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Nucleation in Protein Crystallization

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Abstract

In this paper protein crystallization is regarded as a process starting with phase separation in a two-component system. The nucleation time of a lysozyme solution is measured by recording the NMR spectra of crystallizing solutions as a function of time. It is found that there is an appreciable induction time before the first nuclei appear in the solution and that this induction time depends strongly on the degree of supersaturation owing to the protein concentration at a given ionic strength or owing to the temperature. From the experimental data it is evident that (at least for lysozyme) crystallization under the prevailing experimental conditions is a transient process with an induction time and not a steady-state process.

1. Introduction

The study of protein-crystal growth usually concentrates on practical aspects (Ducruix & Giegé, 1992; Giegé *et al.*, 1995). A theoretical basis for protein-crystal growth is much less developed than for small molecules (Chernov, 1997; Chernov & Komatsu, 1995; Kam *et al.*, 1978; Rosenberger, 1996; Wolde & Frenkel, 1997). Light scattering (Kam *et al.*, 1978; Mikol *et al.*, 1989; Georgalis *et al.*, 1993; Rosenberger, 1996), fluorescence spectroscopy (Crosio & Jullien, 1992), microscopy (Durbin & Carlson, 1992; Pusey, 1993; McPherson *et al.*, 1995) and small-angle X-ray scattering (Ducruix *et al.*, 1996; Tardieu, 1997) are the most commonly used techniques for studying protein-crystal growth. In this paper we present protein nucleation as a consequence of fluctuations in the supersaturated solution, and report measurements of the nucleation time of aqueous protein solutions using NMR as a diagnostic tool.

2. Materials and methods

The protein preparation was crystalline hen egg-white lysozyme from Boehringer Mannheim. According to our analysis, it contained 4% salt, 4% loosely bound water and less than 1% protein impurities. The lysozyme solutions were prepared in 0.2 *M* Na acetate buffer, pH 4.7 and 7%(*v*/*v*) D_2O. They were brought to supersaturation by adding NaCl to a final concentration of 6%(*w*/*v*). Experimental data reported in the literature (Howard *et al.*, 1988; Gripon *et al.*, 1997) indicate a solubility of 4–6 mg ml^{-1} for lysozyme in an aqueous solution with 6% NaCl and pH 4.7 in the temperature range 293–303 K. The effect of D_2O on the solubility is small (Gripon *et al.*, 1997). The solutions were centrifuged in a table-top centrifuge to remove solid particles† and subsequently crystallized in a batch experiment in a small-bore NMR tube with 0.5–0.8 ml of the lysozyme solution. Spectra were collected at or near room temperature in a 500 MHz instrument 45 min after supersaturation was established and then registered every 15 min; the integrated intensity of a peak near 0 p.p.m. was followed as a function of time. The integrated intensity is proportional to the concentration of the compound in solution, according to the Beer–Lambert absorption law.

3. Phase separation

3.1. *The free-energy curve for a protein solution*

The solution of the protein in a buffer-containing water solution can be regarded as a two-component system: the solvent and the solute. In Fig. 1 the free-energy curves for such a system at two different temperatures are drawn. Fig. 2 shows a corresponding phase diagram for a protein–water system (Wolde & Frenkel, 1997; Haas & Drenth, 1998). Above the critical temperature T_c the free-energy curve of the liquid phase has a simple minimum and separation into a liquid phase c_{sat} and a solid phase c_{cryst} occurs. At lower temperatures, $T < T_c$, the free-energy curve of the liquid phase has two minima and the phase diagram shows a metastable liquid–liquid immiscibility region where the solution is not stable and separates into two liquid phases with concentrations c_1 and c_2. The crystalline phase is more stable than the concentrated liquid phase with concentration c_2, and this fluid phase c_2 is easily converted to the crystalline phase (Taratuta *et al.*, 1990; Broide *et al.*, 1991).

† The length of the centrifugation procedure may influence the relative concentration of oligomers (Kashchiev, 1969) and have an effect on the induction time. Such an effect was not observed in our experiments.

