Studies on Precrystalline Aggregates of 2Zn-Insulin by a Self-assembly Theory

Kenji Waizumi,* Takami Matsumoto, Nobuhiro Fukushima,† and Hideki Masuda††
Department of Chemistry, Faculty of Education, Yamaguchi University, Yamaguchi 753-8513
†*Nihon SiliconGraphics•Cray K.K., 4-20-3 Ebisu, Shibuya-ku, Tokyo 150-6031*
††*Department of Applied Chemistry, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555*

(Received June 29, 1998; CL-980486)

Concentration changes of supersaturated solutions of 2Zn-insulin during crystallization have been measured for 50~100 h at 20 °C by monitoring absorbance at 276 nm. The self-assembly theory has been applied for the analysis, which revealed that aggregate of insulin at precrystallization stage is composed of four insulin hexamers.

The crystal growth in proteins is quite complicated as compared with that in low molecular weight substances. In general understanding of the crystal growth in low molecular weight substances, ions or molecules continuously repeat the formation and dispersion in the order more than ten or hundred particles before reaching a "critical size" which is governed by a supersaturation ratio. They are thermodynamically understandable by a combination of the concentration-dependent free energies such as the negative bulk and positive surface energies.[1] However, Ataka et al.[2] previously indicated that the critical size of aggregates of protein is not governed by the supersaturation ratio of protein solution and that the classical thermodynamic model is inapplicable to analysis of the nucleation of lysozyme. For analysis of the nucleation and crystal growth of lysozyme protein, they applied a self-assembly theory developed by Oosawa et al.[3] and successfully analyzed its concentration changes of supersaturated solutions. They concluded that the nucleation of lysozyme is induced by aggregation of four protein molecules into particular geometry and that the crystal growth of lysozyme proceeds via attachment of lysozyme monomers. The application of this theory to other protein crystals has been awaited.

Insulin, which is stored in the secretory granules of pancreatic β-cell as a crystalline array of hexamers,[4] is one of the most important and thoroughly studied hormone proteins, because it is cssential for controlling the amount of sugar in the blood and is utilized as a medicine for diabetes.[5] Previously, Kadima et al.[6] have examined the aggregation of 2Zn-insulin prior to crystallization by dynamic light scattering method, which revealed that only the formation of aggregates with an average hydrodynamic diameter of 59 Å have been observed. The measured size is good agreement with the dimensions of the insulin hexamer estimated from the crystal structure. Thus, they concluded that the crystallizing growth unit of insulin is a hexamer. However, they have not succeeded in the detection of particular aggregates of the crystallizing hexamers as nucleation centers in solution.

In this communication, in order to estimate the number of crystallizing hexamers in precrystalline aggregates of 2Zn-insulin, the concentration changes in supersaturated solutions of 2Zn-insulin during the precipitation have been measured, which have been analyzed by the self-assembly theory.

Bovine insulin, obtained from Wako Pure Chemical Industries was purified according to the usual manner.[7] Other reagents were of the highest purity grade available and used without further purification. Precipitation of 2Zn-insulin aggregate was induced by mixing sodium citrate (2.0 cm^3, 0.10 M, pH 8.2) into the insulin solutions (2.0 cm^3). The insulin concentrations of the supersaturated solutions of insulin were 0.21, 0.26, 0.45, 0.49, 1.03, 1.05, 1.13, 2.29, and 2.41 mg/ml as monomer. After mixing, the measurements were immediately carried out for the nine cases on standing at 20 °C for about 50~100 h by monitoring UV absorption using the molar absorption coefficient of 1.05 at 276 nm, in which an inhomogeneity of solutions was calibrated with a background correction.[8] The pH of the supersaturated solutions obtained was all about 6.5. Since the experiment was performed in the sealed cell, the solvents are not evaporated and the concentration of salts and pH of the solutions were held constant.

The rates of precipitation of 2Zn-insulin crystals significantly depend on the initial concentrations. There appeared an interesting relationship between the initial concentration of 2Zn-insulin solution, c_0, and the time by which half the initial concentration of the supersaturated solution have been transferred from a solution phase to a crystalline one, $t_{1/2}$: A plot of log $t_{1/2}$ vs. log c_0 gave a straight line with a slope of -2 as shown in Figure 1.

