Analysis of the nucleation and crystal growth kinetics of lysozyme by a theory of self-assembly

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ABSTRACT Concentration changes in supersaturated solutions during the nucleation and growth of the orthorhombic form of hen egg-white lysozyme crystals have been observed for 121 d at 35°C and pH 4.6, and with 3% NaCl. The effect of a variation in the initial protein concentration on the rate of approach to solubility in equilibrium is analyzed, by applying a model, originally developed for the understanding of protein self-assembly. It is shown that the observed kinetics can be explained fairly well by this model, whose basic assumptions are that (*a*) the nucleation is induced by aggregation of i_0 molecules into particular geometry, and (*b*) the growth proceeds via attachment of a monomer. The i_0 value for this process is four, which agrees with the number of molecules in a unit cell. Similarity and dissimilarity of the observed crystal growth to that of low molecular weight substances are discussed.

INTRODUCTION

Although crystallization of proteins has been used repeatedly in various epoch-making works in biochemistry and molecular biology (Sumner, 1948; Perutz, 1969; Michel, 1983), the physics of protein crystal growth remains largely unknown. Systematic efforts started only rather recently toward an understanding of the underlying mechanisms of protein crystal growth (Kam et al., 1978; Feigelson, 1986; Giegé 1988). These efforts are necessitated by drug design and protein engineering requirements, as well as those in molecular biology, to determine protein structures by x-ray crystallography (Bugg, 1986). It was emphasized that there exists a large gap at present between the physical understanding of the crystal growth of low molecular weight substances and that of proteins (Feigelson, 1986).

We have previously studied the conditions necessary to allow reliable and reproducible determination of the solubility of protein crystals, and presented phase diagrams of two crystal forms of hen egg-white lysozyme (Ataka and Tanaka, 1986; Ataka and Asai, 1988). It was also shown that the crystal size is dominantly governed by the degree of supersaturation which can be derived from the phase diagrams. In this communication, we use an experimental method, developed to determine solubility in thermodynamic equilibrium, to study the nucleation and crystal growth kinetics. The experiments were then analyzed by applying a model (Oosawa and Kasai, 1962; Oosawa and Asakura, 1975), originally developed to understand protein self-assembly. This theory has been applied successfully to account for the morphogenesis of F-actin, flagella, microtubules, etc. from their constituent protein molecules, but, as long as we know, has not been applied to crystal growth. Unlike conventional nucleation theories of the crystal growth of smaller substances (Lewis, 1980), the positive surface energy and the negative bulk energy are not considered to be simple and smooth functions of the number of molecules in the crystals. Instead, for some geometrical or chemical reason, the size of a critical nucleus is considered to be a priori given in Oosawa's theory.

MATERIALS AND METHODS

Hen egg-white lysozyme, obtained from Seikagaku Kogyo (1-5, Nihonbashi-Honcho 2-chome, Chuo-ku, Tokyo, Japan), was crystallized batchwise at 35°C as previously described (Ataka and Asai, 1988). The protein concentration remaining in the supernatant was determined optically by a Cary 17D spectrophotometer. The effect of a change in the initial protein concentration, with all the other conditions (pH 4.6, NaCl 3.0%, 1 atm) kept constant, was compared.

RESULTS

Fig. 1 shows the concentration changes observed in the supernatant during the initial 8 d. The concentrations start to decrease after the crystals become observable under an optical microscope. The crystals then increase both in number and in size. Even after the number of the crystals almost stops increasing, the concentration continues to decrease considerably due to the growth of the crystals. Therefore, the concentration change as shown in Fig. 1 is a consequence of both nucleation and growth, occurring more or less simultaneously. The only solid phase in the solutions throughout the 121 d experiment

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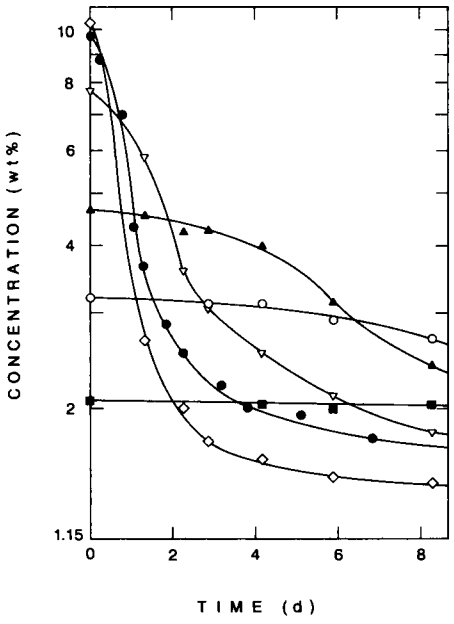

