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A role for destabilizing amino acid replacements in light-chain amyloidosis

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ABSTRACT **Light-chain (L-chain) amyloidosis is characterized by deposition of fibrillar aggregates composed of the N-terminal L-chain variable region (V_L) domain of an immunoglobulin, generally in individuals overproducing a monoclonal L chain. In addition to proteolytic fragmentation and high protein concentration, particular amino acid substitutions may also contribute to the tendency of an L chain to aggregate in L-chain amyloidosis, although evidence in support of this has been limited and difficult to interpret. In this paper we identify particular amino acid replacements at specific positions in the V_L domain that are occupied at frequencies significantly higher in those L chains associated with amyloidosis. Analysis of the structural model for the V_L domain of the Bence–Jones protein REI suggests that these positions play important roles in maintaining domain structure and stability. Using an *Escherichia coli* expression system, we prepared single-point mutants of REI V_L incorporating amyloid-associated amino acid replacements that are both rare and located at structurally important positions. These mutants support ordered aggregate formation in an *in vitro* L-chain fibril formation model in which wild-type REI V_L remains soluble. Moreover, the ability of these sequences to aggregate *in vitro* correlates well with the extent to which domain stability is decreased in denaturant-induced unfolding. The results are consistent with a mechanism for the disease process in which the V_L domain, either before or after proteolytic cleavage from the L-chain constant region domain, unfolds by virtue of one or more destabilizing amino acid replacements to generate an aggregation-prone nonnative state.**

Amyloidosis is a disease characterized by the extracellular deposition of fibrillar aggregates of particular proteins leading to cell loss, tissue disruption, and, in many cases, death (1, 2). Although fundamental disease mechanisms are still unclear, a number of factors, such as elevated concentrations and/or limited proteolysis of the constituent protein, seem to interact synergistically in most types of amyloidosis. Extrinsic physiological factors often also play a major role (2). In some forms, such as familial amyloidosis (1) and hereditary cerebral amyloid angiopathy (3), critical amino acid replacements are also important.

In light-chain (L-chain) amyloidosis (AL) (4–6), fibrils composed of L-chain variable region (V_L) domains lead to malfunction of the kidney, heart, liver, and/or peripheral nervous system, with death generally occurring between 1 and 4 years after clinical diagnosis (7). AL is associated with various conditions generating monoclonal L-chain overproduction, such as multiple myeloma. However, only 10–15% of myeloma patients develop AL (6). Since L chains associated with AL differ in sequence from other L chains, disease mechanisms have been proposed that include a potential role for particular amino acid replacements (8–14). Critical replacements have been difficult to confirm, however, and the mechanisms of any such sequence effects have remained conjectural. One impediment to analysis is the general background of sequence polymorphism characteristic of the immunoglobulin family. In addition, those replacements that do stand out as being particularly unusual tend to be located in different parts of the molecule.

Localization of amyloid-related replacements to a particular part of a protein molecule might be expected if these replacements function by modifying a region of the folded molecule's surface—increasing its tendency to aggregate, as has been suggested for the polymerization of hemoglobin S (15). However, the mutations in transthyretin that are linked to amyloid formation in familial amyloidosis are distributed throughout the three-dimensional structure of the molecule (1). Such a distribution resembles more closely that found for mutations that alter the folding stability of globular proteins (16, 17). These and other observations have led to the speculation that particular mutations dispose proteins to amyloid formation by enhancing formation of a partially/completely unfolded state that is responsible for fibril formation (18). In fact, good evidence has recently been provided for the importance of an unfolding step in fibrillogenesis by transthyretin in familial amyloidosis (19, 20).

In this paper we report experiments that support the hypothesis that specific amino acid replacements play an important role in the development of AL by facilitating protein unfolding to generate an aggregation-prone nonnative state.

