

Thermodynamic Modulation of Light Chain Amyloid Fibril Formation*

(Received for publication, October 7, 1999, and in revised form, November 19, 1999)

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To obtain further insight into the pathogenesis of amyloidosis and develop therapeutic strategies to inhibit fibril formation we investigated: 1) the relationship between intrinsic physical properties (thermodynamic stability and hydrogen-deuterium (H-D) exchange rates) and the propensity of human immunoglobulin light chains to form amyloid fibrils *in vitro*; and 2) the effects of extrinsically modulating these properties on fibril formation. An amyloid-associated protein readily formed amyloid fibrils *in vitro* and had a lower free energy of unfolding than a homologous nonpathological protein, which did not form fibrils *in vitro*. H-D exchange was much faster for the pathological protein, suggesting it had a greater fraction of partially folded molecules. The thermodynamic stabilizer sucrose completely inhibited fibril formation by the pathological protein and shifted the values for its physical parameters to those measured for the nonpathological protein in buffer alone. Conversely, urea sufficiently destabilized the nonpathological protein such that its measured physical properties were equivalent to those of the pathological protein in buffer, and it formed fibrils. Thus, fibril formation by light chains is predominantly controlled by thermodynamic stability; and a rational strategy to inhibit amyloidosis is to design high affinity ligands that specifically increase the stability of the native protein.

The amyloidoses represent a group of diseases characterized by the pathological deposition of insoluble fibrils, having a characteristic non-native intermolecular β -sheet structure, which are formed from proteins that originally have soluble, native conformations (1–3). At least 18 different amyloidogenic proteins have been identified, and among human disorders associated with their deposition are Alzheimer's disease, Parkinson's disease, adult (type 2) diabetes, chronic inflammation, and monoclonal plasma cell dyscrasias (1–4). The continual accumulation of fibrils within tissues leads to organ dysfunction and, with rare exception, is an irreversible process leading to death (5, 6).

* This work was supported by National Science Foundation Grant BES9816975 (to J. F. C. and T. W. R.) and National Institutes of Health Grant CA10056 (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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To obtain further insight into the pathogenesis of amyloidosis and develop therapeutic strategies to inhibit fibril formation, our current research involves investigation of the intrinsic thermodynamic stabilities of human amyloid precursor proteins and the effects of extrinsic factors on stability and fibril formation. We have focused on molecules found in the deposits of patients with primary or light chain-associated amyloidosis that consist of monoclonal immunoglobulin light chains and related variable domain (V_L)¹ fragments (5, 7). We and others have shown, using recombinant V_L s with sequences based on amyloidogenic and nonpathological light chains, that the propensity to form fibrils *in vitro* is inversely correlated with the free energy of unfolding (8–11), as has also been found for amyloid-associated variants of transthyretin and lysozyme (12, 13).

In this report, we present the results of studies in which we compared thermodynamic stabilities of two human monoclonal light chains, an amyloid-associated protein, BIF, and a homologous nonpathological light chain, GAL. We have demonstrated that the amyloidogenic light chain was inherently less thermodynamically stable than its nonpathological counterpart and that the protein hydrogen-deuterium (H-D) exchange rates, which reflect relative levels of partially folded protein molecules, correlated directly with the propensity of these molecules to form fibrils. Further, we have shown, employing sucrose as a nonspecific thermodynamic stabilizer (14, 16), that fibril formation can be inhibited by extrinsic factors that increase thermodynamic stability of the native state and reduce the levels of partially folded molecules. Conversely, we found that urea destabilized the proteins and promoted protein aggregation and fibril formation, which has implications for the frequently observed deposition of light chain amyloid in the kidney (6, 17).

EXPERIMENTAL PROCEDURES

Protein Preparation—The monoclonal light chains, *i.e.* Bence Jones proteins, BIF and GAL were isolated and purified (18) from urine specimens obtained from a patient with light chain amyloidosis and multiple myeloma, respectively. Patient BIF had extreme amyloid deposition throughout the entire gastrointestinal tract, heart, spleen, bladder, lungs, liver, and kidneys (18). In contrast, patient GAL, despite the excretion of up to 100 g of Bence Jones protein daily, did not have any detectable pathologic protein deposits.

