrophobic interactions were determined with the Protein Interaction Server using criteria based on interatomic distances of 5.3 Å and 4.0 Å, respectively, and the Protein Interaction Calculator server (55). Dashes, hydrogen bonds or hydrophobic interactions are not present in corresponding proteins.
3r mutants can be ordered as follows: 3rJL2/Y > 3rJL2/YA > 3rJL2/YA/P40S > 3rJL2/YA/I48M > 3rJL2/YA/P7D. The mutants had different transition slopes (m and ΔH) values and different chemical and thermal unfolding curves (Table 2 and Fig. 2, C and D). In other words, although the global stability was not significantly altered, the cooperativity of the unfolding process was modified in each mutant. The thermodynamic parameters as a whole showed that the more unstable mutants were 3rJL2/YA/P7D and 3rJL2/YA/I48M.

3rJL2/YA/P7D and 6aJL2 Have Similar Kinetics during in Vitro Fibrillogenesis—One of the characteristics of amyloidogenic proteins is their capacity to form fibers in vitro (18, 52).
Biophysical Characterization of λ3 Germ Lines

TABLE 5

Stability of λ light chains at different protein concentrations

| Concentration | Parameter   | 3mJL2 | 3rJL2/Y | 3rJL2/YA | 6aJL2 |
|---------------|-------------|-------|---------|----------|-------|
| 20 µg         | C° (s)      | 1.7 ± 0.1 | 1.8 ± 0.1 | 1.4 ± 0.0 | 1.3 ± 0.2 |
|               | ΔG°(H₂O) (KJ/mol) | 24 ± 0.8 | 21 ± 3.6 | 21 ± 2.2 | 11 ± 0.4 |
|               | m (KJ/mol) | −14 ± 0.0 | −11 ± 2.6 | −15 ± 1.6 | −9 ± 1.6 |
|               | T°m (°C) | 61.3 ± 2.3 | 63.5 ± 0.6 | 56.9 ± 0.2 | 49.5 ± 0.5 |
|               | ΔG°m (KJ/mol) | 29.5 ± 4 | 36 ± 0.6 | 30.5 ± 4.2 | 25.5 ± 1.4 |
|               | ΔH°m (KJ/mol) | 273 ± 52.4 | 316.2 ± 10 | 316.2 ± 45.3 | 342 ± 31.6 |
| 50 µg         | C° (s)      | 1.6 ± 0.1 | 1.8 ± 0.0 | 1.4 ± 0.1 | 1.6 ± 0.0 |
|               | ΔG°(H₂O) (KJ/mol) | 21 ± 0.9 | 24 ± 2.1 | 23 ± 2.3 | 21.4 ± 0.9 |
|               | m (KJ/mol) | −12 ± 1.2 | −13 ± 1.3 | −16 ± 1.0 | −14 ± 1.2 |
|               | T°m (°C) | 60 ± 0.8 | 63 ± 0.1 | 56 ± 0.5 | 49 ± 0.5 |
|               | ΔG°m (KJ/mol) | 33 ± 0.7 | 37 ± 2.9 | 31 ± 0.2 | 27 ± 1.3 |
|               | ΔH°m (KJ/mol) | 317 ± 1.8 | 331.3 ± 26 | 332 ± 4.9 | 359.5 ± 19.4 |
|               | Growth rate (h⁻¹) | NA | NA | 0.08 ± 0.01 | 1.20 ± 0.24 |
|               | t°lag (h) | NA | NA | 11.25 ± 5.22 | 5.17 ± 0.84 |
| 200 µg        | C° (s)      | 1.7 ± 0.0 | 1.8 ± 0.0 | 1.5 ± 0.1 | 1.5 ± 0.1 |
|               | ΔG°(H₂O) (KJ/mol) | 24.4 ± 0.2 | 18 ± 0.3 | 21 ± 0.6 | 20 ± 3.1 |
|               | m (KJ/mol) | −15 ± 0.1 | −10 ± 0.3 | −14 ± 1.1 | −14 ± 1.2 |
|               | T°m (°C) | 61.6 ± 1 | 63 ± 0.6 | 56.5 ± 0.0 | 49 ± 0.0 |
|               | ΔG°m (KJ/mol) | 31.6 ± 0.5 | 37 ± 1.2 | 30 ± 1 | 25.5 ± 0.8 |
|               | ΔH°m (KJ/mol) | 289 ± 2.7 | 328 ± 6.4 | 321 ± 9.8 | 339 ± 11.3 |

FIGURE 7. Aggregation properties of 3rJL2 and 3mJL2 predicted from their amino acid sequences. Four regions with intrinsic aggregation propensity were identified on the λ3 sequences using the TANGO, PASTA, and Aggrescan servers. Only the representative TANGO prediction profiles are shown. The regions are identified with letters in the 3rJL2 profile as a (residues 32–37), b (residues 44–50), c (residues 70–76), and d (residues 93–100). Region b, which includes strand C′, has the highest aggregation score. The replacement of Ile-48 by Pro or Met diminished or eliminated the aggregation score, respectively. The fibril extension rate of mutant 3rJL2/YA/I48M was slower than that of 3rJL2/YA, confirming the importance of position 48 in β-sheet aggregation. Surprisingly, there were no predicted aggregation regions in the 6aJL2 sequence.

