NASA-CR-197682

FINAL REPORT

NASA GRANT NAG8-931

BREADBOARD ACTIVITIES FOR ADVANCED PROTEIN CRYSTAL GROWTH

Period of Performance
8/3/92 - 1/6/95

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(NASA-CR-197682) BREADBOARD ACTIVITIES FOR ADVANCED PROTEIN CRYSTAL GROWTH Final Report, 3 Aug. 1992 - 6 Jan. 1995 (Alabama Univ.) 8 p

N95-70685

Unclas

Z9/76 0039723

1. Statement of Work and Deliverables

The proposed work entailed the design, assembly, testing and delivery of a turn-key system for the semi-automated determination of protein solubilities as a function of temperature. The system utilitizes optical scintillation as a means of detecting and monitoring nucleation and crystallite growth during temperature lowering (or raising, with retrograde solubility systems).

The deliverables of this contract are:

1) Turn-key scintillation system for the semi-automatic determination of protein solubilities as a function of temperature,
2) Instructions and software package for the operation of the scintillation system,
3) Semi-annual and final reports including the test results obtained for ovostatin with the above scintillation system.

This final report summarizes the work performed by UAH personnel under this grant.

2. Turn-key Scintillation System

The turn-key scintillation system, instructions and software package, together with appropriate spare parts, and has been delivered to MSFC personnel.

The purpose of this apparatus is to determine the solubility points of a protein solutions at given concentrations. This is achieved by monitoring the scattered light from crystallites in the solution. After inducing nucleation by an appropriate temperature change, the crystals are slowly redissolved until the scattered signal reaches the solution background. This apparatus requires sufficient temperature dependence for the solubility, but works for either normal or retrograde systems . However, it should be noted that this apparatus works poorly in solutions that nucleate at low supersaturations. In these type of solutions only a few, rather than hundreds of crystals are formed and the reflected light is not of high enough intensity for the system to work properly.

The apparatus consists of a clear glass cell which holds the solution and an appropriately sized stir bar. The glass cell is surrounded by a high thermal conductivity block which is heated or cooled by a thermoelectric device that is controlled and programmed via a PC, which also keeps a time-record of the temperature. A light beam from a small solid state laser passes through the cell and the scattered light is monitored at 90° by a high-efficiency photodiode and the photosignal is recorded by the PC. Prior to temperature-induced nucleation, the low scattering intensity is

recorded as background signal. After nucleation occurs, the scattering signal increases due to reflection by the crystallites. Preprogrammed temperature changes are used to dissolve the small crystals until the scattering signal decreases to near-background. This final temperature represents the solubility point.

The scintillation system is a stand-alone unit requiring only room-temperature cooling water and power, and utilizes parts that are available commercially. MSFC personnel have been trained in the use of this system. Scintillation Cell Instructions and Scintillation Cell Program Documentation are presented in the Sections 2.1 and 2.2, respectively. A parts list with supplier information is given in Section 2.3.

2.1 Scintillation Cell Instructions

1. Re-zero the scintillation cell photodiode. This is done, without scattering cell in place, by putting the photodiode in its holder with the laser off and adjusting the 25-turn potentiometer until the output voltage is less than ± 1 mV. Turn the laser on. The signal should not exceed 3 mV as long as the photo-black inside the isothermal brass block is unscratched.

2. Turn on the power to the electronics and laser 30 minutes before the start of a run.

3. Fill the pre-cleaned scattering cell. Add magnetic stir bar.

4. Stopper scattering cell with window-sleeve-O-ring assembly.

5. Place stoppered cell into isothermal brass block. Add photodiode holder, ensuring that the scattering cell is centered via the O-ring.

6. Insert photodiode and check the signal (<5 mV) and the reference (≈ 1 V) voltages. If the signal voltage is greater than 10 mV then the cell is too dirty or precipitation/crystallization has already occurred.

7. Start magnet rotation. You may need to physically spin the magnet to get it going.

8. Start the computer program and respond to its questions (see separate scintillation cell program documentation sheet). Start the Monitor function and observe its behavior for a few minutes. The signal and temperature outputs should be constant. Set the temperature to a value at which crystals should form (if you are going to temperatures of less than 5°C a temperature ramp-rate of 0.5°C/min or less is suggested). Start the Crystallization function. Toggle back to the Monitor function, the data from the initial monitoring will not be lost.

2.2 Scintillation Cell Program Documentation

Start the program by typing GWBAS LONGTIM5 at the C> prompt or GWBASIC LONGTIM5 at the C\DOS> prompt. The program starts by asking for various inputs.

