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Investigating the nucleation of protein crystals with hydrostatic pressure

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Abstract

Hydrostatic pressure in the 0.1–75 MPa range has been used as a non-invasive tool to study the crystallization process of the tetragonal crystal form of the protein thaumatin (M_r 22 200). Crystals were prepared within agarose gel and at temperatures in the range from 283 to 303 K. The solubility, i.e. the concentration of soluble macromolecules remaining in equilibrium with the crystals, decreases when the pressure increases and when the temperature decreases. High pressure was used to probe the nucleation behaviour of thaumatin. The pressure dependence of the nucleation rate leads to an activation volume of $-46.5\ \mathrm{cm^3\ mol^{-1}}$. It is shown that an increase in pressure decreases the enthalpy, the entropy and the free energy of crystallization of thaumatin. The data are discussed in the light of the results of crystallographic analyses and of the structure of the protein.

1. Introduction

The thermodynamic parameter pressure has become increasingly important for basic and as well as for applied physics studies of solid-state phenomena such as crystal growth from homogeneous solutions. Despite numerous industrial applications employing crystallization to separate inorganic molecules, similar experiments on biological macromolecules are rare [1]. Preliminary studies on model proteins have suggested that pressure might influence their crystallization as much as temperature, supersaturation, or ionic strength (see e.g. [2–7]).

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However, it is not clear whether the observed effects result only from compression of the polypeptide chains or also from subtle changes in their solvation. Although the effects of pressurization on the crystallization outcome apparently vary from protein to protein, pressure is a useful tool to analyse the steps of this process.

Proteins are biopolymers that are formed by one or several chains made of well-defined sequences of amino acids linked by covalent peptide bonds. In aqueous solution, the surface of the native (i.e. not denatured) conformations of soluble polypeptides is preferentially hydrated. As with the crystallization of small molecules, that of proteins occurs in two steps: (i) nucleation during which critical sized nuclei form (see e.g. [8] for a review of current theories), and (ii) growth during which the latter gain in volume and attain their definitive habit. The driving force for crystallization is supersaturation. When it exceeds a critical value, the activation energy of nucleation is sufficient to create attractive interactions between solute macromolecules. At the surface of the protein, solvent molecules and counter-ions are displaced to expose charged chemical groups or hydrophobic surfaces that become involved in non-covalent packing contacts during the assembly. Nuclei are unstable intermediates resulting from the ordered aggregation of a small number of unitary particles. In the case of proteins, light (see e.g. [9–13] and references therein) and x-ray scattering analyses (see e.g. [14, 15]) have shown that monodisperse solutions have a greater propensity to nucleate, while polydisperse ones tend to form disordered aggregates. (This phenomenon is different from the denaturation that is observed beyond a critical pressure.) Further, the best crystals (in terms of volume and perfection) are obtained when the mother solution has an initial composition corresponding to that in the metastable zone (located between the solubility and the supersolubility curves) of the 2D phase diagram representing protein versus precipitant concentration [16].

This paper reports the effect of hydrostatic pressure on the nucleation of tetragonal thaumatin crystals within agarose gel. Kinetic and thermodynamic data are described and discussed in the light of the crystallographic properties of the crystals and of the 3D structure of this protein. The goal of these investigations is dual: first, to understand the physics of the crystallization process under pressure, and second, to find a way to produce crystals of enhanced crystallographic quality (i.e. having fewer defects). Preliminary results of this study have been presented as a poster at the *Joint 19th AIRAPT–41st EHPRG International Conference on High Pressure Science and Technology (Bordeaux, France, 7–11 July 2003).*

2. Experimental details

2.1. Proteins and chemicals

All solutions were made with ultra-pure sterile water and filtered through membranes of 0.22 μm pore diameter. For the purpose of comparison, a single batch of lyophilized thaumatin I (Cat. No T-7638, Lot 108F00299, Sigma; M_r 22 204) was used throughout a great variety of crystallogenesis studies (i.e. crystallization in various media like solutions and gels, or environments including microgravity). The protein was dissolved in water and used without any further purification. Analyses by electrophoresis have shown that thaumatin is accompanied by contaminants that do not exceed in total a few per cent (m/m). Mass spectrometry analyses have confirmed the presence of traces of thaumatin II that differs in M_r by only 84 (or 0.4%) [17]. Protein concentration was determined from absorbance at 280 nm using an extinction coefficient of 2.6 units per 1 mg ml^{-1} solution (1 cm path length). Tartaric acid (Cat. No T-5259) and *N*-[2-acetamido]-2-iminodiacetic acid (ADA, Cat. No A-9883) were purchased from Sigma. Low gelling temperature agarose powder was a gift from So.Bi.Gel (Hendaye, France).

