

Practical Physics Behind Growing Crystals of Biological Macromolecules

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Abstract: The aim of this review is to provide biocrystallographers who intend to tackle protein-crystallization with theory and practical examples. Crystallization involves two separate processes, nucleation and growth, which are rarely completely unconnected. Here we give theoretical background and concrete examples illustrating protein crystallization. We describe the nucleation of a new phase, solid or liquid, and the growth and transformation of existing crystals obtained by primary or secondary nucleation or by seeding. Above all, we believe that a thorough knowledge of the phase diagram is vital to the selection of starting position and path for any crystallization experiment.

Keywords: Crystal growth, nucleation, phase transition, ripening solubility, supersaturation.

1. INTRODUCTION

Unlike the crystallization of small inorganic molecules, the problem of protein crystallization was first approached by trial and error methods without any reference to theory. Later, a physico-chemical approach was chosen because crystallographers and biochemists needed criteria to rationally select crystallization conditions, as well as to optimize the crystallization conditions in order to obtain single crystals for structural purposes. In fact, the problem of producing homogeneous and structurally perfect protein crystals is the same in the production of crystals for pharmaceuticals as in opto-electronics or nanomaterials, because in all these cases crystal growth mechanisms are the same. That is to say, biological macromolecules and small organic or mineral molecules follow the same rules [1] concerning crystallization even though each material exhibits specific characteristics.

In practice, the usual approach to solution crystallization is to study the respective influence of temperature, supersaturation, medium (chemical composition) 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. This approach is usually named protein crystallization screening [2]. This paper introduces the fundamental phenomena in protein crystallization: supersaturation, nucleation, growth and transformation of crystals.

2. SOLUBILITY AND SUPERSATURATION: A GOOD START

2.1. Solubilizing the Protein

Before obtaining any nucleation or growth, it is necessary to dissolve the biological macromolecules under considera-

tion in a good solvent. In crystallization, a good solvent is defined by high solubility of material and/or easy control of nucleation and/or fast growth of crystals exhibiting the appropriate habit [3] (see definition in part 4). In practice, the (soluble) protein is solubilized in an aqueous buffer, which cannot be considered the crystallization medium. The crystallization medium is defined by the chemical composition of the medium used for crystallization, that is to say after the addition of the agents of crystallization, the so-called precipitants, to the solution. Note that the term precipitant is ill-chosen, because the aim is to control crystallization, not precipitation.

The first step is the choice of buffer, for which there are three possibilities: (i) using the elution buffer, (ii) collecting biological data such as stability of the protein (assessed by emission fluorescence for instance [4]) or its pI (isoelectric point). For instance, the solubility of protein is generally lowest at pI, and this generally leads to precipitation or aggregation of proteins rather than to a well-controlled crystallization. Different authors have observed that the pH of crystallization solutions is correlated with the pI of the molecule [5-6]: basic proteins are more likely to crystallize at pH below their pI (from 0 to 3 pH units), whereas acidic proteins are more likely to crystallize at pH above their pI (from 0 to 3 pH units). (iii) The third possibility consists of testing the protein-aggregation behavior by light scattering. As stated by Zulauf and D'Arcy: "Proteins showing a tendency to form aggregates in dilute solution (and in the absence of precipitating agents) do not crystallize in the majority of cases." [7].

2.2. Supersaturation

Once the material is in solution, this solution must be supersaturated in order to observe nucleation or growth. The solution is supersaturated when the solute concentration exceeds its solubility, namely the concentration for which crystals and solution are at equilibrium. Supersaturation is the driving force for nucleation and growth. Supersaturation is the difference between the chemical potential of the solute

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molecules in the supersaturated state (μ) and in the saturated state (μ_s), respectively. For one molecule the expression of this difference is:

$$\Delta\mu = \mu - \mu_s = kT \ln \beta \quad (1)$$

where k is the Boltzmann constant and T the temperature. To simplify, activities are considered equal to the concentrations and can be written here without specifying the units:

$$\beta = C/C_s \quad (2)$$

where β is the supersaturation ratio, C is the concentration of the solute in solution and C_s its saturated or equilibrium concentration. Moreover, if $\beta > 1$, the crystal grows, if $\beta < 1$ the crystal dissolves, and if $\beta = 1$ crystals and solution are at equilibrium. Obviously this ratio is dimensionless but its value depends somewhat on the concentration units (g/l, mol/l, mol fraction, activities, etc.). For protein crystallization, the concentrations are mostly expressed as mg/ml, i.e. g/l, which is the easiest, but probably not the best way to explain crystallization kinetics. For the sake of simplicity, supersaturation is usually defined as β , the ratio defined in Equation 2 or as another dimensionless ratio $\sigma = \beta - 1$:

$$\sigma = (C - C_s)/C_s \quad (3)$$

It is also worth noting that supersaturation is sometimes defined as the difference $C - C_s$. In this case, its value drastically depends on the concentration units. The difference $C - C_s = 100\text{g/L}$, for example, reduces to about $4.5 \cdot 10^{-3}$ mole/L if the concentrations are expressed as mole/L fractions for Thaumatin having a molar weight of 22 204g/mole.

However, this may conceal the specific influence of the concentration on crystallization. As an example, let us consider the case of a protein whose solubility decreases with the concentration of the crystallization agent, i.e. a salt or a poor solvent (the opposite of a good solvent). Thus, in the case of BPTI in NaCl solutions [8] (Fig. 1), a supersaturation of three times the solubility $\beta = 3$, can be achieved in different areas of the solubility diagram: for instance at a temperature where solubility is high (44mg/ml in 1.4 M NaCl solutions at 25°C), or low (3mg/ml in 2.3 M NaCl solutions at 25°C). In

these cases the mass of solute crystallized is either 88 mg/ml or only 6 mg/ml, respectively. Consequently, despite the same β value, nucleation and growth will be favored in the first case.

