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Crystal quality and differential crystal-growth behaviour of three proteins crystallized in gel at high hydrostatic pressure

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Pressure is a non-invasive physical parameter that can be used to control and influence protein crystallization. It is also found that protein crystals of superior quality can be produced in gel. Here, a novel crystallization strategy combining hydrostatic pressure and agarose gel is described. Comparative experiments were conducted on hen and turkey egg-white lysozymes and the plant protein thaumatin. Crystals could be produced under up to 75–100 MPa (lysozymes) and 250 MPa (thaumatin). Several pressure-dependent parameters were determined, which included solubility and supersaturation of the proteins, number, size and morphology of the crystals, and the crystallization volume. Exploration of three-dimensional phase diagrams in which pH and pressure varied identified growth conditions where crystals had largest size and best morphology. As a general trend, nucleation and crystal-growth kinetics are altered and nucleation is always enhanced under pressure. Further, solubility of the lysozymes increases with pressure while that of thaumatin decreases. Likewise, changes in crystallization volumes at high and atmospheric pressure are opposite, being positive for the lysozymes and negative for thaumatin. Crystal quality was estimated by analysis of Bragg reflection profiles and X-ray topographs. While the quality of lysozyme crystals deteriorates as pressure increases, that of thaumatin crystals improves, with more homogeneous crystal morphology suggesting that pressure selectively dissociates ill-formed nuclei. Analysis of the thaumatin structure reveals a less hydrated solvent shell around the protein when pressure increases, with ∼20% less ordered water molecules in crystals grown at 150 MPa when compared with those grown at atmospheric pressure (0.1 MPa). Noticeably, the altered water distribution is seen in depressurized crystals, indicating that pressure triggers a stable structural alteration on the protein surface while its polypeptide backbone remains essentially unaltered.

1. Introduction

Like crystallization of other materials, crystallization of proteins is influenced by physical parameters (Ducruix & Giegé, 1999; McPherson, 1999; Giegé & McPherson, 2001). Surprisingly, the effects of pressure have been neglected for a long time (reviewed by Suzuki, Sazaki, Miyashita *et al.*, 2002). After the first report of the application of pressure in the industrial crystallization of glucose isomerase (Visuri *et al.*, 1990), a series of systematic studies on hen egg-white lysozyme (HeL) appeared (*e.g.* Gross & Jaenicke, 1991; Schall *et al.*, 1994; Lorber *et al.*, 1996; Takano *et al.*, 1997; Sazaki *et al.*, 1999). To date, however, apart from the studies on HeL and the recent investigation on glucose isomerase from *Streptomyces rubiginosus* (Suzuki, Sazaki, Visuri *et al.*, 2002), only three other proteins have been crystallized under hydrostatic pressure, namely bacterial subtilisin (Waghmare *et al.*, 2000), turkey egg-white lysozyme (Kadri *et al.*, 2002) and the sweet plant protein thaumatin (Kadri *et al.*, 2002).

Initially, crystallization attempts with biomacromolecules were always performed in aqueous solutions. For various reasons, however, some investigators decided to crystallize their proteins in transparent gel matrices, media in which the crystals should be of improved quality because convection is minimized (Robert & Lefaucheux, 1988). The additional advantage is the immobilization of the crystals in the gel, with the consequence that crystals can be counted and measured more easily (Kadri *et al.*, 2002). However, it took many

years before the improvement of crystal quality in gel was experimentally verified (see, for example, Robert *et al.*, 1999; Lorber, Suater, Ng *et al.*, 1999; Vidal *et al.*, 1999) and despite this breakthrough crystallization in gel is not of current use in structural biology laboratories.