2.2. Crystallization assays

To prepare crystallization assays, buffer, precipitant, agarose and protein solutions were mixed following this order. The mixture was then filled without air bubbles in small glass tubes (i.d. 5 mm, o.d. 0.7 mm, length 13 mm, volume 250 μl) that were previously coated inside with the organosilane Prosil 28 (PCR, Gainesville, FL). The precipitant was tartaric acid buffered with 0.1 M ADA adjusted at pH 6.5 with NaOH. Agarose gel was used at a final concentration of 0.3% (m/v).

For kinetics measurements, duplicate samples of thaumatin were incubated under 0.1 or 75 MPa and at 293 K for 1–9 days by increments of one day. Other samples were incubated in duplicate for a period of 9 days at different temperatures between 283 and 303 K by increments of 5 K. Hydrostatic pressure experiments were performed as described previously [5]. Pressure was applied at a rate of 50 MPa min^{-1}, kept constant for a given time, and released at the same rate to minimize protein denaturation or perturbations. Experiments at atmospheric pressure (0.1 MPa) were performed in parallel under otherwise identical conditions. The content of the cells was analysed immediately after depressurization. For crystal counting, size measurements and photography, the solution was transferred from the tube onto a glass plate placed under a binocular stereomicroscope (model SZH10, Olympus). Under the present experimental conditions, thaumatin crystallizes as tetragonal dipyramids belonging to space group $P4_12_12$ with unit cell parameters $a = b = 58.6$ Å and $c = 151.5$ Å [18]. These crystals typically diffract x-rays to a resolution of 1.3 Å on a synchrotron source. Owing to these crystals, the structure of the protein is known at a resolution of 1.2 Å [19].

2.3. Solubility measurement

After depressurization, some solution containing a minimum of crystalline material was withdrawn from each sample tube and centrifuged for 2 min to remove crystal and gel fragments. The clear solution was then filtered on a membrane with pore diameter of 0.22 μm (Ultrafree-MC; Cat. No UFC30GV00, Millipore) and 20 μl filtrate was diluted 30-fold with sterile water prior to concentration measurement.

2.4. Static light scattering analyses

Measurements were performed with a dp-801 dynamic light scattering instrument (Protein Solution Ltd, High Wycombe, UK). Undersaturated solutions containing 2–5 mg ml^{-1} thaumatin (by increments of 0.5 mg ml^{-1}), 0.73 M tartrate and 50 mM ADA were transferred in 12 μl cuvettes and centrifuged for 3 min at 4500 rpm (1350 $\times$ g) prior to measurements.

Static light scattering data were used to determine the second virial coefficient that is related to protein concentration and molecular mass by Kartochvil's equation [20]:

$$\frac{KC}{R_\theta} = \frac{1}{M} + 2B_2C + \cdots \tag{1}$$

with R_θ the Rayleigh ratio (i.e. the excess intensity of scattered light at the angle θ) and K the optical constant of the experimental set-up that is given by

$$K = \frac{4\pi^2 n_0^2 (\mathrm{d}n/\mathrm{d}C)^2}{N\lambda^4} \tag{2}$$

where n_0 is the refractive index of the solvent, N is Avogadro's number and λ the wavelength of the incident light in vacuum. The second virial coefficient B_2 is obtained from the slope of the graph of $KC/R\theta$ versus C.

3. Analysis of solubility data

3.1. Kinetic concept

When the crystallizing system relaxes into a state that is thermodynamically more stable by forming a new phase, the process can be represented by the reaction

$$\text{Protein}_{\text{solution}} + \text{Water}_{\text{various pools}} \rightarrow \text{Transition state} \rightarrow \text{Protein}_{\text{nuclei}} + \text{Water}_{\text{various pools}}.$$

