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Solubility and prenucleation of aprotinin (BPTI) molecules in sodium chloride solutions

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Abstract

In the present study we describe the first stage of the crystallization process of the bovine pancreatic trypsin inhibitor BPTI from sodium chloride solutions, with special attention to the polydispersity of the particle size distribution. First, we measured the solubility of BPTI at four NaCl concentrations (1.4 to 2.3 mol/l) and four temperatures (5 to 25°C). The solubility of the protein decreases with increasing temperature and increasing ionic strength, the effect of temperature being more pronounced at low ionic strength. Second, we investigated the behaviour of the BPTI molecules by quasi-elastic light scattering (QELS) measurements at different concentrations in protein (15 to 110 mg/ml), and ionic strengthes (0 to 2.3 mol/l NaCl). The solutions become monodisperse, but not monomeric, in the vicinity of the solubility curve. They are stable until nucleation takes place but it is impossible to decide whether these aggregates are the growth units of the crystals.

1. Introduction

The bovine pancreatic trypsin inhibitor BPTI or Aprotinin, is a rather small protein, the molecular weight of which is 6511 Da. Among the different polymorphs which have been identified [1–6] we are especially interested here in the hexagonal form growing in the presence of sodium chloride [1,2]. Contrarily to porcine pancreatic α-amylase, a good model substance because it grows in the presence of very low salt concentrations [7], this polymorphic modification of BPTI needs about 1.7 to 2.3 mol/l NaCl for growing at convenient solute concentrations (20 to 30 mg/ml). BPTI is therefore, in contrast to α-amylase whose molecular weight is 55 kDa, another good model substance, as both of them give large and good crystals under very different growth conditions.

In the present study, we are interested in the first stages of the crystallization process. They are investigated using the quasi-elastic light scattering (QELS) technique for measuring diffusion coefficients and molecular aggregate sizes. Spe-

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cial attention is paid to the polydispersity of the particle size distribution, which seems to be a very important parameter for crystallization. In the case of α-amylase [8] prior to nucleation, the protein is strictly monodisperse (polydispersity $< 6\%$).

2. Materials and methods

BPTI was supplied as a lyophilized powder by Bayer AG and used as received. Prior to powder dissolution, the proper amount of sodium chloride was dissolved in pure water (obtained by double-distillation on quartz tubes and passed through a milliQ), the pH of the solutions being adjusted to 4.50 by addition of 50 mM of acetic acid and NaOH 1M. After dissolution of BPTI the pH was ~ 4.9 and all experiments were carried out at this final value. From solutions supersaturated by temperature variation, large crystals of the hexagonal modification nucleate and grow very rapidly. These crystals are not yet used for structure determination since there are too many molecules in the unit cell [1,2]. However, this is not a drawback for the present study since we are only dealing here with pre-nucleation problems.

It is noteworthy that BPTI was thoroughly investigated by QELS [9] in the 2.59–9.92 pH range in KCl 0.3M solutions and also in NaCl 0.1M to 0.5M solutions at pH = 7.0, the highest protein concentration being 65 mg/ml. We will see in the sequel that all these solutions were undersaturated. Actually, the authors [9] were not interested in crystallization problems, but only aimed at clarifying the long dated problem of BPTI dimerization. They concluded that BPTI behaves as a hydrated monomer of hydrodynamic radius of 14.9 Å if the solution is very pure while Scholtan and Lie [10] previously showed that impurities of low molecular weight (purines, pyrimidines, ADP and ATP) causes BPTI to behave like a dimer.

In the present paper, the QELS measurements were carried out using a Sematech SEM 633 light scattering apparatus (Sematech, Nice) and a Spectra Physics 2017 5 W argon ion-laser, running at a power ranging from 50 to 500 mW and operating at 514 nm. The data were collected at a scattering angle of 30° with a sample time of 0.8 μs, and were processed through a RTG correlator (Sematech, Nice). Some of the measurements were also performed at 90° with a sample time of 0.3 μs and processed through a multi-τ correlator (UNICOR). Prior to measurements, the samples were filtered through a 0.5 μm Millex LCR single-use membrane (Millipore), before being poured in a 12 mm diameter cylindral glass cuvette with a flat bottom. The analysis volume was about 300 μl.