In Vitro Fibril Formation and Sample Analyses—The lyophilized proteins were reconstituted into 10 mM potassium phosphate, pH 7.4 at 37 °C, with 100 mM NaCl (PBS) to a protein concentration of 1 mg/ml (unless otherwise indicated), filtered through a 0.2- μ m syringe filter, and incubated at 37 °C. Samples were prepared in PBS alone, PBS plus 1.0 M sucrose and PBS plus 1.0 M urea. Fibrils were generated *in vitro*

¹ The abbreviations used are: V_L , immunoglobulin light chain variable domain; H-D, hydrogen-deuterium; PBS, phosphate-buffered saline.

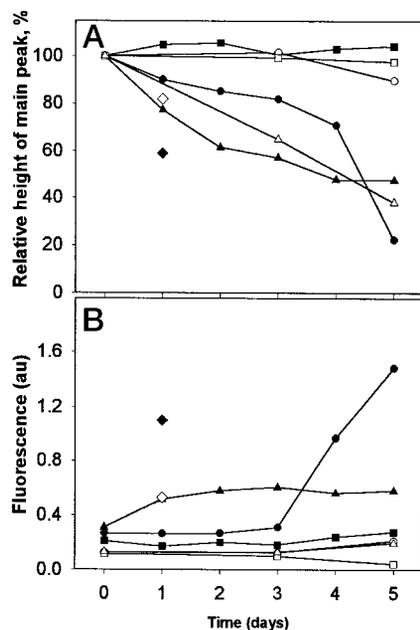


FIG. 3. Effect of duration of incubation at 37 °C on soluble protein levels and thioflavin T fluorescence. *A*, relative height of main protein peak on size exclusion chromatogram. Results for samples incubated for ≥ 1 day in 1.0 M urea are based on the actual absorbance of the shoulder occurring at the retention time of the original main peak. This analysis was necessitated because the formation of soluble aggregates shifted the retention time of the largest peak. The shoulder area for which a value is given most likely represents both native and aggregated protein, but the actual levels of each cannot be determined. Thus, the value given represents an overestimate of the actual level of native protein. *B*, thioflavin T fluorescence of 10 $\mu\text{g/ml}$ protein samples (see "Experimental Procedures"). Results are shown for samples incubated at 1 mg/ml: BIF in PBS alone (\bullet); BIF in PBS with 1.0 M sucrose (\blacksquare); BIF in PBS with 1.0 M urea (\blacktriangle); GAL in PBS alone (\circ); GAL in PBS with 1.0 M sucrose (\square); GAL in PBS with 1.0 M urea (\triangle). Results are also shown for samples incubated at 10 mg/ml: BIF in PBS with 1.0 M urea (\blacklozenge); GAL in PBS with 1.0 M urea (\diamond).

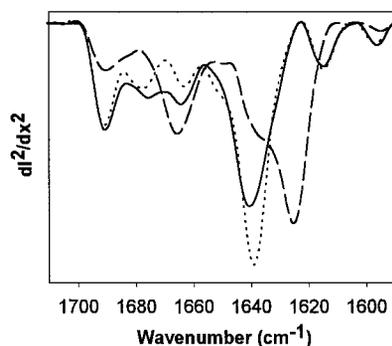


FIG. 4. Second derivative amide I infrared spectra for native BIF (solid line), BIF fibrils (dashed line), and native GAL (dotted line).

ing, the fibrils exhibited green birefringence when viewed under cross-polarizers (data not shown). The fibrils had a characteristic intermolecular β -sheet structure as documented by a strong band at 1625 cm^{-1} (24) in their second derivative infrared spectrum (Fig. 4). There was also a partial loss of native intramolecular β -sheet structure, as indicated by the decrease in absorbance at 1638 cm^{-1} . Quantitative analysis of the spectra (16) showed that the fibrils contained 38% intermolecular β -sheet, 27% intramolecular β -sheet, 32% turns, and 3% random elements. The native BIF protein contained 62% intramolecular β -sheet, 5% α -helix, and 33% turn structures (Fig. 4). Native GAL had 61% intramolecular β -sheet, 13% α -helix, and 26% turn structures (Fig. 4). The two proteins also had similar