Here, we have combined the potential positive effects of high hydrostatic pressure and agarose gel on the crystallization of thaumatin and the two lysozymes from hen egg white (HeL) and turkey egg white (TeL). The choice of these proteins was dictated by the fact that thaumatin and the lysozymes show significant architectural differences, but also because the lysozymes are structurally related, differing by only seven amino acids. Thus, experiments were designed so that general and possibly specific effects of pressure and agarose gels on the crystallizability of these proteins could be detected. Another aim was to find whether the peculiar crystallization conditions at high pressure and in gel would provide an alternative means to produce crystals of improved (or modified) quality for structural studies. Results reviewed in this report demonstrate that pressure affects nucleation of the three proteins and has specific effects on crystal growth parameters and crystal quality. Thus, solubility of the two lysozymes increases with pressure while that of thaumatin decreases. These differential effects, which are correlated with negative and positive variations of the crystallization volumes of the proteins, are discussed in relation to the structural modifications that occur in the hydration shell of depressurized thaumatin.

2. Materials and methods

2.1. Proteins and crystallization under high hydrostatic pressure

All chemicals and proteins were as described elsewhere (Charron *et al.*, 2002; Kadri, Jenner *et al.*, 2003). Solutions were made with ultrapure sterile water and filtered through membranes of 0.22 µm pore size (Millex, Millipore). Thaumatin was crystallized in the presence of tartaric acid and 0.1 *M* *N*-[2-acetamido]-2-iminodiacetic acid adjusted to pH 6.5. Lyophilized turkey and hen lysozyme were dissolved in 0.05 *M* sodium acetate buffer adjusted to pH 4.5 with NaOH. Both proteins were crystallized in the presence of NaCl. In some experiments, pH was varied as indicated in Table 1. Low-gelling temperature agarose from So.Bi.Gel (Hendaye, France) was used at a final concentration of 0.3%(*w*/*v*). Other conditions for batch crystallization were as in Kadri, Jenner *et al.* (2003).

Crystallization components were filled in small glass tubes (inner diameter 5 mm, outer diameter 0.7 mm, length 13 mm, volume 250 µl). Samples were pressurized under 25–250 MPa for 1–9 d at $T = 293 \pm 1$ K. Controls at 0.1 MPa were performed under otherwise identical conditions. Limits to obtain HeL, TeL and thaumatin crystals were 75, 100, 250 MPa, respectively. For pressurization and depressurization protocols, see Kadri, Jenner *et al.* (2003).

2.2. Nucleation and growth-rate measurements

Experimental conditions were as published elsewhere (Kadri, Jenner *et al.*, 2003; Kadri, Damak *et al.*, 2003). Summarizing, after depressurization the content of each 250 µl sample was transferred onto a glass plate for visual examination and crystal counting. The length along the *c* axis and the width along the *a* axis of the lysozyme crystals were also measured. Supersaturations were calculated from residual protein concentrations measured at 293 K after depressurization. For thaumatin, nucleation rates (N) and crystal-growth rates were obtained by plotting respectively the number of crystals or the crystal length *versus* time (by increments of 1 d) (Kadri, Damak *et al.*, 2003). Notice that N is related to supersaturation (σ) by the equation

Table 1
Solubility variations with pressure and pH of thaumatin, HeL and TeL.

Solubility values at the pH at which their variation with pressure is maximal, leading either to increased (***S***↑) or decreased (***S***↓) solubilities, are emphasized in bold characters.

(*a*) HeL.

	Solubilities (mg ml^{-1})				
Pressure (MPa)	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0
0.1	6.7	6.6	5.5	4.0	**2.6**
25	7.0	6.9	5.7	4.3	**2.8**
50	7.2	7.1	5.9	4.5	**3.1**
75	7.4	7.3	6.2	4.7	**3.6**
					S↑1.4-fold

(*b*) TeL.

	Solubilities (mg ml^{-1})				
Pressure (MPa)	pH 4.0	pH 4.5	pH 5.0	pH 5.5	pH 6.0
0.1	6.0	**4.5**	3.6	2.3	2.0
25	6.9	**5.3**	4.4	2.6	2.0
50	7.2	**6.2**	4.9	2.6	2.1
75	8.2	**7.1**	5.0	2.7	2.2
100	9.0	**8.0**	5.1	2.7	2.2
		S↑1.8-fold			

(*c*) Thaumatin.

