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Protein crystallization and phase diagrams

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Abstract

The phase diagram is a map which represents the state of a material (e.g., solid and liquid) as a function of the ambient conditions (e.g., temperature and concentration). It is therefore a useful tool in processing many different classes of materials. In this article, methods to determine the phase diagram of an aqueous solution of a globular protein are described, focusing on the solid (crystal) and condensed liquid states. The use of the information contained in the phase diagram for protein crystallization is also discussed.

Keywords: Protein crystals; Liquid–liquid phase separation; Phase diagrams; Cloud-point method; Solubility

1. Introduction

A protein will stay in solution only up to a certain concentration. Once this limiting concentration is reached, the solution will no longer remain homogeneous, but a new state or phase will appear. This phenomenon forms the basis of all protein crystallization experiments. By changing the solution conditions, the crystallographer tries to exceed the solubility limit of the protein so as to produce crystals [1].

This plan rarely runs smoothly. After changing the solution conditions, one of several difficulties is usually encountered: (i) nothing happens, i.e., the protein solution remains homogeneous; (ii) a new phase appears, but it is not a crystal. Instead, it is an aggregate or a liquid; or (iii) crystals do form, but they are unsuitable for structure determination because they give a poor X-ray diffraction pattern.

It is often possible to overcome these difficulties by trial and error—repeated crystallization attempts with many different conditions—but this strategy does not always work. Even when it is successful, the lessons learned cannot be easily generalized; the conditions which work with one protein are not necessarily optimal for a different protein.

The problems associated with producing protein crystals have stimulated fundamental research on protein crystallization. An important tool in this work is the phase diagram. A complete phase diagram shows the state of a material as a function of all of the relevant variables of the system [2]. For a protein solution, these variables are the concentration of the protein, the temperature and the characteristics of the solvent (e.g., pH, ionic strength and the concentration and identity of the buffer and any additives). The most common form of the phase diagram for proteins is two-dimensional and usually displays the concentration of protein as a function of one parameter, with all other parameters held constant [3]. Three-dimensional diagrams (two dependent parameters) have also been reported [4] and a few more complex ones have been determined as well [5].

In this article I describe methods for measuring the phase diagram of an aqueous solution of a globular protein, focusing on the crystalline (Section 2) and liquid phases (Section 3) which form. In Section 4 I describe

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how the phase diagram provides information which can be useful for protein crystallization. I briefly discuss protein aggregation in Section 5 and offer concluding remarks in Section 6.

2. Solubility curve

2.1. Definitions

When a protein crystal (Fig. 1A) is placed in a solvent which is free of protein, the crystals will begin to dissolve. If the volume of solvent is small enough, the crystal will not dissolve completely; it will stop dissolving when the concentration of protein in solution reaches a specific value. At this concentration, the crystal loses protein molecules at the same rate at which protein molecules rejoin the crystal—the system is said to be at equilibrium. The concentration of proteins in the solution at equilibrium is the solubility.

The solubility of a protein varies with the solution conditions. A schematic diagram of a solubility curve, illustrating how the solubility varies with the concentration of a precipitant (e.g., polyethylene glycol (PEG) or a salt), is shown in Fig. 1B. Crystals dissolve in the undersaturated region—where the concentration is below the protein solubility—and grow in the supersaturated region. The three subdivisions of the supersaturated region (the metastable, labile, and precipitation zones) will be discussed in Section 2.3.

2.2. Methods

A straightforward procedure for measuring protein solubility is given below. Variants and alternatives are described in the literature [6–10].

1. Place some crystals in a protein-free solution under the conditions of interest.
2. Stir the solution continuously to ensure thorough mixing of the components.
3. As the crystals melt, monitor regularly the concentration of protein in the solution. This is usually done by removing aliquots and measuring the absorbance at 280 nm.
4. When the concentration of the solution reaches a constant value, assume the system has reached equilibrium. Therefore, the final concentration is the solubility.

It typically takes a couple of days to about a week to measure a point on the solubility curve. This method has been used to measure the solubility of more than 40 proteins including lysozyme [11–14], several γ crystallins [15,16], hemoglobin [17], β lactoglobulins A and B [18], chymotrypsinogen A [19], and ovalbumin [20]. One difficulty is ensuring that the initial proportion of crystals to solution is correct: too few crystals, and they will all melt before equilibrium is established; too little solution, and the aliquots removed will exhaust the solution before equilibrium is reached. Since the crystals are usually the more precious commodity—once they have melted, it is hard to get them back— it is better to be conservative with the amount of solution used and to remove the smallest aliquots possible for concentration measurements.

