THE JOURNAL OF PHYSICAL CHEMISTRY Letters

Letter

pubs.acs.org/JPCL

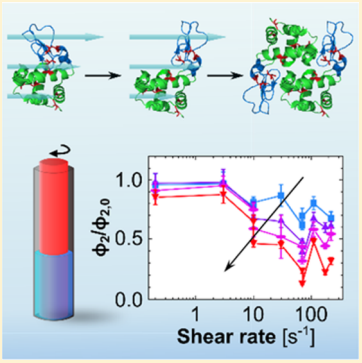
Protein Conformational Flexibility Enables the Formation of Dense Liquid Clusters: Tests Using Solution Shear

Michael C. Byington,[†] Mohammad S. Safari,[†] Jacinta C. Conrad,*[,†] and Peter G. Vekilov*[,†,‡]

[†]Department of Chemical and Biomolecular Engineering and [‡]Department of Chemistry, University of Houston, 4726 Calhoun Road, Houston, Texas 77204-4004, United States

Supporting Information

ABSTRACT: According to recently proposed two-step nucleation mechanisms, crystal nuclei form within preexisting dense liquid clusters. Clusters with radii about 100 nm, which capture from 10^{-7} to 10^{-3} of the total protein, have been observed with numerous proteins and shown to host crystal nucleation. Theories aiming to understand the mesoscopic size and small protein fraction held in the clusters have proposed that in solutions of single-chain proteins, the clusters consist of partially misfolded protein molecules. To test this conjecture, we perturb the protein conformation by shearing solutions of the protein lysozyme. We demonstrate that shear rates greater than a threshold applied for longer than 1 h reduce the volume of the cluster population. The likely mechanism of the observed response involves enhanced partial unfolding of lysozyme molecules, which exposes hydrophobic surfaces between the constituent domains to the aqueous solution.



Nucleation of crystals from solution underpins myriad environmental, physiological, and industrial processes. Classical approaches assumed that crystals nucleate from solution through a single-step process, in which monomers directly assemble into a properly structured array.[1,2] Predictions of the nucleation rate based on this picture, however, deviate by ten and more orders of magnitude from careful experimental determinations, suggesting that the classical nucleation mechanism does not operate in all systems.[3] Hence, alternative nonclassical mechanisms that lead to nucleation of crystals in solution remain vigorously debated.[4]

One important outcome of recent studies was the proposal that ordered nuclei form within clusters, in which the solute concentration exceeds that in the solution.[3,5−14] These precursor clusters were directly observed[9,12,14−16] and the nucleation of crystal inside them monitored[11,14,17,18] across a range of systems. Importantly, experiments demonstrated that larger volume of the cluster population correlates with higher nucleation rate.[3,19−22] The two-step nucleation mechanism successfully captures crucial features of crystal nucleation in solution; still, there remains limited insight into the pathways by which the precursor clusters form.

Proteins represent a convenient model system for studies of multistep nucleation, owing to the large size and slow dynamics of protein molecules. Solute-rich clusters, thought to serve as precursors to nucleation, have been reported for many proteins.[3,5,7,15,16,18−21] Their existence, however, challenges our understanding of phase ordering. Although the clusters are likely liquid,[15,16,20] they exist in the homogeneous region of the protein solution phase diagram, away from the conditions of liquid−liquid coexistence.[15,16,23] Typical sizes are on the order of 100 nm,[22,24,25,33,34,36−39] which is much larger than predicted from a colloid aggregation scenario based on the balance of van der Waals attraction and Coulomb repulsion.[24] Moreover, the responses of cluster size and of cluster population volume are decoupled upon variations in ionic strength, pH, and additive concentration.[25−27] These properties indicate that the mesoscopic clusters represent a novel class of protein condensate that forms by a fundamentally different mechanism than other protein aggregates, such as crystals and amyloid fibrils. The available data suggest that the clusters represent regions to which protein molecules diffuse and combine to form transient oligomers (dimers, trimers, etc.), which in turn diffuse out of the clusters and decay to monomers.[23,28] According to theory, the cluster size R_2 is related to the diffusivity D_{oligo} (similar in value to the monomer diffusivity) and decay rate constant k_{oligo} of oligomers as $R_2 = \sqrt{D_{oligo}/k_{oligo}}$.[23,28] Importantly, the cluster size does not depend on the rate of oligomerization.[23,28] Whether and how the conformation of the protein affects oligomerization is not understood.

Here, we test the premise that partial protein unfolding is a part of the oligomerization that underlies cluster formation in solutions of single-chain proteins.[23] As a model protein, we use lysozyme, one of the best-studied soluble enzymes with a robust structure, moderate size (molecular weight 14 300 g mol^{-1}), and easy availability at high purity.[29−32] Nucleation rates data and direct observations suggest that lysozyme follows a two-step mechanism of crystal nucleation[3,7,18] and a population of mesoscopic protein-rich clusters that may be

