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Thermal Optimization of Growth and Quality in Protein Crystals
NASA Grant Number NAG8-1159
FINAL REPORT

Executive Summary

Experimental evidence suggests that larger and higher quality crystals can be attained in the microgravity of space; however, the effect of growth rate on protein crystal quality is not well documented. This research is the first step towards providing strategies to grow crystals under constant rates of growth. Controlling growth rates at a constant value allows for direct one-to-one comparison of results obtained in microgravity and on earth. The overall goal of the project was to control supersaturation at a constant value during protein crystal growth by varying temperature in a predetermined manner. Applying appropriate theory requires knowledge of specific physicochemical properties of the protein solution including the effect of supersaturation on growth rates and the effect of temperature on protein solubility. Such measurements typically require gram quantities of protein and many months of data acquisition. A second goal of the project applied microcalorimetry for the rapid determination of these physicochemical properties using a minimum amount of protein. These two goals were successfully implemented on hen egg-white lysozyme. Results of these studies are described in the attached reprints.

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Enthalpy of crystallization of hen egg-white lysozyme

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Abstract

The validity of the van 't Hoff calculation of crystallization enthalpy from the temperature dependence of solubility was proven for hen egg-white lysozyme. The enthalpy of crystallization of hen egg-white lysozyme in two 0.05M acetate buffers (5% NaCl, pH = 4.6 and 3% NaCl, pH = 5.2) was determined at 15°C using isothermal calorimetry and was found to be -17.1 ± 3.2 kcal/mol (5% NaCl) and -10.5 ± 2.3 kcal/mol (3% NaCl). These values were found to agree within experimental error with the enthalpy of crystallization determined from a van 't Hoff plot of solubility.

1. Introduction

In crystallization, control of the level of supersaturation throughout the crystallization process is essential if the crystal size is to be optimized. In protein crystallization the supersaturation is normally controlled by use of precipitants or pH. An alternate approach to optimize crystallization conditions is through temperature control. For inorganic crystallization, controlled cooling can result in fewer nuclei and larger crystals formed in batches under temperature control designed to allow for control of the nucleation rate [1]. Such control strategies require knowledge of the solubility as a function of temperature, nucleation kinetics and growth kinetics. More recently, DeLucas and coworkers [2] and Wiencek and coworkers [3] have employed temperature control to protein crystallization in batch microdrop systems. DeLucas [2] utilizes an active feedback (i.e. light scattering) to determine the onset of nucleation and then relies on scanning a variety of temperature profiles to arrive at an optimum temperature control algorithm. Wiencek [3] characterizes key features of the system including critical nucleation temperatures, solubility and growth rates to arrive at a predetermined temperature control algorithm. Both approaches benefit tremendously from an accurate knowledge of the temperature dependence of the protein's solubility.

In experiments reported here, calorimetry is utilized to obtain phase diagram information for the tetragonal crystal form of a model protein, hen egg-white lysozyme. Utilizing the van 't Hoff relationship, a single point measurement of the equilibrium concentration of protein and the enthalpy of crystal-

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lization will yield the local temperature dependency of the solubility of protein in solution. In turn, this information can be used to develop strategies for controlling the crystallization process using temperature as the control parameter.

It is recognized that such information may be obtained via solubility measurements at two distinct temperatures (followed by van 't Hoff analysis) in a fairly simple microdevice such as the device developed by Pusey and coworkers [4,5]. An accurate estimate of solubility requires not only growth of a crystal from supersaturated solution but also dissolution of crystals into an undersaturated solution. Convergence between the two methods (i.e. dissolution and crystallization) can take months by conventional methods. Pusey and coworkers [4,5] devised a microvolume apparatus which utilizes a small volume of crystallites in a semi-batch configuration to obtain estimates for solubility. The method requires some preknowledge of solubility since both under- and over-saturated solutions are required for this device. In order to ascertain whether a solution is under- or over-saturated requires advanced knowledge of the solubility which the experiment intends to measure. However, after one solubility measurement is completed, systematic studies away from that point become trivial. Although the design of Pusey and coworkers [4,5] greatly accelerates the determination of solubilities, there are systems which will display very slow rates of dissolution and prevent accurate measurements in a reasonable timeframe. In such cases, the use of isothermal calorimetry, requiring on the order of 10 to 50 h to complete an experiment, may prove advantageous. In any case, the present study certainly justifies the use of van 't Hoff analysis in protein systems, at least over a local temperature range.

There are many ways that microcalorimetry may be used in the investigation of the protein crystallization process. For example, recent work by Sibille and Pusey [6] has focused on the use of stopped-flow techniques to assay the heat of binding between lysozyme and sodium chloride ions. In addition, differential scanning microcalorimetry has been utilized to detect the onset of nucleation [7] of lysozyme as a function of solution variables such as protein and salt concentration. Such studies help define the labile/metastable boundary for protein systems. The temperature control methods of DeLucas and coworkers [2] and Wiencek and coworkers [3] are only useful if the candidate protein's solubility is sensitive to temperature. Isothermal analysis of the heat of crystallization will lead to a direct measurement of this sensitivity to temperature as well as the nature of the dependence (i.e. normal or retrograde solubility behavior). Thus, the potential value of microcalorimetry in the study of protein crystallization is becoming apparent although reported utilization of the technique is still minimal.

An important first step is to justify the use of the van 't Hoff analysis for describing the temperature dependence of protein solubility. Although one would not normally question such an analysis, previous calorimetric studies have cast some doubt on the validity of such an analysis. Howard and coworkers [8] found that their solubility measurements yielded van 't Hoff enthalpy of crystallization that varied significantly from the values measured calorimetrically by Takizawa and Hayashi [9]. Since the Howard et al. solubility data [8] were not measured at the same pH as the calorimetric data of Takizawa and Hayashi [9], the comparison of ΔH_{cryst} required some interpolation by Howard et al. [8]. In order to alleviate any questions arising from methods of interpolation or thermodynamic analysis, the present work measures ΔHcryst under a solubility condition for lysozyme as reported by Howard et al. [8] (i.e. pH = 4.6, 5% NaCl). In addition, a second condition (i.e. pH 5.2, 3% NaCl) was tested to show the general utility of the method. A series of measurements were made using isothermal calorimetry to determine the enthalpy of crystallization of hen egg-white lysozyme in 5% NaCl and 0.05M acetate buffer, final pH 4.6 and in 3% NaCl, 0.05M acetate buffer, final pH 5.2 at 15°C. Solubility over a range of temperatures were determined for both of these conditions, with the pH 4.6, 3% solubility data being used in addition to the data of Howard et al. [8]