EXPERIMENTAL PROCEDURES

Production of V_L Sequence Variants. The construction of DNA encoding residues 1–108 of REI V_L from chemically synthesized oligonucleotides has been described (21). This DNA was used to construct a vector encoding expression of the V_L domain in *Escherichia coli* with secretion into the periplasmic space, using the *tac* promoter and the *pelB* signal sequence (W.C., G. Lee, and R.W., unpublished data). The protein contains the N terminus expected for correct processing of the signal peptide, the expected intradomain disulfide bond, and a parent ion in plasma desorption mass spectrometry consistent with the programed amino acid sequence (W.C., D. McNulty, and R.W., unpublished data). All of the mutants constructed were stably expressed. Some of the mutants were deposited in periplasmic inclusion bodies after signal peptide processing and disulfide formation (W.C. and R.W., unpublished data). These were isolated by centrifugation after native lysis, solubilized in guanidine hydrochloride (Gdn HCl), dialyzed into 1 M urea, and purified by HPLC (W.C. and R.W., unpublished data). After purification and dialysis, all of the V_L domains have good solubility in native buffer at 1 mg/ml or greater, and in appropriate solvent conditions, all exhibit the unusual highly quenched tryp-

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Abbreviations: L chain, light chain; AL, L-chain amyloidosis; V_L, L chain variable region; Gdn HCl, guanidine hydrochloride.

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tophan fluorescence characteristic of properly folded immunoglobulin domains.

Stability and Aggregation of V_L Domains. All mutants were purified to homogeneity, as determined by SDS/PAGE, by C_4 reverse-phase HPLC before *in vitro* analysis. Thermodynamic stabilities of V_L domains were determined by monitoring the dependence of intrinsic tryptophan fluorescence on Gdn HCl concentration (22). The *in vitro* fibril formation system of Linke *et al.* (23) was modified for microtiter plate assay to minimize material requirements. Purified protein was incubated partially covered at pH 3.5 and 37°C and allowed to slowly concentrate by vaporization. After 48 h, water was added to restore the original volume. At this point, aggregate is visible by light microscopy. Aggregation extent was measured by a microplate assay developed for quantification of amyloid by virtue of its Congo red binding (S. J. Wood, R.W., and M.R.H., unpublished data). To the aggregate was added 4 vol of a filtered solution of 200 mM Congo red. After 2 h, absorbance was read at 480 nm and 540 nm and converted to the molar concentration of bound Congo red by the method of Klunk (24).

RESULTS AND DISCUSSION

Sequence Analysis. Our approach to sequence analysis was guided by the hypothesis that if amyloidogenic replacements affect the stability and/or folding kinetics of the L chain, they are more likely to occur at structurally important positions. In addition we assumed that sequences at these positions are likely to be conserved across species and that replacements that destabilize the protein significantly are likely to be rare. Therefore, we analyzed the primary sequences of all κ L chains in the database (25) for the frequencies at which each of the 20 amino acid residues occurs at each position in κ chain and separately analyzed the λ chain database. If a residue was present at a position in <1% of the sequences, it was defined as rare. A collection of 36 amyloidogenic sequences (18 κ and 18 λ) of amyloid-associated L chains was analyzed for the presence of such mutations. Fig. 1 demonstrates that such rare mutations occur more frequently in amyloidogenic L chains than in the L-chain database as a whole and that they appear to be localized to defined positions. As discussed below, the locations of many of these rare replacements within the three-dimensional model of the REI V_L domain (Fig. 2) are consistent with a role in destabilization of the folded structure.

To test the hypothesis that these rare replacements play important roles in AL and do so by weakening domain stability, we constructed a series of six AL-associated single-point mutants in the V_L domain of the Bence–Jones protein REI. Table 1 lists the sequence variants produced in this study and the names and subtypes of the amyloid-associated L chains in which they are found. Table 1 also lists the frequencies with which these residue positions are conserved in the κ and λ L-chain database and their frequency of replacement among AL L chains. In all six cases, the residue found at the position in wild-type REI is the expected highly conserved amino acid.

