Effect of Association State and Conformational Stability on the Kinetics of Immunoglobulin Light Chain Amyloid Fibril Formation at Physiological pH

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Light chain amyloidosis involves the systemic deposition of fibrils in patients overproducing monoclonal immunoglobulin light chains. The kinetics of fibril formation of LEN, a benign light chain variable domain, were investigated at physiological pH in the presence of urea. Despite the lack of in vivo fibril formation, LEN readily forms fibrils in vitro under mildly destabilizing conditions. The effect of low to moderate concentrations of urea on the conformation, association state, stability, and kinetics of fibrillation of LEN were investigated. The conformation of LEN was only slightly affected by the addition of up to 4 M urea. The fibrillation kinetics were highly dependent on protein and urea concentrations, becoming faster with decreasing protein concentration and increasing urea concentration. Changes in spectral probes were concomitant to fibril formation throughout the protein and urea concentration ranges, indicating the absence of off-pathway oligomeric species or amorphous aggregates prior to fibril formation. Reducing the amount of dimers initially present in solution by either decreasing the protein concentration or adding urea resulted in faster fibril formation. Thus, increasing concentrations of urea, by triggering dissociation of dimeric LEN, lead to increased rates of fibrillation.

Amyloidoses arise from the formation of insoluble ordered aggregates (fibrils with cross-β structure) from normally soluble proteins. Fibril formation has been shown to originate from partially folded intermediates following either the partial destabilization of physiologically folded proteins, in the case of globular proteins (1), or the partial stabilization (i.e. folding) of random coil polypeptide chains, in the case of natively unfolded proteins (2). Light chain amyloidosis arises from the overproduction and abnormal deposition of light chain protein aggregates in various organs and more particularly in the kidneys (3). LEN is the variable domain of an immunoglobulin light chain (κLV Bence Jones protein) originally isolated from the urine of a patient suffering from multiple myeloma, with no sign of renal dysfunction or amyloidosis (4). However, LEN was shown to form fibrils in vitro under vigorous stirring. It is composed of 114 residues with, under native conditions, the classical sandwich fold of the immunoglobulin subunits. In addition, variable domains usually self-associate to form relatively tight dimers. Under native conditions, the self-dissociation constant of LEN was determined to be 10 µM at neutral pH (4). The importance of the variable domains in the association process of immunoglobulin light chains has long been recognized (5, 6). Furthermore, the critical role in the association process of the Pro96 of the CDR3 region of the variable domain has been demonstrated (7). Dissociation constants between 1 µM and 1 mM have been measured for various light chains and for the self-association of variable domains (8). Based on our previous study (9) of LEN fibrillogenesis, the monomer/dimer equilibrium appeared to play a critical role in both the kinetics of fibril formation and the selective formation of different aggregated species along a complex branched pathway. Despite the poor understanding of in vivo factors responsible for the abnormal formation of insoluble fibrils (10), the involvement of accessory and physiological factors is almost certain (11). However, the presence of such factors is often under-investigated during in vitro experiments. In this study, we studied the effect of low to moderate concentrations of urea (up to 4 M) on the conformation, association state, and stability of LEN as a mean of dissociating and destabilizing the protein. Urea was chosen in this study not only for its destabilizing effect on globular proteins (12) but also its normal physiological presence in the kidneys. Various biophysical methods, such as near- and far-UV CD, intrinsic and ANS fluorescence, acrylamide quenching, small-angle X-ray scattering, and thermal stability were used to monitor conformational and association changes. Furthermore, these observations were correlated with the kinetic parameters of fibril formation (monitored by an increase in ThT fluorescence intensity).

MATERIALS AND METHODS

Chemicals—Ultra-pure urea was purchased from ICN Biomedicals. Acrylamide was purchased from Ampresco. HEPES was purchased from Sigma. Peptone and yeast extract used in the medium were purchased from Difco. All other chemicals were purchased from Fisher and were of the highest grade available. The water was doubly deionized.

LEN Purification—LEN was overexpressed using a frozen stock of Escherichia coli JM83 cloned with the plasmid pKIVlen004, the expression system generously given by Dr. F. Stevens (4). LEN was purified using the procedure described previously (9). The sucrose and water extracts were pooled together, dialyzed against a 10 mM Tris buffer, pH 8.0, and eluted through a Mono Q column (Bio-Rad) to remove some protein impurities. After dialysis against a 10 mM acetate buffer, pH 7.0.

The abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; ThT, thioflavin T; SAXS, small angle x-ray scattering; ATR-FTIR, attenuated total reflectance-Fourier transform infrared spectroscopy.

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4.0, the flow-through was loaded on a Mono S column (Amersham Biosciences), and LEN was eluted using a NaCl gradient from 0 to 120 mM over 20 min. The purest fractions were pooled, dialyzed against 10 mM phosphate buffer solution, pH 8.0, and concentrated by ultrafiltration to about 4 mg/ml. LEN purity and conformation were assessed by SDS-gel electrophoresis, mass spectroscopy, near-UV CD, and thin film ATR-FTIR. No impurity bands could be detected on SDS gels when loaded at a concentration of ~4 mg/ml.

