Conformational Prerequisites for α-Lactalbumin Fibrillation†

John Goers,‡,§ Sergei E. Permyakov,|| Eugene A. Permyakov,|| Vladimir N. Uversky,*,‡,|| and Anthony L. Fink‡

Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, Institute for Biological Instrumentation, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia, and California Polytechnic State University, San Luis Obispo, California 93407

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ABSTRACT: Bovine α-lactalbumin, a small acidic Ca^{2+}-binding milk protein, formed amyloid fibrils at low pH, where it adopted the classical molten globule-like conformation. Fibrillation was accompanied by a dramatic increase in the *β*-structure content and a characteristic increase in the thioflavin T fluorescence intensity. *S*-(Carboxymethyl)-α-lactalbumin, a disordered form of the protein with three out of four disulfide bridges reduced, was even more susceptible to fibrillation. Other partially folded conformations induced in the intact protein at neutral pH, either by the removal of Ca^{2+} or by the binding of Zn^{2+} to the Ca^{2+}−protein complex, did not fibrillate, although Zn^{2+}-loaded α-lactalbumin precipitated out of solution as amorphous aggregates. Our data suggest that the transformation of a protein into an essentially unfolded (thus, highly flexible) conformation is required for successful fibril formation, whereas more rigid (but still flexible) species may form amorphous aggregates.

A number of human diseases, known as amyloidoses, originate from the deposition of stable protein aggregates, amyloid fibrils. In each of these pathological states, a specific protein or protein fragment changes from its native soluble form into insoluble fibrils, which accumulate in a variety of organs and tissues (*1*−*6*). Although approximately 20 different proteins have been found to be involved in these amyloidoses, most of them are unrelated structurally or at the level of primary structure. Despite these differences, all the amyloid fibrils from different pathologies display common properties, typically two to six unbranched protofilaments 2−5 nm wide associated laterally or twisted together to form fibrils that are 4−13 nm wide (e.g., see refs *7* and *8*). Fibrils with similar morphology have also been formed in vitro from disease-associated (*9*−*13*) and disease-unrelated proteins (*14*−*23*). It has been proposed that amyloid fibril formation can occur when the native structure of a protein is destabilized, favoring formation of partially folded conformations (*1*−*6*, *24*, *25*).

α-Lactalbumin (α-LA)[1] is a small acidic Ca^{2+}-binding protein involved in the regulation of lactose biosynthesis as a component of lactose synthetase (*26*, *27*). α-LA possesses a single Ca^{2+}-binding site and is frequently used as a model Ca^{2+}-binding protein distinct from the EF-hand family (*28*, *29*). In addition, α-LA is very attractive for studies of partially folded conformations because at either acidic pH, moderate guanidinium chloride concentrations, or elevated temperatures (apo form) it adopts the classic molten globule state (*29*−*31*). Interestingly, it has been found that some associative forms of α-LA can induce apoptosis in tumor cells (*32*, *33*).

Crystallographic analysis revealed that the three-dimensional structure of α-LA is very similar to that of lysozyme, i.e., α-LA is comprised of a large α-helical domain and a small *β*-sheet domain connected by a calcium-binding loop (*34*, *35*). The α-helical domain is composed of three major α-helices (residues 5−11, 23−34, and 86−98) and two short 3_{10}-helices (residues 18−20 and 115−118). The small domain is composed of a small three-stranded antiparallel *β*-pleated sheet (residues 41−44, 47−50, 55, and 56), a series of loops, and a short 3_{10}-helix (residues 77−80). As in lysozyme, the two domains are divided by a deep cleft held together by two (Cys73−Cys91 and Cys61−Cys77) of the four disulfide bridges (the others being Cys6−Cys120 and Cys28−Cys111 bridges).