Number of total points: The maximum number of data points to be taken during this experiment. A temperature and signal data is the average of eight readings taken over approximately a minute. The largest number of data points available is 2000, and the minimum is 30.

Reference voltage: The reference voltage read earlier, usually about one volt.

Starting (background) level: Signal voltage read from the detector before the experiment starts. The crystallization/dissolution program ("crystallization function") will stop when the signal voltage returns to less than twice this level after signal saturation occurs.

Temperature increments: The change in the controller setpoint each time the temperature and signal curves flatten out during the dissolution steps.

Output file: A file will be created with this name and with no extension in the C/DOS directory. All signal and temperature data points will be recorded in this file. If this file already exists, then the data will be appended onto the previous data.

Solution: A string of 25 characters or less that is printed in the output file to identify the experiment.

Trigger voltage and ramp start temperature: When the signal reaches the ramp start voltage for the first time, the temperature is rapidly changed to the ramp start temperature.

Low and high temperature display limits: These are the high and low limits on the temperatures shown on the video display. Any temperatures outside this region will not be shown, but will still be recorded in the output file. (The high and low signal limits are 0 and 100 percent of the reference signal). These are ***not*** limits on the controller setpoint.

Once the above values are given, the program asks for a choice of subprograms:

Monitor: This subprogram merely records the values for the signal and reference voltages and the temperature to the output file about every minute. The last thirty values of the temperature and the signal/reference percentage are shown on the screen. Press [Esc] to exit this subprogram at any time.

Change parameters: This subprogram alters the temperature controller's setpoint and ramp rate. Exit when done.

Crystallization program: This main routine should be started when the controller setpoint is at or ramping to a temperature where crystals should form. When the signal reaches the ramp start voltage, the controller's setpoint is changed to the ramp start temperature. Each time the signal and temperature curves flatten in response to a temperature increment, the temperature is incremented again. When the signal drops to twice the background level, the routine stops. Press [F1] to change the temperature increment, ramp start voltage or controller parameters at any time, but you will have to wait until the next data point is taken.

A temperature increment is instigated according to the following criterion: The flatness of the temperature and signal curves is determined by taking the average of the last ten data points and subtracting that from the average of the next to last ten data points. If the absolute difference between the two temperature averages is less than half the temperature increment, and the difference between the signal averages is less than 10 millivolts or 7 percent of the reference signal, the temperature is incremented if there have been more than 10 data points taken since the last increment.

2.3 Parts List and Suppliers

(1) Thermoelectric device and controller, MI1062T-03AC and Model SE5010, Marlow Industries, Dallas, TX 75238

(2) Cell, WG-37-G-1, cylinderical glass cell, Wilmad Glass, Buena, NJ 08310

(3) Photodiode, UDT 455 photops, UDT Sensors, Hawthorne, CA 90250

(4) Laser, VLM 25L,SMW visiable laser diode 670 nm, Applied Laser Systems, Grants Pass, OR 07526

(5) Motor, Hurst 542-0200 synchronous motor, Allied Electronics, Huntsville, AL 35816

(6) Magnet, AM302-RT horseshoe magnet, Magnetic Fabricators, Wizom, MI 48393

(7) Power supply, ElPac WM220-S regulated power supply, Newark Electronics, Huntsville, AL 35805

(8) Op amps, Burr Brown OP27-E, Newark Electronics, Huntsville, AL 35805

3. Ovostatin

Initially, preliminary conditions for the crystallization of ovostatin from high molecular weight polyethyleneglycol (PEG) solutions were given to us by MSFC personnel. However, exact solution conditions for ovostatin crystallization were not established. It was also reported that crystallization was highly dependent on a specific manufacturer and (low) purity of PEG. We found this statement to be true in general. Since MSFC personnel observed what appeared to be temperature dependent solubility behavior, both the MSFC and UAH groups were confident that reproducible crystallization conditions could be established. Unfortunately, this did not occur. Therefore, because of the close contact that existed between the UAH and MSFC groups, UAH personnel worked on establishing proper solution conditions for the reproducible temperature dependent crystallization of ovostatin. The ovostatin used in these studies was supplied purified and lyophilized from MSFC.

