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University of Alabama in Huntsville
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Final Report for NASA Grant **NAG8-984** Titled:
***Analysis of Monomer Aggregation and Crystal Growth Rates of
Lysozyme***

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Period of Performance: **1/1/94 - 12/31/96**

1. Achievements of the Project

This project was originally conceived to analyze the extensive data of tetragonal lysozyme crystal growth rates collected at NASA/MSFC by Dr. Marc L. Pusey's research group. At that time the lack of analysis of the growth rates was hindering progress in understanding the growth mechanism of tetragonal lysozyme and other protein crystals. After the project was initiated our initial analysis revealed unexpected complexities in the growth rate behavior. This resulted in an expansion in the scope of the project to include a comprehensive investigation of the growth mechanisms of tetragonal lysozyme crystals.

Much has been achieved during the three years of the project. The work on the project resulted in the growth mechanisms of tetragonal lysozyme crystals being determined at the macroscopic level, as well as at the molecular level. Periodic Bond Chain theory was applied to protein crystals to determine growth mechanisms for the first time. Additionally, new applications for the use of Atomic Force Microscopy were developed to determine the molecular packing arrangements on crystal surfaces and to determine growth mechanisms at the molecular level. Research was also initiated in other areas: understanding the role of anions in the polymorphism exhibited by lysozyme crystals; use of fluorescence spectroscopy to relate chaotropicity of anions to the crystal form obtained; determination of bound anion locations in lysozyme crystals. This research is still ongoing and should eventually lead to an understanding of the fundamental forces driving protein crystallization. The research has resulted in the publication of 6 papers with 3 more in preparation.

This project also contributed significantly towards the education of several students and fostered extensive collaborations between investigators in the Department of Chemical and Materials Engineering at UAH and the Biophysics Branch at NASA/MSFC. Three undergraduate students, four graduate students and three faculty members at UAH were supported on the project at various times. As a result of this there is a continuing exchange of researchers and students and the sharing of facilities between the two institutions. In many ways this grant could serve as a model for future collaborative agreements between UAH and NASA.

2. Task Descriptions

2.1 Analyzing Tetragonal Lysozyme Growth Rates

The large collection of growth rate data collected at NASA/MSFC for the (110) and (101) faces of tetragonal lysozyme was analyzed. The data sets were collected at three sets of pH and salt concentration conditions and were collected at a variety of temperatures and protein concentrations. By analyzing the data in terms of supersaturations, a characteristic growth behavior was discovered. The lysozyme growth rates asymptotically approached zero at low supersaturations. As the supersaturation was increased the growth rates initially increased, reached a maximum and then started decreasing.

It was argued that these results could be explained if lysozyme aggregation occurred in solution and the growth unit was a higher order lysozyme aggregate. These results were first published for the (110) face [1], and subsequently for the (101) face [6]. Although this task as outlined in the research proposal has been completed, future work will focus on the effect of varying the pH and the salt concentration on the growth rates.

2.2 Modeling Tetragonal Lysozyme Aggregation and Crystal Growth Rates

A model was developed to explain the unusual growth rate behavior observed for tetragonal lysozyme crystals with supersaturation described above. The growth was assumed to proceed by the formation of lysozyme aggregates in solution followed by their attached to the crystal faces by screw dislocation growth and 2D nucleation growth. The growth rates calculated from this model were then compared with the measured growth rates of the (110) and (101) faces.

The model calculations and the measured growth rates showed remarkable agreement for an octamer growth unit for the (110) face and for a tetramer or an octamer growth unit for the (101) face. The enthalpy of aggregation and growth unit attachment obtained for the best least squares fit with the data agreed very closely with the same values determined from solubility measurements, indicating the validity of this model. These results were published for the (110) face [2,4] and for the (101) face [7]. Although this task as outlined in the research proposal has been completed, future work will focus on modeling the effect of varying salt concentration and pH on the growth rates.

