Second Annual Technical Report

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NUCLEATION AND CONVECTION EFFECTS IN PROTEIN CRYSTAL GROWTH

Period of Performance
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Work during the second year under this grant (NAG8-1161) resulted in several major achievements. We have characterized protein impurities as well as microheterogeneities in the proteins hen egg white lysozyme and horse spleen apoferritin, and demonstrated the effects of these impurities on nucleation and crystallization. In particular, the purification of apoferritin resulted in crystals with an X-ray diffraction resolution of better than 1.8 Å, i.e. a 1 Å improvement over earlier work on the cubic form. Furthermore, we have shown, in association with studies of liquid-liquid phase separation, that depending on the growth conditions, lysozyme can produce all growth morphologies that have been observed with other proteins. Finally, in connection with our experimental and simulation work on growth step bunching, we have developed a system-dependent criterion for advantages and disadvantages of crystallization from solution under reduced gravity. In the following, these efforts are described in some detail.

1. Heterogeneity and purification of lysozyme, consequences for crystal growth

Hen egg white lysozyme (HEWL) is widely used as a model protein, although its purity has not been adequately characterized by modern biochemical techniques. We have identified and quantified the protein heterogeneities in three commercial HEWL preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis with enhanced silver staining, reversed-phase fast protein liquid chromatography (FPLC) and immunoblotting with comparison to authentic protein standards. Depending on the source, the contaminating proteins totaled 1-6% (w/w) and consisted of ovotransferrin ovalbumin, HEWL dimers, and polipeptides with approximate Mr of 39 and 18 kDa. Furthermore, we have obtained gram quantities of electrophoretically homogeneous [> 99.9%(w/w)] HEWL by single-step semi-preparative scale cation-exchange FPLC with a yield of 50%. Parallel studies of crystal growth with this highly purified material showed a fourfold increase in the growth-step velocities and significant enhancement in the structural homogeneity of HEWL crystals.

This work has been published; see Thomas et al., 1996, in the list of refereed publications.

The above SDS PAGE studies had indicated that commonly utilized HEWL preparations contained 0.2 - 0.4 mol% covalently bound dimers To explore the origin of this microheterogeneous contaminant, we have oxidized purified HEWL (PHEWL) with hydrogen peroxide (0.0026-0.88 M) at various pH between 4.5 - 12.0. Optical densitometry of oxidized PHEWL (OHEWL) bands in SDS PAGE gels shows that hydrogen peroxide at 0.88 M in acetate buffer pH 4.5 increased the amount of dimers about six-fold over that in commercial HEWL. High performance capillary electrophoresis (HPCE) revealed that OHEWL contained four differently charged monomer forms, compared to two in the original HEWL, and that ~ 50% of the monomers were oxidized by the hydrogen peroxide treatment, while only a small proportion formed dimers. SDS PAGE analysis of OHEWL yielded two closely spaced dimer bands with Mr

= 28,000 and 27,500. In addition, larger HEWL oligomers with Mr = 1.7 million and 320,000 were detected by Gel Filtration Fast Protein Liquid Chromatography with multiangle laser light scattering detection. Nondissociating PAGE in large pore size gels at pH 4.5 confirmed the presence of these large oligomers in HEWL and OHEWL. Increased microheterogeneity resulted in substantial effects on crystal growth and nucleation rate. On addition of 10 μg - 1 mg/ml OHEWL to 32 mg/ml HEWL crystallizing solutions, both the number and size of forming crystals decreased roughly proportional to the concentration of the added microheterogeneity. The same effect was observed in HEWL solutions on addition of 0.03 - 0.3 M hydrogen peroxide. Repartitioning of the microheterogeneities during crystallization at various temperatures between 4 and 20 °C was analyzed by SDS PAGE. The crystals contained ≤ 25 %w/w of the oligomers in the solution, with no apparent temperature dependence of the repartitioning.

This work is in print in Acta Crystallogr. D; see the list of refereed publications.