**In Vitro Fibil Formation Procedure—**In vitro fibril formation was studied by incubating, at 37°C with stirring, solutions of different concentrations of LEN at pH 7 (20 mM HEPES and 100 mM NaCl) and various urea concentrations (from 0 to 4 M urea). Sodium azide (0.02%) was added to the solutions to avoid bacterial growth. The stirring rate was maintained constant throughout this study and was estimated at ~660 rpm. Solutions of 3 (237 μM), 1 (79 μM), 0.5 (40 μM), 0.1 (7.9 μM), and 0.01 mg/ml (0.79 μM) were the solutions. The solutions, with volume ranging from 500 to 650 μl, were stirred in 2-ml high pressure liquid chromatography glass vials (Fisher) containing micro-stirrer bars (8 × 1.5 mm, Fisher). Aliquots of different volumes (depending on the analytical method used and the protein concentration) were withdrawn at predetermined time points to follow the kinetics of fibril formation.

**Protein Stability toward Urea—**The stability of LEN at pH 7 (20 mM HEPES and 100 mM NaCl) was measured at two different protein concentrations, 0.01 (0.79 μM) and 0.5 (40 μM) mg/ml. Protein stability was assessed by following LEN intrinsic fluorescence intensity after incubation in increasing amounts of urea (from 0 to 8 M). At 0.01 mg/ml (0.79 μM), each point was determined on a different solution prepared volumetrically. The samples were incubated for 1 h at room temperature to ensure complete unfolding equilibrium. The final protein concentration was 0.01 mg/ml. The fluorescence measurements were performed using a FluoroMax-2 fluorescence spectrophotometer (Jobin Yvon-Spek) with an excitation wavelength of 280 nm (5 nm band pass) and recording the emission spectra from 300 to 420 nm (5 nm band pass).

At 0.5 mg/ml (40 μM), all points were measured on the same protein solution, small aliquots of solid urea being added stepwise to the solution. After each addition of solid urea, the solution was gently shaken until complete dissolution of the urea (~30 s) and then was incubated for 45 min at room temperature to ensure unfolding and temperature equilibria. The fluorescence measurements were performed using a FluoroMax-2 fluorescence spectrophotometer (Jobin Yvon-Spek) with an excitation wavelength of 280 nm (3 nm band pass) and recording the emission spectra from 300 to 420 nm (3 nm band pass). The urea concentration of the protein solution was measured before each new addition of solid urea by measuring the refractive index (Spectronic Instrument Inc.) on 5-μl aliquots.

Assuming a two-state folding mechanism, the fraction of the unfolded conformation (f_u) was obtained using Equation 1,

\[ f_u = \frac{y_i - y_f}{y_f - y_i} \]

where \( y_f \) and \( y_i \) represent the tryptophan fluorescence intensity characteristic of the folded and unfolded conformations, respectively, under the conditions where \( y \) is being measured (13). The values of \( y_f \) and \( y_i \) were obtained by linear regression on the data points before and after the unfolding transition, respectively. A sigmoidal curve-fit was subsequently used to determine the midpoints of the unfolding transition (C_{u0}).

**Protein Thermal Stability—**The thermal stability of LEN at pH 7 (20 mM HEPES and 100 mM NaCl) was studied at various urea concentrations (from 0 to 4 M urea). At 0.01 mg/ml (0.79 μM) was the solution. The solutions, with volume ranging from 500 to 650 μl, were stirred at 37°C with stirring to ensure complete equilibrium. The final protein concentration was 0.01 mg/ml. The fluorescence measurements were performed using a FluoroMax-2 fluorescence spectrophotometer (Jobin Yvon-Spek) with an excitation wavelength of 280 nm (3 nm band pass) and recording the emission spectra from 300 to 420 nm (3 nm band pass).

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**Circular Dichroism Measurements—**CD measurements were performed using an Aviv model 60DS spectropolarimeter (Aviv). Far-UV CD spectra of LEN solutions at pH 7 (20 mM HEPES and 100 mM NaCl) and various urea concentrations (from 0 to 4 M urea) were recorded at a protein concentration of 3 mg/ml (237 μM) from 250 to 200 nm using a circular cell with a 0.1-cm path length. Near-UV CD spectra of LEN solutions at pH 7 with various urea concentrations were recorded from 320 to 250 nm at 3 (237 μM) and 0.1 (7.9 μM) mg/ml using a rectangular cell with a 0.1-cm path length and a circular cell with a 1-cm path length, respectively. After buffer subtraction, the CD spectra were corrected for protein concentration and smoothed using the Savitsky-Golay function (using a second order polynomial function with 5 points).