The way supersaturation is achieved will define the crystallization method to be used [9]. The simplest is to partly evaporate the solvent, the drawback being that all species in the solution (salts, additives, impurities) concentrate as well. This is what happens with the hanging or sitting drop method, namely the **vapor diffusion method**. An even simpler method consists of mixing different solutions, protein with agent of crystallization for instance, in order to rapidly reach the supersaturated stage, and then waiting for crystallization: this is the **batch method**. However, another way of achieving supersaturation is using **temperature, which provides** better control of crystallization. The solution is cooled or heated, depending on whether the solubility decreases with decreasing temperature (Fig. 1b) or conversely (Fig. 1a). This is an easy way to control supersaturation at constant composition and the effect is reversible [10]. Nevertheless, this method is not recommended when the temperature dependence of solubility is too low.

Diagrams in (Fig. 1) illustrate the general rule about the temperature dependence of solubility, which is higher at lower ionic strength. Moreover, increasing ionic strength decreases solubility, which diminishes the control of nucleation and growth, thereby risking fast and uncontrolled nucleation, that is to say precipitation. This rule is also true for crystallization with PEG as agent of crystallization [11]; in this case it may be favorable to decrease the percentage of PEG or to use a PEG of smaller molecular weight.

Instead of directly mixing solutions, a better way of achieving supersaturation is the **counter diffusion method**. Solutions are placed facing each other, generally separated by a physical barrier, agarose gel for instance. Mixing is obtained by diffusion allowing different crystallization conditions to be screened in a single experiment [12]: this is the principle of the Granada crystallization box.

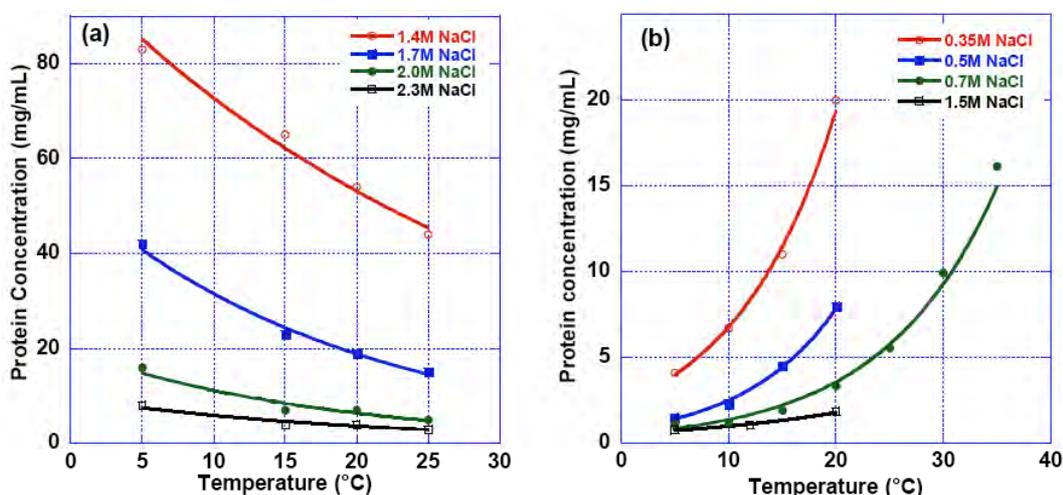


Figure 1. Solubility of (a) BPTI [8] and (b) Lysozyme as a function of NaCl [13] concentrations for different temperatures at pH=4.5. Note the reverse solubility with temperature for BPTI and the direct solubility for Lysozyme.

Lastly, **dialysis** is an elegant way to increase supersaturation by increasing, for instance, ionic strength at constant protein concentration. Supersaturation can also be achieved by pH variation, chemical reaction, and addition of a poor solvent. However, the evolution of the system is often more difficult to control with these methods.

3. NUCLEATION, THE BIRTH OF CRYSTALS

When a solution is supersaturated, the solid phase forms more or less rapidly depending on crystallization conditions: concentration of solute, crystallization agent, pH, supersaturation, temperature, nature and concentration of impurities, stirring (uncommon for protein crystallization), presence of solid particles [14]. Primary nucleation occurs in a solution that is clear, without crystals. It is called homogeneous nucleation if the nuclei form in the bulk of the solution. It is called heterogeneous if the nuclei preferentially form on substrates such as the wall of the crystallizer or solid particles (such as dust particles). Conversely, secondary nucleation is induced by the crystals of the same phase.

It is noteworthy that in the case of **seeding experiments** where crystals formed in a previous experiment are added to the solution [15-17], their growth and secondary nucleation are in competition. However, if supersaturation is high enough, the added seed can launch a nucleation wave, namely secondary nucleation. Thus the solution has to be metastable with respect to secondary nucleation (see paragraph 3.3. for definition of nucleation metastability).

3.1. Nucleation Mechanisms

Until recently, solution nucleation has been described solely by the classical nucleation theory, a theory derived from nucleation of droplets in the bulk of pure supersaturated vapors. It considers that once a cluster has reached the critical size r^* , given by the Gibbs-Thomson equation (equation (4)), nucleation starts. While this theory has the advantage of simplicity, some discrepancies have been observed with experiments [18-19].

$$r^* = \frac{2\Omega\gamma}{kT \ln \frac{C}{C_s}} \quad (4)$$

with γ the crystal-solution interfacial free energy ($\text{J}\cdot\text{m}^{-2}$) and Ω the volume of a molecule inside the crystal. Note that the larger the supersaturation, the smaller the critical nuclei.

