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Characterization of amyloidogenesis of hen egg lysozyme in concentrated ethanol solution

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ABSTRACT

We show that hen egg white lysozyme [HEWL] reproducibly forms amyloid fibrils in 80% ethanol at 22 °C with constant agitation. Fibril formation occurs over a time course of approximately 30 days, displays polymerization nucleation kinetics, and demonstrates a marked decrease in α-helical structure. Seeding with as little as 0.05% v/v of fibrils cleaved into smaller seed fragments by sonication abolishes the lag phase. Thioflavin T assays confirm the amyloid nature of the fibrils. Atomic force microscopy reveals unbranched amyloid fibrils with lengths varying between 1 and 3 μm and heights ranging from 6–12 nm. The formation of amyloid fibrils in predominantly organic solvents demonstrates that the basic principles guiding fibril formation arise from interactions of the peptide backbone rather than from interactions between the amino acid side chains.

The ability of proteins to fold into and maintain a functional conformation or three-dimensional structure is essential for organismal viability. Despite advances in our knowledge of protein folding, we know and understand much less about protein aggregation and misfolding, two related processes that have undesirable consequences both *in vivo* and *in vitro*. One of the most common triggers for misfolding and subsequent aggregation is protein instability which can be caused by mutation, chemical modification, and/or changes in environmental conditions such as pH, temperature or solvent. Aggregation by physical association is also triggered by high protein concentrations and can be a major problem under laboratory and industrial conditions.

Protein misfolding and aggregation have gained a great deal of attention because of their association with several pathological medical disorders. To date, over 25 disorders have been identified that are characterized by the deposition of amyloid in a variety of organs and tissues [1]. Although many amyloidogenic peptides and proteins share no sequence or structural similarity, they all possess the ability to fold into the ultimately insoluble, β-sheet rich, fibrillar, highly ordered amyloid structure.

Within the past 10 years, several groups have demonstrated that proteins not associated with disease also can form amyloid. These studies suggest that while all proteins have the potential to aggregate, evolutionary pressure causes them to avoid unproductive folding pathways [2]. Since its discovery in 1922, lysozyme and its homologs have served as model molecules for the study of protein folding, structure, and function [3]. The native lysozymes are highly soluble, monomeric, stable globular proteins. Hen egg white lysozyme (HEWL), one of the most commonly studied lysozymes, has been shown to form amyloid fibrils under a variety of conditions that promote partial unfolding and/or fragmentation [4–6]. Addition of denaturants has also been shown to induce fibril formation when concentrations promote partial unfolding rather than complete denaturation [7]. Data from studies of lysozyme and a variety of other proteins under aqueous conditions demonstrate a correlation between aggregation and physicochemical properties such as charge, hydrophobicity, and secondary structure propensity [8].

To date, many fewer studies have been performed in predominantly organic solvents. Most of these studies involve co-solvents containing fluorinated alcohols such as 2,2,2-trifluoroethanol (TFE) or 3,3,3,3′,3′,3′-hexafluoro-2-propanol (HFIP). The effects of these alcohols on protein structure depend upon environmental conditions and vary from having a helix inducing effect [9,10] to destabilization of unfolded structures [11]. Intermediate concentrations of TFE are conducive to amyloid formation as demonstrated by studies on acylphosphatase [12] and α-chymotrypsin [13,14]. Goda has demonstrated that high concentrations of ethanol in the presence of salt promote formation of amyloid fibrils of HEWL [15]. Our study examines the kinetics of aggregation in a predominantly organic solvent system in the absence of cations and anions. We have developed an experimental system using HEWL in which we achieve reproducible formation of amyloid under controlled conditions. Fibril formation is slow enough that

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changes in protein conformation can be probed using a variety of techniques. Our system lets us make a comparison of the conformational events and transitions that occur during amyloid formation in both aqueous and organic solvents.