It is possible to determine the solubility of a protein by starting with a supersaturated solution instead of an undersaturated one [13]. In this case, the solution would reach equilibrium through the growth of the crystals (and possibly the formation of new crystals) rather than melting. Regardless of the approach, the protein concentration in the supernatant should converge to the same value (Fig. 2A). It is, however, more difficult to establish equilibrium when starting with supersaturated solutions (Fig. 2B). The reason is that as the crystals grow their surface is poisoned by impurities or improperly oriented proteins [21,22]. This poisoning eventually halts any further growth before a true equilibrium between the crystals and the solution is established. If clean crystal surfaces are exposed (for example, by shaking the sample), crystal growth can continue, and eventually the measurements from the undersaturated and supersaturated

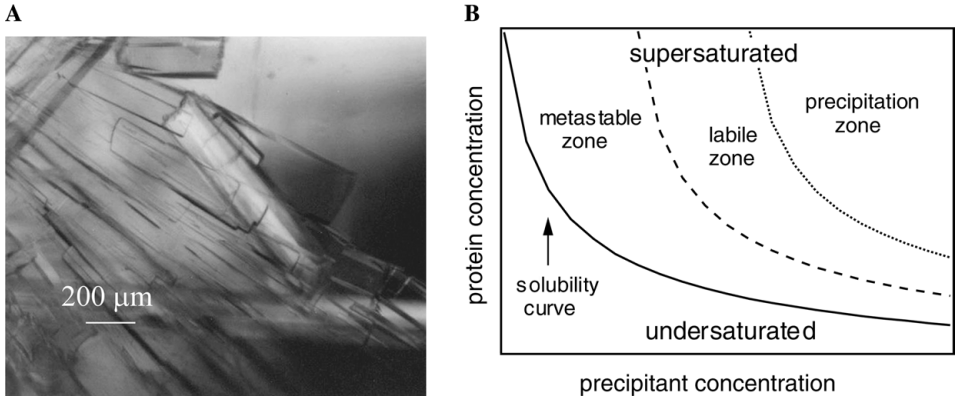

Fig. 1. (A) Crystals of wild-type bovine γB crystallin. (B) A schematic phase diagram showing the solubility of a protein in solution as a function of the concentration of the precipitant present [1].

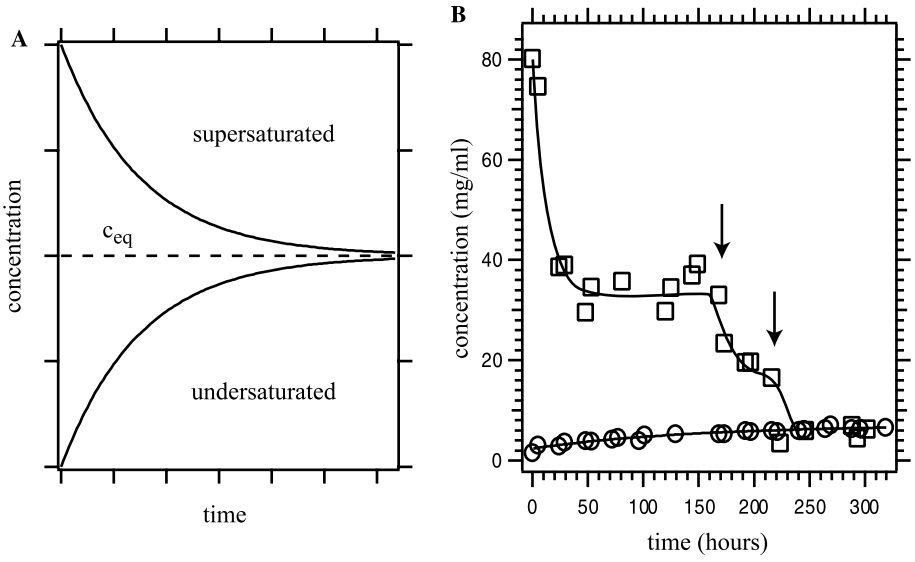

Fig. 2. Solubility measurements. (A) The idealized approach to equilibrium in supersaturated and undersaturated solutions. c_{eq} is the equilibrium concentration (the solubility) [6]. (B) The concentration of protein in solution for a sample of lysozyme crystals as a function of time. The squares correspond to a sample which is initially supersaturated; the circles are for a sample which is initially undersaturated. The samples are at 4 °C and the solution also contains 0.15 M sodium chloride with no buffer. The arrows indicate the points at which the supersaturated sample was vigorously shaken with a touch-mixer (vortexer) to renew crystal growth. The lines are guides to the eye.

solutions do converge to the same value. Nevertheless, it is easy to mistake one of the plateaus of arrested crystal growth in the supersaturated solution for the equilibrium solubility. Therefore, if time and amounts of material are limited, the solubility measurements should be made from undersaturated solutions.

