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Temperature and pH Effect on the Polymorphism of Aprotinin (BPTI) in Sodium Bromide Solutions

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ABSTRACT: In this contribution, we present the phase transition of protein crystals in suspension as a function of temperature and pH. We measure the solubility of two polymorphs of aprotinin (BPTI) in 2 M NaBr solutions at different acidic pH values between 4.50 and 5.13 at 20 °C. We observe a major increase in solubility when the pH is increased from 4.5 to 5.13. The temperature effect between 5 and 35 °C on the solubility of the two BPTI polymorphs in 2 M NaBr solutions is investigated at pH 4.5 and 4.75. We observe two polymorphs in the same solution with direct and reverse solubility curves. We show that an adequate control of temperature around the transition temperature between the two polymorphs can be used to obtain the polymorph desired.

1. Introduction

One of the most challenging tasks in crystallization in solution is to control the solid-phase crystallized, for instance, the problem of polymorphism in the industrial crystallization of pharmaceutical compounds.[1−4] In the case of crystallization of biomolecules, it is now evident that an understanding of the phase diagram is useful for the production of suitable crystals for structural determination; in particular, Boistelle et al.[5] have shown the importance of temperature on the crystallization of polymorphs A and B of α-amylase. More recently, Lafont et al.[6,7] have shown the effect of temperature on the solubilities of different polymorphs of aprotinin or bovine pancreatic trypsin inhibitor (BPTI). Hexagonal BPTI crystals obtained in NaCl at pH 4.5 and hexagonal crystals obtained in $(NH_4)_2SO_4$ at pH 4.5 have reverse solubility curves, the solubility decreasing with increasing temperature. However, the monoclinic polymorph obtained in KSCN at pH 4.5 has a direct solubility curve. It is noteworthy that for all the polymorphs crystallized under acidic pH in the presence of thiocyanate, chloride, and sulfate ions the same decamer is found in the packing and in solution.[8]

In the present study, we measure the solubility of the chloride hexagonal polymorph and of a new BPTI polymorph in 2 M NaBr solutions at different acidic pH between 4.50 and 5.13 at 20 °C. A drastic effect of the pH on the solubility is observed. The effect of temperature, between 5 and 35 °C, on the solubility in 2 M NaBr solutions is investigated at pH 4.5 and 4.75. In addition, we show that with an adequate control of temperature and because of the enantiotropy of the system the polymorph desired can be obtained.

2. Materials and Methods

2.1. Protein Solutions. BPTI (6511 Da, pI = 10.5) was supplied as a lyophilized powder by Bayer and used as received; the purity was checked by molecular sieving FPLC (see below). BPTI is employed as an anticoagulant to reduce blood loss during cardiac surgery. Proper amounts of BPTI and NaBr were dissolved in pure water (ELGA UHQ reverse osmosis system) to obtain stock solutions needed for solubility measurements and crystallization experiments. The different solutions were buffered with 80 mM acetic acid, adjusted to the desired pH with NaOH (1M) and filtered through 0.22 μm Millipore filters. After dissolution of BPTI, the pH was checked (pH meter Tacussel ISIS 20000 and micro pH electrodes radiometer pHC3359-8) and adjusted when required. The BPTI concentration was controlled by optical density measurements (Kontron UVKON810) using an extinction coefficient of 0.786 cm^{-1} mL mg^{-1} at 280 nm.[6] Throughout the study, the NaBr concentration was 2 M.

FPLC experiments were carried out at 20 °C on the AKTA basic 10 FPLC system (Amersham Pharmacia Biotech A. B.). The protein (40 mg mL^{-1}) was incubated with buffer containing acetic acid and 2 M NaBr (pH 4.5, 4.65, 4.8, and 5.0) at 20 °C. A total of 10 μL of each solution was passed over an analytical molecular sieve chromatography column (3.2 × 300 mm) of Superdex 75 preequilibrated with the incubation buffer. The protein was eluted with the same buffer at a flow rate of 50 μL min^{-1}. Detection was performed at 280 nm, and the peak area was calculated between the curve and the baseline, between the peak start and the peak end. Decamer and monomer proportion were defined by the percent of the sum of all integrated peaks with the UNICORN 4.0 program.

2.2. Crystal Identification. The diffraction data for both polymorphs of BPTI were measured with Cu Kα X-rays using a Rigaku RV-200 rotating anode, operating at 40 kV and 80 mA, with a graphite monochromator. Data were collected with a MAR research imaging plate detector. The space group of the NP was determined at 100 K at the European Synchrotron Radiation Facility (Grenoble, France) on beamline ID14-EH1 using an ADSC Quantum 4R CCD detector. The crystals were flash-frozen in mother-liquor containing 20% glycerol prior to the data collection.

