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Amyloid Fibril Formation and Other Aggregate Species Formed by Human Serum Albumin Association

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Under in vitro solution conditions where the native state is destabilized, many proteins present an abnormal structure and metabolism associated with a strong tendency to self-aggregation into a polymeric amyloid fibril structure, suggesting that this ability is a generic feature of the polypeptide chains. Such structures play a key role in different pathogenesis of neurodegenerative diseases such as Alzheimer, Parkinson, or Creutzfeldt-Jakob. Here, we report the formation of amyloid fibrils in the plasma protein human serum albumin under different in vitro conditions monitored using a combination of spectrophotometric and microscopic tecnhiques. Amyloid fibril formation, therefore, is also allowed in a protein with a high degree of structural complexity. We also infer from experimental data the existence of other protein aggregated species than fibrils, some of which seem to be formed by a structural rearrangement of the proper fibrils.

Under in vitro solution conditions where the native state is destabilized, many proteins present an abnormal structure associated with a strong tendency to self-aggregation into a polymeric amyloid fibril structure, suggesting that this ability is a generic feature of the polypeptide chains.[1,2] These amyloid fibrils are polypeptide aggregates with a core formed by β-strands extend transversely to the main fibril axis.[1,3] Such structures play a key role in different pathogenesis of neurodegenerative diseases[4,5] such as Alzheimer, Parkinson, or Creutzfeldt-Jakob and, recently elucidated, in other nonpathogenic phenomena as the transfer of genetic information or synaptic changes associated with memory.[6,7] Here, we report the formation of amyloid fibrils in human serum albumin (HSA) under different in vitro conditions monitored using a combination of spectrophotometric and microscopic techniques. Amyloid fibril formation, therefore, is also allowed in a protein with a high degree of structural complexity.[1,8] We also infer from experimental data the existence of other protein aggregated species than fibrils, some of which seem to be formed by a structural rearrangement of the proper fibrils. Thus, the observations lead to the suggestion that the aggregated form may represent a generic form of the protein, and point out the necessity of a complete knowledge of these self-association phenomena in order to design strategies to prevent pathologycal amyloidogenic conditions.

HSA is an all-α-protein which constitutes approximately half of the total blood protein, acting as a carrier from bloodstream to tissues, but also maintains the osmotic pressure and plays a key role in coagulation and thrombosis. It consists of 585 amino acids in a single polypeptide chain with a molar mass of 66 411 g mol^{-1} (see Supporting Information for further details). Thus, native HSA lacks any properties suggesting a predisposition to form amyloid fibrils, since most of its sequence (>60%) is arranged in an α-helix structure, with the subsequent tightening of its structure through intramolecular interactions such as hydrogen bonds.

In a screening process, pH, temperature, and ethanol concentration as a cosolvent were varied in order to determine the type of aggregates formed under different conditions, with special emphasis on those where partial destabilization of the nativelike structure of HSA occurred, which are thought to be a crucial factor in the amyloid formation by globular proteins.[1,2] The formation of these protein aggregates would suggest that, under proper solution conditions, inter- and intramolecular interactions are conveniently modified involving that the thermodynamic stable state would be the aggregated one, whereas the supposed correct folding of HSA would not signify more than a metastable state, as suggested by Ganzit in a pioneering paper.[9]

Fibril formation was found (a) in the presence of ethanol concentrations between 40 and 60% (v/v) at protein concentrations larger than 1% (w/v) at room temperature, (b) after incubation at 65 °C for 6 h, and (c) at pH 2 and 7 after incubation at 65 °C for 6 h, followed by 7- or 30-day incubation at ambient temperature (Figure 1a). The kinetics of fibril formation was not analyzed in detail. Other kinds of aggregates were found under other solution conditions (see below). Previous studies had shown the formation of fibril structures in bovine serum albumin (BSA) after incubation at high temperatures, although the compositions of these aggregates were not analyzed.[10,11] However, the formation of amyloid fibrils in the presence of ethanol is less known.[12] Ethanol increases the α-helix content of HSA up to concentrations of 20% (v/v), enhancing intramolecular interactions. However, at higher

