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A kinetic model explaining the effects of hydrostatic pressure on nucleation and growth of lysozyme crystals

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Abstract

The pressure-induced inhibition of lysozyme crystal growth has been examined with regard to the time scale of events in the interplay of volume changes and effects of high hydrostatic pressure. The increase in molar volume derived from high pressure experiments is confirmed by dilatometry and found to take place rapidly in comparison with the occurrence of detectable crystals. However, pressure reduces crystal yields even if it is applied in the later phase of crystallization, i.e. after nucleation and volume increase are virtually complete. This indicates that nucleation is not the only pressure-dependent process in crystallisation experiments. The overall kinetics can be interpreted in terms of the Oosawa theory of protein self-assembly, assuming a fast pressure-dependent pre-equilibrium governing nucleation and crystal growth. The equilibrium involves two conformations of the protein, one capable of being incorporated into nuclei or crystals and one that is not.

Keywords: Crystallization; Dilatometry; Hen egg-white lysozyme; High pressure; Molar volume; Oosawa theory

1. Introduction

Protein crystal growth is sensitive to changes in a variety of physical parameters, which may alter the thermodynamic properties of both the crystal or the solution [1]. Among these, high pressure has been neglected for a long time, although it is a thermodynamic variable of fundamental importance, and a condition much more easily obtained than, for instance, microgravity.

The growth of glucose isomerase crystals has been shown to be enhanced by pressures up to 2000 bar [2]. In contrast, we found that the crystallization of lysozyme is slowed down considerably by pressures ranging from 500 to 1000 bar [3]. According to these results, both, the solubility of lysozyme and the half time of its crystallization must be higher at 1000 bar than at atmospheric pressure. The corresponding reaction volume amounts to 12.5 ml/mol; on the other hand, the decreased rate of crystallization may be ascribed to the reduced number of nuclei formed at high hydrostatic pressure [3].

In this study, a kinetic analysis of the volume changes involved in crystallization is presented, combining high pressure crystallization and atmo-

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spheric pressure dilatometry of the two-phase-system. Summarizing available date, it is shown that the effects of high pressure on crystal growth can be quantitatively described by Oosawa's theory of protein self-assembly, if a pressure-sensitive pre-equilibrium is assumed between two forms of the protein, one that is competent for crystallization and one that is not.

2. Experimental procedures

The standard buffer for all experiments was 0.02 *M* sodium acetate, 0.02 *M* acetic acid pH 4.66. Hen egg-white lysozyme (Boehringer Mannheim) was dissolved in buffer, passed through a filter membrane (pore size 0.45 μm) and used without further purification. The precipitating agent was NaCl, dissolved in standard buffer, with a standard final concentration of 0.83 *M*. For the determination of protein concentration in the supernatant, the samples were centrifuged (10 min, 14000 rpm; ca. 15000 g) [1]. Aliquots of 10 μl were diluted 60-fold in water and subjected to photometric absorption measurement at 280 nm. The extinction coefficient was taken to be 2.56. The time was read at the moment when the sample was diluted.

Protein crystallization at high hydrostatic pressures was performed in the equipment described previously [3]. Since in this study the exact definition of the zero time is crucial, the diffusion technique was replaced by a batch procedure. Pressurization was started at defined times after mixing equal volumes of protein and salt solution, each containing twice the concentration required for the final solution. The agarose seal (which permitted the entry of the salt by diffusion in the original device), was replaced by parafilm, and the salt solution in the outer reservoir by water.

[1] For particles of a given size to be removed quantitatively from the solution, they must travel at about 1.1×10^{-5} m/s. Hence, their Svedberg coefficient must be above 760 S. Roughly estimated from this result, the critical size of crystals to be separated is about 61000 kD or 4000 molecules of lysozyme.

The standard protein concentration in the high pressure experiments was 20 mg/ml, corresponding to an oversaturation of 14 mg/ml and of 12 mg/ml at atmospheric pressure and at 1000 bar, respectively, as can be derived from the solubilities determined in [3].

