Shear Flow Induces Amyloid Fibril Formation

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Shear flow is indirectly implicated in amyloid formation in vitro. Despite the association between amyloid fibrils and disease, and the prevalence of flow in physiological systems, the effect of this parameter is uncharacterized. We designed a novel Couette cell to quantitatively investigate shear exposure during fibrillogenesis. Amyloid formation by β-lactoglobulin was monitored in situ with real-time fluorescence measurements across a range of shear rates. We demonstrate shear-induced aggregation of spheroidal seed-like species. These seeds enhance fibril formation in native β-lactoglobulin, thereby demonstrating that shear flow generates an amyloidogenic precursor. Furthermore, preformed fibrils are degraded by exposure to high shear rates. Our results have implications for the mechanism of amyloid formation in physiological flow conditions.

The relationship between amyloid fibril formation and disease is well established[1−4] yet the detailed mechanism of fibrillogenesis awaits conclusive determination.[5,6] Considerable anecdotal evidence exists that shear flow affects amyloid formation in vitro. Despite ubiquitous physiological flow in vivo, the effect of shear on fibril formation has remained largely unexamined. Several workers have commented that stirring, shaking, or mechanical agitation accelerate the rate of fibril formation.[6−8] Similar shearing processes have also been reported to influence fibril morphology.[6,9] Sonication can enhance amyloid-like aggregation of proteins[10] and is widely used to fracture fibrils and generate seeds.[6,9] Fibrils can also be broken up by injection through a narrow aperture.[6,11] These examples span a diverse range of proteins, environments, pH, temperature, and flow regimes, with one unifying theme: the solutions are exposed to some form of shearing flow. These effects suggest that a shear-related mechanism may be involved in amyloid formation.

Simple shear flow (Couette flow) is generated when two parallel plates separated by a liquid move relative to each other, creating a uniform velocity gradient in the fluid. The rates, nature, and duration of shear exposure of the protein solutions were not quantified in earlier observations.[6−11] Consequently, we have used the model amyloid-forming protein β-lactoglobulin (β-lg) to investigate the effect of well-defined Couette shear flow on amyloid fibril formation. β-lg is well characterized[12] and is readily available in high purity. It can be induced to form amyloid by pH adjustment and heat treatment[11,13] and has been used as a model system for in vitro studies with physiological relevance. Amyloid formation was detected by Thioflavin T (ThT) fluorescence[14,15] and further substantiated using atomic force microscopy (AFM).[16]

Experimental Methods

Materials. Bovine β-lactoglobulin was obtained from Sigma Aldrich, product reference L0130, batch code 033K7003, and comprised a mixture of A&B variants. It was refrigerated on receipt and used without further purification. Thioflavin T was also sourced from Sigma Aldrich and used as received. Control measurements were undertaken to ensure that the dye did not degrade within the experimental time frame. Native protein solutions were prepared at 6.7 and 20.0 mg/mL by direct dissolution into 250 μM ThT(aq), and the pH was adjusted to 2 or 7 as required using 1.00 M hydrochloric acid and 1.02 M sodium hydroxide solutions. Preformed β-lg fibrils were generated by incubation of 20.0 and 40.0 mg/mL solutions at pH 2, low ionic strength, and 80 °C for 24 h. These samples formed gels which were gently agitated to disperse the aggregates prior to measurement. The fibrils were subsequently diluted in ThT stock solutions to yield a final composition of 6.7 mg/mL protein and 208 μM ThT, and the pH was readjusted to 7.1. The ionic strength of the solutions was then $\sim 10^{-2}$ M. Solutions were refrigerated until required at 5 °C for periods of up to one week. No effect of refrigeration was observed during this time.

Instrumentation. All fluorescence measurements were recorded in a Cary Eclipse fluorescence spectrometer (Varian Inc.) equipped with a magnetic stirrer and Peltier heating accessory. Temperature equilibration to 80 °C occurs in approximately 2 min from injection of the sample into the cell. A 7 mm stirrer bar was used in the cuvette at a rotational speed of 120 rpm. Assuming a gap of 10 μm between surfaces yields a shear rate of 10^4 s^{-1} at the stirrer bar tip. 2 mm and 10 mm quartz cuvettes were used for all quiescent measurements. Emission spectra were excited at 442 nm, and the evolution of ThT emission intensity was continuously monitored at 484 nm using the Varian "Kinetics" module. Atomic force microscopy was undertaken in TappingMode on a Dimension 3100 instrument (Digital Instruments), using silicon nitride probes sourced from MikroMasch. The spring constants and resonance frequencies were 40 $N \cdot m^{-1}$ and 325 kHz, respectively. Solution viscosity was determined using a Schott Ubbelohde capillary viscometer equilibrated at 25.0 ± 0.1 °C. The reference solvent was distilled water, $\eta = 0.89$ mPa·s.

