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Growth inhibition of lysozyme crystals at high hydrostatic pressure*

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The influence of high hydrostatic pressures on the crystallization of hen egg-white lysozyme has been determined by growing lysozyme crystals with a capillary technique adapted to high pressure conditions and monitoring the residual protein concentration remaining in the supernatant. Pressure is found to reduce the rate of crystallization and to enhance the solubility of lysozyme in 1 M NaCl, pH 4.66. The volume change calculated from the latter result amounts to 12.5 ml/mol. This value is too small to be detectable by comparison of total molar volumes.

Crystallization; Hen egg-white lysozyme; High pressure; Molar volume

1. INTRODUCTION

Pressure as an intensive thermodynamic variable is expected to influence nucleation and growth of protein crystals as strongly as temperature or solute concentrations do. However, in a recent review on macromolecular crystallization, A. McPherson stated that 'there have been virtually no systematic studies of such factors as pressure ... on the rate of growth or final quality of protein crystals' [1]. Previous papers dealing with both pressure and protein crystallization were limited either to large-scale growth of small crystals for technical purposes [2], or to high pressure X-ray analysis of crystals grown at atmospheric pressure [3,4]. The obvious pendant to the latter studies, namely the analysis of pressure grown crystals at ambient and at growth pressure has not yet been undertaken.

Here we present a simple technique for the slow growth of protein crystals at pressures up to 2000 bar. We used this method to study the influence of high hydrostatic pressures on the kinetics and thermodynamics of lysozyme crystallization in acidic solution.

2. MATERIALS AND METHODS

The buffer used in all experiments was 0.02 M sodium acetate, 0.02 M acetic acid, pH 4.66 (Merck). Hen egg-white lysozyme (Boehringer Mannheim, Germany) was dissolved in buffer (50 mg/ml), passed through a filter membrane and then used without further purification. The precipitating agent was NaCl dissolved in buffer at concentrations ranging from 0.8 M to 1.4 M.

Fig. 1 illustrates the device used for high pressure crystallization. Three such silicon tubes could be accomodated in one autoclave; four autoclaves could be pressurized and depressurized independently. Reference samples were incubated at atmospheric pressure in the same kind of silicon tubes.

The autoclave equipment is as described in [5], except for the electric heating, which has been replaced by a waterbath device. The standard temperature was 20°C.

Depressurization was performed at a rate of approximately 400 bar/min and was completed within 3 min. After depressurization, the mother liquor was removed within less than 10 min. A 10 μl aliquot was diluted 60-fold in water, centrifuged and subjected to photometric concentration measurement at 280 nm. The $A^{0.1\%}_{280\,nm,\,1cm}$ was taken to be 2.56.

3. RESULTS

First attempts to crystallize lysozyme at high hydrostatic pressure yielded a reduced number of crystals compared to the normal pressure reference, but both kinds of crystals grew to the same size within the same incubation time. This was taken as a hint to an inhibition of nucleation by pressure. An effect of pressure on the rate of crystal growth could not be detected by this simple qualitative means. This interpretation was corroborated by a cross-exchange experiment. Samples that had formed few nuclei under high pressure conditions were still capable of forming many crystals, when depressurized after half of the incubation time (that is, after 18 days) ; on the other hand, the large number of nuclei formed in normal pressure samples did not dissolve but showed normal growth when incubated at 1000 bar for the second half of the incubation period.

In order to quantitize these observations and to check, whether there was no effect of pressure on growth rates, we measured the A_{280} of the mother liquor, varying all three, pressure, incubation time and salt concentration. After short-term incubation at different pressures (Fig. 2, left), the residual protein concentration remaining in the mother liquor was found to depend linearly on the pressure applied. The slope, that is the pressure sensitivity of crystallization, decreased with increasing salt concentration.