***In Vitro* Aggregate Formation and Thermodynamic Stability.** Glenner *et al.* (34) showed that an AL-associated Bence–Jones L-chain incubated with pepsin at pH 3.5 produces amyloid fibrils, suggesting that features of the molecule itself might be sufficient to account for much of the disease mechanism. Later it was shown (23) that the effect could be duplicated by generating V_L domains via limited trypsin digestion at pH 7 and then incubating these fragments at low pH to produce fibrils or other aggregates. We used a modification of this low pH procedure to test the ability of the *E. coli*-produced V_L domains to aggregate *in vitro*. Importantly, the wild-type domain produces minimal aggregate by this procedure (Table 1), as determined both by dark-field light microscopy (data not shown) and by quantifying the spectral shift of the dye Congo red when it binds to aggregate (ref. 24; S. J. Wood, R.W., and M.R.H., unpublished data). In contrast, the point mutants G57E, G68D, A84T, and R61N and the AL-unrelated mutant REI-RGD23 aggregate strongly in the *in vitro* model (Table 1).

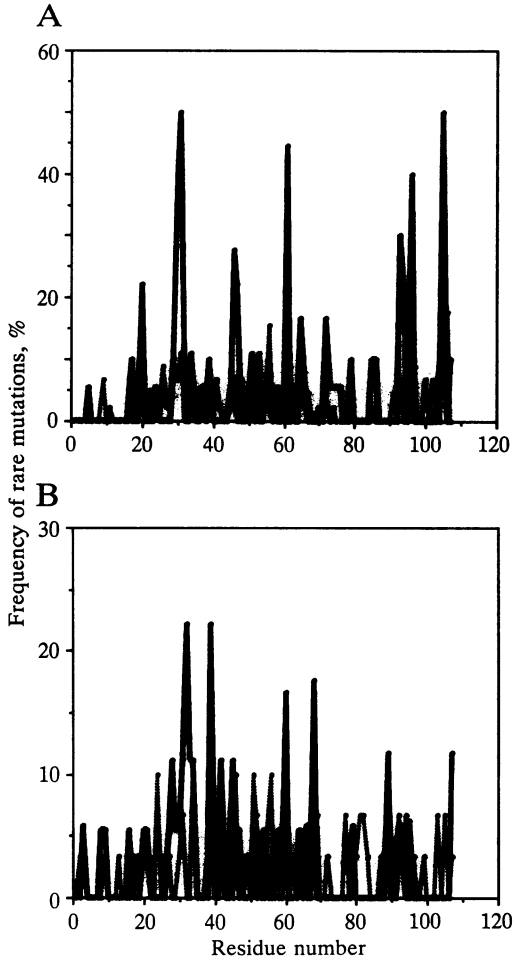

FIG. 1. Positional dependence of the frequency of rare mutations in immunoglobulin κ (*A*) and λ (*B*) chains for amyloid (solid lines) and nonamyloid (dashed lines) proteins. Rare residues were defined as residues that appear in <1% of the respective class (≈800 κ and ≈200 λ chains) as summarized by Kabat *et al.* (25). The set of amyloid proteins consisted of 18 κ chains (BAN, AND, MEV, AM81, AM107, AM113, NIE, COZ, MUM, CRU, BRE, EPP, HAC, COL, TEW, INC, AL700, ALSO124) and 18 λ chains (DIA, NIG77, NIG51, NIG84, ZIM, EZI, POL, EPS, MC, NOV, ES492, HAR, MOL, GIL, BAK, AR, WLT, SUT). (Alignments of these sequences and their literature citations are available from the authors on request.) The control set of nonamyloid proteins consisted of all human L chains in release 25.0 of the Swiss-Prot protein sequence database, excluding those associated with amyloid disease. Each data point in these graphs represents the sum of the frequencies of occurrence of all rare sequence replacements at that position.

