ELSEVIER

Biophysical Chemistry 58 (1996) 355–363

Biophysical Chemistry

Small angle neutron scattering from lysozyme solutions in unsaturated and supersaturated states (SANS from lysozyme solutions)

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Received 20 April 1995; accepted 26 May 1995

Abstract

Small angle neutron scattering (SANS) method was used to study lysozyme solutions, with particular interest in an understanding of the crystallization process at the initial stage. It is found that (1) in the unsaturated solution, the protein molecules aggregate with a continuous increase in size when NaCl concentration is increased, and (2) in the supersaturated solution, an irreversible change, superimposed on the former process, occurs when the supersaturation is realized. These facts indicate the usefulness of SANS in detecting changes of protein molecules in solution on the nanometer scale. The reliability of the SANS results are indicated by (1) comparing them with those of small angle X-ray scattering (SAXS), and (2) comparing the effect of D_2O and H_2O as solvent. Since the interparticle interaction is essential in the crystallization process and a simple Guinier plot analysis is not allowed, a more rigorous framework of analyzing data with interference function is developed, through which both average interparticle distance and particle size are estimated.

Keywords: Small angle neutron scattering; Small angle X-ray scattering; Lysozyme; Pre-crystallization; Aggregates; Time evolution

1. Introduction

The success in the structure analysis of biological macromolecules using either X-ray or neutron depends on the availability of proper single crystals. However, protein single crystals have traditionally been grown in trial-and-error experiments. Rational design of the crystal growth based on basic understanding of the growth mechanism is more and more called for [1,2]. In particular, the understanding of the very initial stage of crystallization, the stage at which nucleation occurs and the crystals of nanometer size start growing, is important, since the crystalline form (symmetry) is determined. It is also highly probable that the size and perfection of the resulting macroscopic crystals are largely determined at this stage. In other words, this embryo stage is considered to determine the ultimate fate of the crystal.

The initial process of protein crystallization has usually been studied by light scattering [3–13], fluorescence anisotropy [14,15], and dialysis equilibrium [16,17] methods. It has been revealed for the first time that the SANS has splendid advantages in

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SSDI 0301-4622(95)00078-X

studying such a process. We also carried out SAXS to confirm the SANS measurements. Both results are reported and compared. The SANS results obtained in the presence of interaction between particles, that is unavoidable in the crystallization process, were interpreted by considering the interference function. We also compared the results in D_2O and H_2O as solvent to show that only little difference was found. Preliminary parts of this study have been already published [18,19].

2. Experiments and sample preparation

The SANS experiment was carried out by using SANS-U installed in the guide hall at JRR-3M of JAERI. Neutrons with a wavelength of 7.0 Å were used with a sample-to-detector distance of 4000 mm to cover the momentum transfer Q range between 0.005 and 0.1 $Å^{-1}$.

The SAXS experiment was carried out in order to establish the reliability of SANS results in two aspects: (1) interference phenomena of the small angle scattering from solution and (2) the effect of D_2O and H_2O as solvent. We have used the SAXS instrument installed at BL-10C beam line in the Photon Factory of KEK. X-rays with wavelength of 1.0 Å were used with a sample-to-detector distance of 1000 mm to cover the range of 0.01 and 0.17 $Å^{-1}$.

Ataka and Asai studied the crystallization kinetics of hen egg-white lysozyme where the concentration of uncrystallized lysozyme, normalized by its initial concentration c_0, is plotted as a function of time for different initial concentrations of lysozyme [20]. The un- or super-saturated conditions of lysozyme solutions were selected by referring to their results on equilibrium [21]. These information was used to determine the experimental conditions.

Hen egg-white lysozyme from Seikagaku Kogyo (purified by 6 times crystallization, and certificated by centrifugation and electrophoresis) was dissolved, as purchased, in either D_2O or H_2O solvent. The solution was treated to remove dust particles with membrane filters. Although the lysozyme solution so prepared was shown, from the crystal growth kinetics studies, to be purer than other preparations, it still contained some impurity that was difficult to identify by most analytical methods [22]. The protein concentration of the supernatant was determined spectrophotometrically. Since we will study the effect of 20 mM (0.12%) NaCl, the coexistence of other salts as a buffer should be avoided. Therefore, the pH of the solution was adjusted by adding about 10 μl 1 N HCl per ml solution and mixing rapidly. The necessary amount of HCl was pre-determined with the aid of a pH meter. Owing to the auto-buffering action of the protein, the pH value was kept constant within ± 0.01 pH units during the experiment.