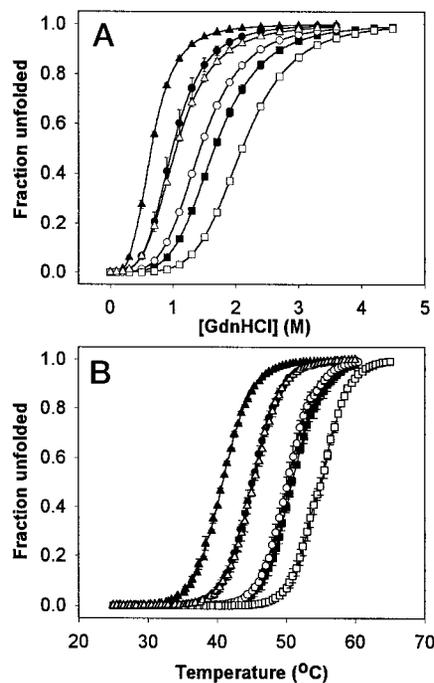


FIG. 5. Guanidinium HCl (*A*) and thermal (*B*) unfolding curves for BIF and GAL. Solution conditions and symbols used are the same as those described in the legend for Fig. 3.

secondary structural contents based on far UV circular dichroism spectroscopy (data not shown).

Comparison of Physical Properties of BIF and GAL—In PBS, BIF unfolded at lower guanidinium concentrations and had a lower free energy of unfolding compared with GAL (Fig. 5; Table I). Also, the apparent midpoint and onset temperatures for thermal unfolding were about 5 °C lower for BIF than for GAL (Fig. 5; Table I).

The H-D exchange rates of the two proteins also differed. Immediately upon placing the proteins into 75% D_2O PBS, there were shifts in the amide I bands because of the exchange of hydrogen for deuterium in residues at the protein surface (Figs. 4, 6). Over the time course of 24 h, there was continued exchange, which can be ascribed to residues that are near the surface of individual molecules (16, 25, 26). The exchange rate was much more rapid for BIF than GAL (Fig. 6), indicating that BIF had a higher level of partially folded species in its molecular population (13, 16, 25, 27).

Effect of Sucrose on Fibril Formation and Protein Thermodynamic Stability—With 1.0 M sucrose, the thermodynamic stability of BIF was increased to that measured for GAL in PBS alone (Fig. 5 and Table I). As a result, there was complete inhibition of loss of native BIF protein during the same experiment in which 80% of the native protein was lost in PBS alone (Fig. 3A). Furthermore, no fibrils were detected (Fig. 3B). Sucrose (1.0 M) also increased the thermodynamic stability of GAL (Fig. 5 and Table I), and loss of neither native protein nor fibrils was detected (Fig. 3).

In addition to increasing the free energy barrier between native and unfolded states (14, 15), sucrose also shifts the equilibrium between protein states toward the most compact native conformation and away from expanded species and, hence, reduces the H-D exchange rate (16, 27, 28). With BIF, 1.0 M sucrose reduced the rate of exchange to approximately that noted with nonpathological GAL in PBS alone (Fig. 6). Sucrose also reduced the exchange rate for GAL (Fig. 6).

Effect of Urea on Fibril Formation and Protein Thermodynamic Stability—To test whether reducing thermodynamic sta-

TABLE I
Thermodynamic parameters of BIF and GAL determined from unfolding curves

Protein	Buffer	$-m$	C_m^a [GdnHCl]	ΔG_{unf}^b	T_m^c
		Kcal/mol/M		Kcal/mol	°C
BIF	Buffer	2.51 ± 0.04	1.05 ± 0.06	2.63 ± 0.18	45.1 ± 0.35
	1.0 M sucrose	1.70 ± 0.08	1.70 ± 0.02	4.25 ± 0.08	51.0 ± 0.06
	1.0 M urea	3.33 ± 0.18	0.72 ± 0.01	1.80 ± 0.05	40.8 ± 0.10
GAL	Buffer	1.85 ± 0.04	1.48 ± 0.01	3.71 ± 0.08	50.2 ± 0.25
	1.0 M sucrose	1.47 ± 0.13	2.13 ± 0.02	5.35 ± 0.07	54.6 ± 0.23
	1.0 M urea	2.01 ± 0.08	1.09 ± 0.02	2.72 ± 0.08	45.8 ± 0.17

^a C_m was calculated by complex sigmoid, nonlinear regression analysis.

^b ΔG_{unf} was calculated using BIF in PBS buffer as reference state with: $\Delta G_{\text{unf}} = \Delta G_{\text{unf}}^{\text{BIF in PBS}} + (m^{\text{BIF in PBS}} \times (C_m - C_m^{\text{BIF in PBS}}))$.