**Intrinsic, ANS, and ThT Fluorescence—**Fluorescence measurements were performed using a FluoroMax-2 fluorescence spectrophotometer (Jobin Yvon-Spek). All measurements were performed at 37°C with final protein concentrations ranging from 0.015 to 0.001 mg/ml, depending on the initial experimental protein concentrations. Intrinsic fluorescence measurements were performed using an excitation wavelength of 280 nm and recording the emission spectra from 300 to 420 nm. ANS fluorescence measurements were performed using an excitation wavelength of 380 nm and recording the emission spectra from 420 to 600 nm. ThT fluorescence measurements were performed using an excitation wavelength of 450 nm and recording the emission spectra from 465 to 560 nm. Solutions of 10 μM ANS and ThT at pH 7 (20 mM HEPES and 100 mM NaCl) were used throughout the study. The ANS/protein and ThT/protein molar ratios varied from 8.4 to 1140, depending on the initial experimental protein concentrations. Among different kinetic experiments, band pass adjustments were performed to compensate for eventual changes in the final protein concentrations. The various fluorescence measurements (intensity and \( \lambda_{max} \) position) were plotted versus time and fitted by a sigmoidal curve allowing slope variations of the initial and final phases (14). Comparison of the kinetics among different samples was performed by using the midpoints of the transition (C_i) obtained from curve fitting.

**Small Angle X-ray Scattering—**X-ray scattering data were collected at the Stanford Synchrotron Radiation Laboratory beam line 4-2. The instrument was configured with a MoCB4 multilayer monochromator, an 18-mm beam stop, and a 218-μm sample-to-detector distance. All measurements were performed using a static cell with a 1.3-mm path length. A radial integration routine (N-terminitely, the N-oxide), at a final concentration of 10 mM, was added to the protein sample before each measurement to eliminate radiation damage. All measurements were performed at room temperature. The radii of gyration were calculated from the Guinier approximation using Equation 2.

\[ I(S) = I(0) \exp \left(-\frac{4\pi^2 R_i^2 S^2}{3}\right) \]

where \( S \) is the scattering vector, \( S = 2\sin(\theta/2) \) is the x-ray wavelength, and \( I(0) \) the scattering amplitude from the sample in the opposite direction to that of the incident beam (15).

**Effect of Urea on Conformation and Association State of LEN—**SAXS measurements were performed on LEN solutions of 3 mg/ml (237 μM) at pH 7 (20 mM HEPES and 100 mM NaCl) and various urea concentrations (from 0 to 4 M urea). Samples were incubated for 1 h at 37°C in 2-ml glass vials. SAXS measurements were performed on aliquots withdrawn at regular intervals over the first 9 h of stirring.

**Hydrated Thin Film Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)—**FTIR spectra were recorded using a Nicolet 800 spectrophotometer from 4000 to 400 cm\(^{-1}\) at 4 cm\(^{-1}\) resolution and an accumulation of 512 interferograms. The system was continuously purged with dry air. 28-μl aliquots of LEN solution at 3 mg/ml (237 μM) and pH 7 (20 mM HEPES and 100 mM NaCl) and pH 7 (20 mM HEPES and 100 mM NaCl) were evenly dried on the surface of a germanium crystal using nitrogen at predetermined time points during kinetic experiments. Background and water vapor subtractions were performed until a straight base line was obtained between 2000 and 1750 cm\(^{-1}\).

**Acrylamide Quenching—**Quenching experiments were performed on LEN samples at pH 7 (20 mM HEPES and 100 mM NaCl) and various urea concentrations (from 0 to 4 M urea) by following the intrinsic fluorescence intensity of protein solutions (0.15 mg/ml or 12 μM) upon adding a series of 5-μl aliquots of a 3 M acrylamide solution. The fluorescence measurements were performed using a FluoroMax-2 fluorescence spectrophotometer (Jobin Yvon-Spek) with an excitation wavelength of 280 nm (3 nm band pass) and recording the emission spectra from 320 to 420 nm (3 nm band pass). Fluorescence intensities were
further corrected for dilution due to the stepwise addition of acrylamide. The values of the Stern-Volmer constant ($K_{SV}$) or dynamic quenching were obtained by curve-fitting the experimental data to Equation 3,

$$F/F_0 = (1 + K_{SV}[A]) \times \exp(K_{SV}[A])$$

(Eq. 3)

where $F_c$ and $F$ are the fluorescence intensities in the absence and presence of acrylamide, respectively; $[A]$ is the concentration of acrylamide, and $K_{SV}$ represents the static quenching constant responsible for a slight upward curvature. Because similar protein/acrylamide ratios were used throughout the experiment, the presence of similar static quenching between acrylamide and LEN (under the various urea concentrations) was assumed. Thus, the same value of $K_{SV}$ was used for the curve fitting of all data.

