Two polymorphs of lysozyme nitrate: temperature dependence of their solubility

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Two crystallographic forms of lysozyme nitrate are known, namely monoclinic and triclinic. Having previously determined the temperature dependence of the solubility of the monoclinic form (0.2 M $NaNO_3$ solutions at pH = 4.5) [Legrand *et al.* (2001). *J. Cryst. Growth*, **232**, 244-249], we focus here on the solubility of the triclinic form. The temperature dependence of the solubility of this crystallographic form has been measured with a static light device developed in our laboratory. This device allows to observe of the dissolution of one phase and/or the occurrence of a new one by varying the temperature with a sweep rate as low as 0.6 degree/hour. The new solubility data are complemented with crystallographic data of the triclinic form for the sake of completeness. The faces of a triclinic crystal are indexed. The crystallisation enthalpy of the triclinic form is deduced from these new results. These new solubility data allow us now to discuss (1) the publishedprotocols used to obtain the monoclinic and triclinic forms of lysozyme nitrate and (2) the phase transformation.

Keywords: lysozyme; solubility; polymorphism; anions; nitrate; temperature; crystallisation enthalpy.

1. Introduction

An enormous effort has been made over the last decades to improve the quality of protein crystals. The reason is the need of protein crystals large enough and diffracting to high enough resolution to determine the three dimensional structure by X-ray crystallography. Indeed structural biology is one of the most rapidly expanding fields in life science and aims to correlate protein structures with their functions. Therefore studies in material sciences have been initiated, involving practical and fundamental approaches to better understand the crystallisation processes of biomacromolecules. Among the parameters of nucleation and crystal growth, solubility is the most important for small molecules as well as for biomacromolecules. Indeed as expressed by J. A. Littlechild (1991), the more that is known about the solubility properties of the proteins to be crystallised, the easier will be the monitoring and the control of the process. Hen egg white lysozyme has been widely used as a model of soluble proteins for crystallogenesis studies. Crystals of lysozyme salts can be grown in numerous forms, namely tetragonal, orthorhombic, hexagonal, monoclinic and triclinic ones (Steinrauf, 1959, and references therein; Steinrauf, 1967; Vaney *et al.*, 2001, and references therein).

A variety of conditions to obtain the monoclinic and triclinic forms of lysozyme nitrates have been reported. Generally the monoclinic lysozyme nitrate is obtained in the absence of acetate buffer, whereas the triclinic one grows in its presence. Nevertheless qualitative protocols have been described for the phase transition from the monoclinic to the triclinic form in the presence of sodium acetate buffer.

Monoclinic lysozyme nitrate has been grown from a 1% protein solution adjusted to pH 4.5 by addition of nitric acid, and 0.35 M sodium nitrate (Steinrauf, 1959). Although the temperature is not specified by the author, it is assumed to be room temperature. The same crystals can also be obtained from a 3% protein solution in 0.23 M sodium nitrate at pH 4.5 at 4 °C and 23 °C (Sieker, 1988). The nature of the buffer is missing in their experimental protocol, but it is undoubtedly acetate to fix the pH at 4.5. More recently the solubility of monoclinic lysozyme nitrate at 18 °C from 0 to 0.15 M $NaNO_3$ has been extensively studied by Retailleau *et al.*, using deionised lysozyme solutions (pH = pI ≈ 10.8) adjusted to pH 4.3 and 8.3 by addition of nitric acid (Retailleau, 1996; Retailleau *et al.*, 2002). In a previous paper we have presented solubility data for monoclinic lysozyme nitrate grown in 0.2 M $NaNO_3$, 50 mM acetate buffer (pH 4.5) in light and heavy water solutions (Legrand *et al.*, 2001). Recently we complemented the solubility curve of this form in light water with additional data, and deduced a corrected value for the crystallisation enthalpy, -150 ± 2 kJ.mol^{-1}, instead of the previous value of -145 ± 2 kJ.mol^{-1} (Legrand *et al.*, 2001).

Concerning the triclinic form of lysozyme nitrate, Steinrauf mentioned its preparation for the first time (Steinrauf, 1959), from a 1% protein solution with 0.23 M $NaNO_3$ in acetate buffer (25 mM sodium acetate and 25 mM acetic acid). The pH was finally adjusted to 4.5 by the addition of diluted nitric acid. Guilloteau *et al.* used dialysed lysozyme in sodium nitrate and 50 mM acetate buffer solutions at pH 4.5 and 18 °C to grow triclinic crystals with the following cell parameters (Guilloteau *et al.*, 1992): a = 34.3 Å, b = 31.8 Å, c = 27.1 Å, α = 111.6 °, β = 108.3 °, γ = 88.8 °.

