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Monitoring protein crystallization by dynamic light scattering

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Crystallization of lysozymes induced by temperature lowering has been monitored by dynamic light scattering from the onset of supersaturation to the growth of protein crystals up to a noticeable size (150–200 μm). The apparent size of the scatterers was found to increase up to a maximum value as supersaturation proceeded, then to decrease down to its initial value. Apparitions of crystals (20–30 μm) were observed during this decrease. Light scattering is thus proved to be a sensitive technique to follow protein crystallization and to provide useful information on the process.

Protein crystallization; Nucleation; Light scattering; Lysozyme

1. INTRODUCTION

X-ray crystallography has revealed structure-function relationships of many biological systems, but production of biological macromolecule crystals suitable for high-resolution diffraction analysis is still based upon 'trial and error' methods. This situation is no longer satisfying and there is a growing interest in understanding protein crystallization [1] in order to grow crystals in a more reliable way.

From small molecule crystal growth theories [2], it is known that crystallization is a two-step process which involves nucleation (formation of a crystalline embryo up to a critical size) and growth (addition of molecules to this crystallite). Supersaturation conditions which favor nucleation are usually different and much higher than those which promote a stable growth. These two stages should thus be uncoupled. In protein crystallization experiments, whatever the technique (vapor diffusion, dialysis, batch, for a review see e.g. [3]), this uncoupling is almost never done consciously, but sometimes occurs through good fortune.

Elastic and quasi-elastic light scattering are sensitive techniques to detect variations in size and interactions of macromolecules. It has already been successfully used to investigate the condensation polymerization process of biological macromolecules (for a review see e.g. [4], to distinguish between protein solutions leading to crystallization or to amorphous precipitation [5] and to probe the prenucleation state in protein crystallization [6,7].

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In the present study, light scattering was applied to monitor the protein crystallization process. Lysozyme was chosen as a model system, because its crystal structure is well established to high resolution [8] and it has been the subject of some previous crystal growth studies (e.g. [9] and references included). Because lysozyme is less soluble at low temperature [10] and in order to accurately control supersaturation as a function of time, crystallization was induced by temperature lowering in a precise and reproducible way. The hydrodynamic properties of lysozyme were followed over three days from the onset of supersaturation to the growth of tetragonal lysozyme crystals up to a noticeable size.

In this preliminary communication, we report an interesting property of the apparent hydrodynamic radius of the scatterers in a lysozyme crystallizing solution. It increases up to a maximum as supersaturation proceeds and decreases down to its initial value as crystal growth takes place. Apparition of crystals can then be observed during the decrease of the measured apparent hydrodynamic radius.

2. MATERIALS AND METHODS

2.1. *Light scattering measurements*

An undersaturated solution of protein containing hen egg white lysozyme from Sigma (20 mg/ml), sodium chloride (2.5% w/v) and sodium acetate pH 4.6 (40 mM) was prepared. One milliliter of the solution was then placed in an Eppendorf tube and centrifuged at 10000 rpm at a constant temperature of 20°C for 15 min to clarify it from dust particles. Eighty percent of the supernatant was then removed and placed in a cylindrical scattering cell which had been previously carefully cleaned. The scattering tube was then set at the center of a temperature-controlled cylindrical glass cell which was filled with ethanol. Ethanol was used in place of the usual water in the bath because at −5°C it does not freeze. A laser beam of green light (488 nm) with a power of 600 mW, provided by an argon ion laser

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(Spectra Physics 2000), was focused on the center of the cell containing the protein solution. The scattered light was collected at 90° and sometimes at 45° with a photomultiplier. The digital autocorrelation function yielded from a B130 autocorrelator (Brookhaven Instruments, USA) was then fed to a microcomputer. The autocorrelation function was analysed using the cumulants method [11] in order to obtain the average decay time $<\Gamma>$ and the variance (ν) often referred to an index of polydispersity. For nearly spherical particles, when the ν is lower than 0.1, it indicates a rather moderate size distribution of the particles [12]. In some cases a single exponential fitting procedure was used to analyse the data as well. The diffusion coefficient of the scatterers (D) is given by the first reduced cumulant $<\Gamma>/2K^2$ where K is the scattering wave vector ($K = 4\pi n/\lambda \cdot \sin(\theta/2)$, where λ is the wavelength, n the refractive index and θ the scattering angle). In order to check the presence of any potential distribution of the scatterers, different sample times were used to measure the autocorrelation function for some experiments. The apparent hydrodynamic radius $R_{h,app}$ was then computed using the Stokes-Einstein equation: $R_{h,app} = kT/6\pi\eta D_t$, where k is the Boltzmann's constant, T the absolute temperature and η the viscosity of the solvent. The latter parameter was measured using a standard capillary viscometer at different temperatures.