It is well-known that α-LA is a member of the lysozyme family of proteins. Lysozyme and its variants have been shown to form amyloid fibrils under a variety of conditions. For instance, two nonconservative amino acid substitutions (Ile65Thr and Asp67His) in human lysozyme destabilized (*36*−*39*) and accelerated its fibrillation in vitro (*37*, *40*). These mutations were amyloidogenic, causing autosomal dominant hereditary amyloidosis (*36*). Further, mutant hen egg white lysozymes Ile55Thr and Asp66His (which are analogous to human amyloidogenic mutant lysozymes Ile65Thr and Asp67His, respectively) were shown to be remarkably destabilized and less soluble than the wild-type protein (*41*). Even wild-type hen lysozyme has been converted from its soluble native state into highly organized amyloid fibrils under conditions promoting partial unfolding of the native globular fold (this includes heating of low-pH solutions and

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* To whom correspondence should be addressed: Department of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. Telephone: (831) 459-2915. Fax: (831) 459-2935. E-mail: uversky@hydrogen.ucsc.edu.

‡ University of California.

§ California Polytechnic State University.

|| Russian Academy of Sciences.

[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; CD, circular dichroism; TfT, thioflavin T; α-LA, α-lactalbumin; 1SS-α-LA, *S*-(carboxymethyl)-α-lactalbumin.

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addition of organic solvents) (*17*). Finally, a novel variant of human lysozyme (Trp64Arg) has recently been reported to cause a combination of amyloid deposition with sicca syndrome and amyloid nephropathy (*42*). From the studies described above, many members of the lysozyme family may be destabilized and able to form amyloid fibrils. In this study, we report that another representative of the lysozyme kindred, α-lactalbumin, forms fibrils either at low pH or by partial disulfide reduction, conditions which induce a partially folded conformation.

MATERIALS AND METHODS

Materials

Thioflavin T (TfT), bovine α-lactalbumin, and *S*-(carboxymethyl)-α-lactalbumin were obtained from Sigma (St. Louis, MO). All other chemicals were analytical grade and from Fisher Chemicals.

Methods

Circular Dichroism (CD) Measurements. CD spectra were obtained on an AVIV (Lakewood, NJ) 60DS spectrophotometer using an α-LA concentration of 1 mg/mL at 37 and 55 °C in varying buffers. Near-UV (250−320 nm) and far-UV (190−250 nm) CD spectra were recorded in 0.4 and 0.01 cm path length cells, respectively, with a step size of 1.0 nm, a bandwidth of 1.5 nm, and an averaging time of 10 s. An average of five scans was obtained for each protein spectrum, followed by subtraction of the buffer spectrum and calculation of the ellipticity.

FTIR Measurements. Data were collected on a Thermo-Nicolet Nexus 670 FTIR spectrometer equipped with an MCT detector and an out-of-compartment 72 mm × 10 mm × 6 mm, 45° germanium trapezoidal internal reflectance element (IRE). The hydrated thin films were prepared as described previously (*43*, *44*). Typically, 256 interferograms were co-added at 2 cm^{-1} resolution. Data analysis was performed with GRAMS32 (Galactic Industries). The secondary structure content was determined from curve fitting to spectra deconvoluted using second-derivative and Fourier self-deconvolution to identify component band positions.

Fibril Formation and Kinetic Evaluation. Fibrillation of α-lactalbumin was monitored using a Fluoroskan Ascent fluorescence plate reader as previously described (*45*). Fibril formation was detected by the characteristic increase in TfT (20 μM) fluorescence emission at 482 nm when excited at 450 nm. The assays were performed at 37 °C with α-LA either in 100 mM NaCl (pH 2.0) or in 100 mM NaCl, 20 mM Tris-HCl buffer (pH 7.4) with or without 5 mM EDTA or 5 mM $ZnCl_2$. TfT fluorescence intensities were plotted as a function of time and fitted to a sigmoidal curve (*45*).

Electron Microscopy. Transmission electron micrographs were collected using a JEOL JEM-100B microscope and Formvar-coated 300 mesh copper grids (Ted Pella Inc., Redding, CA). Samples at 1.0 mg/mL were applied to the grids for 4 min followed by one washing with buffer, one washing with water, and two washings with 1% uranyl acetate.