^c The midpoint of the thermal transition is referred to as the apparent midpoint, because thermally induced unfolding was only ~80% reversible.

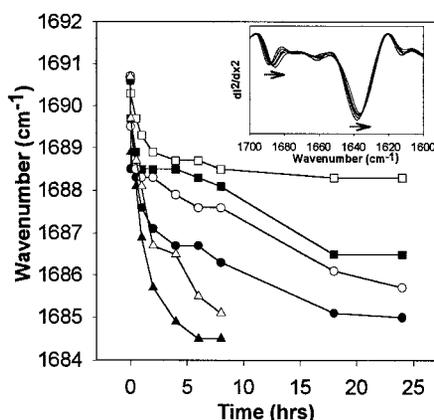


FIG. 6. Changes in the position of the high frequency β -sheet band as a function of time of exposure to 75% D_2O . Solution conditions and symbols used are the same as those described in the legend for Fig. 3. *Inset*, changes in the second derivative amide I spectra of BIF in PBS as a function of time of exposure to 75% D_2O PBS. *Arrows* indicate the direction of shifts in component bands.

bility would foster protein aggregation and fibril formation, we investigated the effects of 1.0 M urea on BIF and GAL. Size exclusion chromatography of BIF and GAL immediately after exposure to 1.0 M urea resulted in elution profiles identical to those determined for respective protein samples in PBS alone, documenting the fact that urea did not cause any immediate formation of soluble or insoluble aggregates (Fig. 2 and data not shown). With BIF, after 1–4 days of incubation at 37 °C, there were greater losses of native, soluble protein than noted in samples prepared in PBS alone (Figs. 2 and 3A), which were due to formation of insoluble fibrils (Fig. 3B). From days 2–5, the levels of fibrils remained constant. However, there was a progressive decrease in the level of soluble native protein (Figs. 2B and 3A), attributed to the formation of higher molecular weight soluble aggregates, which eluted earlier during size exclusion chromatography than did native protein molecules (Fig. 2B). During incubation of GAL at 37 °C, the elution profile was shifted to earlier time points, but minimally changed in shape and intensity (Fig. 1C), and fibrils were not detected (Fig. 3B). These results suggest that the protein GAL was forming higher molecular weight soluble aggregates but was not precipitating from solution.

Urea (1.0 M) reduced the thermodynamic stability of BIF and GAL (Fig. 5 and Table I). The unfolding curves for GAL in 1.0 M urea were almost at the same positions as those for BIF in PBS alone. In addition, 1.0 M urea greatly accelerated the H-D exchange rate of both proteins (Fig. 6). Data are shown only for 8 h of incubation, because for the 18- and 24-h time points, the infrared spectra of both BIF and GAL indicated the presence of non-native protein aggregates containing intermolecular β -sheet (data not shown). During the 24-hour incubation, intermolecular β -sheet bands were not detected in spectra for

samples of either protein in PBS alone or in 1.0 M sucrose (data not shown). The H-D exchange experiments were conducted at protein concentrations of 10 mg/ml. To determine how aggregates formed at this concentration compared with those formed in the initial experiments at 1 mg/ml protein concentration, 10 mg/ml solutions of BIF and GAL in 1.0 M urea (prepared in H_2O -PBS) were incubated at 37 °C for 24 h. Analysis of the BIF sample by size exclusion chromatography documented a substantial fraction of higher molecular weight soluble aggregates (data not shown). There was also a relatively high level of insoluble fibrils (Fig. 3B) and a 40% loss of native, soluble protein (Fig. 3A). With GAL, there was a 20% loss of native, soluble protein (Fig. 3A) because of fibril formation (Fig. 3B) but not formation of higher molecular weight soluble aggregates (data not shown).