2.3. Solubility. Solubilities were obtained by seeding supersaturated solutions with small BPTI crystallites. Solubilities were measured in 2 M NaBr solutions at 20 °C at different acidic pH between 4.50 and 5.13 and at 5, 13, 20, 29, and 35 °C at pH 4.5 and 4.75. Equilibration between crystals and solution is observed by regularly withdrawing 10 μL aliquots of supernatant from the cell to measure variation in protein concentration. We used thermostated centrifuge (EPPENDORF, Centrifuge 5804R, 4mn at 10000 rpm) to avoid

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Table 1. Solubilities of BPTI Polymorphs, Needle and Bipyramid, as a Function of pH at 20 °C

pH	needle polymorph solubility (mg mL^{-1})	bipyramid polymorph solubility (mg mL^{-1})
4.50	12	17.6[c]
4.60	20	25
4.72		34
4.75	38	35[d]
4.83		48
5.01	92.5[a]	79
5.10	157[b]	108
5.13		116

[a] Obtained by the bracketing method ± 1.5 mg mL^{-1}. [b] Obtained by the bracketing method ± 3 mg mL^{-1}. [c] Obtained by the bracketing method ± 5 mg mL^{-1}. [d] Obtained by dissolution.

temperature variation which could provoke nucleation of another polymorph. We used the growth technique for most of the experiments, unless specified in the text. The solution volumes were about 500 μL; solutions and crystals were placed in a glass vessel inserted in a thermostated cell under an optical microscope (Nikon Diaphot). The whole setup has been previously described.[5] Special attention was paid to determining solubilities of the metastable polymorphs; we observed continuously to check that there were only crystals of one polymorph. The suspension was stirred manually to disperse all the crystals in the cell before observation.

Sometimes it was impossible to directly measure the solubility of the metastable polymorph because of the undesired nucleation of the stable polymorph. In those cases (a, b, and c in Table 1), we used a bracketing method, measuring the solution concentrations before the beginning of the dissolution and after the complete dissolution of the metastable polymorph. The solubility is between these values.

The experiments, however, could not be performed in the entire temperature range. When the metastable polymorph, which has a higher solubility, is investigated far from the temperature of equal solubility, the stable polymorph may spontaneously precipitate and grow. This changes the concentration in the solution, which leads to incorrect results. In the case of nucleation of small crystals of the stable polymorph, temperature variation can be used to dissolve the small crystals (kinetic ripening).

3. Results and Discussion

3.1. Identification of Crystals. In acidic conditions of crystallization,[8,9] we observed only two BPTI polymorphic modifications whose crystal habit looked like those of the two hexagonal polymorphs. To avoid any ambiguity with respect to other polymorphs, we measured the cell parameters of both polymorphs. Whereas the bipyramid polymorph (BP) was solved in the $P6_422$ space group with cell parameters: a = b = 95.39 Å, c = 159.66 Å, which corresponds to the chloride hexagonal polymorph,[8] the needle polymorph (NP) was solved in the $C222$ or $C222_1$ space group with cell parameters: a = 101.52 Å, b = 263.36 Å, c = 107.47 Å, which correspond to a new BPTI polymorph. Clearly, the crystal habits of both polymorphs are different and they can easily be distinguished by optical microscopy (see section 3.3).

3.2. Solubility. In Tables 1−3 and Figures 1−3 data concerning the solubility of the two polymorphs at different pH and temperature are collected and plotted. The accuracy of the solubility values, unless specified in the table, is ±1 mg mL^{-1}.

3.2.1. pH Effect. Figure 1 shows that a variation in the pH from 4.50 to 5.13 at 20 °C has a drastic effect on solubility. When the pH is increased from 4.5 to 5.10 the solubility of the NP increases by more than 1 order of magnitude. This result is unexpected, because it is known that solubility is minimal at the pI of a protein,[10] which for the BPTI is 10.5. In addition, outside the range of the pK_a values of charged residues, solubility

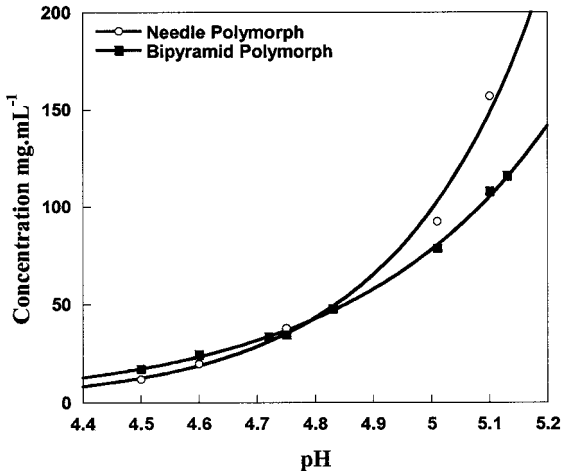

Figure 1. Variation of the solubility of the two BPTI polymorphs in 2 M NaBr at 20 °C versus pH.