Received: April 16, 2016
Accepted: June 7, 2016

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

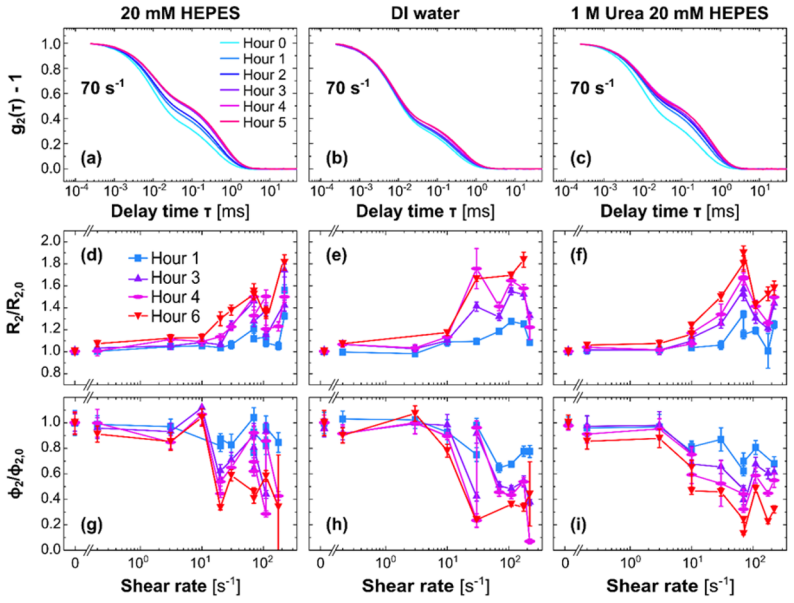


Figure 1. Response of the cluster populations in lysozyme solutions with concentration 100 mg mL^{-1} in three solvents, listed at the top, to continuous shearing extending to 6 h. (a)−(c) Evolution of the intensity correlation functions of scattered light with time of shearing at shear rate 70 s^{-1} in solutions of three compositions. (d)−(f) Variation of the cluster radius R_2, scaled with that in unsheared solutions $R_{2,0}$, with increasing shear rate after shearing for times indicated in (d). (g)−(i) Variation of the fraction of the solution volume occupied by the cluster population ϕ_2, scaled with that in unsheared solutions $\phi_{2,0}$, with increasing shear rate after shearing for times indicated in (d).

the nucleation precursors has been identified and characterized.[23,26,33] We use solution shear to induce partial protein unfolding. Laminar flow at relatively low shear rates, from 10 to 700 s^{-1}, induces reversible conformational changes in relatively stable globular proteins, including lysozyme.[34,35] Shear rates as fast as 10^5 s^{-1} lead to complete unfolding, detectable by intrinsic fluorescence,[36] calorimetry,[37] and aggregation.[38] We compare its effects to those of chemical additives (urea and mercaptoethanol) known to unfold proteins

We sheared lysozyme solutions with concentration 100 mg mL^{-1} in a custom-built Couette cell. At select times after the start of shearing, solution aliquots were extracted from the Couette cell, filtered to remove dust and debris, and analyzed with dynamic light scattering (DLS) to determine the characteristics of the cluster population. The intensity−intensity correlation functions $g_2(\tau)$, where τ is the lag time, recorded after shearing for up to 6 h, all possess two distinct shoulders, indicating the presence of two populations of scatters (Figure 1a). The corresponding intensity distribution functions, computed using the CONTIN algorithm (Figure S1), indicate that both scattering populations are relatively monodisperse. We fit the correlation functions with

$$[g_2(\tau) - 1]^2 = A_1 \exp\left(-\frac{\tau}{\tau_1}\right) + A_2 \exp\left(-\frac{\tau}{\tau_2}\right) + \varepsilon$$

where the times τ_1 and τ_2 characterize the diffusion of the two scattering populations;[15,26,39,40] A_1 and A_2 are the respective amplitudes, which are proportional to the intensity scattered by the respective scatterers; and ε accounts for mechanical, optical, and electronic noise in the signal.[15,39] Using τ_1, τ_2, A_1, and A_2 and independently measured solution viscosities, refractive index increments, and protein intermolecular interaction parameters,[26] we evaluate the average hydrodynamic radius of each population, R_1 and R_2, and the fraction of the total solution volume occupied by the slow scatterers, ϕ_2.[15,26,39] The average radius R_1 of the smaller scatterers is 1.7 nm, in good agreement with that of individual lysozyme molecules. The larger scatterers have an average radius R_2 of about 30 nm and occupy about 5×10^{-5} of the solution volume, consistent with the values measured for lysozyme dense liquid clusters in previous studies.[23,26,33,41] We conclude that the small and large scatterers are lysozyme monomers and clusters, respectively.

The evolutions of R_2 and of ϕ_2 in solutions buffered with HEPES at shear rates varying from 0.3 to 200 s^{-1} are displayed in Figures 1d,g. In quiescent solutions, the only source of shear is buoyancy-driven convection; the flow velocity in a quiescent solution that is held in a cuvette of diameter 1 cm at a height of about 1 cm with temperature gradients of about 0.1 K is on the order of 1 μm s^{-1}, leading to shear rates in the range 0.001−0.01 s^{-1}.[42,43] Hence, the enforced shear rates are much larger than those induced by buoyancy-driven convection. Because we find that in quiescent solutions both R_2 and ϕ_2 are constant over 7 h (Figure S2), equal to the longest duration of shearing in Figure 1b,c, we scale R_2 and ϕ_2 by the corresponding values in quiescent solutions, $R_{2,0}$ and $\phi_{2,0}$.

The lowest enforced shear rate, 0.3 s^{-1} consistently induces a small increase in R_2 and a small decrease in ϕ_2. This effect increases at longer exposures to shear but is always limited to less than 10% of $R_{2,0}$ and $\phi_{2,0}$. Exposures to shear rates lower than a threshold value do not induce additional deviations of R_2 and ϕ_2 from their values in quiescent solutions. Interestingly, shear-induced partial unfolding also exhibits a threshold shear rate, below which only minor conformational modifications were observed.[35] In Figure 1b,c, after the threshold is reached, however, R_2 increases and ϕ_2 decreases as a function of increasing shear rate. Whereas the threshold shear rate is mostly unaffected by the duration of shearing, longer exposure to shear above the threshold amplifies the effects of faster shearing. At shear rates faster than 100 s^{-1}, the solution remained turbid even after filtering through a 0.22 μm filter, suggesting

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

irreversible denaturation and aggregation of a part of the dissolved protein.