Therefore, a more complicated two-step mechanism has been proposed for protein crystallization [20]: first, formation of a dense phase of clusters on the model of a liquid-liquid phase separation and second, organization of these clusters into structured clusters (Fig. 2). This second step is rate-limiting in the case of protein crystallization, explaining why it is often more difficult to nucleate proteins than small molecules.

3.2. Nucleation Rate

The nucleation rate or nucleation frequency J is the number of crystals that form in a supersaturated solution per unit of time and unit of volume [22-25]. Hence, J is proportional to n times the solubility expressed in number of molecules per unit of volume. Here, we only need to recall that:

$$J = nN_0\nu \exp\left(-\frac{f\Omega^2\gamma^3}{(kT)^3 \ln^2 \beta}\right) \quad (5)$$

$N_0\nu$ is the frequency with which nuclei of critical size r^* become supercritical by addition of a molecule and develop into crystals. The term $nN_0\nu$ can be simply described as a pre-exponential factor K_0 . f is the nuclei form factor ($16\pi/3$ for a spherical nuclei). Equation (5) shows that the frequency of nucleation depends not only on the supersaturation β but also on the concentration of molecules nN_0 . All things being equal, included supersaturation, the higher the probability of intermolecular contact, the easier nucleation appears. Systems with high solubility meet this condition. For systems with low solubility, the solute molecules are separated by greater distances and by a greater number of solvent molecules. The probability that the molecules will come into contact and form a nucleus is thus lower.

3.3. Properties of J

Let us proceed to a numerical application with nucleation of lysozyme in 0.7M NaCl at pH=4.5 according to the data of Ildefonso *et al.* [26], at 20°C $C_s=3.17\text{mg/mL}$ in 0.7M NaCl at pH=4.5 [13]. Thus Equation 5 becomes:

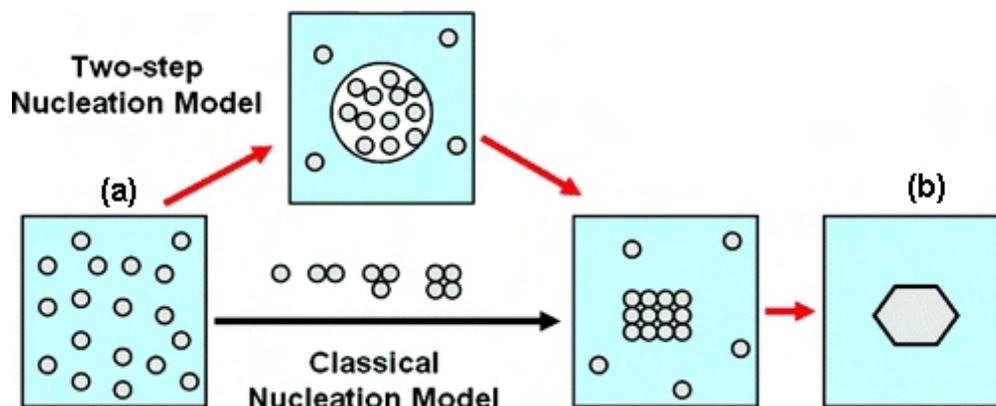


Figure 2. Schematic representation of the different nucleation mechanisms, starting from (a) a supersaturated solution to (b) a crystal. Reprinted with permission from Erdemir *et al.* [21]. Copyright 2009 American Chemical Society.

$$J = 2608 \exp\left(-\frac{58.1}{\ln^2 \beta}\right) (\text{nb.mL}^{-1}.\text{s}^{-1}) \quad (6)$$

In order to have a nucleation rate J of 1 nucleus /mL/s, the supersaturation β has to be ~ 15 ; whereas if β were only 6.3 (lysozyme concentration of 20mg/mL) then the nucleation rate would be catastrophically low: $J=9.3 \cdot 10^{-5}$ nucleus /mL/s, i.e. a nucleation time of $J^{-1}=10\ 800\text{s}$. This demonstrates that nucleation is highly dependent on supersaturation. In the low supersaturation range the solution remains metastable over a long period of time, whereas in the high supersaturation range nucleation occurs spontaneously. For protein crystallization, this means that a condition which leads to a large number of crystals can be improved by decreasing supersaturation.

Another parameter greatly affecting nucleation rate is the crystal-solution interfacial free energy of the nucleus. For instance the smaller the solubility in a solvent, the higher the interfacial free energy. Increasing γ by 50% using a poor solvent, that is to say $\gamma'/\gamma=1.5$, according to equation (4) the nucleation frequency ratio (all other parameters being unchanged) is:

$$\frac{J'}{J} = \exp(-1.5^3) = 0.035 \quad (6)$$

With γ' and J' the new values of interfacial free energy and nucleation frequency respectively.

Thus the nucleation frequency is reduced 30 times by diminishing the solubility. Furthermore, the metastable zone, where no nucleation occurs after a reasonable time lag, drastically widens out as interfacial free energy is increased.

As a concluding remark, it should be emphasized that the objective of crystal growers is to separate nucleation and growth phases. This task is extremely challenging because growth of crystals is optimal in the metastable zone, at low supersaturation, where nucleation is kinetically inactive. To overcome this problem and because primary nucleation is a stochastic phenomenon, seeding techniques are often used. However, some authors recently proposed unusual approaches using external fields to control crystallization from metastable solutions, for instance magnetic [28-33], [34-41] or electromagnetic [42-51]. Moreover, spatial and temporal location of nucleation can also be reached by confining the nucleation volume [52].

4. CRYSTAL GROWTH, THE LIFE STORY OF THE CRYSTAL

Once the nuclei are formed and exceed the critical size, they become crystals; hereafter we recall the basic principles of crystal growth.

