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ARTICLE

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Lysozyme crystal growth, as observed by small angle X-ray scattering, proceeds without crystallization intermediates

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Abstract A combination of small angle X-ray scattering and gel techniques was used to follow the kinetics of protein crystal growth as a function of time. Hen egg white lysozyme, at different protein concentrations, was used as a model system. A new sample holder was designed, in which supersaturation is induced in the presence of salt by decreasing the temperature. It had been shown previously that a decrease in temperature and/or an increase in crystallizing agent induces an increase in the attractive interactions present in the lysozyme solutions, the lysozyme remaining monomeric. In the present paper we show that similar behaviour is observed in NaCl when agarose gels are used. During crystal growth, special attention was paid to determine whether oligomers were formed as the protein in solution was incorporated in the newly formed crystals. From these first series of experiments, we did not find any indication of oligomer formation between monomer in solution and crystal. The results obtained are in agreement with the hypothesis that lysozyme crystals in NaCl grow by addition of monomeric particles.

Key words Crystallization kinetics · Small angle X-ray scattering · Lysozyme · Interactions

Introduction

The process of macromolecular nucleation and crystal growth is still not well understood, although a variety of investigations have been conducted (Kam et al. 1978; Riès-Kautt and Ducruix 1989; Wilson 1990; Pusey 1991; Guilloteau et al. 1992; Wilson 1992; Boué et al. 1993; Niimura et al. 1995; Muschol and Rosenberger 1995; Ducruix et al. 1996). The analysis of Hen egg white (HEW) lysozyme in solution, as observed with small angle X-ray scattering (SAXS), demonstrated that at pH 4.5 in acetate buffer, lysozyme is monomeric and presents overall repulsive interactions. The addition of salt, which screens the charges, progressively leads to attractive interactions, even when crossing the solubility curve (before the beginning of crystallization). The attractive potential is reinforced by decreasing the temperature. Comparison with solubility curves, measured as a function of salt type and concentration, demonstrated that crystallization occurs in the attractive regime. The strength of the attraction follows, like the solubility, the reverse order of the Hoffmeister series (Riès-Kautt and Ducruix 1989). In order to further analyse what happens with time in a supersaturated lysozyme solution where crystallization occurs, agarose gels were used in combination with SAXS (Bonneté et al. 1996). Such gels, which suppress convection, are known to favor the nucleation of good quality crystals (Robert and Lefaucheux 1988; Robert et al. 1992). Our purpose, however, was to trap the newly formed nucleation centers in the gel network so that they remain at the same place during crystal growth. Since the same amount of material stays in the X-ray beam at all times, it becomes possible to follow the kinetics of crystal growth as a function of time. In previous experiments in capillary tubes (Bonneté et al. 1996), supersaturation of lysozyme (45 mg/ml) in NaCl (0.3–0.6 M) was induced by lowering the temperature. Conditions were found where crystals good enough to give Bragg reflections at small angles could be obtained in a few hours.

In the present study, a new sample holder was used in order to improve the temperature control and prevent radiation damage previously encountered (samples irradiated for too long were no longer able to crystallize). The sample holder was designed with the idea that to record spectra on the same sample as a function of time is equivalent to recording spectra on different samples, so long as the different samples are identical, with identical histories. The result is a multi-cell container, placed in a thermostat-

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ted sample holder. The system is motorized and remotely controlled with a PC. In a series of control experiments, the lysozyme association state and interactions in solution in the gel were measured as a function of temperature prior to crystallization and were compared with lysozyme interactions in solution under equivalent conditions. The X-ray spectra were put on the same intensity scale and analysed with Guinier plots. As in solution, the lysozyme was found to be monomeric, with attractive interactions increasing with decreasing temperature. In another series of X-ray experiments, the kinetics of crystal growth were followed. Optical microscopy was used to define the salt and temperature conditions under which a high and homogeneous density of crystal nucleation begins within about half an hour, and crystal growth then regularly proceeds for some hours. Special attention was paid to find conditions where about 50% of the protein initially in solution is incorporated in the crystals at the end. Therefore, the X-ray signal arising from the protein in solution decreases when the protein is incorporated in the crystals during crystal growth. The amount of protein remaining in solution can thus be deduced from the X-ray spectra. Furthermore, the evolution with time of the protein interactions could be analysed from Guinier plots. Data obtained in several studies of lysozyme nucleation and crystal growth using other techniques have been interpreted as indicating the formation of aggregates, or of oligomeric species, between monomer and crystal. The whole of our experimental results, however, and especially the comparison between solutions and gels, can be quantitatively interpreted on the basis of attractive interactions between lysozyme monomers. No sign of crystallization intermediates between monomers and crystals could be observed during the process of lysozyme crystal growth.