The negatively coupled response of R_2 and ϕ_2 to increasing shear is in contrast to conventional phase transformations, such as solidification or liquefaction, in which the domain size of the incipient phase increases concurrently with its overall volume in response to variations of the external parameters. To understand the mechanism that underlies the surprising trends observed with the protein-rich clusters, we compare the effects of shear in solutions of three compositions: HEPES buffer (Figure 1a,d,g), water (Figure 1b,e,h), and urea (Figure 1c,f,i). HEPES (sodium 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonate) is a zwitterionic organic molecule that is used to maintain near-physiological pH. Binding of the HEPES solution species to proteins is extremely rare,[44] suggesting that its action on the cluster properties would be through the hydrogen ion concentration, adjusted to pH = 7.8. Protonation of basic and acidic surface amino acid groups leads to a +8 net charge of the lysozyme monomer at pH = 7.8,[45] the balance of 17 positive and nine negative groups.[46] If lysozyme is dissolved in water and dialyzed to remove excess precipitant used in purification, pH sets at 5.4. At the lower pH = 5.4, the net charge increases to +12.[45] The change from HEPES buffer to water induces stronger sensitivity to shear: the threshold shear rate for increased R_2 and lowered ϕ_2 decreases from 10 s^{-1} to 3 s^{-1}, as revealed in Figure 1e,h. A possible explanation for the effects of solution composition on the response to shear is that the higher molecular charge increases repulsion between intramolecular domains and hence destabilizes the molecular conformation.

To test the correlation between molecular destabilization and response to shear, we studied the effects of shear on the cluster population in urea-containing solutions. Urea is known to perturb the native structure of most proteins; addition of 8 M urea to aqueous solutions completely unfolds proteins.[47−49] Urea is currently thought to be a universal denaturant because it interacts favorably with the peptide backbone.[50] The amino acid side chains further assist the action of urea by interacting preferentially with it and by diluting the effective concentration of the backbone amides.[51−53] The accumulation of urea at nonpolar protein patches and the accompanying destruction of the water structures are described as chaotropic action.[54] The addition of 1 M urea to a lysozyme solution in 20 mM HEPES buffer preserves the pH at 7.8. Nonetheless, adding urea reduces the threshold for enhanced response to shear from 10 to 3 s^{-1}, Figure 1f,i, suggesting that partial protein unfolding contributes to the cluster population response to solution shear. Partial unfolding could expose to the solvent the hydrophobic interface between the α and β domains of the lysozyme molecule (Figure 2), and induce the formation of dimers (or trimers), in which one domain of a monomer is replaced by the same domain from another molecule. Such oligomers are described as domain swapped.[25,26,55]

The oligomer mechanism of cluster formation suggests two pathways by which shear flow could increase the cluster size. First, solution flow may accelerate the exodus of oligomers from the clusters, effectively increasing D_{oligo}. With oligomer diffusivity of order 10^{-10} m^2 s^{-1} (comparable to that of the monomer), diffusion over the cluster radius of about 30 nm would have a characteristic time of about 10 μs. This is nearly 3 orders of magnitude faster than oligomer transport enhanced by shear rates slower than 200 s^{-1}, which have characteristic times longer than 5 ms. This estimate suggests that the second

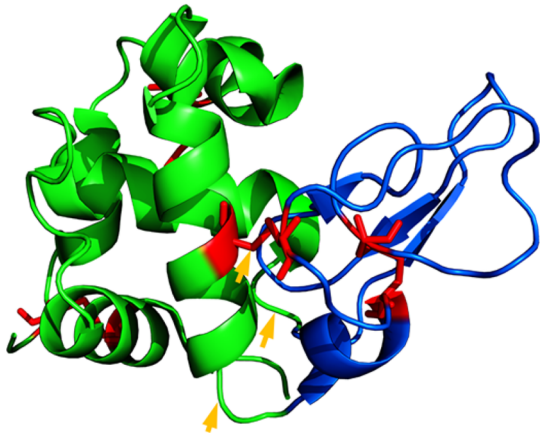


Figure 2. Structural domains in lysozyme. The α and β domains are highlighted in green and blue, respectively, and the S−S bridges in red. The domains are linked by two peptide chain loops and a S−S bridge (indicated with arrows) that are aligned in a hinge, which allows domains to open and hence expose the nonpolar interdomain interface to the solution. Hydrophobic attraction between internal domain surfaces from different molecules leads to domain-swapped oligomers. Protein structure drawn using PyMOL and atomic coordinates from Wang et al.;[31] α and β domains identified as in McGamon et al.[30]

mechanism, involving shear-induced unfolding, dominates. The unfolding exposes to the solvent hydrophobic surfaces heretofore tucked inside the molecule, which slows the decay rate of oligomers k_{oligo} and, hence, increases the cluster size. This mechanism is supported by the stronger response to shear in the presence of urea and at lower pH, both of which destabilize the native protein conformation (Figure 1). Furthermore, because the cluster population volume is determined by the free-energy balance between clusters and solution,[33] the decrease in ϕ_2 is likely due to stronger attraction between lysozyme molecules with exposed nonpolar interdomain surfaces in a partially unfolded conformation. The unfolding lowers the chemical potential of the protein in the solution, the driving force of monomers into clusters and, hence, ϕ_2. Importantly, the cluster population response to mechanical unfolding, by shearing, opposes that of chemical unfolding, by urea.[26] Urea weakens the hydrophobic interaction between newly exposed nonpolar patches. This chaotropic effect decreases the oligomer lifetime and increases the solution free energy, inducing *smaller* clusters and *larger* cluster populations.

The suggested conformational destabilization suggests an explanation of the nonmonotonic responses of R_2 and ϕ_2 to higher shear observed in Figure 1d−i. In other experiments under identical conditions, we found that, overall, R_2 always increased and ϕ_2 always decreased at faster shear rates and longer exposures to shear. The threshold shear rates for these trends were faithfully reproduced. Nonmonotonic behaviors, however, were observed at varying shear rates above the threshold, or not at all. We conclude that after the native protein conformation is destabilized by shearing faster than the threshold rate the degree of induced partial unfolding may vary in response to minor inconsistencies in the system parameters.