	Solubilities (mg ml^{-1})			
Pressure (MPa)	pH 5.7	pH 6.5	pH 7.0	pH 8.0
0.1	10.8	9.7	9.0	**8.2**
50	9.1	8.3	7.3	**5.9**
100	7.5	6.8	5.2	**4.4**
150	6.0	5.4	4.6	**3.5**
200	4.4	4.0	3.4	**2.2**
230	3.3	3.2	2.2	**1.5**
				S↓5.5-fold

$N = k_n\sigma^a$, where k_n is the nucleation kinetic constant and a is the nucleation rate exponent (Kadri, Damak *et al.*, 2003). Crystal fragments and gel were eliminated by centrifugation (2 min, 300*g*) and ultrafiltration on membranes of 0.22 µm pore diameter (Ultrafree-MC, Millipore). Filtrates were diluted in water prior to absorbance measurements and protein concentrations calculated assuming one $A_{280\,nm}$ unit corresponds to 0.8 mg ml^{-1} thaumatin or 0.385 mg ml^{-1} lysozyme when the optical pathway is 1 cm.

2.3. Access to crystallization volume

As discussed by Kadri *et al.* (2002), the pressure-dependence of protein crystallization is related to the crystallization volume ΔV (with $\Delta V = V_{\text{protein in crystal}} - V_{\text{protein in solution}}$), or more precisely to the volume change of crystallization expressed in cm^3 mol^{-1}, by the relationship

$$[\partial \ln K/\partial P]T = -\Delta V/RT, \quad (1)$$

where K is the equilibrium constant between the protein in the crystalline state and in solution, R the gas constant and T the absolute temperature. Notice that crystallizations accompanied by a decrease in volume are favoured under pressure. Furthermore, ΔV is related to the solubility S *via*

$$\Delta V = -RT \ln(S_P/S_0)/(P_P - P_0), \quad (2)$$

where S_P and S_0 are solubilities at a given pressure P_P and at atmospheric pressure P_0, respectively. Thus, ΔV can be obtained from $\ln(S_P/S_0)$ *versus* ΔP plots.

Notice that one deals here with apparent instead of real solubilities. They were obtained as residual protein concentrations (see

Table 2
Pressure-dependent mosaicity changes in thaumatin and HeL crystals as evaluated by FWHMs of Bragg reflection profiles of a series of crystals oriented with their *c* axis parallel to the plane of the X-rays (w_1 values) or perpendicular to the plane of the wave (w_2 values).

	FWHM w_1			FWHM w_2	
Protein	Pressure (MPa)	No. crystals analyzed	w_1 range (arcsec)	No. crystals analyzed	w_2 range (arcsec)
Thaumatin	0.1	14	10–45	6	10–28
	15	8	12–40	—	—
	30	9	7–32	6	9–26
	45	7	8–22	5	12–26
	75	7	7–18	4	18–26
HeL	0.1	4	7–21	5	13–29
	15	3	6–22	5	12–42
	60	4	14–22	5	67–85

above), *i.e.* concentrations in equilibrium with the crystals after a given time. It was verified that after 9 d equilibration, the real solubilities (that would be obtained after an infinite time) were almost reached (Kadri *et al.*, 2002).

2.4. Methods to assess crystal quality and protein structure

Bragg reflection profiles of depressurized and unpressurized crystals were recorded on beamline D25 of the synchrotron facility LURE at Orsay (France). Full-widths at half-maximum (FWHM) w_1 and w_2 were measured on oriented crystals with their *c* axis parallel (w_1) or perpendicular (w_2) to the vertical plane of the incident X-ray beam. Topographs were recorded under an angular position corresponding to the maximum of reflected intensity (Lorber, Sauter, Robert *et al.*, 1999). For crystal quality comparison, diffraction data were collected at 293 K on the wiggler beamline DW 32 at LURE and were reduced according to standard methods (Collaborative Computational Project, Number 4, 1994; Otwinowski & Minor, 1997). For structure determination of thaumatin, diffraction data were collected at 100 K on beamline ID14-3 at ESRF (Grenoble, France). Data were recorded on crystals of similar size, either grown at 150 MPa and depressurized or on controls prepared at 0.1 MPa. The water molecules were defined using the *CNS* package (Brünger *et al.*, 1998). For the purpose of comparison, water sites located within a radius of 1.4 Å of their closest neighbour were assumed to be equivalent. For more details, see Charron *et al.* (2002).