2. Materials and methods

Hen egg-white lysozyme was purchased from Sigma Chemicals (catalog number L-6876, lot number 89F8276). The lysozyme was first dialyzed against deionized water followed by dialysis against

the buffer solution (0.05M sodium acetate, pH 4.5). Stock solutions of lysozyme were then concentrated using a 5000 molecular weight cut-off microconcentrator (Amicon). Protein stock solution was mixed with appropriate quantities of 10% NaCl in buffer to obtain a final salt concentration of 5% or 3%. Lysozyme concentrations were determined using an extinction coefficient of 26.35 for a 1% solution at 280 nm [10]. All stock solutions were passed through 0.2 μm syringe filters. The pH values of the crystallizing solutions were measured using Baker PHix pH papers (3.8 to 5.5). The final pH values of the 5% NaCl solutions were found to be 4.6, and the final pH of the 3% NaCl solutions were found to lie between 5.2 and 5.5.

To supplement literature data [8], the solubility of hen egg-white lysozyme was determined for several temperatures and the following precipitant conditions: 5% NaCl with pH 4.5, and 3% NaCl with pH 5.2, both in a 0.05M sodium acetate buffer. Hen egg-white lysozyme was dialyzed against deionized water, followed by a buffer solution of 0.05M sodium acetate, pH 4.5 or 5.2. Solutions dialyzed against buffer with pH 5.2 were also passed through a PD-10 (Pharmacia LKB Biotechnology) desalting column with 0.05M sodium acetate, pH 5.2, as the exchange buffer. The protein solutions were then combined with appropriate quantities of 10% NaCl stock solution buffered at a pH matching the protein solution. The pH of the final mixture was measured with a pH meter (Orion, model 720A) and found to be 4.69 for the 5% salt solution and 5.31 for the 3% salt solution. Five 1.5 ml Eppendorf tubes were filled with 1 ml of solution for each temperature and precipitant concentration. The tubes were then immersed in constant temperature baths (VWR model 1187, ±0.01°C) for 11 to 14 weeks. The supernatant was then sampled and residual lysozyme concentrations were taken as the equilibrium solubility. The pH of the solutions measured with pH paper were unchanged at the time of solubility determination and were found to be 4.6 for the 5% salt solutions and 5.2–5.5 for the 3% salt solutions.

The heat signal from a crystallizing solution of lysozyme was recorded using the 2277 Thermal Activity Monitor (TAM) isothermal calorimeter manufactured by Thermometric (Allied Chemical Technology, Burtonsville, MD). The measuring and reference cells were both 3 ml capacity stainless steel screw top ampoules. The reference cell was filled with 3 ml of distilled water. The sample cell was filled with 3 ml of a lysozyme and sodium chloride mixture (the crystallizing solution). The calorimeter was operated isothermally at 15°C. The measurement recorded by the calorimeter was power (in μW) versus time as shown in Fig. 1. The total enthalpy change was calculated by summing the area under the power versus time curve. Areas were calculated using the Thermometric software, correcting for the initial baseline and baseline drift. The quantity of lysozyme crystallized was assumed to be the difference between the initial quantity of lysozyme in solution minus the quantity of lysozyme in solution at the conclusion of the calorimetry experiment. Lysozyme crystals formed primarily on the walls and the bottom of the ampoule and were examined with an optical microscope. The crystals exhibited a morphology typical of the tetragonal form of lysozyme.

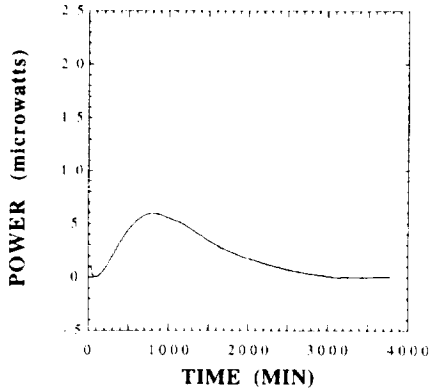

Fig. 1. The measured signal from the TAM calorimeter for a crystallizing solution of lysozyme in 3% NaCl, 0.05M sodium acetate buffer, pH = 5.3 at 15°C.

3. Results

A summary of the experimentally determined lysozyme solubility values is given in Table 1. The data collected at 3% NaCl, pH 5.2 are not available in the current literature and represent an independent test of the validity of the van 't Hoff analysis of the data. The data collected at 5% NaCl, pH 4.6 are intended to supplement the data of Howard et al. [8].

Table 1
Solubility of hen egg-white lysozyme (average of five samples at each condition)

	Temperature (°C)	Solubility (mg/ml)
3% NaCl, pH = 5.2	5	2.55 ± 0.05
	10	3.83 ± 0.05
	15	4.35 ± 0.06
5% NaCl, pH = 4.6	5	1.42 ± 0.03
	10	1.93 ± 0.18

Since the isothermal instrument used in this study was a shared instrument, these studies were limited to 15°C. The data of Howard et al. [8] provided only two solubility data points (15 and 20°C) below the tetragonal to orthorhombic phase transition. These data points were not centered around the calorimetric measurement temperature and were, thus, supplemented with the data of Table 1. The two sets of solubility data were plotted in the typical van 't Hoff fashion and yielded fairly straight lines as shown in Fig. 2.

Calorimetric results for four experiments with crystallization conditions of 5% NaCl, and final pH of 4.6 are shown in Table 2. The enthalpy of crystallization for lysozyme at 15°C based on these four experiments is -17.1 ± 3.2 kcal/mol. A z-test does not indicate a significant difference between the enthalpy of crystallization measured calorimetrically and the value of -15.1 ± 1.3 kcal/mol regressed from a van 't Hoff plot of solubility versus temperature (Fig. 2).