2. *Horse spleen apoferritin microheterogeneity - determination and effect on crystallization*

The effect of naturally occurring apoferritin (APO) oligomers on the crystallization of isolated, microhomogeneous APO monomers (24 subunits, Mr = 440,000) was investigated with modern analytical biochemical techniques, batch crystallization in microwell plates and X-ray diffraction analysis of crystals. SDS PAGE analysis with silver staining and immunoblotting showed that unpurified APO was free of foreign heterogeneity (>99.9% w/w) and was composed of light (L) and heavy (H) subunits with Mr = 20,000 and 22,000, subunit dimers with Mr = 40,000, and two proteolytic fragments of the subunit with Mr ≈ 14,000 and 6,000. The quaternary structure of APO oligomers that form prior to the addition of precipitant was analyzed with pore-limiting separations in native 4-15% T (1-2% C) gradient PAGE. Optical densitometry of scanned gels showed that oligomers (Mr > 440,000) constituted approximately 45% w/w of the total APO. The primary oligomeric contaminants were dimers (48 subunits, Mr = 880,000) with 35% w/w of the APO, and several bands constituting trimers (~72 subunits, 880,000 < Mr ≤ 1,300,000) with 10% w/w. Thus, monomers comprised only 55% w/w of the preparation. Direct physical molecular weights (Mw) and conformational data for oligomers obtained by analytical gel filtration Fast Protein Liquid Chromatography separations of APO utilizing UV and multi-angle laser light scattering detectors (GF-FPLC-MALLS) confirmed and expanded the native PAGE results: trimers were polydisperse with 900,000< Mw ≤ 1,300,000 and with a mixture of rod shaped and spherical shaped oligomers, dimers were monodisperse with Mw = 900,000 in a spherical conformation, monomers were monodisperse with Mw = 450,000, and very large oligomers (Mw = 5,000,000 and 80,000,000) were present in concentrations < 1% w/w. Semi-preparative GF-FPLC was used to reduce oligomer contamination to 5% w/w, and to produce 0.25 g of microhomogeneous monomers in a single separation of 0.5 g APO. Crystallization from

microhomogeneous monomer solutions yielded much fewer and larger crystals, which, on proper choice of pH and cadmium sulfate concentration, exceeded 0.5 mm in size. These crystals gave an X-ray diffraction resolution of better than 1.8 Å, i.e. a 1 Å improvement over earlier work on the cubic form. Reconstitutive experiments in which isolated oligomers were added to monomer preparations showed that dimers perturb the growth habit and reduce the crystal growth rate, without significantly affecting the nucleation rate. On trimer addition, the nucleation rate was increased and the crystal growth rate decreased. Addition of cadmium sulfate precipitant to unpurified APO did not affect the nature or quantity of the oligomers. The improvement in diffraction resolution and the effects of oligomers on crystallization underline microheterogeneity as a critical factor in protein crystallization.

This work has been submitted for publication to Acta Cryst. D; see the list of refereed publications.

3. Liquid-liquid phase separation in supersaturated lysozyme solutions and associated precipitate formation/crystallization

Using cloud point determinations, the phase boundaries (binodals) for metastable liquid-liquid (L-L) separation in supersaturated hen egg white lysozyme solutions with 3, 5 and 7% (w/v) NaCl at pH = 4.5 and protein concentrations c between 40 and 400 mg/ml were determined. The critical temperature for the binodal increased approximately linearly with salt concentration. The coexisting liquid phases both remained supersaturated but differed widely in protein concentration. No salt repartitioning was observed between the initial and the two separated liquid phases. After the L-L separation, due to the presence of the high protein concentration phase, crystallization occurred much more rapidly than in the initial solution. At high initial protein concentrations, a metastable gel phase formed at temperatures above the liquid binodal. Both crystal nucleation and gel formation were accelerated in samples that had been cycled through the binodal. Solutions in the gel and L-L regions yielded various types of precipitates. Based on theoretical considerations, previous observations with other proteins, and our experimental results with lysozyme, a generic phase diagram for globular proteins is put forth. A limited region in the (T,c)-plane favorable for the growth of protein single crystals is delineated.

This work is in print in the Journal of Chemical Physics; see the list of refereed publications.

