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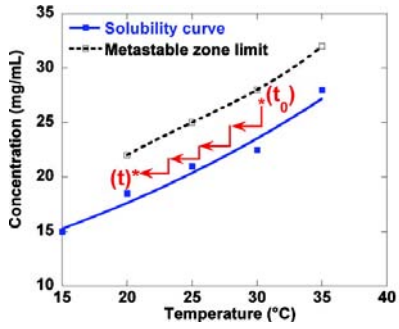

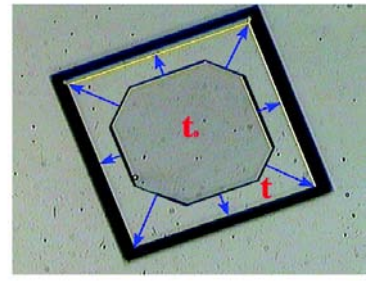

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Using Temperature To Crystallize Proteins: A Mini-Review†

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ABSTRACT: The aim of this overview of the work of Marseille Nanoscience Center is to provide biocrystallographs that intend to tackle crystallization with practical examples of the effect of temperature on protein phase separation and crystallization. Crystallization involves two separate processes: nucleation and growth, which are rarely completely unconnected. Here we give four concrete examples illustrating how temperature can be used to crystallize proteins. We describe the nucleation of a new phase, solid or liquid and the growth and transformation of existing crystals obtained by seeding or primary or secondary nucleation. The advantages of temperature as a crystallization parameter are constant composition, ease of control and monitoring and reversibility.

1. Introduction

The aim of this overview of the work of Marseille Nanoscience Center is to provide biocrystallographs that intend to tackle crystallization with practical examples of the effect of temperature on protein phase separation and crystallization. It is now clearly established that nucleation and growth mechanisms are the same for any crystalline material.[1] The usual approach of solution crystallization is to study the respective influence of temperature, supersaturation, medium (chemical conditions), and hydrodynamics. However, for protein crystallization due to the chemical complexity of solutions, most studies look at solution composition: pH, salt type and concentration, and additives such as polymers or polyols. Although the importance of temperature has long been recognized, not many papers dealing with temperature[2−4] have been published in the field of protein crystallization, and the majority of them focused on crystal quality,[5,6] solubility[7] and nucleation rate[8,9] measurements. The advantages of temperature as a crystallization parameter are constant composition, ease of control and monitoring and reversibility. Supersaturation can be readily controlled (by temperature) because heat diffuses at least 2 orders of magnitude more rapidly than species.[2] Since crystallization involves two separate processes, nucleation and growth, which are rarely completely unconnected, we divide this paper into two parts: crystal growth and phase separation. We describe, first, the growth and transformation of existing crystals obtained by seeding or nucleation[10] and second, the nucleation of a new phase, solid or liquid.

2. Materials and Methods: A Synthesis of Our Experimental Practice

2.1. Materials. Porcine pancreatic α-amylase and bovine pancreatic trypsin inhibitor (BPTI) are used. Porcine pancreatic α-amylase molecular form I (55 kDa, pI = 5.25)[11] is delivered in aqueous solution at pH = 8 (10 mM Tris-HCl) containing 6 mM NaCl, 1 mM $CaCl_2$ and 3 mM NaN_3, in the presence of 0.1 mM phenylmethylsulfonate fluoride: it crystallizes in this medium. For details on purification, crystallization, and solubility of porcine pancreatic α-amylase, see previously published studies.[3] BPTI (6511 Da, pI = 10.5) is supplied as a lyophilized powder by Bayer and used as received. The purity of BPTI is checked by molecular sieving. Proper amounts of BPTI and salt (KSCN and NaBr) are dissolved in pure water (ELGA UHQ reverse osmosis system) to obtain stock solutions needed for crystallization experiments. The different solutions are buffered with 80 mM acetic acid, adjusted to the desired pH, with NaOH (1 M) and filtered through 0.22 μm Millipore filters. The pH is checked with a pH meter Tacussel ISIS 20000 equipped with a micro pH electrode (Radiometer pHC3359-8). α-Amylase and BPTI concentrations are measured by optical density measurements (Biochrom, Libra S22) using an extinction coefficient of 2.5 mL cm^{-1} mg^{-1}[12] and 0.786 cm^{-1} mL mg^{-1}[13] at 280 nm for α-amylase and BPTI, respectively.

2.2. Methods. All crystallization experiments are performed in a glass vessel inserted in a thermostatted cell by Peltier elements under an optical microscope.[14,15] Using this setup, we can perform, monitor and control batch crystallization in volume as low as 15 μL and temperature can be ramped or changed instantaneously from 0 to 80 °C.