RESULTS AND DISCUSSION

α-LA is known to adopt different partially folded conformations under mildly denaturing conditions (*29*−*31*, *46*−*48*). For instance, acidic pH converts α-LA to the classical molten globule state (*30*, *31*), while release of Ca^{2+} at neutral pH reduces the thermal stability of bovine α-LA so that moderate heating transforms the apoprotein into a molten globule-like conformation (*30*, *31*, *46*, *48*). Furthermore, the binding of Zn^{2+} ions to Ca^{2+}-loaded α-LA decreased protein thermal stability, caused aggregation, and increased its susceptibility to protease digestion. These results also showed that α-LA is in a partially unfolded but aggregated state in the presence of high Zn^{2+} concentrations (*50*, *51*). In addition, disulfide bridges were shown to play a very important role in the stabilization of α-LA structure (*52*−*56*). For example, when two of the four disulfides were reduced, α-LA retained approximately half of the secondary and tertiary structure of the native protein (*52*, *53*), while the fully reduced form had little tertiary structure but still retained an appreciable amount of secondary structure (*54*).

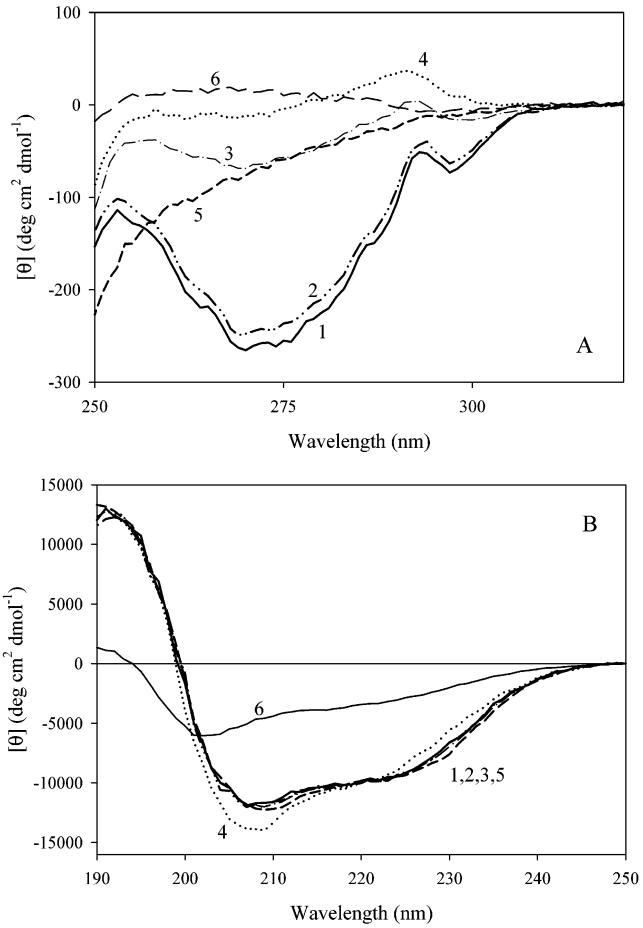

FIGURE 1: Structural characteristics of α-LA under different experimental conditions. Near-UV (A) and far-UV (B) CD spectra of 1 mg/mL α-LA measured at 37 °C: 20 mM Tris-HCl and 100 mM NaCl at pH 7.4 (1), 20 mM Tris-HCl, 100 mM NaCl, and 5 mM EDTA at pH 7.4 (2), 20 mM Tris-HCl and 5 mM EDTA at pH 7.4 (3), 100 mM NaCl at pH 2.0 (4), 20 mM Tris-HCl, 100 mM NaCl, and 5 mM $ZnCl_2$ at pH 7.4 (5), and 1 mg/mL 1SS-α-LA in 100 mM NaCl at pH 2.0 (6).

In agreement with the above observations, we found that changes in either the environment or the disulfide structure of α-LA resulted in pronounced reductions in tertiary structure levels but not in secondary structure levels. In particular, the near-UV CD signal was completely absent for 1SS-α-LA, the form in which three disulfide bonds have

Table 1: Secondary Structure Content of Bovine α-Lactalbumin in Different Conformational States As Determined by FTIR, Far-UV CD, and X-ray Analysis

	FTIR					
	native, pH 7.4		molten globule, pH 2		fibrils	
structural assignment	wavenumber[a] (cm^{-1})	%[b]	wavenumber[a] (cm^{-1})	%[b]	wavenumber[a] (cm^{-1})	%[b]
turn	1685	3.8	1685	5.7	1685	6.2
turn	1669	30.5	1669	24.0	1667	12.7
α-helix	1650	47.8	1653	43.2	1651	20.3
β-sheet	1635	9.6	1637	16.5	1631	55.1
β-sheet/side chains	1621	8.3	1621	10.6	1614	5.7