Since low pH is known to be destabilizing to most proteins (35), the positive response of these mutants in the *in vitro* model system supports the hypothesis that critical amyloidogenic replacements function by destabilizing domain structure. To quantify this destabilization, we characterized the response of the mutants to Gdn HCl, by monitoring the unfolding-dependent increase in Trp-35 fluorescence due to the loss of quenching by the proximal disulfide bond (Fig. 2) (22). Fig. 3 shows the results of these analyses as a series of plots representing the extent of unfolding of each protein as

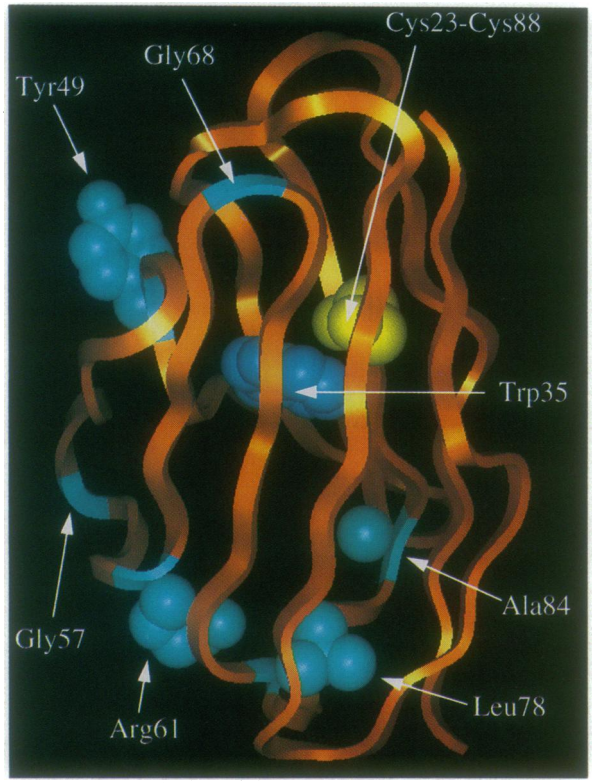

FIG. 2. Ribbon diagram of the polypeptide backbone of the immunoglobulin V_L domain REI from the x-ray crystallographic coordinate set 1REI in the Brookhaven data base (26). Side chains of selected residues are shown in Corey–Pauling–Koltun model format. Note the close spacial proximity of the tryptophan and the disulfide bond that quenches its fluorescence in the native state.

a function of Gdn HCl concentration, and Table 1 lists the denaturation midpoints, stabilities, and stability differences from this analysis. The point mutants L78T and Y49F and the wild type exhibit very similar stabilities. In contrast, the mutants G57E, R61N, A84T, and G68D are between 1.0 and 3.1 kcal/mol less stable than wild-type REI, as calculated at

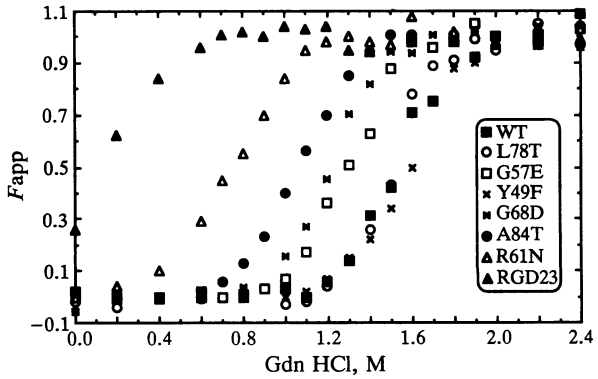

FIG. 3. Gdn HCl unfolding curves of proteins. Proteins were allowed to equilibrate in solutions of various concentrations of Gdn HCl in 10 mM sodium phosphate (pH 7.5) at room temperature for 4 h before fluorescence was read in a thermostated cuvette at 25°C with excitation at 295 nm and emission at 350 nm. Data from the SLM4800C phase fluorometer was converted to F_{app} (mol fraction of molecules unfolded) (33) to generate the plots shown.

the midpoint of unfolding of the wild-type domain (36). The sequence RGD23, in which the six residues of CDR3 (YQSLPY) are replaced by eight nonnative amino acids (RIPRGDMP) (21), is the least stable protein examined; Fig. 3 shows that an unfolded state is significantly populated in RGD23 even in the absence of denaturant.

