The influence of pH on nucleation, solubility and structure of lysozyme protein crystals

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The Influence of pH on Nucleation, Solubility and Structure of
Lysozyme Protein Crystals

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
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THE INFLUENCE OF PH ON NUCLEATION, SOLUBILITY AND CRYSTAL STRUCTURE OF LYSOZYME PROTEIN CRYSTALS

Marc Apgar

ABSTRACT

X-ray diffraction from protein crystals remains the most reliable way to determine the molecular structure of proteins, and how this structure relates to biological function. However, we still lack the ability to predict solution conditions that support the nucleation and growth of high-quality protein crystals for X-ray diffraction studies. The overall goal of this thesis is two-fold: (a) determine the nucleation behavior and solubilities for lysozyme crystals with two distinct crystal structures (orthorhombic vs. tetragonal) and (b) investigate whether these changes in crystal habit and crystal solubility correlate with any discontinuities in the liquid-liquid phase boundary of lysozyme that occurs under the same solution conditions.

We measured lysozyme crystal solubility by nucleating and subsequently dissolving very small lysozyme crystals in highly supersaturated solutions. The presence of crystals in our samples is detected and monitored by measuring the light scattered off the micron-sized crystals. These "turbidity measurements" are repeated across a range of protein concentrations, for pH 4.6 and 5.6, thereby
yielding the crystal solubility boundary. Changes in crystal structure are assessed at the end of the experiments by microscopic inspection of the distinct crystal habits.

Attractive protein interactions in solution also induce liquid-liquid phase separation. Similar to the crystal solubility measurement, we use the turbidity increase associated with liquid-liquid phase separation to map out this phase boundary. Since both crystal formation and liquid-liquid phase separation are driven by attractive protein interactions, we investigated whether the dramatic changes in crystal solubility associated with different protein crystal structures lead to any discernable “discontinuities” in the liquid-liquid phase boundaries.
1. INTRODUCTION

1.1. Motivation

The objective of this thesis is to contribute to our understanding of conditions that promote or interfere with the nucleation and growth of high-quality protein crystals from solution. Study of the solution conditions that influence protein crystal growth leads to further understanding of the nucleation and growth process. In this study, crystals are nucleated and subsequently dissolved by respective cooling and warming samples of hen egg white lysozyme. A static light scattering apparatus is used to detect the presence of crystals or liquid droplets that form in samples that initially contain lysozyme monomers. The measurements are repeated across a range of protein concentrations, and for two separate pH values (pH = 4.6 and 5.6) to obtain phase diagrams. The specific measurement protocol had to be adjusted to accommodate differences in the phase nucleation and equilibration behavior of the liquid-liquid phase boundary, tetragonal crystal solubility, and orthorhombic crystal solubility.
1.2. Hen-Egg White Lysozyme: Interaction Forces in Solution

Hen egg white lysozyme is a globular protein with a molecular weight of 14,388 g/mol. Lysozyme is comprised of a single chain of 129 amino acids that, because of hydrophobicity, compacts into an ellipsoidal shape approximately 45Å across. The net charge of Lysozyme depends on solution pH and will carry a 10-12 positive charges at pH 4.6. It is important to recognize, however, that proteins are “zwitterionic” by nature, i.e. they will carry a combination of both positive and negative charges at any given pH. This bipolar charge distribution gives rise to a permanent dipole moment (and higher charge distribution moments) that contribute to the attractive protein-protein interactions.

\[ U_c(r) = k \frac{q_1q_2}{r} \]

\[ U_H = \infty \]

Figure 1 – Three dimensional model of Lysozyme with acid and base residues indicated with red and green respectively (Source: 2LYZ from pdb.org).

Figure 2 - Illustration of the interaction potentials of Lysozyme at three ranges.
1.3. Protein Phase Diagrams

Proteins can undergo a variety of phase transitions in solution, including the formation of crystalline phases and liquid-liquid phase separation, i.e. the separation of the solution into two liquid phases at vastly different concentrations. These transitions can occur when the long-range repulsion between the charged protein molecules is reduced by the presence of salt ions. Negative ions from dissociated salts attract to a positively charged protein to create an ion cloud with a net charge that is lower than the protein alone. The electrostatic fields become screened from neighboring proteins and short-ranged attractive forces become noticeable.

Molecular dynamics calculations of particles interacting via such short-range interactions have provided important insights into the expected shape of protein phase diagrams\(^3\). In these models, globular proteins under crystallization conditions are represented as spherical particles interacting via attractive, short ranged forces between them\(^5,11\). These short range attractions can be approximated with a Yukawa interaction potential\(^3\),

\[
\begin{align*}
    u(r) = \begin{cases} 
    \infty (r < \sigma) \\
    e^{\delta(r-\sigma)} - \varepsilon \frac{\delta(r-\sigma)}{r/\sigma} (r \geq \sigma)
    \end{cases}
\end{align*}
\]  

(1)

where \(\sigma\) is the diameter of the hard core sphere, while \(\delta\) and \(\varepsilon\) characterize the range and well-depth for the attractive interaction, respectively. The infinite potential produced when coming in contact with one another \((r = \sigma)\) is the nature of hard spheres, and a reasonable assumption for lysozyme\(^3,4\). For atoms or small molecules, Monte Carlo simulation with this interaction potential generate a phase diagram comparable to typical van-der Waals gases (Figure 3, left
panel\textsuperscript{22}. At low concentrations and high temperatures, the system is in the gas phase. Upon lowering the temperature, the gas condenses and enters the gas-liquid coexistence region (G+L), with the gas-phase and liquid-phase concentrations in that region given by the left and right branch of the parabolic coexistence curve. These two branches merge at the critical point (CP). At higher concentrations, we cross the sublimation phase boundary into the gas-solid coexistence region, with the sublimation and gas-liquid coexistence curves merging at the triple point (TP).