A summary of four experiments with crystallization conditions of 3% NaCl, and final pH of 5.2 is shown in Table 2. An example of the measured signal from the TAM calorimeter is presented in Fig. 1. The enthalpy of crystallization for lysozyme at 15°C based on the four TAM experiments is -10.5 ± 2.3 kcal/mol. A z-test does not indicate a significant difference between the enthalpy of crystalliza-

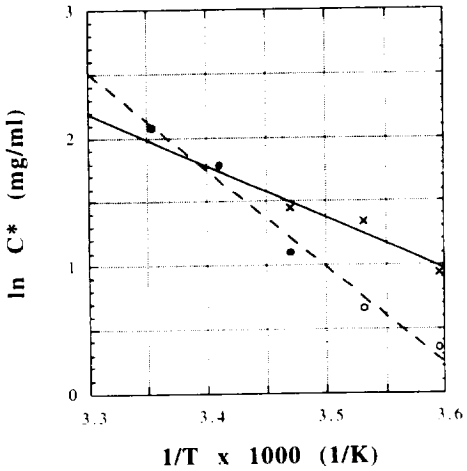

Fig. 2. Van 't Hoff plot of lysozyme equilibrium solubility as a function of temperature. 3% NaCl, 0.05M sodium acetate, pH = 5.2 (×) from Table 1; 5% NaCl, 0.05M sodium acetate, pH = 4.6 (●) from Table 1; 5% NaCl, 0.05M sodium acetate, pH = 4.5 (○) from Howard et al. [8]. The slope of the solid line corresponds to an enthalpy of crystallization of -8.1 ± 2.7 kcal/mol for 3% NaCl ($r = 0.9482$). This compares with a value of -10.5 ± 2.3 kcal/mol determined by calorimetry. The slope of the dashed line corresponds to an enthalpy of crystallization of -15.1 ± 1.3 kcal/mol for 5% NaCl ($r = 0.9894$). This compares with a value of -17.1 ± 3.2 kcal/mol determined by calorimetry.

Table 2
Enthalpy of crystallization of lysozyme measured by isothermal calorimetry (all solutions were made using 0.05M sodium acetate buffer at the indicated salt concentration and final pH

Buffer conditions	Initial protein conc. (mg/ml)	Final protein conc. (mg/ml)	$-\Delta H_{cryst}$ (kcal/mol)	Number of days in calorimeter
5% NaCl, pH = 4.6	41.0 ± 0.8	9.8 ± 0.2	14.8	3
	36.2 ± 1.0	14.5 ± 1.2	15.8	4
	26.6 ± 0.4	8.0 ± 0.2	16.1	5
	41.3 ± 1.1	8.6 ± 0.4	21.8	4
3% NaCl, pH = 5.2	78.3 ± 1.6	46.5 ± 1.8	10.8	2
	77.2 ± 1.4	33.2 ± 0.5	12.3	2.5
	84.5 ± 2.5	36.7 ± 1.9	11.8	3
	122.1 ± 5.0	33.4 ± 0.5	7.2	4

tion measured calorimetrically and the value of -8.1 ± 2.7 kcal/mol regressed from a van 't Hoff plot (Fig. 2).

The crystals removed from the ampoules at the conclusion of the crystallization experiments were examined using an optical stereomicroscope and exhibited a morphology typical of that observed for the tetragonal form of lysozyme crystals [11].

4. Discussion

First and foremost, the validity of the van 't Hoff analysis to protein solubility is apparent for this lysozyme system. Takizawa and Hayashi measured the enthalpy of crystallization of a lysozyme solution in 3% NaCl, adjusted to a pH of 4.2, at 15°C and report a value of -25.1 ± 5.0 kcal/mol [9]. Howard et al. [8] found that their solubility data would predict an enthalpy of crystallization of -18.9 kcal/mol based on interpolation of the solubility data. Although this data is arguably within the error of the measurement, a second data set displayed large discrepancies (13 ± 3.0 kcal/mol by calorimetry versus 5.3 kcal/mol by van 't Hoff analysis of solubility) indicating that one of the two techniques were suspect. Obvious points of contention include the assumptions of the van 't Hoff analysis (i.e. temperature independent enthalpy of crystallization) and the interpolation method. However, experimental protocols of Takizawa and Hayashi [9] are also a source of error. The Takizawa and Hayashi value for the enthalpy of crystallization at 3% NaCl, pH 4.2 is significantly higher than our measured value in 5% NaCl at pH 4.5 as well as the estimate of Howard et al. [8]. Utilizing the data of Howard et al. [8] as well as the data reported in Table 1, the effect of salt concentration on the enthalpy of crystallization at pH 4.5 is shown in Fig. 3. The magnitude of the enthalpy of crystallization tends to increase dramatically as salt concentration increases at a pH of 4.5. Because the lysozyme in Takizawa and Hayashi's experiment was not dialyzed, the salt content of the crystallizing solution was most likely higher than the reported value of 3% due to the large amount of buffer salts typically found in lyophilized proteins. Assuming the same qualitative behavior at pH = 4.2

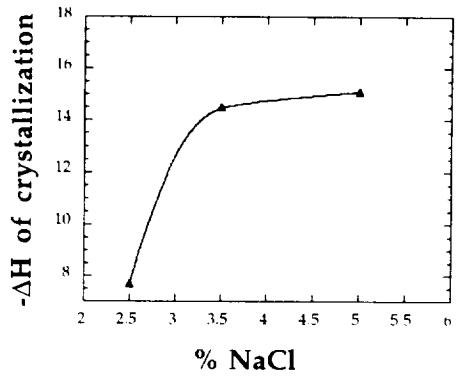

Fig. 3. Enthalpy of crystallization for hen egg-white lysozyme versus salt concentration at pH = 4.5 in 50mM acetate buffer. The enthalpy of crystallization was calculated by measuring the slope of a van 't Hoff plot of solubility as a function of temperature using solubility from Table 1 and Howard et al. [8]. The enthalpy of crystallization is strongly affected by salt concentration under these conditions.

as that depicted in Fig. 3 for pH = 4.5, one would expect that Takizawa and Hayashi would report a larger magnitude for ΔH_{cryst} than the true value due to the miscalculated salt concentration. This would account for the discrepancy between the results of Takizawa and Hayashi [9] and van 't Hoff analysis of Howard et al. [8].

The enthalpy of crystallization of lysozyme measured by calorimetry is not significantly different than the enthalpy of crystallization calculated from a van 't Hoff plot of equilibrium solubilities for both 3% and 5% NaCl solutions. This study justifies the use of such van 't Hoff analysis for the analysis of protein solubilities in the future.