3. Results and Discussion: The Lessons to Be Learned

One of the difficulties in designing crystallization experiments is to select the initial conditions, that is, a starting position in the phase diagram; therefore, an important step is the solubility measurement. An alternative is the colloidal approach in which solubilities are qualitatively estimated via the correlation between molecular interactions between macromolecules in solution, solubilities and crystallization conditions,[16−20] thus allowing us to select conditions yielding crystals. In the following, solubilities were obtained by seeding supersaturated solutions with small crystallites by the method previously described by Boistelle et al.[3] For metastable phases, because of the undesired nucleation of the stable phase we used a

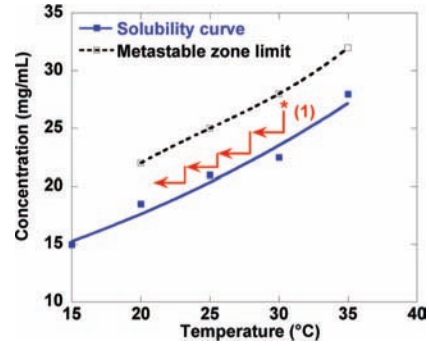

Figure 1. Solubility and metastable zone curves of BPTI in 350 mM KSCN at pH = 4.9 (after ref 12). Point (1) represents the starting point and arrows represent the path in the phase diagram.

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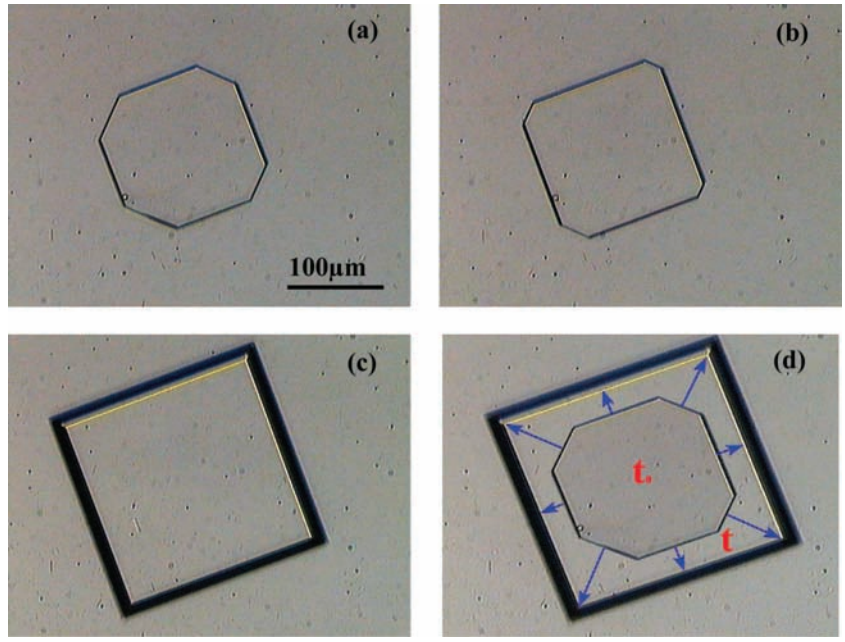

Figure 2. Growth of a BPTI crystal in 350 mM KSCN at pH = 4.9 across the trajectory shown in Figure 1. Panels a−c are frames of a time sequence obtained at different temperatures showing the evolution of the growth form as illustrated in (d), in which arrows indicate the face displacement with time.

bracketing method, measuring the solution concentrations before the beginning of the dissolution and after the complete dissolution of the metastable phase. The solubility of the metastable phase is in between these values.[21]

3.1. Three Examples on Crystal Growth. The principle of the experiments is to grow a seed crystal in the metastable zone in order to avoid nucleation. The supersaturation is low, near the equilibrium state, and kept in the correct range by fine-tuning the temperature (Figure 1). Although this method requires the protein solubility to be sensitive to temperature, the problem can be easily solved. When solubility is not or is little sensitive to temperature, a method consists of increasing protein solubility: this means, for a salting-out effect diminishing the crystallization agent concentration, temperature dependence being more pronounced at low ionic strength than at high ionic strength.[6,13] For salting-in, the reverse applies. We can hypothesize that the same is expected for nonadsorbing polymers, such as PEG. Note that, since crystallization conditions using screening kits are often complicated, a mixture of salts and additives, it is advisable to simplify the medium of crystallization.