Materials and methods

Gel and protein solutions

All chemicals were purchased from Sigma. Two batches of HEW lysozyme, 3 times crystallized, dialyzed and lyophilyzed were used (Sigma n° 111H7010 and n° 65H7025). The second one contained a large amount of residual salt and was desalted before the X-ray experiments using strong cation- and anion-exchange resins (Biorad AG 50W-X8 20–50 mesh, H^+ form and AG 1-X8 20–50 mesh, OH^- form) as described by Riès-Kautt and Ducruix (1994). The pH of the protein was verified to be close to its isoionic point, i. e. pH 11.3. The protein solution was then buffered to obtain a 50 mM NaOAc/AcOH buffer at pH 4.5. pH values were measured with a Sentron pH system and the protein concentration adjusted and checked by absorbance measurements at 280 nm in Hellma cells (0.2, 0.5 or 1 cm) with a UV spectrophotometer Perkin-Elmer Lambda Bio. The absorbance of a 1 mg/ml lysozyme solution in a 1 cm Hellma cell was known to be 2.66 at 280 nm. For all preparations, the water was obtained from an UHQ-II device.

A stock solution of lysozyme at a concentration between 300 and 400 mg/ml was prepared in sodium acetate 50 mM pH 4.5. The stock solution was then diluted with various salt solutions (NaCl, NaOAc) to obtain the different protein and salt concentrations required for the solution experiments, Agarose type V (gel point 42 °C) was prepared using the method developed by Robert and Lefaucheux (1988). As in previous experiments (Bonneté et al. 1996) a final agarose concentration of 0.1% wt/vol was used. The lysozyme solutions in gel are prepared by mixing at 42 °C the agarose gel and the protein solution The mixture gelifies in a few minutes upon decreasing the temperature.

Sample holder and plate preparation for kinetics measurements

A new sample holder was especially designed (Fig. 1) at the Laboratoire de Minéralogie-Cristallographie (L. M. C. P.) for the instrument D 24 at the synchrotron radiation facility L. U. R. E. (Orsay). Thanks to the viscosity of the gel, a very simple container can be used. It consists of a plexiglas plate with 25 holes to be filled with gel or with lysozyme gel, closed by two mica windows and inserted between two thermostatted jaws. The system is driven by motors and computer controlled. A simple subroutine indicates which holes, in which order and when, are observed with X-rays. The tricky part of the experiment is to fill the holes of the plexiglass plate with the lysozyme solution, previously mixed at 42 °C with agarose gel, without making bubbles. In the present experiment, some holes were filled with the mixture of lysozyme and gel and some only with gel in order to use the latter to determine the background. Uniaxial mica sheets are convenient to close the plates since they are rigid and transparent. Moreover, they can be cleaved easily and do not diffract X-rays. Each row of holes is isolated from its neighbours with grease, which also serves to attach the sheets of mica to the plate. Once the agarose is gelified, the plexiglass plates can be manipulated easily. Usually, as soon as the filling of the plate is complete, it is immediately inserted between the thermostatted jaws and equilibrated at the desired temperature.

Choice of the crystallization conditions with optical microscopy

Various experimental conditions – nature and concentration of salts, supersaturation β of lysozyme ($\beta = c/s$, c = lysozyme concentration, s = solubility) – inferred from published solubility curves (Howard et al. 1988; Pusey and Gernert 1988; Riès-Kautt and Ducruix 1989; Cacioppo et al. 1991; Guilloteau et al. 1992), were tested to find out which ones were able to give a sufficient density of large enough crystals within a few hours (the duration of one run at L. U. R. E. is 24 hours). The high nucleation density is required to ensure that the part of the sample in the X-ray beam (2 mm×0.5 mm) is representative. A sufficient crystal size at the end of the experiment is required to ensure