In order to explain such a linearity, we applied the self-assembly theory.[2,3] The crystallization begins with aggregation of individual crystallizing particles in solution and crystal growth proceeds as an ordered aggregation process. At the first stage, the concentration of nuclei, m, changes with time, t, which is expressed as

$$dm/dt = k_1 \cdot c^i \quad (1)$$

where k_1 is a constant, c the concentration of crystallizing particles, growth units, in supersaturated solution, and i the number of the entities constituting a nucleus. At the next stage, the crystal growth is propagated by the attachment of crystallizing

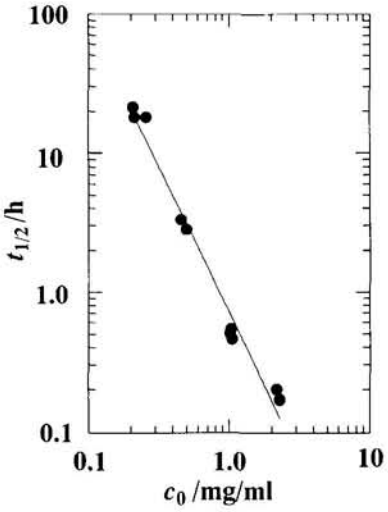

Figure 1. A plot of log $t_{1/2}$ vs. log c_0. The graph yields a slope of -2.

units to a nucleus. The decrease in c is presented as

$$-dc/dt = k_2 \cdot m \cdot c \quad (2)$$

where k_2 is another constant. In Eqs. 1 and 2, the backward processes such as the dissociation of nuclei and the dissolution of crystals are neglected. This approximation is valid for the system far from equilibrium that the supersaturated solutions used in this study are much higher concentration than the equilibrium one of 0.09 mg/ml.[2] With initial conditions that $m = 0$ and $c = c_0$, the following equation is derived from Eqs. 1 and 2.

$$c = c_0 \cdot \mathrm{sech}^{2/i}\{(i \cdot k_1 \cdot k_2/2)^{1/2} \cdot c_0^{i/2} \cdot t\} \quad (3)$$

This equation expresses a change in c with t as a result of both nucleation (Eq. 1) and crystal growth (Eq. 2). When the concentration becomes half the initial one, $c/c_0 = 1/2$, Eq. 3 can be reduced as follows.

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

The equation indicates that the number of growth units constituting the nucleus will be derived from a plot of $\log t_{1/2}$ vs. $\log c_0$.

As described above, the slope obtained from a plot of $\log t_{1/2}$ vs. $\log c_0$ for the nine solutions studied (Figure 1) is -2, indicating that the number of growth units of 2Zn-insulin hexamers in the critical aggregate is $i = 4$. According to Eq. 3, the decrease in c with t should be monotonic and sigmoidal, if i, k_1, and k_2 are all constant. A plot of (c/c_0) vs. $\log t$ was applied for four typical cases of supersaturated solutions, which are shown in Figure 2 together with the theoretical curves estimated from Eq. 3 by use of $i = 4$. Figure 2 indicates that the experimental data obtained for four cases are in fair agreement with the theoretical data. At higher initial concentrations, they deviate from the theoretical curves. The deviation may be explained in terms of the backward process: At the earlier stage of crystallization, proteins should precipitate as amorphous particles and then crystals are formed and start to grow up. In general, the solubility of an amorphous particle is higher than that of a crystalline one, as reported by Schlichthrull,[9] and the amorphous particles once precipitated begin to dissolve with decrease of the concentration. However, the good agreement between the experimental and theoretical values in Figure 2 indicates that the self-assembly theory is applicable to the precipitation phenomenon and the effect of backward process is essentially negligible to the precipitation from the saturated solutions being far from equilibrium.