The solutions which were used for SANS measurements are as follows: in the case of the unsaturated solutions, lysozyme concentration is 30 mg/ml, pH 4.7, temperature 18.1°C and NaCl concentration varies from 0 to 1.8 wt.-%. In the case of time evolution of crystal growth for one day and for 2 days, lysozyme concentration is 60 mg/ml and 40 mg/ml, respectively, pH is 4.6, temperature 18.0°C and NaCl concentration 3.0 wt.-%. As the control of time evolution, unsaturated solution of lysozyme of 20 mg/ml was tried, where NaCl concentration is 3.0 wt.-%, pH 4.6 and temperature 18.0°C.

3. Results and discussion

3.1. Changes in unsaturated solutions

Fig. 1 shows the SANS results from unsaturated solutions of lysozyme in D_2O at different sodium chloride concentrations. The lowest curve is the SANS from the lysozyme solution at the concentration of 30 mg/ml without salt. If sodium chloride was added to it even at small concentrations, the scattering intensity in the small Q region was enhanced, and the position of the peak shifted to the smaller Q values. It can be seen that the peak position moves rapidly when even a slight amount of the salt is added. If the salt concentration is increased beyond 1.8 wt.-%, crystals start to grow at this protein concentration. The result was quite reproducible in repeated experiments.

3.2. Interpretation of the SANS spectral profile: the interference function

In the Guinier region, SANS intensity is written by the formula (1),

$$Is(Q) = I(0)\exp\left(-\frac{R_g^2}{3}Q^2\right), \qquad (1)$$

where R_g and Q are the radius of gyration and momentum transfer, respectively.

When the interaction between particles is small, for example, in the case of dilute solution and $QR_g < 1$, the above formula is rigorously valid, and the scattering curve corresponds only to the structure form factor of the particle. When the Guinier plot is carried out, R_g can be obtained from the inclination angle of the slope. However, when the interparticle interaction is not negligibly small as in the crystallization process, the SANS intensity must be considered as the product of two quantities, the squared form factor of a particle itself and the interference function between particles. When the target of the study is the structure of the individual solute, we must, as a rule, avoid such interference by preparing a specimen in a proper manner. However, since the interaction between particles is the driving force of the crystallization process, we must attack the interference problem frontally if we would like to extend the SANS ability to such systems.

The concept of the interference function was first introduced by Ehrenfest [23], and afterwards it was applied for the analysis of the scattering function from the colloidal system [24–26]. The interference function is written as Eq. 2 [27]:

$$Y(Q;D,\sigma) = \frac{1 - H(Q,\sigma)\cdot\cos(Q\cdot D)}{1 - 2\cdot H(Q,\sigma)\cdot\cos(Q\cdot D) + H^2(Q,\sigma)} \quad (2)$$

where $H(Q,\sigma) = \exp(-Q^2\cdot\sigma^2)/4$ and D and σ are the preferred distance between particles and its fluctuation, respectively. The interference functions $Y(Q;D,\sigma)$ are demonstrated at different values of σ/D in the literature [25]. When σ/D is close to zero, the long range ordering of molecules exists and the system resembles a crystal. As the σ/D increases, the system becomes liquid-like and the interference function explains the appearance of the halo peak at around $QD = 2\pi$. Therefore, the average preferred distance between the first neighbor particles will be guessed from the peak position, but a more probable value should be obtained by the convolution of the squared form factor of a particle itself and the interference function between particles.