3.1.1. Growth Form (Example 1). A crystal is limited by a number of faces. The set of equivalent faces resulting from the crystal symmetry is a form. The morphology of the crystal is represented by all the forms present on a crystal. The concept of morphology does not fully cover the external form of the crystal. This is determined by the habit. The concept of habit includes the notion of face extension. It is important to underline that the growth form of the crystal only includes the faces which have the slowest growth rates. Figure 2a−c represents the growth in the metastable zone of a seeded monoclinic BPTI crystal in KSCN solution. This experiment consists of gradually adjusting the temperature as the growth of the crystal is observed by videomicroscopy. Temperature is set at an initial value, and the crystal is seeded and grown in the metastable zone: when the growth rate is low (for instance, no faces displacement observable by optical microscopy for 1 h) the temperature is decreased and this process is repeated until the crystal reaches the right size for X-ray diffraction, for instance. The path in the phase diagram is shown in Figure 1. From time t_0 to time t, all the faces will have migrated parallel to themselves and crossed distances proportional to their growth rates (vectors in Figure 2d). Obviously, the growth form is different at time t and time t_0. The slowest faces develop at the expense of the fastest faces, which entirely disappear. Moreover, some very slow faces appear because their growth rates are slower than the others. The growth form thus depends on kinetic factors, that is to say, crystallization conditions.

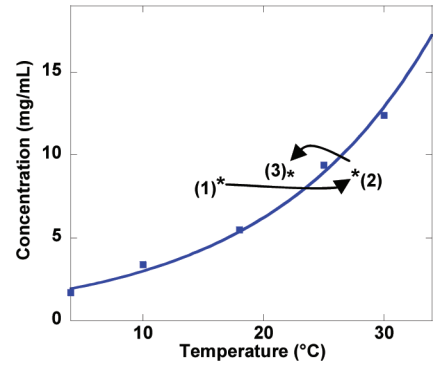

Figure 3. Solubility of B polymorph of α-amylase (after ref 3), points (1), (2), and (3). Arrows represent the path in the phase diagram.

3.1.2. Kinetic Ripening (Example 2). Different empirical approaches have been developed to produce X-ray-quality crystals, based on screening and optimization.[22] Here we use the kinetic ripening method (Figures 3 and 4) in experiments in which the crystal size distribution is wide due to nucleation or seeding (Figure 4a, point (1) in Figure 3). Temperature fluctuations in the neighborhood of the equilibrium temperature induce dissolution of the smallest crystals and growth of the largest. This method is also applicable to precipitates.

Figure 4 presents the complete kinetic ripening process for α-amylase crystals. In the first stage, temperature is increased by a few degrees. Small and large crystals dissolve (Figure 4b, point (2) in Figure 3), but as small crystals have less matter to

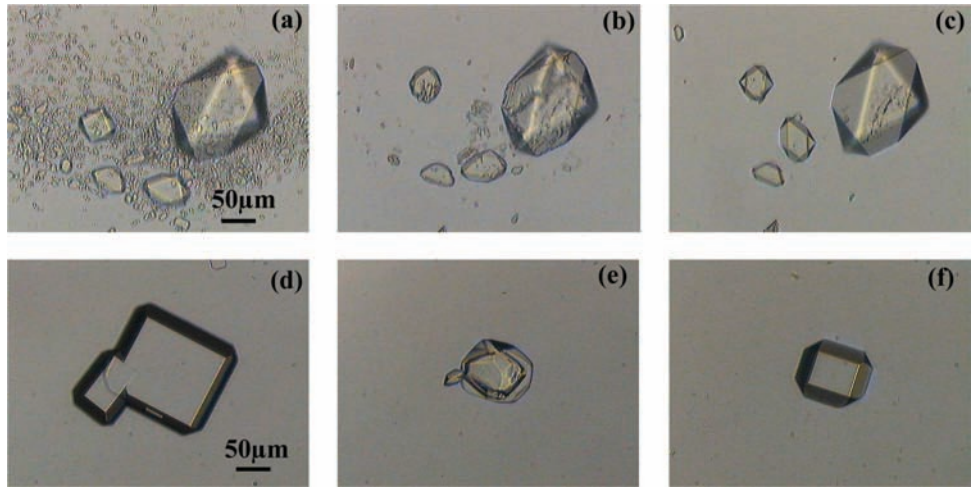

Figure 4. Kinetic ripening of B polymorph of α-amylase crystals shown in (a), by (b) partial dissolution and (c) regrowth. Elimination of a macrodefect observed in (d) by (e) dissolution and (f) growth.