FIGURE 1 Concentration changes due to the growth of hen egg-white lysozyme crystals, observed in six solutions of different initial lysozyme concentrations during the first 8 d. The concentrations dissolved at $t = 0$ were 10.2 (◇), 9.7 (●), 7.7 (▽), 4.7 (▲), 3.2 (○), and 2.1 (■) wt %. The experiments were carried out at 35°C, at pH 4.6, with 3.0 wt % NaCl, under 1 atm, in water without buffer, and with the existence of the orthorhombic form crystals as the only solid phase. Under these conditions, the solubility is uniquely determined to be 1.15%, to which all the supernatant concentrations approach as time elapses. The lines are drawn merely to assist in following concentration changes with time.

was the orthorhombic (Jollès and Berthou, 1972) crystals. The concentration of each solution gradually approaches 1.15%, which is the solubility of this crystal form at 35°C and pH 4.6, and with 3% NaCl (Ataka and Asai, 1988). Although the initial protein concentration is 2–9 times the solubility, which means that the degree of supersaturation is surprisingly high when compared with small molecule crystallization, the time needed both for the appearance of crystals and for their growth is extremely large. Also, the rate of approach to equilibrium varies greatly with the initial protein concentration.

THEORY AND ITS APPLICATION

To interpret the above result, we apply a theory, widely accepted to explain the self-assembly of proteins (Oosawa and Asakura, 1975). Protein molecules are considered to be dispersed monomolecularly at $t = 0$ with an initial concentration of c_0. For self-assembly to start, it is considered that i_0 molecules have to assemble and have a defined geometry. In other words, this is the rate determining step for nucleation of these molecules. Mathematically, the concentration of nuclei, m, changes with time t as

$$dm/dt = k_1 \cdot c_1^{i_0}, \tag{1}$$

where k_1 is a constant, and c_1 that part of the monomer concentration which is supersaturated. The attachment of molecules to a nucleus is considered to proceed via monomers, i.e., the decrease in c_1 is expressed as

$$-dc_1/dt = k_2 \cdot m \cdot c_1 \tag{2}$$

with k_2 being another constant. In both Eqs. 1 and 2, the backward process is neglected, the system being thought to be far from equilibrium. With initial conditions that $m = 0$ and $c_1 = c_0$, Eqs. 1 and 2 can be integrated to give

$$\ln \frac{1 + [1 - (c_1/c_0)^{i_0}]^{1/2}}{1 - [1 - (c_1/c_0)^{i_0}]^{1/2}} = (2i_0)^{1/2} \cdot (k_1 \cdot k_2)^{1/2} \cdot c_0^{i_0/2} \cdot t. \tag{3}$$

This equation expresses a change in c_1 with t as a result of both nucleation (Eq. 1) and growth (Eq. 2). If i_0, k_1, and k_2 are all constant, the decrease in c_1 with t is monotonic and sigmoidal, which, when plotted in a (c_1/c_0) vs. log t scale, is mutually superimposable by translation along the log t axis (see also the theoretical curves in Fig. 3). If we denote the time at which $c_1/c_0 = 1/2$ as $t_{1/2}$, we have from Eq. 3

$$\log t_{1/2} = \text{const.} - (i_0/2) \cdot \log c_0. \tag{4}$$

The existence of a simple relation between $t_{1/2}$ and c_0 is one of the major outcomes of the theory; hence we carried out the experiments which would evaluate an effect of c_0 on $t_{1/2}$. Fig. 2 is a plot of log $t_{1/2}$ vs. log c_0 for the six solutions studied. All the data fall on a line whose slope is -2 as expected from Eq. 4, which means that i_0 in this case can actually be regarded as a constant, and is equal to 4. This number exactly agrees with the number of molecules in a unit cell of these orthorhombic crystals. This result is also consistent with the original meaning of i_0 that it is the particular number of molecules needed to form and work as a nucleus. It must be emphasized that the assumption that i_0 in Eq. 1 is a constant is different from the one usually postulated in the nucleation of atoms and small molecules (Lewis, 1980), and may only be verified by an experiment such as shown in Fig. 2. Instead of the constancy of i_0, the conventional nucleation theory assumes that the positive surface energy per unit surface (G_S) and the negative bulk energy per unit volume (G_B) are constant, and that the total free energy change upon crystallization is obtained as a sum of these quantities, multiplied by either the surface area or the total volume.