Materials and methods

Protein preparation. Highly purified lysozyme from chicken egg white was purchased from Sigma-Aldrich and used without further purification. All solutions were prepared in deionized water and ethanol (ACS/USP grade) and filtered through a 0.22 μm filter before use. Protein solutions were made by first dissolving lysozyme in deionized water followed by slow addition of the appropriate amount of ethanol. Concentrations of lysozyme were confirmed by amino acid analysis.

Light scattering assays. Lysozyme solutions at 3 mg/mL were prepared as above and immediately placed in conical polypropylene tubes with a Teflon®-coated magnetic stir bar. Experiments were carried out at room temperature with constant agitation. Samples were periodically removed for assay at 400 nm using a Shimadzu UV–vis spectrophotometer. At least six independent experiments were performed.

Thioflavin T assays. Thioflavin T (ThT) shows enhanced fluorescence at 482 nm when bound to amyloid fibrils [16,17]. Aliquots of the lysozyme solution were removed at various timepoints and assayed for the presence of amyloid. Assays were performed in a total volume of 3 mL of 0.050 mM ThT in phosphate buffer (10 mM potassium phosphate, 150 mM NaCl, pH 7.0); lysozyme solution was added to a final concentration of 0.003 mM. Measurements were taken immediately following sample preparation. The fluorescence intensities of the sample and a blank containing just 0.050 mM ThT were compared by measuring intensity in a Photon Technology International fluorimeter by excitation at 440 nm (5 nm slitwidth) and emission at 482 nm (5 nm slitwidth). Data were collected at 1-nm intervals with a 1-s integration time; 60 scans were averaged. A negative control [3 mg/mL lysozyme in PBS] was included with each assay to establish background levels of fluorescence due to non-specific binding of ThT [18]. ThT stock solutions contained 2.5 mM ThT in phosphate buffer [10 mM potassium phosphate, 150 mM NaCl, pH 7.0] and were filtered through a 0.22 μm filter. They are light-sensitive and should be stored in the dark at 4 °C for no more than a week.

Seeding experiments. Seeding was done either with pre-formed fibrils or with fibrils that had been sonicated for 5 min [Branson model 1510]. Seeds were added to lysozyme solutions so that final concentrations ranged from 0.05% to 5% v/v (seeds to native lysozyme). Light scattering, and ThT assays were performed as described above; the experiment was ended once the absorbance reached 1 in the light-scattering experiments.

Atomic force microscopy. Tapping mode atomic force microscopy (AFM) was carried out using a Nanoscope IIIA, multimode scanning force microscope (Digital Instruments, Veeco Metrology, Inc.). AFM probes were silicon with a nominal length of 160 μm, a resonance frequency of ~300 kHz, and a nominal force constant of 40 N/m. They were purchased from Veeco Probes. Samples were prepared as follows. Protein solutions were diluted as necessary with 80% ethanol and 10 μL was placed on a freshly cleaved mica disc. Samples were air dried, washed twice with 50 μL of 80% ethanol, and then stored at room temperature in a desiccator. Samples were scanned in air at a 1-Hz scan rate.

Circular dichroism spectroscopy. Lysozyme solutions were diluted 100-fold for circular dichroism (CD). Samples were centrifuged at 20,000*g* to remove particulate matter immediately prior to analysis. CD analysis of lysozyme samples was performed on either a Jasco J-600 or J-810 at 25 °C. Far ultraviolet (190–260 nm) spectra were recorded in a 0.1 cm quartz cell using a step size of 1 nm. For each sample, 4 scans were averaged and solvent background was subtracted. A mean residue weight of 111 g/mol was used to calculate ellipticities.

Results

Because there is no single defining characteristic of amyloid or a single assay for its detection, it is common practice to use a combination of techniques to identify amyloid fibrils. We followed the recommendations of Nilsson and used multiple criteria to monitor amyloid fibril formation *in vitro* [19]. We used a combination of light scattering to determine the onset and progress of aggregation, ThT assays to confirm the amyloid nature of the aggregates, and a final technique of AFM to image the structure and morphology of the fibrils.