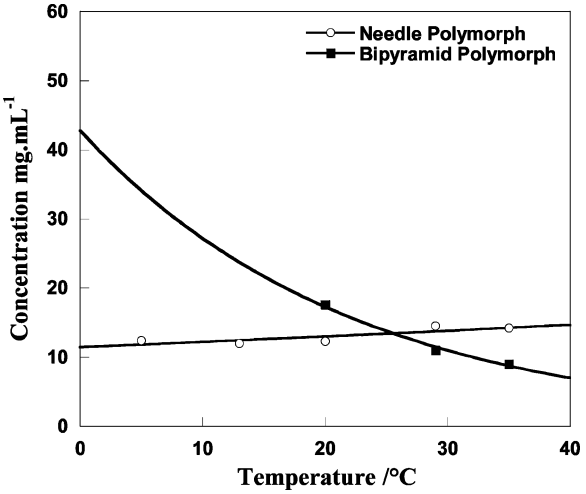

Figure 2. Solubility curves of the two BPTI polymorphs in 2 M NaBr versus temperature at pH 4.5. Solid lines are exponential extrapolations and are guidelines.

Table 2. Solubilities of BPTI Polymorphs, Needle and Bipyramid, as a Function of Temperature for 2 M NaBr at pH 4

temp °C	needle polymorph solubility (mg mL^{-1})	bipyramid polymorph solubility (mg mL^{-1})
5	12.4	
13	12	
20	12.3	17.6
29	14.5	11
35	14.2	9

Table 3. Solubilities of BPTI Polymorphs, Needle and Bipyramid, as a Function of Temperature for 2 M NaBr at pH 4.75

temp °C	needle polymorph solubility (mg mL^{-1})	bipyramid polymorph solubility (mg mL^{-1})
5	32	
13	33	
20	38	35
29		23.2
35		15.6

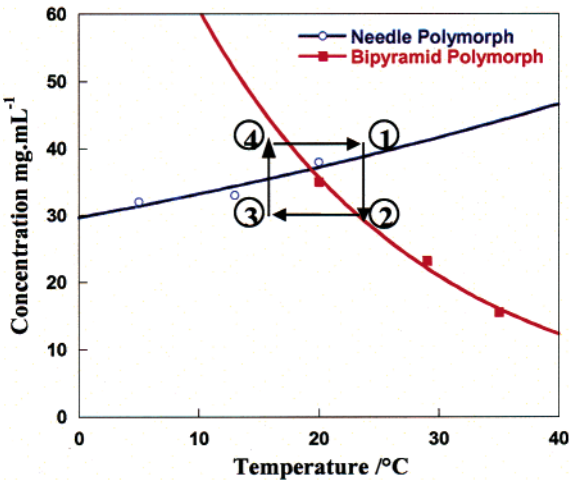

Figure 3. Solubility curves of the two BPTI polymorphs in 2 M NaBr versus temperature at pH 4.75. Solid lines are exponential extrapolations and are guidelines.

Table 4. Fraction of Monomer and Decamer, Given in Surface Fraction of the Detection Peak (±2%), from FPLC Experiments as a Function of pH at 20 °C

pH	% monomer	% decamer
4.50	13.4	86.6
4.65	14.0	86.0
4.80	15.3	84.7
5.00	17.1	82.9

scarcely changes, which is true for a monomeric protein. In the case of multimeric proteins, it must be known which charged residues are accessible and which are buried at the interface. Since for BPTI we showed the coexistence of two different BPTI particles in solution, a monomer and a decamer,[8] the decamer being the growth unit for the acidic BPTI polymorphs,[11] the pH for a net charge of zero of the BPTI decamer is bound to be different at 10.5. Moreover, solubilities of the NP and of the BP are related to the fraction of decamers in solution. For instance, the fraction of decamers was found to increase with increasing salt concentration, for KSCN, NaCl, and $(NH_4)_2SO_4$ [8], leading to a decrease in BPTI solubility. In our previous dynamic light scattering experiments, in NaCl solutions, we observed decamer formation only at an acidic pH, whereas the BPTI solutions were monomeric at a neutral or basic pH.[12] This result is confirmed by recent small-angle X-ray scattering experiments (not shown). However, our results disagree with a recent study by Gottschalk et al.,[13] who observe that the decamer fraction increases with increasing pH. This discrepancy will be discussed in a paper in preparation.