RESULTS

Stability of LEN at pH 7, Urea Unfolding Transitions—The urea unfolding of LEN at pH 7 was studied at two different protein concentrations: 0.01 and 0.5 mg/ml (Fig. 1). At 0.01 mg/ml (0.79 $\mu$m), where LEN is mostly monomeric (based on a $K_u$ value of 10 $\mu$m), the $C_m$ was 5.4 $\pm$ 0.1 $\mu$m. On the other hand, at 0.5 mg/ml (4 $\mu$m), a concentration at which about 70% of LEN exists as a dimer under native conditions, the $C_m$ was 6.0 $\pm$ 0.1 $\mu$m. Furthermore, in both cases, no significant changes in intrinsic fluorescence intensity were observed below 4 $M$ urea.

Temperature Denaturation Transitions—The temperature denaturation of LEN at pH 7 in various urea concentrations (up to 4 $M$) was investigated at a protein concentration of 0.01 mg/ml (0.79 $\mu$m) (Fig. 2). The reversibility of the denaturation transition was also studied by following the fluorescence intensity of the protein samples upon cooling (data not shown). Without urea, the $T_m$ value of LEN was 69.9 $\pm$ 0.1°C. Upon cooling, a similar transition temperature was observed (61.0 $\pm$ 0.1°C), indicating the reversible character of the denaturation transition. With increasing urea concentrations from 1 to 4 $M$, the $T_m$ values were 55.6 $\pm$ 0.1, 53.0 $\pm$ 0.1, 47.2 $\pm$ 0.6, and 39.1 $\pm$ 1.9°C, respectively, indicating a significant and progressive decrease in the stability of LEN. Interestingly, the transition temperatures observed upon cooling LEN solutions in urea (data not shown) were 5–10°C lower than the corresponding $T_m$ values obtained upon heating the samples. The presence of hysteresis between the heating and cooling transition temperatures measured in urea is probably due to high temperature-induced self-association, although it could also reflect slow refolding in the presence of urea. It is noteworthy that in the absence of urea the transition is completely reversible, with no hysteresis.

Structural Characterization of LEN at pH 7 and Various Urea Concentrations, LEN Conformation—The effect of urea (up to 4 $M$) on LEN conformation at pH 7, 23°C, was investigated using near- and far-UV CD, intrinsic fluorescence, acrylamide quenching, and small angle x-ray scattering.

The near-UV CD spectra of LEN were recorded at 3 mg/ml (Fig. 3a) and 0.1 mg/ml (data not shown). The near-UV CD spectrum of LEN at pH 7, 0.1 M urea) was characterized by several maxima (Fig. 3a). Upon adding increasing urea concentrations, a significant and progressive decrease of the intensities of three maxima at 297, 289, and 280 nm was observed. These maxima were, however, still observed in the spectrum obtained for the sample containing 4 $M$ urea, indicating that the tertiary structure of LEN, although slightly modified, was still maintained under these conditions. Similar changes with increasing urea concentrations were also observed for the CD spectra obtained at 0.1 mg/ml (7.9 $\mu$m) (data not shown).

The far-UV CD spectrum of LEN was not significantly affected by the addition of urea (Fig. 3b). The spectrum of native LEN (pH 7, 0 $M$ urea) was characterized by two minima at 219 and 235 nm. The 235 nm minimum, due to the presence of aromatic clusters (16, 17), was not changed upon adding urea. However, in the native state the fluorescence contribution of Trp$^{35}$ is significantly solvent-exposed (18). Thus, in the native state, the fluorescence contribution of Trp$^{35}$ is completely quenched by the spatial proximity of the disulfide bridge Cys$^{23}$–Cys$^{94}$. Thus, under native conditions, the intrinsic fluorescence of LEN results from the solvent-exposed Trp$^{35}$, as illustrated by the high $\lambda_{max}$ value of 349 nm at pH 7 in the absence of urea (Fig. 3c). The intrinsic fluorescence of LEN was not affected by the addition of 4 $M$ urea (Fig. 3c); neither the fluorescence intensities nor the $\lambda_{max}$ positions were significantly different. For comparison, the
respectively. This slight and progressive increase of the Stern-Volmer constant indicated the increased solvent accessibility of the fluorophores. However, these values were significantly lower than the value obtained previously ($8.99 \pm 0.06 \text{m}^{-1}$) for unfolded LEN (9), indicating that only a slight rearrangement of the environment of the fluorophores occurred upon adding up to 4 M urea.

Association State of LEN at pH 7—Small angle x-ray scattering (SAXS) profiles were obtained for LEN solutions at pH 7 and increasing urea concentrations. The radii of gyration ($R_g$) and forward scattering values ($I(0)$) were obtained from the Guinier and Kratky plots (Fig. 3c). The $R_g$ value of LEN at 3 mg/ml, pH 7, without urea was 18.1 ± 0.3 Å, corresponding to the size of the native dimer of LEN (2). The addition of urea (up to 4 M) led to a decrease in the $R_g$ values; the values of the samples containing 1–4 M urea were 18.1 ± 0.3, 17.6 ± 0.5, 17.0 ± 0.5, and 16.4 ± 0.6 Å, respectively. Concurrently, a progressive and significant decrease in $I(0)$ was observed with increasing urea concentrations (from 0.0167 ± 0.0002 in the absence of urea to 0.0163 ± 0.0002, 0.0127 ± 0.0002, 0.0101 ± 0.0002, and 0.0079 ± 0.0002 in 1–4 M urea, respectively), clearly indicating the urea-induced dissociation of LEN dimers.