An essential question both for the general mechanism of protein-rich clusters and to understand the effects of conformational flexibility on the cluster population is whether intramolecular S−S bridges are disrupted during the unfolding leading to clusters. The lysozyme structure in Figure 2 suggests

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

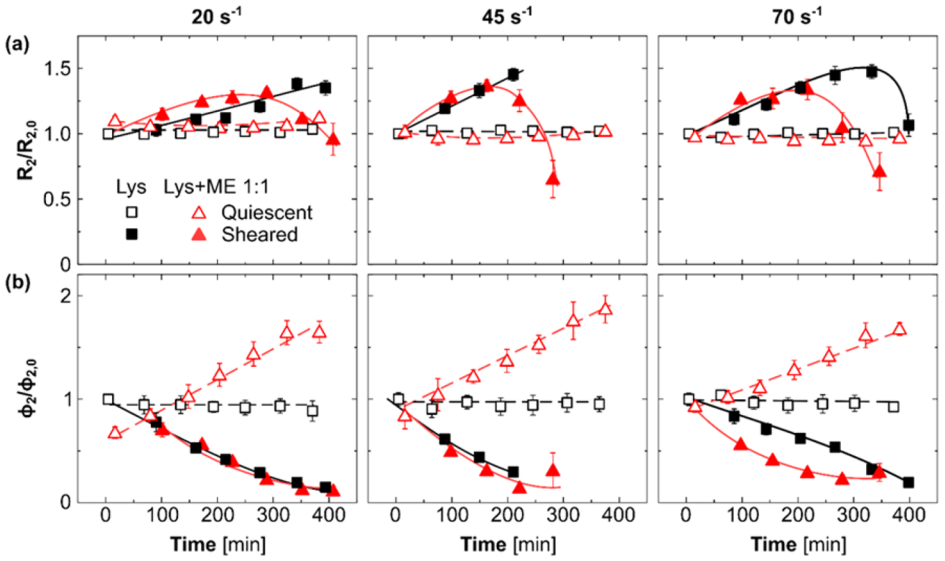


Figure 3. Effects of mercaptoethanol (ME) on the response of lysozyme clusters to shear. Evolution of (a) average cluster radius R_2 and (b) cluster population volume ϕ_2, scaled by their respective values in quiescent solutions in the absence of ME, $R_{2,0}$ and $\phi_{2,0}$, at three shear rates indicated above the plots and in quiescent controls carried out in parallel with each shearing experiment.

that the formation of domain-swapped lysozyme oligomers does not require the breaking of S−S bridges. To answer this question, we partially reduced the S−S bridges with mercaptoethanol (HSC_2H_4OH, ME; results in the literature indicate that preventing the formation of disulfide bridges by either chemical modification of the cysteines or mutating them equally affects lysozyme folding dynamics[56,57]) and monitored the response of the cluster population in quiescent and sheared solutions. Preliminary tests revealed that 4-fold molar excess of ME denatures lysozyme, whereas using 0.5 mol equiv insignificantly affects the characteristics of the cluster population. Hence, we used a molar concentration of ME equal to that of lysozyme, 6.9 mM. Previous experiments with ethanol, a reagent whose structure, polarity, and chaotropic activity are similar to those of ME, demonstrated that ethanol weakly affects the cluster population characteristics at concentrations as high as 2.5 M.[26] Hence, we expect that ME affects clusters only through the reduction potential of the HS group.

In quiescent solutions in the absence of ME, the cluster radius and population volume are steady (Figure 3). Shearing in pure lysozyme increases R_2 and reduces ϕ_2, in agreement with the trends observed in Figure 1d,e,g,h. The decrease in R_2 after 350 min of shearing at 70 s^{-1} is likely due to incipient irreversible denaturation. In quiescent solutions, ME does not affect the cluster size and induces a slow increase of the cluster population volume. These observed responses dramatically differ from those caused by significantly higher concentrations of two chaotropic agents, urea (Figure 1f,i) or ethanol,[26] suggesting that ME induces chemical and conformational changes in the lysozyme molecule that are distinct from exposure of the interdomain interface. These responses are compatible with disruption of the S−S bridges in the α and β domains (Figure 2), which creates disordered chain segments. The modified molecular surface leads to enhanced attraction at short intermolecular separations, which lowers the free energy of the cluster phase and, hence, increases the cluster population volume. These newly created attractive patches may be distant from the interdomain interface; in this case, they would not affect the stability and decay rate of domain-swapped oligomers, leaving the cluster size unchanged.

The response of the cluster population to shear in the presence of ME is similar to that in pure lysozyme solution: the cluster size increases and the cluster population volume is reduced. In the presence of ME, R_2 increases to reach a local maximum at an intermediate shearing time and decreases at longer shearing times. This trend is similar to the decrease of R_2 after shearing for 350 min at 70 s^{-1}, suggesting that it is due to ME-induced irreversible denaturation of the protein. Overall, the effects of solution shearing and ME are dissimilar, indicating that the mechanisms of cluster response to solution shear and to addition of ME are distinct. Importantly, this observation indicates that disruption of S−S bridges and the structure of the two lysozyme domains are not parts of the general mechanism of cluster formation in lysozyme.

Previous studies of lysozyme clusters in quiescent solutions indicated that the clusters are in equilibrium with the monomeric protein and respond to variations of the monomer concentration as predicted by the Boltzmann relation.[23,33] In this context, a relevant question is whether the shear-modified cluster population remains in equilibrium with the lysozyme monomers. To address this issue, we sheared a lysozyme solution for 280 min at 30 s^{-1} and monitored the evolution of R_2 and ϕ_2 at quiescent conditions for 15 h after cessation of shearing. The increased R_2 and lowered ϕ_2 persist (Figure 4a). The irreversibility of the cluster population characteristics may indicate either that the clusters converted to irreversible protein aggregates or that shear modified the equilibrium between monomers and clusters. To distinguish between these two scenarios, we tested the response of the clusters to decreasing protein concentration from 100 to 50 and 25 mg mL^{-1} after shearing for 2 or 4 h at 30 s^{-1}. We find that R_2 does not depend on the protein concentration (in agreement with previous observations)[23,33] and this behavior is not altered by shearing (Figure 4b). If the clusters were irreversible aggregates, the ϕ_2/ϕ_1 ratio would not depend on the solution dilution. Instead, ϕ_2/ϕ_1 strongly decreases upon solution dilution (Figure 4b), in sharp contrast with this expectation. This observation indicates

