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Cloud-point temperature and liquid–liquid phase separation of supersaturated lysozyme solution

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Abstract

The detailed understanding of the structure of biological macromolecules reveals their functions, and is thus important in the design of new medicines and for engineering molecules with improved properties for industrial applications. Although techniques used for protein crystallization have been progressing greatly, protein crystallization may still be considered an art rather than a science, and successful crystallization remains largely empirical and operator-dependent. In this work, a microcalorimetric technique has been utilized to investigate liquid–liquid phase separation through measuring cloud-point temperature T^{cloud} for supersaturated lysozyme solution. The effects of ionic strength and glycerol on the cloud-point temperature are studied in detail. Over the entire range of salt concentrations studied, the cloud-point temperature increases monotonically with the concentration of sodium chloride. When glycerol is added as additive, the solubility of lysozyme is increased, whereas the cloud-point temperature is decreased.

Keywords: Biocrystallization; Microcalorimetry; Cloud-point temperature; Liquid–liquid phase separation

1. Introduction

Knowledge of detailed protein structure is essential for protein engineering and the design of pharmaceuticals. Production of high-quality protein crystals is required for molecular structure determination by X-ray crystallography. Although considerable effort has been made in recent years, obtaining such crystals is still difficult in general, and predicting the solution conditions where proteins successfully crystallize remains a significant obstacle in the advancement of structural molecular biology [1].

The parameters affecting protein crystallization are typically reagent concentration, pH, temperature, additive, etc. A phase diagram can provide the method for quantifying the influence of solution parameters on the production of crystals [2,3]. To characterize protein crystallization, it is necessary to first obtain detailed information on protein solution phase behavior and phase diagram. Recently physics shows that there is a direct relationship between colloidal interaction energy

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and phase diagram. Gast and Lekkerkerker [4,5] have indicated that the range of attraction between colloid particles has a significant effect on the qualitative features of phase diagram. A similar relationship should hold for biomacromolecules, i.e. the corresponding interaction potentials govern the macromolecular distribution in solution, the shape of the phase diagram and the crystallization process [6]. Many macromolecular crystallizations appear to be driven by the strength of the attractive interactions, and occur in, or close to, attractive regimes [7,8].

Recent intensive investigation has revealed that protein or colloidal solution possesses a peculiar phase diagram, i.e. liquid–liquid phase separation and sol–gel transition exists in general in addition to crystallization [9,10]. The potential responsible for the liquid–liquid phase separation is a rather short range, possibly van der Waals, attractive potential [11,12]. The measurement of cloud-point temperature T^{cloud} can provide useful information on the net attractive interaction between protein molecules, namely, the higher the cloud-point temperature, the greater the net attractive interaction. Herein Taratuta et al. [13] studied the effects of salts and pH on the cloud-point temperature of lysozyme. Broide et al. [14] subsequently measured the cloud-point temperature and crystallization temperature for lysozyme as a function of salt type and concentration. From these works the cloud-point temperature was found to be typically 15–45 °C below the crystallization temperature. Furthermore, Muschol and Rosenberger [15] determined the metastable coexistence curves for lysozyme through cloud-point measurements, and suggested a systematic approach to promote protein crystallization. In general, an effective way to determine the strength of protein interactions is to study temperature-induced phase transitions that occur in concentrated protein solutions.

Liquid–liquid phase separation can be divided into two stages [11]: (1) the local separation stage at which the separation proceeds in small regions and local equilibrium is achieved rapidly; and (2) the coarsening stage at which condensation of these small domains proceeds slowly to reduce the loss of interface free energy [16]. The coexisting liquid phases both remain supersaturated but differ widely in protein concentration.