Controlled Shear Experiments. Real-time measurements in controlled shear flow were undertaken in a custom-built Couette cell optimized for fluorescence studies. The instrument was configured as previously described, but incorporated a quartz inner cylinder and protective PTFE spacer at the base of the rod.[17] (See Figure 1.) Samples were recovered from the shear cell at the end of the experiments and retained for subsequent analysis. Photodegradation of ThT was not observed.

Seeding Experiments. Native protein solutions comprising 6.7 mg/mL β-lg and 250 μM ThT at pH 2.0 were sheared continuously at 200 s^{-1} for 15 h and recovered from the Couette cell. Aliquots were mixed with further unsheared native protein and incubated at 80 °C in the

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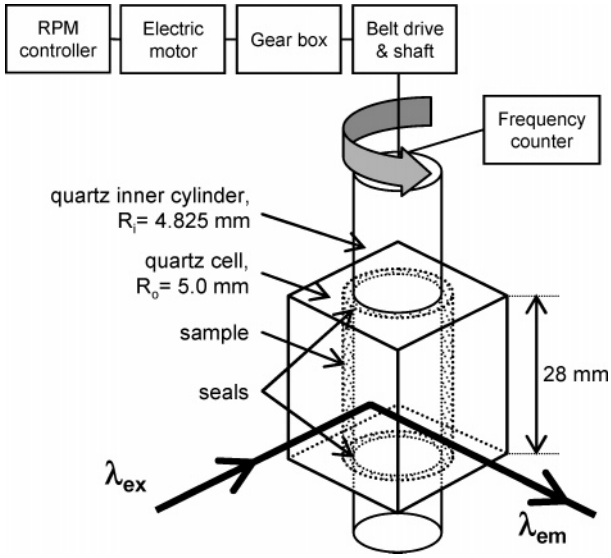

Figure 1. Schematic diagram of the quartz Couette rheofluorescence cell used to study β-lg amyloid solutions in a controlled shear environment. The shear rate $\dot{\gamma}$ is determined by $\dot{\gamma} = 2\Omega/(1 - (R_i/R_o)^2)$ where Ω is the angular frequency, and R_i and R_o are the inner and outer cylinder radii, respectively. The shear field in a Newtonian fluid is effectively uniform across the cell for the specified gap ratio.

fluorescence spectrometer or oven. The concentration of the seed solutions was adjusted to be approximately 6.7 mg/ml β-lg.The rate of amyloid formation was followed in situ by ThT emission, and the morphology of the final product was imaged with atomic force microscopy.

AFM Sample Preparation. All samples were prepared in accordance with standard procedures. 20 or 50 μL of 1 mg/mL solutions were deposited onto freshly cleaved mica, allowed to adsorb for ~30 min in a covered Petri dish, and rinsed twice with 1 mL of Milli-Q water. Excess fluid was drained, and the surfaces were dried in air for ~30 min before final drying in a slow stream of nitrogen. Samples were imaged immediately using AFM. The scans shown are representative of a number of samples and images.

Results and Discussion

Initial data was obtained using a magnetically stirred, incubated cuvette placed in a fluorescence spectrometer. This configuration revealed the impact of continuously applied variable shear in conditions that emulated previous observations. The increase in ThT emission during incubation is compared for quiescent and stirred solutions of bovine β-lg in Figure 2. A small positive slope for the unstirred control indicates slow formation of amyloid fibrils. In contrast, the stirred solution exhibits a pronounced increase in ThT emission (30-fold rise in 18 h). Examination of the relative fluorescence enhancement (ratio of stirred: unstirred emission intensities) indicates that agitation markedly accelerates fibril formation in the first 5 h of incubation. Interestingly, there is no significant enhancement during the first 30 min, an observation also made by Collins et al. using Congo Red in seeded solutions of Sup35 prion fragments.[6]