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

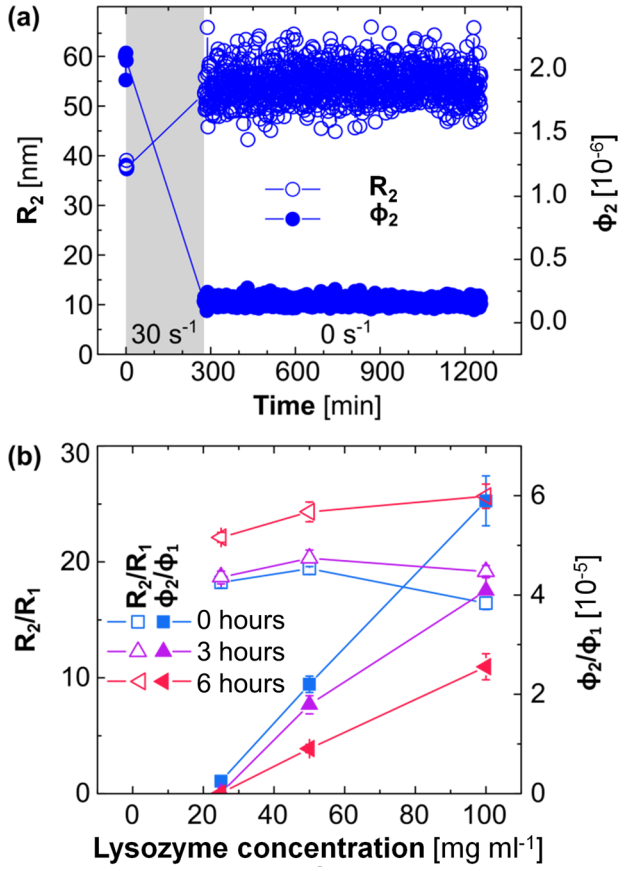


Figure 4. Reversibility of shear effects on cluster formation. (a) Evolution of the cluster radius R_2 and population volume ϕ_2 in a quiescent lysozyme solution in 20 mM HEPES buffer after 280 min of shearing at 30 s^{-1}. Values of R_2 and ϕ_2 prior to shearing are shown for comparison. (b) Variation of R_2 and ϕ_2, scaled by the respective values for the monomer R_1 and ϕ_1, in an unsheared solution and solutions sheared at 30 s^{-1} for 3 and 6 h, respectively, upon sequential dilution from 100 mg mL^{-1} to 50 mg mL^{-1} and 25 mg mL^{-1}.

that the clusters retain their reversibility after shearing. The surprising conclusion is that the shear-induced suppression of the cluster population volume is at least partially due to a permanent conformational modification of the monomers, with which the clusters are in equilibrium.

To evaluate the magnitude of the shear-induced conformational modification, we recorded the spectra of intrinsic fluorescence of lysozyme, due to emittance of the three aromatic amino acid residues, tryptophan, tyrosine, and phenylalanine. The fluorescence spectra sensitively respond to the environment of the reporter amino acids in the folded protein chain.[58,59] The spectra in Figure S5 demonstrate that shearing does not induce significant conformational modifications at the core of the α domain, where the majority of the fluorescence reporter groups are located.[31] The fluorescence tests do not characterize the relative positions of the α and β domains.

In additional tests, we compared the activity of lysozyme in degradation of *Micrococcus lysodeikticus* bacteria in quiescent solution and after shearing. Lysozyme hydrolyzes a tetrasaccharide found in Gram-positive bacteria and breaks the glycosidic bond between *n*-acetylmuramic acid and *n*-acetylglucosamine.[60] We observed (Figure S6) that the activity of lysozyme is not affected by shearing. As the active center of lysozyme consists of amino acid residues that belong to both domains, these observations suggest that the configuration of the α and β domains of lysozyme is not affected in the majority of the solute molecules.

The conformational integrity of lysozyme after shearing was tested using the 1-anilino-8-naphthalenesulfonate (ANS) and Thioflavin T (ThT) assays. ANS is a fluorescent probe for the detection of partially unfolded states. ANS binds to buried hydrophobic sites of proteins, resulting in a blue shift of the fluorescence emission maximum and increase of the fluorescence intensity.[61,62] ThT is employed for selectively staining and identifying amyloid structures as ThT binding to β structures enhances its fluorescence emission.[63] Figure S7 demonstrates that shearing does not affect the fluorescence spectra in solutions of lysozyme and each of the two probe molecules.

The preservation of the enzymatic activity after shearing and the unmodified fluorescence spectra in the presence of ANS and ThT suggest that the partial unfolding, which exposes sufficient hydrophobic areas of the interdomain interface to drive reduction of the cluster population volume, affects only a small fraction of the protein molecules.

In summary, we demonstrate that in solutions of the protein lysozyme shear flow increases the size and suppresses the volume of the population of protein-rich clusters, which may be precursors to crystal nucleation. The likely mechanism of the observed shear response involves partial unfolding of the lysozyme molecules, which exposes to aqueous solution the nonpolar interfaces between the constituent α and β domains. The extended hydrophobic surfaces lower the chemical potential of the lysozyme in solution and, per the oligomer mechanism of cluster formation,[23,25,26,33] stabilize a domain-swapped oligomer. The former outcome lowers the volume occupied by the cluster phase, whereas the latter increases the cluster radius. These observations indicate that enhanced partial unfolding of the lysozyme molecules is a part of the mechanism underlying the formation of mesoscopic protein-rich clusters. It is likely that similar conformational modification may be a general mode of transient oligomerization for other single-chain proteins and underlie the formation of the precursors to protein crystal nuclei.

Protein aggregation has been divided into two distinct classes: amyloid fibrillation, where unfolding precedes aggregation, and crystallization, in which native structure is preserved. We demonstrate that the two classes share common mechanisms, notably, partial unfolding underlies the formation of the crystal nucleation precursors.