These crystals are isomorphous with the form first described by Steinrauf (1959).

Many more lysozyme solubility measurements have been devoted to the tetragonal and orthorhombic forms (see for example Ataka & Asai, 1988; Howard *et al.*, 1988; Riès-Kautt & Ducruix, 1989; Cacioppo & Pusey, 1991; Elgersma *et al.*, 1992; Guilloteau *et al.*,1992; Rosenberger *et al.*, 1993; Gripon *et al.*, 1997; Retailleau *et al.*, 1997; Bénas *et al.*, 2002) than to the monoclinic and triclinic ones (Riès-Kautt & Ducruix, 1989; Guilloteau *et al.*, 1992; Retailleau, 1996; Legrand *et al.*, 2001; Retailleau, 2002). The temperature dependence of the triclinic form has not been quantified so far by solubility measurements.

Solubility of the triclinic lysozyme nitrate presented here was measured *versus*temperature in 0.2 M $NaNO_3$ and 50 mM acetate buffered solutions at pH 4.5 in light water.Included are crystallographic data of the triclinic form. The various protocols used to obtain the monoclinic and triclinic forms of lysozyme nitrate in the presence of acetate buffer are discussed in the light of these new data.

2. Methods and materials

2.1. Light detection method

A 100 µl to 1500 µl solution is set up in a cell to crystallise at 4 °C for about 12 hours. The sample is then installed in a temperature controlled sample holder and undergoes a temperature sweep (0.6 degree/hour) while stirring to maintain the microcrystallites in suspension (Rosenberger *et al.*, 1993; Gripon *et al.*, 1997). The intensity of a red laser light scattered at 90 ° is recorded and drops rapidly to zero when the crystallites dissolve. At the equilibrium temperature at which the crystallites are completely dissolved, the solubility is equal to the protein concentration of the solution.

2.2. Sample preparation

We used three times crystallised, dialysed and lyophilised hen egg white lysozyme (M=14300) from SIGMA (Cat. N° L6876, batch 65H 7025). The purity of the batch used without further purification has been checked byelectrophoresis showing a very small amount of species of mainly 30 000 (lysozyme dimers) and 45 000 (ovalbumin) molecular weight (Legrand *et al.*, 2001).

Lysozyme has beendeionised according to published procedures (Riès-Kautt & Ducruix, 1994; Riès-Kautt & Ducruix, 1997). The lysozyme powder was then stored freeze-dried. To prepare the samples, the protein was dissolved in Na acetate buffer solutions (50 mM) prepared with deionised water and the required $NaNO_3$ is added. The solutions were filtered through a 0.22 mm Millipore filter (Millex-GV4) and the lysozyme concentration determined by UV absorption at 280 nm, using 26.6 for the absorbance of a 1% solution. We corrected for the absorbance due to $NaNO_3$ ($3.81\ M^{-1}.\ cm^{-1}$) at this wavelength. The solutions were prepared at room temperature and then set up to crystallise at 4 °C during at least 12 hours in order to obtain a samplethat scatters sufficiently to permit the necessary measurements.

3. Results and discussion

3.1. The triclinic form

Fig. 1 shows a crystal grown in a 1 mm diameter capillary filled with a solution of 7 mg/ml protein, 0.2 M $NaNO_3$ kept overnight at 4 °C and then at 25 °C for about two months.

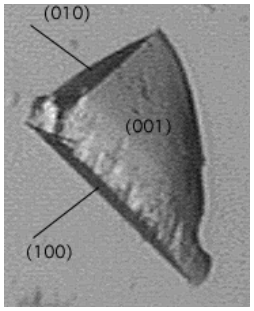

Figure 1
Optical view of a triclinic crystal obtained in a 0.2 M $NaNO_3$, 50 mM acetate buffer solution at room temperature after one night at 4 °C; the faces are indexed according to optical views of wellfaceted triclinic crystals grown in agarose gel.

The cell parameters obtained are as follows: a = 28.5 Å, b = 32.7 Å, c = 35.1 Å, α = 88.2 °, β = 108.9 °, γ = 111.9 °.

This corresponds to the known triclinic unit cell (Steinrauf, 1959; Riès-Kautt & Ducruix, 1989; Guilloteau *et al.*, 1992; Salemme *et al.*, 1992; Vidal *et al.*, 1999). Angular measurements on well faceted triclinic crystals grown in agarose gel with 0.23 M $NaNO_3$ and 50 mM acetate buffer at pH 4.5 and 18 °C allow us to index the faces of the crystal shown in Fig. 1.