The shear field generated by magnetic stirring in a cuvette is highly heterogeneous and poorly controlled. To elucidate the effect of controlled shear flow on an amyloid-forming model system, we designed a novel all-quartz Couette cell to measure fluorescence intensities in flow in real time (Figure 1). We have previously used this instrument to examine shear-induced deformation and orientation of synthetic polymers.[17] The

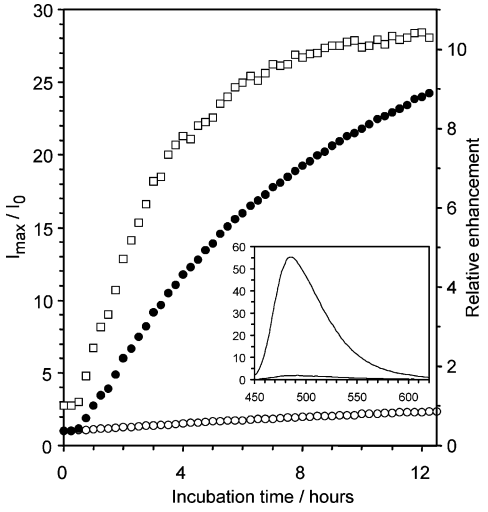

Figure 2. Increase in ThT emission intensity as a function of incubation time for stirred and unstirred β-lactoglobulin solutions in a cuvette. Inset shows emission spectra of stirred sample at 0 and 18 h. ○, unstirred solution; ●, stirred continuously; □, relative enhancement (ratio of stirred: unstirred intensities). 20.0 mg/mL β-lg in 250 μM ThT, pH 2.0, incubated at 80.0 °C, $\lambda_{ex} = 442$ nm, $\lambda_{em} = 484$ nm, emission spectra recorded every 15 min.

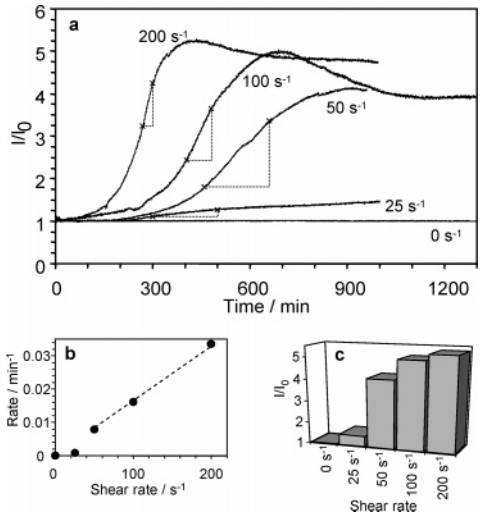

Figure 3. (a) Increase in ThT emission intensity as a function of shear exposure for β-lactoglobulin solutions sheared at a range of controlled rates in the rheofluorescence cell. 6.7 mg/mL β-lg in 250 μM ThT, pH 2.0, $\lambda_{ex} = 442$ nm, $\lambda_{em} = 484$ nm, ambient temperature (20 °C). (b) Effect of shear rate on maximum amyloid formation rate. (Gradient of steepest part of curve, as indicated by dotted lines in 3a.) (c) Effect of shear rate on apparent quantity of fibrils formed (maximum emission intensity I/I_0).

advantage of a narrow gap Couette cell lies in the ability to expose the protein solution to a uniform, well-defined shear rate for a controlled period. The instrument is mounted in the sample compartment of a spectrometer to enable continuous real-time measurement of fluorescence emission from ThT in the presence of β-lg. The kinetics of fibril formation in defined shear flow may therefore be determined accurately.

Shear-induced formation of amyloid-like material from β-lg in the presence of ThT is shown in Figure 3. The control measurement (0 s^{-1}) showed no increase in ThT emission during the experiment. At rates between 25 and 200 s^{-1} shear flow induced the formation of species that bind ThT, indicated by increased fluorescence emission. Each curve has a characteristic sigmoidal form that resembles kinetic data for other amyloidogenic systems,[6,18] an initial lag time is followed by a growth