■ EXPERIMENTAL METHODS

Solution shearing was performed using a Couette cell (Figure 5a), in which the rotor, a Teflon rod, was driven by an electric motor via a gear wheel assembly. The shear rates at the rotor and at the periphery of the Couette cell differed by less than 20% (Figure 5b). At chosen intervals, solution aliquots were removed from the shear cell and filtered through a 0.22 μm syringe filter into a cuvette. Cluster populations were characterized by dynamic light scattering, following established procedures.[15,39,40] At least ten correlation functions of light scattered over 60 s were collected for each aliquot. From each correlation function, the sizes of the lysozyme monomers and protein-rich clusters and the total volume of the cluster population were evaluated using established procedures.[15,39] The values extracted from individual correlation functions are

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

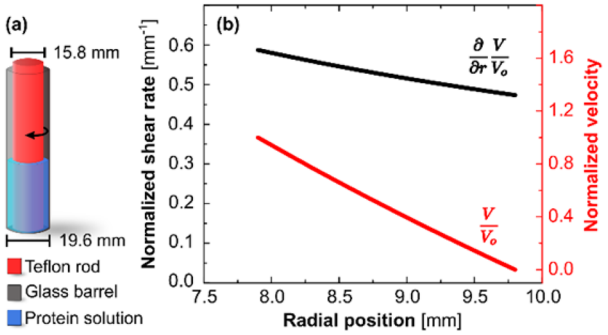


Figure 5. Solution shearing. (a) Schematic and dimensions of a Couette cell, consisting of a static glass barrel and concentric rotating Teflon rod. (b) Shear rate (upper curve) and flow velocity (lower curve) distributions along the radius of the Couette cell, normalized by the tangential velocity of the Teflon rod surface V_0, computed assuming no-slip at the surface between the rotor and the barrel, negligible edge effects at the bottom, and a laminar steady-state Newtonian flow. The Reynolds number was less than 800 in all experiments.

displayed in Figures S3 and S4, and the averaged sizes and volumes and the respective standard deviations in Figures 1 and 3, respectively. Additional details on materials, solution preparation, experimental methods, and data processing are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b00822.

Seven supporting figures and details about the used materials, solution preparation, experimental methods, and data processing procedures. (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: jcconrad@uh.edu.

*E-mail: vekilov@uh.edu.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Maria A. Vorontsova for helpful suggestions on the experiments and result interpretation. Funding was generously provided by NASA (Grants NNX14AE79G and NNX14AD68G) and NSF (Grants MCB-1518204).

■ REFERENCES

(1) Volmer, M. *Kinetik Der Phasenbildung*; Steinkopff: Dresden, Germany, 1939.

(2) Kashchiev, D. *Nucleation. Basic Theory with Applications*; Butterworth-Heinemann: Oxford, U.K., 2000.

(3) Vekilov, P. G. Nucleation. *Cryst. Growth Des.* **2010**, *10*, 5007−5019.

(4) De Yoreo, J. Crystal Nucleation: More Than One Pathway. *Nat. Mater.* **2013**, *12*, 284−285.

(5) ten Wolde, P. R.; Frenkel, D. Enhancement of Protein Crystal Nucleation by Critical Density Fluctuations. *Science* **1997**, *277*, 1975−1978.

(6) Talanquer, V.; Oxtoby, D. W. Crystal Nucleation in the Presence of a Metastable Critical Point. *J. Chem. Phys.* **1998**, *109*, 223−227.

(7) Galkin, O.; Vekilov, P. G. Control of Protein Crystal Nucleation around the Metastable Liquid-Liquid Phase Boundary. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 6277−6281.

(8) Vekilov, P. G. Dense Liquid Precursor for the Nucleation of Ordered Solid Phases from Solution. *Cryst. Growth Des.* **2004**, *4*, 671−685.

(9) Gebauer, D.; Kellermeier, M.; Gale, J. D.; Bergstrom, L.; Colfen, H. Pre-Nucleation Clusters as Solute Precursors in Crystallisation. *Chem. Soc. Rev.* **2014**, *43*, 2348−2371.

(10) Gebauer, D.; Volkel, A.; Colfen, H. Stable Prenucleation Calcium Carbonate Clusters. *Science* **2008**, *322*, 1819−1822.

(11) Pouget, E. M.; Bomans, P. H. H.; Goos, J. A. C. M.; Frederik, P. M.; de With, G.; Sommerdijk, N. A. J. M. The Initial Stages of Template-Controlled Caco3 Formation Revealed by Cryo-TEM. *Science* **2009**, *323*, 1455−1458.

(12) Dey, A.; Bomans, P. H. H.; Müller, F. A.; Will, J.; Frederik, P. M.; de With, G.; Sommerdijk, N. A. J. M. The Role of Prenucleation Clusters in Surface-Induced Calcium Phosphate Crystallization. *Nat. Mater.* **2010**, *9*, 1010−1014.

(13) Wallace, A. F.; Hedges, L. O.; Fernandez-Martinez, A.; Raiteri, P.; Gale, J. D.; Waychunas, G. A.; Whitelam, S.; Banfield, J. F.; De Yoreo, J. J. Microscopic Evidence for Liquid-Liquid Separation in Supersaturated $CaCO_3$ Solutions. *Science* **2013**, *341*, 885−889.

(14) Harano, K.; Homma, T.; Niimi, Y.; Koshino, M.; Suenaga, K.; Leibler, L.; Nakamura, E. Heterogeneous Nucleation of Organic Crystals Mediated by Single-Molecule Templates. *Nat. Mater.* **2012**, *11*, 877−881.

(15) Pan, W.; Galkin, O.; Filobelo, L.; Nagel, R. L.; Vekilov, P. G. Metastable Mesoscopic Clusters in Solutions of Sickle Cell Hemoglobin. *Biophys. J.* **2007**, *92*, 267−277.