Except for the measurements of the solubility at 26.2 °C (3.4 mg/ml) and 18.5 °C (1.44 mg/ml), the measurements of the triclinic form have been obtained without seeding the solutions. As already mentioned, all solutions have been prepared by mixing the salt and the protein buffered solutions at room temperature (25-27 °C). Then the solutions have been kept overnight at 4 °C. They finally underwent scintillation experiments under a temperature sweep as slow as 0.6 degree per hour.

In order to dispose of solubility measurements of the triclinic form below 5 mg/ml, two solutions have been prepared at room temperature, a solution with 3.4 mg/ml protein (solution 1) and a second one with 3 mg/ml protein (solution 2). They both have been precipitated at 4 °C and then seeded at 18.5 °C with triclinic crystals. The two solutions have been kept at 18.5 °C during four weeks. After this period, a scintillation experiment performed on solution 1leads to the temperature of complete dissolution, 26.2 °C. During the four weeks the protein concentration of the supernatant of the precipitated solution 2, kept at 18.5 °C, has been periodically measured, leading finally to the 1.44 mg/ml solubility at this temperature.

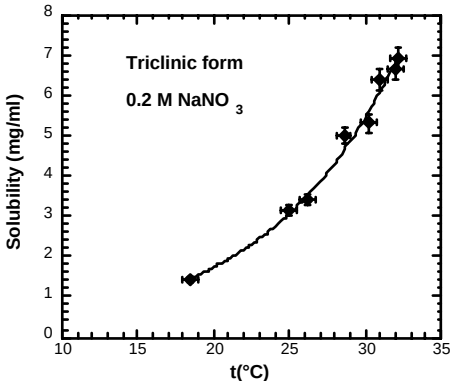

Figure 2
Temperature dependence of the triclinic form lysozyme solubility at pH = 4.5 (50 mM NaAc) for 0.2 M $NaNO_3$; the error bar on temperature is ± 0.5°C (Gripon *et al.*, 1997). Error bars on solubility values correspond to the maximum dispersion on the concentrations measured by absorption at 280 nm (measured after the light scattering experiments) and the nominal concentrations. The solid line is the best exponential fit (see text).

Fig. 2 presents the solubility data corresponding to the triclinic form of lysozyme nitrate. The molar crystallisation enthalpy, ΔH, of a phase can be deduced from the temperature variation of its solubility ϕ_S by an Arrhenius plot:

$$\ln(\phi_S) = A_1 + \frac{\Delta H}{RT} \qquad (1)$$

In equation (1), the solubility is expressed in volume fraction $\phi_S$and A_1 includes the entropic terms. Fig. 3 presents the variation of the solubility logarithm as a function of *1000/T*, showing that the set of data can reasonably be fitted by Eq. (1).

The crystallisation enthalpy, measured for the first time, is – 85 ± 2 $kJ.mol^{-1}$ for the triclinic form of lysozyme nitrate.

The solid line generated in Fig. 2 is the best exponential fit, the use of which can be theoretically justified by considering the dependence of solubility versus temperature in standard models leading to the equation (1). Indeed a linearisation of the absolute temperature above 273 K is justified and leads to the exponential dependence of solubility versus temperature expressed in degrees Celsius, because of the narrow temperature range in which the protein remains stable.

3.2. Polymorphism

Fig. 4a shows the solubility measurements of both polymorphs obtained in 0.2 M $NaNO_3$ at pH 4.5 in acetate buffer solutions. The data of the monoclinic form are from (Legrand *et al.*, 2001).

Fig. 4b shows the extrapolations of the two curves to higher protein concentrations by the two best exponential fits, in order to map the two protocols described hereafter in § a and § b.

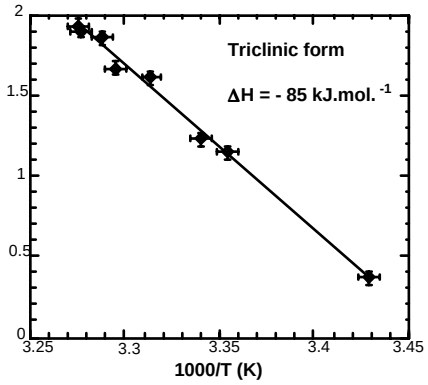

Figure 3
Logarithms of lysozyme solubility data of Fig. 2 plotted versus 1000/T(K).