The effect of a metastable liquid–liquid phase separation on crystallization remains ambiguous [17]. Molecular dynamics simulations and analytical theory predict that the phase separation will affect the kinetics and the mechanisms of protein crystal nucleation [18]. tenWolde and Frenkel [19] have demonstrated that the free energy barrier for crystal nucleation is remarkably reduced at the critical point of liquid–liquid phase separation, thus in general, after liquid–liquid phase separation, crystallization occurs much more rapidly than in the initial solution, which is typically too rapid for the growth of single crystal with low defect densities [15]. The determination of the location of liquid–liquid phase separation curve is thus crucial for efficiently identifying the optimum solution conditions for growing protein crystals.

Microcalorimetry has the potential to be a useful tool for determining: (1) the metastable-labile zone boundary; (2) the temperature-dependence of protein solubility in a given solvent; and (3) the crystal-growth rates as a function of supersaturation [20]. Microcalorimeters can detect a power signal as low as a few microwatts whereas standard calorimeters detect signals in the milliwatt range. Because of this greater sensitivity, samples with small heat effects can be analyzed. In addition, microcalorimetry has the advantage of being fast, non-destructive to the protein and requiring a relatively small amount of material. The present work is concerned with the analysis of the transient heat signal from microcalorimeter to yield liquid–liquid phase separation information for lysozyme solutions at pH 4.8. To further examine the role of salt and additive on interprotein interactions, cloud-point temperature T^{cloud} has been determined experimentally as a function of the concentrations of salt, protein and glycerol.

2. Materials and methods

2.1. Materials

Six times crystallized lysozyme was purchased from Seikagaku Kogyo, and used without further

purification. All other chemicals used were of reagent grade, from Sigma Chemical Co.

2.2. Preparation of solutions

Sodium acetate buffer (0.1 M) at pH 4.8 was prepared with ultrafiltered, deionized water. Sodium azide, at a concentration of 0.05% (w/v), was added to the buffer solution as an antimicrobial agent. Protein stock solution was prepared by dissolving protein powder into buffer. To remove undissolved particles, the solution was centrifuged in a Sigma centrifuge at 12 000 rev./min for 5–10 min, then filtered through 0.22-μm filters (Millex-VV) into a clean sample vial and stored at 4 °C for further experiments. The concentration of protein solution was determined by measuring the absorbance at 280 nm of UV spectroscopy (Shimadzu UV-2550), with an extinction coefficient of 2.64 ml/(mg cm) [21]. Precipitant stock solution was prepared by dissolving the required amount of sodium chloride together with additive glycerol into buffer. The pH of solutions was measured by a digital pH meter (Mettler Toledo 320) and adjusted by the addition of small volumes of NaOH or HAc solution.

2.3. Measurement of solubility

Solubility of lysozyme at various temperatures and precipitant/additive concentrations was measured at pH 4.8 in 0.1 M acetate buffer. Solid–liquid equilibrium was approached through both crystallization and dissolution. Dissolving lasted 3 days, while the period of crystallization was over 2 weeks. The supernatant in equilibrium with a macroscopically observable solid was then filtered through 0.1-μm filters (Millex-VV). The concentration of diluted supernatant was determined spectroscopically and verified by refractive meter (Krüss) until refractive index remained unchanged at equilibrium state. Solubility of each sample was measured in duplicate.

2.4. Differential scanning microcalorimetry

Calorimetric experiments were performed with a micro-differential scanning calorimeter with an ultra sensitivity, micro-DSC III, from Setaram SA, France. The micro-DSC recorded heat flow in microwatts vs. temperature, thus can detect the heat associated with phase transition during a temperature scan. The sample made up of equal volumes of protein solution and precipitant solution was filtered through 0.1-μm filters to remove dust particles further. To remove the dissolved air, the sample was placed under vacuum for 3 min while stirring at 500 rev./min by a magnetic stirrer.

The degassed sample was placed into the sample cell of 1.0 ml, and a same concentration NaCl solution was placed into the reference cell. The solutions in the micro-DSC were then cooled at the rate of 0.2 °C/min.