Aggregation kinetics

Moderate agitation of lysozyme solutions (3 mg/mL) in 80% ethanol led to the prototypical sigmoidal curve indicative of nucleation-dependent polymerization kinetics (Fig. 1). The lag phase, determined by fitting a straight line to the slope of the curve during the growth phase and locating the point of intersection on the time axis, ranged from five to nine days for independent experiments (avg = 7 ± 2 days). In Fig. 1 it was determined to be 8.5 days. In the absence of agitation, there was no fibril formation even after 60 days.

One of the hallmarks of nucleation polymerization kinetics is that such processes are subject to seeding. We performed seeding experiments using either pre-formed fibrils or seeds generated by sonication of pre-formed fibrils. The final concentration of seeds ranged from 0.05% to 5% v/v. The addition of sonicated fibrils at the lowest concentration tested (0.05% v/v) consistently and completely abolished the lag phase, while a 20-fold higher concentration of pre-formed fibrils was required to consistently abolish the lag phase (Fig. 1).

Thioflavin T assays

Thioflavin T is a dye that has traditionally been used as an indicator for the presence of amyloid in tissue and its utility arises from reports that it is specific for amyloid [16,17]; the cross β-sheet structure is thought to be the key feature responsible for

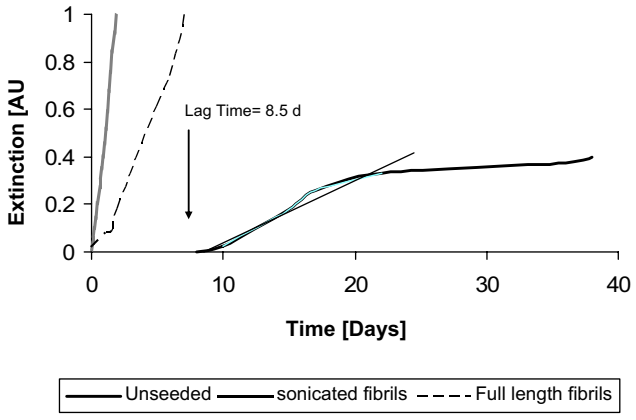

Fig. 1. Aggregation kinetics of HEWL (3 mg/mL) in 80% ethanol with agitation. Light-scattering intensity was measured as a function of time at 400 nm. The length of the lag phase was determined by fitting a straight line to the slope of the curve during the growth phase and determining the point of intersection on the time axis. Seeding of HEWL solutions using either pre-formed-then-sonicated or full-length HEWL fibrils. Data are shown for HEWL (3 mg/ml) in 80% ethanol with agitation, 0.05% v/v sonicated fibrils (dotted line), and 1% pre-formed fibrils (dashed line).

the specific binding [20,21]. Advances in fluorescence microscopy and spectroscopy have made fluorescence techniques extremely sensitive. The use of this assay provides additional verification of amyloid formation.

The appearance of amyloid fibrils followed a time dependence that was similar to that seen for the aggregation kinetics and is most evident from the results of the ThT assays where fluorescence saturation exhibits a sigmoidal time dependence that mirrors that of the light scattering data. Maximal fluorescence was seen during the plateau phase. There was no indication that amyloid fibrils formed during the lag phase and assay results confirmed that the onset of aggregation corresponded with the growth phase as determined from the light-scattering assays. A representative experiment is shown in (Fig. 2). In some instances the lag phase was shorter than that seen in light scattering (data not shown). This could be due to either the higher sensitivity of fluorescence assays or to non-specific binding of ThT to intermediate species or to a combination of the two.

