Are the protein pre-nucleation clusters equilibrium structures or irreversible aggregates?

P.G. Vekilov[1,2], M.A. Vorontsova[1], M. Safari[1], M. Byington[1]

[1] *Department of Chemical and Biomolecular Engineering, University of Houston, Houston TX 77204-4004, USA*

[2] *Department of Chemistry, University of Houston, Houston TX 77204-5003, USA*

vekilov@uh.edu

Protein-rich clusters of steady submicron size and narrow size distribution exist in protein solutions in apparent violation of the classical laws of phase equilibrium. Even though they contain a minor fraction of the total protein, evidence suggests that they may serve as essential precursors for the nucleation of ordered solids such as crystals, sickle-cell hemoglobin polymers, and amyloid fibrils. The cluster formation mechanism remains elusive. We use the highly basic protein lysozyme at nearly neutral and lower pH as a model and explore the response of the cluster population to the electrostatic forces, which govern numerous biophysical phenomena, including crystallization and fibrillization. We tune the strength of intermolecular electrostatic forces by varying the solution ionic strength *I* and pH and find that despite the weaker repulsion at higher I and pH, the cluster size remains constant. Cluster responses to the presence of urea and ethanol demonstrate that cluster formation is controlled by hydrophobic interactions between the peptide backbones, exposed to the solvent after partial protein unfolding that may lead to transient protein oligomers. These findings reveal that the mechanism of the mesoscopic clusters is fundamentally different from those underlying the two main classes of ordered protein solid phases, crystals and amyloid fibrils, and partial unfolding of the protein chain may play a significant role.

The role of partial protein unfolding in cluster formation suggests that the clusters may represent irreversible aggregates of denatured protein. We determine the enzyme activity of lysozyme in cluster-containing solutions and demonstrate that it is equal to that of native lysozyme. Fluorescence spectroscopy reveals that the structures of the α and β lysozyme domain are intact, i.e., the partial unfolding that underlies cluster formation is constrained to the interdomain contacts. Upon solution dilution, the ratio of the cluster concentrations to that of protein monomers decreases exponentially (it should be constant for irreversible aggregates), in agreement with an equilibrium model of cluster formation. In their sum, these results demonstrate that the clusters are not irreversible aggregates, but represent equilibrium high-concentration protein domains.

1. M.A. Vorontsova, H.Y. Chan, V. Lubchenko, P G. Vekilov, *Biophysical Journal*, **109**, (2015), 1959-1968.

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