After every run, the cells were cleaned by sonicating for 10–15 min in several solutions in the following order: deionized water, methanol, ethanol, acetone, 1 M KOH and finally copious amounts of deionized water. This protocol ensured that lysozyme was completely removed from the cells. The cells were then placed in a drying oven for several hours. The rubber gaskets were cleaned in a similar manner except acetone and 1 M KOH were omitted and they were allowed to dry at low temperature.

3. Results and discussion

A typical micro-DSC scanning experiment is shown in Fig. 1. The onset of the clouding phenomenon is very dramatic and easily detected. The sharp increase in the heat flow is indicative of a liquid–liquid phase separation process producing a latent heat. This is much consistent with many recent investigations of the liquid–liquid phase separation of lysozyme from solution [22,23]. In fact, such a liquid–liquid phase separation is a phase transition with an associated latent heat of demixing. In this work, the cloud-point temperatures at a variety of lysozyme, NaCl and glycerol concentrations are determined by the micro-DSC at the scan rate of 12 °C/h.

3.1. Effect of protein concentration

In semilogarithmic Fig. 2 we plot the solid–liquid and liquid–liquid phase boundaries for lyso-

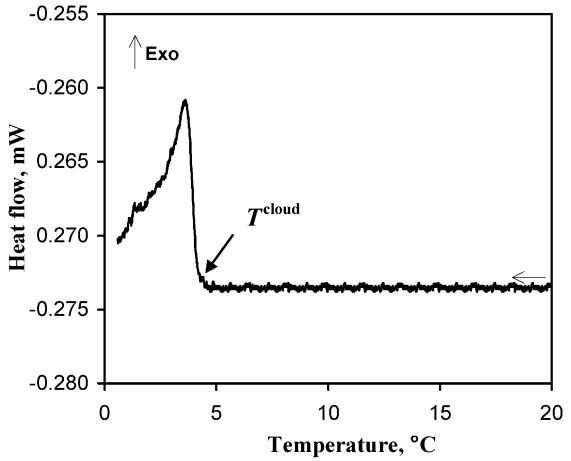

Fig. 1. Heat flow of a typical micro-DSC scan of lysozyme solution, 50 mg/ml, 0.1 M acetate buffer, pH 4.8, 3% NaCl. The scan rate 12 °C/h is chosen referenced to the experimental results of Darcy and Wiencek [23]. Note the large deflection in the curve at approximately 4.3 °C indicating a latent heat resulting from demixing (i.e. liquid–liquid phase separation) process.

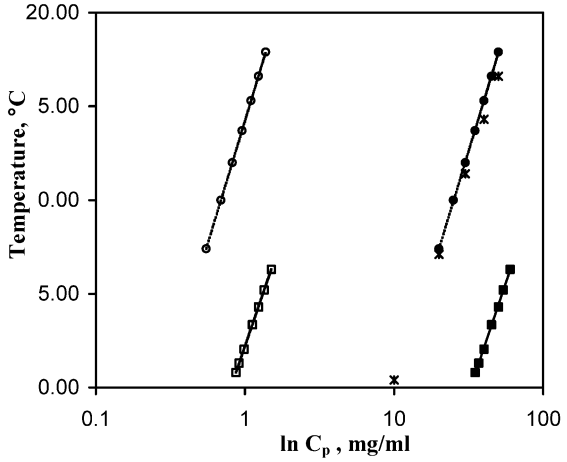

Fig. 2. Cloud-point temperature and solubility determination for lysozyme in 0.1 M acetate buffer, pH 4.8: solubility (5% NaCl) (○); T^{cloud} (5% NaCl, this work) (●); T^{cloud} (5% NaCl, the work of Darcy and Wiencek [23]) (*); solubility (3% NaCl) (□); T^{cloud} (3% NaCl) (■).

zyme in 0.1 M acetate buffer, pH 4.8, for a range of protein concentrations. It is worth noting that, at 5% NaCl, our experimental data of T^{cloud} from micro-DSC are quite consistent with those from laser light scattering and DSC by Darcy and Wiencek [23], with difference averaging at approximately 0.8 °C. This figure demonstrates that liquid–liquid phase boundary is far below solid–liquid phase boundary, which implies that the liquid–liquid phase separation normally takes place in a highly metastable solution. In addition, cloud-point temperature T^{cloud} increases with the concentration of protein.