![Figure 3 - Schematic temperature-density phase diagrams for hard spheres of diameter $\sigma$ with Yukawa attraction of range $\tilde{\sigma}$. Left: Standard phase diagram with a stable G+L coexistence region below the critical point (CP). Right: Phase diagram for short attraction ($\tilde{\sigma} \ll \sigma/7$) which results in G+L coexistence that is metastable with respect to G+S. (Based on Muschol\textsuperscript{22})](image)

The shape of this “traditional” phase diagram changes dramatically as the size of the molecule is increased beyond approximately seven times the range of the attractive force. Large molecules exhibit a sublimation curve that “hops over” the G+L coexistence curve (dashed), and results in a metastable phase boundary below a sublimation curve that is more thermodynamically stable\textsuperscript{3}.

To interpret these results for particles interacting in the gas phase to proteins suspended in solution, we need to define the gas-phase as protein uniformly
dispersed in solution. The gas-liquid coexistence, in turn, represents the separation of the protein solution into two liquid phases with very different protein concentration. Proteins will tend to coalesce into dense liquid droplets, as shown in the liquid-liquid phase separation photograph in Figure 4 below. Crystal solubility, i.e. the coexistence curves of protein crystals in equilibrium with proteins dispersed in solution, should be identified with the G+S coexistence curve (sublimation curve) in the above model. Several investigators have shown that this simplified model predicts the phase separation boundaries seen with supersaturated protein solutions\textsuperscript{4,22,23}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{(A) Liquid-liquid phase separations of bovine \textgreek{E} crystalline in sodium phosphate for a sample at 22\textdegree\textcelsius for a few minutes. (B) Liquid-liquid phase separation of thaumatin at -9\textdegree\textcelsius. The initial protein concentration was 229 mg/ml (source: Asherie\textsuperscript{5})}
\end{figure}

1.4. Solubility

Consider a system of solid protein crystals in solution. Protein crystals in a protein-free solvent will lose protein molecules to the surroundings and will increase the protein concentration of the solution. Alternatively, a protein-rich solvent will act as a source of protein molecules that will be consumed during crystal growth. As long as the crystals don’t entirely melt, the system will reach an equilibrium concentration at which crystals lose protein molecules at the same
rate as they are gained. The concentration of proteins in the solution at equilibrium is the solubility\(^5\).

The van’t Hoff law is a general thermodynamic relationship that can be used to relate the equilibrium solubility concentration to the solubility temperature. Suppose a solution containing crystals that is kept at temperature \(T_1\) reaches an equilibrium concentration \(C_1\). As the solution temperature is changed to \(T_2\) a new equilibrium is reached at \(C_2\). Two equilibrium points are related by the van’t Hoff expression for solubility\(^6,7\):

\[
\ln \left( \frac{C_2}{C_1} \right) = -\frac{\Delta H}{R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right)
\]  

(2)

Where \(\Delta H\) is the enthalpy of crystallization and \(R\) is the gas constant. The relationship can be used to predict all other equilibrium points along the solubility curve. To plot a solubility curve, \(C_1\) and \(T_1\) are fixed to a single point and all other points \(C_2\) are calculated by plugging in different temperatures \(T_2\). To simplify the evaluation, equation (2) is rearranged like so:

\[
C_2 = C_1 \exp \left( \frac{\Delta H}{R} \left( \frac{T_2 - T_1}{T_1 T_2} \right) \right)
\]  

(3)

Assuming \(T_2\) is not too far from the reference temperature \(T_1\), the denominator inside the exponential can be approximated as \(T_1 T_2 \approx T_1^2\) which allows further simplification,

\[
C_2 = C_1 \exp(\alpha(T_2 - T_1))
\]  

(4)
where $C_1$ is the solubility concentration at the reference temperature $T_1$ and the constant $\alpha$ is given by

$$\alpha = \frac{\Delta H}{RT_1^2}$$

(5)

Figure 5 – van’t Hoff plots for typical values of enthalpy. From equation 4 $C_1=5.16\,\text{mg/ml}$, $T_1=25\,^\circ\text{C}$

Solubility curves are typically two-dimensional graphs that plot concentration against temperature, with all other conditions held constant. Protein solubilities can be plotted against temperature$^{8,22}$, salt concentration$^{22}$, pH$^8$, or ionic strength of the buffer$^9,10$. Various experimental conditions can be presented with multiple plots on a single temperature versus concentration graph. Protein point-mutations can be studied by measuring changes in solubility and some mutations have been shown to shift or invert the solubility line$^{11}$.

1.5. Crystal Nucleation and Growth

Crystals will grow at conditions below the solubility curve and will melt at conditions above. Solutions more concentrated than the solubility are said to be supersaturated and the degree of supersaturation can be expressed as fractional difference from $C_{\text{sat}}$. In principle, all supersaturated solutions ($C>C_{\text{sat}}$) should
nucleate crystals. In general, nucleation of crystals requires some additional driving force because there is an energy barrier associated with the surface free energy for small crystalline clusters\textsuperscript{12}. Protein crystals will hardly ever nucleate unless concentration exceeds solubility by a factor of least three\textsuperscript{5} (C>3C\textsubscript{sat}). Once protein crystals have nucleated, they will grow at a rate proportional to the square of supersaturation\textsuperscript{20}, but have been found to not grow at all if supersaturation is too low (C<2C\textsubscript{sat})\textsuperscript{20}. For concentrations in the range 2-3 C\textsubscript{sat}, crystals grow readily\textsuperscript{13,20} and become large enough to be seen with optical microscope in a few hours. Highly concentrated (>3C\textsubscript{sat}) solutions grow too quickly and new protein molecules attach anywhere instead of the energetically favorable sites resulting in poorly formed crystals with numerous defects\textsuperscript{14}. Extremely high supersaturation can result in aggregation of protein into amorphous liquid droplets that lack any long-range order, i.e. liquid-liquid phase separation.