3.2. *Supersaturation and the metastable state*

A supersaturated solution with concentration $c_s < c_{sp}$ in the unstable region between c_1 and c_2 should separate into two phases corresponding to the concentrations c_1 and c_2 (Fig. 1). In fact, from crystallization experiments, we know that the supersaturated solution often has a very long lifetime and must be regarded as a metastable state (Feher & Kam, 1985). It may take hours, days or even weeks before crystal growth sets in. The metastability is a consequence of the concave shape of the free-energy curve in the region of the supersaturated solution with $c < c_{sp}$ (Fig. 1). Going further to the right the curve passes an inflection point and becomes convex. The inflection point is called the spinodal point and corresponds to a concentration c_{sp}. The metastability of the supersaturated solution in this concave region with $c < c_{sp}$ can be explained as follows. The concentration of protein in the solution is not constant throughout the solution, as thermally induced local fluctuations in concentration occur. These local-concentration fluctuations can be observed by dynamic light scattering (Huang *et al.*, 1974; Guenoun *et al.*, 1987). Suppose $\bar{c}$ is the average protein concentration and there is fluctuation between c_{low} and c_{high} (Fig. 3). The free energy of such a fluctuating system is A, not B. The conclusion is that fluctuations increase the free energy; this is unfavourable and, therefore, they are damped and the system does not separate into two phases. The fluctuations in the solution are of a stochastic nature; fluctuations appear and disappear continuously. It is only if a fluctuation appears that is large enough to penetrate into the convex region that nucleation sets in. In the convex part of the free-energy curve the system is no longer metastable but unstable. In this region fluctuations lower the free energy and phase separation may set in spontaneously. Fluctuations with a large amplitude are required for nucleation. These large-amplitude fluctuations are responsible for many physical processes; they occur only rarely and, as a consequence, are difficult to detect experimentally. An accurate and quantitative theoretical description of large-amplitude fluctuations is difficult (Binder & Stauffer, 1976; Binder *et al.*, 1978; Dykman *et al.*, 1992; Luchinski & McClintock, 1997).

It is clear that if supersaturation is high and c_s is close to the inflection point (Fig. 1), the chance of nucleation

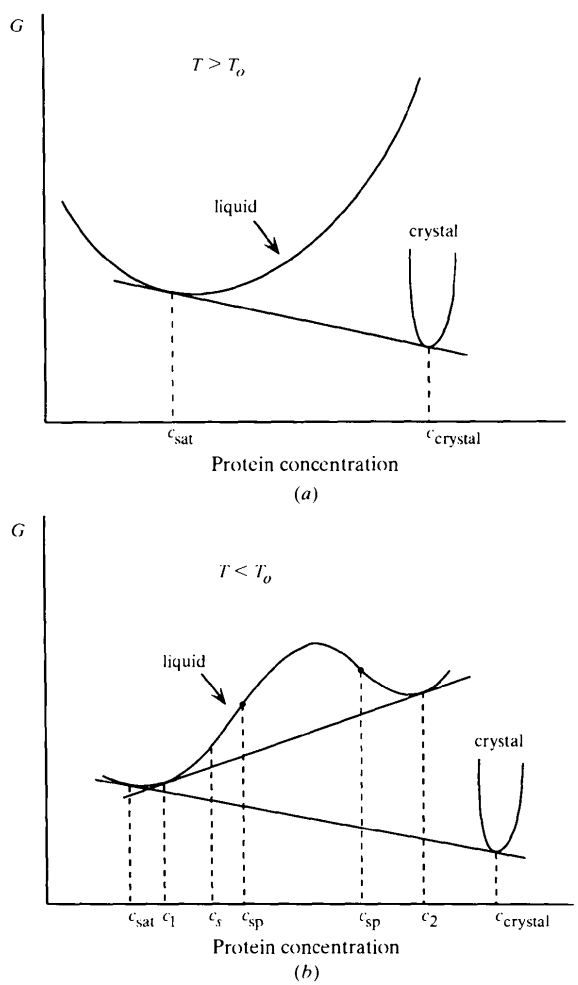

Fig. 1. Free energy G as a function of the protein concentration for the liquid and crystalline phases of a two-component system for a temperature (*a*) above and (*b*) below the critical temperature T_o. The saturated solution with concentration c_{sat} is in equilibrium with the crystalline phase. For $T < T_o$ there is a metastable liquid–liquid immiscibility region between the concentrations c_1 and c_2; the spinodal concentrations are indicated by c_{sp}.