3. Results and discussion

3.1. General effects on protein crystallizability and nucleation

Systematic and comparative studies of the crystallizability of thaumatin, TeL and HeL under pressure and in agarose gel have revealed different behaviours of the three proteins (Kadri *et al.*, 2002; Kadri, Jenner *et al.*, 2003). Thaumatin was found to crystallize up to 250 MPa ($P4_12_12$ crystals) whereas TeL and HeL form precipitate at this pressure and yield crystals only up to 100 MPa ($P6_122$) and 75 MPa ($P4_32_12$), respectively. This indicates that thaumatin is more robust under pressure than the two lysozymes. Further differences were seen when the number, the morphology and the shape of the crystals were compared. As a general trend one notices an increase of the number of crystals of the three proteins when pressure increases, whatever the pH, indicating that pressure favours nucleation. However, the number of crystals (after 9 d), which gives an estimate of the nucleation rate, is more enhanced for thaumatin (∼1300 crystals per 250 µl sample at 200 MPa) than for TeL or HeL (at most ∼100 crystals per 250 µl sample at 75 MPa). These numbers should be compared with the ∼10–20 crystals per 250 µl sample grown at atmospheric pressure. For thaumatin, the enhanced pressure-dependent nucleation could be explicitly quantitated with the determination of the kinetic parameters of the nucleation process (a and k_n) that are increased 1.9-fold and 4.5-fold at 75 MPa compared with their values at 0.1 MPa (0.8 and 53.4 for a and k_n, respectively; Kadri, Damak *et al.*, 2003). Assuming the pressure-dependence of nucleation rate is linear (which is not strictly the case), one finds an approximately sevenfold stronger increase of this rate per pressure unit for thaumatin than for the two lysozymes. This stimulated nucleation, *i.e.* the increased number of crystals at high pressure, is accompanied by a significant decrease in crystal size. Even though the two structurally related lysozymes behave globally similarly, they nevertheless show specific features, with the morphology of TeL crystals more influenced by pressure then that of HeL crystals.

3.2. Effects on protein solubility and supersaturation

Crystallization phase diagrams in gel were established for the three proteins with pressure and pH as variables (Kadri, Jenner *et al.*, 2003). Under all pH conditions tested (pH range 5.7–8.0 for thaumatin and 4.0–6.0 for the lysozymes) pressure provokes a solubility decrease of thaumatin and a solubility increase of HeL and TeL. Since solubility (S) and supersaturation (σ) are related by $\sigma = (C - S)/C$ (with C the starting protein concentration), it can be concluded that supersaturation increases with pressure in the case of thaumatin while it decreases in that of the two lysozymes. Changes of both parameters can be dramatic. For thaumatin, S varies in the range 1.5–10.8 mg ml^{-1} (for C = 35 mg ml^{-1}) with $S_{minimal}$ occurring at 230 MPa and pH 8.0 and $S_{maximal}$ at 0.1 MPa and pH 5.7. Solubility variations in the range 2.0–9.0 and 2.6–7.1 mg ml^{-1} (for C = 15 and 40 mg ml^{-1}) occur for TeL and HeL, respectively. However, and in contrast with thaumatin, $S_{minimal}$ values were found at low pressure and $S_{maximal}$ values at higher pressure. A comparative summary of the solubility variations with pressure and pH is given in Table 1. Notice the differential behaviour of the two lysozymes, with the largest variations occurring at pH 6.0 for HeL and at pH 4.5 for TeL.

3.3. Effects on crystal quality

The different effects triggered by pressure on thaumatin and lysozyme crystallizations, with in particular thaumatin being more robust at high pressure, raises the question of the existence of possible correlations between crystallizability and crystal quality under such conditions. To that aim diffraction experiments were carried out (Charron *et al.*, 2002). No significant variations in unit-cell parameters were found, except slight changes along the *c* axis of HeL crystals and the *a* and *c* axes of TeL crystals. Furthermore, diffraction limits did not vary significantly with pressure. Topographs of thaumatin and HeL crystals prepared under normal pressure (0.1 MPa) and under high pressure (60–75 MPa) gave essentially similar contrasts. However, significant pressure-dependent changes were found when the mosaicity of the crystals was analyzed. Data on thaumatin and HeL crystals are summarized in Table 2.