Nucleation is assumed to begin when two monomers bind to create a dimer\textsuperscript{15}. The second aggregation step will either be binding of an additional monomer to the dimer of binding of dimer to dimer. Subsequent steps could occur via additional aggregation pathways because of the increased variety of particle populations in solution. Some crystal growth has been found to proceed by addition of higher order aggregates that have preformed in the bulk solution prior to attachment\textsuperscript{15}. There is some evidence that nucleation begins with low-order liquid droplets until it reaches a critical size and rearranges into a crystal\textsuperscript{12}. 
2. MATERIALS AND METHODS

2.1. General Approach

Our approach to measure protein phase separation is to take advantage of the strong temperature dependence of protein solubility and of the dramatic changes in light scattering intensity associated with phase separation. During our experiments, we quench the temperature of protein solutions while measuring changes in light scattering intensity caused by phase separation\textsuperscript{16,22}.

\begin{equation}
\frac{I_s}{I_i} = \frac{4 \pi n^2}{\lambda^2 R^2} \left( \frac{\partial n}{\partial C} \right)^2 \frac{CM}{N_a} \approx \frac{10^{-2}}{R^2}
\end{equation}

*Figure 6 - Illustration showing that scattering increases when proteins aggregate into larger particles. Left: Diffuse protein (G) scatters weakly according to Raleigh’s law. Right: Aggregates (L or S) are larger than incident light wavelength, so that Mie scattering and refraction occurs and scattering increases.*

The basic principle of the measurement is readily explained by looking at the theoretical phase diagram of proteins in solutions (see Figure 7). The starting point (point 1) of our measurements is the uniform solution phase, equivalent to the gas phase (G). The sample is then cooled to point 2, which is below the gas-
liquid phase boundary (dashed curve) and two coexisting liquid phases form (Xs). The phase change can be detected as a rapid increase of light scattering and confirmed by re-warming the sample above the phase boundary. The sample is slowly warmed toward point 3 until scattering reduces and the temperature at which this occurs is recorded.

The temperature is maintained at point 3 until scattering increases again which is attributed to a phase change into crystals (squares in Figure 7). Protein crystals can form in the G+S coexistence region, and these crystals will also contribute to scattering. For crystal solubilities, we had to proceed cautiously with our measurements since (a) crystal nucleation requires very high values of supersaturation and (b) crystals, once formed, have to be given long time periods to equilibrate with their surrounding solutions. Hence temperature changes were performed in small steps, and subsequent step increases were not taken until the temperature and scattering intensity during the previous step had stabilized. Orthorhombic crystals grow and melt slower than tetragonal crystals so these can be differentiated by the rate of change of scattering intensity. Finally, the crystals are melted as the sample is slowly warmed from point 3 to point 1, and the solubility temperature is recorded.
2.2. Illumination and Detection

To implement these measurements, we modified and improved a temperature controlled light scattering instrument previously built in our laboratory\textsuperscript{17}. A schematic overview of the hardware used for the detection of temperature-induced phase separation in protein solutions is shown in Figure 8. The light source was a high intensity, AlGaInP light emitting diode (superbrightleds.com, model RL5-RD1560) which had a center wavelength $\lambda = 638$nm, and power output of about 1mW. The LED was powered by a 6 volt DC battery in series with an $R = 330$ $\Omega$ resistor to deliver an operating voltage of 1.84 V. A power meter measured the light output at $P = 1$mW and confirmed that it was stable and noise-free. The light was focused with an $f = 15$cm lens on the center of the sample cuvette. Light scattered from the samples was collected with an $f = 30$mm lens at a right angle to the incident illumination and was focused onto a silicon photodiode (UDT sensors, model 11-05-001-1).
The LED, temperature controller and photodiode detector were covered with a box to prevent ambient room light from entering the photodetector. The photo current, which is proportional to the incident light intensity, was converted into a voltage using a high-impedance ($R = 100\, \text{M}\Omega$) current-to-voltage amplifier (Femto, model DLCPA-200). The amplifier gain was set to $10^8$ and an internal 10 Hz low-pass filter was enabled. The output voltage from the amplifier was digitized with a data acquisition board (National Instruments, model PCI6221 and BNC2090 breakout box). Voltage was sampled 100 times per second, and the average voltage for each one second interval was calculated to create another filter in software, that was low-pass below 1Hz.

2.3. Temperature Control

The temperature of the protein solutions was controlled by placing the sample cuvette inside a temperature controller (Quantum Northwest, model TLC50F).
The temperature controller has a water-cooled peltier element connected to an electronic control module that adjusts the current to provide the proper rate of cooling/heating for a chosen sample temperature. Measurements of the protein phase separation required that (a) the sample temperature could be changed under computer control (b) scattering intensities could be acquired and correlated to actual sample temperatures and (c) sample temperature could be automatically readjusted in response to changes in light scattering intensity indicating phase separation. Tap water was run through the peltier cooler for heat transfer from the controller to maintain temperature of the cuvette holder to within 0.1°C. A calibrated thermocouple probe was inserted into the top of the cuvette to measure sample temperature throughout the entire experiment. These readings were typically 0.1-0.5°C different from the temperature reported by the controller and lagged behind the set temperature due the thermal equilibration time required.

The quartz cuvette (Starna, model 9F-Q-10-MS) held 0.8 to 1.7 ml of sample solution in a rectangular column. It also had a conical cavity at the bottom to allow a stirring rod to rotate freely. The stirring rod (Fisher 14-512-152) was actuated with a magnet located at the bottom of the cuvette holder and could be turned on or off via software. The stirring rod was found to become stuck when samples became excessively viscous so the drive magnets were upgraded by the manufacturer early in the experiments. Trials requiring temperatures below 10°C were performed while flushing dry nitrogen gas through the holder to prevent condensate from forming on the cuvette.
2.4. Preparation of Stock Solutions

All stock solutions were made with deionized water obtained from a four-stage water filtration system (Barnstead E-Pure model D4641). We typically used two stock solutions for any given trial: a buffer solution adjusted to the appropriate pH and a buffer/salt solution with salt concentration at twice its final value. Solutions for measurements were generated by dissolving the protein at twice its final concentration in the buffer stock solution and mixing it in equal proportions with the twice-concentrated salt/buffer solution.