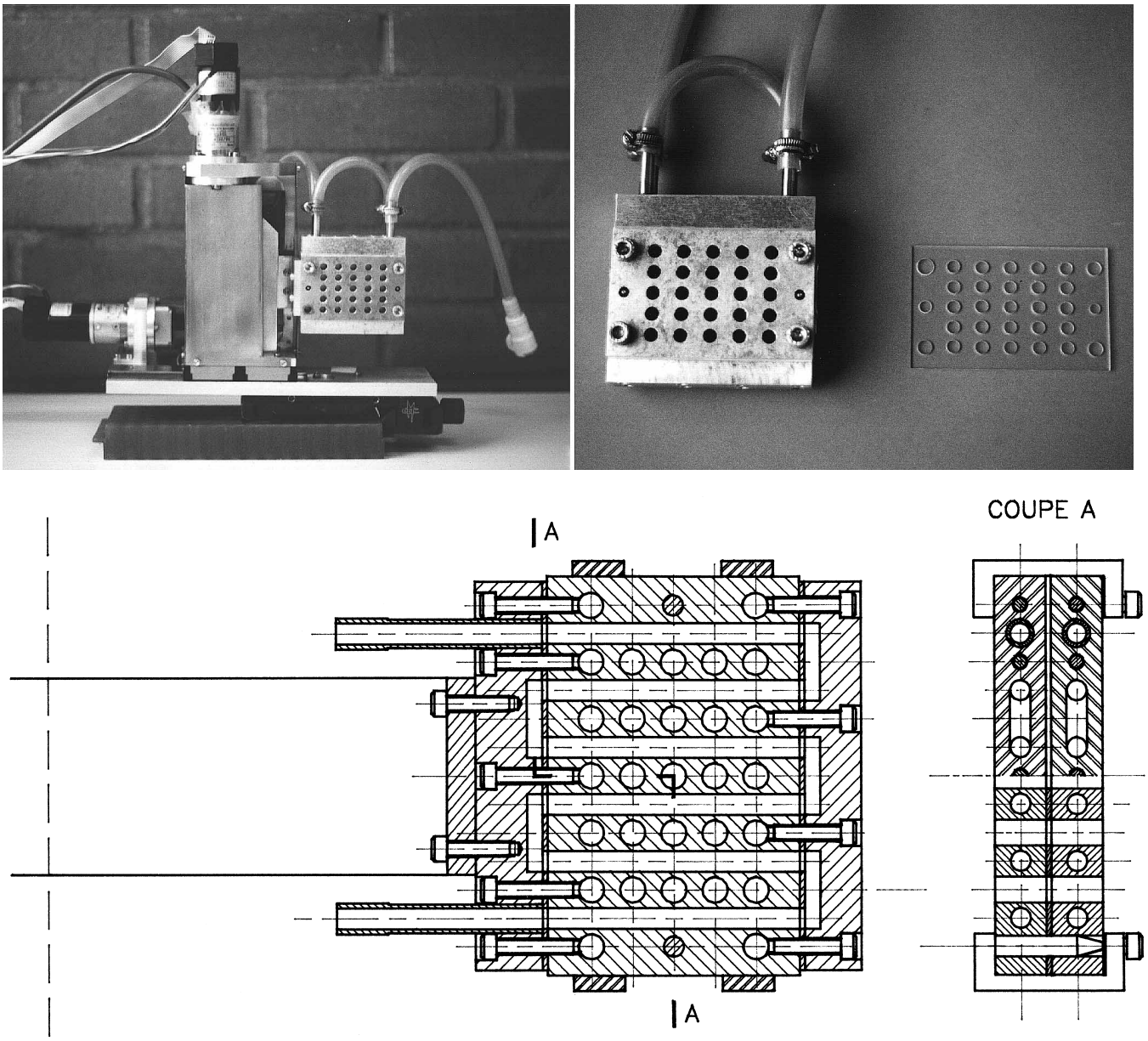

Fig. 1 Thermostatted sample holder with its motors (on the *left*). Thermostatted jaws with plexiglass plate (on the *right*) and corresponding line drawing. The line drawing for the motor part is available on request

that a large fraction of the protein which was initially in solution is now incorporated into crystals.

Using the same sample holder, optical observations were performed at regular periods of time (Fig. 2) to determine under different conditions the time required for crystals to become visible (the smallest observable crystal size was 10 μm) and to follow their growth. For each observation, crystals were numbered in the different holes and their size measured in two directions perpendicular to the observation direction, corresponding to the smallest dimension of the crystal. A mean nucleation rate and a mean characteristic crystal size in each hole were calculated. Using the space group, $P4_32_12$ for lysozyme crystallised in the presence of NaCl, the lattice parameters, a = b = 78.54, c = 37.77, and the HEW lysozyme molecular weight, 14 300 Da, an estimation of the mean protein concentration remaining in solution could then be deduced.

Small angle X-ray scattering experiments

Experiments were carried out using the small angle instrument D24 (Depautex et al. 1987) at the Laboratory for Synchrotron Radiation, L. U. R. E. (Orsay) where measurements can be performed in a short time, typically in a few minutes. The experimental conditions were the same as in previous experiments (Bonneté et al. 1996). The sample to detector distances were 1324 and 1235 mm for solutions and gels respectively. The angular increment ds/channel

were therefore 2.26×10^{-4} and 2.44×10^{-4} Å^{-1} respectively. The solution experiments were performed with a specially designed quartz cell operating in a vacuum (Dubuisson et al. 1997). All the experiments in agarose gels were made with the new sample holder described above. Sample exposure times were from 2 to 5 min, depending upon protein concentration, and buffer exposure time was 5 min.

The intensity curves, I(c,s), measured as a function of the scattering vector s, where $s = 2\sin\theta/\lambda$ and 2θ is the scattering angle, were subtracted for background and scaled on the same relative value with normalisation for concentration, c, and direct beam intensity (Luzzati and Tardieu 1980).

The intensity curves, I(c,s), are the product of two terms:

$$I(c,s) = I(0,s) \cdot S(c,s)$$

where the form factor, I(0,s), is the sum of the scattering of the individual particles (Luzzati and Tardieu 1980) and the structure factor S(c,s) is related to the particle distri-