4. System-dependent criterion for advantages and disadvantages of crystallization from solution under reduced gravity

In-situ high-resolution interferometry on horizontal facets of the protein lysozyme reveal that the local growth rate R, vicinal slope p and tangential (step) velocity v fluctuate by up to 80 %

of their average values. The time scale of these fluctuations, which occur under steady bulk transport conditions through the formation and decay of step bunches (macrosteps), is of the order of 10 min. The fluctuation amplitude of R increases with growth rate (supersaturation) and crystal size, while the amplitude of the v- and p- fluctuations changes relatively little. Based on a stability analysis for equidistant step trains in the mixed transport-interface kinetics regime, we argue that the fluctuations originate from the coupling of bulk transport with nonlinear interface kinetics. Furthermore, step bunches moving across the interface in the direction or opposite to the buoyancy-driven convective flow increase or decrease in height, respectively. This is in agreement with analytical treatments of the interaction of moving steps with solution flow. Major excursions in growth rate are associated with the formation of lattice defects (striations). We show that, in general, the system-dependent kinetic Peclet number, Pe_k, i.e., the relative weight of bulk transport and interface kinetics in the control of the growth process, governs the step bunching dynamics. Since Pe_k can be modified by either forced solution flow or suppression of buoyancy-driven convection under reduced gravity, this model provides a rationale for the choice of specific transport conditions to minimize the formation of compositional inhomogeneities under steady bulk nutrient crystallization conditions.

Based on the above experimental findings of growth rate fluctuations during the crystallization of the protein lysozyme , we have developed a numerical model that combines diffusion in the bulk of a solution with diffusive transport to microscopic growth steps that propagate on the interface of a finite crystal facet. Nonlinearities in layer growth kinetics arising from step interaction by bulk and surface diffusion, and from step generation by surface nucleation, are taken into account. On evaluation of the model with properties characteristic for the solute transport, and the generation and propagation of steps in the lysozyme system, growth rate fluctuations of the same magnitude and characteristic time as in the experiments are obtained. The fluctuation time scale is large compared to that of step generation. Variations of the governing parameters of the model reveal that both the nonlinearity in step kinetics and mixed transport/kinetics control of the crystallization process are necessary conditions for the fluctuations. On a microscopic scale, the fluctuations are associated with a morphological instability of the vicinal face, in which a step bunch triggers a cascade of new step bunches through the microscopic interfacial supersaturation distribution.

This efforts resulted in two papers in the Physical Review E; see the list of refereed publications.

Refereed Publications of Research Results obtained under this Grant

/, P.G. Vekilov, L.A. Monaco and F. Rosenberger, *Facet morphology response to nonuniformities in nutrient and impurity supply. I. Experiments and interpretation.*, J. Crystal Growth **156** (1995) 267-278.

H. Lin, P.G. Vekilov and F. Rosenberger, *Facet morphology response to nonuniformities in nutrient and impurity supply. II. Numerical modelling,* J. Crystal Growth **158** (1996) 552-559.

P.G. Vekilov and F. Rosenberger, *Dependence of lysozyme growth kinetics on step sources and impurities,* J. Crystal Growth **158** (1996) 540-551.

M. Muschol and F. Rosenberger, *Interactions in undersaturated and supersaturated lysozyme solutions: static and dynamic light scattering results*, J. Chem. Phys. **103** (1995) 10424-10432.

B.R. Thomas, P.G. Vekilov and F. Rosenberger, *Heterogeneity determination and purification of commercial hen egg white lysozyme*, Acta Cryst. D **52** (1966) 776-784.

P. Vekilov, L. A. Monaco, B. R. Thomas, V. Stojanoff and F. Rosenberger, *Repartitioning of NaCl and protein impurities in lysozyme crystallization,* Acta Cryst. D **52** (1996) 785-798.

M. Muschol and F. Rosenberger, *Lack of evidence for prenucleation aggregate formation in lysozyme crystal growth solutions*, J. Crystal Growth **167** (1996) 738-747.

F. Rosenberger, *Protein crystallization,* J. Crystal Growth **166** (1996) 40-54.

F. Rosenberger, P.G. Vekilov, M. Muschol and B.R. Thomas, *Nucleation and crystallization of globular proteins - what do we know and what is missing?*, J. Crystal Growth **168** (1996) 1-27.

P.G. Vekilov, J.I.D. Alexander and F. Rosenberger, *Nonlinear dynamics of layer growth in the mixed kinetics-bulk transport regime*, Phys. Rev. E **54** (1996) 6650-6660.

H. Lin, P.G. Vekilov and F. Rosenberger, *Unsteady crystal growth due to step-bunch cascading*, Phys. Rev. E **55** (1997) 3302-3214.