3.3. Appearance of a peak

Thus, the appearance of a peak in the SANS spectra in Fig. 1 was explained by the existence of a cos $(Q\cdot D)$ term in the interference function. The origin and its movement of a peak in the SANS spectra attracted interest for synthetic polymer solutions. In some cases, the variation of the peak position was ascribed to that of the shape of the structure form factor [28]. However, it must be emphasized that this mechanism cannot be applied to interpret Fig. 1. In our case, the form factor of a single lysozyme molecule should always be the same, and, nevertheless, the total scattering intensity changed greatly with the NaCl concentrations. This is the reason why we were led to consider the interference function, without which we could not explain the changes shown in Fig. 1.

3.4. SAXS results

In order to confirm the general relation between the peak position in small angle scattering and the preferred distance between particles, we have measured the SAXS of lysozyme solution without NaCl by changing the protein concentration. Fig. 2 shows

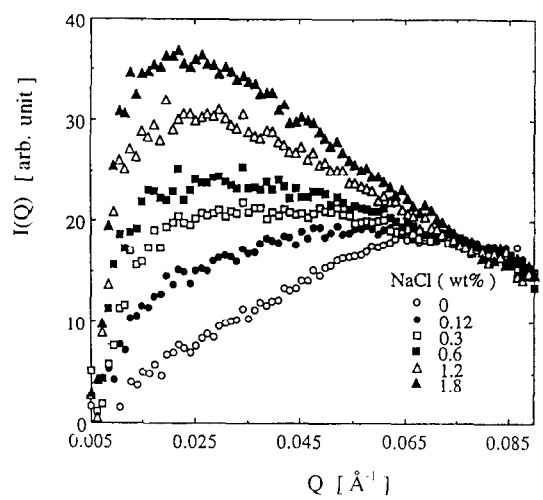


Fig. 1. SANS results from unsaturated solutions of lysozyme in D_2O. Various curves correspond to different sodium chloride concentrations. The lysozyme concentration and pH are fixed as 30 mg/ml and 4.7 in each measurement.

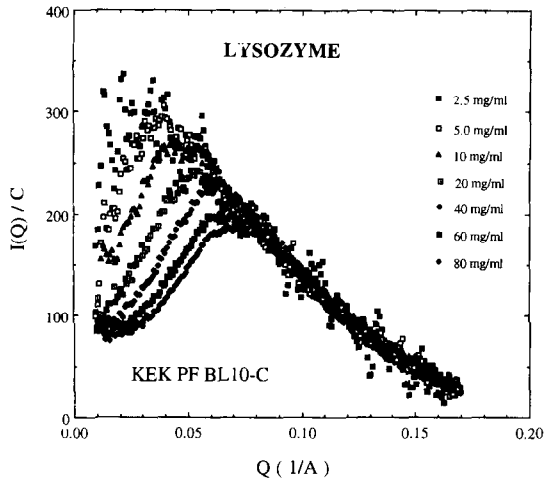


Fig. 2. SAXS of lysozyme solutions of different concentration. The intensity is normalized by the lysozyme concentration. Since the X-ray is strongly absorbed by salt, the solutions in this experiment contain no NaCl at all.

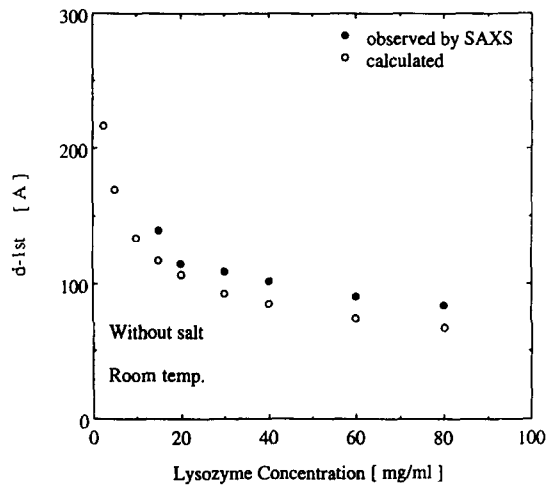


Fig. 3. The comparison of values related to the inter-molecular distances in the lysozyme solution estimated from the lysozyme concentration (○) and SAXS interference peak position (●).

the results, where the intensity is normalized by the lysozyme concentration. As the lysozyme concentration decreases, the peak shifts toward the low Q region. When the Guinier plot was exploited (not shown), the linear part of all the curves had the same slope in strong contrast with the case in Fig. 1. This means that the size of the particles does not change, and is identical with the monomer size when the concentration is varied in the absence of NaCl. Since we are allowed to consider the lysozyme molecules exist as monomers, we can estimate the interparticle distance by pure calculation from the number of lysozyme molecules per unit volume, if we assume that they are distributed homogeneously in the solution. The results are shown in Fig. 3. We have also determined D_{app} by dividing 2π by experimental values of peak position as a first approximation. The results are also indicated in Fig. 3. The values obtained in completely different ways are nearly identical. The results support the idea that the peak position is a proper measure of the real interparticle distances.