The thermodynamic analysis supports the hypothesis that domain stability plays an important role in the response of these proteins in the *in vitro* fibrillization model. Less-stable domains form more aggregate, and the most-stable domain, the wild type, forms essentially none. This relationship is shown graphically in Fig. 4, a plot of *in vitro* aggregate formation vs. Gdn HCl denaturation midpoints. The figure shows that the sequence variant RGD23, constructed in an unrelated line of experiments (21), is an extreme example of the general trend of increasing aggregate formation with decreasing protein stability. The similar trend exhibited by the destabilizing, non-AL-associated mutations included in the figure suggests that any mutation that sufficiently decreases domain stability may have the capability to generate AL.

Table 1. Properties of REI V_L containing predicted amyloidogenic replacements

Variant	Source	Residue conservation, % WT	Residue conservation, % Amyloid	Congo red	C_m (Gdn HCl), M	ΔG°_{unf}, kcal/mol	$\Delta\Delta G_{unf}$, kcal/mol
WT	—	—	—	0.07	1.55	−6.8 ± 1.2	—
Y49F	AND (κ)	90.6	1.0/4.3	0.14	1.59	−6.2 ± 0.9	−0.2
G57E	WLT (λ)	99.6	0.3/1.3	0.32	1.30	−5.8 ± 0.5	1.0
R61N	BAN (κ)	99.4	0.0/0.0	0.46	0.75	−2.9 ± 0.2	3.1
G68D	ZIM (λ)	94.7	0.0/0.9	0.20	1.22	−6.7 ± 0.5	1.8
L78T	MOL (λ)	29.8 (97.1)	0.3/4.8	0.07	1.43	−6.0 ± 0.6	0.5
A84T	Nig77 (λ)	68.1 (96.8)	0.9/1.4	0.49	1.08	−4.3 ± 0.7	2.1
RGD23	—	—		0.54	0.1	—	—

Name and class of the associated L chain (10, 27–31) are given. For wild-type (WT) residue conservation, the percent of sequences in the κ chain database (≈800 sequences) (ref. 25, pp. 2134–2136) containing the wild-type residue at this position is shown. For amyloid residue conservation, the percentages of sequences in the κ chain database (≈800 sequences) and the λ chain database (≈200 sequences) containing the amyloid-associated residue at these positions are shown. Data for Congo red are mol of Congo red bound per mol of total REI V_L domain in assay (24). Since the binding capacity of aggregate is assumed to be constant, this value reflects the amount of aggregate formed. C_m is the midpoint of the Gdn HCl unfolding transition obtained from fitting the fluorescence data by the method of Santoro and Bolen (32). The denaturation midpoint for RDG23 was estimated manually from the denaturation curve since there were no native baseline points to fit a formal F_{app} plot (Fig. 3). The cooperativity parameter (33), A, for the wild type was −4.4 kcal per mol per molar Gdn HCl and ranged from −3.9 to −5.5 for the mutants; it can be obtained for any of these proteins by dividing ΔG by C_m. The difference between the ΔG_{unf} of the mutant and the ΔG_{unf} of the wild type was calculated at the C_m of the wild type. A positive value means that the mutant is less stable than wild type. Values in parentheses are for sequence conservation if conservative replacements are allowed: at position 78, 97.1% contain Leu, Val, or Met; at position 84, 96.8% contain Ala or Gly. Thermodynamic parameters could not be determined for RGD23 because an incomplete denaturation curve was obtained (Fig. 3).