A simple batch procedure was used for the crystallization kinetics at 10 different protein concentrations under atmospheric pressure. Equal volumes of protein and salt solution were mixed and the supersaturated solution was incubated in 100 μl fractions.

For the dilatometric measurements a 23.8 ml glass vessel with a capillary of 1 mm inner diameter was applied. The ratio of the volume change to the height of the liquid column in the capillary (determined gravimetrically) was 0.767 μl/mm. The dilatometer was immersed in a 70 l Tamson thermostate adjusted to 20°C using a buffer reservoir permanently kept at 15°C in order to obtain a heat flow that can be balanced precisely by the heating of the thermostate. The temperature in the bath was found to fluctuate less than 0.05°C. Deviations from the standard temperature, measured with a Beckman thermometer with less than 0.002°C error, were corrected with respect to their volume effect. The height of the meniscus was read by means of a cathetometer with a precision of 50 μm (38.4 nl), corresponding to 1.6×10^{-4}% of the total volume. Thus, the total error of the method amounts to less than 1.9 nl per ml of solution.

3. Experimental results

In our previous report [3], we calculated a positive volume change of 12.5 ml/mol for the crystallization of lysozyme. In order to verify this result by an independant method and to determine the kinetics of the transition from the supersaturated solution to the crystallized state of the protein, we used dilatometric measurements.

Figure 1 illustrates the time course observed for lysozyme concentrations ranging from 17 to 28 mg/ml (20.4 mg/ml in this instance). As becomes clear, a fast increase in volume ranging from 29 to 48 ml/mol (35 ml/mol in this in-

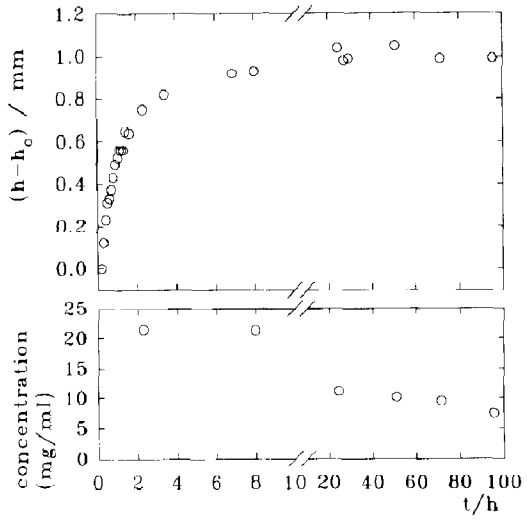


Fig. 1. Top panel: Kinetics of the rapid volume change in the early phase of crystallization of hen egg-white lysozyme in 0.83 *M* NaCl at 20°C, total protein concentration 20.4 mg/ml. Bottom: Protein concentration in the supernatant as determined from a second sample of the same solution incubated in a reference vessel. In control experiments with undersaturated or merely saturated solutions, no volume change has been observed (data not shown).

stance) is typical for the first phase of the reaction [2]. This is virtually completed before crystals have reached a size sufficiently large to allow sedimentation in the centrifuge, which is necessary for the spectroscopic determination of crystallization.

It may be tentatively ascribed to the nucleation reaction in the overall process of crystallization. If this assumption were correct, the effect of pressure on the kinetics of crystal growth would take place mainly in the nucleation phase of crystallization. From this, one would predict the existence of a point of no return, after which pressurization would not interfere with crystallization any longer, except for the change in equilibrium solubility.

In order to test this hypothesis, batch crystallization experiments were performed over a constant time range of four days, applying a constant pressure of 1000 bar after varying times t_0 along the time span of crystallization. The results (Fig. 2) show that there is no instantaneous loss of pressure-induced inhibition of crystallization but rather a continuous decrease of the effect with increasing time t_0. As will be shown, the results may be explained on the basis of the Oosawa theory, assuming two equilibrium conformations of the protein.