The water pools include bulk water and hydration water that is bound to the amino acids residues either directly or by H-bonds. The nucleation rate is defined as the number of crystals appearing per unit volume of supersaturated solution per unit time. The dependence of the nucleation rate N upon supersaturation is given by the relationship

$$N = k_{\text{n}}\left(\frac{C - C_{\text{e}}}{C_{\text{e}}}\right)^{a} \tag{3}$$

where k_{n} is the nucleation kinetic constant and a is the nucleation rate exponent. The ratio $(C - C_{\text{e}})/C_{\text{e}}$ is the supersaturation, with C being the initial protein concentration and C_{e} the solubility, i.e. the concentration remaining in equilibrium with the crystals at the end of their growth. For the purpose of comparison, in this study solubility is actually replaced by the residual protein concentration that is in equilibrium with crystals after a given time. We have verified that after the longest equilibration time (9 days), the real solubility is almost reached [21]. Nevertheless, and because of the experimental errors and the presence of small amounts of impurities and insoluble material, we consider the measured values to be apparent solubilities.

At constant temperature and composition, the nucleation kinetics constant is affected by pressure according to the relationship

$$[\partial(\ln k_{\text{n}})/\partial(P)]_{T,n} = -\Delta V_{\text{n}}^{*}/RT \tag{4}$$

where ΔV_{n}^{*} stands for the activation volume of nucleation. The latter is defined as the difference between the partial molar volume of the species in the transition state and the partial molar volume of the reaction [22].

3.2. Thermodynamic concept

The value of Gibbs free energy for a reaction is based upon its enthalpy and entropy. Both factors are either negative or positive when a reaction can occur spontaneously or not at all, respectively. For reactions occurring at constant temperature, the change in free energy is given by

$$\Delta G = \Delta H - T\Delta S \tag{5}$$

where ΔH and ΔS represent the variation of the enthalpy and of the entropy of crystallization, respectively.

When protein molecules in a solution are in equilibrium with those in a crystalline state, the standard free energy of crystal formation is expressed by

$$\Delta G^{0} = G^{0}_{\text{crystal}} - G^{0}_{\text{solution}} = -RT \ln K_{\text{e}} \tag{6}$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}) and T the absolute temperature in kelvins. $K_{\text{e}} = 1/\gamma C_{\text{e}}$ is the equilibrium constant in which C_{e} is the solubility of the protein and γ the activity coefficient.

Supersaturated protein solutions are far from being ideal, and non-ideality was repeatedly confirmed by the values of the virial coefficient determined by osmometry or by static light

scattering measurements (see, for example, [23, 24]). The solubility C_e and second virial coefficient B_2 are related by

$$\Delta\mu/k_BT = \ln(C/C_e) + 2B_2M(C - C_e) + \cdots \tag{7}$$

where $\Delta\mu$ is the difference in chemical potential between the crystalline and the solution state, k_B the Boltzmann constant (1.3807×10^{-23} J K^{-1}), T temperature and M the molecular mass [25]. In the case of an ideal solution, equation (7) can be written as

$$\Delta\mu/k_BT = \ln(C/C_e) \tag{8}$$

and the activity coefficient γ can be approximated to unity since attractive and repulsive interactions between molecules are balanced. Further, equation (6) can be written as

$$G^0_{\text{crystal}} - G^0_{\text{solution}} = RT \ln C_e. \tag{9}$$

In the specific case of crystallization, enthalpy and entropy can be obtained from van't Hoff plots representing $\ln C_e$ as a function of $1/T$. An alternative approach to estimate these properties of state is to consider the effect of temperature on protein solubility. Indeed, the combination of equations (5) and (9) leads to the equation

$$\ln C_e = \frac{\Delta H}{R}\left(\frac{1}{T}\right) - \frac{\Delta S}{R}. \tag{10}$$

The enthalpy changes associated with the crystallization are generally expressed as the number of heat units liberated by the system when the process takes place isothermally.

4. Results and discussion

4.1. The effects of pressure and temperature on thaumatin nucleation in a hydrogel

In these experiments the role of the agarose gel was to reduce convection in the crystallization solution, to immobilize nuclei, and to favour the diffusive transport of molecules towards the surfaces of nuclei and growing crystals in order to produce a crystalline material of superior quality [18, 21]. From a practical point of view, the gel also facilitates crystal counting and size measurements.

Figure 1 displays tetragonal crystals of thaumatin having grown after 216 h (=9 days) at 0.1 or 75 MPa and 293 K. At ambient pressure, the apex-to-apex length of the dipyramids increases by a factor of ∼4.6 from samples that were incubated for 72 h to those incubated for 216 h. At 75 MPa and in the same time interval, the increase factor is similar (∼4) but the final volume of the crystals is on average 6–7-fold smaller. On the other hand, pressurization is accompanied by an augmentation of the number of crystals (not shown in figure 1; see the caption), as reported previously for lysozyme [5]. By extending the duration of pressurization from 72 to 216 h, the average number of crystals increases by ∼520 and 460% at 0.1 and 75 MPa, respectively.