	Far-UV CD[c]			
	native, pH 7.4		molten globule, pH 2	
structural assignment	%, KS[d]	%, LG[d]	%, KS[d]	%, LG[d]
α-helix	36	47	47	56
β-sheet	9	21	11	14

	X-ray data[c]	
	native, pH 7.4	
structural assignment	%, KS[d]	%, LG[d]
α-helix	31	47
β-sheet	8	25

[a] The estimated error in the frequencies is ±2 cm^{-1}. [b] The estimated error in the percent contribution to total secondary structure is ±1.5%. [c] Data taken from ref *57*. [d] KS and LG, secondary structure content determined by the methods of Kabsh and Sander (*67*) and Levitt and Greer (*68*), respectively.

been reduced and the six cysteines have been S-carboxymethylated, and significantly reduced for the intact protein incubated at pH 2.0. Further, at neutral pH, 5 mM EDTA in the absence of salt (100 mM NaCl) or addition of 5 mM $ZnCl_2$ produced a moderate decrease in the magnitude of the signal, while 5 mM EDTA with salt showed little change in native ellipticity (Figure 1A). In contrast to the almost universal change in tertiary structure seen by the diminished near-UV CD spectra, the far-UV CD spectra revealed that the helical secondary structure of α-LA was largely maintained under the above conditions. Even acidic pH produced only a slight distortion in secondary structure, which was further confirmed by FTIR analysis (cf. panels A and B of Figure 2 and Table 1). These results are in a good agreement with recently published data for far-UV CD analysis of molten globules (*57*) (see Table 1). The 1SS-α-LA form of α-LA was the only sample that appeared to be significantly unfolded, showing only approximately one-third of the normal far-UV CD signal. Thus, α-LA appeared to lose considerable tertiary structure while maintaining most secondary structure under these defined conditions. Such forms may lend themselves to fibril formation.

Incubation of α-LA at 5 mg/mL in 100 mM NaCl at pH 2.0 and 37 °C with constant stirring resulted in a dramatic change to a viscous and slightly turbid solution, indicative of fibril formation. A similar appearance was seen with solutions of 1SS-α-LA. FTIR analysis of the intact protein solutions produced at pH 2.0 showed the transformation of the highly helical α-LA (~50%) into a predominantly *β*-structural conformation (~55%) (see Figure 2 and Table 1). Formation of extensive *β*-structure is characteristic of protein aggregation, particularly in the formation of *β*-rich amyloid fibrils. There was no evidence of incubation-induced conformational perturbations for α-LA with intact disulfide