The theory, technique and methods of QELS have already been fully described [11–13]. In short, the technique consists in measuring the mutual diffusion coefficient D of molecules dispersed in a solvent undergoing Brownian motion. In the case of particles of arbitrary shape, which are optically isotropic and small enough, as compared to the reciprocal of the scattering vector q^{-1}, the effect of rotational motion on the exponential correlation function can be neglected. The diffusion coefficient D and the polydispersity v of the system are directly related to the first and second cumulants [14]. When polydispersity was high ($v > 6\%$), we used a data analysis method based on the singular system and exponential-sampling method [15–17]. The algorithm directly determines a particle size distribution from the QELS data, but it must be borne in mind that this problem, i.e. the inversion of the Laplace transform in photon correlation spectroscopy, is an ill-posed problem so that no single solution exists. The diffusion coefficients D obtained from the cumulant or singular system method are mutual diffusion coefficients and to obtain the self-translational coefficients D_0, they must be extrapolated to zero concentration. From D_0 it is then possible to deduce the hydrodynamic radius R_h of the molecule by means of the Stokes-Einstein equation $D_0 = k_B T / 6\pi\eta_0 R_h$, where k_B is the Boltzmann constant, T the absolute temperature and η_0 the solution viscosity. More details on experimental set-up and data analysis were given previously [8]. In this whole paper, polydispersity and monodispersity of solutions are determined from QELS measurements performed at 30° and 90°.

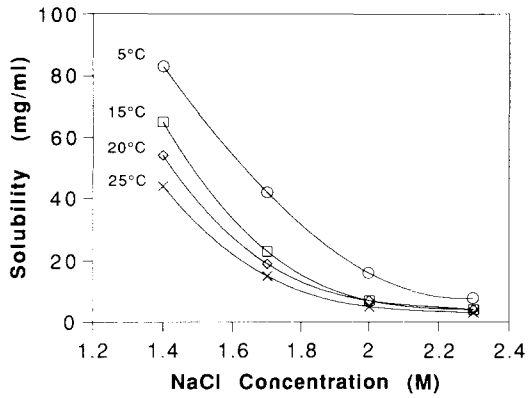

Fig. 1. Solubility of BPTI as a function of NaCl concentrations for different temperatures.

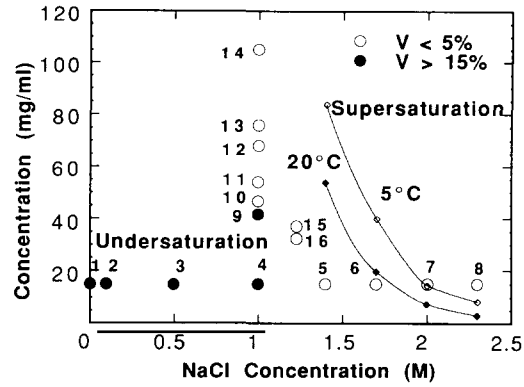

Fig. 2. Protein solutions investigated by quasi elastic light scattering and corresponding polydispersity v:

3. Results

3.1. Solubility

The first stage of our work consisted in measuring the solubility of BPTI at four differents NaCl concentrations and four temperatures. For doing this, we seeded supersaturated solutions with small BPTI crystallites and followed the diminution of the protein concentration by spectrophotometry till it remained unchanged for at least two weeks. Protein concentrations were determined from the UV absorbance at a wavelength of 280 nm using an extinction coefficient of 0.786 ml/mg · cm. The results are plotted in Fig. 1 and the data are summarized in Table 1. We see that solubility decreases rapidly with increasing temperature and increasing ionic strength. In addition, the temperature dependence is much more pronounced at low ionic strength than at high ionic strength.