(16) Gliko, O.; Neumaier, N.; Pan, W.; Haase, I.; Fischer, M.; Bacher, A.; Weinkauf, S.; Vekilov, P. G. A Metastable Prerequisite for the Growth of Lumazine Synthase Crystals. *J. Am. Chem. Soc.* **2005**, *127*, 3433−3438.

(17) Savage, J. R.; Dinsmore, A. D. Experimental Evidence for Two-Step Nucleation in Colloidal Crystallization. *Phys. Rev. Lett.* **2009**, *102*, 198302.

(18) Maes, D.; Vorontsova, M. A.; Potenza, M. A. C.; Sanvito, T.; Sleutel, M.; Giglio, M.; Vekilov, P. G. Do Protein Crystals Nucleate within Dense Liquid Clusters? *Acta Crystallogr., Sect. F: Struct. Biol. Commun.* **2015**, *71*, 815−822.

(19) Uzunova, V.; Pan, W.; Lubchenko, V.; Vekilov, P. G. Control of the Nucleation of Sickle Cell Hemoglobin Polymers by Free Hematin. *Faraday Discuss.* **2012**, *159*, 87−104.

(20) Sleutel, M.; Van Driessche, A. E. Role of Clusters in Nonclassical Nucleation and Growth of Protein Crystals. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, E546−E553.

(21) Kashchiev, D.; Vekilov, P. G.; Kolomeisky, A. B. Kinetics of Two-Step Nucleation of Crystals. *J. Chem. Phys.* **2005**, *122*, 244706.

(22) Pan, W.; Kolomeisky, A. B.; Vekilov, P. G. Nucleation of Ordered Solid Phases of Protein Via a Disordered High-Density State: Phenomenological Approach. *J. Chem. Phys.* **2005**, *122*, 174905.

(23) Pan, W.; Vekilov, P. G.; Lubchenko, V. The Origin of Anomalous Mesoscopic Phases in Protein Solutions. *J. Phys. Chem. B* **2010**, *114*, 7620−7630.

(24) Hutchens, S. B.; Wang, Z.-G. Metastable Cluster Intermediates in the Condensation of Charged Macromolecule Solutions. *J. Chem. Phys.* **2007**, *127*, 084912.

(25) Vorontsova, M. A.; Maes, D.; Vekilov, P. G. Recent Advances in the Understanding of Two-Step Nucleation of Protein Crystals. *Faraday Discuss.* **2015**, *179*, 27−40.

(26) Vorontsova, M. A.; Chan, H. Y.; Lubchenko, V.; Vekilov, P. G. Lack of Dependence of the Sizes of the Mesoscopic Protein Clusters on Electrostatics. *Biophys. J.* **2015**, *109*, 1959−1968.

(27) Vekilov, P. G.; Vorontsova, M. A. Nucleation Precursors in Protein Crystallization. *Acta Crystallogr., Sect. F: Struct. Biol. Commun.* **2014**, *70*, 271−282.