2.3. Nucleation

How do you produce the crystals needed to measure the solubility curve? In principle, crystals will form in any protein solution that is supersaturated i.e., when the protein concentration exceeds the solubility. In practice, crystals hardly ever form unless the concentration exceeds the solubility by a factor of at least three [23]. The large supersaturation is required to overcome the activation energy barrier which exists when forming the crystal. This barrier represents the free energy required to create the small microscopic cluster of proteins—known as a nucleus—from which the crystal will eventually grow [24].

Since there is an energy barrier, nucleation (the process of forming a nucleus) takes time. If the supersaturation is too small, the nucleation rate will be so slow that no crystals form in any reasonable amount of time. The corresponding area of the phase diagram is known as the "metastable zone." In the "labile" or "crystallization" zone, the supersaturation is large enough that spontaneous nucleation is observable. If the supersaturation is too large, then disordered structures, such as aggregates or precipitates, may form. The "precipitation zone" is unfavorable for crystal formation, because the aggregates and precipitates form faster than the crystals.

The three zones are illustrated schematically in Fig. 1B. Since these zones are related to kinetic phenomena, the boundaries between the zones are not well-defined (this in contrast to the solubility line which is unambiguous description of the equilibrium between solution and crystal). Even though the division in zones is qualitative, the different behaviors serve as guide when searching for the appropriate conditions to produce crystals [25,26].

3. Liquid–liquid phase separation

3.1. Definitions

When a precipitant is added to a protein solution to help produce crystals, liquid drops sometimes form. These drops—also referred to as "oils" or "coacervates"—can occasionally be observed by simply changing the temperature, pH, or other solution condition (Fig. 3A). Such drops generally contain a high concentration of protein. Under the influence of gravity, the drops may separate from the rest of the solution. Eventually, two liquid phases will form (Fig. 3B), and the concentrations of the various components of the original solution will be different in the two phases.

This phenomenon is known as liquid–liquid phase separation (LLPS). It occurs with mixtures of small molecules (e.g., aniline and cyclohexane) and is analogous to water condensing from steam [28]. LLPS is important

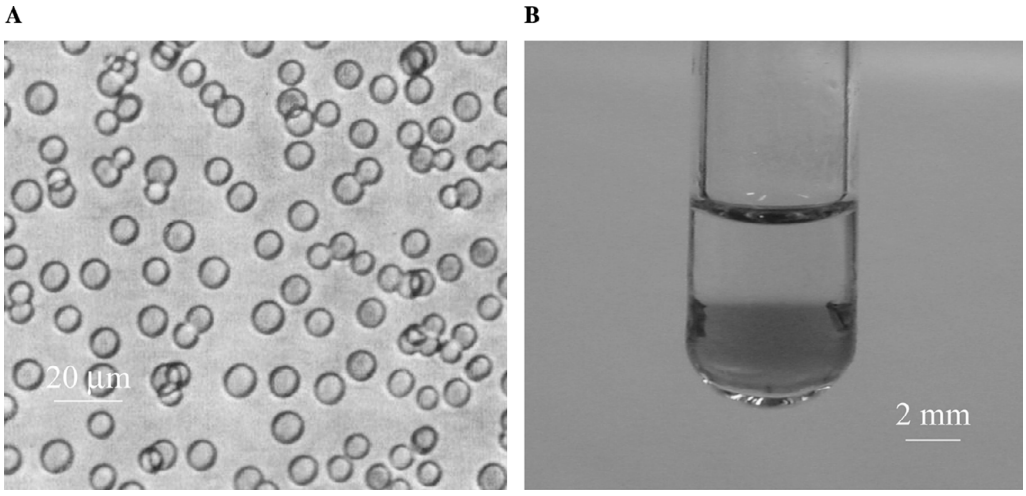

Fig. 3. (A) Liquid–liquid phase separation of bovine γE crystallin in sodium phosphate (pH 7.1; ionic strength = 0.24 M). The picture was taken after the sample was at 22 °C for a few minutes [27]. (B) Liquid–liquid phase separation of thaumatin. The initial protein concentration was 229 mg/ml. The solution also contained 1.9% (w/w) PEG (average molecular weight 1450) and 0.1 M sodium phosphate buffer (pH 9; ionic strength = 0.24 M). The picture was taken after the sample was at −9.5 °C for 42 h. The protein concentrations in the two phases are 84 mg/ml (A) and 383 mg/ml (B).