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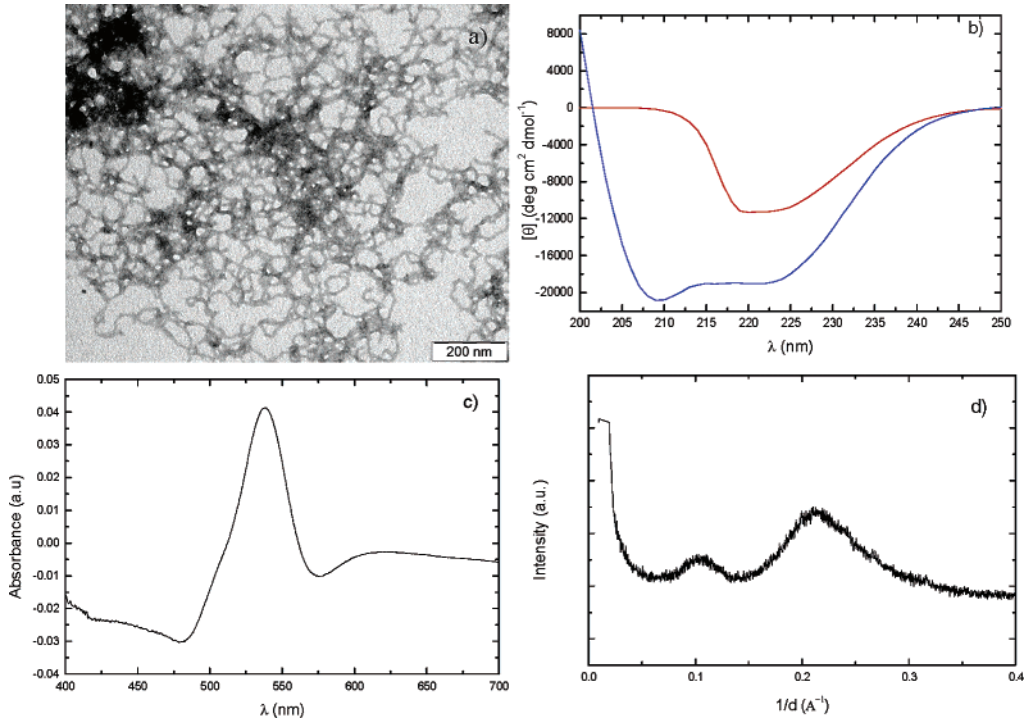

Figure 1. (a) TEM image of amyloid fibrils of HSA after 6 h of incubation at 65 °C and further incubation at room temperature for a week. (b) Far-UV CD spectra of native HSA (blue) and HSA aggregates in fibril form in 50% (v/v) ethanol solution in the presence of 20 mM NaCl after incubation for a month (red). (c) Difference absorbance of Congo Red in the presence of amyloid fibrils of HSA formed in a solution of pH 2 after incubation for 6 h at 65 °C and subsequent incubation at room temperature for a week. (d) Difference radial intensity distribution profile (solvent background scattering subtracted) of X-ray diffraction pattern from amyloid fibrils in 50% (v/v) ethanol solution in the presence of 20 mM NaCl after incubation for a month.

concentrations, alcohol molecules associate[13] and this change in solvent quality also involves a larger exposure of nonpolar areas of the protein to the solvent, resulting in a conformational change favoring protein aggregation and enabling fibril formation. Other partially folded states, as the partial ethanol induced refolding of HSA after denaturation under acidic conditions,[14] did not result in fibril formation due to an increase of α-helix structure involving intramolecular hydrogen bonds and, thus, reducing the probability of intermolecular interactions. This evidences the need of a delicate balance between intra- and intermolecular interactions for fibril formation.