4. Application of the Oosawa theory

The Oosawa theory, originally developed for the polymerization kinetics of linear biopolymers such as actin and tubulin [4], has been successfully applied to the growth kinetics of orthorhombic lysozyme crystals [5]. Here we show that, in a limited range of concentrations, it can also account for the growth kinetics of the tetragonal crystal form of the enzyme. The kinetic profiles generated by the theory may be used to examine which parameters must be changed (or introduced) in order to explain the effect of high pressure on the nucleation and growth of tetragonal lysozyme crystals.

In the Oosawa theory, a process of two consecutive one-directional reactions is considered. Nu-

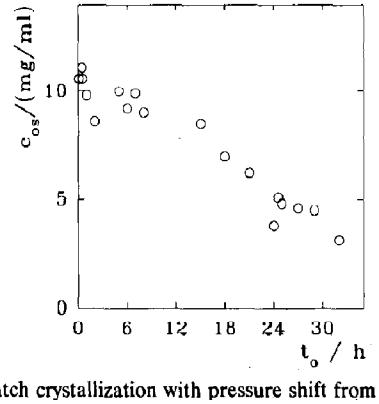


Fig. 2. Batch crystallization with pressure shift from 1 to 1000 bar. Residual protein concentration in the supernatant as a function of the time t_0 of pressure application; 20 mg/ml lysozyme at 20°C.

[2] There is a linear relationship between volume change and initial concentration, which, however, cannot be extrapolated through zero. From the slope of this line, a smaller molar volume change of 8 ml/mol is obtained.

cleation and growth (propagation) are described by

$$dm/dt = k_1 c_{os}^{i_0} \quad (1)$$

and

$$-dc_{os}/dt = k_2 m c_{os} \quad (2)$$

respectively, with m, as number concentration of nuclei (including larger crystals); k_1, k_2, as rate constants; c_{os} as protein concentration in oversaturation, and i_0, as number of molecules per nucleus [3]. Assuming the initial conditions $m = 0$ and $c_{os} = c_0$, both equations can be integrated to yield

$$\ln((1+x)/(1-x)) = \left(2 i_0 k_1 k_2 c_0^{i_0}\right)^{1/2} t \quad (3)$$

with

$$x = \left[1 - (c_{os}/c_0)^{i_0}\right]^{1/2}$$

The Oosawa theory provides a simple equation predicting the dependence of half-times on the starting concentration:

$$\log t_{1/2} = \text{const.} + (i_0/2) \log c_0 \quad (4)$$

From the kinetics of batch crystallizations at 10 different protein concentrations ranging from 11 to 26 mg/ml (Fig. 3), the linear $\log t_{1/2}$ vs. $\log c_0$ relationship is found to be valid between 11 and 21 mg/ml (Fig. 3, insert). From the slope, $i_0 = 5.8 \pm 0.4$ is calculated. The data summarized in Fig. 3 illustrate the fit of the kinetic results based on eq. (3) with

$$i_0 = 6 \quad \text{and} \quad k_1 k_2 = 1.09 \times 10^{-16} (\text{mg/ml})^{-6} \text{s}^{-2}$$

as parameters.

Applying these equations to the kinetic data published previously, (Fig. 3 in [3]), we found that

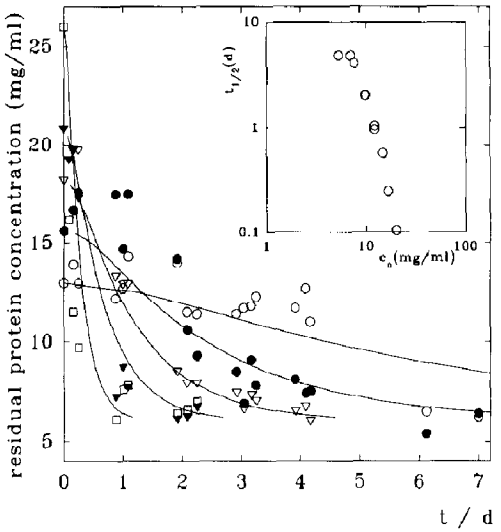


Fig. 3. Crystallization kinetics with different starting concentrations c_0 (○) 13.0, (●) 15.6, (▽) 18.2, (▼) 20.8, (□) 26.0 mg/ml. Insert: double-logarithmic plot of $t_{1/2}$ vs. $c_{os,0}$

the atmospheric pressure curve can be fitted fairly well with the parameters

$$i_0 = 4; \quad k_1 k_2 = 3.5 \times 10^{-17} (\text{mg/ml})^{-4} \text{s}^{-2}$$

assuming a certain delay time for the equilibration of salt concentrations in the capillary and in the outer reservoir (Fig. 4) [4]. Assuming the same constants to hold at high pressure, the high pres-