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Application of temperature control strategies to the growth of hen egg-white lysozyme crystals

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Abstract

Solubility data were combined with mass balances and growth kinetics to derive a temperature control algorithm which maintains a constant level of supersaturation. This constant supersaturation control (CSC) algorithm attempts to maximize the size of protein crystals by maintaining the growth conditions in the metastable zone. Using hen egg-white lysozyme as a model protein system, four temperature programming strategies were employed in seeded and unseeded systems: the CSC algorithm, a linear ramp derived from the CSC algorithm, isothermal 20°C, and isothermal 4°C. Both the CSC-derived linear and the CSC temperature programs yielded large, well-formed crystals which were significantly larger than crystals grown isothermally at 20 and 4°C. The isothermal 4°C program resulted in poorly formed crystals due to the high initial growth rates. The seeded systems displayed much higher levels of nucleation than the unseeded systems which is attributed to secondary nucleation. The results indicate that moderate deviations ($\sim 20\%$) from constant supersaturation can be tolerated, while still producing large, well-formed crystals appropriate for X-ray crystallography.

1. Introduction

Control of the level of supersaturation throughout the crystallization process is essential if crystal size is to be optimized for the purpose of producing large, well-formed crystals for X-ray crystallographic structure determination. In protein crystallization, supersaturation is normally controlled through the use of precipitants or pH. Typically, protein in a hanging drop is equilibrated against a reservoir of precipitant solution of higher concentration to produce protein crystals. This device offers no control of the level of supersaturation beyond setting the initial conditions. In contrast, Gernert et al. [1] devised a hanging drop apparatus in which the reservoir concentration could be changed over time and found that maintaining lower levels of supersaturation led to fewer protein

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crystals of a larger size than crystals grown under highly supersaturated conditions.

An alternative approach to controlling supersaturation is through temperature control. For inorganic crystallization, fewer nuclei and larger crystals are formed in batches using temperature control to minimize nucleation rate while maintaining a finite growth rate [2]. Temperature control of crystal growth has been underutilized in protein systems, perhaps because such strategies require knowledge of the solubility as a function of temperature, as well as nucleation and growth kinetics. Although the use of temperature control in protein crystallization has been strongly advocated [3], only recently have studies utilized this strategy to control nucleation and crystal growth [4–9].

Predictive temperature control of supersaturation in protein solutions has not been reported in the literature. With that in mind, we have used engineering analysis to construct a temperature control strategy designed to optimize crystal growth by maintaining a constant level of supersaturation in the metastable zone. In this regime of the phase diagram, existing crystals will grow but only minimal homogeneous nucleation will occur. Our approach utilizes knowledge of mass balances, growth rate kinetics and temperature-dependent protein solubility to generate a control algorithm which decreases the temperature in a predicted fashion. The model must be very simple for two reasons. First, the model will require knowledge of some thermodynamic (i.e. solubility as a function of temperature) and kinetic (i.e. growth rate as a function of supersaturation) processes. Layers and layers of sophistication can be added to such models (e.g. temperature dependent rate constants for the growth kinetics) with a marginal improvement in predicting the "best" temperature profile required to achieve a desired result, large terminal crystal size in this case. Any realistic protein crystallization system will not have such information available.

Thus, the intent is to minimize the amount of data gathering required to obtain a reasonable temperature algorithm. The second reason for a simple model is the need for the solution to be in explicit algebraic form. Such a simple mathematical expression is easily implemented by off-the-shelf temperature controllers. This ease-of-use should accelerate the use of such temperature programming strategies for protein crystal growth in the labs of practicing protein X-ray crystallographers. As an initial test of this idea, the tetragonal form of lysozyme was investigated because of the extensive amount of thermodynamic and kinetic information already available.

1.1. Temperature program for constant supersaturation control (CSC)

The goal of our temperature programming strategy is to maintain a constant level of supersaturation in the metastable zone. A single small seed crystal (approximately 100 μm on one arbitrary side) is placed in a vessel initially at temperature, T, and supersaturation, $\Delta C = (C - C^*)$, where C is the concentration in the bulk solution and C^* is the solubility of the protein at T. Assuming only one crystal is present, a mass balance on the total protein mass (M) yields:

$$M = \rho k_v L_0^3 + V_{soln} C_0 = \rho k_v L(t)^3 + V_{soln} C(t), \tag{1}$$

where ρ is the protein density in crystal (i.e. mass of protein per volume of crystal), k_v the volume shape factor of the crystal, L_0 the initial value of characteristic crystal dimension, V_{soln} the solution volume (does not include crystal), C_0 the initial protein concentration in solution, $L(t)$ the characteristic length at any given time t, $C(t)$ the protein concentration in solution at any given time t, t the time.

It is assumed that the volumetric growth can be characterized by the rate of growth in one dimension or the linear growth rate (G). Choosing an arbitrary dimension of a crystal, L, to characterize its growth, a power law growth model is often adequate:

$$G = \frac{dL}{dt} = k(C - C^*)^n. \tag{2}$$

Values of the parameters k and n for both the [110] and [101] faces have been determined experimentally by Durbin and Feher [10] for a variety of tetragonal lysozyme crystallization conditions.

An algorithm which maintains supersaturation at a constant value will also maintain a constant rate of growth; thus, the first two terms of Eq. (2) can be integrated to yield $L(t) = L_0 + Gt$. Placing this func-

tion into Eq. (1) gives the protein concentration in solution as a function of time (in terms of the chosen growth rate):

$$C(t) = C_0 - \frac{\rho k_v}{V_{soln}}\left[(L_0 + Gt)^3 - L_0^3\right]. \quad (3)$$

The solubility of the protein as a function of time, $C^*(t)$, can be obtained by substituting Eq. (3) into the growth rate expression. For the case of the power law expression shown in (2)

$$C^*(t) = C_0 - \frac{\rho k_v}{V_{soln}}\left[(L_0 + Gt)^3 - L_0^3\right] - {}^n\sqrt{G/k}\,. \quad (4)$$

Finally, a relationship between temperature and solubility is required. The van 't Hoff equation works well for lysozyme:

$$\ln C^* = (\Delta H_{cryst}/R)(1/T - b), \quad (5)$$

where ΔH_{cryst} is the enthalpy of formation of crystals, b is a constant, and R is the gas constant. Combining Eqs. (4) and (5) yields an expression relating temperature to growth rate:

$$T(t) = \left[b - \frac{R}{\Delta H_{cryst}}\ln\left(C_0 - \frac{\rho k_v}{V_{soln}} \times \left[(L_0 + Gt)^3 - L_0^3\right] - {}^n\sqrt{G/k}\right)\right]^{-1} \quad (6)$$

This temperature control algorithm maintains supersaturation, $\Delta C = (C - C^*)$, at a constant level provided the simplifying assumptions hold. This further implies that growth is also constant per Eq. (2).