Circular dichroism

At the beginning of the experiment, the far-UV CD spectra of native lysozyme and of lysozyme in 80% ethanol appear very similar (data not shown). Both exhibit minima at 208 and 222 nm—these minima are characteristic in proteins that contain α-helix as the predominant secondary structure [22]. Over time, there is a loss of the prominent minimum at 208 nm along with the appearance of a minimum around 216 nm that is indicative of β-sheet [22] (Fig. 3). Both changes indicate a loss of α-helix secondary structure. This change is quite pronounced at 2 weeks and corresponds to the growth phase in the kinetic profile. Congo red and ThT assays indicate the presence of amyloid fibrils. In the absence of agitation there was essentially no change in secondary structure of HEWL as a function of time (data not shown).

Atomic force microscopy

Samples of lysozyme were imaged at varying time intervals during the kinetics experiments. AFM confirmed the absence of particulate matter at the beginning of the experiment [data not shown] and confirmed the presence of amyloid fibrils during the latter part of the exponential growth phase and during the plateau phase of the kinetics experiments. Images clearly show the presence of mature, unbranched, amyloid fibrils with varying lengths (1–3 μm) and heights ranging from 6 to 12 nm. AFM images of multiple samples clearly show that fibrils are plentiful and that there is an absence of amorphous material (Fig. 4). This indicates that there is little competition from random aggregation processes. The conversion to amyloid fibrils is very efficient under these conditions.

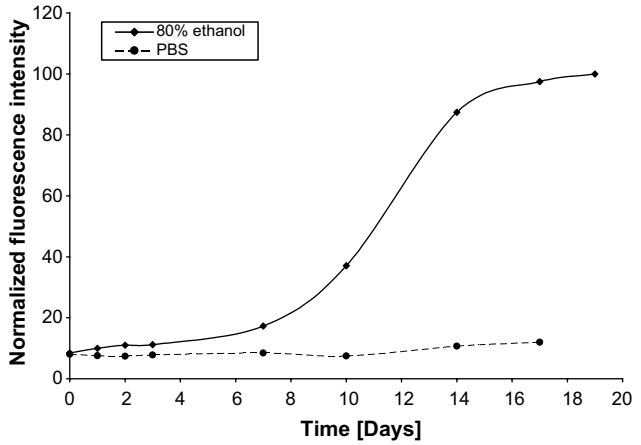

Fig. 2. Amyloid fibril formation monitored as a function of time using ThT fluorescence. Lysozyme concentrations were approximately 3 mg/mL in 80% ethanol (filled diamonds) and PBS (filled circles). The standard deviations are 1% or less.

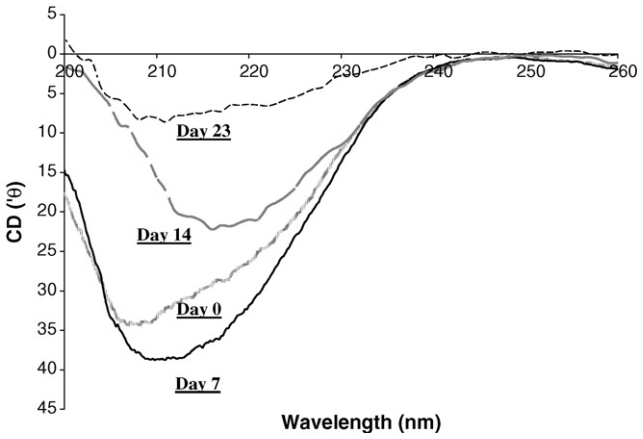

Fig. 3. Conformational analysis of lysozyme in 80% ethanol as a function of time. The concentration of lysozyme was approximately 30 μg/mL. Data were collected on a J-600 CD spectrophotometer.