Table 1
Solubility data of BPTI as a function of temperature for different concentrations in NaCl

NaCl (M)	Solubility at 5°C (mg/ml)	Solubility at 15°C (mg/ml)	Solubility at 20°C (mg/ml)	Solubility at 25°C (mg/ml)
1.4	83	65	54	44
1.7	42	23	19	15
2.0	16	7	7	5
2.3	8	4	4	3

3.2. Diffusion coefficients and polydispersity

For studying the behaviour of BPTI molecules in solution, we first prepared two sets of 8 solutions at constant protein concentration (15 mg/ml) but different salt concentrations. One set was stored at 5°C and the other one at 20°C. The solutions are represented by circles in Fig. 2 and marked, with respect to the solubility curves at 5 and 20°C. Six of them are undersaturated at both temperatures (Nos. 1 to 6), while No. 8 is supersaturated at both temperatures. Solution No. 7 is in an intermediate situation, clearly supersaturated at 20°C, but only very slightly at 5°C. All undersaturated solutions were stable for several days and no significant change of the diffusion coefficient and of the polydispersity could be detected. On the contrary, we rapidly observed a slow but clear evolution of the solutions No. 8. The diffusion coefficients and polydispersities measured 3 h after the beginning of experiments Nos. 1 to 7 and 2 h after the beginning of experiments No. 8 are displayed in Fig. 3. The uncertainty on D is $\pm 0.2 \times 10^{-7}$ cm^2 s^{-1} while the uncertainty on v is about 2%. The values of D obtained at 5 and 20°C were corrected to those expected for a solvent having the viscosity of water. Both D and v decrease rapidly with increasing NaCl concentration, especially between 0 and 1.4 mol/l. Beyond this value, D at 20°C tends more slowly towards about 7×10^{-7} cm^2 s^{-1}, while polydispersity remains nearly constant

between 3% and 8%. However, and more important, for NaCl concentrations equal or higher than 1.4 mol/l, all solutions are monodisperse but not monomeric. The hydrodynamic radius R_h is about 25 Å. As R_h of a monomer, hydrated with a water monolayer, is 14.9 Å [9], we conclude that the aggregates in our solutions could be made of about 2 to 6 monomers. This is an order of magnitude.

To obtain a better insight into the variation of the polydispersity we show in Fig. 4 the evolution of the particle size distribution for three solutions at different NaCl concentrations (0M, 1.0M and 1.7M). We see that there are at least two protein populations for the two lowest NaCl concentrations, whereas there is only one population for the highest concentration. Accordingly, polydispersity is high in the two former cases and we consider that the values of the particle sizes are not reliable at all. The reverse is valid for the solutions highly concentrated in NaCl as the peak of the particle size distribution is very narrow.

In the second step of this QELS study, we investigated solutions at higher protein concentration but at constant (1.0M) NaCl concentration. These solutions are numbered from 9 to 14 in Fig. 2. Solutions Nos. 15 and 16 were added for having analyses closer to the solubility curves. We see that the protein is monodisperse not only at high salt concentration but also at high protein concentration.

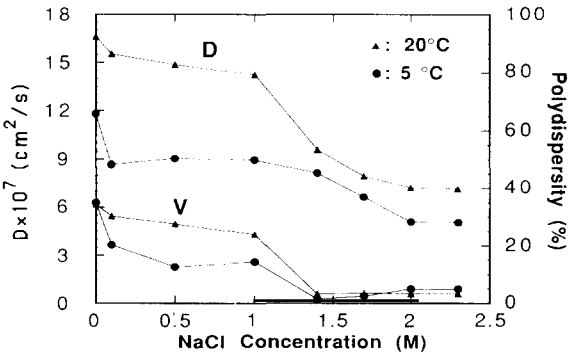

Fig. 3. Evolution of BPTI diffusion coefficient and polydispersity as a function of NaCl concentration.