3mJL2 and the proteins derived from the 3r germ line were continually stirred to induce fibril formation. The fibril formation kinetics is illustrated in Fig. 8A. 3mJL2 and 3rJL2/Y samples did not show any change in ThT fluorescence after 70 h (Fig. 8A). These protein samples only became slightly turbid as the experiment progressed. The transmission electron micrographs showed the presence of amorphous deposits but no amyloid fibrils (data not shown). This is in agreement with the thermodynamic data, which indicate that both 3mJL2 and 3rJL2/Y are stable variable domains. Although the 3rJL2/YA and 3rJL2/YA/I48M mutants are borderline stable, they formed fibrils, as indicated by the significant increase in ThT fluorescence after 10 h of stirring (Fig. 8A). The 3rJL2/YA and 3rJL2/YA/I48M mutant fibril formation kinetics showed slightly different nucleation times (t°lag) and growth rates. 3rJL2/YA displayed a t°lag of 11.25 h and required 40 h for fiber extension. 3rJL2/YA/I48M showed a t°lag of 11.74 h and required a longer time for fiber extension (56 h). 3rJL2/YA and 3rJL2/YA/I48M showed similar growth rates, with a tendency of 3rJL2/YA/I48M to form fewer fibers with slower kinetics (Table 2).

3rJL2/YA/P8S and 3rJL2/YA/P40S did not form fibrils despite being marginally less stable than their parental protein (Fig. 8A). The transmission electron microscopy of 3rJL2/YA samples after fiber formation confirmed the presence of thin, unbranched, individual fibrils, and amorphous aggregates (Fig. 8B). 3rJL2/YA/P7D fibers appeared to be long and slightly curved, which is an unusual morphological feature. Smooth
bundles containing two to four well defined fibrils were easily observed in this preparation (Fig. 8B). This mutant was the least stable with a t_{lag} of 9.2 h and a fibril formation plateau at 20 h (Table 2 and Fig. 8A). In the 3rJL2/YA/I48M mutant, there were fewer fibrils compared with the amorphous deposits, but the fibrils were organized as wide, rod-like bundles containing several linear fibrils (Fig. 8B). 6aJL2 also formed fibrils under the same conditions (Fig. 8B). According to the kinetics of fibrillogenesis, 3rJL2/Y and 3rJL2/YA should be as stable as 3mJL2, a model protein that corresponds to a nonamyloidic 3r germ line (Table 2). We expected that 3rJL2/Y would form fibrils in vitro because 3r is among the germ lines most frequently associated with AL (6). However, we did not find any fibrils in the recombinant protein sample (data not shown). The relatively high stability of 3rJL2/Y may be attributed to the new interactions formed by residue Tyr-34 (Table 2 and Fig. 4D), which may have also increased the expression level of the protein. However, these results did not directly answer whether the 3r germ line is intrinsically amyloidogenic.

Although 3mJL2, 3rJL2/Y, and 3rJL2/YA share similar thermodynamic properties, 3rJL2/YA was the only mutant that formed fibrils in vitro. Structural comparisons indicate that the W91A mutation in 3rJL2/YA decreased its hydrophobic interactions compared with the other 3r structures (Table 3). The results indicate that mutations at positions Cys-34 and Trp-91 had opposite effects in the 3rJL2 derivatives. The mutation in position 34 may have reinforced the interactions among the β-strands C, F, and G, resulting in a more stable domain. The second mutation, in position 91, may have diminished these interactions rendering a less stable variant and more prone to fibril aggregation (Fig. 4D).

The analysis of the triple mutants revealed the critical role of Pro-7 and Ile-48 on the stability and fibril formation of the 3r domain. Our results suggest a relationship between cooperativity and fiber formation, with m and ΔH being key parameters that inhibit fibrillogenesis. A previous report stated that the sheet switch at the N terminus of the light chains may impede and edge strand-mediated aggregation (8). The 3rJL2/YA/P7D mutant was less thermodynamically stable than 3rJL2/YA. The decrease in the stability of mutant 3rJL2/YA/P7D is comparable with the decrease in that of mutant 6aJL2/P7S (13). The P8S mutation, despite causing a much lower effect on the stability of 3rJL2/YA/P8S, abolished fibril formation (Table 2). These
results suggest that Pro-8 is important for fibril formation. The analysis of the 6aJL2-derived mutants P7S and H8P and the double mutant P7S/H8P revealed that only the mutation in position 7 decreased the stability of the protein (13). Our results confirm that position 7 contributes to the protective role of the β-bulge of β-strand A (sheet switch) against fibril formation may depend on the combinatorial amino acid sequence of residues 7 and 8. Furthermore, the similar features observed in the mutants studied in the context of the 6a and 3r scaffolds suggest that the protective role of the β-sheet against fibril formation may be conserved in the λ family.