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345

(28) Lutsko, J. F.; Nicolis, G. Mechanism for the Stabilization of Protein Clusters above the Solubility Curve. *Soft Matter* **2016**, *12*, 93−98.
(29) Sophianopoulos, A. J.; Rhodes, C. K.; Holcomb, D. N.; VanHolde, K. E. Physical Studies of Lysozyme. *J. Biol. Chem.* **1962**, *237*, 1107−1112.
(30) McCammon, J. A.; Gelin, B. R.; Karplus, M.; Wolynes, P. G. The Hinge-Bending Mode of Lysozyme. *Nature (London, U. K.)* **1976**, *262*, 325−326.
(31) Wang, J.; Dauter, M.; Alkire, R.; Joachimiak, A.; Dauter, Z. Triclinic Lysozyme at 0.65 a Resolution. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2007**, *63*, 1254−1268.
(32) Thomas, B. R.; Vekilov, P. G.; Rosenberger, F. Effects of Microheterogeneity on Hen Egg White Lysozyme Crystallization. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1998**, *54*, 226−236.
(33) Li, Y.; Lubchenko, V.; Vorontsova, M. A.; Filobelo, L.; Vekilov, P. G. Ostwald-Like Ripening of the Anomalous Mesoscopic Clusters in Protein Solutions. *J. Phys. Chem. B* **2012**, *116*, 10657−10664.
(34) Ashton, L.; Dusting, J.; Imomoh, E.; Balabani, S.; Blanch, E. W. Shear-Induced Unfolding of Lysozyme Monitored in Situ. *Biophys. J.* **2009**, *96*, 4231−4236.
(35) Ashton, L.; Dusting, J.; Imomoh, E.; Balabani, S.; Blanch, E. W. Susceptibility of Different Proteins to Flow-Induced Conformational Changes Monitored with Raman Spectroscopy. *Biophys. J.* **2010**, *98*, 707−714.
(36) Jaspe, J.; Hagen, S. J. Do Protein Molecules Unfold in a Simple Shear Flow? *Biophys. J.* **2006**, *91*, 3415−3424.
(37) Maa, Y.-F.; Hsu, C. C. Effect of High Shear on Proteins. *Biotechnol. Bioeng.* **1996**, *51*, 458−465.
(38) Di Stasio, E.; De Cristofaro, R. The Effect of Shear Stress on Protein Conformation: Physical Forces Operating on Biochemical Systems: The Case of Von Willebrand Factor. *Biophys. Chem.* **2010**, *153*, 1−8.
(39) Li, Y.; Lubchenko, V.; Vekilov, P. G. The Use of Dynamic Light Scattering and Brownian Microscopy to Characterize Protein Aggregation. *Rev. Sci. Instrum.* **2011**, *82*, 053106.
(40) Gliko, O.; Pan, W.; Katsonis, P.; Neumaier, N.; Galkin, O.; Weinkauf, S.; Vekilov, P. G. Metastable Liquid Clusters in Super- and Undersaturated Protein Solutions. *J. Phys. Chem. B* **2007**, *111*, 3106−3114.
(41) Safari, M. S.; Vorontsova, M. A.; Poling-Skutvik, R.; Vekilov, P. G.; Conrad, J. C. Differential Dynamic Microscopy of Weakly Scattering and Polydisperse Protein-Rich Clusters. *Phys. Rev. E* **2015**, *92*, 042712.
(42) Lin, H.; Petsev, D. N.; Yau, S.-T.; Thomas, B. R.; Vekilov, P. G. Lower Incorporation of Impurities in Ferritin Crystals by Suppression of Convection: Modeling Results. *Cryst. Growth Des.* **2001**, *1*, 73−79.
(43) Pusey, M.; Witherow, W.; Naumann, R. Preliminary Investigation into Solutal Flow About Growing Tetragonal Lysozyme Crystals. *J. Cryst. Growth* **1988**, *90*, 105−111.
(44) Brejc, K.; van Dijk, W. J.; Klaassen, R. V.; Schuurmans, M.; van der Oost, J.; Smit, A. B.; Sixma, T. K. Crystal Structure of an Ach-Binding Protein Reveals the Ligand-Binding Domain of Nicotinic Receptors. *Nature (London, U. K.)* **2001**, *411*, 269−276.
(45) Roxby, R.; Tanford, C. Hydrogen Ion Titration Curve of Lysozyme in 6 M Guanidine Hydrochloride. *Biochemistry* **1971**, *10*, 3348−3352.
(46) Chan, H. Y.; Lankevich, V.; Vekilov, P. G.; Lubchenko, V. Anisotropy of the Coulomb Interaction between Folded Proteins: Consequences for Mesoscopic Aggregation of Lysozyme. *Biophys. J.* **2012**, *102*, 1934−1943.
(47) Das, A.; Mukhopadhyay, C. Atomistic Mechanism of Protein Denaturation by Urea. *J. Phys. Chem. B* **2008**, *112*, 7903−7908.
(48) Caballero-Herrera, A.; Nordstrand, K.; Berndt, K. D.; Nilsson, L. Effect of Urea on Peptide Conformation in Water: Molecular Dynamics and Experimental Characterization. *Biophys. J.* **2005**, *89*, 842−857.
(49) Hua, L.; Zhou, R.; Thirumalai, D.; Berne, B. J. Urea Denaturation by Stronger Dispersion Interactions with Proteins Than Water Implies a 2-Stage Unfolding. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 16928−16933.
(50) Auton, M.; Holthauzen, L. M. F.; Bolen, D. W. Anatomy of Energetic Changes Accompanying Urea-Induced Protein Denaturation. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 15317−15322.
(51) Courtenay, E. S.; Capp, M. W.; Record, M. T. Thermodynamics of Interactions of Urea and Guanidinium Salts with Protein Surface: Relationship between Solute Effects on Protein Processes and Changes in Water-Accessible Surface Area. *Protein Sci.* **2001**, *10*, 2485−2497.
(52) Diehl, R. C.; Guinn, E. J.; Capp, M. W.; Tsodikov, O. V.; Record, M. T. Quantifying Additive Interactions of the Osmolyte Proline with Individual Functional Groups of Proteins: Comparisons with Urea and Glycine Betaine, Interpretation of M-Values. *Biochemistry* **2013**, *52*, 5997−6010.
(53) Holehouse, A. S.; Garai, K.; Lyle, N.; Vitalis, A.; Pappu, R. V. Quantitative Assessments of the Distinct Contributions of Polypeptide Backbone Amides Versus Side Chain Groups to Chain Expansion Via Chemical Denaturation. *J. Am. Chem. Soc.* **2015**, *137*, 2984−2995.
(54) Sagle, L. B.; Zhang, Y.; Litosh, V. A.; Chen, X.; Cho, Y.; Cremer, P. S. Investigating the Hydrogen-Bonding Model of Urea Denaturation. *J. Am. Chem. Soc.* **2009**, *131*, 9304−9310.
(55) Schlunegger, M. P.; Bennett, M. J.; Eisenberg, D. Oligomer Formation by 3d Domain Swapping: A Model for Protein Assembly and Misassembly. *Adv. Protein Chem.* **1997**, *50*, 61−122.
(56) Guez, V.; Roux, P.; Navon, A.; Goldberg, M. E.Role of Individual Disulfide Bonds in Hen Lysozyme Early Folding Steps. *Protein Sci.* **2002**, *11*, 1136−1151.
(57) Eyles, S.; Radford, S.; Robinson, C.; Dobson, C. Kinetic Consequences of the Removal of a Disulfide Bridge on the Folding of Hen Lysozyme. *Biochemistry* **1994**, *33*, 13038−13048.
(58) Chen, Y.; Barkley, M. D. Toward Understanding Tryptophan Fluorescence in Proteins. *Biochemistry* **1998**, *37*, 9976−9982.
(59) Vivian, J. T.; Callis, P. R. Mechanisms of Tryptophan Fluorescence Shifts in Proteins. *Biophys. J.* **2001**, *80*, 2093−2109.
(60) Ibrahim, H. R.; Matsuzaki, T.; Aoki, T. Genetic Evidence That Antibacterial Activity of Lysozyme Is Independent of Its Catalytic Function. *FEBS Lett.* **2001**, *506*, 27−32.
(61) Gasymov, O. K.; Glasgow, B. J. Ans Fluorescence: Potential to Augment the Identification of the External Binding Sites of Proteins. *Biochim. Biophys. Acta, Proteins Proteomics* **2007**, *1774*, 403−411.
(62) Hawe, A.; Sutter, M.; Jiskoot, W. Extrinsic Fluorescent Dyes as Tools for Protein Characterization. *Pharm. Res.* **2008**, *25*, 1487−1499.
(63) Biancalana, M.; Koide, S. Molecular Mechanism of Thioflavin-T Binding to Amyloid Fibrils. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 1405−1412.

DOI: 10.1021/acs.jpclett.6b00822
J. Phys. Chem. Lett. 2016, 7, 2339−2345