Figure 2 displays the effect of temperature on thaumatin crystallization at two constant pressures. The striking feature is that the size of the crystals gradually increases with temperature at both pressures. Their apex-to-apex length decreases by factors of 2.75 and 2.25 at 283 and 303 K, respectively when the pressure goes from 0.1 to 75 MPa. On the other hand, the length of the crystals increases by factors of 3.1 and 3.8 between 283 and 303 K at 0.1 and 75 MPa, respectively. The average maximal crystal volume is 12 times greater at 303 K at 0.1 MPa than under pressure. Under pressure, there are fewer large crystals at 303 K. Some of them exhibit defaults such as cracks that are likely a consequence of the gel–sol transition of agarose. In addition, there is a smaller amount of insoluble (i.e. aggregated) protein with the crystals across the temperature range. Thus, the effect of temperature is comparable but under pressure the crystals are always smaller.

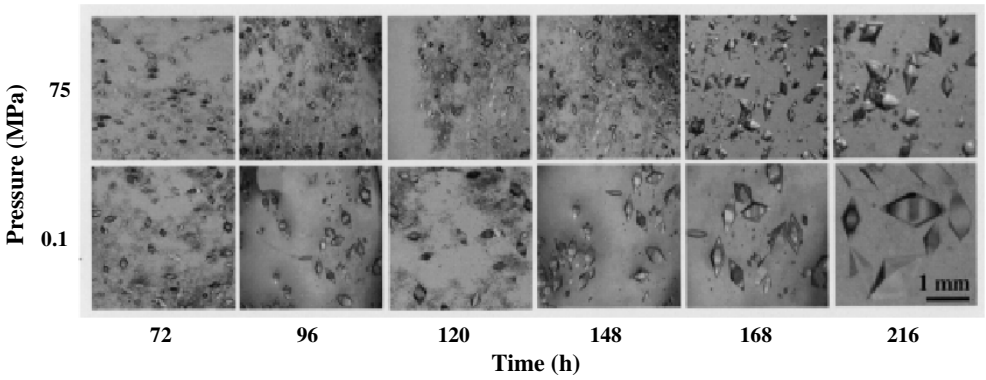

Figure 1. The effect of pressure (0.1 and 75 MPa at 293 K) on the crystallization of the tetragonal crystal form of thaumatin. All images are at the same scale but each one displays only a part of the sample tube to show the crystals with largest dimensions. Thus it does not reflect the actual number of crystals in the entire sample.

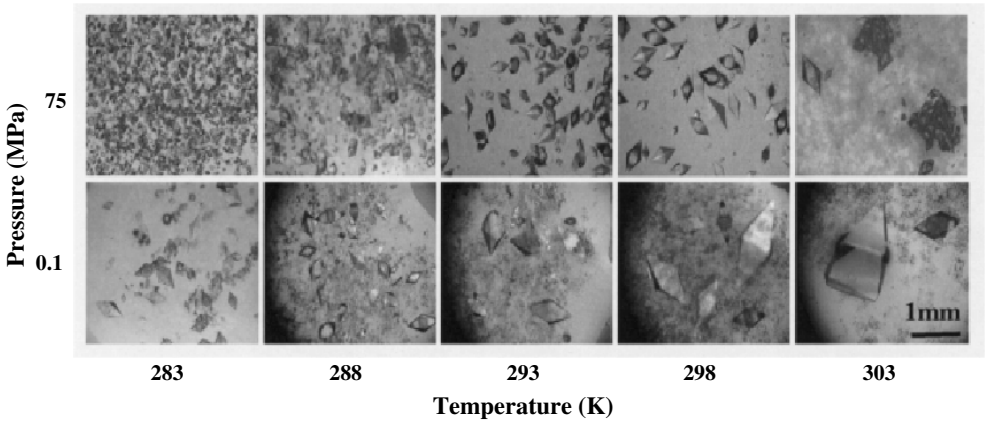

Figure 2. The effect of temperature (between 283 and 303 K) on the crystallization of thaumatin. The same remarks apply as for figure 1.