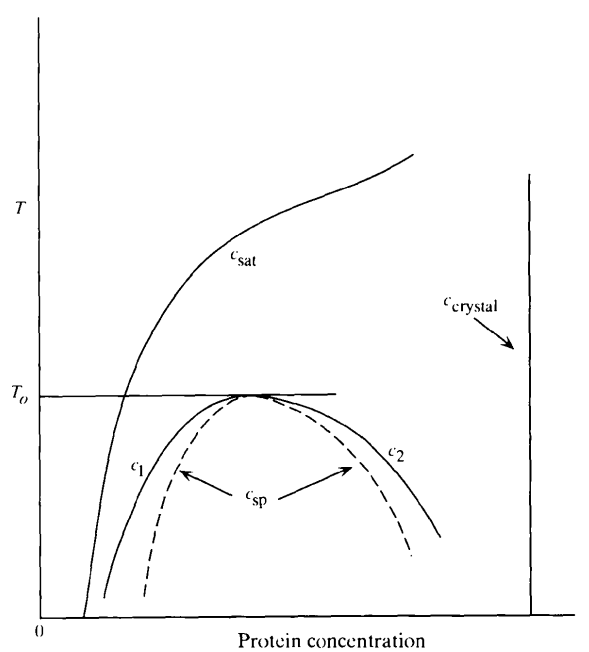

Fig. 2. Phase diagram of a protein–water system with a metastable liquid–liquid immiscibility region below the critical temperature T_o.

occurring is high. This can also be considered in the following way. In concentration fluctuations with small amplitude, the distance between the protein molecules is relatively large and the attractive forces between them are relatively weak. Entropy is then the dominating term. Chaos prevails and the protein molecules diffuse away. For concentration fluctuations with large amplitude, in which the molecules become closer, the attractive forces dominate over the entropy and these large fluctuations grow by an uphill diffusion (Fig. 4), and a new phase begins to form. As a consequence an additional energy term, surface energy, starts to play a role as the new phase develops. The attractive forces must not only overcome the entropy but also the surface energy.

4. Nucleation

In the mechanism described above, the large concentration fluctuations are the origin of nucleation. The just-formed nuclei do not yet have a crystalline order. Support for this mechanism comes from experiments by Taratuta *et al.* (1990) and Broide *et al.* (1991). They observed a liquid–liquid phase separation and not a liquid–solid phase separation for lysozyme and calf-lens proteins when they worked carefully under very clean conditions. However, the highly concentrated liquid phase is unstable with respect to the crystalline phase, and after a while crystals start to grow in this highly concentrated phase.

Recent work by Wolde & Frenkel (1997) also supports liquid–liquid phase separation before crystal-nuclei formation. They showed, using numerical simulations, that the free-energy barrier to crystal nucleation is strongly reduced by the presence of a metastable fluid–fluid critical point. It could be argued that the potential for interaction between the protein molecules as used by ten Wolde & Frenkel is not very realistic; for example, it does not take into account the anisotropy of the interactions and the non-spherical shape of the molecules. However, the general form of the phase diagram is not influenced by details of the interaction potential (Haas & Drenth, 1998). Asherie *et al.* (1996) showed that data available for protein–water phase diagrams can be obtained with simple square-well potentials. We expect that the general conclusions of ten Wolde & Frenkel, *i.e.* the reduction of the energy barrier for nucleation by the presence of a metastable liquid–liquid immiscibility region, will also be valid for more realistic potentials.

In real experiments where protein crystals are grown for X-ray diffraction purposes two coexisting liquid phases are not usually observed, the crystals instead growing straight from the solution. We assume that (at least for many proteins) every crystal originates from a tiny drop of a highly concentrated liquid phase. If the transformation process from the liquid to solid state proceeds too fast, the molecules have no time to order themselves properly and the system is quenched from the disordered liquid state to the amorphous solid state. We have assumed that seeds are absent in the solution.