4.1. Growth Form

A crystal is limited by its faces. The set of equivalent faces resulting from the crystal symmetry is a **form**. All the forms present on a crystal represent the **morphology** of the crystal. However, in order to describe the external form of a crystal, morphology is not sufficient and the concept of **cryst-**

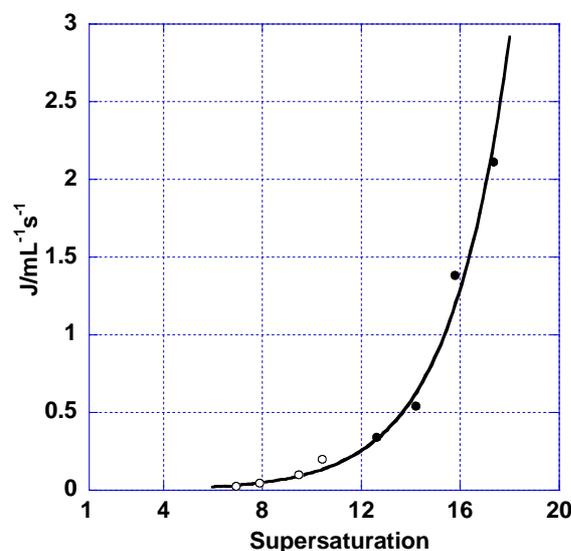


Figure 3. Lysozyme primary nucleation rate vs. supersaturation, at 20°C, NaCl=0.7M and pH=4.5 after Ildefonso *et al.* [27].

tal habit is needed, entailing the notion of face extension. But it is important to point out that the growth form of the crystal is defined by the faces with the slowest growth rates. This is shown by (Fig. 4) which represents the growth in the metastable zone of a seeded monoclinic BPTI crystal in KSCN solution [10]. During the experiment all the faces migrate parallel to themselves and cross distances proportional to their growth rates. Obviously, the growth forms are different in frames a and c. The slowest faces develop at the expense of the fastest faces, which entirely disappear. The growth form thus depends on kinetic factors, that is to say crystallization conditions. This is the reason why changing the crystallization conditions induces the observation of different crystal habits.

4.2. Growth Medium and Kinetics

Growth kinetics and mechanisms depend on external factors (medium or chemical composition, temperature, supersaturation and hydrodynamics) and on internal factors (structure, bonds and defects). The growth medium influences the growth kinetics of the faces in different ways. First of all, the solvent is more or less adsorbed by the faces and selectively slows down their growth rates. Moreover growth rate increases with the solubility. The growth medium also influences solvation, desolvation and complex formation [53]. Furthermore, variations in temperature produce extremely different growth rates. Lastly, hydrodynamics, the relative velocity of the solution compared to the crystal [54], is an important parameter. Indeed if the solution is quiescent, the face grows slowly at a rate determined by molecular diffusion and convection of the solute towards the crystal. The growth rate of the face increases with the flow velocity of solution to the crystal. However, there is still a diffusional limitation: this growth rate tends very quickly towards a plateau and thus reaches an upper limit determined by phenomena at the crystal surface. At the laboratory scale, thermal

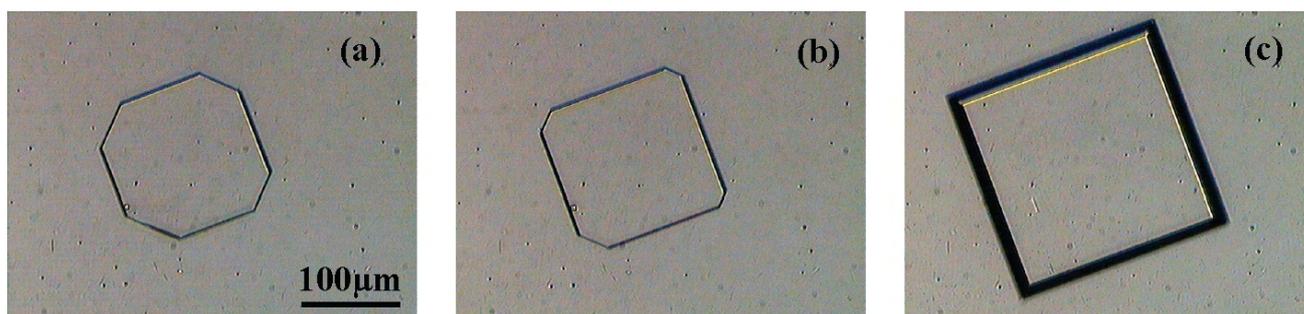


Figure 4. Growth of a BPTI crystal in 350mM KSCN at pH=4.9 (a) to (c) are frames of a time sequence showing the evolution of the growth form. Reprinted with permission from Astier and Veessler [10]. Copyright 2008 American Chemical Society.

and mass convections are reduced using either gel as medium of crystallization [55] or small volumes as in microfluidics experiments [26, 56-59].

4.3. Growth Mechanisms

The theories of crystal growth mechanisms are extensively discussed elsewhere [60-62]. Here, we give a rapid survey. Depending on crystallization conditions, different growth mechanisms can occur: through direct incorporation of molecules or through layer-by-layer building. Usually, since the slowest faces develop at the expense of the fastest faces, we only observe growth layer by layer and thus crystals have flat faces (Fig. 5a). However, very high supersaturation renders the crystal rough [60] (Fig. 5b) because faces which grow by direct incorporation display growth rates equivalent to those of flat faces, growing layer by layer. This phenomenon can also occur above a roughening temperature threshold. This mechanism is, a priori, unfavorable when crystals of good quality are required.