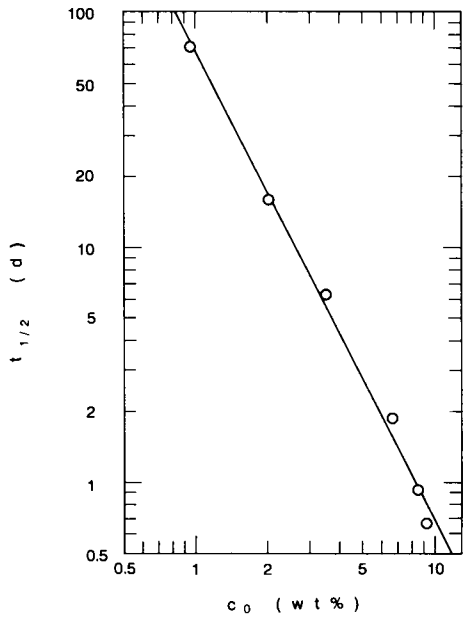

FIGURE 2 Relation between the time by which half the molecules in supersaturation have been transferred from the solution into the crystalline phase ($t_{1/2}$), and the initial lysozyme concentration in supersaturation (c_0; actual concentration minus solubility). The line has the slope of -2.0, which means that Eq. 4 in the text holds with $i_0 = 4$.

Simulated curves of Eq. 3 with the best selected value for $k_1 \cdot k_2$ are compared with the full data (Fig. 3). The best value for $k_1 \cdot k_2$ was selected by a curve fitting procedure, in which the data set of (c_1, t) was compared with theoretical values ($c_1^{\text{theoretical}}, t$), and the total deviation ($\Sigma[\Delta c_1]^2$) was minimized. Having known that $i_0 = 4$ and $k_1 \cdot k_2 = 3.97 \times 10^{-4}$, we substituted, into c_0 in Eq. 3, the actual initial protein concentrations of the six solutions to calculate the theoretical $c_1(t)$ curves in Fig. 3. The c_1 value in this figure corresponds to the experimentally determined protein concentration minus solubility. It can be seen that all the data almost fall on the theoretical curves.

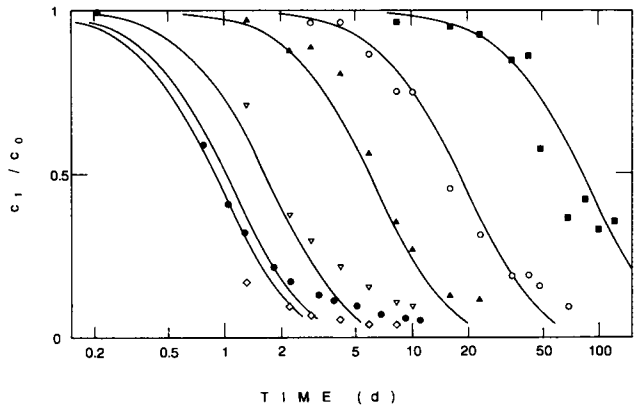

FIGURE 3 Full kinetic data obtained during 121 d, plotted into a (c_1/c_0) vs. log t scale, where c_1 and c_0 are the concentrations in supersaturation at $t = t$ and $t = 0$, respectively. The symbols are the same as in Fig. 1. The lines are the theoretical curves of Eq. 3, with $i_0 = 4$, and with the six c_0 values of the decreasing order from the left. The value of $k_1 \cdot k_2$ used is 3.97×10^{-4}, in a unit in which t is measured in day, and c_1 in wt %. The data for the initial 8 d are the same as used in Fig. 1.

DISCUSSION

Fig. 3 shows that the six theoretical curves obtained can reproduce the data collected over a wide range between several hours and 121 d. It must be noted that, in the curve fitting, it was only one parameter, $k_1 \cdot k_2$, that was adjustable. The agreement between theory and experiment becomes less perfect when the experiment is carried out with more concentrated solutions, or when the supernatant concentrations approach equilibrium. Possible reasons for this include (*a*) some crystals may stop growing after reaching a definite size (Kam et al., 1978), and (*b*) the Eq. 2 neglects the backward process of dissolution of molecules from crystals into the solution.