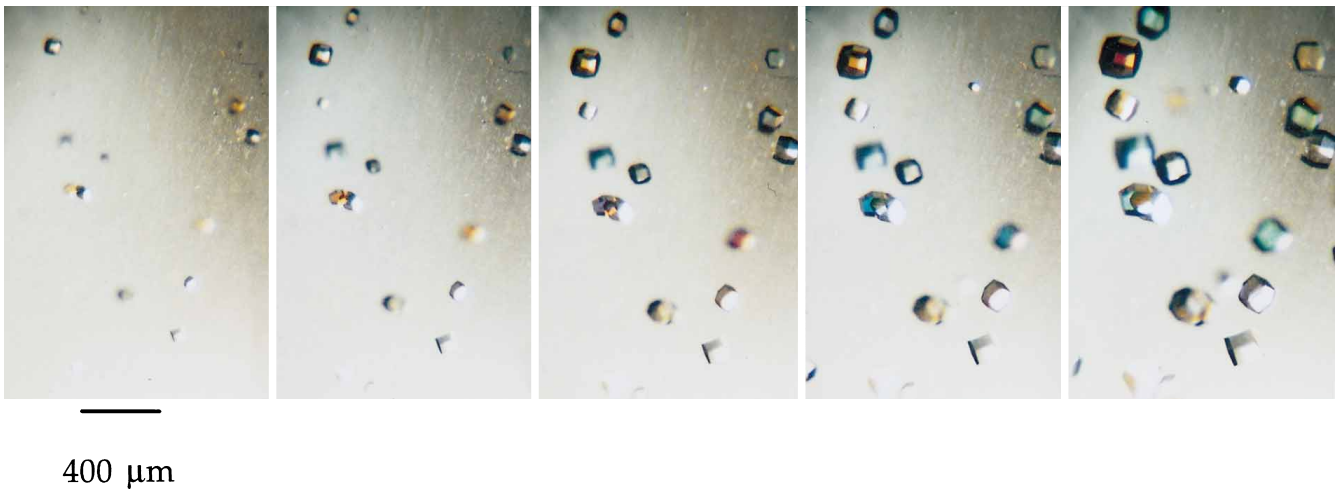

Fig. 2 Kinetics of lysozyme crystal growth (40 mg/ml, 0.5 M NaCl, 50 mM acetate, pH 4.5, 0,1% agarose gel, 10 °C), as observed with optical microscope. From left to right, the pictures correspond to 2 h, 3 h, 4 h, 5 h and 7 h. 1 cm = 400 µm

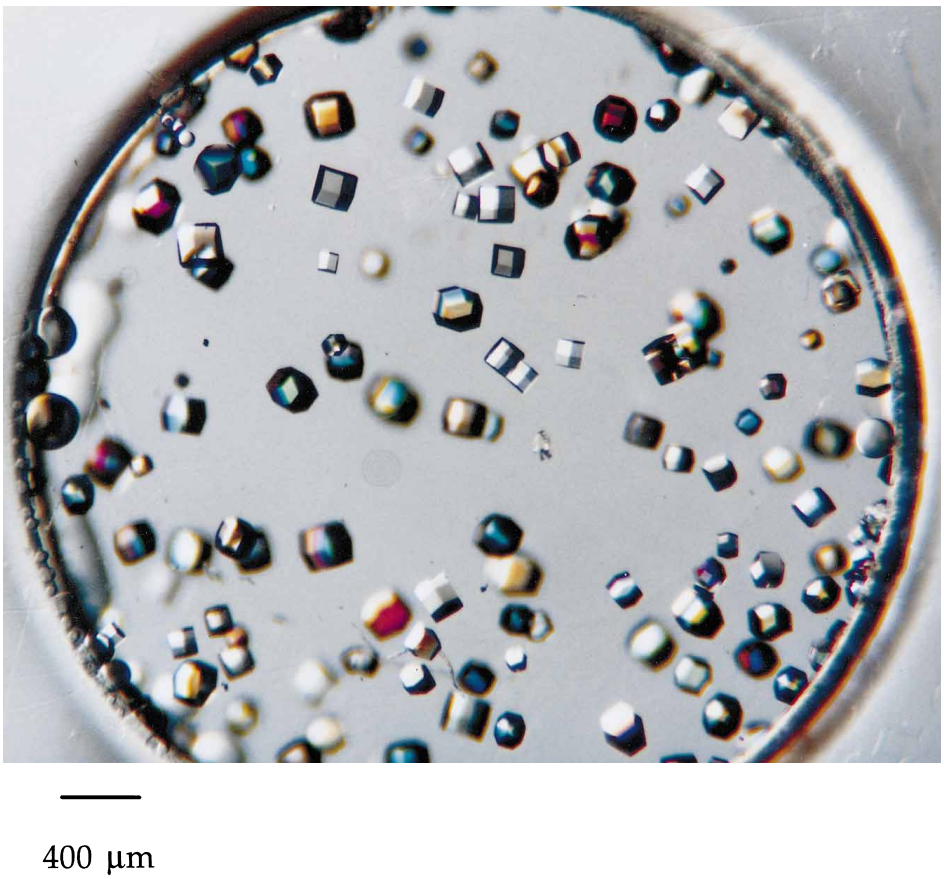

Fig. 4 Crystals of lysozyme (40 mg/ml, 0.5 M NaCl, 50 mM acetate, pH 4.5, 0.1% agarose gel) in a hole (diameter 5 mm) of the plexiglass plate after 10 hours in the thermostatted holder at 10 °C

bution in solution (Tardieu 1994). With repulsive or attractive interactions, S(c,0) is respectively lower or higher than 1. With ideal solutions, i. e. in the absence of interactions, the intensity obtained, I(0,s), can be analysed in terms of the radius of gyration of the particle, R_g, by using the Guinier approximation (Guinier and Fournet 1955):

$$I(0,s) = I(0,0) \cdot \exp(-4\pi^2 R_g^2 s^2/3)$$

R_g is obtained from a linear fit of Log I(0,s) as a function of s^2. By extension to solutions with attractive interactions, where Log I(c,s) usually shows a linear variation with s^2, the intensity was written as:

$$I(c,s) = I(c,0) \cdot \exp(-4\pi^2 R_{ga}^2 s^2/3 + \ldots)$$

where R_{ga} is an apparent radius of gyration which depends upon both the form of the particle and the particle interactions. Another advantage of this formulation is to give a direct determination of I(c,0) by extrapolation of the Guinier plot as of function of the concentration c.