2.2. *Crystallization procedure*

To induce supersaturation, the temperature of the bath, which contained the scattering cell, was cooled down, starting from 20°C to −2°C by a 2°C-step lowering every 30 min. Accordingly, 330 min elapsed before the temperature of the cell had reached its final value which was then maintained until the end of the experiment. During temperature variations, light scattering measurements were performed 25 min after the temperature setting (i.e. 5 min were available for measurement). Once the final temperature was attained, light scattering measurements were carried out until crystals grew up to a noticeable size (150–200 μm). Experiments were at least triplicate.

2.3. *Concentration and viscosity measurements*

To determine the variations of lysozyme concentration, a protein solution, with a composition identical to that used for the light scattering experiment, was placed in a scattering cell. The tube was then cooled down according to the same procedure as for light scattering experiment. Aliquots were pipetted out of the solution at given times and centrifuged at 10000 rpm for 15 min to clarify them from eventual crystallites. The concentration of the supernatant was determined by absorbance measurement (assuming $E^{1\%w/v} = 26.3$ for a 1 cm optical path at 280 nm).

Viscosity measurements of the protein solution were performed as a function of temperature. Temperature changes were made according to the same cooling procedure as for inducing supersaturation. This was done to investigate the presence of eventual large aggregates within the solution. Measurements were only carried out until time 400 min.

3. RESULTS AND DISCUSSION

Fig.1. represents a typical plot of the apparent hydrodynamic radius as a function of time. The apparent hydrodynamic radius increases as supersaturation proceeds. The crystal solubility of lysozyme, in 2.5% (w/v) sodium chloride, 40 mM sodium acetate, pH 4.6, was measured at 20°C, and found to be equal to 22 mg/ml. Since the initial concentration of lysozyme was 20 mg/ml, the protein solution at 20°C was slightly undersaturated. It must be stressed that the initial value of the hydrodynamic radius measured here was close to that obtained from a previous study ($R_h = 21.2$ Å) [5], where extrapolations to infinite dilu-

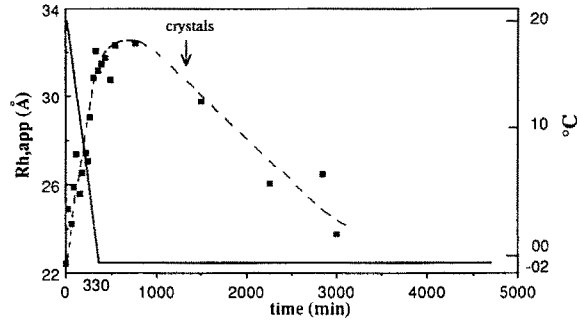

Fig.1. Variations of the apparent hydrodynamic radius of lysozyme as a function of time during a crystallization experiment induced by temperature lowering. The temperature variations during the experiment are indicated by a solid line, although it should be represented by successive steps (see section 2.2.). The scattered light was collected at 90°. The index of polydispersity remained lower than 0.1 until time 2000 min, and then lower than 0.2. The time of appearance of visible crystals (the dimensions of which exceeded 20–30 μm) is shown.

tion were carried out. It suggests that the protein solution at the beginning of the experiment is essentially composed of non-interacting free-lysozyme molecules. The distribution of species within the solution remained rather low as indicated by the values of ν. This latter parameter was always lower than 0.1. Indeed, the autocorrelation function analysed by a single exponential fitting procedure yielded similar decay times with a 2.5% average difference. Once the final temperature (−2°C) was set for 300 ± 40 min, the apparent radius kept on increasing up to its maximum value and attained it at a time called t_{max} (i.e. $t_{max} = 630 \pm 40$ min). Then, the radius started to vary and to tend towards its initial value. About 700 ± 100 min after $R_{h,app}$ had attained its maximum value at a time called t_{cryst} (i.e. $t_{cryst} = 1330 \pm 140$ min), a pattern of light and dark rings was observed on a screen, placed perpendicular to the incident beam and after it had gone through the scattering cell. This typical diffraction pattern indicated the presence of large particles which were then proved to be protein crystals of about 30 μm long by examination with a microscope. These protein crystals grew up to about 150–200 μm length within two days. Examination under an optical microscope showed that they had exactly the same morphology as that of the tetragonal crystal form of lysozyme, commonly obtained from sodium chloride solutions at a temperature lower than 25°C [13]. To avoid scattering from static crystals which grew on the glass wall of the cell, much care was exercised: to perform measurements, the cell was rotated and set in a position where the incident laser beam did not fall upon protein crystals. Measurements at 45° were then impossible. Nevertheless, at the end of the experiment, some static scattering coming from the crystals could not be totally avoided, leading to an eventual heterodyning of the signal. Hence, the observed trend should be taken as a qualitative one. The variance increased but remained lower than 0.2. When

measurable, data collected at 45° are shown to follow the same trend as that obtained at a 90° scattering angle.