The above analytical Eq. (6) was used in our experiments as the model temperature control algorithm and is referred to as the constant supersaturation control (CSC) algorithm. Lysozyme crystal geometry was approximated as a cube, with an appropriate shape factor $k_v = 1$. For the crystals grown in this study, we have measured bounds on k_v and found that it lies between 0.9 and 1.1. Thus, the cube geometry approximation is reasonable. The consumption of mass by the growing crystal is characterized by k_v and the growth of the [110] face through Eqs. (1) and (2). Approximate rate parameters were taken from the work of Durbin and Feher [10].

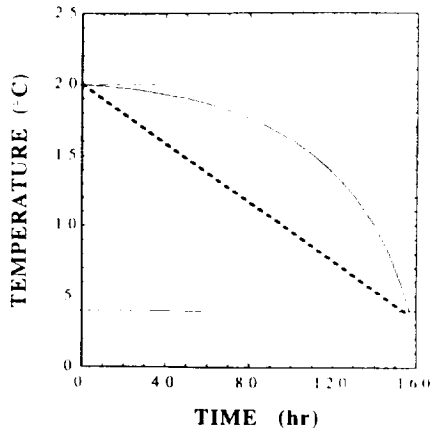

Fig. 1. Temperature programs applied to solutions of lysozyme. A lysozyme seed crystal (~100 μm) was placed in one well of a 24-well Linbro plate with 10 μl of lysozyme solution (5% NaCl, 0.05M acetate, pH = 4.5, 12 mg/ml lysozyme) and overlaid with mineral oil. The plates were then placed in a cooling water bath with one of the temperature programs shown above: isothermal 20°C (– – –), constant supersaturation (CSC) model (——), CSC-derived linear ramp (- - -), or isothermal 4°C (——).

The rate constant of 2.54×10^{-10} cm s^{-1} (mg/ml)$^{-2.6}$ and a power dependence $n = 2.6$ was taken from data collected at 24°C. It is known that the rate constant, k, will vary with temperature. This effect is secondary to the effect of temperature on solubility. In order to minimize the need for many parameters, the temperature dependence of k is ignored. The lysozyme solubility data at 50mM sodium acetate buffer, pH = 4.5, 5% NaCl were obtained from the literature [11] and supplemented with data from our lab. These data were regressed to Eq. (5) to yield $\Delta H_{cryst} = -15.6$ kcal/mol and $b = 0.0036$ K^{-1}. In order to ascertain whether such a simple model can yield significant improvements in lysozyme crystal size and quality, seed crystals of approximately 100 μm across the [110] face were grown under one of four different temperature control schemes: isothermal at 20°C, isothermal at 4°C, a linear temperature ramp (from 20 to 4°C) derived from the CSC algorithm, or CSC algorithm (operating from 20 to 4°C) given by Eq. (6) (see Fig. 1). The initial temperature was chosen due to the phase transition in the crystal space group which occurs when lysozyme is crystallized above 25°C [12]. At the conclusion of the growth experiments, crystal

growth was measured by observing the change in the chosen characteristic length ([110] face). Nucleation was quantified by counting the total number of crystals and crystallites in images of the drop of crystallizing solution taken at the end of each experiment.

2. Materials and methods

Hen egg-white lysozyme solutions (Sigma Chemicals, lots 89F8276 and 111H7010) were prepared by dialysis against deionized water and then against 0.05M sodium acetate buffer, pH = 4.5, with two buffer changes. The final concentration of the lysozyme stock solutions was 40–60 mg/ml. All solutions were passed through disposable 0.2 μm sterile syringe filters before use. Lysozyme stock solution was then mixed with the appropriate quantities of acetate buffer and 10% or 20% NaCl (in acetate buffer) to obtain the desired final concentration of salt and lysozyme for seed crystal formation or for crystal growth experiments. Lysozyme concentrations were determined using a published value for molar absorptivity [13].

Seed crystals were prepared using a 27 to 30 mg/ml lysozyme solution in 5% NaCl and 0.05M acetate buffer at pH = 4.5. Approximately 200 μl of solution was placed in wells of a 9-well pyrex spot plate (Fisher Scientific), then sealed with high vacuum grease and a 25 mm square microscope cover slip. The glass plate was refrigerated at approximately 7°C for 30 min and then transferred to room temperature for several hours, resulting in the formation of numerous seed crystals. The seed crystals selected were approximately 100 μm in size and exhibited the normal morphology of tetragonal lysozyme crystals [14].

To retrieve a seed crystal from a well, a suction device was constructed by attaching a 1 mm diameter non-heparinized microhematocrit capillary tube (Fisher Scientific) to a length of 3/32″ diameter silicon rubber tubing, which was connected in turn to a tuberculin syringe (Becton Dickinson and Co.). The hematocrit tubes were rinsed with filtered deionized water and dried before use, to remove debris. The seed crystal was drawn up into the capillary tube as the plunger on the syringe was displaced. This device allowed the retrieval of a single seed crystal, selected on the basis of size, morphology, and accessibility from a well containing many crystals. Seed crystals were transferred to a well containing a lysozyme wash solution (12 mg/ml lysozyme, 5% NaCl in 0.05M acetate buffer, pH = 4.5). The seed crystal was washed twice by transferring to fresh lysozyme wash solutions. The seed crystal was then placed into a well of a Linbro 24-well plate (Flow Laboratories) containing 10 μl of a 11.7 to 12.2 mg/ml filtered lysozyme solution with 5% NaCl, 0.05M acetate buffer at pH = 4.5. The wells of the Linbro plate were cleaned using an Ultra-Jet compressed gas cylinder (Fisher) and examined under a stereomicroscope to ensure that dust particles were removed before addition of the crystal. The 10 μl solution with the seed crystal was then overlaid with approximately 500 μl of light mineral oil (after Ref. [15]) to prevent evaporation of the crystallizing solution and to ensure adequate heat transfer between the bath coolant and the solution. Each crystal-containing well was sealed with high-vacuum grease and a 22 mm square cover slip. The Linbro plate was then placed into a pre-programmed temperature bath.