Lysozyme concentration measurement after the kinetic experiment

Immediately after the kinetic experiment, the concentration of the protein remaining in solution was determined biochemically. The content of each hole (lysozyme in solution, crystals and agarose gel) is removed with a pipetman, put in a Micropure separator (Amicon) and centrifuged for one minute at 14 000 g. The filtrate is diluted with acetate buffer and used for an optical density measurement at 280 nm.

Results and discussion

Crystallization conditions

The challenge was to find conditions in which a high and homogeneous density of nucleation could be obtained, yet leading to good quality crystals, in a few hours. For instance, the protein concentration had to be high enough to follow protein interactions with X-rays, but low enough to be compatible with good quality. Since many studies including our previous experiments (Bonneté et al. 1996) had already been performed with monovalent salts, sodium chloride, sodium acetate and sodium nitrate were first tested as crystallizing agents. Series of plates were prepared at different protein and salt concentrations at 8 °C. In all cases, rapid nucleation was obtained when the supersaturation was varied from about 1 to a value between 10 and 20 by lowering the temperature (Table 1). The example shown in Fig. 3 a (HEWL 40 mg/ml, NaCl 0.5 M, 10 °C) is typical of conditions acceptable for further scattering experiments. At the temperature of 10 °C, the nucleation occurred in less than half an hour, whereas it takes a few days at room temperature. The density of crystals is uniform in the hole and high enough (4–5 crystals per mm^2) so that

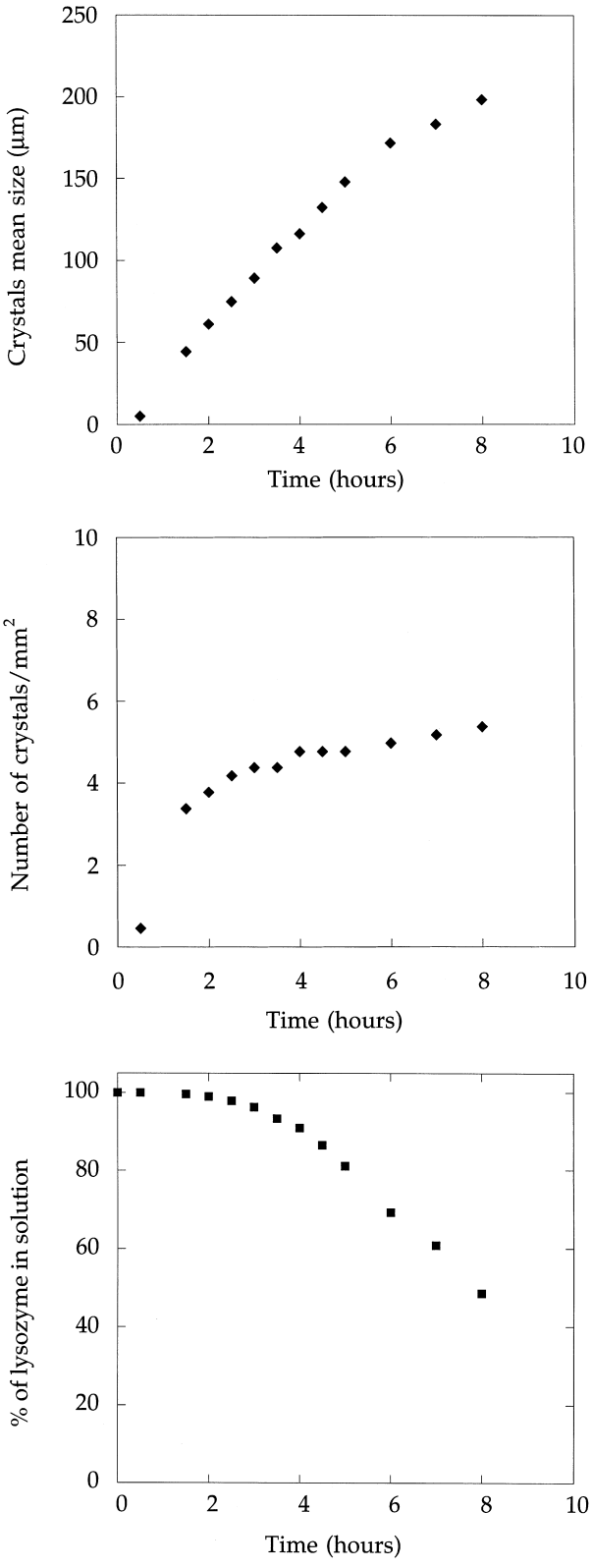

Fig. 3 **a** Evolution as a function of time of lysozyme crystal size (initial concentration 40 mg/ml) in NaCl 0.5 M acetate buffer 50 mM pH 4.5 in agarose gel at 0.1% at 10 °C. **b** Density of lysozyme crystals (initial concentration 40 mg/ml) in NaCl 0.5 M acetate buffer 50 mM pH 4.5 agarose gel 0.1% at 10 °C versus time. **c** Percentage of lysozyme remaining in solution as crystals grew, in NaCl 0.5 M, sodium acetate buffer 50 mM pH 4.5 at 10 °C