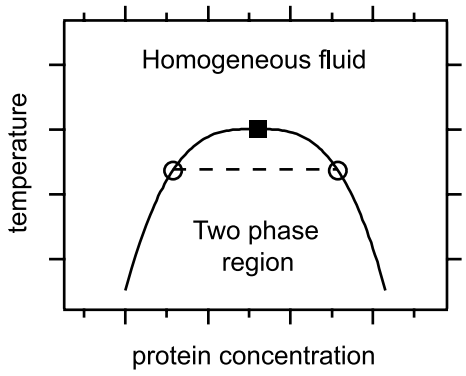

Fig. 4. A schematic liquid–liquid coexistence curve [30]. The circles correspond to two different concentrations of protein which are in equilibrium with each other. The solid square is the critical point. In this example the protein is the only component which partitions significantly between the two phases, so the critical point is at a maximum on the coexistence curve.

for protein crystallization because crystals sometimes form in the protein-rich phase [29].

Just like crystallization, LLPS is also sensitive to the solution conditions. A schematic diagram of a liquid–liquid coexistence curve, illustrating how the coexisting concentrations of protein vary with temperature is shown in Fig. 4. The coexistence curve is the boundary between the region where the protein solution remains homogeneous and the region in which droplets form. Unlike crystallization, in which there are always two distinct phases, in LLPS there is a critical point at which the two phases become identical. Beyond this point, LLPS is not possible.

3.2. Methods

When the conditions are reached so that a protein solution begins to undergo liquid–liquid phase separation, the many, small drops which form scatter light. The solution will therefore no longer be transparent, but becomes turbid. The drop in the light intensity transmitted through the solution can be used to determine the coexistence curve, and a detailed description of the "cloud-point method" has been published [30]. This method is summarized below.

1. Shine a light upon a solution of known protein concentration as it is cooled. The temperature at which transmitted intensity falls to half of its initial value is defined as the clouding temperature T_{cloud}.
2. Once the transmitted intensity reaches zero, warm the solution. The temperature at which the transmitted intensity returns to half of its initial value is defined as the clearing temperature T_{clear}.
3. To calculate the phase separation temperature T_{ph}, take the average of the clouding and clearing temperatures. The coexistence curve (see Fig. 4) represents T_{ph} as a function of protein concentration.

Typical measurements of transmitted intensity versus temperature, taken using the cloud-point method, are shown in Fig. 5. For a solution with a protein concentration which is either much less or much greater than the concentration at the critical point ("the critical concentration"), T_{cloud} lies well below T_{clear} (Fig. 5A). As the concentration of the solution approaches the critical concentration, the difference between T_{cloud} and T_{clear} decreases. At the critical concentration, this difference is zero and the change of transmitted intensity versus temperature is very gradual (Fig. 5B). When making such measurements, it is important to verify that the source of turbidity is indeed liquid droplets and not some other transition, such as the formation of crystals or aggregates. This can be done by looking at the sample under a microscope (Fig. 3A) or by eye (Fig. 3B).