To make a 100mM sodium acetate buffer, an appropriate amount of dry sodium acetate (Fisher cat# BP333-500, purity > 99%) was weighed and dissolved into water. The pH was adjusted with glacial acetic acid (Fisher cat# BP1185-500, purity > 99.9%). Solution pH was measured with a calibrated and temperature compensated pH probe (Fisher model 13-620-185) and pH meter (Fisher model AR15) until the desired pH was reached. Stocks solutions were stored in sealed glass bottles that were refrigerated at 5°C.

A stock of buffer with 8% w/v NaCl was prepared by dissolving 80 mg of NaCl (Fisher cat# BP358-212, purity 99.5%) in 1 ml deionized water and heating it
above 80°C to ensure the salt is thoroughly dissociated. Then the solution was cooled and 100 mM of NaAc is added. The pH was set in a similar manner as the buffer stock. Setting the pH of the salt solution separately ensured that any effect the salt has on the pH or the pH probe was eliminated.

2.5. Preparation of Measurement Samples

Lyophilized lysozyme protein (Worthington Biochemical, Lakewood, NJ, cat# LYSF) was weighed out on a scale, gently combined with 1 ml of buffer stock and warmed to 45°C for at least 15 minutes. Then the solution was filtered through a 0.22 μm syringe filter (Fisher cat# 09-720-3) and transferred to sealed centrifuge tubes and stored at 45°C prior to the trial.

The actual protein concentration of the sample was measured from the uv-absorption of the sample. A small portion of the sample (typically 10 μl) was diluted 150-fold into the buffer stock. A spectrophotometer (Thermo Electron, model UV1) was used to measure the optical absorption of the sample at $\lambda = 280$ nm, where the absorption coefficient for lysozyme is known to be $\alpha_{280} = 2.64$ ml/mg cm. Prior to the concentration measurement, the spectrophotometer was zeroed with a lysozyme-free buffer solution. The absorption measurement was multiplied by the dilution factor and divided by the absorption coefficient to obtain the actual lysozyme concentration in mg/ml. The dilution and concentration measurement was performed at least twice to improve accuracy and to detect mistakes if and when they occurred.

The accurately measured lysozyme solution was combined, in equal parts, with the buffer stock containing 8% NaCl to achieve final solution containing half the measured concentration of lysozyme at 4% NaCl. 400-1000 μl of sample
was transferred into the quartz measurement cuvette that was placed into the peltier temperature controller. The sample was allowed to equilibrate to temperature for several minutes while turning the stirring bar to ensure thorough mixing of the solution.

The specific sequence chosen for sample preparation was not accidental. Figure 10 below depicts alternative sample preparation pathways within a typical protein vs. salt concentration phase diagram. Line A1 (vertical line) represents adding solid-phase lysozyme directly to a 4% NaCl/buffer solution. Method A1 is not desirable because the sample will pass through the solid-phase region briefly which might risk crystal nucleation prior to the onset of measurements. Lines B1 and B2 represent the method employed for these experiments. Note that the sample is much less likely to cross into the solid-phase region. B1 represents the 8% NaCl stock solution and B2 represents the lysozyme at twice the final target concentration.

Figure 10 – Protein Concentration vs. Concentration of NaCl with a typical crystal solubility region shaded. Arrows indicate pathways for solution preparation.
2.6. IGOR Software

Automated computer control was achieved with IGOR Pro 5.03 mathematical software that was purchased from www.wavemetrics.com. Custom written IGOR routines communicate with the temperature controller, with the data acquisition card and with the user via a small graphical user interface (GUI). A second-by-second log was kept for measured scattering intensity, controller temperature and thermocouple temperature. The program initially determines the scattering intensity of the homogeneous sample solution while the sample is kept above the crystal solubility temperature for the given sample composition. This scattering intensity is considered the minimal scattering intensity, or \( V_{\text{Clear}} \). The IGOR macro “start new trial” is invoked to bring up a small GUI window which is used to set the specific parameters for the trial. Figure 11 below is an image of the GUI at the beginning of a typical trial.

The settings shown in Figure 11 were for trial 539 which resulted in the data shown in Figure 14 below. Trial 539 was typical for most trials performed but specific settings vary from trial to trial. The clouding step is skipped for conditions where these measurements are not possible, i.e. low protein concentrations. The crystal growth and the melting steps are usually possible if the cloud point measurements are possible.
The trial is started by clicking “start” in the GUI and all the steps that the user has checked are performed sequentially from top to bottom. In this example, the first step is to preheat the sample for 5-10 minutes at 45°C, while stirring to ensure that the sample is homogeneous. Then, the stirring rod is turned off and temperature is lowered to 30°C which is still above the temperature where liquid-liquid phase separation is expected to occur. When stabilized, background intensity, $V_{\text{Clear}}$, is measured and recorded as baseline intensity for the remainder of the trial. The value of $V_{\text{Clear}}$ will be used as the basis for thresholds in subsequent steps in the trial.
Figure 12 - Record of the solution temperature (red) and scattering intensity (blue) vs time for a lysozyme solution (Clys = 97mg/ml, 4% NaCl, pH = 4.6) undergoing temperature-induced phase separation. At t = 15 min (T = 11°C) the solution undergoes liquid-liquid phase separation which persists until the solution is rewarmed to 12.25°C. At t = 60 minutes, the stirring rod is turned on which immediately induces crystal nucleation. Crystals persisted in solution until the temperature was raised to 47°C. (Source: trial #330)

2.7. Liquid-Liquid Phase Separation

Liquid-liquid phase separation is induced by stepping the temperature down until a rapid increase in scattering is detected, which occurred at 15 minutes in Figure 12 above. The temperature that induced the scattering increase is recorded as $T_{\text{cloud}}$. After clouding, $T_{\text{clear}}$ is determined by slowly stepping the temperature back up until the scattering returns close to the baseline intensity, $V_{\text{clear}}$.