Table 1 Conditions of crystallization in agarose gel, at 8 °C in a cold room or 10 °C (*) with the sample holder

Salt	Molarity (mM)	Lysozyme (mg/ml)	Observations
NaCl	100	250	Strong nucleation after 20 hours
		180/90 *	No crystals after 10 days
	200	200	Strong nucleation after 20 hours
		90/54	No crystals after 10 days
	300	200 *	Crystals after 15 minutes
		80	Crystals after 20 hours
		54	Crystals after 2 days
	500	40 *	Crystals after 30 minutes
		20 *	Crystals after 4 hours
NaOAc	400	100 *	No crystals after 3 days
	850	200 *	Crystals after 45 minutes

what happens in the beam size reflects what happens in the whole hole (Fig. 3b). Furthermore, more than 70% of the crystals nucleated within the two first hours. The calculated decrease in protein concentration remaining in solution is of the order of 5% after 3 hours and 40% after 7 hours (Fig. 3c). The nucleation density, usually uniform throughout the sample (as shown in Fig. 4), can, of course, be modulated with the protein concentration and the temperature. The rate of growth varies at the same time. One advantage of the high supersaturation, between 10 or 20, which seems necessary for rapid nucleation, is that most of the nuclei are formed at the same time and subsequent time evolution is essentially growth. During the first hours, the crystal size varies linearly with time. Then, the rate of growth is reduced as the lysozyme concentration remaining in solution is decreased. After 20 hours, the growth process is stopped. From the linear dependance observed at the beginning of the experiment, it may be inferred that the growth process remains the same when growth proceeds. In the present experiments we did not try to exhaustively analyse the effects of protein and salt concentration or of temperature and we only give a brief account of trials in Table 1. The important point for us was to check that the gel technique was effective in allowing us to have a high and homogeneous density of nucleation (Fig. 4) and that the nucleation and the growth process were sufficiently separated in time to allow us to follow both processes independently. Conditions close to the conditions shown in Fig. 3(a–b) were adopted for the X-ray experiments.

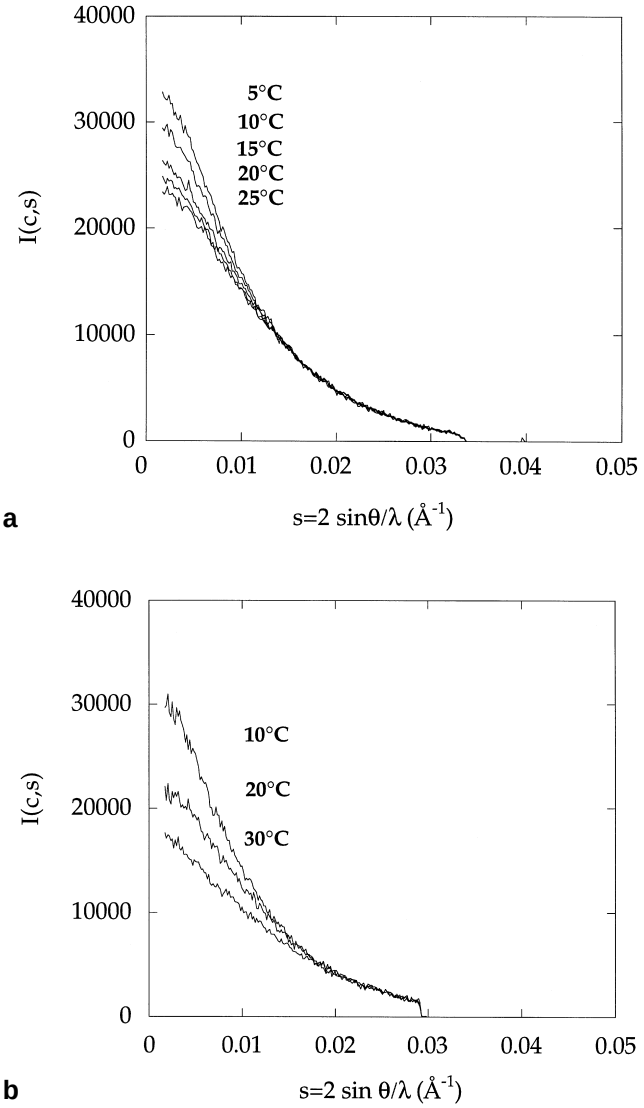

Fig. 5 **a** Scattered intensity of lysozyme solutions (40 mg/ml) in NaCl 0.5 M acetate buffer 50 mM pH 4.5 versus scattering angle as a function of temperature; intensities were substracted for background and put on a relative scale but were otherwise uncorrected for concentration. **b** Scattered intensities of lysozyme solutions (40 mg/ml) in NaCl 0.5 M acetate buffer 50 mM pH 4.5 in agarose gel 0.1% versus scattering angle as a function of temperature

