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Final Report

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Effects of Convective Transport of Solute and Impurities on Defect-Causing Kinectics Instabilities in Protein Crystallization

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Objective

The objective of the proposed research is to obtain further insight into the onset and development of the defect-causing instabilities that arise due to the coupling of the bulk transport and non-linear interfacial kinetics during growth in the mixed regime, utilizing the reduction of the convective contribution to the bulk transport under microgravity. These studies will build upon the data on the effects of quantitative variations of the forced convection velocity on the averaged and time-dependent kinetic behavior of protein crystal growth systems that have recently been obtained in our laboratory.

Results

Thermodynamics and interactions in aqueous solutions of proteins. We used chromatographic, static and dynamic light scattering techniques, and atomic force microscopy (AFM) to study the structure of the protein species and the protein-protein interactions in solutions containing two apoferritin molecular forms, monomers and dimers, in the presence of NaAc buffer and $CdSO_4$. The sizes and shapes of the monomers and dimers, separated by size-exclusion chromatography, were determined by dynamic light scattering and AFM. While the monomer is an apparent sphere with a diameter corresponding to previous x-ray crystallography determinations, the dimer shape corresponds to two, bound monomer spheres. Static light scattering was used to characterize the interactions between solute molecules of monomers and dimers in terms of the second osmotic virial coefficients. The addition of even small amounts of Cd^{2+} causes strong attraction between the monomer molecules, but does not lead to oligomer formation, at least at the protein concentrations used. Furthermore, we found that the second virial coefficient and the protein solubility do not noticeably depend on temperature in the range from 0 to 40 °C. This suggests that the enthalpy for crystallization of apoferritin is close to zero, and the gain of entropy is essentially constant in this temperature range. We also found that in solutions of the apoferritin dimer, the molecules attract even in the presence of acetate buffer only, indicating a change in the surface of the apoferritin molecule. In view of the repulsion between the monomers at the same conditions, this indicates that the dimers and higher oligomers form only after partial unfolding of some of the apoferritin subunits. These observations suggest that aggregation and self-assembly of protein molecules or molecular subunits may be driven by forces other than those responsible for crystallization in the protein solution.

Dynamics of the molecular-level processes in the crystallization from solution. The self-assembly of apoferritin molecules into crystals is a suitable model for protein crystallization and aggregation; these processes underlie several biological and biomedical phenomena, as well as for protein and virus self-assembly. We use the atomic force microscope *in-situ*, during the crystallization of apoferritin to visualize and quantify at the molecular-level the processes responsible for crystal growth. To evaluate the governing thermodynamic parameters, we image the configuration of the incorporation sites, "kinks", on the surface of a growing crystal. We show that the kinks are due to thermal fluctuations of the molecules at the crystal-solution interface. This allows evaluation of the free energy of the intermolecular bond $\phi = 3.0\ k_B T = 7.3$ kJ/mol.

The crystallization free energy, extracted from the protein solubility, is – 42 kJ/mol. Published determinations of the second virial coefficient and the protein solubility between 0 and 40 °C revealed that the enthalpy of crystallization is close to zero. Analyses based on these three values suggest that the main component in the crystallization driving force is the entropy gain of the waters bound to the protein molecules in solution and released upon crystallization. Furthermore, monitoring the incorporation of individual molecules in to the kinks, we determine the characteristic frequency of attachment of individual molecules at one set of conditions. This allows a correlation between the mesoscopic kinetic coefficient for growth and the molecular-level thermodynamic and kinetic parameters determined here. We found that step growth velocity, scaled by the molecular size, equals the product of the kink density and attachment frequency, i.e., the latter pair are the molecular-level parameters for self-assembly of the molecules into crystals.

Imaging of critical nuclei in apoferritin crystallization. Using atomic force microscopy (AFM) *in situ* during the crystallization of the protein apoferritin from its solution, we image the arrangement of the molecules in near-critical clusters, larger or smaller than the crystal nucleus, that are representative of the nucleus structure. At supersaturations $\Delta\mu/k_BT$ of 1.1 – 1.6 – 2.3, the nuclei contain about 50 – 20 – 10 molecules. The molecular arrangement within the nuclei is similar to that in the crystal bulk. Contrary to the general belief, the observed nuclei are not compact molecular clusters, but are planar arrays of several rods of 4-7 molecules set in one or two monomolecular layers. Similarly unexpected nuclei structures might be common, especially for anisotropic molecules. Hence, the nucleus structure should be considered as a variable by advanced theoretical treatments.

Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization. We apply *in-situ* atomic force microscopy to the crystallization of ferritins from solutions containing ~5% (w/w) of their inherent molecular dimers. Molecular resolution imaging shows that the dimers consist of two bound monomers. The constituent monomers are likely partially denatured resulting in increased hydrophobicity of the dimer surface. Correspondingly, the dimers strongly adsorb on the crystal surface. The adsorbed dimers hinder step growth and upon incorporation by the crystal initiate stacks of up to 10 triple and single vacancies in the subsequent crystal layers. The molecules around the vacancies are shifted by ~ 0.1 molecular dimensions from their crystallographic positions. The shifts strain the lattice and, as a consequence, at crystal sizes > 200 μm, the accumulated strain is resolved by a plastic deformation whereupon the crystal breaks into mosaic blocks 20 to 50 μm in size. The critical size for the onset of mosaicity is the similar for ferritin and apoferritin and close to the value for a third protein, lysozyme; it also agrees with theoretical predictions. Trapped microcrystals in ferritin and apoferritin induce strain with a characteristic lengthscale equal to that of a single point defect, and, as a consequence, trapping does not contribute to the mosaicity. The sequence of undesired phenomena that include heterogeneity generation, adsorption, incorporation and arising lattice strain and mosaicity in this and other proteins systems could be avoided by improved methods to separate similar proteins species (microheterogeneity), or by increasing the biochemical stability of the macromolecules against oligomerization.

Lower Incorporation of Impurities in Ferritin Crystals by Suppression of Convection: Modeling Results. We have developed an axi-symmetric time-dependent numerical model of the diffusive-convective transport of a crystallizing protein and an impurity, in an isothermal crystal growth system *at standard and zero gravity*. We model crystallization of ferritin, a protein with M_w=440,000, and its most abundant impurity, the protein's native dimer. The diffusivities of the protein and the dimer and the kinetic coefficients for crystallization and impurity incorporation are available. The model assumes the geometry of a cylindrical vessel used in protein crystallization trials on earth and in space, the DCAM. At terrestrial gravity, buoyancy-driven convection with a maximum velocity of 12 μm/s enhances the supply of both protein and impurity. In the absence of convection, e.g., at 0g, the diffusion depletion zone is wider and the interfacial concentrations drop significantly. The lower diffusivity of the larger dimer results in its incorporation at 0g lower by factors of 2 – 3 than on earth. The three-dimensional computational scheme used here allows direct comparisons of these results with space and laboratory experimental data. The two data sets agree quantitatively, suggesting that in some cases, the improved quality of space grown crystals as compared to the earth grown controls may be due to the suppressed supply of larger impurities.

Bibliography:

Book Chapters

1. P.G. Vekilov, *Cover Pictures and Story*, in **Advances in Crystal Growth Research,** edited by K. Sato, Y. Furukawa, and K. Nakajima. (Elsevier, Amsterdam, 2001).

Journals

1. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for the self-assembly of apoferritin molecules into crystals*. J. Mol. Biol. **303** (2000) 667-678.
2. H. Lin, D.N. Petsev, S.-T. Yau, B.R. Thomas and P.G. Vekilov, *Lower incorporation of impurities in ferritin crystals by suppression of convection: modeling results,* Crystal Growth and Design **1** (2001) 73-79 **(invited paper)**.
3. S.-T. Yau and P.G. Vekilov, *Direct observation of nucleus structure and nucleation pathways*, J. Am. Chem. Soc. **123** (2001) 1080-1089.
4. S.-T. Yau, B.R. Thomas, O. Galkin, O. Gliko, and P.G. Vekilov, *Molecular mechanisms of microheterogeneity-induced defect formation in ferritin crystallization,* Proteins **43** (2001) 343-352.
5. M.D. Serrano, O. Galkin, S.-T. Yau, B.R. Thomas, R.L. Nagel, R. E. Hirsch, and P.G. Vekilov, *Are protein crystallization mechanisms relevant to understanding and control of polymerization of deoxyhemoglobin S?* J. Crystal Growth **232** (2001) 368-375.
6. O. Galkin and P.G. Vekilov, *Nucleation of protein crystals: critical nuclei, phase behavior, and control pathways*. J. Crystal Growth **232** (2001) 63-76.
7. S.-T. Yau, B.R. Thomas, and P.G. Vekilov, *Real time, in-situ, monitoring of apoferritin crystallization and defect formation with molecular resolution.* J. Crystal Growth **232** (2001) 188-194.