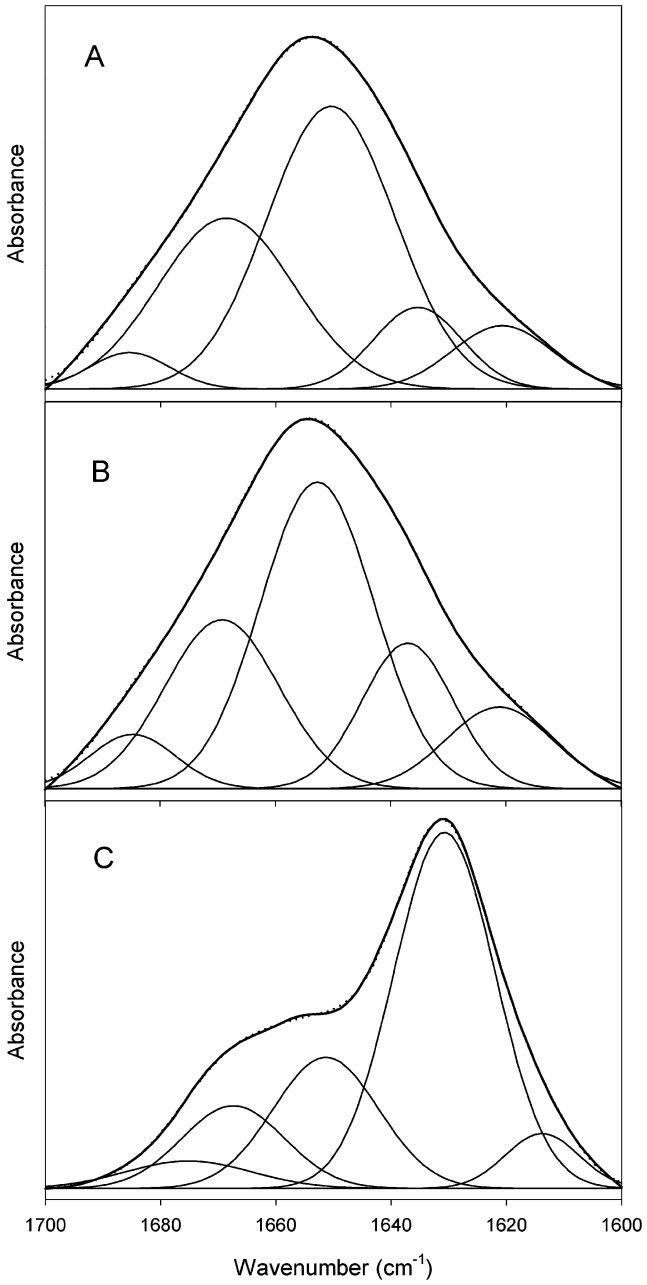

FIGURE 2: FTIR secondary structure analysis of α-LA in different conformational states: native at pH 7.4 (A), acid-induced molten globule at pH 2.0 (B), and fibrillar at pH 2.0 after overnight incubation at 37 °C with stirring (C). FTIR spectra of the amide I region are shown as solid lines. Curve fit spectra are shown as dotted lines.

bridges under the other experimental conditions (5 mM EDTA in the absence or presence of salt, or 5 mM $ZnCl_2$ at neutral pH).

Further evidence of α-LA fibrillation under conditions of low pH, or fibrillation of 1SS-α-LA at neutral pH, was obtained with the TfT binding assay. TfT is a fluorescent dye that interacts with amyloid fibrils, leading to a major increase in the fluorescence intensity in the vicinity of 480 nm (*58*). The time courses of TfT fluorescence for both α-LA at pH 2 and 1SS-α-LA display typical sigmoidal curves, with fibrillation of 1SS-α-LA characterized by an extremely short lag time. There was no evidence in the TfT assay of fibril formation by either the native protein or apo-α-LA at neutral pH on the time scale that is shown. In fact, at neutral pH (in

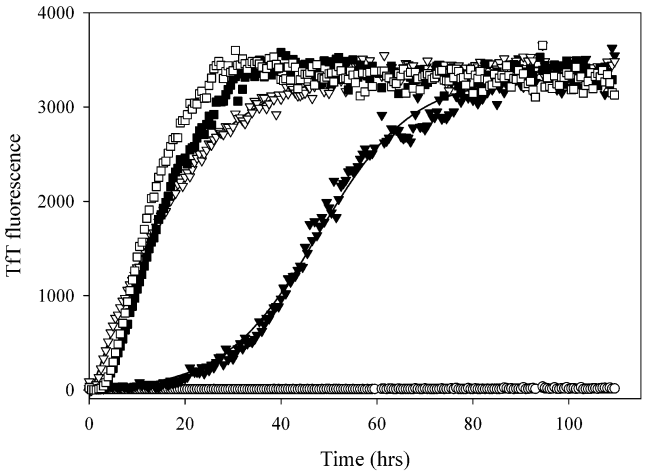

FIGURE 3: Fibrillation of α-lactalbumin monitored by the enhancement of thioflavin T fluorescence intensity at 37 °C with stirring: intact native α-LA protein in 20 mM Tris-HCl and 100 mM NaCl at pH 7.4 (●, overlapping with ○ at baseline), apoprotein in 20 mM Tris-HCl and 5 mM EDTA at pH 7.4 (○), molten globule protein in 100 mM NaCl at pH 2.0 (▼), 1SS-α-LA in 100 mM NaCl at pH 2.0 (▽), 1SS-α-LA in 20 mM Tris-HCl and 100 mM NaCl at pH 7.4 (■), and 1SS-α-LA in 20 mM Tris-HCl, 100 mM NaCl, and 5 mM EDTA at pH 7.4 (□).

the presence or absence of EDTA as well as in the presence of $ZnCl_2$), fibrils were not formed even after incubation of α-LA for 14 days. However, increased turbidity was observed in the neutral α-LA solutions containing 5 mM $ZnCl_2$, reflecting the formation of insoluble aggregates that are different from fibrils.