* This paper is dedicated to Tabitha Naomi Groß, born 29 March 1991

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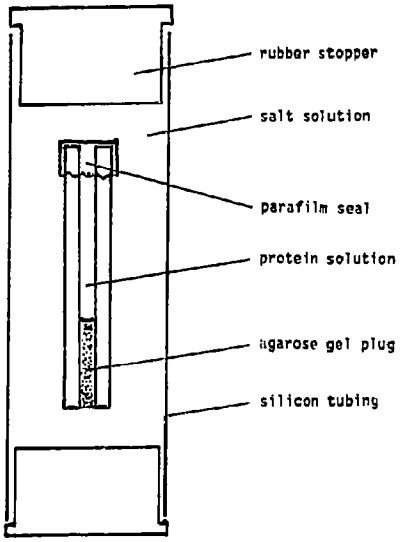

Fig. 1. Equipment for protein crystallization at high hydrostatic pressures. The inner volumes of the capillary and the silicon tube are 0.11 ml and 4.5 ml, respectively.

Long-term incubation yielded a different pressure dependence characterized by a positive curvature (Fig. 2, right). The residual protein concentration remaining after 21 days of incubation is assumed to be very near to the solubility. E.g., in the kinetics shown in Fig. 3, the concentrations after 21 days are 1.2 times the solubilities at 1000 bar as well as at atmospheric pressure. Obviously, the influence of pressure on the equilibrium of crystallisation is determined by more complicated laws than the simple linearity of the early crystallization period, which might be governed by the number of nuclei that can form at different pressures.

Lysozyme crystals grown at atmospheric pressure in 0.83 M NaCl have been reported to crack when being pressurized at 1000 bar, unless the salt concentration is

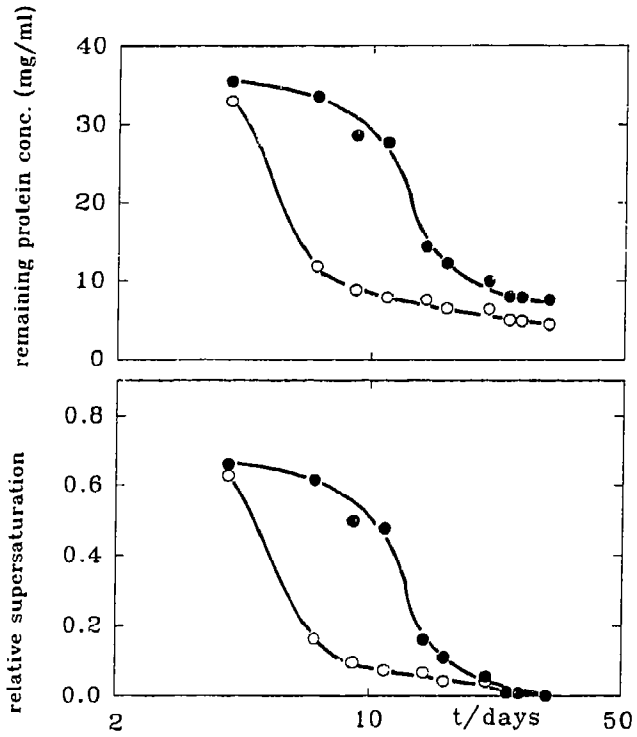

Fig. 3. Kinetics of lysozyme crystallization at 1 bar (○) and at 1000 bar (●) and 1.0 M NaCl. Top: absolute protein concentration (c) in the supernatant; bottom: relative supersaturation ($s = (c - c_{\infty})/c_{\infty}$), both plotted vs incubation time (logarithmic time scale). c_0 is the starting concentration, c_{∞} the final concentration (solubility) of lysozyme.

raised to 1.4 M before pressurization [3]. Fig.2 (right side) possibly provides an explanation for this finding, showing that at 0.83 M NaCl there is a strong increase in solubility between 1 and 1000 bar, which is not the case at 1.4 M salt.