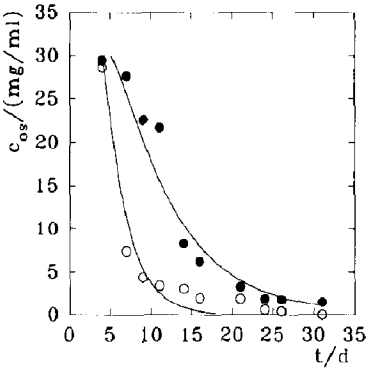
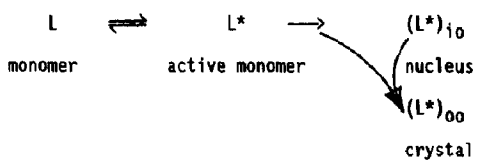


Fig. 4. Comparison of simulated kinetics with experimental data taken from Fig. 3 in [3]. (○) atmospheric pressure, (●) 1000 bar kinetics. The simulated curves were calculated using eq. (3), with $i_0 = 4$, $k_1 k_2 = 3.5 \times 10^{-17}$ (mg/ml)$^{-4}$ s^{-2} as parameters. For the high pressure curve, $c_0' = 19.4$ mg/ml was used instead of $c_0 = 32$ mg/ml.

[3] The exact function of i_0 is to indicate the reaction order of the initial step in crystallization. This may differ from the number of molecules involved. Having no structural data about the nature of the nucleus, we shall henceforth use this word for the kinetically defined intermediate in crystallization without implying the i_0 to be an exact stochiometric value.



Scheme 1.

sure profile can be fitted equally well if the concentration of "crystallization-active" lysozyme is assumed to be reduced significantly; e.g., for the simulated high pressure kinetics in Fig. 4, $c_0' = 19.4$ mg/ ml was used instead of $c_0 = 32$ mg/ml.

The underlying idea was that there might be an equilibrium between the conformation (L*) that can form crystal nuclei and others (L) that cannot (Scheme 1). The pressure-induced inhibition of crystallization may then be explained by the reduced concentration of L* at higher pressures. The rapid volume increase observed by dilatometry is ascribed to the pull that incorporation of L* into nuclei or crystals exerts on the equilibrium. Thus, an almost complete conversion of L to L* is achieved before crystallization reaches equilibrium. The ratio of L*/L, which is governed by the (pressure-dependent) pre-equilibrium remains constant during the crystallization procedure. According to the result of Fig. 4, the equilibrium concentration of L* is reduced by a factor of 32/19.4 = 1.65 at 1000 bar. From this, the volume change between L and L* can be calculated to amount to 11.9 ml/mol. This result is in remarkably good agreement with the value 12.5 ml/mol calculated from the difference in solubilities [3].

If the Oosawa theory is valid for the kinetics of the appearance of crystals, then Scheme 1 predicts that it should also account for the disappearance of the inactive conformation L, as monitored by dilatometry (Fig. 1). The kinetic data in Fig. 1, with the parameters

$$i_0 = 6;\ k_1 k_2 = 5.0 \times 10^{-14} (\mathrm{mg/ml})^{-6} \mathrm{s}^{-2}$$

yield a good fit for the first four hours of the experiment (Fig. 5). The product of the two rate constants is two orders of magnitude larger than in the experiment based on concentration measurement after centrifugation. This is easily explained by the fact that the latter method can only detect crystals exceeding a certain critical size. Thus both rate constants k_1, (nucleation) and k_2, (propagation) are apparently reduced, because only part of the nuclei are bound to reach the critical size, and only part of the material incorporated during propagation is found in crystals beyond the critical size. However, the log $t_{1/2}$ vs. log c_{os}-graph for six dilatometric experiments does not reveal a clear picture, as i_0 is found to range between 2 and 6, depending on the interpretation (data not shown).