4.2. The effects on thaumatin solubility

By working under different pressures and/or temperatures, some thermodynamic data can be identified and the flow of matter from one phase to another can be monitored. Both pressure and temperature affect the solubility of thaumatin when they are kept constant. At ambient pressure, the increase in the solubility with temperature is biphasic (result not shown). The slope is 0.21 mg ml^{-1} K^{-1} between 283 and 298 K and 0.96 mg ml^{-1} K^{-1} between 298 and 303 K. At 75 MPa, the variation is monotonic, with a slope of 0.29 mg ml^{-1} K^{-1} between 283 and 303 K.

4.3. The effect of pressure on the nucleation kinetics

The kinetic parameters of the nucleation of thaumatin crystals are listed in table 1. As mentioned above, the number of crystals increases at higher pressure and thus at higher supersaturation since protein solubility decreases. For this protein, the nucleation rate exponent

Table 1. Kinetic parameters for the nucleation process of thaumatin.

	a	k_n	ΔV_n^*
0.1 MPa	0.84	53.38	
			-46.50 ± 2.30
75 MPa	1.63	239.9	

Table 2. The second virial coefficient and the thermodynamic driving force of thaumatin crystallization at 0.1 and 75 MPa.

	B_2 (mol ml g^{-2})	$\Delta\mu/k_BT$[a] (ideal solution)	$\Delta\mu/k_BT$[a] (real solution)	$\frac{\Delta(\Delta\mu_{ideal} - \Delta\mu_{real})}{\Delta\mu_{ideal}}$ (%)
0.1 MPa	-4.34×10^{-5}	1.25	1.23	1.6
75 MPa	-4.34×10^{-5}	1.55	1.49	3.9

[a] For a protein concentration $C = 35$ mg ml^{-1} and a solubility $C_e = 9.99$ mg ml^{-1} at 0.1 MPa and $C_e = 7.43$ mg ml^{-1} at 75 MPa, respectively.

is 0.84 and 1.63 at 0.1 and 75 MPa, respectively. The nucleation rate constant is 4–5 times higher at 75 than at 0.1 MPa. The activation volume of nucleation is -46.50 cm^3 mol^{-1}. This is in favour of a lower volume for the activated state, meaning that high pressure promotes the nucleation of thaumatin crystals. The greater number of crystals is ascribed to the combined effect of the presence of the agarose gel (promoting nucleation) and of the decrease of thaumatin solubility under pressure. The latter point was confirmed by the comparison of the number of ordered water molecules inside the solvation shell of the protein. Indeed, there are 17% fewer such molecules in the structure model at 1.8 Å resolution that was built from diffraction data collected on a crystals grown under a pressure of 150 MPa and subsequently depressurized [18].

In the case of the protein glucose isomerase the nucleation rate was 17 times greater at 200 MPa than at ambient pressure [1]. In the case of lysozyme, a protein widely studied under pressure and serving as a model, the solubility increases [5, 26, 27] with pressure and the nucleation rate decreases [27]. The same trend was found for a third protein named subtilisin [22, 28].

4.4. Thermodynamic characteristics of thaumatin crystallization

A second virial coefficient $B_2 = -4.34 \times 10^{-5}$ mol ml g^{-2} was derived from static light scattering data obtained on undersaturated thaumatin solutions (see table 2). In equation (7) the terms $\ln(C/C_e) = 1.253$ and $2B_2M(C - C_e) = 0.0192$. Hence, the second term represents only 1.5% (at 0.1 MPa) and 3.4% (at 75 MPa) of the first one and can be considered to be negligible with regard to experimental errors. The thermodynamic driving force of crystallization, expressed by $\Delta\mu/k_BT$, is slightly greater under pressure (see table 2). The relative increment in chemical potential confirms that the protein solution can be assumed to be an ideal one. Consequently, thermodynamic ideality can be used to characterize protein crystallization.