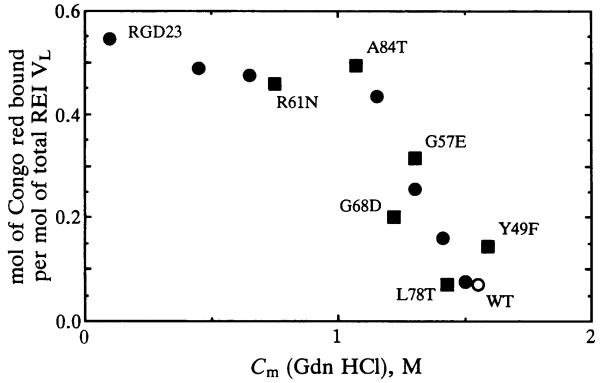

FIG. 4. Plot of extent of formation of Congo red binding aggregate vs. protein stability [as assessed using the midpoint of Gdn HCl unfolding transition (C_m) values] for each of the proteins in this study (Table 1) (○, ■) as well as other REI V_L mutants (●) constructed and analyzed by the same methods (L.R.H., W.C., G. Lee, and R.W., unpublished data).

Thermodynamic and *in vitro* aggregation data for four of the six replacements chosen for analysis, G57E, R61N, A84T, and G68D, support the hypothesis. The x-ray crystal structure of REI (Fig. 2) suggests reasonable explanations for their destabilizing effects, although a true accounting must await further experiments. The most destabilizing disease-related replacement, R61N, would be expected to eliminate or decrease the potential for a conserved salt bridge/H-bond with Asp-82 in an adjacent loop. The A84T mutation is expected to place a hydrophilic hydroxyl group in a tight hydrophobic environment involved in the packing of the two β-sheets of the molecule into a β-sandwich. The two mutated Gly residues are located at positions in the native structure with unusual ϕ,ψ angles, suggesting peptide backbone conformations that are most energetically favored when occupied by Gly (35). Any other residue at these positions might be expected to destabilize the protein due to the constraints of peptide bond configuration.

Although the minor effects on stability and aggregation of L78T and Y49F were initially disappointing, in retrospect these amino acid replacements should not have been chosen as strong candidates. The six replacements were originally selected for testing based on their rarity against only the κ L-chain database. When the λ chain database comparison was made later, the L78T (from an amyloidogenic λ chain) and Y49F (from a κ) replacements proved to be not so unusual, occurring at frequencies of 4.8 and 4.3%, respectively (Table 1). Structurally, the surface-exposed side chain of residue 49 does not interact with other groups and is thus expected to play a relatively minor role in stability. The side chain of residue 78, however, makes van der Waals contact with Ala-13 in an adjacent loop in the wild-type structure and thus might have been expected to be more important.

A Model for the Role of Amino Acid Sequence in AL. A number of possible explanations for the hypothetical role of amino acid replacements in AL, such as hydrophilic → hydrophobic surface effects, conformational changes to generate altered states with enhanced tendencies to aggregate, enhanced susceptibility of the L chain to the proteolysis that generates V_L, or enhanced stability of an amyloidogenic V_L dimer, have been discussed (8–14, 27, 28). This variety of speculations reflects the difficulties in assigning importance to residue replacements.

The data presented here suggest an alternative explanation for the role of amino acid replacements in AL based on the recognized *in vitro* relationship between unfolding and aggregation in many globular proteins (18, 37, 38). In our model, replacements that decrease the energetic barrier to unfolding would be expected to increase the population of a nonnative state (which may or may not be fully unfolded) required for amyloid fibril formation. Although the linkage of rare destabilizing replacements with AL supports this hypothesis, there are a number of outstanding issues, discussed below, that should be addressed in future studies.

The in vitro *model for fibril formation.* The low pH conditions of the *in vitro* fibrillization conditions are clearly nonphysiological, and we thus view this system simply as a useful heuristic tool. At the same time, it should be noted that involvement in amyloidogenesis of acidic lysosomal compartments has been suggested for AL (34, 39) and other amyloid systems (19, 40, 41). It is also possible that some other, as yet poorly understood, denaturing conditions are involved in amyloidogenesis *in vivo*. In addition, less-stringent denaturing forces might be capable of inducing amyloid formation at very high protein concentrations or in the presence of severely destabilizing amino acid replacements.