3.2. Effect of salt concentration

Fig. 3 shows how cloud-point temperature changes as the concentration of NaCl is varied from 2.5 to 7% (w/v). The buffer is 0.1 M acetate (pH 4.8); the protein concentration is fixed at 50 mg/ml. Over the entire range of salt concentrations studied, the cloud-point temperature strongly depends on the ionic strength and increases monotonically with the concentration of NaCl.

Crystallization is driven by the difference in chemical potential of the solute in solution and in the crystal. The driving force can be simplified as [24]

$$\phi = -\Delta\mu = kT \ln(C/C_{eq}) \quad (1)$$

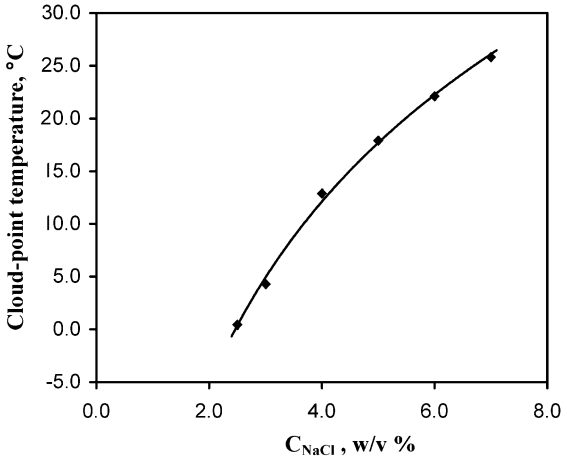

Fig. 3. Cloud-point temperature determination for lysozyme as a function of the concentration of sodium chloride, 50 mg/ml, 0.1 M acetate buffer, pH 4.8.

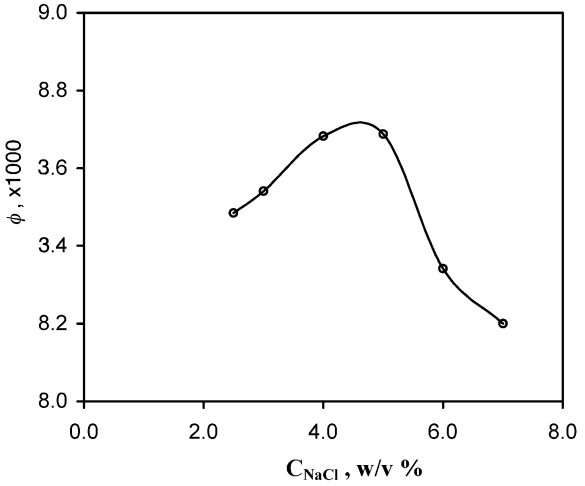

Fig. 4. The driving force required by liquid–liquid phase separation as a function of the concentration of sodium chloride, 50 mg/ml lysozyme solution, 0.1 M acetate buffer, pH 4.8.

In the same way, we plot the driving force, ϕ, required by liquid–liquid phase separation as a function of the concentration of sodium chloride in Fig. 4. At the moderate concentration of sodium chloride, the driving force required by liquid–liquid phase separation is higher than that at low or high salt concentration.

As shown in Fig. 3, with NaCl concentration increasing, the cloud-point temperature increases, which is in accord with the results of Broide et al. [14] and Grigsby et al. [25]. It is known that protein interaction is the sum of different potentials like electrostatic, van der Waals, hydrophobic, hydration, etc. The liquid–liquid phase separation is driven by a net attraction between protein molecules, and the stronger the attraction, the higher the cloud-point temperature. Ionic strength is found to have an effect on the intermolecular forces: attractions increase with ionic strength, solubility decreases with ionic strength, resulting in the cloud-point temperature increases with ionic strength.