The morphology of the insoluble material formed by both 1SS-α-LA and intact protein at low pH (high-magnitude TfT signals) and by intact α-LA at neutral pH in the presence of 5 mM $ZnCl_2$ (no TfT signal) was analyzed by transmission EM. Figure 4 shows that both 1SS-α-LA and the intact protein at low pH formed typical amyloid fibrils, whereas protein incubated in the presence of 5 mM $ZnCl_2$ formed amorphous aggregates.

Several important points concerning α-LA fibrillation should be emphasized here.

(i) Fibrillation of both α-LA forms was extremely sensitive to the ionic strength of the solution. For instance, the lag time of α-LA molten globule (pH 2.0) fibrillation decreased from 48.9 ± 0.5 h in the presence of 25 mM NaCl to 19.9 ± 0.3 h in the presence of 150 mM NaCl. Similarly, in the absence of salt, 1SS-α-LA had a lag time of 32.7 ± 0.2 h, while the addition of 100 mM NaCl decreased the lag time to 1.6 ± 0.1 h.

(ii) Fibrillation of intact α-LA occurred within a narrow pH range, while fibrillation of 1SS-α-LA occurred over a wide pH range. The results of Lowry protein assays showed that 13 ± 1, 95 ± 3, and 19 ± 2% of the α-LA formed insoluble fibrils when incubated for 60 h at pH 1.5, 2.0, and 2.5, respectively, but fibrillation of 1SS-α-LA was almost insensitive to pH from 2.0 to 7.4 (and to the presence of EDTA, Figure 3). In fact, according to the Lowry protein assays, 99 ± 3% of 1SS-α-LA was insoluble when incubated for 60 h under the all conditions that were studied.

(iii) Fibrillation of the α-LA molten globule was shown to have a "normal" dependence on protein concentration; i.e., a 20-fold increase in the protein concentration resulted in a 2.5-fold decrease in the lag time and a commensurate increase in the rate constant for fibril growth (data not shown).

(iv) The lag time of 1SS-α-LA fibrillation was considerably shorter than that of the molten globule of the intact protein at pH 2.0 (1.6 ± 0.2 and 28.2 ± 0.3 h for 2 mg/mL 1SS-α-LA and intact protein in 100 mM NaCl at pH 2.0, respectively) (Figure 3).

(v) Both forms of α-LA showed comparable elongation rates (0.10 ± 0.01 h^{-1}) at low pH (Figure 3).

(vi) An increase in the incubation temperature up to 55 °C did not change the fibrillation profiles of intact α-LA compared to those at 37 °C. Thus, α-LA at pH 2 still formed fibrils, while holo-α-LA or apo-α-LA at neutral pH did not. Further, in agreement with previous studies (*30*, *31*, *46*, *48*), we found the near-UV CD spectra were almost completely abolished at 55 °C for all forms of the intact protein, while the nativelike far-UV CD spectra were maintained. Together, the UV CD data reflect a temperature-induced transformation of the proteins into a molten globule-like conformation at 55 °C. However, these molten globule forms were not sufficient for either holo- or apoproteins at neutral pH to form fibrils or any other insoluble aggregated material for the 4 weeks that were measured (data not shown).

Our results allow some conclusions about the structural prerequisites of α-LA fibrillation to be made. First, of the various conditions that were tested, only low pH converted