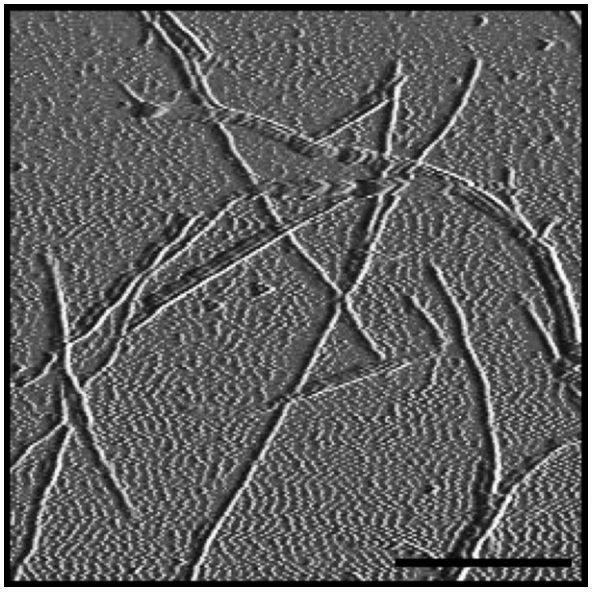

Fig. 4. AFM images of lysozyme fibrils generated by agitation of lysozyme in 80% ethanol at 22 °C. The image is representative of a sample in the plateau phase of the kinetic profile. The scan size is 3.1 μm × 3.1 μm. The scale bar represents 1 μm.

Discussion

We have developed an experimental system in which amyloid fibrils reproducibly form from HEWL in 80% ethanol in the absence of salts. Fibril formation is initiated by agitation and occurs at ambient temperatures over a time frame of approximately 30 days with a lag phase of 7 ± 2 days. Conversion to amyloid is quite efficient as there is very little evidence of amorphous aggregation by atomic force microscopy. HEWL is an excellent model system for the study of amyloid formation because it has been induced to form fibrils under so many different conditions. Fibrils form at very acidic conditions (pH 2.0) and elevated temperatures (50–70 °C), in the presence of denaturants, and in the presence of alcohols

[4–7,15]. The common themes that emerge from these studies can begin to answer some of the major questions about the formation of amyloid. At the initial conditions of the studies cited, the structure of lysozyme is very similar to that of its native structure. Over a given time course that varies with experimental conditions, lysozyme undergoes a conformational change which allows for the formation of a more β-sheet rich structure. While it has been suggested that some proteins can form amyloid directly from the native state, [23] experimental data indicate that this is highly unlikely for lysozyme and its analogs. Studies in aqueous conditions and now our study in a predominantly organic solvent suggest that amyloid formation for lysozyme involves partially folded states. In our experimental system, agitation is the event that initiates partial unfolding as we clearly see a lack of aggregation without agitation. We conclude that for lysozyme, partial unfolding is an obligatory step in the formation of amyloid fibrils as it is for many other fully folded proteins [24].

Partial unfolding makes proteins more susceptible to fibril formation and can be induced under laboratory conditions by the addition of organic co-solvents, salts and/or denaturants. This leads to exposure of the hydrophobic core and subsequent rearrangement of the protein or peptide to a variety of different conformational states, some of which are well-defined fibrillar structures. Our CD study indicates that in 80% ethanol, HEWL adopts a relatively stable structure that resembles the native state. The major effect of alcohol as a co-solvent is to reduce hydration in the starting state and as a result α-helical conformations may expand slightly. Beyond that, there is very little interaction with the hydrophobic core and consequently, there is little effect on the structure or mobility of HEWL in the beginning of the experiment [25]. Our results confirm that ethanol alone is not sufficient to induce significant partial unfolding of HEWL. Moderate agitation over the course of a few weeks, however, does destabilize HEWL enough to induce partial unfolding. Studies on the aggregation of insulin suggest that both agitation and interaction with hydrophobic surfaces are positive effectors of aggregation. Observations of Sluzky and colleagues suggest that insulin monomers become destabilized by adsorption at hydrophobic surfaces [26]. Such partially unfolded species associate with one another and eventually form a "nucleus" that initiates the growth process by interacting with more stable species [26]. Agitation seems to increase the probability that monomers will encounter a hydrophobic surface. Our results with HEWL are consistent with Sluzky's observations. The lag phase seems to represent a transition to a partially unfolded state and CD spectroscopy supports this. After 7 days in 80% ethanol with agitation, HEWL exhibits a significant change in structure as seen by CD, yet there is no measurable aggregation by light scattering, Congo red or ThT assays. Thus, agitation, in combination with ethanol solvent, can promote interactions that promote nucleation and fibril deposition.