3.5. Interpretation of SANS results

We have tried to reproduce the SANS intensity profile of Fig. 1 by using the formula including the interference function. We tried a range of σ/D values between 0.4 and 0.9, and found that about 0.7 σ/D could reproduce the intensity profile well. We assume that the particle (lysozyme or its aggregates) is a uniform sphere with a radius R and the ratio σ/D is fixed to 0.7. The results are shown in Fig. 4, which explains the experimental results in Fig. 1 reasonably.

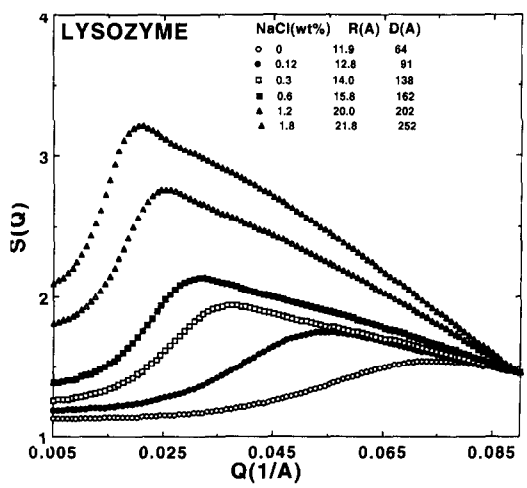


Fig. 4. The calculated SANS intensity by using the formula including the interference function. The particle (aggregate lysozyme) is assumed to be a uniform sphere, and the ratio σ/D is fixed to 0.7. The radius of particles, R, and the preferred distance between particles, D, were parameters used for curve fitting.

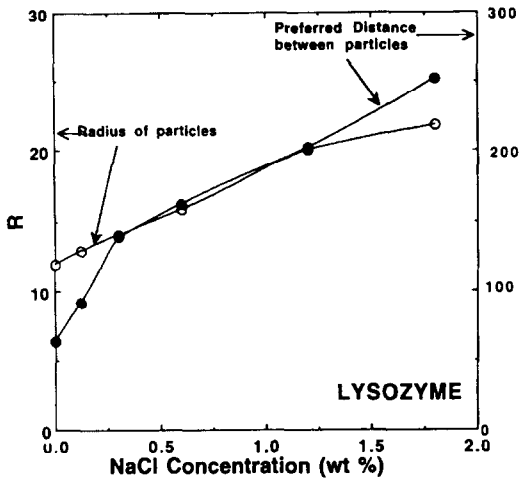


Fig. 5. Radius of particles and preferred distance between particles, which were calculated by using the formula including interference function, are plotted as a function of NaCl concentration (wt.-%). The apparent R_g obtained by the Guinier plot of Fig. 7 was overplotted.

The radius of the particles and preferred distance between particles thus obtained are plotted as a function of NaCl concentration (wt.-%) in Fig. 5. The results are interpreted as follows: by adding NaCl, lysozyme molecules start to aggregate. As NaCl concentration increases, the aggregation proceeds, and the radius of particles increases. As a result, the total number of particles (lysozyme aggregates) decreases, since the number of lysozyme molecules in solution is constant in the experiment. So, the preferred distance between particles increases and the peak of SANS shifts toward the low Q region. The argument is schematically illustrated in Fig. 6.

By using the dialysis equilibrium technique, Wilson et al. recently showed that the dimer and higher oligomers are progressively formed in lysozyme solutions when the solution condition approaches the solubility limit from the unsaturated side [17]. We consider that they have observed a similar phenomenon as we demonstrated in Figs. 5 and 6.