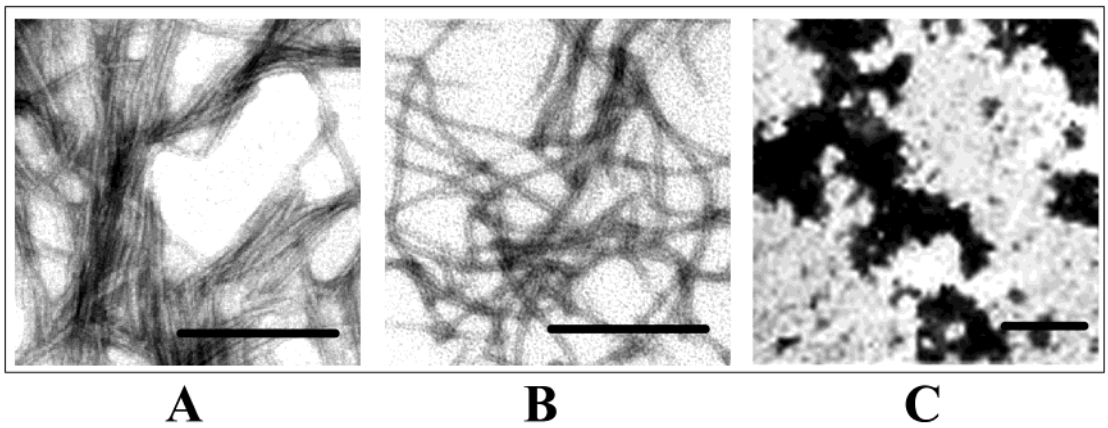

FIGURE 4: Negatively stained transmission electron micrographs of α-LA fibrils prepared from the acid-induced molten globule (A) or 1SS-a-LA at pH 7.4 (B) or amorphous aggregates prepared from Zn^{2+}-loaded holo-α-LA in the presence of 5 mM $ZnCl_2$ (C). The bars are 100 nm in length.

intact α-LA into a molten globule that was able to fibrillate. This may be due to the fact that the acid-induced molten globule was essentially less ordered and much more flexible than the molten globule forms induced by either the removal of Ca^{2+} (*59*) or high temperatures. Second, our data are consistent with a highly flexible partially folded conformation representing the important prerequisite for successful fibril formation (molten globule at low pH or 1SS-α-LA), whereas a more rigid but still flexible conformation may interact to form amorphous aggregates but not fibrils. As an example, while Zn^{2+}-loaded Ca^{2+}-bound α-LA acquired a molten globule-like form (Figure 1A,B), it was still sufficiently rigid to form amorphous aggregates rather than fibrils. On the other hand, the reduction and carboxymethylation of three of four disulfide bridges in 1SS-α-LA produced an essentially unfolded flexible conformation (Figure 1B), which was able to form fibrils. Third, the formation of an essentially unfolded conformation may represent an important primary step of intact α-LA fibrillation. For example, fibrillation of 1SS-α-LA (essentially unfolded) was characterized by a very short lag time, while its elongation rate was comparable with that of intact α-LA fibrillation at pH 2.0. This suggests that the major event during the lag phase of intact α-LA fibrillation would be the partial unfolding of its molten globule-like state into a conformation with properties similar to those of 1SS-α-LA, after which rapid fibril formation occurs.

It is interesting to compare our results with the data retrieved for other amyloidogenic systems. Amyloid-like fibrils may be formed in vitro from both disease-associated and disease-unrelated proteins (e.g., see ref *2*). It has been proposed that amyloid fibril formation from native proteins occurs via a conformational change leading to a partially folded intermediate conformation, the subsequent association of which is a key step in fibrillation (*1*−*6*, *24*, *25*). Detailed structural analyses of the early events during the fibrillation of several proteins have uncovered the vital role of substantially unfolded forms as fibril precursors. Examples include fibronectin type III (*15*), the SH3 domain (*14*), monellin (*16*), immunoglobulin light chains SMA (*60*) and LEN (*61*, *62*), and several others. Interestingly, it has been noticed that depending on the environmental conditions, SMA can be converted into one of the two partially folded intermediates, nativelike I(N) and substantially unfolded I(U). The I(U) intermediate readily formed amyloid fibrils, whereas I(N) preferentially led to amorphous aggregates (*60*). On the other hand, partial folding was shown to be a primary step in the fibrillation of so-called natively unfolded proteins (*63*−*65*), such as α-synuclein (*13*), islet amyloid polypeptide (*66*), and prothymosin α (*23*). These data strongly indicate that essentially unfolded and highly flexible conformations (notably distinct from the native, molten globule-like, and completely unfolded ones) might represent key species in the fibrillation process. As comparable behavior has been observed for globular and natively unfolded proteins, both disease-associated and disease-unrelated, we assume that essential unfolding might be a structural prerequisite for fibril formation in all systems.

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