When crystals grow layer by layer, two growth mechanisms can occur depending on the supersaturation and the crystal quality. The first mechanism concerns crystal faces which are perfectly flat, without any defect. In this case, molecules adsorb, randomly diffuse on the surface, encounter each other and coalesce into a two-dimensional nucleus which spreads across the crystal face if its size exceeds a critical size: this is the 2D mononuclear growth mechanism. If several nuclei spread at the same time across the crystal face: this is the 2D polynuclear mechanism (Fig. 6a). As with 3D nucleation, there is a critical supersaturation below which the growth rate is zero or nearly zero. Hence the diagram of growth rate versus β shows a dead zone at low supersaturation once this critical supersaturation is exceeded, the growth rate drastically increases with increasing supersaturation and growth is difficult to control.

The second mechanism is observed in pure medium, where growth occurs even at very low supersaturation due to the presence of defects. For instance when a screw dislocation emerges on a crystal face, it generates a growth spiral (Fig. 6b). Because this involves a parallel sequence of steps, growth can take place even at low supersaturation since the growth units which adsorb onto the crystal face easily find growth sites where they are incorporated into the crystal. To conclude, at low supersaturation, growth by spiral mecha-

nism is predominant and at higher supersaturation, it is the 2D growth mechanism which is predominant.

Depending on the influence of the different parameters, growth varies with volume diffusion, surface diffusion, kink integration kinetics and so on. In practice, for protein crystallization experiments are generally carried out in stagnant systems. The reason for this is the lack of instrumentation, as well as the fragility of protein crystals. Moreover, the diffusion coefficient of proteins is two orders of magnitude smaller than that of small molecules. Accordingly, growth rates are mainly controlled by volume diffusion. However, sometimes growth rates are controlled by kink integration kinetics. The growth units reach the growth sites but take some time finding the proper conformation before being incorporated into kinks. This time is called relaxation time. Such a mechanism is likely to occur with large molecules such as proteins, which have to reorient themselves in order to be incorporated into the growth sites.

4.4. Role of Impurities

Crystallization is a purification process and because impurities are often present in the crystallization medium, they tend to concentrate during crystallization. This increase in impurity concentration is more pronounced for evaporation-based methods such as the vapor diffusion method. It is worth noting that impurities can be chemical or biological. In practice, impurities adsorb on the crystal faces. Depending on the energy of the bonds between impurity and adsorption sites, adsorption is more or less reversible. Thus, while growth proceeds, there is competition between the kinetics of molecule incorporation and the kinetics of impurity adsorption and desorption. Accordingly, impurities hinder the crystallization processes so that nucleation and growth rates are sometimes drastically slowed down. Vekilov [64] measured HEWL growth rates 5-6 times lower in presence of impurities than in pure media, at low supersaturation. When impurity adsorption selectively occurs on a crystal face, the growth rate of this face is selectively reduced and its relative development rapidly increases at the expense of the development of the other faces. This behavior also induces changes in habit or influences the crystal quality. Lorber *et al.*, [65] have observed an increase in the proportion of twinned crystals correlated with the addition of ovalbumin or bovine serum albumin to pure HEWL. When impurity adsorption takes place on all crystal faces, and is irreversible,

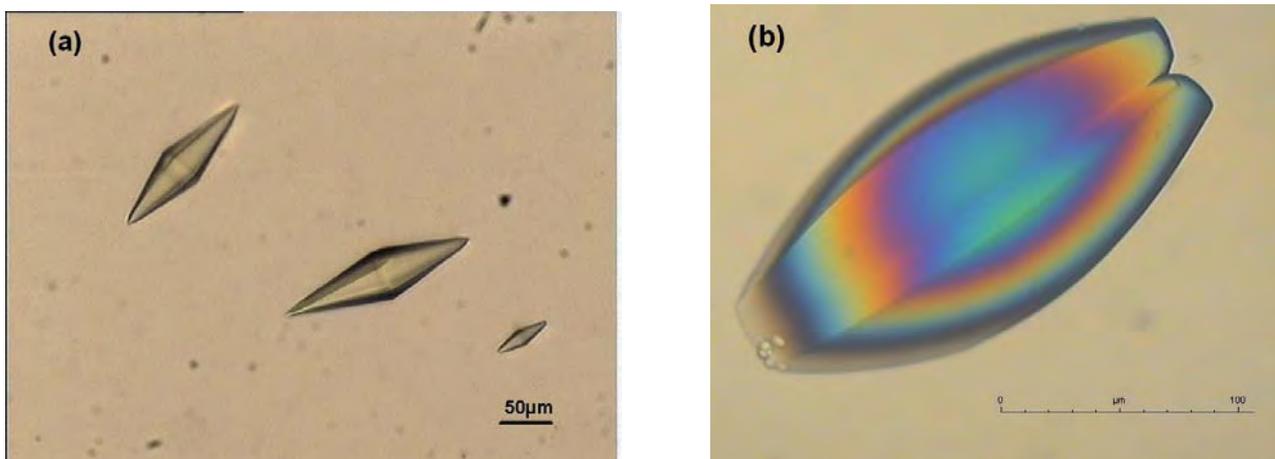


Figure 5. Crystals of BPTI at pH=4.5 in 350mM KSCN grown, (a) at low supersaturation and (b) at higher supersaturation.