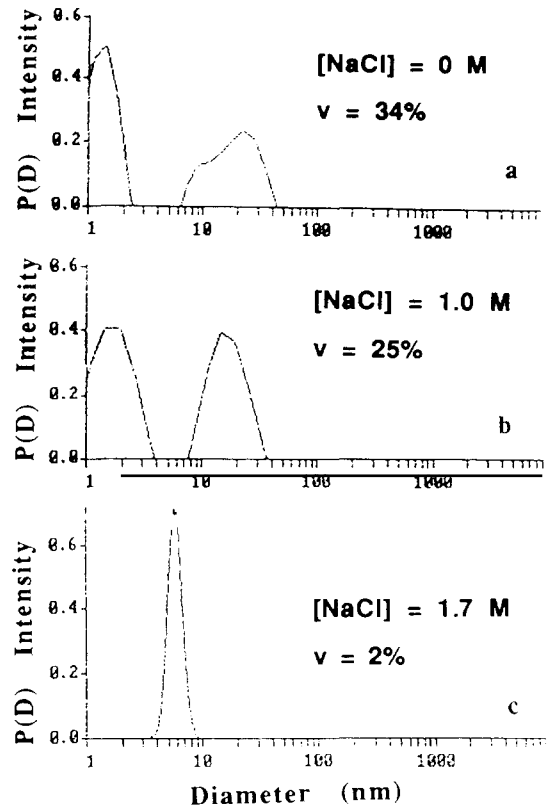

Fig. 4. Particle size distribution of BPTI (15 mg/ml) for three solutions at (a) 0M, (b) 1.0M, and (c) 1.7M NaCl concentrations at 20°C.

3.3. Pre-nucleation and nucleation

Looking more in detail at the evolution with time of the supersaturated solution (No. 8) at 20°C, we found exactly the same behaviour as that observed with α-amylase [8]. The experiment was carried out at 20°C with a protein concentration fixed at 15 mg/ml (NaCl 2.3M), so that the supersaturation, expressed as the ratio of actual concentration over equilibrium concentration $\beta = C/C_e$, was $\beta = 4.8$. In Fig. 5 we have displayed the results obtained. Three zones can be distinguished:

– In the prenucleation zone 1 (from 0 to 3 h), the diffusion coefficient D remains nearly constant with increasing time. The protein remains

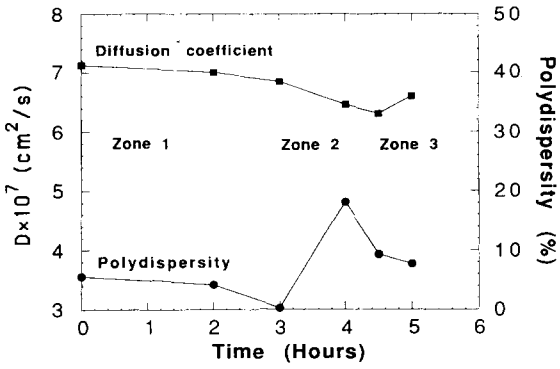

Fig. 5. Evolution of BPTI diffusion coefficient and polydispersity of a solution where nucleation occurs after 3 to 4 h at 20°C ($C = 15$ mg/ml, 2.3M NaCl).

monodisperse, the polydispersity being less than 5%.

– In the nucleation zone 2 (around 4 h), polydispersity increases up to 18%, the particle size distribution showing several populations of aggregates with apparent radii larger than 25 Å. On the other hand, the diffusion coefficient only decreases a little bit in this zone which obviously is the nucleation zone. This rapid association is in agreement with the theory outlined by Kam et al. [18]: once the energy barrier to nucleation is reached, the process is co-operative:

– In the growth zone 3 (for $t > 4$ h) some crystals are visible in the glass cell. Once they have deposited, on the bottom of the cell the diffusion coefficient has slightly increased but polydispersity has diminished to about 10%. In this zone measurements are difficult because of the presence of small crystals in solution.

4. Discussion

The behaviour of the BPTI samples we have investigated was completely unexpected. As a matter of fact, it was previously observed [9,10] that BPTI molecules are monodisperse and even monomeric in NaCl solutions at pH = 7 provided that both protein and solution are very pure. This is valid for NaCl concentrations ranging from 0 to 0.5 mol/l. On the other hand, if the solutions contain some low molecular weight contaminants or ions that function as cross-linkers, the protein becomes dimeric. From QELS data, it was even possible to determine, a value for the dimerization constant [9,19]. On the other hand, in our case, we always observed the presence of aggregates coexisting with monomers or dimers. Moreover, at constant protein concentration, the number of aggregates increased with increasing precipitant concentration, while, at the same time, the population of aggregate became monodisperse when the NaCl concentration reached 1.4 mol/l. Under the latter conditions, using the UNICOR correlator (0.3 μs/90°), we obtained the same D values as those obtained with the RTG correlator (0.8 μs/30°).