In 80% ethanol, interactions between side chains and solvent become more favorable, as do intermolecular interactions between molecules of HEWL. The hydrophobic effect is a major factor in the folding and maintenance of protein structure in aqueous environments, but it is less significant in predominantly organic solvents due to weakening of hydrophobic interactions between side chains [27]. Dynamic light-scattering techniques indicate that ethanol in high enough concentrations changes the nature of intermolecular interactions between proteins [28]. In aqueous solutions, HEWL molecules exhibit strong repulsive interactions [28]. As the ethanol concentration increases to 80%, a variety of structures form ranging from "temporary clusters" at lower ethanol concentration to intermediates with a "broken rod" conformation at 80% ethanol. The "broken rod" conformation is flexible, easily entangled, and can lead to intermolecular β-sheet formation [28]. Small angle X-ray and neutron scattering studies of HEWL in ethanol indicate that a prominent intermediate structure in the formation of amyloid is a dimeric species formed from monomers that have undergone a structural transition [29]. We assume that the increase in β-sheet we observe in CD from day 0 to day 7 corresponds to formation of dimers. The formation of any β-sheet rich structure requires extensive hydrogen bonding involving the peptide backbone. In alcohol solvent, such hydrogen bonds are stronger than in an aqueous environment. The structural changes continue until finally, the dimers associate to a critical mass, and the growth phase begins. At this point, we begin to detect larger aggregates that also have the properties of amyloid.

Goda and colleagues also observed fibrils in concentrated ethanol solutions, but the nature of their experimental system makes it difficult to determine whether peptide backbone interactions are a major determinant in the formation of amyloid. Goda's system consisted of lysozyme (10 mg/mL) in 90% ethanol with 10 mM NaCl and no agitation [15]. They observed fibrils after one week. The more rapid fibril formation is almost certainly due to the addition of salt, which, in the presence of organic solvents, promotes association of lysozyme molecules due to an enhanced hydrophobic effect. More rapid fibril formation can also occur because the initial state of HEWL in Goda's studies is a non-native state; they indicate that HEWL adopts a structure that is rich in β-sheet in 90% ethanol [15]. Their study, along with the present study, is consistent with partial unfolding as an obligatory step in the formation of amyloid fibrils from HEWL. Goda's studies were performed with significantly higher protein concentrations. In such a concentrated organic solvent, molecular crowding of partially folded protein molecules can also make a major contribution to aggregation, especially amorphous aggregation. By performing studies in the absence of salt and using lower protein and ethanol concentrations, the present study is able to focus more on the role of interactions of the peptide backbone.

Studies in which polyamino acids were induced to form amyloid under aqueous conditions indicate that neither amino acid side chain interactions nor specific sequence patterns play a large role in amyloid formation [30]. We infer from the present study that intermolecular interactions of the peptide backbone are a major determinant in the formation of amyloid in organic solvent as in aqueous solvent. In organic solvents, the effects of specific side chain interactions become less pronounced and it becomes more favorable for proteins to associate via extensive hydrogen bonding of the peptide backbone. Regardless of physicochemical conditions or primary structure, peptide backbone interactions can consistently form in all proteins because of their polymeric properties. Appropriate physicochemical conditions, however, enhance this process and can result in amyloid formation. We believe that more detailed studies of experimental systems of amyloidogenic proteins in organic solvents can lead to a better appreciation of the underlying role of peptide backbone interactions.

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