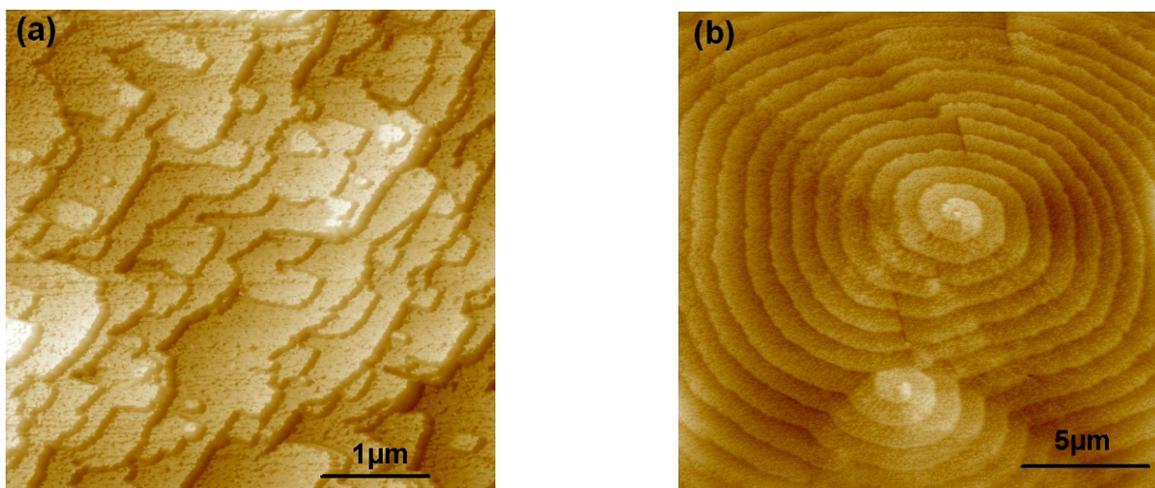


Figure 6. AFM images showing surfaces of α -amylase, (a) 2D islands and (b) spirals after Astier *et al.* [63].

i.e. without exchanges with the surrounding solution, growth is completely inhibited. Then the so-called growth cessation that often occurs with protein crystal is observed. One way to overcome this difficulty is to drastically increase supersaturation, which sometimes leads to new surface nuclei, which means that growth starts again. Another option is to start to dissolve crystals, by increasing the temperature for instance, and then go back to growth conditions. However, if the crystal surface is too energetically "poisoned" by impurities [62], 3D nucleation becomes easier than growth. Thus, the solution nucleates fresh crystals.

As a general rule, the habit and/or kinetic change results from impurity adsorption and not from impurity incorporation. However, impurity incorporation can take place especially when the molecule of the impurity resembles the molecule of the crystal. This was first observed in the case of small molecules (e.g. glutamic acid incorporated into asparagine monohydrate crystals for example) and later on in the case of biological macromolecules, e.g. contamination of turkey-egg-white lysozyme crystallizing solutions by HEWL [66]. Consequently, pure materials are difficult to grow when impurity and crystal molecules are homologues.

5. PHASES AND POLYMORPHISM, THE CRYSTAL FAMILY

Different polymorphs of a chemical compound have the same composition but different crystal structures. In contrast, different phases of a compound have both different compositions and different crystal structures. From these definitions, the crystallization of a protein in different solutions, i.e. in the presence of different crystallization agents, gives rise not to real polymorphs but to different crystalline phases of the same protein. The crystallization agents, salts in general, belong to the crystal structure, so that the phases of the same protein have different compositions. However, for the sake of simplicity, these different phases are in practice called polymorphs.

5.1. Phase Transition

Let us consider a system constituted by two polymorphs I and II. At a specific temperature, polymorph II is more stable than polymorph I. The more stable polymorph has the lower free energy G , in other words the more stable polymorph of

the two always has lower solubility, whatever the solvent in contact with the solid.

Metastable phases form for kinetic reasons and are favored by high supersaturation. When several phases are possible in the same solution, each of them has its own solubility so that the solution can be supersaturated with respect to several phases at the same time. These unstable phases may stay in a metastable state for a few seconds or several centuries. Ostwald [67] established in 1897 the rule that a chemical system does not directly tend towards equilibrium but rather towards the closest metastable state. In other words, nature prefers to follow a sequence of nucleations, growths and phase transitions rather than reaching a high energy level, directly nucleating the most stable phase. The metastable phase later undergoes a phase transition as soon as nuclei of a more stable phase, i.e. a less soluble phase, appear. Several phases or polymorphs may temporarily coexist but all except one are subject to transformation. In most cases, the phase transition occurs by dissolution of the metastable phases and recrystallization into the stable phase, called a solution-mediated phase transformation.

5.2. Control of Polymorphism

Control of polymorphism or phase selection is 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. Hence solution-mediated phase transitions can be used to grow large crystals of the stable phase at the expense of the metastable phase [16, 68]. In the following, we observe the concomitant nucleation of the bipyramid [69] and needle polymorphs of BPTI in 2M NaBr at pH 4.75 (Fig. 7B_a). 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.

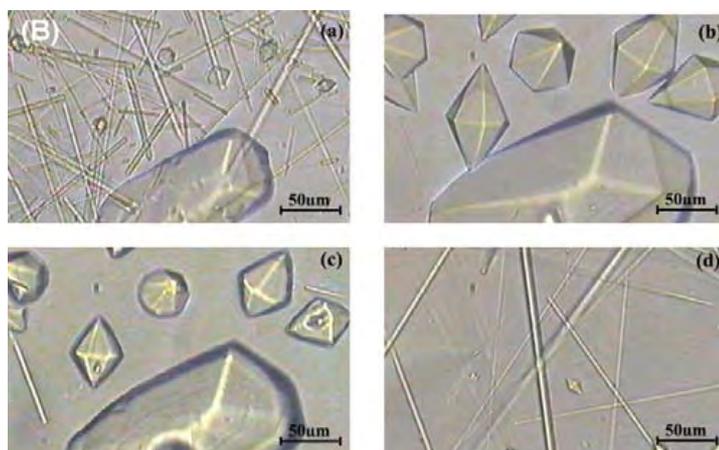
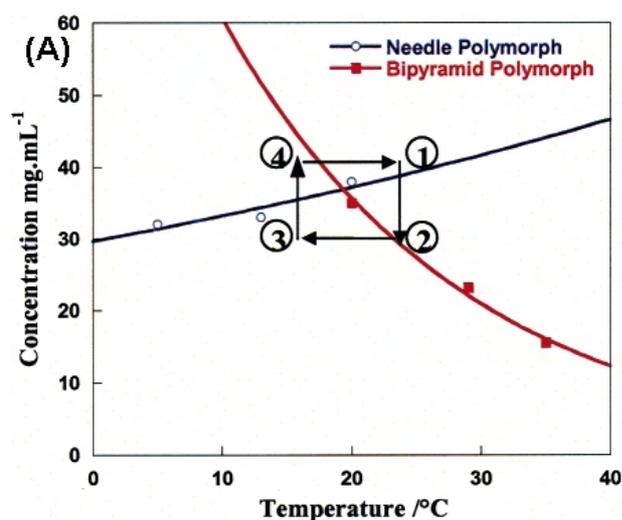