Kinetics of crystal growth at 1000 bar and at atmospheric pressure were monitored over a time range of 31 days (Fig.3, top). Each of the kinetic results is the

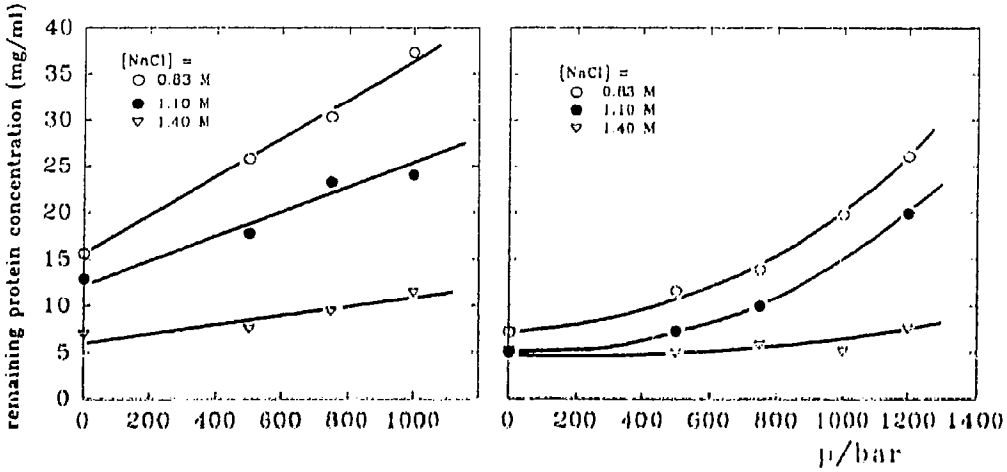

Fig. 2. Decrease of protein concentration in the mother liquor as a function of pressure and salt concentration after 10 days (left) and 21 days (right) incubation.

mean value of a triple determination. In order to compile sufficient data over the whole time range and to determine the end points exactly, three independent experiments under the same conditions were necessary.

The plot of relative supersaturation vs log t (Fig.3) shows that the half-time of crystallization is 12 days at 1000 bar as compared to 6 days at atmospheric pressure. The end points of both graphs differ significantly. Thus, the solubility of lysozyme in 1 M NaCl is found to be higher at 1000 bar than at atmospheric pressure by a factor of 1.7, corresponding to a ΔG of 1250 J/mol and a ΔV_m of 12.5 ml/mol, calculated on the assumption that ΔV_m is independent of pressure.

Conclusions from the half-times of crystallization in Fig.3 can only be drawn, if the effects of nucleation can be separated from the growth kinetics. The most simple approach to this problem is counting the crystals after long-term incubation. After 14 and 19 days of incubation the number of crystals was determined. In both cases it was roughly two-fold larger in atmospheric pressure samples than in the high pressure ones. Assuming that every nucleus contributing to the kinetics of the decrease in protein concentration will have reached a size visible under the light microscope by then, we conclude from this result that the two-fold reduction of growth rate is mainly an effect of the inhibition of nucleation by high hydrostatic pressure.

4. DISCUSSION

The effect of pressure on the equilibrium between protein crystals and the protein concentration in the mother liquor is governed by four molar volumes represented schematically in Fig. 4. Kundrot and Richards found that the compressibility of the solvent in lysozyme crystals does not differ significantly from the compressibility of bulk solvent [4]. This allows us to discuss the pressure-induced change of solubility in terms of molar volumes and compressibilities of the protein component in the solution or in the crystal alone, keeping in mind that the solvent still may contribute to the volume changes in the vertical direction of Fig. 4, although it is excluded from those in the horizontal direction.

Molecular volumes determined from solution measurements are by roughly 10% larger than those calculated by Kundrot and Richards from the crystal structure [3]. If this difference represented real physical changes rather than the sum of the systematic errors of both methods, crystallization ought to be strongly