A photo of a lysozyme solution that has undergone liquid-liquid phase separation is shown in Figure 13. The separated solution reveals small droplets of fluid at highly elevated protein concentration observed during the clouded phase of trial 539, between 40-50 minutes in Figure 14. The droplets are large relative to wavelength so that incident light undergoes multiple-scattering and
refraction/reflections at the interfaces of the two solution phases. As a result, the solution displays a turbid, milky-white color.

![Lysozyme solution after liquid-liquid phase separation. Photo was taken during the intensity peak at 40 minutes in trial 488](Image)

**Figure 13 – Lysozyme solution after liquid-liquid phase separation. Photo was taken during the intensity peak at 40 minutes in trial 488**

2.8. Crystal Nucleation and Solubility Determination

After the sample clears, a constant temperature is maintained while crystals are allowed to nucleate. The stirrer is turned on because, in previous trials, the stirrer was found to enhance the crystal nucleation rate. At that point, the solution is below the expected crystal solubility temperature but above the cloud temperature for liquid-liquid phase separation (point 3 in Figure 3). Thus, any dramatic intensity increase can be attributed to the nucleation of many small crystals. The instrument simply waits at this temperature until there is an adequate intensity increase, usually 10 times $V_{\text{Clear}}$. Next, the temperature is stepped up slowly while the resulting scattering intensity is monitored. The temperature is raised until the sample scattering reduces to the background scattering intensity, indicating that all crystals have melted. This point is recorded as the solubility temperature $T_{\text{Sol}}$. 
2.9. Dilution Used to Measure Solubility at Low Concentrations

For lower protein concentrations, crystal nucleation requires excessively long time periods. To obtain crystal solubilities at low protein concentrations, crystals are nucleated at higher lysozyme concentrations, then samples are diluted with 4% NaCl buffer solution and the experiment is resumed. Figure 15 below illustrates two alternatives for diluting these solutions. The open circle represents the starting point where high concentration crystals are nucleated and the solid circle represents the target condition after dilution. Method D1 involves diluting first and then cooling which is not desirable because the sample will pass outside the solid-liquid coexistence region and the crystals will melt. Method D2 was used in these experiments because cooling the sample first ensures that the crystals do not melt during dilution.
Note that crystals will grow at the filled circle in the figure but kinetic factors result in very slow nucleation rates. Growing crystals in these regions was attempted but abandoned after several hours of waiting for nucleation.

Figure 15 – Two different diluting methods for crystals in solution represented by two arrows from the open circle to the filled circle. D1 occurs when diluting first then cooling. D2 occurs when cooling first.

2.10. Freezing Point of a Typical Sample

A typical sample (Clys = 22.2mg/ml, 4%NaCl, pH = 4.6) was used for determination of the freezing point. The freezing point is important for establishing a lower measurement limit for the SLS experiments. The sample was frozen inside the cell until a thermocouple probe was firmly entrenched and could not be removed. The temperature was slowly increased until the probe was freed. The probe temperature at this point was -5.4°C.

2.11. Denaturing Temperature of Lysozyme

The denaturing temperature of lysozyme was measured by steadily increasing temperature until scattering increased. The assumption is that the increased scattering is a result of lysozyme uncoiling and aggregating. Scattering increased slightly at 63°C and significantly at 65°C as shown in Figure 16 below. Broide⁴ obtained similar results and reports that lysozyme irreversibly precipitates
at 65 °C. The denaturing temperature is important because it sets the upper temperature limit for these experiments.

Figure 16 - Scattering and temperature versus time for 61.4 mg/ml lysozyme in 4% NaCl. Sample denatured at 65 °C (trial # 458).

The upper temperature limit results in an upper concentration limit because a portion of the solubility curve exceeds the denaturing temperature. Denatured proteins scatter light in a way that cannot be differentiated from light scattered from crystals. So if crystals do not melt when temperature is raised to ~62 °C, solubility measurement is impossible, as exemplified in Figure 17 below.

Liquid-liquid phase measurements are also difficult at high concentration because of the tendency of nucleate crystals before the measurement can be made. The presence of “unmeltable” crystals acts as background scatterering that cannot be differentiated from liquid droplets, as can be seen in the elevated intensity at 50 minutes in Figure 17, which occurred before liquid-liquid separation. When highly-concentrated trials nucleated too many unmeltable
crystals before liquid-liquid separation, the trial was aborted because no valid data points could be obtained.

![Graph showing temperature over time](image)

**Figure 17 – Record for a concentrated lysozyme solution (Cl stagnant=252mg/ml, 4% NaCl, pH=4.6) that undergoes aggregation early in the trial. Raising the temperature to 60 °C did not reduce scattering which made solubility determination impossible. (Source: Trial 444)**

### 2.12. Temperature Gradients inside the Sample Cuvette

The vertical temperature gradients inside two types of sample cuvette were measured by inserting a thermocouple probe into the cuvettes containing water. The cell holder was set to a constant temperature and allowed to equilibrate before measuring. An Extech 421305 thermocouple meter was used for these measurements. Temperature variations in the large cell (Starna 9F-Q-10-MS) were measured with a small stirring rod at the bottom set to medium speed.

<table>
<thead>
<tr>
<th>Depth (mm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.0</td>
<td>44.0</td>
</tr>
<tr>
<td>30.0</td>
<td>31.2</td>
</tr>
<tr>
<td>15.0</td>
<td>17.2</td>
</tr>
<tr>
<td>5.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

*Table 1 – Temperature at four depths inside the Large Starna cell (9F-Q-10-MS). The top temperature column corresponds to the set temperature of the controller.*
Table 2 – Temperature at four depths inside the Small Starna cell (Starna,3-3.45-Q-3) and aluminum carrier. Each column corresponds to a setting on the temperature controller.

<table>
<thead>
<tr>
<th>Depth (mm)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>44.6  30.1  16.3  6.9</td>
</tr>
<tr>
<td>20</td>
<td>45.2  30.5  16.1  6.5</td>
</tr>
<tr>
<td>30</td>
<td>45.3  30.5  16.1  6.4</td>
</tr>
<tr>
<td>40</td>
<td>45.4  30.5  16.0  6.3</td>
</tr>
</tbody>
</table>

Typical thermocouple probe depth during phase separation experiments was chosen to be 30 mm; a position close to but slightly above the light beam passing through the sample.