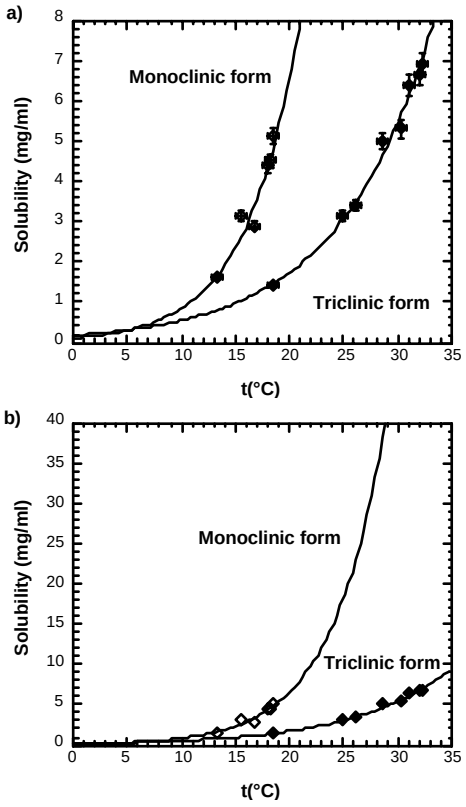

Figure 4
(a) Lysozyme solubility as a function of the temperature for 0.2 M $NaNO_3$. Empty diamonds correspond to the monoclinic form of lysozyme nitrate, full diamonds to the triclinic form. Same remark concerning the error bars as in Fig. 2; (b) Extrapolations of the two solubility curves to higher protein concentrations; the solid lines generated are exponential extrapolations (see text).

(a) At 18-25 °C constant temperature and protein concentrations higher than 5 mg/ml.

According to Sieker, from a 30 mg/ml protein solution probably buffered with acetate at pH 4.5, "*monoclinic lysozyme crystals (nitrate form only) will form initially at 23 °C and will be stable for weeks to months, but will dissolve slowly as the triclinic form of crystal develops. An initial crystallisation attempt for triclinic crystals will typically produce a yield of about 10% triclinic and 90% monoclinic crystals both appearing very similar in morphology*". Sieker observed that the transition from the monoclinic to the triclinic form does not appear to occur at 4 °C.

Because solubility of both forms decreases with increasing the sodium nitrate concentration, the solubilities at 0.23 M $NaNO_3$ are slightly lower than our values for 0.2 M reported in Fig. 4a and 4b. At 23 °C, the 30 mg/ml solution used by Sieker is therefore slightly more supersaturated with respect to the two phases than in our conditions.

According to R. Boistelle & J. P. Astier (1988), if a solution is simultaneously supersaturated with respect to two phases, it is often the phase of highest solubility, i.e. the less supersaturated one, which nucleates and crystallises first and rarely the most stable one. Indeed a high solubility leads to "*more monomer encounters when they are closer to each other*". Moreover, "*the interfacial free energy γ between nucleus and solution decreases when the solubility increases by changing the solvent or the solution composition*". In other words, the larger the solubility, the smaller γ and the higher the nucleation rate. When solubility is high, nucleation occurs therefore at relatively low supersaturation, and *vice versa*: the width of the metastable zone, between the solubility curve and the so-called supersolubility curve (critical supersaturation), is thus narrow when solubility is high, broad when solubility is low. The nucleation domains, above the metastable zones, overlap when polymorphism exists.

When growth of the first nucleated phase proceeds in a solution supersaturated with respect to two phases, the solute concentration decreases until it reaches the solubility of this phase. Further evolution of the solution depends on nucleation of the most stable phase. If no new crystal nucleates, the first nucleated phase remains in a metastable state with respect to the most stable phase. If the stable crystal forms, the solute concentration decreases again, the first nucleated phase dissolves, feeds the solution and then contributes to the growth of the stable phase.

The observations of Sieker at 23 °C are compatible with this scenario. Indeed, as seen in Fig. 4b, above about 5 mg/ml and temperatures between 18-25°C, the lysozyme solutions are supersaturated for both monoclinic and triclinic phases, as shown by the curves. We underline that we have also observed the monoclinic-triclinic transition at 20 ± 1 °C.

The fact that Sieker does not observe the monoclinic-triclinic transition at 4 °C can be explained by the low solubility values of the monoclinic and triclinic forms which are almost equal at this temperature, as shown in Fig. 4a.

(b) Use of a precipitation step at 4°C.

Another protocol (Ducruix, A. F, private communication) to obtain triclinic lysozyme nitrate consists in keeping a solution of 7-10 mg/ml with 0.2-0.23 M $NaNO_3$, 50 mM acetic acid/sodium acetate buffer (pH = 4.5) overnight at 4 °C to induce a substantial precipitation. Triclinic crystals appear after raising the temperature to 18 °C. This procedure has been used to make samples for X-ray topography studies (Vidal *et al.*, 1999). If the precipitation step is omitted, only monoclinic crystals appear at 18 °C and may be stable for weeks as already pointed out in § a.