It is worth noting that, the effect of ionic strength on cloud-point temperature depends strongly on the specific nature of the ions [13]. Kosmotropic ions bind adjacent water molecules more strongly than water binds itself. When a kosmotropic ion is introduced into water, the entropy of the system decreases due to increased water structuring around the ion. In contrast, chaotropes bind adjacent water molecules less strongly than water binds itself. When a chaotrope is introduced into water, the entropy of the system increases because the water structuring around the ion is less than that in salt-free water. This classification is related to the size and charge of the ion. At high salt concentration (>0.3 M), the specific nature of the ions is much more important [25].

The charges on a protein are due to discrete positively and negatively charged surface groups. In lysozyme, the average distance between these charges is approximately 10 Å [26]. As to the salt NaCl used as precipitant, Na^+ is weakly kosmotropic and Cl^- is weakly chaotropic [27]. At low NaCl concentrations, as the concentration of NaCl increases, the repulsive electrostatic charge–charge interactions between protein molecules decrease because of screening, resulting in the increase of cloud-point temperature. While at high NaCl concentrations, protein molecules experience an attraction, in which differences can be attributed to repulsive hydration forces [14,25]. That is, as the ionic strength increases, repulsive electrostatic or hydration forces decrease, protein molecules appear more and more attractive, leading to higher cloud-point temperature. At various salt concentrations, the predominant potentials reflecting the driving force for liquid–liquid phase separation are different.

Fig. 4 shows that the driving force, ϕ, is parabolic with ionic strength, while Grigsby et al. [25] have reported that ϕ/kT is linear with ionic strength for monovalent salts. The possible reasons for that difference include, their model is based on a fixed protein concentration of 87 mg/ml, which is higher than that used in our study, yet ϕ/kT is probably dependent on protein concentration, besides the solutions at high protein and salt concentrations are far from ideal solutions.

3.3. Effect of glycerol

Fig. 5 compares cloud-point temperature data for 50 mg/ml lysozyme solutions in absence of glycerol and in presence of 5% glycerol, respec-

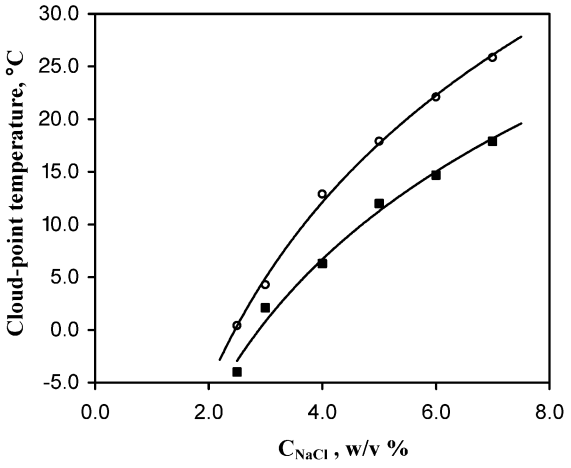

Fig. 5. Comparison of cloud-point temperatures for lysozyme at different glycerol concentrations as a function of the concentration of sodium chloride, 50 mg/ml, 0.1 M acetate buffer, pH 4.8: 0% glycerol (○); 5% glycerol (■).

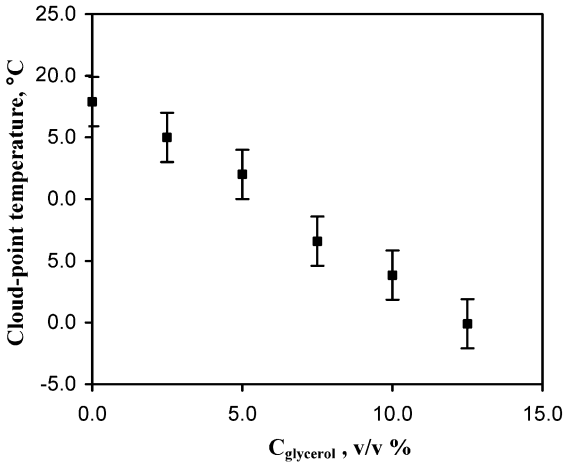

Fig. 6. Cloud-point temperatures for lysozyme at different glycerol concentrations, 50 mg/ml lysozyme, 5% NaCl, 0.1 M acetate buffer, pH 4.8.

tively. Fig. 6 shows the cloud-point temperature as a function of the concentration of glycerol. The cloud-point temperature is decreased as the addition of glycerol.