Invited presentations

1. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Phase transitions is protein solutions: structures, dynamics and control pathways.* 2000 Biology Retreat, Guntersville, Alabama, USA, September 29-30, 2000.
2. P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport.* Texas Christian University, Dallas, Texas, November 2, 2000.
3. P.G. Vekilov, O. Galkin, and S.-T. Yau, *Structures, dynamics and control pathways of protein crystal nucleation.* Southern Methodist University, Fort Worth, Texas, November 3, 2000.
4. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *Real-time in situ monitoring with molecular resolution of the elementary processes of crystallization of apoferritin.* 2000 Annual Meeting of the American Institute of Chemical Engineers, Los Angeles, California, November 12-17, 2000.
5. P.G. Vekilov, S.-T. Yau, D.N. Petsev and B.R. Thomas, *What do we learn about biological molecules from watching them partake in phase transitions?* Seminar, Department of Biological Sciences, University of Alabama in Huntsville, Huntsville, Alabama, USA, November 29, 2000.
6. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into crystals?* Seminar, Hokaido National Industrial Research Institute, Sapporo, Japan, December 8, 2000.
7. P.G. Vekilov, S.-T. Yau, H. Lin, D. Petsev, B. Thomas *Characteristic lengthscales of the protein crystallization processes: where can gravity affect growth,* Japan Space Utilization Promotion Center Tokyo, Japan, December 12, 2000.
8. P.G. Vekilov, S.-T. Yau, O. Galkin, D. Petsev, B. Thomas, *How do molecules arrange themselves into protein crystals?* Seminar, Tohoku University, Sendai, Japan, December 13, 2000.
9. P.G. Vekilov, O. Galkin, S.-T. Yau, M. Wu, D.N Petsev, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies.* Department of Chemical Engineering, University of Illinois, Champaign, IL, February 1, 2001
10. S.-T. Yau, D.N. Petsev, B.R. Thomas, and P.G. Vekilov, *Tracking Individual Molecules as They Attach Themselves to Crystals: Statistics, Dynamics and Mechanisms.* Physics Colloquium, University of Alabama in Huntsville, Huntsville, Alabama, February 7, 2001.
11. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N Petsev, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies.* Department of Chemical Engineering, University of Houston, February 16, 2001
12. P.G. Vekilov, *Molecular mechanisms of crystallization of proteins.* Marshal Space Flight Center, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, February28, 2001.
13. P.G. Vekilov, S.-T. Yau, O. Galkin, D.N. Petsev, B.R. Thomas, *Phase Transitions in Protein Solutions: Dynamics, Structures and Control Strategies*, University of Alabama in Huntsville, Research Council Meeting, Huntsville, Alabama, April 2, 2001. P G. Vekilov, D.N. Petsev, S.-T. Yau, and K. Chen, *Crystallization of Small and Large Molecules*, 9th Inhalation Technology Seminar, Orion Pharma, Espoo, Finland, June 6, 2001

15. P.G. Vekilov, *Mechanisms of crystallization form solution: a short course.* VTT (Technology Research Center of Finland) Helsinki, Finland, June 7-8, 2001.
16. P.G. Vekilov, O. Galkin, M. Wu, K. Chen, *Phase transition in protein solutions: dynamics and control strategies*; Colloquium, Institute of Physical Chemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, June 12, 2001.
17. P.G. Vekilov, O. Galkin, D.N. Petsev, M. Wu, *Dynamics of phase transition in proteins solutions*, Albert Einstein College of Medicine, Department of Medicine, Division of Hematology, The Bronx, NY, June 27, 2001.
18. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level parameters for the self assembly of biological macromolecules into crystals*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
19. O. Galkin and P.G. Vekilov, *Liquid-liquid separation in solutions of proteins: implications for the formation of condensed phases.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
20. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Molecular-resolution atomic force microscopy movies of step propagation around surface defects and impurities.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
21. S.-T. Yau, D.N. Petsev, and P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferritin crystallization.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.
22. P.G. Vekilov, S.-T. Yau, and H. Lin, *Characteristic lengthscales of the protein crystallization processes: where can gravity affect growth.* 13th International Conference on Crystal Growth, Kyoto, Japan, July 30 – August 4, 2001.

Contributed Presentations

1. S.-T. Yau and P.G. Vekilov, *Protein crystallization processes at three length scales: molecular, capillary and transport,* First International Symposium on Microgravity Research and Applications, Sorrento, Italy, September 10-15, 2000.
2. A.R. Feeling-Taylor, S.-T. Yau, D.N. Petsev, O. Galkin, R. Nagel, R.E. Hirsch, and P.G. Vekilov, *Molecular Mechanisms of Crystallization of HbC,* 25th Annual Meeting National Sickle Cell Disease Program, New York, NY, April 13 - 17, 2001.
3. O. Galkin, K. Chen, R. Elison Hirsch, R.L. Nagel and P.G. Vekilov, *Liquid-liquid Separation in Solutions of Hemoglobins S and A: Implications for the Polymerization of HbS,* 25th Annual Meeting National Sickle Cell Disease Program, New York, NY, April 13 - 17, 2001.
4. H. Lin, S.-T. Yau, O. Gliko, and P.G. Vekilov, *Dynamics of Trains of Non-interacting Steps Growing under Diffusion Control,* 2001 Spring Materials Research Society Meeting, San Francisco, CA, April 16-21, 2001
5. S.-T. Yau, P.G. Vekilov, *Direct visualization of nucleus structure and nucleation pathways in apoferrtin* crystallization, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001

6. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Phase transition in protein solutions: dynamics and control strategies*, Gordon Conference on Thin Films and Crystal Growth, Williams College, Williamstown, Massachusetts, USA, July 1-6, 2001
7. S.-T. Yau, D.N. Petsev, P.G. Vekilov, *Molecular-level thermodynamic and kinetic parameters for crystallization*, Gordon Conference on Gravitational Effects in Physicochemical Systems, Colby Sawyer College, New London, New Hampshire, USA, July 8-13, 2001
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12. P.G. Vekilov and O. Galkin, *Phase transition in protein solutions: dynamics and control strategies*, Keck Center 2001 Annual Research Conference, Galveston, TX, USA, September 21, 2001.