The temperature dependence of thaumatin solubility is displayed in figure 3. At 0.1 and 75 MPa, $\ln(C_e)$ values decrease linearly with $1/T$ within experimental errors. This linearity is supported by the absence of any detectable phase transition. For instance, neither change in crystal habit (the axial ratio of the dipyramids is constant) nor in space group is observed between 283 and 303 K. The dependence provides, through equation (4),

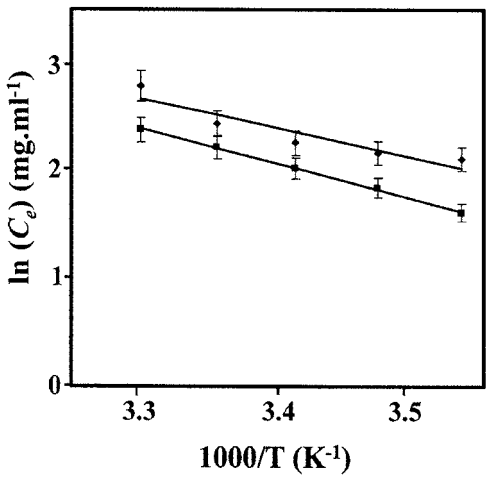

Figure 3. van't Hoff plots of thaumatin solubility as a function of temperature at 0.1 MPa (upper data points) and 75 MPa (lower data points). The error bars on solubility represent 5% of data.

Table 3. Thermodynamic parameters for the crystallization of thaumatin.

	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})
0.1 MPa	-23.10 ± 1.15	-98.05 ± 4.90	$+4.95 \pm 0.25$[a]
			$+7.00 \pm 0.35$[b]
75 MPa	-27.40 ± 1.35	-110.25 ± 5.50	$+3.80 \pm 0.20$[a]
			$+6.00 \pm 0.30$[b]

[a] Values at 283 K.
[b] Values at 303 K.

a value for the enthalpy of crystallization. It is -23.10 ± 1.15 kJ mol^{-1} at 0.1 MPa and -27.40 ± 1.35 kJ mol^{-1} at 75 MPa. Using equation (10), entropies of crystallization of -98.05 ± 4.90 J mol^{-1} K^{-1} and -110.25 ± 5.50 J mol^{-1} K^{-1} are found at 0.1 and 75 MPa, respectively (see table 3).

The variation of the free energy of crystallization can be deduced from equation (9). ΔG decreases with increasing pressure and depends on temperature. It varies from $+4.95 \pm 0.25$ to $+3.80 \pm 0.20$ at 280 K and from $+7.00 \pm 0.35$ to $+6.00 \pm 0.30$ at 303 K, at 0.1 and 75 MPa, respectively (see table 3). From these results, we conclude that the crystallization of thaumatin occurs with negative ΔH and ΔS values and a positive ΔG value whatever the pressure. The decrease in the solubility accompanying an increase in pressure leads to a volume of crystallization of ~ -11 cm^3 mol^{-1} [21].

We ascribe the decrease in enthalpy of crystallization to the decrease in volume of crystallization under pressure. The lower ΔS value indicates that pressure favours the ordered arrangement of thaumatin molecules. The lower value of ΔG is a consequence of the lower value of the equilibrium constant (i.e. the lowest values of the solubility due to the highest amount of crystalline material). On the other hand, a higher pressure results in a lower ΔH indicating that the crystallization of thaumatin is an exothermic reaction (see table 3). A positive ΔG value means that ΔH values are larger than $T\Delta S$ ones and that the crystallization of thaumatin is a non-spontaneous process.

4.5. The crystallographic quality of the crystals

As mentioned under section 4.1, the crystalline material produced under pressure may have some advantages for the determination of the structures of biological macromolecules. Indeed, our x-ray diffraction and topography analyses have shown that thaumatin crystals produced under pressures of 50–75 MPa are less mosaic (see figures 2 and 5 in [18]). The Bragg reflection profiles recorded from such crystals are sharper because poor-quality nuclei are destabilized under pressure. These studies had also confirmed that depressurization does not introduce additional defects in the elastic lattice. The latter point is of practical importance if crystals are prepared under pressure and subsequently analysed at atmospheric pressure.

5. Conclusion

High pressure was used as a probe to understand the nucleation behaviour of thaumatin. A study of its effects as well as of those of temperature has led to the determination of thermodynamic data of the crystallization process of this protein. Pressure causes a decrease in the enthalpy, in the entropy and in the free energy of crystallization. Values for enthalpy and entropy are negative while that for free energy is positive. The nucleation rate exponent and nucleation rate constant are respectively 2 times and 4 times higher at 75 MPa than at atmospheric pressure. The pressure dependence of the nucleation rate provides a negative activation volume indicating that pressure increases thaumatin crystal nucleation. These properties of the system are correlated with results from crystallographic analyses showing that pressure may be applied for selecting high-quality crystals. In the case of thaumatin, the practical use of pressure is to produce crystals with a higher order.

Acknowledgments

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