Figure 7. (A) Solubility curves of the two BPTI polymorphs in 2M NaBr versus temperature at pH 4.75. Solid lines are exponential extrapolations and are guidelines. (B) In situ observation under optical microscopy of the different stage of the BPTI phase transition; (a) mixture of BP and NP crystals in suspension (point 1 in (Fig. 7A)), (b) BP in suspension (point 2 in (Fig. 7A)), (c) dissolution of BP and nucleation and growth of NP (between point 3 and 4 in (Fig. 7A)) and (d) growth of NP and nucleation and growth of BP (between point 4 and 1 in (Fig. 7A)). Reprinted with permission from Veesler *et al.* [68]. Copyright 2004 American Chemical Society.

First, the stable polymorph at $T > T_R$, bipyramid-polymorph (BP), is obtained by an isothermal process (from points (1) to (2) in (Fig. 7A)): dissolution of the metastable phase and growth of the stable phase (Fig. 7B_b). This process is slow and can be activated by temperature fluctuation, as in the case of kinetic ripening (see part 5.3.).

Second, the stable polymorph at $T < T_R$, needle-polymorph (NP), is obtained by decreasing temperature to 15°C (because BP has reverse solubility) (point 3 in (Fig. 7A)), so that crystals dissolve and the concentration increases until NP solubility is crossed. When the concentration corresponding to the limit of the metastable zone-width is reached, NP crystals nucleate and grow. During this time (Fig. 7B_c) dissolution of BP crystals compensates for NP crystallization [70-72]. At the end of this process, all BP crystals have dissolved and NP crystals continue to grow until the now-decreasing concentration nearly reaches the solubility of NP (point 4 in (Fig. 7A) and (Fig. 7B_d)). Lastly, an increase in temperature to 25°C allows the suspension to return to point (1). To summarize, a thorough control of crystallization parameters, and understanding the phase diagram allow the desired polymorph to be obtained.

5.3. Liquid-liquid Phase Separation (LLPS)

The first objective of crystallization screening is to nucleate crystals. In doing so, it is common to observe precipitates which are generally dismissed as disordered phases. It is now clearly established that what is identified as precipitates can correspond to a metastable LLPS [20, 73-81]. The solution becomes cloudy or turbid due to the presence of 2 liquid phases of different compositions. The phase diagram explains this phenomenon. (Fig. 8a) presents the BPTI-phase diagram measured at pH 4.9 in 350mM KSCN. Point (1) in (Fig. 8a) represents an experimental condition leading to crystal nucleation alone (Fig. 8b). Decreasing temperature to 15°C, from point (1) to point (2) in (Fig. 8a), leads to an LLPS (Fig. 8c). Note that in the LLPS zone, both crystal and

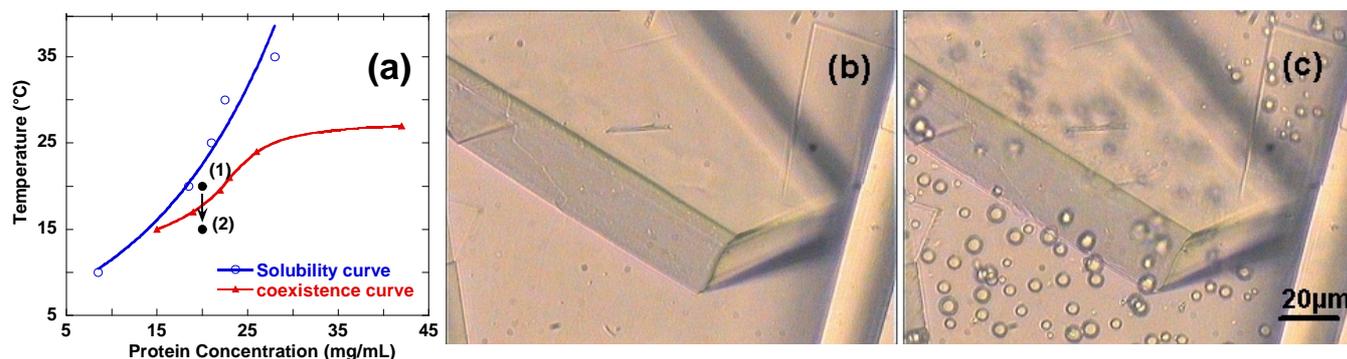


Figure 8. (a) Phase diagram for BPTI (350 mM KSCN at pH=4.9). Open circles: solubility of monoclinic BPTI from Lafont *et al.* [82]. Triangles: cloud point data from Grouazel *et al.* [77]. Observation by optical microscopy of droplets of the protein rich phase in a supersaturated solution of BPTI (20 mg.mL⁻¹, 350 mM KSCN, pH = 4.9) when decreasing the temperature: (b) T = 20°C and (c) T = 15°C, after Grouazel *et al.* [77]. Reproduced with permission of the International Union of Crystallography.

liquid droplets can nucleate, the new liquid phase being metastable. According to Oswald's rule of stage, LLPS occurs prior to crystal nucleation, thus hindering it. Liquid nucleation, which proceeds by density fluctuation alone, is clearly faster and easier than crystal nucleation, which requires both density and structure fluctuation.