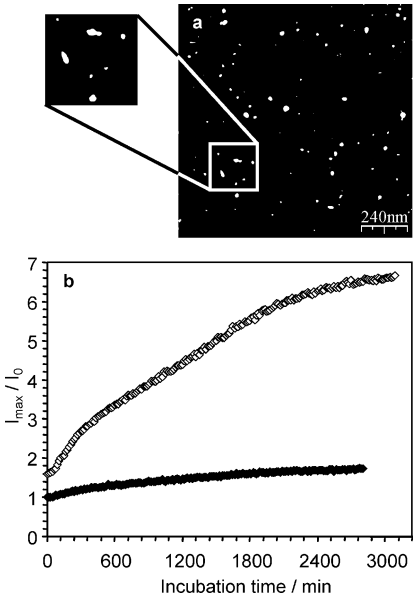

Figure 4. (a) AFM height image of β-lg aggregates induced by shearing at 200 s^{-1} for 15 h. Typical apparent particle width = 28 nm, height = 4−5 nm. The main image is 1.2 μm × 1.2 μm wide; the inset is 250 nm × 250 nm. (b) Enhanced amyloid formation in β-lactoglobulin solutions seeded with sheared native protein to produce seeds and incubated at 80.0 °C. White diamonds show increased ThT binding in β-lg solution seeded with β-lg aggregates generated by shearing at 200 s^{-1} for 15 h. Black diamonds show ThT emission from the unseeded control solution. Emission is normalized to the initial, unseeded intensity. (Sample composition as for Figure 3; seeded solution contains 33% sheared material with the final protein concentration adjusted to be approximately 6.7 mg/mL.)

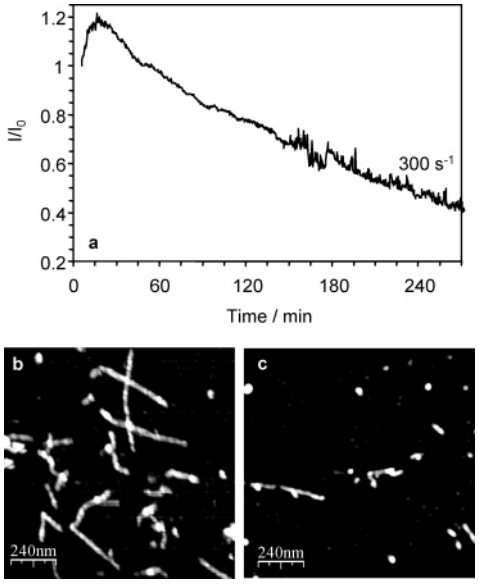

Figure 5. (a) Degradation of preformed amyloid fibrils at high shear (300 s^{-1}) revealed by a decrease in ThT emission intensity as a function of shear exposure in the rheofluorescence cell. (λ_{ex} = 442 nm, λ_{em} = 484 nm). (b and c) AFM height images of preformed and shear-degraded fibrils, respectively. Fibrils were sheared at 500 s^{-1} for 15 h. The typical contour length is reduced from 450 to 100 nm. Heights remain constant at 4−5 nm; the mean apparent width is 29−30 nm. Image sizes are 1.2 μm × 1.2 μm.

phase. At 100 and 200 s^{-1} ThT emission reached a maximum before decaying to a plateau; at 25 and 50 s^{-1}, formation was not completed within the time scale of the experiment (Figure 3a). This is one of the first quantitative data sets that directly shows that the effect of shear is to induce and enhance formation of amyloid-like aggregates in solution.

Several systematic trends appear. The rate of aggregation, indicated by the gradient of the growth phase, increases linearly with shear rates above 50 s^{-1} (Figure 3b). This is consistent with a sheared Newtonian fluid, in which the energy dissipation rate is predicted by shear rate × shear stress.[19] The lag time is progressively reduced at elevated shear rates. The quantity of pre-fibrillar material generated, represented by the maximum value of each kinetic curve, is also shear-dependent. A 5-fold enhancement in ThT emission was achieved in the 200 s^{-1} experiment (Figure 3c). It is reported that oligomeric precursors in β-lg amyloid formation also bind ThT.[20] Solutions were therefore retrieved from the Couette cell in order to establish the nature of the species generated in shear. Atomic force microscopy (AFM) revealed the formation of aggregated material typically 4−5 nm high, as illustrated in Figure 4a. The mean apparent width was 28 nm, although the true values are obscured by tip-broadening artifacts associated with AFM. These remarkably uniform particles were not observed in analogous unsheared solutions. (Figure S1, Supplementary Information.) No other aggregated species were observed in the AFM images. Aliquots of sheared protein were incubated with further β-lg under amyloid-forming conditions. The resultant amyloidogenesis is compared with an equivalent control solution in Figure 4b. Addition of sheared material significantly accelerated and enhanced amyloid formation, i.e., acted like a seed. The application of defined shear flow in these experiments has enabled this amyloid precursor to be identified.