It is clear from this figure that the concentration decrease of supersaturated solutions takes place only after an induction, or a delay, period needed for nucleation to occur, as long as no seeding from outside is performed. The induction period varies greatly with the initial protein concentration, owing to the c_0^2 factor in Eq. 3. These results are consistent with the empirical observation in protein crystal growth (Blundell and Johnson, 1976) that the nucleation rate usually depends greatly on the protein concentration. Although the induction time may be as long as several weeks when c_0 is relatively low, the supersaturated solution is considered to be preparing the nuclei in it according to the same kinetics as in more concentrated solutions. The predictability of the delay period may have practical significance to crystal growers, because it indicates the interval after which visible crystals will be obtained for a given c_0, which, in turn, is closely related to the final crystal size (Ataka and Tanaka, 1986).

In the case of morphogenesis of actin fibers, flagella, microtubules, etc. (Oosawa and Asakura, 1975), it has been shown that protein polymerization is induced only when i_0 molecules assemble into particular geometry. The concept of aggregation of i_0 molecules was also adopted for insulin fibril formation (Waugh, 1959), and deoxyhemoglobin S gelation (Hofrichter et al., 1974). In view of these different observations, all with proteins, it is tempting to consider that accumulation of a definite number of molecules into a particular shape, often of higher symme-

try than the individual, asymmetric molecule, is an essentially important step in various protein aggregation phenomena, and that the crystal growth of orthorhombic lysozyme is still another example. As has been shown above, this is unlike the nucleation of atomic or small molecular crystal growth. It is added that a version of the latter nucleation theory has been used to analyze the growth of tetragonal lysozyme crystals at 20°C (Kam et al., 1978). Our preliminary study on the tetragonal crystal form shows that a simple relation as in Fig. 2 does not hold in this case (Ataka et al., manuscript to be submitted for publication). Our intention is therefore not to argue against the former analysis, but to point out a possibility, using a differing crystal form, that plural nucleation mechanisms may have to be taken into account. The theoretical framework given here allows one to compare and connect the crystal growth with the self-assembly, which has mainly been studied separately so far.

While this communication was in revision, two papers, both dealing with nucleation of proteins by means of light scattering, appeared (Mikol et al., 1989; Kadima et al., 1990). Using lysozyme, Mikol et al. (1989) showed that the critical nucleus was composed of only a few molecules even when the supersaturation was small. Kadima et al. (1990) were aware, using canavalin, that their result was outside the framework given by the energy barrier concept of Kam et al. (1978). Both of these results are consistent with our conclusions described above.

There has been controversy (Kam et al., 1978; Fiddis et al., 1979; Durbin and Feher, 1986; Pusey and Naumann, 1986) as to whether the growth mechanism of tetragonal lysozyme crystals at ~20°C is controlled by diffusion or by surface reaction, mainly using direct observation of the growth rate. Eq. 2 is similar in form to the diffusion control growth (Fiddis et al., 1979). However, we consider that the applicability of Eq. 2 must not be employed as a criterion to discriminate the two possible growth mechanisms. The dependence of the growth rate on the crystal size is another thing remaining to be elucidated. We will treat these points in another paper (Bessho et al., manuscript in preparation). However, the similarity of all the growth curves (Fig. 3) may suggest that the controlling mechanism of growth is basically the same in all the six solutions studied.

CONCLUSIONS

By observing the crystal growth of orthorhombic lysozyme at 35°C, and by combining the data with already determined solubility, we have shown that the kinetics of protein crystal nucleation and growth can be understood quantitatively by a model based on simple, but physically well-characterized, assumptions. The same concepts of solubility, degree of supersaturation (e.g., c_0), nucleation (Eq. 1), and subsequent growth (Eq. 2) can be used as low molecular weight substances. However, reflecting the particular size and shape of the protein molecule, the time needed both for nucleation and growth is much longer, and the nucleation of this crystal form is likely to occur with the formation of tetramers, which are probably identical with the unit cell of the crystal.

We thank Dr. Shin-ichi Aizawa, the late Prof. Yonosuke Kobatake, Prof. Fumio Oosawa, and Prof. Ichiro Sunagawa for valuable discussions.

Received for publication 13 March 1989 and in final form 28 June 1990.

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