Here, to check if the pH range of this study has an effect on the fraction of decamers in solution, we performed FPLC experiments in 2 M NaBr solutions containing 40 mg mL^{-1} BPTI at 20 °C and at different acidic pH values, ranging from 4.5 to 5. Results of these experiments are summarized in Table 4. These values are in good agreement with those previously obtained by small-angle X-ray scattering,[8] which account for a decamer concentration of 66% for 30 mg mL^{-1} BPTI in a 1.8 M NaCl solution at pH = 4.5. If we consider that peak surfaces are proportional to BPTI concentration, we obtain for 40 mg mL^{-1} BPTI in a 2 M NaBr solution at pH = 4.5 a decamer concentration of 86.6%. The increase from 66 to 86.6% is due to a higher ionic strength and BPTI concentration. The fraction of monomer to decamer is correlated with the salt concentration and anion type,[7,8] in relation with the Hofmeister series.[14,15] Since bromide and chloride ions are equivalent in the Hofmeister series, equivalent decamer concentrations are expected for bromide and chloride solutions.

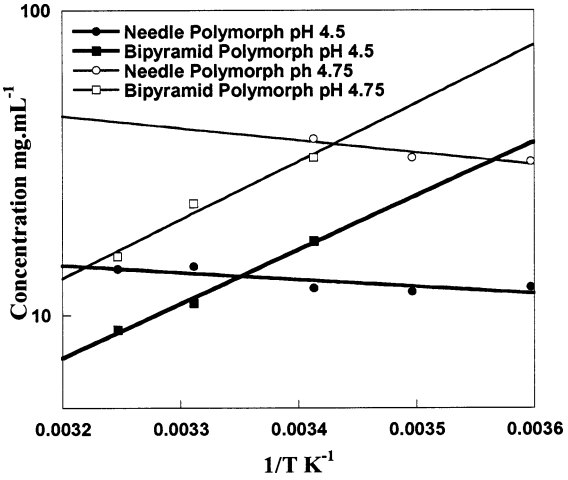

Figure 4. Solubilities of the two BPTI polymorphs in 2 M NaBr versus the inverse of the absolute temperature at pH 4.5 and 4.75.

Table 5. Enthalpy of Dissolution of BPTI Polymorphs for 2 M NaBr at pH 4.5 and 4.75

pH	$\Delta_d H$ (kJ mol^{-1}) needle polymorph	$\Delta_d H$ (kJ mol^{-1}) bipyramid polymorph
4.5	−4.3	34.0
4.75	−7.6	36.7

Results of these experiments clearly show that for the pH considered here it has no effect on the fraction of decamers in solution.

3.2.2. Temperature Effect. The temperature dependence of solubility can be used to calculate dissolution enthalpy from a Van't Hoff plot (Figure 4 and Table 5). From the slope of the straight lines we get a negative value for the enthalpy of dissolution of the NP (direct solubility curve) and a positive value for the BP (reverse solubility curve). However, due to the very narrow temperature range (5−20, 5−35, and 20−35 °C) and to the low solubility variation in this range, the accuracy of the slope of the curves is low. Thus, the $\Delta_d H$ values should be considered only as an order of magnitude. Interestingly, the $\Delta_d H$ values are of the same order of magnitude for each polymorph at different pH, i.e., curves for the same polymorph are parallel in Figure 4.

In a previous paper,[7] we explained that the solubility of a polymorph is reverse because the entropy of dissolution is negative due to a higher association of BPTI molecules with solvent and salt in solution than in the crystal. In this work, we observe two polymorphs in the same solution with direct and reverse solubility curves. That means that the dissolution entropy is negative and positive for BP and NP, respectively.