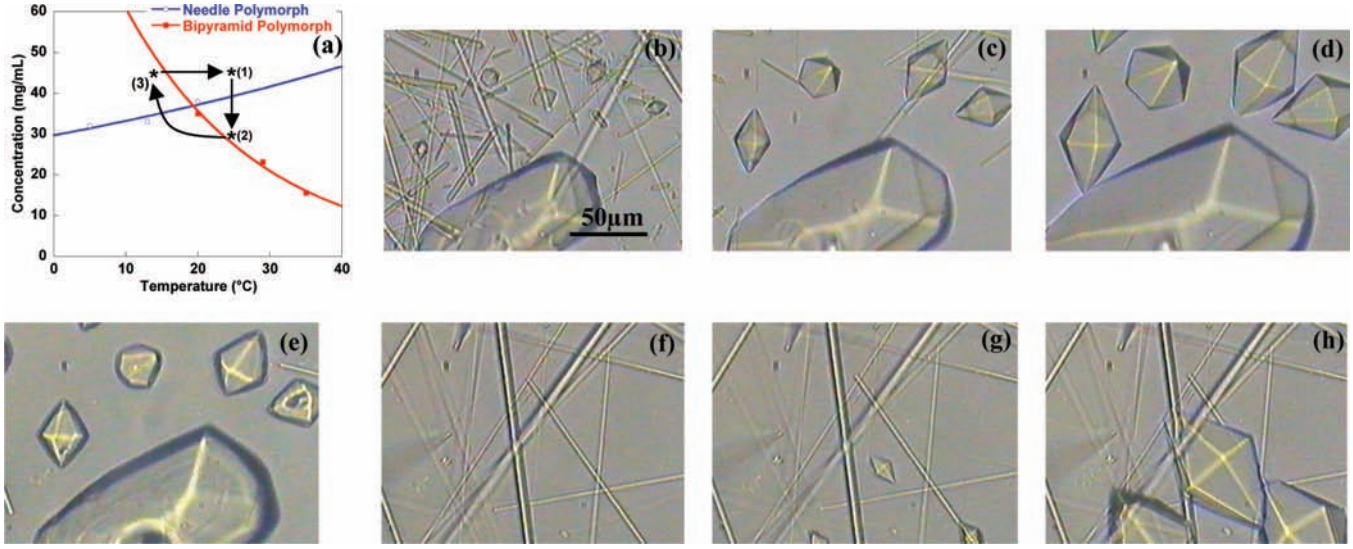

Figure 5. (a) Phase diagram of BPTI at pH 4.75 in 2 M NaBr (after ref 21); (b) in situ observation under optical microscopy of the solution mediated phase transition of BPTI crystals: mixture of both phases (point (1)) at 25 °C; (c) dissolution of the needle-polymorph and growth of the bipyramid-polymorph; (d) stable phase at equilibrium at end of process at $T = 25$ °C (point (2)); (e) dissolution of the bipyramid-polymorph and nucleation and growth of the needle-polymorph at $T = 15$ °C (between point (2) and (3)); (f) growth of needle-polymorph; (g) nucleation and growth of bipyramid-polymorph at $T = 25$ °C; and (h) finally return to point (1).

be transferred they dissolve faster and the process is stopped before complete dissolution of the larger crystals by a temperature decrease (second stage). Finally, large crystals grow and are faceted (Figure 4c, point (3) in Figure 3). This procedure can be easily applied to twinned crystals (Figure 4d−f). Note that crystal habit is also improved by this procedure, due to different growth conditions, here temperature and supersaturation, which induce different crystal growth rates for each face.

3.1.3. Phase Transitions (Example 3). Phase selection is an important topic in industrial crystallization, because different phases for the same molecule induce different physical properties. Phase selection is also important in protein crystallization due to the need to grow crystals of one phase for structural purposes; for instance, nucleation of a metastable phase can hinder the growth of the stable phase. Solution-mediated phase transitions can be used to grow large crystals of the stable phase at the expense of the metastable phase.[3,21] In the following, we observe the concomitant nucleation of the bipyramid[23] and needle polymorphs of BPTI in 2 M NaBr at pH 4.75 (Figure 5a,b). In this example the system is said to be enantiotropic; that is to say, polymorphs can undergo reversible changes from one form to another, meaning that solubility curves cross at a transition temperature T_R, here at 19 °C.