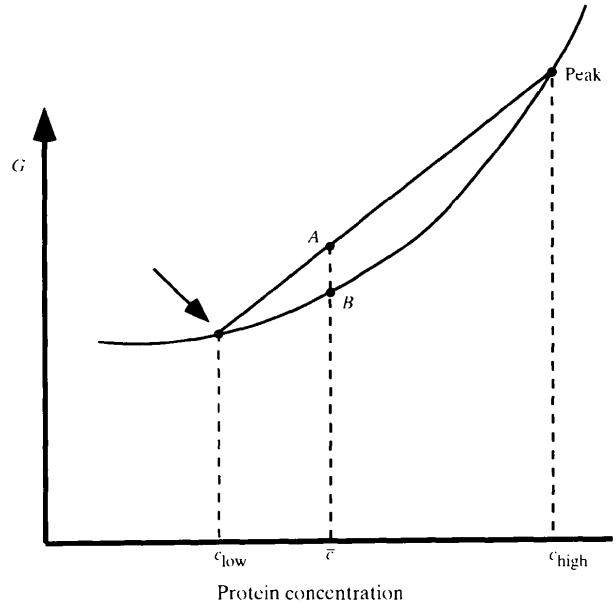

Fig. 3. Fluctuation of the protein concentration between c_{low} and c_{high}; $\bar{c}$ is the average concentration. The free energy of the fluctuating system (*A*) is higher than the free energy of the system without fluctuations (*B*).

5. NMR experiments

If protein crystallization starts from large-amplitude fluctuations in concentration, the appearance of nuclei and the beginning of crystal growth would be expected

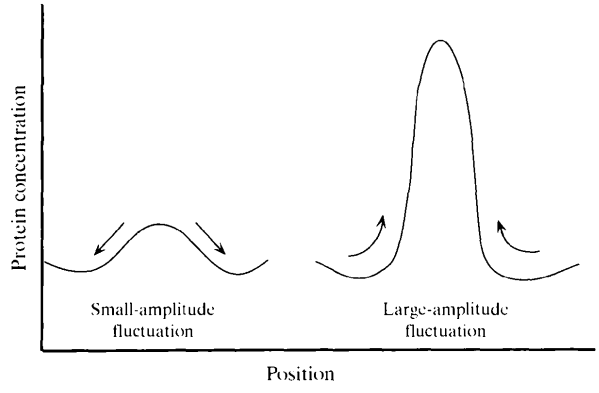

Fig. 4. Fluctuation of the protein concentration as a function of position in space. Small-amplitude fluctuations disappear in time. Large-amplitude fluctuations grow by an uphill diffusion and become nucleation centres. The arrows indicate the direction of protein transport.

to be a stochastic process and to start at different times under the same conditions. To investigate this effect we have applied NMR as a diagnostic tool. Lysozyme was crystallized in a batch experiment in an NMR instrument and the integrated intensity of a peak near 0 p.p.m. was followed as a function of time. The effect of the magnetic field on the crystallization is small and can be neglected (Sazaki *et al.*, 1997; Ataka *et al.*, 1997; Wakayama *et al.*, 1997).

A problem with lysozyme is that there is no complete agreement about the state of lysozyme molecules in solution. Experimental evidence for the existence of aggregates, even in undersaturated solutions, has been presented in the literature (Nadarajah *et al.*, 1995; Georgalis *et al.*, 1993; Eberstein *et al.*, 1993; Tanaka *et al.*, 1996; Strom & Bennema, 1997*a*,*b*). On the other hand, Muschol & Rosenberger (1996) claim that the crystallizing particles are monomers and that the observation of oligomers is due to non-ideality of the solutions. This is supported by Ducruix *et al.* (1996) and Tardieu (1997) on the basis of small-angle X-ray scattering experiments. In the NMR experiments we only observe the appearance or disappearance of monomeric lysozyme molecules from the solution.