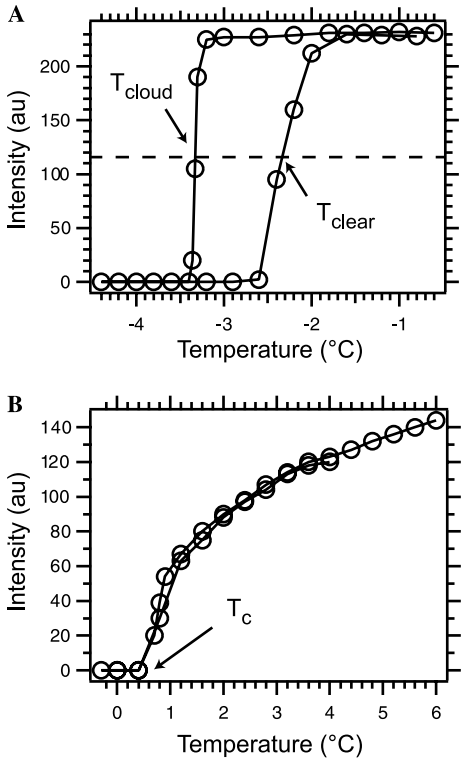

Fig. 5. Cloud-point measurements for the Cys15 to Ser mutant of bovine γB crystallin in a 0.1 M sodium phosphate solution (pH 7.1) with 20 mM dithiothreitol. (A) Sample at low concentration (much less than the critical concentration): $T_{cloud} = -3.3$ °C, $T_{clear} = -2.3$ °C, and $T_{ph} = -2.8$ °C for this sample. The dashed line denotes the intensity which is half of the initial value. (B) Sample at the critical concentration $c_c \approx 300$ mg/ml. The critical temperature is $T_c = 0.4$ °C.

LLPS has been studied for fewer than twenty proteins. Those that have been studied include lysozyme [31,32], the γ crystallins [27,33], 7S and 11S globulins [34], bovine pancreatic trypsin inhibitor [35] and arachin [36]. The number of proteins for which LLPS has been observed is smaller than the number for which solubility data is available. This is partly because LLPS is a metastable transition in protein solutions (see Section 3.3) and therefore it is not always easily seen.

3.3. Crystal nucleation

Although LLPS is analogous to water condensing from steam, there is a significant difference. While liquid water is stable once it has formed, the protein-rich and protein-poor liquid phases are not. The phases may exist for days, even weeks, but LLPS is inherently metastable with respect to crystallization [28]. In other words, the protein-rich liquid phase can convert into crystals [37].

LLPS can promote crystal formation even without the formation of a macroscopic protein-rich phase. For example, several members of the γ crystallins have been crystallized as follows [38]: (i) cool the solution sufficiently so that it becomes turbid and LLPS occurs (the coexistence curve is crossed); (ii) keep the solution at this low temperature for a few minutes; (iii) raise the temperature so that the solution becomes clear again. Crystals then form within a few days. If the coexistence curve is not crossed, crystals either take weeks to form or do not form at all.

The precise mechanism by which LLPS promotes crystal nucleation is still not known. One factor is the high protein concentration which exists in the droplets. This high concentration corresponds to a large supersaturation and so increases the crystal nucleation rate. Another factor may be the wetting of the surface of the crystal by one of the liquid phases [39]. Systematic studies have shown that in the case of lysozyme both factors are important [40].

4. The phase diagram and protein crystallization

The most direct connection between the phase diagram and protein crystallization is through the location of the solubility curve. Crystals can only form in supersaturated solutions, so knowing where the solubility curve is located helps to grow crystals for X-ray structure determination. This may appear to be a specious argument, since crystals are needed to determine the solubility curve in the first place. However, it is rare that the first crystals which are grown are suitable for structural studies. These crystals can be used to determine the solubility curve, which then serves as a guide for refining the conditions to produce better crystals [6].

The phase diagram is also useful because it reveals information about the interactions amongst the components of the solutions. For example, the presence of liquid–liquid phase separation implies that the effective interactions between the proteins are attractive [41,42]; this attraction is a necessary (though not sufficient) condition for crystallization [43]. More quantitative data can be extracted as well: the numerical values of the enthalpies and entropies of the protein in the liquid and solid phases can be determined from the liquid–liquid coexistence curve and the solubility curve, respectively, [16,44,45]. Furthermore, additives change the position of the phase boundaries, providing information on the interactions between the additive and the protein. Also, since the positions of the solubility curve and coexistence curve are related, a shift of one boundary due to an additive implies a shift of the other. Thus, the effectiveness of PEG as a precipitant for crystallization can be gauged from the extent to which it partitions in the two phases of the liquid–liquid phase separation of a protein [46].

Finally, the concept of a phase diagram can help achieve a more ambitious goal: to predict the conditions under which a protein can be crystallized (or at least reduce the number of conditions that initially should be

tried). Predicting crystallization conditions is frequently done for simple fluids [47] and metal alloys [48]. The scheme used to make the predictions is the following: (i) develop a model for the interactions between the particles; (ii) measure some properties of the material; (iii) use these measurements to determine the parameters of the model which characterize the material; and (iv) calculate the phase diagram using the model with the known parameters. The predictions made for simple fluids and metal alloys are fairly successful because the models for the interactions in these systems are well-developed and for each class of materials there is only limited number of possible phase diagrams. For proteins, the models required are more complex and the determination of phase diagrams has begun only relatively recently, so there are opportunities for further work.