When viewed by transmission electron microscopy (TEM), it is clear that discrete fibril structures possess a linear, rodlike morphology with a diameter of ∼9 nm and lengths between 0.5 and 2 μm. The far-UV circular dichroism (CD) spectrum (Figure 1b) of HSA changes from the native state characterized by the presence of two minima at 222 and 208 nm associated with the existence of predominant α-helical structure to one spectrum with a decrease in ellipticity, and the appearance of a single minimum at 219 nm or larger wavelengths as a consequence of β-sheet structure. The shift of the latter minimum, normally expected in the range 215−220 nm, is a result of wavelength-dependent light scattering. In addition, CD spectra of all-β-proteins, such as concavalin, sometimes show minima at these wavelengths.[15]

The fibril precipitates were also subjected to determine whether they were able to bind Congo Red and give apple green birefringence. The characteristic red-shift of its optical absorption from 490 to 540 nm was observed (see Figure 1c), together with the characteristic green birefringence (not shown). Moreover, the X-ray diffraction pattern of precipitates (see Figure 1d) showed two reflections: a larger one at 4.8 Å corresponding to the interstrand spacing in the β-sheets and a second one with lower intensity at 10 Å, characteristic of multilayer β-sheet fibrils.

Under other solution conditions, non-fibrillar aggregates were also found. Some aggregates possess typical minimums in CD spectra at wavelengths close to 220 nm and bind Congo Red, displaying a certain filamentous structure (but not fibril), as suggested by scanning electron microscopy (SEM) images (Figure 2a). Other classes of aggregates were spherical (see Figure 2b), with unordered and α-helix structures, and do not display Congo Red binding. Moreover, these nonamyloidal aggregated structures are also extremely interesting, since this aggregated state may be the actual agent that causes cell death.[16]

The different precursor states that result from different solution conditions can generate fibrillar or non-fibrillar aggregates with similar β-sheet content but with distinct morphological features,[17] for example, flat ribbons (Figure 2c) as compared to more cylindrical forms of amyloid fibrils or relatively thick rodlike specimens (Figure 2d). This involves that regions of a polypeptide sequence involved in triggering the aggregation process from very different initial states could well differ significantly.[18]

Thus, even when interactions involving the polypeptide backbone are key for amyloid structure through the formation of intermolecular hydrogen bonds, the side chains can also exert a significant influence on the assembly.[19]

In this respect, we have observed that lateral interactions of single particles collaborate in growing the fibrils.[20] A scrutiny of the protein aggregates indicates that HSA particles and clusters tend to align within the immediate vicinity of the early fibrils (see Figure 2e), serving the single fiber as a lateral template or scaffold for small protein molecules, and would constitute a subcomponent in the mature fibrils.