In Fig. 5 the integrated intensity of the selected NMR peak is plotted as a function of time. The curve stays horizontal for 11.5 h before it rather sharply bends downwards. This change in the slope of the curve was regarded as the onset of crystallization and the period corresponding to the horizontal part as the induction time.

In an initial series of experiments we found that the induction time for crystallization strongly depends on supersaturation (Table 1). Because we expected a temperature effect on the induction time, in subsequent

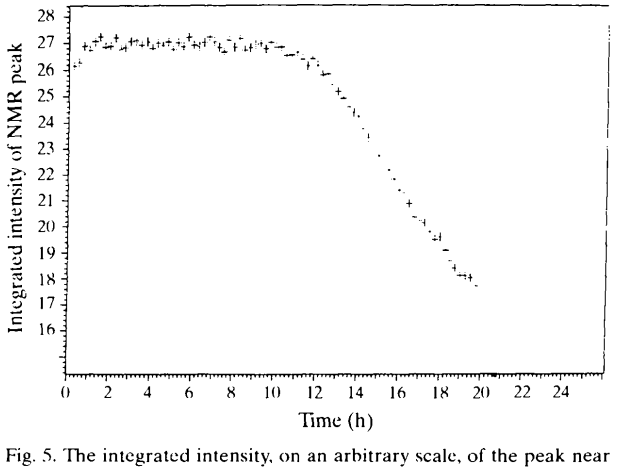

Fig. 5. The integrated intensity, on an arbitrary scale, of the peak near 0 p.p.m. in the NMR spectrum of lysozyme as a function of the time in h. The protein concentration was 25 mg ml $^{-1}$. The temperature was not stabilized and was estimated to be near 296 K. The solution was brought to supersaturation at time zero.

Table 1. *Induction time as a function of the protein concentration*

Experiments were performed near room temperature (296 K), but the temperature inside the NMR instrument was not stabilized.

Lysozyme concentration (mg ml^{-1})	Estimated supersaturation c_s/c_{sat}	Induction time (h)
20	3.6	>24
25	4.6	10.5
30	5.5	4

Table 2. *Induction time as a function of temperature*

The temperature within the NMR instrument was kept constant within 0.1 K.

Lysozyme concentration (mg ml^{-1})	Temperature (K)	Induction time (h)
25	292	7.7
25	294	8.2, 8.9, 9.7
25	296	11.2
25	298	16.1

experiments the temperature inside the NMR instrument was kept constant within 0.1 K. Three further measurements under exactly identical conditions at 294 K and 25 mg ml^{-1} gave induction times of 8.2, 8.9 and 9.7 h with an average value of 8.9 h (Table 2). The variation is within the possible error and the conclusion is that the induction time is constant for the same crystallization conditions. Next we measured induction times at 292, 296 and 298 K (Table 2). We find that with an increase in temperature the induction time becomes longer, from 7.7 h at 292 K to 16.1 h at 298 K. This is again a supersaturation-dependent effect. At higher temperature the solubility of lysozyme is higher and the supersaturation is lower.

We also measured an acetate peak for comparison. This would be expected to stay constant, but this was not the case. The intensity was found to change in the same way as the protein peak but to a much smaller degree. This could be due to an absorption of acetate by the crystals, suppressing its visibility in the NMR. Another explanation is that the magnetic field becomes less isotropic when crystals start to form in the NMR tube. This would then also mean that part of the change in the protein NMR spectrum is due to the formation of crystals as such, and not completely to the disappearance of monomeric lysozyme from the solution, but this does not affect our conclusions.

A nucleus starts as a high concentration fluctuation, the density of the concentrated region being higher than the density of its surrounding region. Gravity will pull this high-density region downwards and a gravity-induced flow will start to develop, at least on earth. In microgravity this flow will be absent. This is a difference in principle between crystal growth on earth and in space, although the effect is expected to be small. However, calculations by Georgalis *et al.* (1993) have

shown that the effect cannot always be neglected. Wakayama *et al.* (1997) have observed some effects from increasing or decreasing the gravitational field on earth by the application of an inhomogeneous magnetic field. Itami *et al.* (1996) found that for a much heavier system, a liquid Bi–Ga alloy, the amplitude of concentration fluctuations differs between microgravity and earth gravity.