Interactions in solution and in gel

After our initial studies (Guilloteau 1991), data have accumulated showing a close relationship between the protein interactions that can be studied in solution – in under and supersaturated conditions – and protein crystallization. The studies on lysozyme, either with light (Muschol and Rosenberger 1995, 1996; Retailleau 1996) or X-ray scattering (Ducruix et al. 1996 and unpublished results) demonstrated that the protein interactions change from repulsive in low ionic strengh buffers at pH 4.5 to more attractive (or less repulsive) regimes as the concentration of salt increases, leading to lysozyme crystallization. If such additives are now known to modify the protein interactions in solution, they are not the only ones; e. g. the addition of D_2O plays a similar role (Gripon et al. 1996). Changing the pH to come closer to the isoelectric point decreases the protein charge and decreases the repulsive interactions (Retailleau 1996; Bonneté et al. 1997). Decreasing the temperature is another way to render lysozyme solutions more attractive. In a previous study, the temperature effect was shown to be due to the temperature dependence of a short range van der Waals attractive potential which, with small compact proteins, may eventually lead at high protein concentration to a metastable phase sep-

aration (Malfois et al. 1996; Lomakin et al. 1996). At lower protein concentration, the effect is well correlated with the decrease in lysozyme solubility when decreasing the temperature (Guilloteau et al. 1992, Gripon et al. 1996).

We therefore decided to induce crystallization by lowering the temperature from 25 °C to 8° – 10 °C. For the sake of comparison, the variation of the interactions with temperature were measured in the conditions chosen for the kinetic experiments, i. e. 40 mg/ml lysozyme solutions at 0.5 M NaCl, 50 mM sodium acetate, pH 4.5.

The results are given in Fig. 5 a. The intensity at low angles increases as the temperature decreases, as expected with a van der Waals potential, whereas the intensity remains unchanged at larger angles where the signal represents the form factor. Of course the temperature decrease and the X-ray exposure had to be made in a few minutes, before the onset of crystallization. We also checked, since it had not been done before, whether the lysozyme interactions were the same in solution and in the gel used to do the kinetic experiment. To make the comparison, X-ray spectra of lysozyme gels were recorded with the new sample holder immediately after the sample preparation in order to avoid crystallization. As can be seen in Fig. 5 b, the X-ray spectra recorded in the gel are similar to those recorded in solution, with the same temperature effect. The radii of gyration in the gel (Fig. 6) calculated from Guinier plots increase with decreasing temperature from 30 °C to 10 °C, from 18 Å up to 25 Å at 40 mg/ml and from 17 Å to 22 Å at 30 mg/ml. These variations are similar to those found in solution. Nevertheless radii of gyration in the gel are found to be slightly higher than in solution as the temperature decreases. We found it difficult, however, to exactly reproduce the absolute values of the intensities from one preparation to another since the few per cent uncertainties inevitable in the protein and salt concentration determinations were sufficient to change the starting point. The relative intensity variations as a function of temperature, salt and protein concentrations were always the same. On average, the lysozyme solution in the gel behaved like a lysozyme solution of slightly higher lysozyme concentration.

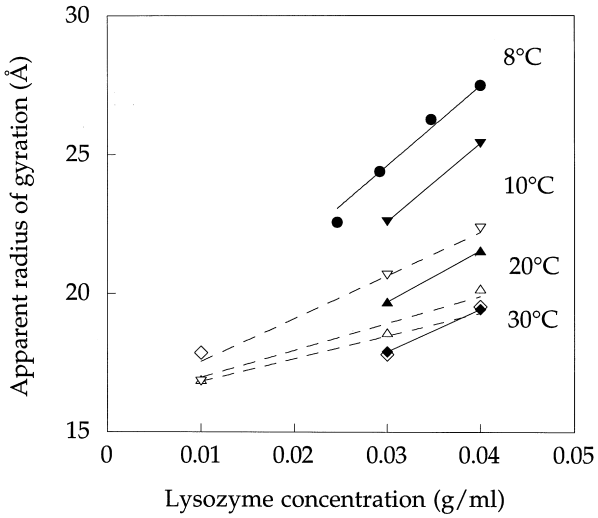

Fig. 6 Variation of apparent radii of gyration, obtained from Guinier plots of normalized scattered intensities, versus lysozyme concentration in static experiments at different temperatures (10 °C ▼, 20 °C ▲, 30 °C ◆), in gel (full symbols), in solution (empty symbols), and in a kinetic experiment at 8 °C (●)

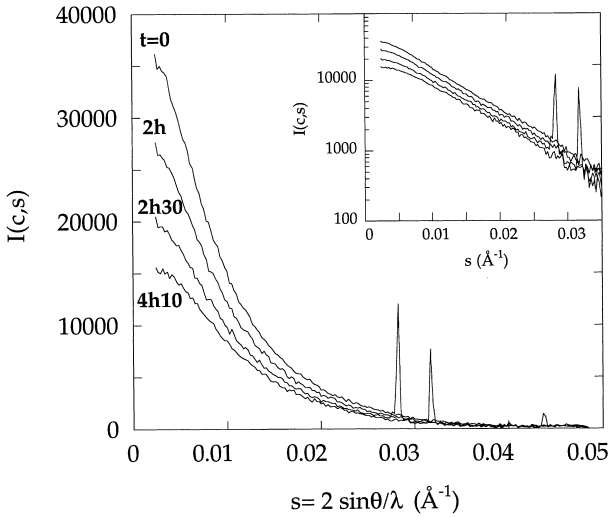

Fig. 7 Scattered intensity of lysozyme solutions (initial concentration 40 mg/ml) in agarose gel 0.1%, NaCl 0.5 M, acetate buffer 50 mM pH 4.5 at 10 °C as a function of time