2.13. Temperature Dependence of the pH for the 100 mM NaAc Buffer

The pH of four batches of stock solutions was measured at four temperatures to determine whether and how much solution temperature altered buffer pH. The NaAc buffer was stabilized to 4.6 or 5.6 pH, stored in glass bottles at 5°C for days-weeks before measurements. A temperature compensated pH probe (Fisher 13-620-185) was used for all of the pH measurements in this trial. The first concern when making this type of measurement was to ensure that the pH changes observed are not simply thermal drift of the pH probe. We used two NIST traceable buffers with known temperature-dependence (Fisher SB107-500 and SB101-500) to calibrate the probe before measurement at a given temperature. The instructions that are supplied with the probe say that the
calibration for elevated pH measurement can be achieved by cycling the probe from hot and cold samples while monitoring the probe voltage. When the voltage measured in the cold bath differs by +/- 2mV or less, the probe is claimed to be calibrated for elevated temperatures. This was performed using two separate vials of pH=4 buffer standard, at 23°C and 60°C respectively. The measurements are in Table 3 below.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>23°C</th>
<th>60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>190 mV</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>198 mV</td>
</tr>
<tr>
<td>3</td>
<td>188.7 mV</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>198.9 mV</td>
</tr>
<tr>
<td>5</td>
<td>189.4 mV</td>
<td></td>
</tr>
</tbody>
</table>

Notice that the reading at the low temperature deviates less than 2mV. After completing this calibration, both buffer standards were measured at 60°C. The standards are labeled to be 4.09 pH and 6.95 pH at these temperatures but the meter measured 3.81 and 6.98. A pH error of 0.38 was observed after calibrating the probe with this technique.

We opted for a more conservative calibration technique, by re-calibrating the probe at the exact temperature where pH measurements were required. To do this, two vials containing pH standards were placed in a 200 mL water bath. Four measurement samples were put into the same beaker to ensure temperature match between calibrations and measurements. The pH probe was calibrated with the two standards using temperature-specific pH values as indicated on the label. This calibration was repeated for each temperature prior to
measuring the four stock solutions. The pH of the stock solutions deviated from
the target pH by at most 0.18 pH units.

Table 4 - pH of four different stock solutions that were used for
many of the trials reported in this work

<table>
<thead>
<tr>
<th>Stock</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target pH</td>
<td>4.5</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>NaCl %</td>
<td>12</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Prep. Date</td>
<td>2/26/07</td>
<td>10/18/07</td>
<td>12/4/07</td>
<td>12/4/07</td>
</tr>
<tr>
<td>pH at 60°</td>
<td>4.60</td>
<td>5.60</td>
<td>5.62</td>
<td>5.64</td>
</tr>
<tr>
<td>pH at 40°</td>
<td>4.62</td>
<td>5.61</td>
<td>5.60</td>
<td>5.61</td>
</tr>
<tr>
<td>pH at 20°</td>
<td>4.53</td>
<td>5.68</td>
<td>5.53</td>
<td>5.66</td>
</tr>
<tr>
<td>pH at 10°</td>
<td>4.59</td>
<td>5.55</td>
<td>5.65</td>
<td>5.61</td>
</tr>
<tr>
<td>pH at 7°</td>
<td>4.58</td>
<td>5.54</td>
<td>5.59</td>
<td>5.62</td>
</tr>
</tbody>
</table>

Figure 18 - Temperature dependent pH changes of four buffer/salt solutions
3. RESULTS AND DISCUSSION

3.1. Enhanced Crystal Nucleation in Response to Solution Agitation

Crystal nucleation is dramatically accelerated when a stirring rod is used to agitate the solutions. Lysozyme samples at a temperature 1-2°C above the liquid-liquid coexistence curve would form crystals nearly immediately after starting the rotation of the stirring rod. One example is shown in Figure 19 above where light scattering remains low until the stirring rod starts. The scattering can be attributed to crystal nucleation because of crystals were observed by microscopic inspection and because scattering reduced when the crystals are melted by warming. At low protein concentration, crystals would not nucleate after waiting 2-3 hours without a stirring rod but would nucleate minutes after...
starting the stirring. At extremely low concentrations (< 5 mg/ml), crystals could not be nucleated with or without a stirring rod. Hence the stirring rod in our experiments serves two purposes: it prevents the small microcrystals we nucleate from settling out and we find that it dramatically enhances nucleation rates.

3.2. Crystal Structure and Habit

Crystal structures are derived from observations of the crystal habits, which are distinctly different from tetragonal vs. orthorhombic crystals. Crystal habit is checked by briefly pausing the trial and removing 10 µl of sample from the cuvette. The removed sample volume is placed onto a microscope slide so that it can be viewed with an Olympus IX-70 inverted microscope.

Figure 20 - Temperature (red) and scattering intensity (blue) versus time for a lysozyme solution (Clys = 54mg/ml, 4% NaCl, pH = 5.6) undergoing liquid-liquid phase separation at 40 minutes, tetragonal crystal growth at 130 minutes and orthorhombic crystal growth at 220 minutes. (Source: trial #488)

shows one of the earlier trials (Trial 488) performed at pH 5.6. This trial utilizes a method similar to the preceding trials to grow and then melt the
tetragonal crystals. At the 200 minute point, the tetragonal crystals are completely melted and the scattering intensity has returned to the baseline observed at the beginning of the trial. But after 200 minutes, the scattering intensity increased again despite a temperature that consistently melted crystals in previous trials. It is clear that a new crystal type was growing, at a slower rate, but with a higher solubility temperature. The high solubility temperature appears to correspond to orthorhombic crystals that grow slower, but are more difficult to melt. Photos taken at various stopping points throughout the trial reveal significant changes in crystal appearance as the trial progressed. Before beginning the trial, the sample was inspected under the microscope and it was confirmed to be clear of any crystals or aggregates. At several points during the trial in, aliquots of the sample were photographed and are included in Figure 13, Figure 21, Figure 22 and Figure 25. The photographs can be compared with other authors that have associated macroscopic crystal habits with crystal structure.