Different temperature programs were programmed into each of four temperature baths (VWR model 1187). Two baths were operated isothermally, one at 20°C and the other at 4°C, for 156 h. A digital temperature control program (DTC10PC program, PolyScience/VWR) applied the CSC-derived linear and the CSC programs (model constant supersaturation temperature program) to the other two baths. The CSC-derived linear temperature program had a constant slope between 20 and 4°C. Fig. 1 shows the four different temperature programs used as a function of time. Images of each seed crystal and the entire protein drop were acquired before and after applying the temperature programs using an Olympus SZ-PT stereomicroscope equipped with an Hitachi KP-M1 CCD camera. Images were then digitized using a Macintosh IIci computer equipped with a RasterOps 24STV frame grabber. At the conclusion of the experiment the largest crystal in the drop was assumed to be the seed crystal. Images were analyzed with NIH Image 1.51 software.

Unseeded crystallization experiments were also performed using the same protocols and the same four temperature programs as above. These experiments were identical to those described above except

that no seed crystal was included in the well. The largest crystal in the drop and the entire drop were imaged for analysis at the conclusion of the temperature program. The SuperANOVA statistical package (Abacus Concepts, Berkeley, CA) was used for statistical analysis.

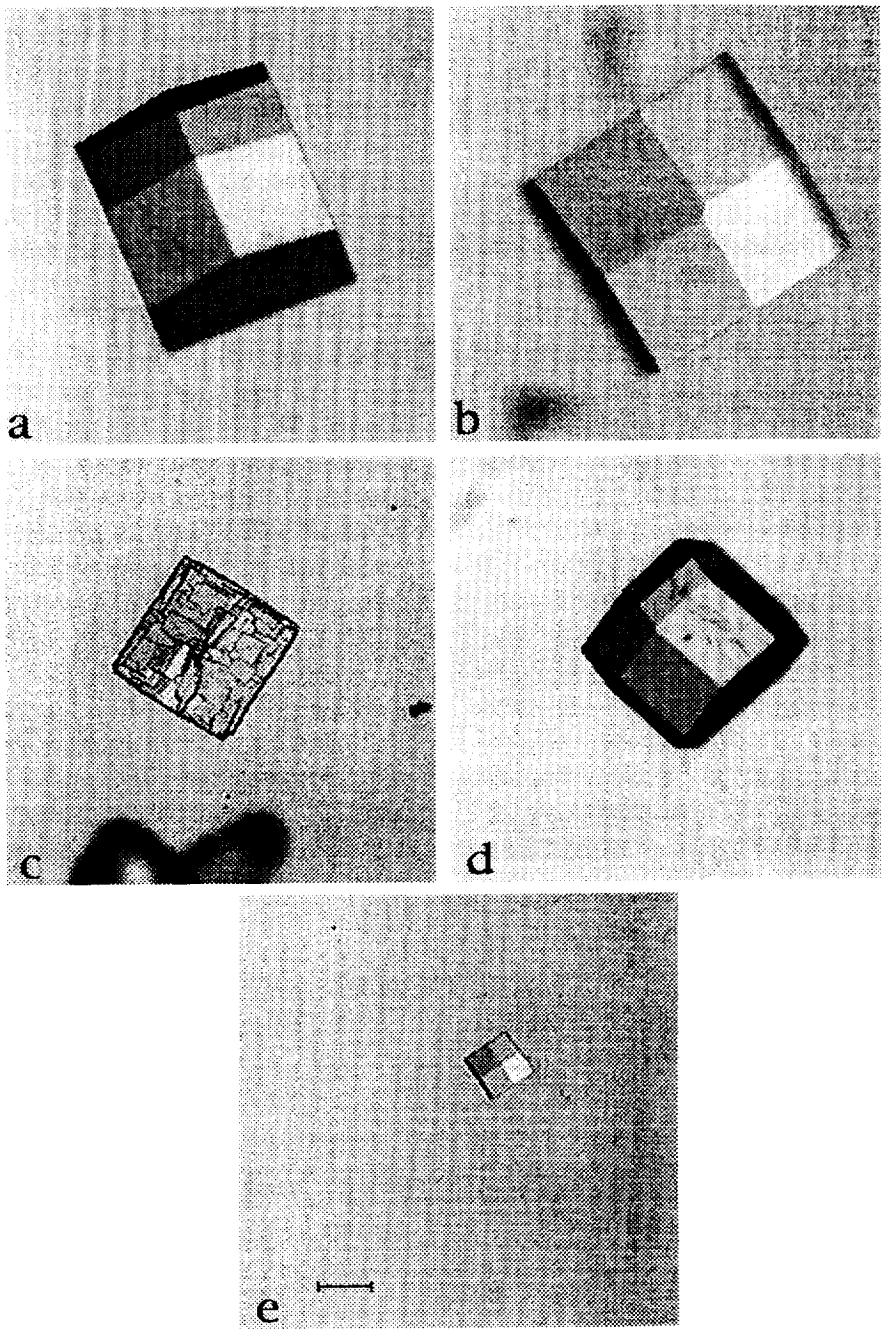

Fig. 2. Images of typical lysozyme crystals after 156 h under each of the 4 temperature programs (as shown in Fig. 1). All images were taken at the same magnification, and in all cases the width of the [110] face was measured. (a) Crystal grown under the CSC-derived linear program (final size, 400 μm). (b) Crystal grown under the CSC (constant supersaturation) program (final size, 449 μm). (c) Crystal grown under the isothermal 4°C program (final size, 222 μm). (d) Crystal grown under the isothermal 20°C program (final size, 290 μm). (e) Typical seed crystal (96 μm). A 100 μm scale is shown in (e).