So far we have taken into account the effect of the interference function. However, if we simply carry out the Guinier plot of the data in Fig. 1, the results of Fig. 7 are obtained. Even in this case we get a straight line at each NaCl concentration as shown in Fig. 7. This means that the distribution of the size of aggregates does not scatter much. The apparent R_g's at each NaCl concentration obtained from the inclination angle of the slope are overplotted in Fig. 5. It is shown that the apparent R_g's are underestimated by about 10% if simply the Guinier plot is applied.

3.6. Time evolution of SANS: Changes in supersaturated solutions

As the NaCl concentration increases the lysozyme aggregation increases, even in the unsaturated solution. This phenomenon continues until and after the supersaturation is realized. However, aggregation in the unsaturated solution is in the equilibrium state. The SANS intensity change was measured every 15 min, and it is found that the intensity does not change with time as seen in Fig. 8(a).

On the other hand, as soon as the solution becomes supersaturated, SANS intensity changes with time as seen in Fig. 8(b), where the lysozyme concentration was 60 mg/ml. When the time evolution experiment with lysozyme concentration of 40 mg/ml was carried out, a similar result was obtained except that the change was slower. It clearly shows that the change in the solution starts to occur when the condition of the supersaturation is fulfilled. Crystals of a visible size were observed in these solutions one or two days after the preparation of the solution.

3.7. Effect of using D_2O as solvent

The results discussed so far are not the consequence of using D_2O as solvent. The following

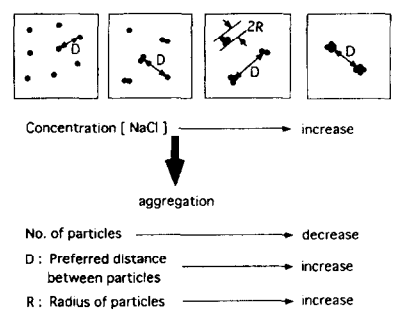
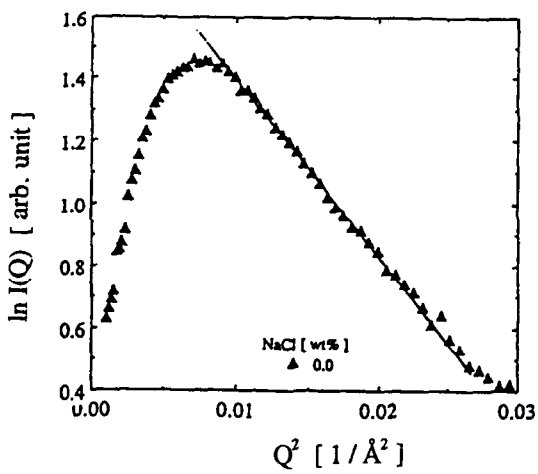
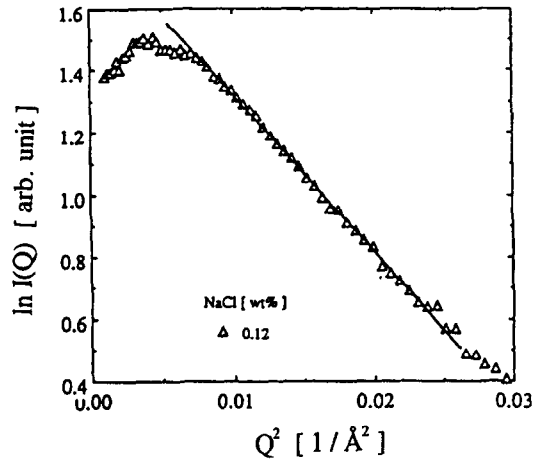
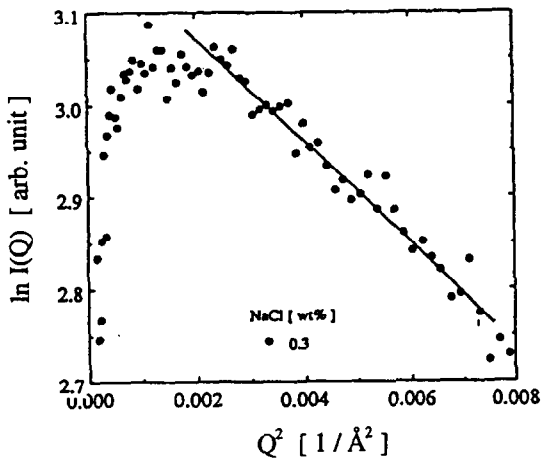
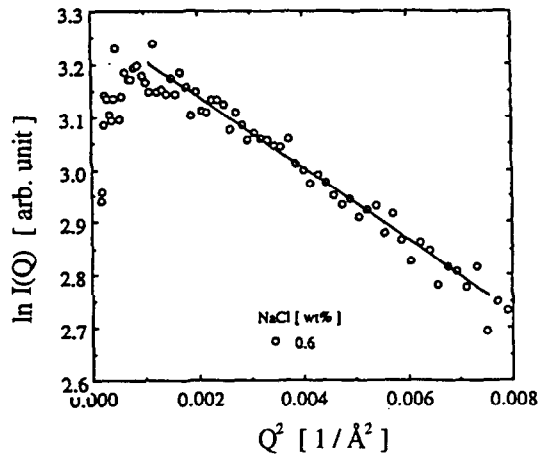