Microscopic examination reveals that the aggregates generated in the *in vitro* model system described here are mixtures predominating in organized fibril-like structures but also containing lesser amounts of amorphous deposits (R.W., unpublished data). This ability of a single V_L domain to give rise to aggregates of differing morphologies mirrors the occasional clinical observation of simultaneous fibril (AL) and amorphous aggregates [L-chain deposition disease (4, 6)] in the same patient (5). It is very likely that domain stability also plays a major role in L-chain deposition disease (L.R.H. and R.W., unpublished data).

Possible roles of sequence and structure beyond domain folding stability. The demonstration of a critical role for destabilizing mutations does not rule out involvement of other sequence elements in other ways. For example, the ability of the proposed misfolded amyloidogenic intermediate to assemble into characteristic amyloid fibrils or other ordered or amorphous aggregates will almost certainly depend on sequence elements that become exposed after partial or complete unfolding (38).

Our unfolding studies were conducted at concentrations at which wild-type REI V_L is >80% monomeric (42) and, hence, primarily monitor the stability of the monomer. Mutations that disrupt or weaken the dimer interface would be expected to also destabilize V_L, under appropriate conditions. For the energy derived from dimer formation to be a factor in amyloidosis, however, the V_L or L chain concentration at the site of fibril formation would have to be quite high, given the K_d of 10^{-5} M typical for the V_L dimer (43).

Database analysis. Although the amyloidogenic replacements implicated by our results are indeed very rare, they are occasionally observed in the database in L chains not known to be involved in deposition disease. There are a number of plausible explanations: (*i*) Some amyloid-associated L chains in the database may have been misassigned as benign. (*ii*) Even a destabilized V_L domain might be benign as part of a molecule circulating at the low levels that obtain for most antibody-related proteins in normal individuals *in vivo*. (*iii*) A single replacement may not always be sufficiently destabilizing to cause AL. (*iv*) Destabilization by rare replacements may be dependent on their structural context.

Given this last possibility, it is in fact remarkable that such strong effects are seen for several of the replacements we tested, despite the fact that they were characterized in the context of REI, and not in the context of the AL-associated L chain in which they were identified. The most destabilizing mutation, R61N, occurs at a residue that is very highly conserved in both λ and κ databases (Table 1). The three next most destabilizing replacements, A84T, G68D, and G57E, are found in amyloid-associated λ L chains, yet their potential to destabilize the immunoglobulin fold is clearly observed in the REI κ background.

If, as shown here, amyloidogenic replacements can be characterized after installation in a "standard" V_L domain, then amyloidogenic L chains themselves should also be recognizable by the low stability of their V_L domains. This should be true whether these domains are destabilized by one mutation—as suggested for some of the replacements discussed here—or the cumulative effects of several. This prediction might serve as the basis for a diagnostic test to determine the AL susceptibility of patients producing high levels of monoclonal L chains and perhaps L-chain deposition disease as well.

Note. While this manuscript was in review, we became aware of a study (44) demonstrating that V_L domains associated with various forms of deposition disease—including amyloidosis—have a tendency to form soluble aggregates under native or mildly denaturing conditions, while benign domains exhibit only the monomer–dimer equilibrium normally associated with V_L domains. These results would be compatible with our folding stability hypothesis if the aggregation observed by Myatt *et al.* (44) were due to the generation of an aggregation-prone nonnative state in native buffer by virtue of one or more destabilizing replacements. That the V_L domain is capable of being destabilized to this extent and is at the same time relatively soluble despite this destabilization, is illustrated by REI-RGD23, which appears to be ≈30% unfolded in native buffer at pH 7 (see Fig. 3). Analysis of the folding stability of authentic amyloid-associated V_L domains should address some of the above issues.

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