More specifically, it is still not known what types of phase diagrams can exist for aqueous protein solutions as very few phase diagrams have been determined. Indeed, there are only two sets of proteins for which complete phase diagrams have been established: lysozyme and the γ crystallins. The phase diagrams of hen egg-white lysozyme [32] and bovine γD crystallin [15,27] are shown in Fig. 6. It has been suggested that since the phase diagrams of these two proteins are very similar, their phase diagrams represent the generic one for aqueous solutions of globular proteins [32]. This suggestion has yet to be verified experimentally. As far as the modeling of interactions is concerned, it has been shown that the phase diagrams of lysozyme and the γ crystallins can be described when the short-range and anisotropic nature of protein interactions is taken into account [44]. When additional experimental data becomes available, these models should be expanded to describe other proteins as well.

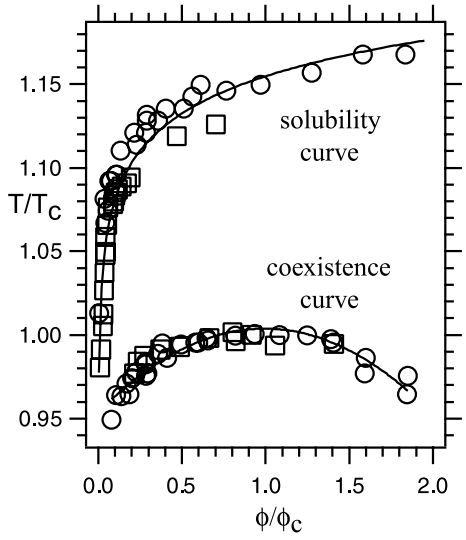

Fig. 6. The phase diagram of lysozyme and bovine γD crystallin. The experimental data for the solubility line and the liquid–liquid coexistence curve are shown for lysozyme (squares) [32] and bovine γD crystallin (circles) [15,27]. To highlight the similarity between the two systems, the results are displayed using the appropriate, dimensionless units [2]: the reduced temperature T/T_c and the reduced volume fraction of protein in solution ϕ/ϕ_c. The volume fraction is given by $\phi = vc$, where v is the specific volume of the protein, and c is the concentration; ϕ_c is the volume fraction corresponding to the critical concentration. The lines are guides to the eye.

5. Aggregation

Aggregation—the assembly of proteins into amorphous clusters—is not a phase transition like crystallization and LLPS. No macroscopic phase forms and therefore there is no aggregation phase boundary. I will discuss it here because aggregation can change the apparent position of the solubility curve and the liquid–liquid coexistence curve, affecting the determination of the phase diagram.

Since aggregation is a kinetic phenomenon, any unusual time-dependence in the measurements of the phase boundaries should be examined carefully. For LLPS, the presence of aggregates changes the temperature at which phase separation happens [33]. Therefore, a drift in T_{cloud} over time for a given sample probably indicates that aggregation is occurring. For solubility measurements, the formation of aggregates alters the concentration of monomers in solution. If the aggregation is irreversible, an equilibrium solubility may never be reached. To check whether aggregation is taking place, techniques such as quasielastic light scattering, high-performance light chromatography and gel-electrophoresis may be used.

Aggregation is commonly observed in crystallization attempts. Unlike LLPS, which is generally a good omen, aggregation is a less favorable sign. Aggregates do occasionally rearrange to form crystals [49], but usually the aggregate is the final state of the system. Ideally, it is best to prevent aggregation from occurring. If the aggregation is the result of some specific reaction (e.g., oxidation of thiol residues leading to the formation disulfide crosslinks between the proteins [16,50]), it may be possible to stop it by chemical means. If the aggregation is non-specific, the simplest remedy is to repeat the experiment at a lower supersaturation, with the intent of moving from the precipitation zone to the labile zone (see Section 2.3).

6. Concluding remarks

In this article I have focused on the phase diagram of a globular protein in an aqueous solution. However, the measurement of phase diagrams can be carried out with other types of proteins as well. In particular, the phase transformations of integral membrane proteins are a focus of intense research since these proteins are very

difficult to crystallize [51]. Finally, phase diagram studies are important not only for protein crystallization, but also for other processes—certain diseases [52], drug delivery [53], microcompartmentation of the cell cytoplasm [54], and industrial separations [55]—in which the collective behavior of proteins is involved.

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