Applying this model to explain the results in Fig. 2, we suppose that the previously mentioned equilibrium is shifted towards inhibition of crystal growth at the time t_0 when pressure is applied. That is, after the pressure-jump, the experiment will follow the trace for 1000 bar (or 19.4 mg/ml) for the remaining time (drawn as a thick line in

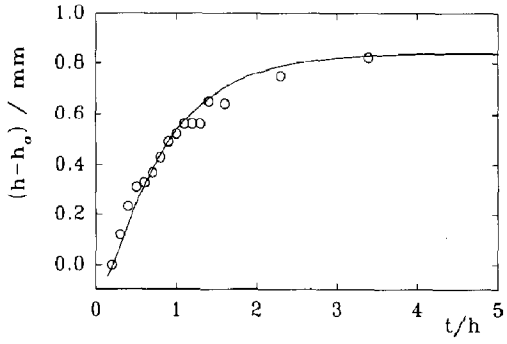


Fig. 5. Curve fit for the kinetics from Fig. 1, making use of the Oosawa formalism with the parameters: $i_0 = 6$, $k_1 k_2 = 5.0 \times 10^{-14}$ $(\mathrm{mg/ml})^{-6}\mathrm{s}^{-2}$. The height at $t = 0$ was assumed to be -0.1. From this plot, the half-time of the volume change and the total molar reaction volume are determined to amount 0.82 h and 35 ml/mol, respectively.

[4] If, however, the value $i_0 = 6$ was supposed to hold at higher concentrations, too, the parameters $i_0 = 6$, $k_1 k_2 = 5 \times 10^{-20}$ $(\mathrm{mg/ml})^{-6}\mathrm{s}^2$ provide an equally acceptable fit. The difference in the parameters between the experiments in Fig. 3, on one hand, and in Fig. 3 of [3], on the other, may be attributed to the different experimental conditions. All experiments reported in [3] rely on the diffusion technique, whereas the experiments in this paper are batch crystallizations.

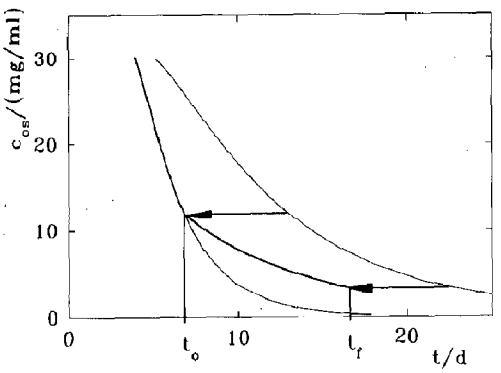


Fig. 6. Procedure for the simulation of the pressure-jump experiments in Fig. 2. At the time t_o of pressure application, the experiment is supposed to be shifted to the high pressure curve, which it follows for the remaining time $t = t_f - t_0$. The final concentration c_f is projected back to the height of t_f for comparison with the final concentrations to be expected for 1 bar or 1000 bar experiments.

Fig. 6). Calculating the final concentration as a function of t_0 for a putative experiment under the conditions underlying Fig. 3 in [3], a curve resembling the experimental curve in Fig. 2 is obtained (Fig. 7); thus, the above model accounts for the experimental results obtained.