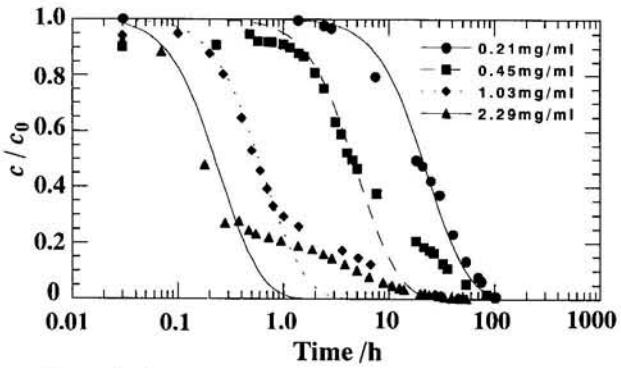

Figure 2. A plot of c/c_0 vs. $\log t$, as observed for typical four solutions of insulin. The lines are the theoretical curves calculated from Eq. 3 with $i = 4$.

The value $i = 4$ obtained here does not coincide with the value of $Z = 3$ (Z is the number of asymmetric units in the unit cell) of the 2Zn-insulin crystal with rhombohedral system, i.e. the asymmetric unit is insulin dimers and in the unit cell the three asymmetric units connected by two zinc ions consist of a hexamer.[10] The unexpected agreement of $i = 4$ between insulin and lysozyme and the disagreement between the values of i and Z may imply that the number of particles forming a critical precrystalline aggregate of protein is generally four without regard to a crystal system. In a model system of the self-assembly theory, the physical meaning of i is that the aggregates of $(i\text{-}1)$ molecules can not play as stable nucleus, but that i molecules form a stable aggregates. The four crystallizing particles, growth units, are liable to form a stable nucleus with a tetrahedral geometry, which may be widely acceptable for crystallization of protein.

References and Notes

1 For example; B. Lewis, in "Crystal Growth," ed by B. R. Pamplin, Academic Press, London (1975), pp. 34-35, K. F. Kelton, in "Solid State Physics," ed by H. Ehrenreich and D. Turnbull, Vol. 45, Academic Press, Boston (1991), p. 86.

2 M. Ataka, *Prog. Crystal Growth and Charact.*, **30**, 109 (1995); M. Ataka and M. Asai, *Biophysical J.*, **58**, 807 (1990).

3 F. Oosawa and M. Kasai, *J. Mol. Biol.*, **4**, 10 (1962); F. Oosawa and S. Asakura, Thermodynamics of the polymerization of Protein, Academic Press, London (1975).

4 M. H. Greider, S. L. Howell, and P. E. Lacy, *J. Cell Biology*, **41**, 162 (1969).

5 J. Brange, "Galenics of Insulin," Springer, Berlin (1987).

6 W. Kadima, A. McPherson, M. F. Dunn, and F. Jurnak, *J. Crystal Growth*, **110**, 188 (1991).

7 C. P. Hill, Z. Dauter, E. J. Dodson, G. G. Dodson, and M. F. Dunn, *Biochem.*, **30**, 917 (1991).
2Zn-insulin has been purified because of the unsettled content of Zn^{2+} ion. Release of Zn^{2+} ion from the sample was followed as described by Hill et al. After releasing the Zn^{2+} ion, appropriate amounts of insulin were dissolved in 0.02 mol dm^{-3} of HCl aqueous solution. To prepare the pure 2Zn-insulin hexamer, $ZnCl_2$ was added to the above solution at 1:1 ratio for the insulin monomer. The insulin solution (pH 2.1) filtered through 0.10 μm Millipore filter was employed in this experiment.

8 B. H. Fank and A. J. Veros, *Biochem. Biophys. Res. Commun.*, **32**, 155 (1968).

9 J. Schlichtkrull, *Acta Chem. Scand.*, **11**, 439 (1957).

10 Recent Work: E. N. Baker, T. L. Blundell, J. F. Cutfield, S. M. Cutfield, E. J. Dodson, G. G. Dodson, D. M. C. Hodgkin, R. E. Hubbard, N. W. Isaacs, C. D. Reynolds, K. Sakabe, N. Sakabe, and N. M. Vijayan, *Phil. Trans. R. Soc. Lond.*, **B319**, 369 (1988).