Such a behaviour cannot be attributed to any pH effect since for BPTI the attractive molecular interactions should be independent of pH over the range 2.6 to 8.5 [9]. In addition it can neither be attributed to the presence of certain ions, since deionized samples (prepared by passing BPTI through a column filled with OH^- and H^+ resin) behave in the same way as non-deionized ones, at least for the solutions we have tested at 15 mg/ml in protein and NaCl concentrations ranging from 0 to 2.3 mol/l (Fig. 6). The diffusion coefficients run nearly parallel and the solutions become monodisperse above the same NaCl concentration. From this point of view, it is difficult to explain why we mainly detect aggregates. As the purity of NaCl is better than 99.5%, it is unlikely that some unknown impurities promote

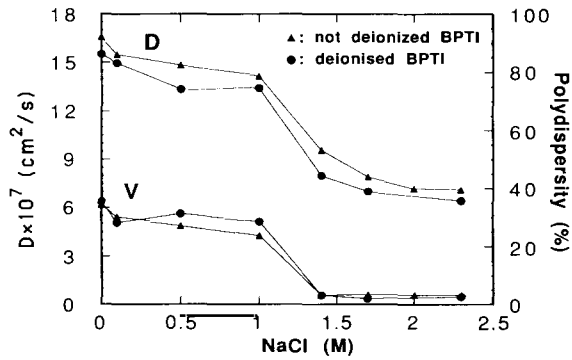

Fig. 6. Evolution of BPTI (15 mg/ml) diffusion coefficient and polydispersity as a function of NaCl concentration at 20°C.

aggregation when the NaCl concentration increases.

Moreover, it is most probable that the state of the BPTI molecules in solution is greatly affected by the chemical potential of the solution, i.e. by the composition of the solution and its localization with respect to the solubility curve. In the protein and NaCl concentration ranges investigated by Gallagher and Woodward [9], the solutions are highly undersaturated, so that polydispersity is high, the molecules reversibly associating and dissociating as it turns out from our experiments. In the 0 to 0.5 mol/l NaCl concentration range, the mean diffusion coefficient at 15 mg/ml in protein varies from 16.6×10^{-7} to 14.9×10^{-7} cm^2 s^{-1}, values not very different from those proposed for the monomer by Gallagher and Woodward who extrapolated D to zero protein concentration. Moreover, as Gallagher and Woodward worked at a scattering angle of 90°, where the QELS technique is not very sensitive to aggregation, it is possible that the weight of the aggregates with respect to the weight of the monomers was too small so that the aggregates were not detected.

Finally, it is noteworthy that in the vicinity of the solubility curves, the aggregates made of about 2 to 6 molecules are monodisperse and stable. Nucleation induces polydispersity as seen in Fig. 5, but the large aggregates, formed during the nucleation process and which do not turn into crystals, are probably unstable and dissociate again once the crystals grow and decrease the supersaturation. Another possibility is that the number of undercritical nuclei larger than 25 Å is so small that they cannot be detected.

5. Conclusion

In the present study, we first determined the solubility of BPTI at four temperatures and four NaCl concentrations, and showed that solubility decreases rapidly with increasing temperature and ionic strength. Second, we investigated the behaviour of the BPTI molecules by QELS measurements. At 15 mg/ml, at 5 and 20°C, the diffusion coefficient decreases with increasing NaCl concentration. The solutions become monodisperse at 5 and 20°C once the NaCl concentration exceeds 1.4 mol/l. At 1.0 mol/l NaCl and 20°C, the solutions are polydisperse unless the protein concentration exceeds 40 mg/ml.

From these measurements we conclude that: (i) Polydispersity of a protein solution can decrease with increasing protein or precipitant concentration. This is in agreement with previous experiments that we carried out on porcine pancreatic α-amylase [8], at least as concerns the protein concentration since α-amylase does not need any precipitant for crystallizing except the buffer of ionic strength about 40 mM [8]. (i) The solutions become monodisperse but not monomeric in the vicinity of the solubility curves. (iii) Supersaturation does not systematically increase polydispersity, at least as long as the solution remains metastable. This was also observed with α-amylase [8]. (iv) Nucleation induces polydispersity, but, once the crystals grow, the solution returns to monodispersity if the supersaturation is not too high. (v) The aggregates that we detected are stable and consist of about 2 to 6 BPTI molecules. However, it is impossible to know whether these aggregates are the growth units of the crystals.

Acknowledgments

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