6. Discussion

We have described the formation of small crystalline nuclei as a complex process, in which the first step is the formation of small liquid droplets with a high protein concentration as a result of a phase-separation process in the solution. The phase separation starts only if concentration fluctuations in the supersaturated solution are large enough to pass the spinodal point. The appearance of the fluctuations is a stochastic process, and it could be expected that the induction time (the time-lag before crystallization begins, Fig. 5) is a consequence of the stochastic nature of the nucleation process. In that case a variable length for the induction time would be expected, even under the same experimental conditions of temperature, concentration, pH *etc.* However, from our NMR experiments it is clear that this is not the case. The induction time is constant within the experimental error (data for 294 K in Table 2) and monomeric lysozyme does not disappear from the solution during the induction time. The explanation is that the formation of nuclei is a transient process and not a steady-state process (Chernov, 1984; Binder & Stauffer, 1976; Feher & Kam, 1985). In the classical steady-state nucleation theory a stationary distribution of fluctuations in the supersaturated solution is assumed, and this leads to a constant nucleation rate, *i.e.* a linear increase of the number of nuclei with time (Fig. 6). However, in the experiment one starts with an unsaturated solution, which is brought to supersaturation by changing the conditions. At the moment supersaturation is reached, the distribution of the fluctuations still corresponds to the distribution characteristic for the original solution. It takes a certain time to transform this distribution to one corresponding to the supersaturated solution and this is the induction time (Dunning, 1964; Kawasaki & Ohta, 1983; Feher & Kam, 1985). Only after the induction time has elapsed does the formation of nuclei proceed according to the classical steady-state nucleation theory (Fig. 6).

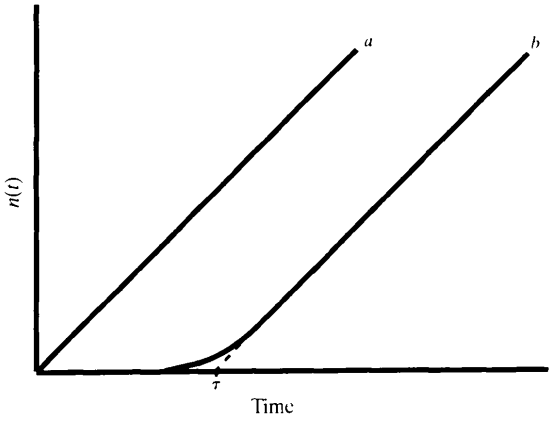

Fig. 6. Number of nuclei $n(t)$ as a function of time in steady-state (a) and transient (b) nucleation. τ is the induction time.

The experiments (Tables 1 and 2) show that the induction time depends strongly on the degree of supersaturation. This is in agreement with experiments by Feher & Kam (1985) who determined the crystallization time (the time elapsed before crystals start to grow) for aqueous solutions of lysozyme, and noted that the crystallization time depends strongly on the degree of supersaturation. The crystallization times reported by Feher & Kam are approximally equal to the induction times reported here.

The strong dependence of the induction time on the degree of supersaturation is in accordance with theoretical considerations (Chernov, 1984; Binder & Stauffer, 1976; Feher & Kam, 1985). This can easily be understood if we compare the formation of concentration fluctuations with the formation of a critical nucleus in classical nucleation theory. This theory states that the rate of nucleation is proportional to $\exp[-\text{constant}/(\ln S)^2]$ where S is the degree of supersaturation: $S = c_s/c_{sat}$ (Mullin, 1961). For large concentration fluctuations the same is true but now $S = c_s/c_l$ (Fig. 1).

Many discussions on protein crystallization are based on the classical steady-state theory of nucleation. However, from the experimental data it is evident that (at least for lysozyme) crystallization under the prevailing experimental conditions is a transient process with an induction time. A theory presenting a quantitative description of the induction time for protein crystallization should be able to explain how the induction time depends on the conditions for crystallization. Unfortunately, such a theory has not yet been developed. We shall expand our studies to the crystallization of other proteins.

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