Information on the globularity of proteins can also be extracted from SAXS profiles when represented as a Kratky plot, a bell-shape curve being representative of a globular protein (Fig. 3f). A bell-shape curve was still observed for the LEN sample containing 4 M urea, clearly indicating the globular character of the species in solution. Interestingly, both the decrease in intensity and the slight shift toward larger angle of the peak maximum between the signals obtained for the samples with and without 4 M urea were another indication of the dissociation of the dimer.

Kinetics of LEN Fibril Formation at pH 7—The kinetics of fibril formation were monitored by following the increase in fluorescence intensity of the fluorescent dye thioflavin T (ThT) upon interaction with fibrils (22–24). Furthermore, the kinetics of conformational changes were also monitored over time using intrinsic fluorescence and ANS binding. LEN solutions were incubated with stirring at 37 °C, pH 7 (20 mM HEPES and 100 mM NaCl), and aliquots were withdrawn at pre-determined time points to follow changes in the various parameters probed by these biophysical techniques.

Kinetics at pH 7 and 0 M Urea—The kinetics of fibril formation of LEN at pH 7 were investigated as a function of protein concentration (0.01, 0.1, 0.5, 1, and 3 mg/ml). As the LEN concentration was increased, the kinetics of fibril formation became slower (Fig. 4b). The midpoint of the ThT transition ranged from 21.1 to 94.1 h with LEN concentration ranging from 0.01 to 3 mg/ml (Table I). No changes in the intrinsic fluorescence nor in the ANS binding were observed before fibril formation throughout the protein concentration range examined. This absence of structural rearrangements of LEN at the beginning of the stirring/incubation period was confirmed by small angle x-ray scattering experiments. No significant changes in either the $R_g$ or $I(0)$ values were detected during the first 9 h of stirring (data not shown). However, concomitant to fibril formation (as monitored by ThT fluorescence), significant changes in intrinsic and ANS fluorescence occurred at all protein concentrations (Fig. 5a, for the 0.1 mg/ml case). Both a significant decrease in the maximum position (from 349 to 338 nm) and an increase in intensity of the intrinsic fluorescence were observed simultaneously to similar changes in ANS fluorescence (Fig. 5a). FTIR spectra were collected during the kinetics of fibril formation of LEN at 3 mg/ml (237 μs). The formation of intermolecular β-structures upon fibril formation is readily detected by FTIR. Despite the fact that LEN is mainly a β-protein, significant changes in the β-peak position were observed in 8 M urea.
during the kinetics experiment, probably due to the rearrangement of the β-sheets to a more hydrogen-bonded structure upon fibril formation (Fig. 6). A significant decrease toward lower wave numbers of the amide I peak maxima (from 1638 to 1632 cm⁻¹) was observed concomitantly to fibril formation, no changes being observed in the early stages of the incubation/stirring period.

**Kinetics at pH 7 and 1 M Urea**—The kinetics of LEN fibril formation in the presence of 1 mM urea were also studied over the protein concentration range from 0.01 to 3 mg/ml. The kinetics of the ThT transition ranging from 0.01 to 1 mg/ml (Fig. 4b). At all protein concentrations, no changes in the conformational probes (intrinsic and ANS fluorescence) were detected before fibrils started to form, significant changes in the intrinsic fluorescence intensity occurring only concurrently with the ThT fluorescence intensity increase (Fig. 5b, for the 0.1 mg/ml case). The absence of formation of oligomeric species in the early stages of the incubation/stirring period was also confirmed by SAXS experiments, no changes in Rg or I(0) values being detected over the first 9 h (data not shown).

**Kinetics at pH 7 and 2 M Urea**—The kinetics of LEN fibril formation in the presence of 2 mM urea were significantly faster than those of ≤1 mM urea (Fig. 4). The midpoints of the ThT transition ranged from 8.9 to 56.0 h with protein concentrations ranging from 0.01 to 3 mg/ml (Table I). Furthermore, the midpoints of the ThT transitions changed only slightly at low protein concentrations (from 0.01 to 1 mg/ml). Again, throughout the protein concentration range, no changes in intrinsic or ANS fluorescence were observed before the formation of fibril monitored by ThT fluorescence. Significant changes in these conformational probes only occurred concomitantly to fibril formation (Fig. 5c, for the 0.1 mg/ml case).

**Kinetics at pH 7 and 3 M Urea**—The kinetics of fibril formation in 3 M urea were faster than those in 2 M (Fig. 4b), the midpoints of the ThT transitions ranging from 5.0 to 35.1 h with concentration ranging from 0.01 to 3 mg/ml (Table I). Interestingly, no significant increase in the midpoint values were observed from 0.01 to 1 mg/ml. Once again, changes in intrinsic and ANS fluorescence were only observed upon fibril formation (Fig. 5d, for the 0.1 mg/ml case).