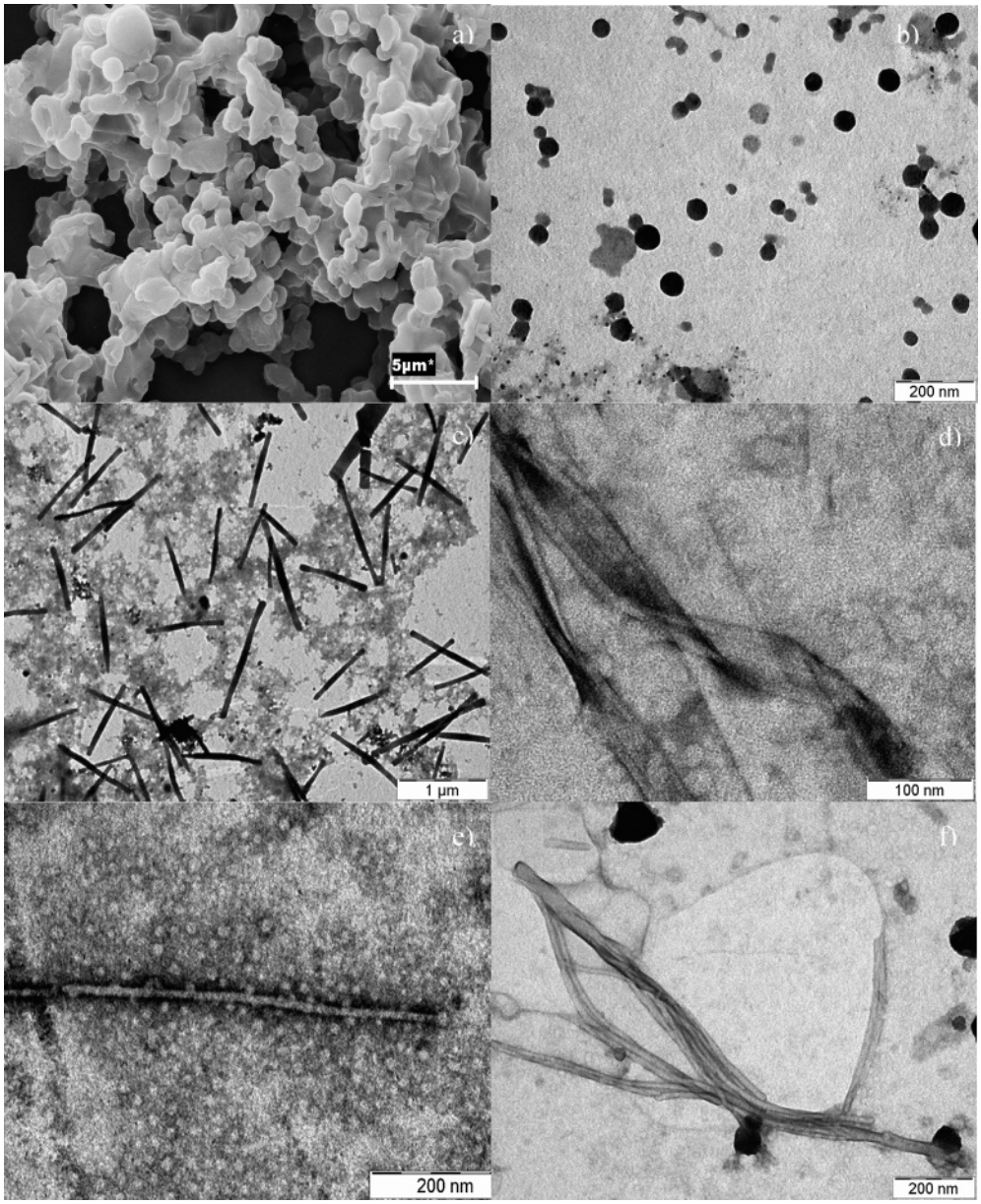

Figure 2. (a) SEM image of elongated HSA aggregates at 60% (v/v) ethanol and pH 2 in the presence of 20 mM NaCl incubated for a week at room temperature. (b) TEM image of spherical HSA aggregates at pH 7.4 in 70% (v/v) ethanol and 20 mM NaCl solution incubated for a week at room temperature. (c) TEM images of rodlike structures of HSA obtained at pH 2 in the presence of 20 mM NaCl after incubation for a week at room temperature. (d) TEM image of a flat ribbon structure obtained from association of protofibrils after incubation for 6 h at 65 °C and for one month at room temperature. (e) TEM micrograph showing lateral interactions between HSA monomers and a HSA fibril. (f) TEM image of aggregation of mature fibrils to form suprafibrillar structures through lateral interactions after incubation for 6 h at 65 °C and for one month at room temperature.

Finally, one of the characteristic traits of the mature amyloid fibrils is their tendency to bend, twist, and agglomerate. Figure 2f shows laterally connected fibers which split over a certain distance or overlap each other. The extent and rate of this growth is dependent on solution conditions and lateral interactions between fibrils, which are responsible for the thickening of the mature fibrils and the formation of suprafibrillar aggregates.

In summary, we have shown that the plasma protein HSA is able to form fibrillar aggregates under adequate solution conditions. The fibril formation involves the normally hierarchical intertwisting of protofilaments and lateral interactions of single particles to form early fibers. The different precursor states that result from different solution conditions can generate fibrillar aggregates with similar β-sheet content but with distinct morphological features. Association of mature fibrils can be interpreted as a way of assembling amyloid fibrils through lateral chain interactions of preformed "discrete" amyloid subunits.

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Supporting Information Available: Experimental details of sample preparation and measurement. This material is available free of charge via the Internet at http://pubs.acs.org.

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