Clearly, for thaumatin crystals the distribution of w_1 FWHM values becomes narrower when pressure increases, with a marked tendency towards smaller values at higher pressure, while both distributions and mean values of w_2 remain unchanged. Interestingly, the behaviour of HeL crystals is the opposite. Here, the w_2 FWHM values increase with pressure, while the w_1 values remain essentially unchanged. Analysis of TeL crystals was more delicate since reflection profiles were generally made of several peaks covering a rather

large angular domain. Nevertheless, for TeL crystals grown at 15 MPa, the distribution of w_1 and w_2 FWHM values was found to be narrower than for controls grown at atmospheric pressure.

Concluding, the tetragonal thaumatin crystals grown at high pressure are of superior quality than the controls. The fact that tetragonal crystals possess only one type of growth sector means that a lower mosaic spread is indicative of better nuclei. Thus, high pressure during crystallization may eliminate poor-quality nuclei. Interpretation of mosaicity data of the lysozyme crystals is more complex since they show two growth sectors and faint changes in unit-cell parameters. Thus the increased w_2 values are indicative of a degradation of crystal quality and the constant w_1 may reflect inhomogeneous incorporation of impurities into the crystals and/or strain formation during depressurization.

3.4. Global effects on protein structure

Since thaumatin crystals grown at high pressure appear to be of better quality than the controls grown at ambient pressure, while for the lysozymes the opposite occurs, this prompted us to verify whether such differential behaviour is reflected by structural alterations in the three proteins. A global answer can be provided by thermodynamics and a comparison of ΔV values, the phenomenological parameter that quantitates the volume changes of the proteins during the crystal-growth process at high and atmospheric pressure. At this stage it is important to emphasize that the crystallizing proteins are in fact polypeptide chains surrounded by a water shell and ions interacting with accessible side chains of charged amino acids. During the transition leading to the nuclei, and especially if pressure is applied, both the distribution of water molecules and ions around the proteins and the protein folding may be altered. The crystallization volume changes ΔV will include these different structural effects.

Results indicate opposite behaviours of thaumatin and the lysozymes, with negative crystallization volume changes for thaumatin ($\Delta V = -11.7$ cm^3 mol^{-1} at pH 6.5) and positive ones for HeL ($\Delta V = +3.2$ cm^3 mol^{-1} at pH 4.5) and TeL ($\Delta V = +13.9$ cm^3 mol^{-1} at pH 4.5). Since ΔV is connected with solubility *via* (2), the opposite ΔV values are directly correlated with the opposite pressure-dependent solubility changes discussed above. Notice that positive ΔV values were reported by others for the pressure-triggered crystallization of HeL (Gross & Jaenicke, 1991) and subtilisin (Webb *et al.*, 1999; Pan & Glatz, 2002). Data on negative ΔV values are scarcer and concern beside thaumatin only glucose isomerase. For this protein the reported positive dissolution volume equals a negative crystallization volume (Suzuki, Sazaki, Visuri *et al.*, 2002).

The negative ΔV values for thaumatin mean that at higher pressure its volume is decreased, while the positive ones for HeL and TeL indicate that their volumes are increased. The precise structural interpretation (perturbation on folding and/or on hydration and ion shell) for these ΔV variations, however, cannot be given at this stage. Note that for each of the three proteins, one observes also a pH-dependence of ΔV that is strongest for TeL (with $\Delta V_{\rm minimal}$ of +2.9 cm^3 mol^{-1} at pH 6.0 and $\Delta V_{\rm maximal}$ of +13.9 cm^3 mol^{-1} at pH 4.5). This dependence is likely to be explained by changes in the protein charges that modify the ion and water surrounding of these proteins.