The growth of the triclinic crystals at 18 °C appears to be conditioned by the precipitation step at 4 °C. Indeed this precipitation permits to produce triclinic crystals at 18 °C within a few days.

The solid phase obtained in our solutions at 4 °C contains spherulites showing a characteristic black centered cross through crossed polarizers, the so-called *zweiblatt* (Morse & Donnay, 1936; Philipps, 1994). This observation shows that the spherulites are small crystals organised radially. From these spherulites, bundles of

monoclinic platelets grow, the bundles having a cone shape after few days (still at 4 °C).

As seen in Fig. 4a, the solubility of the monoclinic and the triclinic forms are very close at 4 °C. Therefore a mixture of both phases is expected at this temperature after precipitation which decreases the protein concentration of the liquid phase. Raising the temperature to 18 °C and gently shaking the solution resets the system under the solubility curve of the monoclinic form which feeds the stable triclinic one. Any amorphous precipitate obtained at 4 °C dissolved as well.

(c) Temperature gradient and weak protein concentration (< 5 mg/ml).

Finally we followed a 3.1 mg/ml (0.2 M $NaNO_3$, acetate) solution prepared at room temperature, and which underwent two successive continuous thermal cyc les at the usual temperature sweep, here ± 0.6 °C/h, illustrated in Fig. 5.

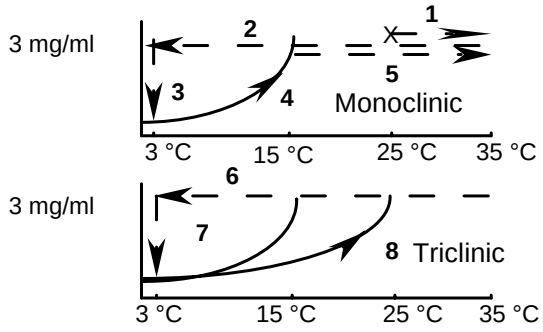

Figure 5
Illustration of the two thermal cycles at ± 0.6 °C/h for a 3.1 mg/ml lysozyme 0.2 M $NaNO_3$, 50 mM acetate buffer solution.

- the first cycle starts by heating the solution to 35 °C (path **1**). Cooling from 35 °C to 3 °C (paths **2** and **3**), the solution becomes slightly turbid around 3.5 °C. The crystallised solution is then slowly heated until 35 °C (paths **4** and **5**). The solid phase dissolves at 15.6 °C, during the three hours of path **4**.

- the second cycle is also from 35 °C to 3 °C (path **6**) with the sweep rate of - 0.6 °C/h. Turbidity of the system takes again place at around 3.5 °C together with crystallisation (path **7**). This time the dissolution temperature is surprisingly measured at 25 °C (path **8**) and the solution remains clear afterwards.

In this way we therefore measured the temperature of dissolution of 15.6 °C for the monoclinic form, and of 25 °C for the triclinic form, with the same 3.1 mg/ml protein solution (Fig. 4a). After the first heating, during path **1**, no solid phase is present in the solution. The crystallisation in the first thermal cycle leads to a majority of monoclinic microcrystallites which then dissolve during path **4** (three hours). During part of path **5** the solution is supersaturated with respect to the triclinic form, this latter can develop. At the end of path **5** at 35 °C, some triclinic form nucleimay still be present in the solution, develop during path **6** when the solution is again supersaturated with respect to the triclinic form (these nuclei are not detected by the light scaterring device), and also during path **7**. At the end of path **7**, the monoclinic form appears: it dissolves while the triclinic crystals grow when temperature raises during path **8**.

4. Summary and conclusions

In this paper we present the first solubility data and the dissolution enthalpy of the triclinic form of lysozyme nitrate grown from buffered 0.2 M $NaNO_3$ (pH = 4.5) solutions. To obtain the solubility data for the monoclinic form below 5 mg/ml and the data for the triclinic above 5 mg/ml we used a precipitation step at 4 °C. To determine the solubility of the triclinic form below 5 mg/ml, the latter protocol is inefficient, therefore we seeded the solutions. This may be due to the relative positions of the nucleation domains of both polymorphs, monoclinic and triclinic forms of lysozyme nitrate. By using the temperature dependences of their solubility, we have interpreted the observations quoted in literature about the crystallisation conditions of the two forms, experimental conditions that this present work contributes to clarify. Moreover, our experiments seems to suggest the presence of submicroscopic nuclei of the triclinic form in a 3.1 mg/ml lysozyme solution which undergoes thermal gradients.

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