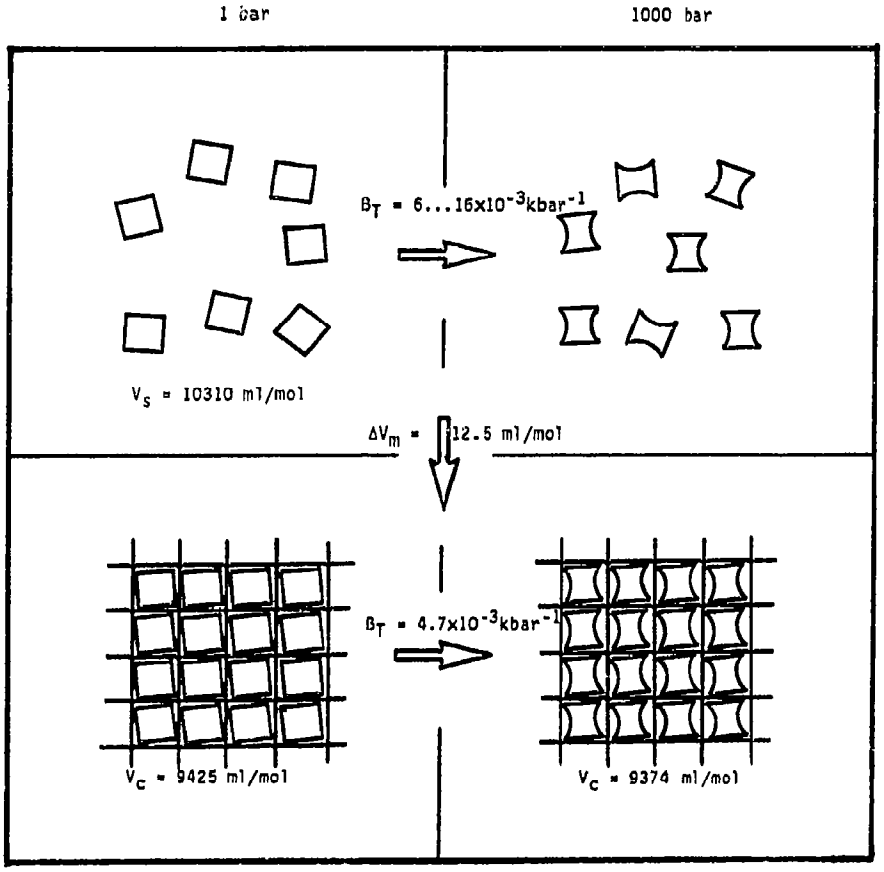

Fig. 4. Schematic representation of the molar volumes involved in the effects of pressure on crystallization. β_T stands for the isothermic compressibility, V_c and V_s are the molar volumes determined from the crystal structure and in solution, respectively. These data have been taken from [3].

favoured by the application of hydrostatic pressure. However, the ΔV_m determined in this study has the opposite sign and is smaller by two orders of magnitude. Actually, it is smaller than the error of the determinations of total molar volumes. Thus, our assumption that ΔV_m is independant of pressure cannot be verified by those methods but only by solubility measurements at several different pressures. Though rather time-consuming, this approach would allow one to complete the scheme depicted in Fig. 4, thus yielding a complete and self-consistent thermodynamic pressure-volume cycle.

The inhibition of nucleation, which according to our results is the main reason for the reduced growth rate, can be related to the general tendency of oligomeric enzymes and assembly systems (such as tubulin or ribosomes) to be dissociable by application of pressures up to 1000 bar [6,7]. Ataka and Asai used Oosawa's theory of protein self-assembly to analyze the growth kinetics of orthorhombic lysozyme crystals (forming above 25°C only) [8]. They conclude from their results that the nuclei of this crystal form are tetramers. If the nuclei of tetragonal lysozyme crystals (growing below 25°C) were well-defined oligomers as well, then the inhibition of nucleation by pressures of up to 1000 bar would be readily understandable in terms of the pressure-induced dissociation of these oligomeric complexes.

Crystallographic characterization of lysozyme crystals grown under pressure is in preparation and will be published separately.

Further applications of the present technique could include systems, where traditional crystallization methods have failed. For example, in the crystallization of ribosomes, pressure might be used to eliminate the more pressure-sensitive and perhaps too flexible loose couples from the 70S population by dissociation [7], thus yielding a homogeneous 70S population containing tight couples only.

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