Figure 21 – Photo of lysozyme after cycling through the LL phase and stirring for 30 minutes at a temperature slightly above this phase. Note numerous aggregated crystallites. (Source: Photo 1500, trial #488, 165 minutes)
Figure 22 – Photo of lysozyme that has been slowly warmed to 42°C taken just before melting. Note two distinct shapes: squares and prisms that resemble Figure 23 and Figure 24. (Source: Photo 1511, trial #488, 206 minutes)

Figure 23 – Photo of tetragonal lysozyme crystals from other research. Left: lysozyme (Clys=100mg/ml, NaCl=4%, 0.1M NaAc, pH=5.0) held at 18.5°C for 30-45 minutes. (Source: Gorti\textsuperscript{18} fig 7) Right: lysozyme (Clys=100-150mg/ml, NaCl=2.5%, 0.05M NaAc, pH=4.6) at 20°C. (Source: Yoshizaki\textsuperscript{19} fig 4)

Figure 24 – Growth habit of tetragonal lysozyme with indices of axis and faces indicated. (Source: Monaco\textsuperscript{20})
3.3. Crystal Solubility

The solubility measurements are fit to the van’t Hoff equation (equation 4) with \( \alpha \) determined by a least squares fit. The IGOR fitting routines perform best when fitting to data that lie along a decaying exponential. Before fitting, temperatures were first multiplied by -1 so that the temperature axis was "flipped" and a decaying exponential was produced (C vs. -T). The fit was performed and temperature was again multiplied by -1 before plotting the fits that are shown in the figures below.
Figure 27 – Phase diagram of lysozyme (100mM NaAc buffer, 4% NaCl, pH=4.6). Squares: solubility measurements, Solid line: least squares fit to a van’t Hoff law (eq. 4), Circles: LL phase measurements plotted for comparison. No orthorhombic phase was observed at this pH.

Figure 28 - Semi-log plot of Figure 27.

At pH 5.6, both tetragonal and orthorhombic crystals occurred and two different solubilities were measured and fit to separate curves (Figure 29). The nucleation rate for orthorhombic crystals increases with increasing protein concentrations which made measurements of tetragonal solubilities impossible above 60 mg/ml. As shown in Figure 31 below, tetragonal crystal solubility at pH 5.6 follows almost exactly the same curve as pH 4.6.
Figure 29 - Phase diagram of lysozyme (0.1M NaAc, pH=5.6, 4% NaCl). Diamonds: orthorhombic, Squares: tetragonal, Circles: LL phase, Lines: least squares fits to a van’t Hoff law (eq. 5).

Figure 30 – Semilog plot of Figure 29. Note the two solubilities are equal at approximately 2 mg/ml.
Figure 31 – Solubility of lysozyme (100mM NaAc, 4% NaCl) tetragonal crystals. Open squares: pH=4.6, solid squares: pH=5.6. Lines: least squares fits to a van’t Hoff law (eq. 5).

The solubility data obtained from these experiments suggests that the notional phase diagram presented in Figure 3 should be modified by adding an orthorhombic solubility curve as shown in Figure 32. The orthorhombic curve crosses the tetragonal curve at a point of equivalent solubility (ES) where the growth of both crystal types reduces free energy equally. At concentrations above ES, orthorhombic crystals are more energetically favored and more stable than tetragonal crystals.
Figure 32 - Phase diagram previously presented in Figure 3 with the orthorhombic phase added (dotted curve). The orthorhombic phase and tetragonal phase are equally soluble where the curves cross (ES). ES is at a concentration lower than the liquid-liquid phase (dashed curve).

To relate the solubility curves to enthalpy of crystal formation, equation 4 can be solved for $\Delta H$:

$$\Delta H = \alpha R T_1^2$$

(6)

Enthalpy is calculated using the solubility fitting coefficients and are presented in Table 5 below.

<table>
<thead>
<tr>
<th>pH</th>
<th>NaCl</th>
<th>$C_1$</th>
<th>$\alpha$</th>
<th>$T_1$</th>
<th>$\Delta H$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.6</td>
<td>4% tetra</td>
<td>5.16</td>
<td>-0.139</td>
<td>298</td>
<td>-24.5 kcal/mol</td>
</tr>
<tr>
<td>5.6</td>
<td>4% tetra</td>
<td>4.96</td>
<td>-0.146</td>
<td>298</td>
<td>-25.7 kcal/mol</td>
</tr>
<tr>
<td>5.6</td>
<td>4% ortho</td>
<td>2.51</td>
<td>-0.052</td>
<td>298</td>
<td>-9.3 kcal/mol</td>
</tr>
</tbody>
</table>

For comparison to other work in this field, data points were obtained from Pusey’s\textsuperscript{24} figure 1, for tetragonal solubility. The Pusey experiments were also performed with 100 mM Acetate buffer, set to a slightly lower pH = 4.0. The figure below shows good agreement with data collected in this study.
Table 6 - Solubility temperatures of tetragonal crystals (0.1M NaAc, pH = 4.0). (From Pusey, figure 1)

<table>
<thead>
<tr>
<th>C (mg/ml)</th>
<th>Tsol (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>30</td>
</tr>
</tbody>
</table>

Figure 33 - Tetragonal solubilities for this work plotted against that of Pusey.

Reported data for orthorhombic crystals are also compared with data collected here. Orthorhombic solubility reported in this paper is a steep function of temperature and the crystal enthalpy is 8.5 kcal/mol while data Pusey 12.6 kcal/mol.

Table 7 - Solubility temperatures of lysozyme (0.1M NaAc, pH=4.0) orthorhombic crystals (From Pusey, figure 4.)

