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. 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. Hence, alternative nonclassical mechanisms that lead to nucleation of crystals in solution remain vigorously debated.

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. These precursor clusters were directly observed and the nucleation of crystal inside them monitored across a range of systems. Importantly, experiments demonstrated that larger volume of the cluster population correlates with higher nucleation rate. 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. Their existence, however, challenges our understanding of phase ordering. Although the clusters are likely liquid, they exist in the homogeneous region of the protein solution phase diagram, away from the conditions of liquid—liquid coexistence. Typical sizes are on the order of 100 nm, which is much larger than predicted from a colloid aggregation scenario based on the balance of van der Waals attraction and Coulomb repulsion. Moreover, the responses of cluster size and of cluster population volume are decoupled upon variations in ionic strength, pH, and additive concentration. 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. According to theory, the cluster size $R_c$ is related to the diffusivity $D_{\text{oligo}}$ (similar in value to the monomer diffusivity) and decay rate constant $k_{\text{oligo}}$ of oligomers as $R_c = \frac{D_{\text{oligo}}}{k_{\text{oligo}}}$. Importantly, the cluster size does not depend on the rate of oligomerization. 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. 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. Nucleation rates data and direct observations suggest that lysozyme follows a two-step mechanism of crystal nucleation and a population of mesoscopic protein-rich clusters that may be...
the nucleation precursors has been identified and characterized.\textsuperscript{23,26,33,36} We use solution shear to induce partial protein unfolding. Laminar flow at relatively low shear rates, from 10 to 700 s\textsuperscript{−1}, induces reversible conformational changes in relatively stable globular proteins, including lysozyme.\textsuperscript{34,35} Shear rates as fast as 10\textsuperscript{5} s\textsuperscript{−1} lead to complete unfolding, detectable by intrinsic fluorescence,\textsuperscript{36} calorimetry,\textsuperscript{37} and aggregation.\textsuperscript{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\textsuperscript{−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–correlation intensity distribution functions, $g_\varepsilon(t)$, where $t$ is the lag time, recorded after shearing for up to 6 h, all possess two distinct shoulders, indicating the presence of two populations of scatterers (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

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

where the times $\tau_1$ and $\tau_2$ characterize the diffusion of the two scattering populations,\textsuperscript{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 $\varepsilon$ accounts for mechanical, optical, and electronic noise in the signal.\textsuperscript{15,39} Using $\tau_1$, $\tau_2$, $A_1$, and $A_2$ and independently measured solution viscosities, refractive index increments, and protein intermolecular interaction parameters,\textsuperscript{36} 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, $\phi_2$.\textsuperscript{15,26,36} The average radius $R_2$ 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 $5 \times 10^{-5}$ of the solution volume, consistent with the values measured for lysozyme dense liquid clusters in previous studies.\textsuperscript{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 $\phi_2$ in solutions buffered with HEPES at shear rates varying from 0.3 to 200 s\textsuperscript{−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\textsuperscript{−1}, leading to shear rates in the range 0.001–0.01 s\textsuperscript{−1}.\textsuperscript{42,45} 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 $\phi_2$ are constant over 7 h (Figure S2), equal to the longest duration of shearing in Figure 1b,c, we scale $R_2$ and $\phi_2$ by the corresponding values in quiescent solutions, $R_{2,0}$ and $\phi_{2,0}$.

The lowest enforced shear rate, 0.3 s\textsuperscript{−1}, consistently induces a small increase in $R_2$ and a small decrease in $\phi_2$. 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 $\phi_2$ 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.\textsuperscript{35} In Figure 1b,c after the threshold is reached, however, $R_2$ increases and $\phi_2$ 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\textsuperscript{−1}, the solution remained turbid even after filtering through a 0.22 μm filter, suggesting
irreversible denaturation and aggregation of a part of the dissolved protein.

The negatively coupled response of $R_2$ and $\phi_2$ 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, 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 $pH = 7.8$. The balance of 17 positive and nine negative groups. 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 $+12$. The change from HEPES buffer to water induces stronger sensitivity to shear: the threshold shear rate for increased $R_2$ and lowered $\phi_2$ decreases from $10^{-3}$ to $3 \text{ s}^{-1}$, 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.

Urea is currently thought to be a universal denaturant because it interacts favorably with the peptide backbone. 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. The accumulation of urea at nonpolar protein patches and the accompanying destruction of the water structures are described as chaotropic action. 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 $3 \text{ s}^{-1}$, 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 $\alpha$ and $\beta$ 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.

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 $D_{\text{oligo}}$. With oligomer diffusivity of order $10^{-10} \text{ m}^2 \text{ s}^{-1}$ (comparable to that of the monomer), diffusion over the cluster radius of about 30 nm would have a characteristic time of about 10 $\mu$s. This is nearly 3 orders of magnitude faster than oligomer transport enhanced by shear rates slower than $200 \text{ s}^{-1}$, which have characteristic times longer than 5 ms. This estimate suggests that the second 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 $k_{\text{oligo}}$ 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, the decrease in $\phi_2$ is likely due to stronger attraction between lysozyme molecules with exposed nonpolar inter-domain 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, $\phi_2$. Importantly, the cluster population response to mechanical unfolding, by shearing, opposes that of chemical unfolding, by urea. 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 $\phi_2$ to higher shear observed in Figure 1d–i. In other experiments under identical conditions, we found that, overall, $R_2$ always increased and $\phi_2$ 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 inconsistences 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...
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 (HSC₂H₄OH, 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 and 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. 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 $\phi_2$ 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, 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 shearing 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. 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 $R_2$ at quiescent conditions for 15 h after cessation of shearing. The increased $R_1$ and lowered $\phi_2$ 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) and this behavior is not altered by shearing (Figure 4b). If the clusters were irreversible aggregates, the $\phi_2/\phi_1$ ratio would not depend on the solution dilution. Instead, $\phi_2/\phi_1$ strongly decreases upon solution dilution (Figure 4b), in sharp contrast with this expectation. This observation indicates that the irreversibility of the cluster population volume may indicate that the clusters converted to irreversible protein aggregates or that shear modified the equilibrium between monomers and clusters.
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. 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. 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. 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. ThT is employed for selectively staining and identifying amyloid structures as ThT binding to β structures enhances its fluorescence emission. Figure S7 demonstrates that shearing does not affect the fluorescence spectra in solutions of lysozyme and each of the two probe 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, 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. 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.
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)

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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).

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