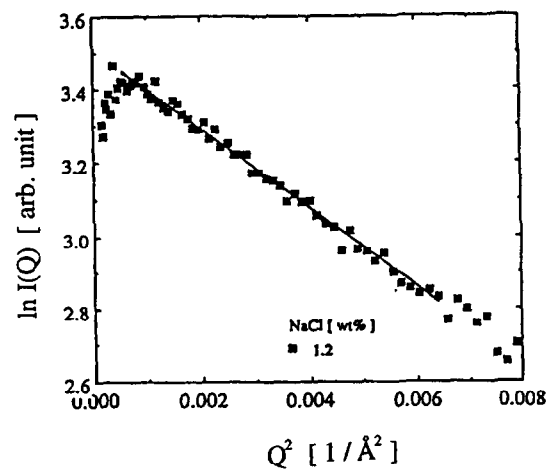
Fig. 6. Schematic illustration of the change of radii of particles and preferred distances between particles when NaCl concentration is increased in lysozyme solutions.

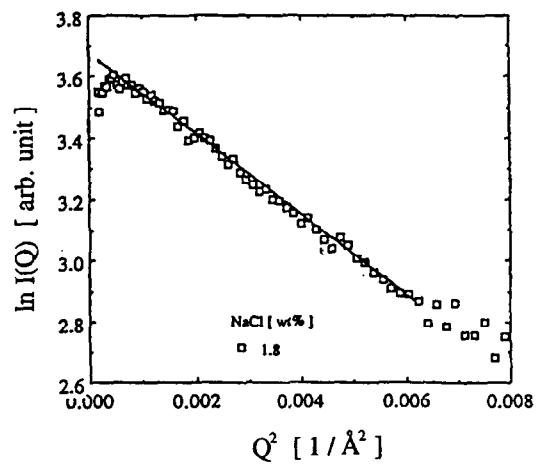

ln I(Q) [arb. unit]
NaCl [wt%]
0.0
Q² [1 / Å²]


ln I(Q) [arb. unit]
NaCl [wt%]
0.12
Q² [1 / Å²]


ln I(Q) [arb. unit]
NaCl [wt%]
0.3
Q² [1 / Å²]


ln I(Q) [arb. unit]
NaCl [wt%]
0.6
Q² [1 / Å²]


ln I(Q) [arb. unit]
NaCl [wt%]
1.2
Q² [1 / Å²]


ln I(Q) [arb. unit]
NaCl [wt%]
1.8
Q² [1 / Å²]