In semilogarithmic Fig. 7 we plot the solid–liquid and liquid–liquid phase boundaries at different glycerol concentrations for lysozyme in 0.1 M acetate buffer, 5% NaCl, pH 4.8, for a range of protein concentration. This figure demonstrates that liquid–liquid and solid–liquid phase boundaries in the presence of glycerol are below those in absence of glycerol, and the region for growing crystals is narrowed when glycerol is added.

Glycerol has the property of stabilizing protein structure. As a result, if crystallization occurs over a long period of time, glycerol is a useful candidate to be part of the crystallization solvent and is often included for this purpose [28]. In addition, glycerol is found to have an effect on the intermolecular forces: repulsions increase with glycerol concentration [29]. Our experiment results of solubility and cloud-point temperature can also confirm the finding.

The increased repulsions induced by glycerol can be explained by a number of possible mechanisms, all of which require small changes in the protein or the solvent in its immediate vicinity. The addition of glycerol decreases the volume of protein core [30], increases hydration and the size of hydration layer at the particle surface [31,32]. In this work, we confirm that glycerol shifts the solid–liquid and liquid–liquid phase boundaries. The effect of glycerol on the phase diagram strong-

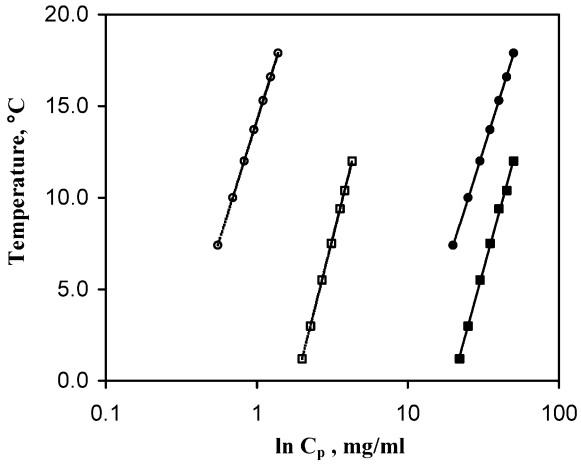

Fig. 7. Cloud-point temperature and solubility determination for lysozyme at different concentrations of glycerol in 0.1 M acetate buffer, 5% NaCl, pH 4.8: solubility (0% glycerol) (○); T^{cloud} (0% glycerol) (●); solubility (5% glycerol) (□); T^{cloud} (5% glycerol) (■).

ly depends on its concentration and this can provide opportunities for further tuning of nucleation rates.

4. Conclusions

Growing evidence suggests protein crystallization can be understood in terms of an order/disorder phase transition between weakly attractive particles. Control of these attractions is thus key to growing crystals. The study of phase transitions in concentrated protein solutions provides one with a simple means of assessing the effect of solution conditions on the strength of protein interactions. The cloud-point temperature and solubility data presented in this paper demonstrate that salt and glycerol have remarkable effects on phase transitions. The solid–liquid and liquid–liquid boundaries can be shifted to higher or lower temperatures by varying ionic strength or adding additives. Our investigation provides further information upon the role of glycerol used in protein crystallization. Glycerol can increase the solubility, and decrease the cloud-point temperature, which is of benefit to tuning nucleation and crystal growth. In continuing studies, we will explore the effects of other kinds of additives like nonionic polymers on phase transitions and nucleation rates. Much more theoretical work will be done to fully interpret our experimental results.

Acknowledgments

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