M. Muschol and F. Rosenberger, *Liquid-liquid phase separation in supersaturated lysozyme solutions: Coupling to precipitate formation and crystallization,* Biophys. J. (in print).

F. Rosenberger, P.G. Vekilov, H. Lin and J.I.D. Alexander, *A rationale for system-dependent advantages and disadvantages of solution crystal growth at low gravity*, Microgravity Sci. Technol. (in print).

B.R. Thomas, P.G. Vekilov and F. Rosenberger, *Effects of microheterogeneity on hen egg white lysozyme crystallization*, Acta Cryst. D. (submitted).

B.R. Thomas, D. Carter and F. Rosenberger, *Effect of microheterogeneity on horse spleen apoferritin crystallization,* Acta Cryst. D. (submitted).

V. Stojanoff, D.P. Siddons, L.A. Monaco, P.G. Vekilov and F. Rosenberger, *X-ray topography of tetragonal lysozyme grown by the temperature controlled technique*, Acta Cryst. D (submitted).

Oral and Poster Presentations on Research under this Grant

H. Lin, "Modeling mass transport and surface kinetics in protein crystal growth." (AIAA 95-3581), AIAA Space Programs and Technologies Conference, Huntsville, AL, September 1995.

H. Lin, P.G. Vekilov and F. Rosenberger, "Simulation of the interplay between diffusive nutrient transport and nonlinear crystal growth kinetics" (poster), Spacebound '97, Montreal, Canada, May 11-14, 1997.

M. Muschol, "Crystallization in protein solutions. What can we learn from light scattering?" AIAA Space Programs and Technologies Conference, Huntsville, AL, September 1995.

F. Rosenberger, "Protein crystallization" (invited lecture), International Space University, Stockholm, Sweden, August 1995.

F. Rosenberger, "Protein crystallization" (seminar), Instituto de Fisica, Universidad Nacional Autonoma de Mexico, Mexico City, September 1995.

F. Rosenberger, "Protein crystallization" (invited paper), Sixth Eastern Regional Conference on Crystal Growth, Atlantic City, NJ, October 15-18, 1995

F. Rosenberger, (invited lectures on protein nucleation and crystallization), Tohoku University, Institute for Materials Research, Sendai, Japan, November 4-8

F. Rosenberger, "Nucleation and crystallization of globular proteins - what do we know and what is missing?" (opening plenary lecture). Sixth International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, Nov.12-17, 1995.

F. Rosenberger, "Crystallization of globular proteins - what do we really know about it?" (colloquium), Universität Berlin, Institut für Kristallographie, Berlin, Germany, February 5, 1996.

F. Rosenberger, "Protein crystallization" (seminar), University of Utah, Department of Physics, Salt Lake City, UT, March 1, 1996.

F. Rosenberger, "Interaction between bulk transport and interface kinetics in crystal growth from solutions" (invited paper), Second European Symposium on Fluids in Space, Naples, Italy. April 22-26, 1996.

F. Rosenberger, "Chemical physics of protein crystallization" (seminar), First University of Rome, Department of Physics, Rome, Italy, May 3, 1996

F. Rosenberger, "Physical chemistry of globular protein crystallization" (invited lecture), Gordon Research Conference on Diffraction Methods in Molecular Biology, Proctor Academy, Andover, NH, June 16-21, 1996.

F. Rosenberger, "Unsteady crystal growth due to step-bunch cascading" (paper), Tenth American Conference on Crystal Growth, Vail, CO, August 4-9, 1996.

F. Rosenberger, "Similarities and differences between inorganic and protein crystallization" (invited paper), JRDC Research Conference on Form, Texture and Function, Tokyo, Japan, November 2, 1996.

F. Rosenberger, "Nonlinear phenomena in protein crystallization" (seminar), Hong Kong University of Science and Technology, Department of Physics, Hong Kong, November 11, 1996.

F. Rosenberger, "Why microgravity is not always beneficial for the perfection of protein crystals" (seminar), Space Science Laboratory, Marshall Space Flight Center, Huntsville, AL, December 11, 1996.

F. Rosenberger, "A rationale for system-dependent advantages and disadvantages of solution growth under reduced gravity" (paper), 35th Aerospace Sciences Meeting, Reno, NV, January 6-9, 1997.