It should be noted, however, that this simulation oversimplifies the implications of the pressure-shift. Actually, when the experiment is shifted to the slower high-pressure profile, the higher m-value (number concentration of nuclei

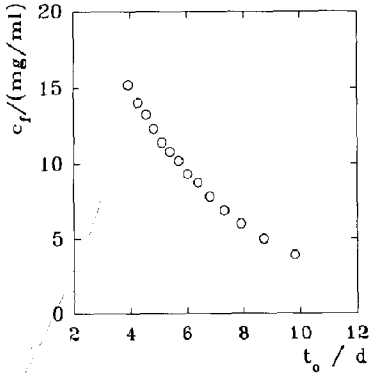


Fig. 7. Simulation of a pressure-jump experiment analogous to the one illustrated in Fig. 2. The simulation is based on the kinetic data from Fig. 3 in [3] (cf. Fig. 4) and was performed according to the procedure explained in Fig. 6.

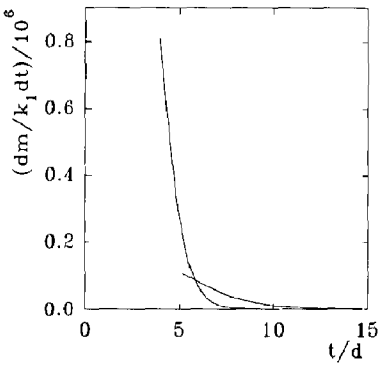


Fig. 8. The rate of nucleus formation as a function of time as predicted by equation (1). The data of the fits in Fig. 4 were readjusted to the "correct" scaling (so that the 19.4 mg/ml curve starts from 19.4 mg/ml) and then raised to the power of 4, thus yielding the term $c_{os}^{i_0} = dm/k_1dt$. According to eq. (1), this represents the rate of nucleus formation, which is plotted versus time. The steep profile is obtained from the atmospheric pressure courve, the other one from the 1000 bar data. The area below a curve represents the amount of nuclei (including larger crystals) present.

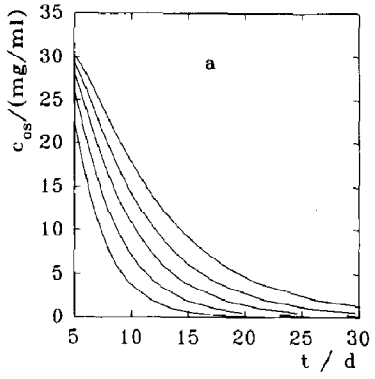
and crystals) of the normal pressure kinetics should be retained. Thus, the incorporation of lysozyme molecules after the pressure jump should proceed more slowly than at atmospheric pressure (because of reduced [L*]), but faster than at high pressure (because of higher m). In order to estimate the effect of nucleation, eq. (1) may be applied to the kinetic data of Fig. 3 in [3], with $i_0 = 4$ and c_{os} starting from $c_0 = 32$ mg/ml and $c_0' = 19.4$ mg/ml. When the term $dm/k_1dt = c_{os}^4$ is plotted versus t (Fig. 8), the number of nuclei is proportional to the area below the curves. It turns out that at high pressure significantly fewer nuclei are formed than at atmospheric pressure. This agrees with the result of visual inspection reported earlier [3]. On the other hand, the rate of nucleation will decrease more slowly in the high pressure profile. Thus, at high pressure, there is still an important formation of nuclei after five days, whereas at atmospheric pressure nucleation does not occur any more.

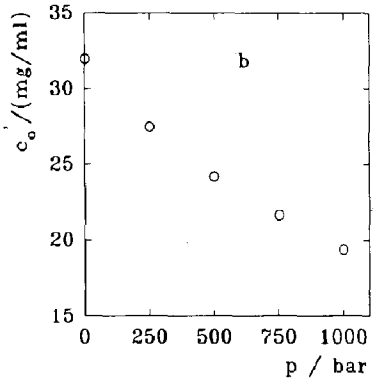
As has been shown previously (Fig. 2 in [3]), the amount of crystallized material changes linearly with pressure in short-term experiments, but shows a pressure dependence with a strong positive curvature after long-time incubation. The