2.3 Determining Molecular Growth Mechanisms of Protein Crystals by PBC Analysis

The application of PBC analysis to protein crystals and their use to determine the growth mechanism was a novel concept. Several difficulties had to be surmounted in order to complete the analysis, the most important of which was the correct determination of all intermolecular bonds in the tetragonal lysozyme crystal structure. It was shown that these crystals constructed by regular helices centered around the 4_3 crystallographic axes. This in turn was shown to explain several observations made on the growth of these crystals, including growth by the addition of higher order aggregates.

The analysis also allowed the molecular packing arrangements on lysozyme crystal faces to be determined. This enabled comparisons with images and packing arrangements obtained from atomic force microscopy (AFM) scans of the crystal faces and also for the structure of lysozyme aggregates in solution to be deduced. These results were reported in two publications which generated considerable interest in the protein crystal growth community [3,4].

2.4 Anion Binding Sites in Tetragonal Lysozyme Crystals

The determination of bound anion locations in protein crystals is important for a number of reasons. In this study the halide binding sites in tetragonal lysozyme crystals were determined by X-ray crystallography combined with ion substitution experiments. Four definite and a fifth possible binding sites were found. Further analysis was carried out to compare these sites with other anion binding sites reported in the Protein Data Bank for various lysozyme crystal structures. The validity of the binding sites found was confirmed by the presence anions at these locations in other studies. These results were recently submitted for publication [5]. Although this task as outlined in the research proposal has been completed, future work will focus on finding the cation binding sites and the anion binding sites in other crystal forms of lysozyme.

2.5 Use of AFM to Determine Molecular Growth Mechanisms of Protein Crystals

This pioneering investigation was begun towards the end of the performance period of the project. The work had two goals. The first was to develop a technique for *high resolution* AFM to determine the precise molecular packing arrangements on protein crystal faces. The second goal was to develop an AFM technique to monitor the discrete steps involved in the protein crystal growth process. Both of these had never been attempted before.

After considerable difficulties a method was found to determine the surface packing arrangements by comparison of high resolutions AFM scans with theoretical images of the crystal surface. These theoretical images were constructed by convoluting the image of the crystal face obtained from crystallographic data with the shape of the AFM tip obtained by scanning a standard sample. The comparisons of the two images allows the correct packing arrangement to be selected. For the second task of monitoring the individual steps in the growth process, a linescan technique was developed to capture the discrete growth and dissolution processes near the saturation limit. Although the both these techniques have been shown to work, more efforts are needed to complete the study and the results will be published shortly [8,9]. It is expected that these will have a far reaching impact not only for crystal growth studies but also in extending the capabilities of atomic force microscopy.

2.6 Other Research

Besides the achievements outlined above and reported in detail in publications [1-9], several other research projects were also initiated. These include the study of polymorphism and precisely determining the phase regions for the existence of the various polymorphs of lysozyme. This was investigated by directly observing the crystals produced at a variety of conditions as well as by using sophisticated probes such as fluorescence spectroscopy. These studies have not progressed to a degree of completion as yet. However, work on these are ongoing and it is expected that in the near future they will lead results that can be reported.

3. Personnel Supported on the Project

Chemical Eng. Undergraduate Students

Craig Morgan
Effy Setiawan
Lori M. Guillion

Chemical Eng. Graduate Students

Meirong Li (Ph.D.)
Gwendolyn C. Campbell (M.S.)
Huayu Li (Ph.D.)
Lisa J. Crawford (Ph.D.)

Chemical Engineering Faculty Members at UAH

Dr. Arunan Nadarajah, Associate Professor
Dr. Jeffery J. Weimer, Associate Professor