F. Rosenberger, "Protein crystallization: A rationale for system-dependent advantages and disadvantages of solution growth under reduced gravity" (seminar), CNRS, Gif-sur-Yvette, France, February 7, 1997.

F. Rosenberger, "A rationale for system-dependent advantages and disadvantages of solution growth under reduced gravity" (seminar), ZARM, University of Bremen, Germany, February 13, 1997.

F. Rosenberger, "A rationale for system-dependent advantages and disadvantages of solution growth under reduced gravity" (invited paper), Spacebound '97, Montreal, Canada, May 11-14, 1997.

B.R. Thomas, "Heterogeneity determination and purification of commercial hen egg white lysozyme" (poster), Protein Crystal Growth Conference, Panama City, FL, April 28-30, 1996.

B. R. Thomas, "Effects of charge and size heterogeneity on protein crystallization" (seminar) Space Science Laboratory, Marshall Space Flight Center, Huntsville, AL, April 23, 1997.

B. R. Thomas, invited lectures on protein purification techniques and applications to protein crystallization, Institute for Materials Research, Tohoku University, Sendai, Japan, January 1996.

B.R. Thomas, "Effects of microheterogeneity on protein crystallization" (invited paper), Spacebound '97, Montreal, Canada, May 11-14, 1997.

P.G. Vekilov, "High-resolution in-situ interferometric studies of lysozyme crystal growth morphology and kinetics." (AIAA 95-3579) AIAA Space Programs and Technologies Conference, Huntsville, AL, September 1995.

P.G. Vekilov, (invited lectures on protein repartitioning and crystallization), Tohoku University, Institute for Materials Research, Sendai, Japan, November 4-8,1995

P.G. Vekilov, "Salt-rich coring in lysozyme crystals" (paper), Sixth International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, Nov.12-17, 1995.

P.G. Vekilov, "Impurities, growth layer sources and kinetics fluctuations in the growth of lysozyme crystals" (poster), Sixth International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, Nov.12-17, 1995.

P.G. Vekilov, "Crystallization under mixed transport and interface control – effects of gravity-driven convection" (seminar), Marshall Space Flight Center, Space Science Laboratory, Material and Crystal Growth Seminar, Huntsville, Alabama, USA, January 31, 1996.

P.G. Vekilov, "New factors for protein crystal perfection on Earth and under reduced gravity" (invited paper), Protein Crystal Growth Conference, Panama City, FL, April 28-30, 1996.

P.G. Vekilov, "Unsteady dynamics of layer growth in the mixed kinetics-bulk transport regime" (invited paper), Tenth American Conference on Crystal Growth, Vail, CO, August 3-9, 1996.

P.G. Vekilov, "Impurities- and salt- rich coring in protein crystals" (seminar), Department of Chemistry, University of Alabama in Huntsville, Colloquium, Huntsville, AL 35899, USA, September 20, 1996.

P.G. Vekilov, "System-specific effects of reduced gravity on protein crystal perfection" (invited paper), Japanese-US Workshop on Protein Crystal Growth in Microgravity, Huntsville, AL, USA, December 16-17, 1996.

P.G. Vekilov, "Transport-kinetics coupling effects in protein crystallization studied by forced flow experiments" (paper), American Physical Society March Meeting, Kansas City, MO, USA, March 17-21, 1997.

P.G. Vekilov, "System-dependent advantages and disadvantages of reduced gravity for the quality of protein crystals" (seminar), Structural Biology Seminars, Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, AL, May 6, 1997.

P.G. Vekilov, "Insight into the transport-kinetics coupling effects in protein crystallization from forced flow experiments" (invited paper), Spacebound '97, Montreal, Canada, May 11-14, 1997.

Honors and Service

F. Rosenberger

Chairman, Advisory Board, Sixth International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, November 1995.

Chairman, Advisory Board, Seventh International Conference on Crystallization of Biological Macromolecules, Granada, Spain, May 1998.

P.G. Vekilov

International Union of Crystallography Young Investigator Award for paper on salt-rich coring in lysozyme, Sixth International Conference on Crystallization of Biological Macromolecules, Hiroshima, Japan, November 1995.

Session Chair, International Workshop on Protein Crystallization, Fukuyama, Japan, November 1995