3.5. Specific effects on protein structures

A more precise interpretation of the negative ΔV of thaumatin came from the comparison of its structure computed from diffraction data obtained from crystals grown at atmospheric pressure and from depressurized crystals grown at 150 MPa (for details on data collection and refinement statistics, see Charron *et al.*, 2002). Comparison of both structures at 1.8 Å resolution did not allow detection of significant changes in protein folding, since the r.m.s. deviations of the superimposed structures were low. In contrast, however, the distribution of water sites in both structures was dramatically changed (Table 3). This means that pressure essentially affects the solvent shell around the thaumatin core. Thus, the negative ΔV is accounted for by a decreased number of water molecules around the thaumatin polypeptide chain at high pressure, which reaches ∼20% in crystals grown at 150 MPa as compared to 0.1 MPa. Noticeably, this altered water distribution is seen in depressurized crystals, indicating that pressure triggers a stable structural alteration on the thaumatin surface while its polypeptide backbone remains essentially unaltered. By analogy, one may propose that the positive ΔV of TeL and HeL crystals is accounted for by an increased number of bound water molecules at high pressure, a view in agreement with former crystallographic evidence that has shown that the number of water molecules increases from 151 to 163 in the HeL structure when pressure is raised from 0.1 to 100 MPa (Kundrot & Richards, 1987).

Table 3
Comparison of structural parameters of thaumatin crystallographic models obtained from unpressurized and depressurized crystals.

Structural parameters	Unpressurized structure (0.1 MPa, pH 6.5)	Depressurized structure (150 MPa, pH 6.5)
R.m.s. deviations (Å)		
C^α positions	0.086	
Main-chain atoms	0.134	
All other atoms	0.140	
Solvation shell		
Total No. of H_2O sites	282	233
Common sites to both structures	213	213
Specific sites	69	20

4. Concluding remarks and perspectives

We emphasize that a major goal of crystallogenesis research in the biological field is to develop methods leading to the production of new or better crystals for structure determination. As shown in this report, combining the effects of pressure and agarose gel is a convenient means to modify crystallizability characteristics of proteins and to obtain under well chosen conditions crystals of excellent diffraction quality. Clearly, the agarose gel that was added in the crystallizing solutions favours the crystallization process by reducing the convection effects around the growing crystals. It has the additional advantage of reducing sedimentation and producing well shaped monocrystals that are easy to handle. As shown previously, crystallization of HeL within agarose gel at ambient pressure is characterized by an increased nucleation rate, the solubility remaining unchanged (Vidal *et al.*, 1998). In contrast, at high pressure but without agarose gel, the nucleation rate of HeL is decreased and the solubility increased (Lorber *et al.*, 1996; Sazaki *et al.*, 1999). As shown here, when the gel is present and pressure applied both nucleation rate and solubility are increased, a behaviour that applies also to the crystallization of TeL. Interestingly, this is accompanied by positive ΔV values, an output that *a priori* contradicts Le Chatelier's principle, since less protein would be present in the crystalline phase (see Kadri *et al.*, 2002). From this viewpoint, the crystallization of thaumatin, characterized by an increased nucleation rate but a decreased solubility and hence a negative ΔV, is as expected with more protein in the crystalline phase at high pressure. Specific effects of pressure on protein structures explain these different behaviours. In agreement with this view, it is shown here that thaumatin is more

robust at high pressure than the lysozymes. Consequently, the structure of thaumatin would be less altered by pressure than that of the two lysozymes. Crystallographic results on thaumatin (this work) and HeL (Kundrot & Richards, 1987) support this view, with thaumatin having only its surface properties tuned when pressure is applied, while HeL undergoes more severe pressure-induced changes that impede its crystallizability and leads to conformational changes in the protein within pressurized crystals. To strengthen these interpretations, it would be interesting to find that glucose isomerase, whose crystallization is characterized by a negative ΔV (Suzuki, Sazaki, Visuri *et al.*, 2002), behaves like thaumatin and has its water shell altered by pressure. As a final remark it can be suggested that application of moderate pressures to proteins could be a means to modify their solvation shell and thus a way to tune their surface properties. This may modify crystallizability features and consequently may lead to crystals of enhanced quality, especially if pressure triggers a solubility decrease. Experimental access to this parameter is rather easy, so that comparison of protein solubilities at ambient and higher pressure could become a convenient indicator for crystal quality.

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