As revealed by concentration determination (fig.2), a slight significant change of protein concentration was observed at about time 900 ± 60 min, that is, 4 h after t_{max}. To check if the maximum of the apparent size of the scatterers corresponded to lysozyme crystal critical nuclei, an experiment was performed with the same solution but cooled down to −5°C according to the same procedure, leading to an increase of supersaturation. The relative amplitude of the apparent size of the scatterers $\Delta R_{h,app}/R_{h,app,i} = (R_{h,app,m} - R_{h,app,i})/R_{h,app,i}$ became 0.75 versus 0.55 in the preceding experiments (where $R_{h,app,m}$ and $R_{h,app,i}$ are the maximum and initial value of the apparent hydrodynamic radius of the scatterers, respectively). The value of t_{cryst} was shortened to about 700 min as well. This indicates that $R_{h,app,m}$ might not only correspond to the critical size of a protein crystal nucleus, since it increased with supersaturation. Indeed, nucleation theory [2] predicts a smaller critical nucleus with an increased supersaturation. Actually, the variations of $R_{app,h}$ might reflect both molecular associations, including the formation of critical nuclei and interactions between molecules. Viscosity measurements showed that the ratio of the viscosity of the protein solution to that of the solvent remained constant as a function of temperature. This could indicate that no numerous large aggregates were present in the solution as supersaturation proceeded, because viscosity is sensitive to changes of hydrodynamic properties.

Intensity of the scattered light, collected at 90°, increased slightly at time 300 min. Significant variations of both apparent radius and scattered light were detected before the concentration of lysozyme had decreased (figs 1 and 2). Dynamic light scattering proves able to monitor an increase and then a decrease of the apparent radius as a function of time before crystals had reached a size observable with a conventional microscope. The diminution of the apparent size is probably accounted for by the decrease of interacions between proteins (decrease of supersaturation) as lysozyme molecules joined the crystallites. The first indication in support of this interpretation is found in the measured decrease of the protein concentration in the solution (fig.2).

Crystals sometimes grew on the glass cell. Different coatings were spread on the scattering cell so as to reduce heterogenous nucleation, but without success. However, the crystals seemed to appear simultaneously whether they grew on the glass walls of the scattering cell or out of the bulk of the solution.

Very few studies have been devoted to the mechanism of protein crystal nucleation and growth. From crystal growth theory [14], the critical nuclei radius of α-chymotrypsinogen crystal was suggested to be about

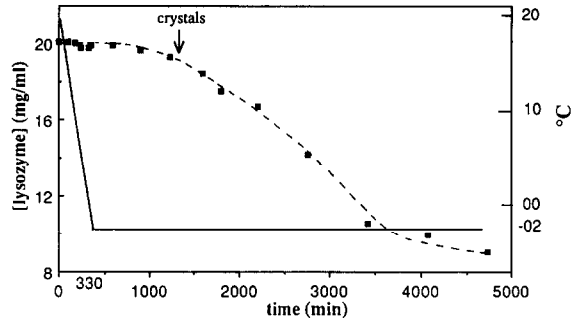

Fig.2. Variations of the concentration of lysozyme as a function of time, during a crystallization experiment induced by temperature lowering. The temperature variations are indicated as in fig.1.

120 Å and it was noticed to correspond approximately to the size of the smaller globules observed from electron microphotographs [15]. Since this protein molecule has an approximate radius of 20 Å, the critical nucleus might comprise about one hundred molecules. In the present study, quasi-elastic light scattering measurements did not provide indication of a large number of such an assembly of lysozyme molecules. At the degrees of supersaturation investigated, we suggest that for lysozyme crystallization, the nucleation phase is a rapid process with respect to growth and that a critical nucleus could only be composed of a few molecules. Taking into account the narrow distribution of species estimated experimentally in the crystallizing solution, we propose that crystal growth proceeds by attachment of either free-lysozyme molecules or very small aggregates to the crystal. Indeed, if growth proceeded by incorporation of large aggregates, they would have been detected because half of the protein molecules joined the crystals (fig.2).

Dynamic light scattering is proved to be a valuable tool to investigate the protein crystallization process. It was recently demonstrated with ribonuclease A as a model system, that fluorescence anisotropy can also be useful to investigate the early steps of crystallization [16]. But, here, light scattering seems to provide further information. It is able to monitor an increase and then a decrease of the apparent size of scatterers, before detection of any protein concentration change and appearance of crystals, observable by a conventional microscope. It may become a useful probe to dynamically control supersaturation in order to uncouple nucleation and growth. Hence, robots may use dynamic light scattering to monitor crystallization. Further work is in progress to analyse the particle distribution and the behaviour of lysozyme molecules within the crystallizing solution in more detail.

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