3. Results and discussion

3.1. Seeded systems

The aim of the constant supersaturation (CSC) temperature control strategy is to maintain the growing crystals within the metastable zone. In this regime, existing crystals will grow but there will be minimal homogenous nucleation of new crystals. Increasing supersaturation will shift crystallization conditions into the labile zone and will cause undesirable homogenous nucleation to occur. If nucleation did not occur at high levels of supersaturation, a temperature control strategy providing the highest levels of supersaturation would be expected to produce the largest crystals over a given time interval, because of the high driving force for crystal growth kinetics. However, high levels of supersaturation result in formation of many crystal nuclei. A mass balance shows that when many crystals are grown in a limited volume of available protein solution, those crystals will grow to a smaller final size than if a single crystal is grown in the same volume of available protein solution. By maintaining the supersaturation level in or near the metastable zone, it should be possible to minimize nucleation and to grow fewer crystals to a larger size. At supersaturations of 15 and 10 mg/ml of lysozyme in pH = 4.5, 5% NaCl (sodium acetate buffer), significant homogeneous nucleation was found to occur. The supersaturation chosen for our experiments was 6 mg/ml since no homogenous nucleation was detected at a supersaturation 6 mg/ml and 20°C, some nucleation occurred in seeded samples and no nucleation occurred in unseeded samples held isothermally, indicating that supersaturation conditions were very close to or within the metastable zone. Nucleation in the presence of the seed crystal was probably due to secondary, rather than homogenous nucleation.

Fig. 2 shows images of a representative seed crystal and of representative crystals grown under each of the temperature control programs (Fig. 1) for the seeded samples. The width of the [110] face was defined as the characteristic dimension L, and was measured from images acquired before and after the seed crystal was subjected to the temperature program. For each crystal, the normalized percentage change in length, $\%\Delta L = 100 \times$ (final length − initial length/initial length), was determined and an average for each temperature control strategy was calculated. The number of crystals and crystallites formed in each well of the Linbro plate was counted from images taken of the entire 10 μl drop. This number is referred to as the nucleation number.

A summary of the average $\%\Delta L$ for seeded samples is found in Table 1. An analysis of variance (ANOVA) was performed on the $\%\Delta L$ grouped by temperature control strategy for the seeded samples. The ANOVA tests the null hypothesis that all temperature profiles produce the same size of crystal.

Table 1
Comparison of predicted versus actual results for the four temperature programs.

Temperature program	Initial size (μm)	Predicted final size (μm) [c]	Actual final size (μm)	Predicted $\%\Delta L$	Actual $\%\Delta L$
Seeded [a]					
Isothermal 4°C	83 ± 20	512	297 ± 84	521	281 ± 135
CSC-derived linear	93 ± 37	488	422 ± 114	426	413 ± 231
CSC	96 ± 29	444	440 ± 141	360	402 ± 243
Isothermal 20°C	114 ± 37	356	321 ± 89	211	201 ± 107
Unseeded [b]					
Isothermal 4°C	0	512	351 ± 86	–	–
CSC-derived linear	0	482	297 ± 275	–	–
CSC	0	427	239 ± 152	–	–
Isothermal 20°C	0	308	0 ± 0	–	–

[a] For the seeded wells, the predicted results assume that only the seed crystal is growing and no other crystals have nucleated.
[b] For the unseeded wells, no $\%\Delta L$ is calculated because no seed crystal is present in the well ($L_0 = 0$).
[c] The predicted final size assumes that a single crystal is nucleated and that there is no nucleation lag time.

The probability that the null hypothesis is true was found to be 0.0013. Thus, it is clear that the average crystal size generated by the different temperature programs are significantly different. The temperature strategy which produced the largest $\%\Delta L$ was the CSC-derived linear temperature program, followed by the CSC, isothermal 4°C, and isothermal 20°C programs. A multiple comparison test allows pairwise comparison of all possible pairs to determine which pairs of temperature algorithms differ significantly from each other. A Fisher's protected least significant difference (FLSD) multiple comparison test was applied to each possible pair of treatments, a total of six pairs. At a 95% confidence level, there is a significant difference in the $\%\Delta L$ between crystals grown under the CSC program (constant supersaturation strategy) or the linear temperature ramp derived from the CSC algorithm when compared to crystals grown isothermally at 4 or 20°C. There was no significant difference in the average $\%\Delta L$ between crystals grown under the CSC or CSC-derived linear programs. Crystals grown under the 4°C isothermal program were poorly formed and would be completely unsuitable for X-ray crystallography (Fig. 2c).

Nucleation numbers for the seeded samples are summarized in Table 2. An ANOVA was performed on the nucleation number. The ANOVA tests the null hypothesis that all temperature profiles produce the same number of nuclei. The probability that the null hypothesis is true was found to be 0.036, indicating significant differences in nucleation exist among the various temperature control strategies. At the 95% confidence limit using the FLSD multiple comparison test, the CSC algorithm and the CSC-derived linear algorithm were found to produce significantly fewer nuclei than the isothermal 4°C program. There were not significant differences between the isothermal 20°C, the CSC and CSC-derived linear algorithms however.

Table 2
Nucleation number for the four temperature programs in seeded and unseeded systems

Temperature program	Nucleation number	
	Seeded systems	Unseeded systems
Isothermal 4°C	49.7 ± 50.4	16.6 ± 19.0
CSC-derived linear	12.3 ± 17.1	0.9 ± 0.7
CSC	25.3 ± 36.7	1.7 ± 1.2
Isothermal 20°C	29.1 ± 48.9	0

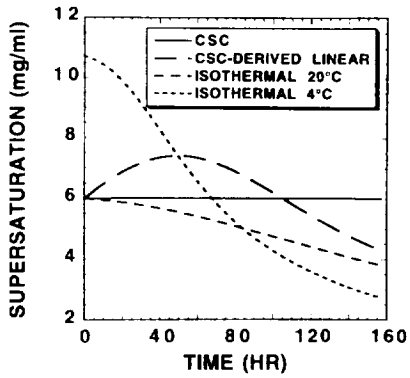

Fig. 3. Calculated supersaturation versus time for the four temperature programs. Supersaturation is defined as $\Delta C = (C - C^*)$. ΔC was calculated by assuming a nominal seed crystal size of 100 μm and that only one crystal was growing during the experiment. A maximum positive deviation of supersaturation from the average ΔC of the CSC program was calculated (see text) for each program resulting in deviations of 23% for the CSC-derived linear program and 78% for the isothermal 4°C program.