First, the stable polymorph at $T > T_R$, bipyramid-polymorph, is obtained by an isothermal process (from points (1) to (2) in Figure 5a): dissolution of the metastable and growth of the stable phase (Figure 5c,d). This process is slow and can be activated by temperature fluctuation as in the case of kinetic ripening (example 2).

Second, the stable polymorph at $T < T_R$, needle-polymorph, is obtained (Figure 5f) by decreasing temperature (because the bipyramid-polymorph solubility is reverse). Therefore, crystals dissolve and the solution concentration increases until it crosses the nucleation curve of the needle-polymorph (between points (2) and (3) of Figure 5a and Figure 5e,f).

Last, an increase in temperature allows the suspension to return to point (1) and the bipyramid-polymorph nucleate (Figure 5g) and thus the two polymorphs are in suspension (Figure 5h).

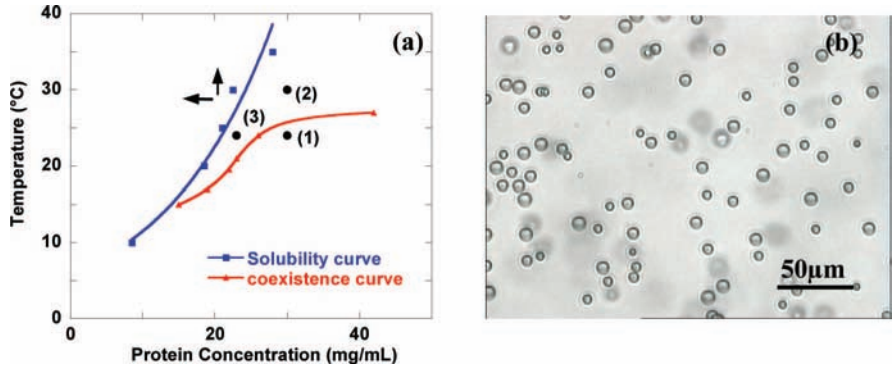

Figure 6. (a) Phase diagram of BPTI at pH 4.9 in 350 mM KSCN in the concentration range 0−50 mg/mL (after refs 12 and 32); and (b) observation by optical microscopy of the nucleation of droplets of the protein-rich phase in a supersaturated solution of BPTI (30 mg/mL) when temperature is decreased to 25 °C (point (1)).

To summarize, an adequate control of temperature, coupled to videomicroscopy, around the transition temperature can be used to obtain the desired polymorph.

3.2. Phase Separation (Example 4). The first objective of crystallization screening is to nucleate crystals. In doing so, precipitates are commonly observed: they are generally dismissed as disordered phases. It is now clearly established that these precipitates are formed of aggregates or gels produced by a (metastable) liquid−liquid phase separation (LLPS).[24−33] The phase diagram provides us with the explanation of this phenomenon. Figure 6a presents the BPTI-phase diagram measured at pH 4.9 in 350 mM KSCN. Point (1) in Figure 6a represents an experimental condition leading to a LLPS (Figure 6b). Here point (1) is in the LLPS zone, meaning that both crystal and liquid nucleations can occur. According to Oswald's rule of stage, LLPS occurs prior to crystal nucleation: for kinetic and thermodynamic reasons, liquid nucleation, which proceeds by density fluctuation alone, is faster and easier than crystal nucleation, which requires both density and structure fluctuation. In practice, an increase in temperature to point (2) in Figure 6a or a decrease in protein concentration to point (3) in Figure 6a leads to supersaturated conditions in which droplets of the dense phase dissolve. This zone in the T−C phase diagram, below the solubility curve and above the LLPS curve, represents the location where the right crystallization conditions can be found. Note that here again, fine-tuning the temperature leads to better control of nucleation and growth.

This example clearly shows that due to the respective position of the phase boundaries for LLPS and crystal nucleation, and as previously suggested by Broide et al.,[25] "LLPS can be used to identify the optimum solution conditions for growing protein crystals".

4. Conclusions

We have given an overview of our work, presenting practical examples of the effect of temperature on protein phase separation and crystallization. The advantages of temperature as a crystallization parameter are constant composition, ease of control and monitoring and reversibility. Since it is essential to separate nucleation and growth processes, our strategy is to form a nucleus at a higher supersaturation value and to grow the nucleus at a lower supersaturation value. The four examples presented here illustrate how this can be done. We have been able to tune the temperature in a small sample, down to 15 μL, to control growth habit and morphology, crystal size and quality, polymorphism and nucleation via solution-mediated phase transitions and liquid−liquid phase separation.

Above all, we believe that a thorough knowledge of the phase diagram is vital to the selection of the starting position and path for any crystallization experiment.

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