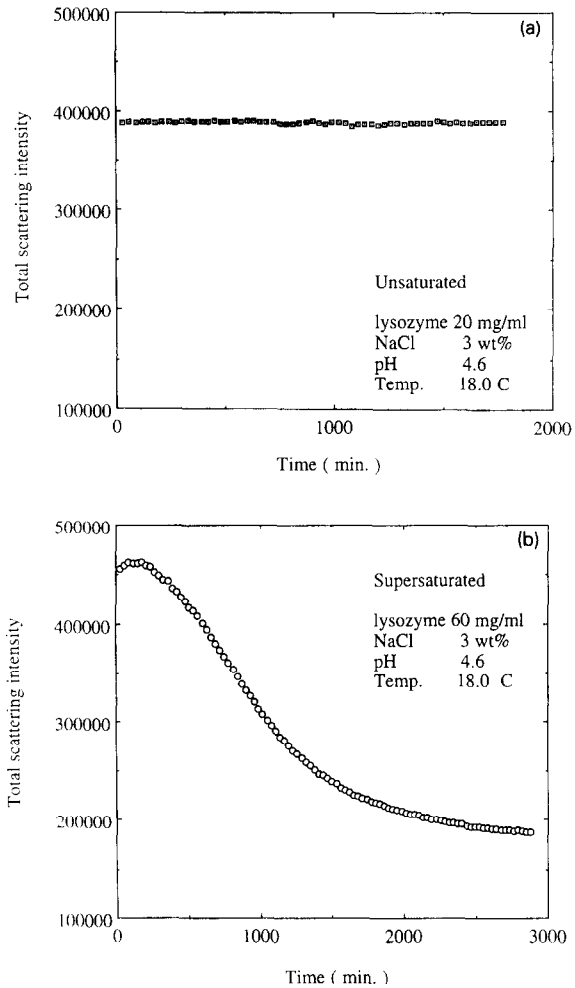


Fig. 8. The time evolution of the total SANS intensity from lysozyme solution, (a) in unsaturated solutions (lysozyme concentration and NaCl concentration are 20 mg/ml and 3.0 wt.-%, respectively) and (b) in supersaturated solutions (lysozyme concentration and NaCl concentration are 60 mg/ml and 3.0 wt.-%, respectively).

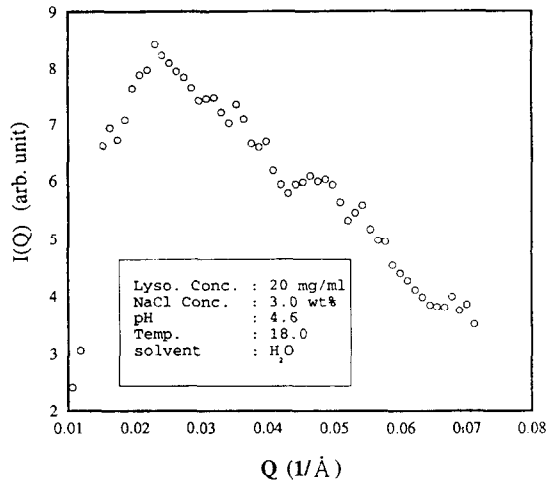


Fig. 9. SANS spectra of lysozyme solution in H_2O solvent, where lysozyme concentration, NaCl concentration, pH and temperature are 20 mg/ml, 3.0 wt.-%, 4.6 and 18.0°C, respectively.

experiments were carried out to reach this conclusion. (1) The crystal growth was observed both in D_2O and H_2O. The tetragonal form crystals of lysozyme grew similarly within an analogous time interval. (2) We carried out the SANS experiment by using H_2O (as in Fig. 9). The quality of the data was poor, due to incoherent background of H_2O, but a similar spectrum as in Fig. 1 with an apparent R_g of 24.5 Å which was larger by about 60% than the monomer value was obtained. (3) In H_2O, SAXS measurement to observe the effect of NaCl concentration in the dilute range was carried out (an example in Fig. 10). A similar peak shift as in Fig. 1 was again confirmed. These facts clearly indicate that the phenomenon we are observing is not specific to D_2O solvent.

The similarity between the crystal growth in D_2O and that in H_2O suggests the general applicability of SANS to the study of the crystal growth of other biological macromolecules.

3.8. Characteristics of SANS for protein crystallization research

The wavelength of the cold neutrons is around 1 nm, that is, the same order of magnitude as the size of protein molecules and their aggregates. In particular, if the protein molecules associate, the change would be expected to be detected very sensitively.