previous explanation for the former effect, namely a reduced rate of nucleus formation at high pressure, is corroborated by the results in Fig. 8 and does not contradict the present model. Assuming a linear relationship (i.e., choosing appropriate concentrations c_0), hypothetical kinetics were computed for the intermediate pressures 250, 500, and 750 bar (Fig. 9a), based on the fits for the atmospheric pressure and 1000 bar data in Fig. 4. As illustrated in Fig. 9b, the pressure dependence of the optimum c_0 values shows a slight deviation from linearity. The final concentration c_f after long-term incubation (21 days) may be taken from the curves in Fig. 9a. It is plotted against pressure in Fig. 9c, which clearly indicates that the best fit for both the c_f vs. p profile, and the curve determined experimentally at 0.83 M NaCl, is of the $a + bx^2$ type. A slight difference in the parameter b is easily explained by the fact that the simulated curve is based on the kinetics performed at 1.0 M rather than 0.83 M NaCl.

5. Discussion

From the application the Oosawa theory of protein self-assembly, it becomes clear that all the effects observed for the pressure dependence of the nucleation and growth of lysozyme crystals can be explained, if a pre-equilibrium between a crystallization-competent and a crystallization-incompetent species (L* and L, respectively, in Scheme 1) is assumed to precede the incorporation of lysozyme molecules into either nuclei or growing crystals of the protein. The pressure-jump experiment reported here (Fig. 2) provides evidence for the assumption that the step connected to the fast and important volume change is distinct from nucleation itself. From this we conclude that L* is not a nucleus but an activated conformation of the monomeric protein.





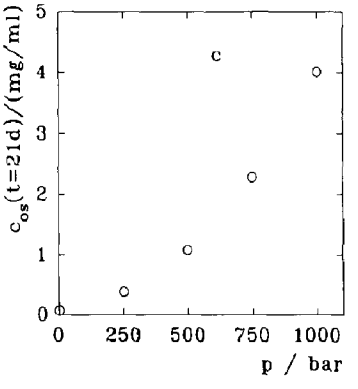


Fig. 9. Simulation of the complex dependence of crystal yields on pressure after long incubation times. (a) simulated kinetics at 1 bar and 1000 bar taken from Fig. 4 together with simulated profiles for intermediate pressures 250, 500, and 750 bars calculated on the assumption of a linear dependence of growth inhibition on pressure after short-term incubation. (b) Apparent concentrations c_0' of crystallization-active lysozyme used for plotting Fig. 9a as a function of pressure. (c) Final concentrations after 21 days of the simulated kinetics from 9a as a function of pressure. The best curve fit is $a + bx^2$ with $a = 0.105$ and $b = 3.891 \times 10^{-16}$. The corresponding experimental results (Fig. 2 in [3]) can be fitted by the same equation. Parameters, however are different, because the experimental conditions are not the same as in the kinetic experiment (Fig. 3 in [3]) on which the simulations rely.

The structural nature of the active monomer L* remains to be investigated. The solution structure of lysozyme having been studied extensively by two-dimensional NMR [6], application of this technique to oversaturated solutions may provide evidence for the existence of this species and reveal structural details.

The only structural transformation of lysozyme by pressure known up to now has been found in a high pressure X-ray study of tetragonal lysozyme crystals grown at atmospheric pressure [7]. This study proved domain 1 (containing the three α-helices) to be compressible, whereas the other domain is essentially incompressible.

There is no reason to exclude that the volume change of the pre-equilibrium may have the reverse sign in the case of other systems, so that the present mechanism does not necessarily contradict the pressure-induced enhancement of crystal growth observed for glucose isomerase [2]. As indicated, the given model for the high-pressure effects on crystal growth allows to explain previous and to devise future experiments. Apart from the perspectives of high pressure biophysics, the volume changes involved in the early phase of protein crystal growth may reveal mechanistic details of this process not satisfactorily understood so far. It seems promising to combine the present approach with the dynamic light scattering technique, which has already been applied to follow pre- and early crystallization events [8,9]. The molecular nature of the volume change in the fast pre-equilibrium reaction still awaits a clear-cut explanation. The generalization of the present findings with respect to crystals of other proteins needs further investigation.

Acknowledgements

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High pressure research would have been near to impossible without the help of the mechanical workshop led by Mr R. Knott.

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