**Kinetics at pH 7 and 4 M Urea**—Except for the 3 mg/ml case, the kinetics of fibril formation in 4 M urea were similar to those observed in 3 M (Fig. 4b). The midpoints of the ThT transitions ranged from 3.2 h at 0.01 mg/ml to 12.1 h at 3 mg/ml (Table I). Changes in intrinsic and ANS fluorescence were detected concomitantly with fibril formation (Fig. 5e, for the 0.1 mg/ml case).

**DISCUSSION**

**Decrease in Stability Versus Dissociation**—Based on a Kd value for LEN of 10 μM (4) under native conditions (pH 7), 70 and 12% of the protein are dimeric at 0.5 (40 μM) and 0.01 (0.079 μM) mg/ml, respectively. The Cm value of LEN at pH 7 was 5.4 ± 0.1 M at 0.01 mg/ml and 6.0 ± 0.1 M at 0.5 mg/ml. This significant difference in urea stability at two different concentrations indicated that (i) not all dimers were dissociated prior to the unfolding of LEN and (ii) that the dimers were significantly more stable than the monomers. Furthermore, a significant decrease in the Tm value of LEN was detected as the concentration of urea was increased from 0 to 4 M urea. The decrease in Tm, which is affected by both dissociation and intrinsic destabilization, probably reflects a larger contribution from dissociation rather than a significant decrease in intrinsic stability, because no significant changes in intrinsic fluorescence were observed up to 4 M urea (as observed from the urea-induced unfolding transitions). Due to the quenching of Trp35, in the native state, by the neighboring disulfide bridge, even a small conformational rearrangement of LEN can be detected by an increase in fluorescence intensity. For example, at pH 2, where LEN still has a native-like structure, a detectable increase in intrinsic fluorescence intensity was observed due to the spatial separation of the disulfide bridge from Trp35 despite the limited conformational rearrangement (9). Thus, in the presence of a small amount of urea, a significant decrease in intrinsic stability without a parallel increase in fluorescence intensity does not seem likely.

Furthermore, the acrylamide quenching results confirmed the limited destabilization of LEN upon adding small to moderate amounts of urea (up to 4 M). The Stern-Volmer constant obtained in the absence of urea was 2.45 ± 0.11 M⁻¹ compared with 3.79 ± 0.05 M⁻¹ in the presence of 4 M urea, reflecting only minor readjustments in the solvent accessibility of the fluorophores. For comparison, the KSV values were 6.51 ± 0.12 M⁻¹ at pH 2 (native-like LEN) and 8.99 ± 0.06 M⁻¹ for the urea-unfolded protein. The absence of a downward curvature of the Stern-Volmer plot, obtained at pH 7 without urea, indicated the presence of only one degree of solvent exposure of the fluorescent groups (25). This observation confirmed the fact
A significant decrease in the ellipticity of LEN at 235 nm was observed with increasing urea concentrations, probably due to the dissociation of the dimers. The dissociation of dimeric LEN also occurred. In any case, because monomers of LEN appeared to be less stable than the dimers, dissociation of LEN indirectly resulted in its slight destabilization.

**Kinetics of Fibril Formation**—It has been recognized that the kinetics of fibril formation are sensitive to numerous parameters such as pH, temperature, ionic strength, speed of stirring, and protein concentration. To avoid reproducibility problems, the speed of stirring and volume of the samples were maintained constant throughout these experiments. The kinetics of fibril formation were monitored by following the increase in fluorescence intensity of the fluorescent dye ThT. LEN fibrillation kinetics at pH 7 appeared to be highly dependent on protein and urea concentrations, the kinetics becoming shorter with decreasing protein concentration and increasing urea concentration (Fig. 4b). In other words, at a given urea concentration, the rate of fibril formation was faster with decreasing LEN concentrations. Similarly, at a given protein concentration, the kinetics were faster with increasing urea concentrations. No significant differences were observed between the transition midpoints obtained in 0 and 1 M urea throughout the protein concentration range, probably indicating that neither the dissociation nor destabilization, induced by 1 M urea, were significant enough to affect the kinetics of fibril formation. On the other hand, the presence of 2 M urea resulted in a significant change in the kinetic parameters of LEN fibril formation at all protein concentrations (Fig. 4b). These significant changes in the kinetics clearly indicated that 2 M urea induced enough changes in LEN quaternary structure and/or destabilization to significantly increase the rate of fibrillation. In 3 M urea, the kinetics were only slightly faster at low protein concentrations (as compared with those obtained in 2 M urea), and where the concentration of monomer was similar, the differences were more pronounced at high protein concentrations (i.e., 3 mg/ml). The fact that the effect of 3 M urea was more dramatic at high protein concentrations seemed to indicate that urea interfered more with the monomer/dimer equilibrium than with the intrinsic stability of LEN. If we assume that the addition of 2 M urea caused the dissociation of most of the dimers present at low protein concentrations, no further significant changes would be expected upon adding more urea, as observed with 3 M urea and low protein concentrations. The preponderant effect of urea on the dissociation of LEN was confirmed by the significant decrease of the transition midpoint observed at high protein concentrations, where significant amounts of dimers were still present in 2 M urea. If, on the other hand, the presence of urea would have had greater effect on the intrinsic stability of LEN, the addition of increasing amounts of urea should have affected the kinetics even at low protein concentrations, which was not the case. The kinetics of fibril formation at 4 M urea also further confirmed the role of