The substitution of residue Pro-40, located in the loop that connects β-strands C and C', has been associated with amyloidosis in several κ light chains, including BRE, ARN, WR, and MCG. In these proteins, the Pro-40 mutation enhanced their fibril formation capacity (53). The 3rJL2/YA/P40S mutant did not exhibit a significant change in stability compared with the double mutant. A plausible explanation can be that residue Pro-40 only establishes stabilizing interactions through the peptide backbone (Table 3). Unexpectedly, this mutant did not form fibrils like the 3rJL2/YA/P8S mutant.

As previously mentioned, the Ile-48 residue is conserved in λ families 3 and 6. The decreased thermodynamic stability of the 3rJL2/YA/I48M mutant is likely related to the presence of the bulky methionine side chain, which may decrease the rate of the fiber formation process without completely inhibiting it. It is likely that the methionine residue induced a structural rearrangement between strands C and C', decreasing the self-complementation between the β-sheets and restricting the correct coupling of the monomers during fiber extension. In summary, position 48 seems to be important for maintaining the stability of the light chain and, when disrupted, favors fiber formation.

Our analysis of the 3rJL2/YA/P40S and 3rJL2/YA/I48M mutants indicates that loop 40–60, which connects the β-sheets, contributes to fibril formation when the protein is destabilized. The protection of loop 40–60 is associated with the canonical V_{H}^{β} V_{L} interaction, which limits its exposure (Fig. 4E).

Importantly, although region 40–60 in germ lines 3m and 3r presented a high β-aggregation profile score, the same region in germ line 6a had a low score (Fig. 7). 6a differs from germ lines 3r and 3m in only three residues at this region (Fig. 1). Consistent with these observations, minimal changes in the loop 40–60 are likely to modify its aggregation profile.

To the best of our knowledge, light chain dimerization has been reported only in proteins isolated from patients, such as the Bence-Jones proteins. Baden et al. (28) reported that the monomeric form of germ line κO18/O8 is more stable than its dimeric form, and the opposite occurs for its amyloidogenic counterpart AL-09, i.e. the dimer of this protein is more stable than the monomer (28). The calculated interface areas of the native dimers in the λ3 crystallographic structures are very similar to the V_{H}^{β} V_{L} and V_{L}^{β} V_{L} interfaces of several other antibodies (Table 4). These observations led us to assess the presence of dimers in the 3mJL2, 3rJL2/C34Y, 3rJL2/YA, and 6aJL2 proteins through various methods. Our results indicated that the variable domains are mainly monomeric at the protein concentrations tested. It is possible that the λ3 germ line proteins exist mainly as monomers, similarly to κO18/O8. This is in contrast to the variable domains isolated from patients with AL, in which both monomers and dimers are present (28). Those variable domains contain several mutations at different regions of the interface, favoring the V_{H}^{β} V_{L} interaction. Additionally, the complete light chain or the variable domain plus a fragment of the constant region may also promote V_{L}^{β} V_{L} interactions leading to dimerization.

In summary, our data indicate that the 3m germ line is relatively stable and did not form fibrils in vitro. This is in agreement with the very low percentage (2%) of 3m-derived proteins in reported AL cases (6). Before a light chain acquires its fibrilogenic potential, it has to accumulate destabilizing mutations to generate new molecular interactions favoring fibril aggregation under micro-environmental conditions, such as pH, temperature, and high local urea concentrations. In the mutants derived from 3r germ line, we identified residues that affect the stability of the protein, influence fiber formation, and alter their anti-aggregation properties, similarly to previously reported mutants (13, 28, 51).

The 6a and 3r germ lines show low polyclonal expression in B lymphocytes (2 and 8%, respectively). The percentage of AL cases in which 6a is the germ line donor precursor can reach up to 40%. Although 3r is expressed at a 4-fold higher level than 6a, the percentage of cases with 3r as the donor gene precursor hardly reaches 20% (6). The differences in polyclonal expression levels, stability, and fibril nucleation times and the differential gain or loss of particular interactions between λ6 and λ3 light chains mutants may explain the different “aggressiveness” of these two λ light chain families, of which 6a is the most pathogenic.

The characterization of other germ lines will lead to a more complete understanding of how changes in the original sequence of a light chain domain can modify its protective effect against aggregation. Determining how a germ line domain gives rise to amyloidogenic variants will allow us to understand why certain light chain subfamilies are highly implicated in AL and to develop new therapeutic strategies.

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