Moreover, solubility curves intersect at 25.5 and 19 °C at pH 4.5 and 4.75, respectively. This variation in transition temperature, which can be considered as

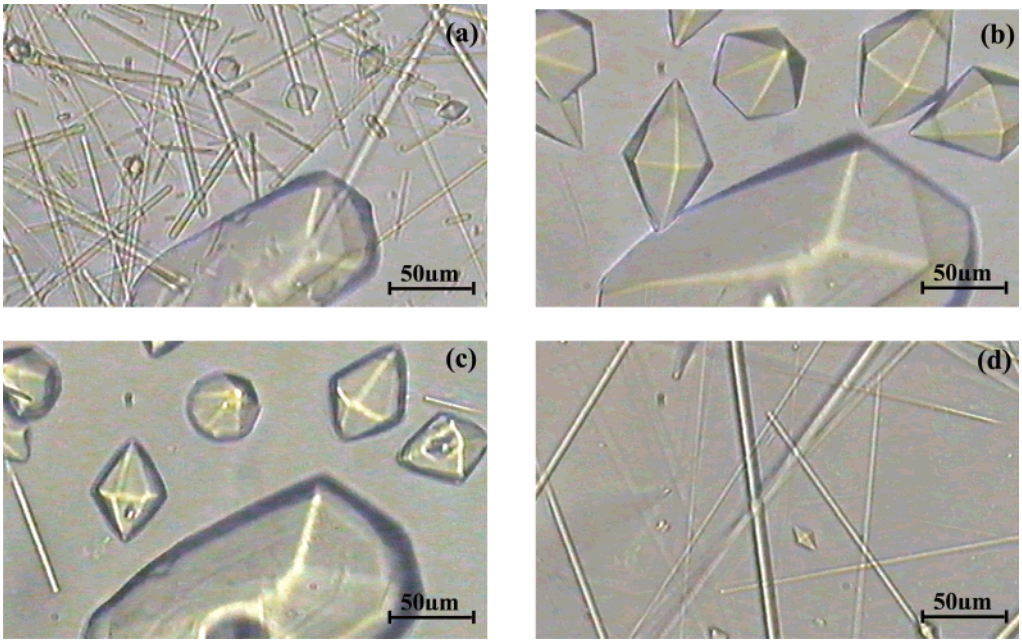

Figure 5. In situ observation under optical microscopy of the different stages of the phase transition described in the text and in Figure 3: (a) mixture of BP and NP crystals in suspension (point 1 in Figure 3); (b) BP in suspension (point 2 in Figure 3); (c) dissolution of BP and nucleation and growth of NP (between point 3 and 4 in Figure 3); and (d) growth of NP and nucleation and growth of BP (between point 4 and 1 in Figure 3).

hydrates, in function of pH is probably because the activity of water varies with pH.

3.3. Nucleation and Phase Transitions. Temperature and protein concentration determine to what extent NP and BP can nucleate and grow, simultaneously or consecutively. In the following we show that an adequate control of temperature around the transition temperature can be used to obtain the polymorph desired, NP or BP. The sequence in Figure 5 shows the different phase transitions obtained by slightly changing the temperature around the transition point.

In the first stage, 25 °C and [BPTI] = 40 mg mL^{-1} (Figure 5a = point 1 in Figure 3), both polymorphs are in suspension. At this point only BP is stable, and thus the metastable phase (NP) must undergo a solution-mediated phase transformation.[16] At 25 °C both polymorphs grow until the solubility of the NP is reached, so that BP crystals grow at the expense of NP crystals, until they completely dissolve. Finally, BP crystals grow until the concentration reaches the BP solubility (point 2 in Figure 3 and Figure 5b).

In the second stage, temperature is decreased to 15 °C (point 3 in Figure 3), so that crystals dissolve and the concentration increases until the NP solubility is crossed. When the concentration corresponding to the limit of the metastable zone-width is reached, NP crystals nucleate and grow. During this time (Figure 5c) dissolution of BP crystals compensates for NP crystallization.[1,17,18] At the end of this process, all BP crystals have dissolved and NP crystals continue to grow until the now-decreasing concentration nearly reaches the solubility of NP (point 4 in Figure 4).

In the third and last stage, before the equilibrium concentration of NP is reached, temperature is increased to 25 °C (point 1 in Figure 3), so that BP crystals nucleate and grow (Figure 5d). Thus, we are back to stage 1.

4. Conclusion

In this paper, we measure the solubility of the chloride hexagonal polymorph (BP) and of a new BPTI polymorph (NP) in 2 M NaBr solutions at different acidic pH between 4.50 and 5.13 at 20 °C. A major increase in solubility is observed when the pH is increased from 4.5 to 5.13.

The temperature effect between 5 and 35 °C on the solubility in 2 M NaBr solutions is investigated at pH 4.5 and 4.75. We find that the BP has a reverse solubility curve and the NP has a direct solubility curve.

We show, for instance, that solubility curves of BP and NP cross at 25 °C for a pH of 4.75. Thus, either polymorph can be obtained at will by slightly changing temperature or concentration.

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