β-lg is monomeric and partially denatured at pH 2.0 and low ionic strength.[21] It retains significant β-sheet structure. We postulate a mechanism in which the protein is aligned and further unfolded in shear flow in a manner that induces formation of spheroidal amyloid precursors. Shear-induced orientation and deformation effects are widespread in synthetic polymers[22] and energy dissipation in a sheared fluid is calculated as viscosity × (shear rate)^2.[19] At 100 s^{-1} we estimate the total energy dissipation in 6.7 mg/mL β-lg to be 25 J·mol^{-1}·s^{-1}, based on a viscosity of 0.91 mPa·s. Average ΔG_0 values for β-lg at pH 2.0 are 20 kJ·mol^{-1} per residue, whereas the most conformationally labile sites have ΔG_0 < 4 kJ·mol^{-1}.[23] The additional energy imparted to the solution during prolonged shear is therefore of similar magnitude to the energy required for protein deformation. Fändrich and Dobson[24] reported that amyloid formation primarily depends on interactions between protein chain backbones, so a process such as shear, which influences the conformation of macromolecular chains, can play a significant role in directing association.

Amyloid degradation in flow was also measured in the Couette cell using preformed β-lg fibrils diluted in ThT. At shear rates above 100 s^{-1} emission from these solutions first increased by up to 20% of the initial intensity and then decreased continuously. Figure 5a illustrates an example of this degradation behavior measured at 300 s^{-1}. Increased emission intensity at the start of shear exposure is likely to be due to initial orientation and partial deformation of the fibrils in the flow field. The overall decline in ThT emission indicates a progressive reduction in fibril length and consequent loss of ThT binding sites.[15] Fibril truncation was independently confirmed using AFM imaging, illustrated in Figure 5, panels b and c. The typical contour length of the amyloid was reduced from 450 nm to less than 100 nm after shearing at 500 s^{-1} for 15 h. Fibril heights remained uniform at 4−5 nm, and the apparent widths were 29 nm. These dimensions are remarkably consistent with the sheared precursors shown in Figure 4. Rogers et al. used birefringence methods to observe alignment of amyloid fibrils in Couette flow at shear

rates in the range 0.1−200 s^{-1}.[25] Fibril fracture did not occur in that study because the cumulative shear duration was considerably shorter.

We have shown that exposure of β-lactoglobulin to controlled shear flow in vitro promotes fibrillogenesis by generating precursors that act as seed-like initiators. The rate and extent of precursor formation increases with shear rate. This phenomenon is distinct from seed generation in fractured fibrils. (No fibrillar species were observed in the relevant AFM images.) Preformed amyloid fibrils degrade in continuous high shear, evidenced by reduced ThT emission intensity and confirmed with AFM imaging. We propose a mechanism in which shear flow imposes conformational change and orientational order on the proteins, to facilitate association. Indirect literature evidence suggests that this mechanism may be general to other amyloidogenic proteins. Physiological shear rates in vivo are significant, and range widely from 100 to 8000 s^{-1} in blood vessels and the exocellular matrix.[26] The rates in this study are therefore physiologically relevant. Clinical amyloidoses are multi-factorial in origin, and shear effects, e.g., in blood, may facilitate amyloid formation in vivo. Amyloid has not been identified in blood, but is deposited in proximal tissue, e.g., vessel walls. Shear flow may form oligomeric precursors in blood which are subsequently taken up in tissue where they nucleate fibrillogenesis. Furthermore, the propagation of amyloid diseases may be promoted by shear-degradation of fibrils to generate new seeds, which can be transported to new deposition sites by fluid flow. Clinical examples of amyloidoses associated with fluid flow include the observation of apolipoprotein A-1-derived fibrils localized in atherosclerotic plaques in the human aorta,[27] and the incidence of haemodialysis-related amyloidosis in patients with renal failure.[28] Although the causal relationship needs to be established, the ability of shear to lower the activation barrier to amyloid formation has significant implications for disease onset.

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Supporting Information Available. AFM height images of unsheared β-lg (Figure S1). TEM images of fibrils seeded by incubation with sheared protein solution (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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