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**Table I**

The effect of urea and protein concentration on the kinetics of LEN fibrillation

| Protein concentration (mg/ml) | Urea concentration (μ) |
|------------------------------|------------------------|
| 0.01                         | 0.01                   |
| 0.1                          | 0.1                    |
| 0.5                          | 0.5                    |
| 1.0                          | 1.0                    |
| 3.0                          | 3.0                    |

The conditions are pH 7, 37 °C, 100 mM NaCl with stirring. The values in parentheses are the standard deviations.

| Protein concentration (mg/ml) | Urea concentration (μ) |
|------------------------------|------------------------|
| 0.01                         | 0.01                   |
| 0.1                          | 0.1                    |
| 0.5                          | 0.5                    |
| 1.0                          | 1.0                    |
| 3.0                          | 3.0                    |

The conformational probes (except intrinsic fluorescence) and stability parameters indicate that LEN at pH 7 was somewhat affected by the addition of increasing amounts of urea (up to 4 M urea). However, these changes appeared to be due mostly to the dissociation of the dimers, although some destabilization of LEN also occurred. In any case, because monomers of LEN appeared to be less stable than the dimers, dissociation of LEN indirectly resulted in its slight destabilization.

**Far- and near-UV CD spectra** have been extensively used to follow changes in protein secondary and tertiary structures, respectively (26–28). Upon increasing urea concentration, the changes detected in the near-UV CD appeared to be more significant than those observed in the far-UV signal. The far-UV CD signal was composed of two minima: one at 235 nm due to aromatic clusters and one at 219 nm, reflecting the β-fold of LEN. An increase in the negative ellipticity at 219 nm was observed with increasing urea concentrations, probably reflecting some subtle changes in the β-fold, although no changes were observed at 235 nm, indicating that the aromatic clusters were not affected by small amounts of urea. The near-UV signal was mainly characterized by three maxima (at 297, 289, and 280 nm) due to the presence of aromatic clusters and tryptophan residues. Upon adding urea, some progressive decrease in ellipticity was observed at all three maxima, changes at 289 nm being the most significant. However, it has been shown that the near-UV CD spectra of Ig light chains are affected by their quaternary structure (29). Thus, partial dissociation of the dimers could be responsible for some of these spectral changes. In fact, similar changes such as those detected in the near-UV CD upon the addition of urea were observed simply upon diluting LEN samples (data not shown), clearly indicating that part of the changes, if not all, was due to the dissociation of the dimers. The dissociation of dimeric LEN upon the addition of increasing urea concentrations (from 0 to 4 M) was confirmed by SAXS experiments at 3 mg/ml (237 μM).

A significant decrease in the I(0) values from 0.0177 ± 0.0002 for the sample without urea to 0.0090 ± 0.0002 for the one in 4 M urea was observed. Because I(0) is sensitive to both concentration and degree of association of proteins, accurate information about the association state of proteins can be extracted by maintaining the concentration constant among protein samples in various conditions. In this case, the significant decrease in I(0) values reflected the dissociation of LEN occurring upon adding increasing amounts of urea. Given that LEN is dimeric at 3 mg/ml in the absence of urea, it was possible to estimate the amount of dimers present at each urea concentration for a particular protein concentration (Fig. 7). A sigmoidal curve was obtained, indicating that 1 M urea did not induce much dissociation, whereas LEN was mostly monomeric in the presence of 4 M urea, although some dimers were still present. The variation in Ksv with increasing urea concentration showed a similar sigmoidal dependence. The calculated Ksv values of native LEN as a monomer and dimer are 14.1 and 17.7 Å, respectively, confirming the preponderance of dimers at 3 mg/ml in the absence of urea and monomer in the presence of 4 M urea. All
FIG. 5. Comparison of the kinetics of LEN fibril formation and conformational changes. LEN fibrillation was monitored by ThT fluorescence intensity (○) and compared with conformational and/or dissociation changes upon stirring LEN solutions at 37 °C at 0.1 mg/ml and pH 7 (20 mM HEPES and 100 mM NaCl) as a function of urea concentration. The conformational changes were monitored using (■) ANS and (□) intrinsic fluorescence \( \lambda_{\text{max}} \) values. a, 0 M urea; b, 1 M urea; c, 2 M urea; d, 3 M urea; and e, 4 M urea. Independent of the urea and protein concentrations, all conformational changes occurred concurrently with the increase in ThT fluorescence intensity. The kinetics of fibril formation increased with increasing urea concentrations.
moderate amounts of urea on the monomer/dimer equilibrium and its critical role on the kinetics of LEN fibril formation. The transition midpoints observed from 0.01 to 1 mg/ml were not significantly different from those obtained in 3M urea. However, the midpoint of the transition at 3 mg/ml was significantly shortened by the presence of 4M urea, consistent with a significant amount of dimers being present in 3 M urea and high protein concentration.