X-ray kinetic experiment

Thus far, we have been able to record 3 series of spectra. We have not tried to record reliable X-ray spectra during the first half hour, during which nucleation occurs. The series in Fig. 7 was chosen since it is the most representative and, in addition, some Bragg peaks of the newly formed crystals are visible. We found it difficult to exactly reproduce the experimental intensities from one series to another, since a change in supersaturation of a few units is sufficient to induce slight changes in crystal nucleation density and growth rate. We could however reproducibly observe the following. At time zero, the spectrum observed is typical of a lysozyme solution in gel in the attractive regime. After some hours (2 h in Fig. 7) the intensity has significantly decreased, reflecting both the decrease of the lysozyme concentration in solution as crystals grew and, consequently, the decrease of the interaction effect. Lysozyme crystal growth is indicated by the presence of some Bragg peaks. When plotted on a Log scale (insert in Fig. 7), the intensity curves recorded as a function of time remain parallel to each other at high angles, in agreement with the hypothesis that the form factor remains the same and that the high angle intensity reflects the change in protein concentration. The lysozyme concentration remaining in solution as a function of time was therefore calculated from the in-

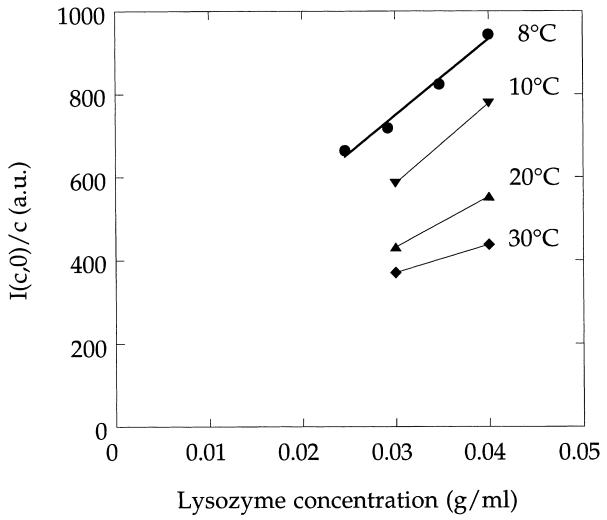

Fig. 8 Variation of the normalized scattered intensities at zero angle versus lysozyme gel concentration as a function of temperature (static experiments), and as a function of time (kinetic experiments at 8 °C)

tensities at large angles. According to Fig. 7, about 40% of the lysozyme has been incorporated in crystals in 4 hours. This value was found to be in good agreement with the biochemical measurements carried out after the kinetic measurements. Guinier plots were used to obtain extrapolated intensities at the origin, I(0,s), and to calculate apparent radii of gyration, R_{ga}. These values, which had been obtained during the kinetic experiment as a function of time, were translated into values as a function of the lysozyme concentration remaining in solution by using the lysozyme concentrations calculated from the high angle intensities. This procedure allowed us to directly compare static and dynamic experiments. The radii of gyration obtained from the static solution experiments and those obtained from the static and dynamic gel experiments are compared in Fig. 6. The intensities at the origin are compared in Fig. 8. It can be seen in the figures that the extrapolated intensity at the origin and apparent radius of gyration in kinetic experiments closely follow their counterparts in static experiments, either in solution or in gel. Therefore, the two parameters simply reflect the difference in interactive behaviour expected with monomers when the protein concentration decreases. The similar behaviour of the measured intensities at the origin and radii of gyration, with protein concentration in solution or in gel experiments, or with time in kinetic experiments, clearly shows that the experimental data are in agreement with the hypothesis that crystals grow from the monomers in solution, without any stable intermediate oligomer formation between monomers and crystals. Indeed, if it were necessary to form oligomers before adding them to the growing crystals, we would expect in our conditions, where 40% of the protein switches from solution to crystals, that the amount of newly formed oligomers would be sufficient to induce a signal change at low angles. Therefore, the whole of our results support the hypothesis that lysozyme crystals grow by the addition of monomeric particles. We may mention that similar experiments on bovine pancreatic trypsin inhibitor (BPTI) are presently under way (S. Veesler, unpublished results). BPTI crystallizes from monodisperse, yet non monomeric solutions. The comparison of the results obtained with different proteins should, in future, allow us to establish which features are of general relevance in the protein field.

Conclusion

Small angle X-ray scattering and gel techniques were combined to follow the kinetics of protein crystal growth as a function of time. The temperature is a convenient parameter to induce supersaturation and in 0.5 M NaCl, it is possible to reproducibly obtain crystal nucleation and growth in a few hours. The lysozyme interactions in solution, and in solution in agarose gels, were followed as a function of temperature and protein concentration. They were demonstrated to be essentially the same. They correspond to monomeric lysozyme interacting through a short range attractive potential. In the presence of crystals, the interactions between the lysozyme remaining in solution were also found to be the same, and to vary in the same way as a function of the protein concentration present in solution. No crystallization intermediates could be observed by small angle X-ray scattering during lysozyme crystal growth.

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