In practice, conditions leading to LLPS can be modified to lead to crystal nucleation. For instance, from point (2) in (Fig. 8a), an increase in temperature to 20°C or a decrease in protein concentration to 15mg/mL 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 crystallization conditions can be found. Note that here again, fine tuning of the crystallization conditions together with a thorough understanding of the phase diagram leads to better control of nucleation and growth.

6. RIPENING, CRYSTALS DIE

6.1. Ostwald Ripening

After nucleation, in a batch or vapor diffusion crystallization experiments for instance, crystals of different sizes are present in suspension depending on the time at which they formed and the velocity at which they grow. We observe a decrease in supersaturation which, in theory should reach solubility. At the end of crystallization a decrease in the number of crystals and an increase in the crystal size can also be observed. Large crystals grow at the expense of small ones due to the fact that smaller crystals have higher solubility: this phenomenon is called **Ostwald ripening** [83]. From equation (4), it appears that each crystal of radius r corresponds to only one concentration C for which the equation stands. Thus the smaller the crystal size r , the greater C .

$$r = \frac{2\Omega\gamma}{kT \ln \frac{C}{C_s}} \quad (4)$$

Ostwald ripening is an isothermal process which is very slow for crystals larger than 1µm and very fast for submicrometer crystals. In protein crystallization, this explains

why sometimes crystals grow from precipitates. In fact, these precipitates are composed of submicrometer crystals, the largest growing and the smallest dissolving. This has been observed in several cases: with thaumatin, concanavalin A, an α -amylase and a thermostable aspartyl-tRNA synthetase by Ng *et al.* [84].

6.1. Kinetic Ripening

For protein crystals, kinetics of dissolution and growth being very low in the vicinity of solubility, Ostwald ripening is not very often observed. Ripening can be activated by temperature: this is the **kinetic ripening method** [16]. Temperature fluctuations in the neighborhood of the equilibrium temperature induce dissolution of the smallest crystals and growth of the largest ones. This method is also applicable to precipitates. (Fig. 9) presents the complete kinetic ripening process for α -amylase crystals in experiments with a wide crystal-size distribution (Fig. 9a). In the first stage, temperature is increased by a few degrees. Small and large crystals dissolve (Fig. 9b), but as small crystals have less matter to 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 (Fig. 9c). This procedure can easily be applied to twinned crystals (Fig. 9d-f).

7. CONCLUDING REMARKS

This paper introduces the fundamental physical concepts in protein crystallization: solubility, supersaturation, nucleation, growth, phase transformation and ripening of crystals. We give an overview of the physics of crystal growth, presenting practical examples of protein nucleation, growth and phase transition. 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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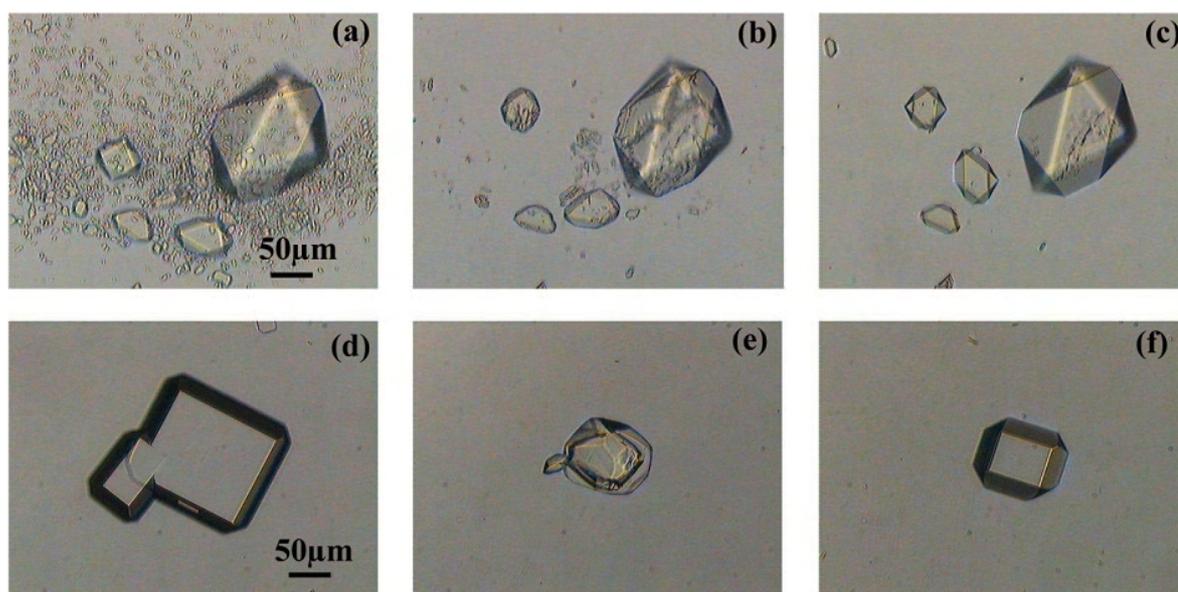


Figure 9. Kinetic ripening of B polymorph of α -amylase crystals shown in (a), by (b) partial dissolution and (c) regrowth. Elimination of a macrodefect observed in (d) by (e) dissolution and (f) growth. Reprinted with permission from Astier and Veessler [10]. Copyright 2008 American Chemical Society.

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