As mentioned above, both groups saw hints of solubility as a function of temperature. We observed both normal and retrograde solubility in approximately the same solution conditions. Overall, ***no reproducible scheme or temperature dependence*** was established. The scope of the reproducibility problem was demonstrated by the fact that the primary precipitation procedure used by MSFC personnel was changed three times during the course of the UAH ovostatin work. Initially, precipitation, and subsequent crystal growth, was carried out using low-grade PEG 8000 from Sigma, it was then changed to high-grade PEG 8000 from Aldrich, then using ammonium sulfate, and finally, a return to low-grade Sigma PEG 8000. The return to low-grade PEG was due to low initial precipitation yields and both crystal growth results with both the high-grade PEG and ammonium sulfate additions. In addition, PEG/buffer/protein solutions showed immiscibility. This behavior is, in general, unacceptable for reproducible crystal growth.

In the following we will report on some of the detailed studies that we made to determine acceptable conditions for ovostatin crystal growth.

3.1 Initial temperature dependence studies

Our first attempts at ovostatin crystal growth were conducted in a temperature-controlled microscope cell using solution conditions (2.5 -6 % PEG addition to buffered ovostatin (10-40 mg/ml) solution) given to us by MSFC. The as supplied ovostatin was initially precipitated with low-grade Sigma PEG-8000. In all the runs conducted, varying both the PEG and ovostatin concentration, large well faceted crystals (several mm^3) were observed. Nucleation typically occurred at room temperature. However, varying the temperature from 4 to 40°C had no observable effect on growth. Dissolution in these solutions was never observed. We did observe

that micelles developed throughout the solution. In addition, we typically observed that the smaller crystals were growing "out-of" or attached to these micelles. Our unconfirmed belief is that the micelles were a PEG-rich phase.

Tests to establish temperature dependent solubility conditions were conducted both in the temperature controllable scintillation cell and in capillary tubes and Linbro plates at 4 °C. The ovostatin concentration was varied between 10 to 50 mg/ml; PEG 1 - 6%; and pH 5.0 - 6.6. Tests were also conducted using NaCl (up to 5 %) in place of the PEG. In the majority of the tests, small crystals were observed when the solutions were cooled to 4°C. These crystals typically dissolved after warming to room temperature. Micelles were again observed in many of the tests. Although, nucleation of crystallites was common in these tests, subsequent growth was not observed.

It was concluded that temperature dependent solubility was not likely to be obtained in the PEG solutions.

3.2 PEG-free counter-ion studies

Because of the above results, a collaborative effort was made to obtain PEG-free ovostatin, by employing a different purification and crystallization (bulk w/ ammonium sulfate) procedures, and by dialysis of ovostatin to remove PEG below its detection limit. We found that if dialysis (> 1 week) was continued, *after* all PEG *appeared* to be removed from the system, the solubility of the ovostatin dropped to one-hundredth of the solubility of "raw" ovostatin in de-ionized water (or buffer). This indicated that a counter-ion is necessary is increase the ovostatin solubility. This is reported to be a common phenomenon in protein solutions leading to "salting-in" and then "salting-out" of the protein. We attempted to determine what counter-ion was dialyzed out of the solution. In the absence of a specific counter-ion we made a mixture of *likely effective* counter-ions (i.e., Mg, Ca, Mn, Fe, Al, Cu, and Zn) which were added in varying concentrations (upto 1 mM of each) to buffered protein solutions. Initial tests using Linbro plates indicated precipitate and crystallite formation, similar to what had been observed in previous studies with PEG. Again, the reproducibility of the results was poor.

3.3 Immiscibility studies

As stated previously we noticed both in the microscope growth cell and in the Linbro plates, that PEG/buffer/protein solutions showed immiscibility evidenced by micelle formation. While the behavior in these solutions is typically unacceptable for *reproducible* crystal growth; we were interested in determining into which phase the ovostatin was partitioned. Due to the fact that ovostatin, imidazole buffer, and PEG have overlapping peaks in the UV, the ovostatin

concentration was determined by IR spectroscopy. Various samples of ovostatin-PEG-imidazole buffer, with and without ammonium sulfate, were allowed to phase separate. The top (PEG-rich) and bottom (PEG-lean) fractions were then analyzed for ovostatin. Ovostatin was present in both phases in approximately a one-to-one correlation with the PEG. We also found considerable amounts of PEG in solutions containing "only" lyophilized ovostatin. This showed that the PEG used in the initial precipitation remains **even after** dialysis and lyophilization. We strongly suspect that this accounts for the variability in crystallization conditions when using "the same" solutions. And hence, the deleterious effects of micelle formation cannot be avoided if PEG is used at all in the purification.