Dr. Douglas G. Hayes, Assistant Professor

4. Presentations and Publications

4.1 Publications from this Research

- [1] A. Nadarajah, E.L. Forsythe and M.L. Pusey, "The Averaged Face Growth Rates of Lysozyme Crystals: The Effect of Temperature," *J. Crystal Growth*, **151**, p163, 1995.
- [2] M. Li, A. Nadarajah and M.L. Pusey, "Modeling the Growth Rates of Tetragonal Lysozyme Crystals," *J. Crystal Growth*, **156**, p121, 1995.
- [3] A. Nadarajah and M.L. Pusey, "Growth Mechanism and Morphology of Tetragonal Lysozyme Crystals," *Acta Crystallographica D***52**, p983, 1996.
- [4] A. Nadarajah, M. Li and M.L. Pusey, "Growth Mechanism of the (110) Face of Tetragonal Lysozyme Crystals," *Acta Crystallographica D*, in press, 1997.
- [5] K. Lim, A. Nadarajah and M.L. Pusey, "Anion Locations in Tetragonal Lysozyme Crystals," *Acta Crystallographica D* (submitted), 1997.
- [6] E.L. Forsythe, A. Nadarajah and M.L. Pusey, "Growth of (101) Faces of Tetragonal Lysozyme Crystals: Measured Growth Rate Trends," *J. Crystal Growth* (submitted), 1997.
- [7] M. Li, A. Nadarajah and M.L. Pusey, "Growth of (101) Faces of Tetragonal Lysozyme Crystals: Determination of the Growth Mechanism," in preparation, to be submitted to *J. Crystal Growth*, 1997.
- [8] H. Li, J.H. Konnert, A. Nadarajah and M.L. Pusey, "Determining the Surface Packing Arrangements on Protein Crystals by High Resolution Atomic Force Microscopy," in preparation, to be submitted to *Biophysical Journal*, 1997.
- [9] H. Li, A. Nadarajah and M.L. Pusey, "Determining the Molecular Growth Mechanisms of Protein Crystals by Atomic Force Microscopy," in preparation, to be submitted to *Science*, 1997.

4.1 Presentations of this Research

- [1] "Growth of Tetragonal Lysozyme Crystals," seminar in the Dept. of Chemical Engineering, Imperial College of Science, Technology and Medicine, London, July 12, 1994.
- [2] "A Fundamental Mechanism for Tetragonal Lysozyme Crystal Growth," seminar in the Center for Macromolecular Crystallography, University of Alabama at Birmingham, July 28, 1994.
- [3] "A Fundamental Mechanism for Tetragonal Lysozyme Crystal Growth," seminar in the Dept. of Biochemistry, University of California at Riverside, September 29, 1994.
- [4] "Modeling of Interactions Leading to Lysozyme Crystal Growth," *Protein Crystal Growth Conference*, Panama City Beach, Florida, April 21-24, 1995.

- [5] "Relationship between Equilibrium Forms of Lysozyme Crystals and Precipitant Anions," *Intl. Conference on Crystallization of Biological Macromolecules*, Hiroshima, Japan, November 12-17, 1995.
- [6] "Modeling the Growth Rates of Tetragonal Lysozyme Crystals," *Intl. Conference on Crystallization of Biological Macromolecules*, Hiroshima, Japan, November 12-17, 1995.
- [7] "Growth Mechanism and Morphology of Tetragonal Lysozyme Crystals," *Intl. Conference on Crystallization of Biological Macromolecules*, Hiroshima, Japan, November 12-17, 1995.
- [8] "Investigations on Lysozyme Crystal Growth," seminar in the Dept. of Chemical Engineering, University of Florida, November 27, 1995.
- [9] "The Mechanism of Protein Crystallization," seminar in the Dept. of Chemical and Biochemical Engineering, Rutgers University, February 2, 1996.
- [10] "Growth Mechanism and Morphology of Tetragonal Lysozyme Crystals," seminar in the Space Sciences Laboratory, NASA/Marshall Space Flight Center, May 15, 1996.
- [11] "Predicting the Growth Rates of Protein Crystals," *AIChE Annual Meeting*, Chicago, November 10-15, 1996.
- [12] "Effect of Precipitant Anions on the Crystal Form of Lysozyme," *AIChE Annual Meeting*, Chicago, November 10-15, 1996.
- [13] "Relating the Growth Mechanism to the Internal Structure of Protein Crystals," *Annual Meeting of the American Physical Society*, Kansas City, March 17, 1997.