Since neutron penetrates into materials deeply in general, a thick solution sample can be used in SANS experiments. As a result, crystallization pro-

Fig. 7. Guinier plot (natural logarithm of intensity vs. Q^2) of the data of Fig. 1 in each NaCl concentration.

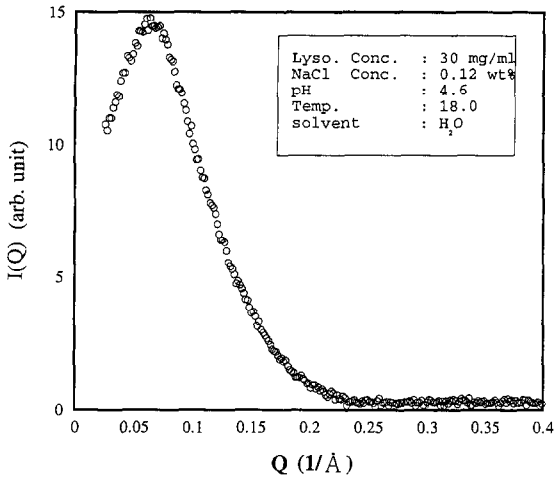


Fig. 10. SAXS spectra of lysozyme solution in H_2O solvent, where lysozyme concentration, NaCl concentration, pH and temperature are 30 mg/ml, 0.12 wt.-%, 4.6 and 18.0°C, respectively.

cesses occurring in the bulk solution can be observed. As a matter of fact, in our SANS experiment, a 4-mm thick solution (lysozyme in D_2O solvent) was used. This fact is very preferable, since the surface of the solution container might affect the crystallization process.

The energy of cold neutrons used in the SANS experiment is low (about 1 meV) compared to that of X-rays (about 20 keV); so non-destructive real time monitoring without disturbing the process is possible. The direct observation of time evolution of crystallization processes lasting more than one day is possible in SANS experiments.

The absorption effect of neutrons by salts is negligibly small compared to that of X-rays. In our experiment, NaCl solution of several wt.-% was prepared to realize the supersaturated condition of lysozyme, and we must remember that the absorption of X-rays becomes more than 90% in this case.

The neutron wavelength is comparable to the size of a protein molecule. The situation is different when light scattering is used, whose wavelength is two orders of magnitude greater than that of individual protein molecules. A mathematical procedure like calculating the autocorrelation function is a smart means to deduce information on nanoscale from the light scattering measurement. Such calculation is not necessary in the case of SANS. Based on light scattering measurements, it was shown that the radius of gyration of the scatterer does not change as the NaCl concentration is increased in the unsaturated region [7]. This seems inconsistent with our results. However, it was already shown that the formation of larger scatterers was easily detected only when the scattering angle was 20° instead of 90° that was used to draw the original conclusion [8]. Also, small changes could have been neglected because of the much more important changes in the presence of solvents other than NaCl. X-ray also has very small wavelengths. However, in the case of X-rays, the energy is much higher so that non-destructive monitoring is impossible, especially in the time evolution of the crystallization process.

SANS measurements similar to ours were reported [29]. The experimental findings are generally consistent with ours when the conditions are similar. For the analysis, only the Guinier plot was used; the range of validity and the justification for its use were given above. Also, it was shown here that the data in the supersaturated region continuously change with time. The time evolution of SANS intensity in the supersaturated solution was successfully analyzed and the results have been already published elsewhere [30].

4. Conclusions

It has been shown that SANS can be used to monitor the following two kinds of changes that occur in the solution of lysozyme. (1) In supersaturated solutions, irreversible time-evolution was shown to occur and continued to the appearance of visible crystals. (2) Even in unsaturated solutions, an increase in an average interparticle distance due to increased interaction between molecules progressed as the salt concentration was increased. For the exact and full understanding of process (1), that of process (2) is required since the former is superimposed on the latter. Is the initial state of a supersaturated solution a mere extension of the change that occurs in an unsaturated solution? The analysis of the time dependent structural change of the aggregates in the supersaturated state, now under way, will give us the answer. The so called contrast variation method in

the SANS experiment should also give us the whole shape of particles in the solution. This experiment is now scheduled.

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