DISCUSSION

Our results document the fact that light chain BIF forms fibrils at 37 °C *in vitro* because of its low free energy of unfolding, relative to protein GAL, which neither formed amyloid deposits *in vivo* nor fibrils *in vitro* in PBS alone. Thus, fibril formation by intact light chains occurs because of the relatively low thermodynamic stability of the native state, as found in studies of recombinant V_L , as well as transthyretin and lysozyme (8–13). BIF also had a much greater rate of H-D exchange than GAL, indicating that BIF molecules have native state conformational fluctuations of greater amplitude (25, 26). Thus, there is an increased probability that BIF molecules will be in the appropriate conformation to foster non-native intermolecular interactions when encountering neighboring molecules. From a state equilibrium viewpoint, as has previously been suggested for amyloid-forming variants of lysozyme, BIF at any instant in time has a greater fraction of partially folded species in its molecular population than does GAL (13, 25, 26). It is well established that non-native protein aggregates are formed from partially folded molecules, which are sparsely and transiently populated relative to the most compact native conformation with which they are in equilibrium (13, 27–31). With the relatively unstable amyloidogenic protein variants, there are sufficiently high levels of partially folded molecules that, during the time scale for amyloid fibril formation of days to weeks or longer, fibrils can form even in nonperturbing environments.

Thus, a rational strategy for inhibiting amyloid fibril formation is to use extrinsic factors to increase native state stability and increase the thermodynamic barrier between the most compact native state and aggregation-competent, partially folded species (27, 28, 32, 33). With BIF, 1.0 M sucrose sufficiently increased the thermodynamic stability so that the resistance of this protein to amyloid fibril formation was equivalent to that of the nonpathological light chain GAL. Sucrose increases the thermodynamic stability of proteins because it is preferentially excluded from their surface, which increases the chemical potential of proteins (14, 15). The magnitudes of ex-

clusion and increase in chemical potential are directly proportional to the surface area exposed to solvent and are independent of the chemical properties of the side chains of exposed residues (14–16, 34). Sucrose increases the free energy barrier between the native and partially or fully unfolded states, because the latter have greater surface area and, hence, greater increase in chemical potential than the native state (14–16, 27, 28, 34). Thus, sucrose shifts the equilibrium between states toward the most compact native state and away from aggregation-competent molecules (16, 27, 28). Because these effects are independent of protein properties, thermodynamic stabilization and inhibition of aggregation by sucrose is generally applicable to soluble, globular proteins (14–16, 27, 28, 34).

Conversely, urea preferentially binds to proteins and lowers their chemical potential (15). Partially and fully unfolded states have a greater surface area for binding than the native state and, hence, are more greatly populated in urea than in buffer alone (15). In 1.0 M urea, the thermodynamic stability and H-D exchange rate of GAL were rendered equivalent to those measured for BIF in PBS alone. During incubation at 1 mg/ml, this destabilization caused a loss of native GAL due to formation of soluble aggregates. Increasing protein concentration to 10 mg/ml led to the formation of amyloid fibrils. Similar results were also noted with BIF. In general, the propensity and rate of forming non-native protein aggregates vary directly with protein concentration (29). This relationship, as well as destabilization by urea, may be important for the frequent renal deposition of light chain amyloid fibrils (6). The protein GAL did not form deposits as amyloid *in vivo*, perhaps because the concentrations of GAL or urea in the extracellular space of the kidney were not sufficiently high. However, there may be cases in which the intrinsic thermodynamic stability of the light chain is low enough that fibril formation will be favored in the kidney because of destabilization by urea. Also, other factors such as relatively high concentrations of NaCl and acidic pH may contribute to renal fibril deposition (17, 35).

In conclusion, amyloid fibril formation by light chains and other amyloidogenic proteins is predominantly controlled by the thermodynamic stability of the protein molecules. Thus, a rational therapeutic strategy to inhibit amyloidosis is to design ligands that bind with high affinity and specificity to the native protein (32, 33). Relative to the effects of 1.0 M sucrose, stoichiometric amounts these compounds greatly increase the thermodynamic stability of the native state and reduce the levels of partially and fully unfolded protein molecules (15, 32, 33, 36, 37). In this regard, naturally occurring stabilizing ligands have been shown to inhibit amyloid fibril formation by transthyretin (32, 33), as well as, for example, the formation of insoluble aggregates by acid-fibroblast growth factor (36). For most amyloidogenic proteins, high affinity ligands have not been identified but can be designed using combinatorial library screening and protein structure-based methods (*e.g.* Refs. 38 and 39). Prior to the development of these compounds, a powerful approach is the use of sucrose for a rapid test of the importance of thermodynamic stabilization for inhibiting fibril formation.

This strategy is applicable to any amyloidogenic system for which the starting material is a structured, globular protein.

Acknowledgments—We thank Dr. Ronald Wetzel for insightful advice and Kim Wightman for assistance in circular dichroism spectroscopic measurements.

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