Fig. 3 is a plot of the calculated supersaturation, $\Delta C = (C - C^*)$, versus time for the various temperature control programs, assuming a single seed crystal is present and growing throughout the experiment. The CSC program exhibits a constant ΔC of 6 mg/ml by definition. It is expected that positive deviations from this supersaturation would push the growth conditions into the labile zone, increasing homogenous nucleation, and thereby decreasing the final size of the seed crystal. As shown in Table 1, the predicted $\%\Delta L$ (which assumes no nucleation) for seeded samples was greatest for the isothermal 4°C program, followed in order by the CSC-derived linear, CSC, and isothermal 20°C programs. Positive deviations from a ΔC of 6 mg/ml most likely caused the actual ranking of $\%\Delta L$ to differ from this predicted ranking due to excursions into the labile zone at higher supersaturation resulting in homogenous nucleation. The CSC-derived linear program resulted in the largest $\%\Delta L$, followed by the CSC, isothermal 4°C, and isothermal 20°C programs. The

isothermal 4°C program did not produce the largest $\%\Delta L$ as predicted, due to the large number of crystals nucleated in the presence of the large initial ΔC (Fig. 3) depleting the amount of protein available for growth of any single crystal. This hypothesis is supported by the observation that the isothermal 4°C program produced statistically more crystals (within a 95% confidence level) than the CSC-derived linear and the CSC temperature programs (see Table 2).

The CSC-derived linear and CSC programs resulted in significantly greater crystal growth (95% confidence level) than either of the isothermal programs, but differences in $\%\Delta L$ for the CSC-derived linear and CSC programs were not significant. The maximum positive deviation (MPD = $100 \times$ (maximum $\Delta C_{\text{program}} - \Delta C_{\text{CSC}})/\Delta C_{\text{CSC}}$) of the CSC-derived linear program from the CSC program was 23%. The deviation of 23% was apparently not great enough to result in significantly greater nucleation in the CSC-derived linear versus the CSC program. As a result, there were no significant differences in crystal growth as measured by $\%\Delta L$ between the CSC-derived linear and CSC programs. Under these conditions, it may be acceptable to substitute a CSC-derived linear temperature ramp derived from the CSC algorithm for the rigorous CSC program. The MPD of the isothermal 4°C was 78%. This was apparently great enough to generate many new nuclei and significantly reduce the final $\%\Delta L$ for the seed crystals.

3.2. Unseeded systems

Although the results from the seeded experiments were encouraging, secondary nucleation produced extraneous crystallites. Most likely, these secondary sites are created during the seeding where the crystal can easily produce small fragments upon suction into and out of the capillary syringe. Ideally, one would like to nucleate one or a very few crystals within the experimental device and then apply the CSC algorithm. Such controlled nucleation is very difficult without the use of specialized devices such as light scattering [7] or a thermal sting [5]. Using the CSC algorithm without a seed is an attractive alternative. In the CSC algorithm, the initial temperature and protein concentration are chosen so that no homogeneous nucleation will take place at the initial temperature. The temperature is gradually reduced, resulting in a concomitant increase in the supersaturation. A small excursion from the metastable to labile zone eventually occurs. At this point, the nucleation event consumes protein which pushes the system back into the metastable zone where the CSC algorithm continues to operate for the duration of the experiment as originally designed. Since no seed handling is required, secondary nucleation should be minimal compared to the seeded experiments.

A summary of the average final crystal size for unseeded samples is found in Table 1. An ANOVA of unseeded samples grown under the four temperature programs indicates significant differences in final crystal size (i.e. probability of null hypothesis being true of 0.0002). The average crystal size was largest for crystals grown under the isothermal 4°C temperature program, followed by the CSC-derived linear and CSC programs. No crystals were formed in the isothermal 20°C samples. Crystals grown using the isothermal 4°C program exhibited significantly greater size (90% confidence level) than those grown using the CSC program but were not significantly different in size than crystals grown using the CSC-derived linear program. The crystals grown using the 4°C isothermal program were poorly formed and not suitable for X-ray diffraction.

The predicted final size for unseeded experiments was calculated assuming that a single crystal was nucleated and there was no nucleation lag time. The experimental results for the unseeded samples reflected the predicted trend for final crystal size from largest to smallest (Table 1): isothermal 4°C, CSC-derived linear, CSC, isothermal 20°C. As with the seeded samples, the high positive deviation (MPD = 79%) of the isothermal 4°C program from the CSC program resulted in the nucleation of significantly more crystals than any other unseeded temperature program. In general, fewer crystals were nucleated in the unseeded systems in comparison to the seeded systems (see Table 2).

Secondary nucleation requires a much smaller level of supersaturation than does homogeneous nucleation [16] which is consistent with the lower level of nucleation seen in all unseeded systems. The seed crystals themselves, along with any submicroscopic nuclei which may detach from the surface of the

crystal (especially during sample handling), provide ample sites for secondary nucleation. This observation is most evident in the isothermal 20°C program where no nucleation occurred in unseeded samples, while ample nucleation occurred in seeded samples. Thus, application of the CSC algorithm to unseeded systems is an attractive method of minimizing homogeneous nucleation and maximizing terminal crystal size.

4. Summary

In this work, we have shown that the use of controlled temperature changes leads to improved size of lysozyme crystals, and can limit homogeneous nucleation in a crystallizing system. These temperature control strategies must have a basis in the physicochemical properties of the system (i.e., solubility, enthalpy of crystallization and growth rate kinetics). Perhaps the major limitation in extending the use of this algorithm to other systems is the limited amount of physicochemical data available for most proteins. However, our results show that there is some flexibility in the application of temperature control, so that a CSC-derived linear temperature ramp may be just as effective as our constant supersaturation algorithm. This indicates that only moderately accurate physical and chemical data may be required to generate an appropriate temperature ramp. Future studies will include evaluation of crystal quality by X-ray diffraction techniques and may indicate differences in crystal quality between crystals grown using differing temperature programs. It is expected that as more information becomes available, this type of predictive temperature control strategy can be applied to a wide variety of systems, facilitating the growth of large, single protein crystals suitable for X-ray crystallography.

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