<table>
<thead>
<tr>
<th>C (mg/ml)</th>
<th>Tsol (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>13</td>
<td>31</td>
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<tr>
<td>15</td>
<td>36</td>
</tr>
<tr>
<td>18</td>
<td>39</td>
</tr>
<tr>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td>23</td>
<td>43</td>
</tr>
<tr>
<td>25</td>
<td>45</td>
</tr>
</tbody>
</table>
Schall\textsuperscript{21} used calorimetry to obtain tetragonal phase diagram information for lysozyme in 50 mM acetate buffer at pH = 4.5. Crystal enthalpies were found for 3% and 5% NaCl concentrations. Schall reports significantly lower enthalpy than this work or Pusey\textsuperscript{24}, which might be attributed to the technique or the lower ionic strength of the buffer.

<table>
<thead>
<tr>
<th>Source</th>
<th>pH</th>
<th>NaCl</th>
<th>C\textsubscript{1}</th>
<th>$\alpha$</th>
<th>$\Delta$H (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apgar</td>
<td>4.6</td>
<td>4%</td>
<td>tetra</td>
<td>5.16</td>
<td>-0.139</td>
</tr>
<tr>
<td>Apgar</td>
<td>5.6</td>
<td>4%</td>
<td>tetra</td>
<td>4.96</td>
<td>-0.146</td>
</tr>
<tr>
<td>Pusey</td>
<td>4.0</td>
<td>4%</td>
<td>tetra</td>
<td>6.73</td>
<td>-0.111</td>
</tr>
<tr>
<td>Schall</td>
<td>5.2</td>
<td>3%</td>
<td>tetra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schall</td>
<td>4.6</td>
<td>5%</td>
<td>tetra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apgar</td>
<td>5.6</td>
<td>4%</td>
<td>ortho</td>
<td>2.51</td>
<td>-0.052</td>
</tr>
<tr>
<td>Pusey</td>
<td>4.0</td>
<td>4%</td>
<td>ortho</td>
<td>8.79</td>
<td>-0.052</td>
</tr>
</tbody>
</table>

3.4. Liquid-Liquid Phase Separation

Liquid-liquid phase separation was induced as described in the methods section above for a range of protein concentrations. The phase boundary plotted
in Figure 35 resembles a portion of the theoretically predicted coexistence curve\textsuperscript{3,23} in Figure 3. Attempts to measure the liquid-liquid (G+L) phase at high concentrations were hampered by the proclivity to nucleate crystals. The few data points above 200 mg/ml were successful only because the trials were performed quickly after sample preparation before the onset of crystals. Muschol\textsuperscript{22} reports that the liquid-liquid phase will reach a maxima at a critical concentration (~255±30 mg/ml), and that this curve follows a mathematical expression for the bimodal of critical phenomena. It is not possible to fit the data in this thesis to a mathematical expression because there are insufficient measurement points near or above the critical concentration.

The liquid-liquid phase measurements at pH 5.6 followed a similar curve, although it appears translated 5-10 °C higher in temperature (Figure 35). The trend to shift the coexistence curve upward with increased pH agrees with simulations\textsuperscript{23}. At pH 5.6, the proclivity for crystal growth was very strong, and it was not possible to measure the liquid-liquid phase change above 150 mg/ml.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{lysozyme_liquid_liquid_phase.png}
\caption{Liquid-Liquid phase clearing temperatures for lysozyme in 4\% NaCl solution at 4.6 and 5.6 pH}
\end{figure}
3.5. **Hysteresis in Liquid-Liquid Phase Separation**

Using the data recorded by the thermocouple probe, temperature was plotted against scattering intensity as shown in Figure 36 below. The temperature required to induce separation (clouding) is several degrees lower than the temperature for clearing - hysteresis. The same hysteresis can be repeated when temperature cycles are repeated.

![Intensity plotted against temperature showing hysteresis](image)

*Figure 36 - Intensity plotted against temperature that clearly shows hysteresis of liquid-liquid phase separation in lysozyme solution (Clys = 42mg/ml, 4% NaCl, pH = 5.6). The trial began at 30°C and was slowly cooled until it clouded at 8°C. As the sample was rewarmed, it cleared after temperature rose above 11°C. (Source: trial #539)*

Using the analysis employed by Asherie\(^5\), a series of clouding temperatures are subtracted from the clearing temperatures and are plotted in Figure 37 below. The temperature difference between \(T_{\text{clear}}\) and \(T_{\text{cloud}}\) decreases as the solution concentration approaches the critical concentration\(^5\). An exponential fit to these points is extrapolated to the \(T=0\) axis which can be used to estimate critical concentration. Using this approach, the critical point (CP in Figure 3) is estimated to be \(C_{\text{crit}}=219\) mg/ml, approximately correct\(^22,24\).
Figure 37 - Temperature difference as measured by thermocouple probe between clouding and clearing, (4% NaCl, pH = 5.6). The solid line is a best fit: 
$T = -2.27 \ln(C) + 12.25$ which intersects with the $T=0$ axis at 219 mg/ml.
4. CONCLUSION

The phase diagram of lysozyme can be traced out using a conceptually straightforward light scattering arrangement. When the data include an adequate range of concentrations, it is possible to fit the data to the van’t Hoff expression and fitting parameters yield the enthalpy of crystallization. The enthalpy of crystallization of tetragonal crystals does not appear to be effected by pH and the solubility curve is unchanged. However, at pH = 5.6 orthorhombic crystals can also occur with significantly different solubility and enthalpy.

There were not enough liquid-liquid phase separation data points to perform a fit to the theoretically expected coexistence curve. At pH=5.6, liquid-liquid phase separation occurred at higher temperatures which is expected because of the reduced net charge on Lysozyme. There were no obvious indications that changes in crystal structure correlate to changes in the coexistence curve for liquid-liquid phase separation. A discontinuity in the coexistence curve might occur at the concentration of equivalent solubility (ES in Figure 32) which is too low in this study to make a determination.

The pronounced effect of the stirring rod on crystal nucleation rates is highly intriguing. Investigation of this mechanism, however, is beyond the scope of this thesis.
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