At 3 mg/ml, where the initial amounts of dimers in the various urea concentrations could be accurately estimated from the I(0) values, the ThT transition midpoints were plotted versus the initial amounts of dimers (Fig. 8). A linear relationship was observed, indicating that the effect of urea on dissociation was far more significant than on LEN destabilization. The structural changes probed by intrinsic and ANS fluorescence occurred concomitantly to the increase in ThT fluorescence throughout the protein and urea concentration ranges (Fig. 5). Interestingly, at pH 2, changes in the conformational probes were observed at high protein concentration prior to ThT increase, reflecting the formation of off-pathway soluble oligomers (9). The formation of these off-pathway species was directly correlated with the amount of dimers present at the beginning of the incubation period; these species were not formed at very low protein concentrations (0.01 mg/ml). However, at pH 7, the formation of such off-pathway species was not observed, even at high protein concentration and in the absence of urea (conditions in which the concentration of dimers was the largest). Reducing the amount of dimers initially present by decreasing the protein concentration and/or increasing urea concentration did not result in the formation of off-pathway species, and changes of all molecular probes occurred simultaneously. This lack of off-pathway oligomeric species prior to fibril formation was confirmed by small angle x-ray scattering experiments. SAXS measurements were performed during the initial period of stirring LEN solution at 3 mg/ml with and without 1 M urea. No significant changes in either the Rs and I(0) values were detected during the first 9 h of stirring. Interestingly, at 3 mg/ml in the absence of urea, the changes in intrinsic and ANS fluorescence appeared slightly faster than the
changes observed by ThT, and the changes in the amide I peak position occurred slightly slower than fibril (Fig. 6). In fact, while the fluorescence changes started at the beginning of the ThT fluorescence intensity increase, changes in the amide I peak position only appeared at the end of the fibril formation process. This time difference in the appearance of the changes suggests that the fluorescence (tryptophan and ANS) and FTIR monitor different aspects of fibril formation. The changes in the conformational probes, with slightly faster kinetics than the increase in the ThT signal, most probably reflect the formation of fibrils, although they could result from conformational or other changes associated with formation of transient oligomers immediately prior to fibril formation. In fact, this is likely to be the case, because the transition monitored by the conformational probes was more cooperative and corresponded to the initial transition observed with ThT. On the other hand, the transition monitored by FTIR (Fig. 6) began after the initial ThT transition, was also more cooperative than that observed by ThT, and appeared to correlate with events in the second half of the ThT transition. Thus, it seems that there are two components to the ThT transition, accounting for its lower apparent cooperativity than that observed by FTIR or fluorescence. This could represent the initial formation of filaments, followed later by protofibrils and fibrils, resulting from intertwining of the filaments (30).

The fact that the amide I peak position values, observed during the kinetic experiment at 3 mg/ml in the absence of urea, shifted from 1639 to only 1632 cm⁻¹ (Fig. 6) seems to indicate the specific formation of fibrils. For example, at pH 2 (9), where off-pathway species were formed prior to fibrils, the amide I peak position values shifted initially from 1639 to 1628 cm⁻¹ before returning to 1632 cm⁻¹ upon fibril formation. These previous observations indicated that an amide I peak position value of 1632 cm⁻¹ seems to be characteristic of LEN fibrils, whereas lower values seem to be characteristic of off-pathway aggregated species. These significantly different amide I peak positions between various types of aggregated species could be explained by different interatomic distances and angles between hydrogen bond donors and acceptors in the different structures.

The simplest kinetic scheme consistent with the data is that shown in Scheme 1, in which \( D_{\text{nat}} \) and \( M_{\text{nat}} \) correspond to the native conformations of the dimeric and monomeric forms of LEN, and \( I \) represents the critical partially folded intermediate (31). For comparison, the situation at pH 2 is summarized in Scheme 2. The latter is significantly more complex due to the presence of urea, shifted from 1639 to only 1632 cm⁻¹ during the kinetic experiment at 3 mg/ml in the absence of urea, resulting in significantly faster fibril formation. Urea appeared to affect, although to different extents, the association state, the dynamic stability, and the conformation of LEN. However, the changes observed in both stability and conformational probes appear to be a result of the dissociation of the dimers, the monomers being significantly less stable than dimers. Reducing the amount of dimers initially present in solution by either decreasing protein concentrations and/or increasing urea concentrations resulted in faster fibril formation. Thus, the formation of fibrils originates from a monomeric form of LEN, probably from a distorted monomeric intermediate.

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