Crystallization phase diagram, the growth of large single crystals of bovine β-Lactoglobulin A

D Yagi1, Y Ohnishi2, I Tanaka2 and N Niimura3,4

1Graduate School of Science and Engineering, Ibaraki University, Japan
2College of Engineering, Ibaraki University, Japan
3Frontier Research Center for Applied Atomic Sciences, Ibaraki University, Japan
4E-mail: niimura@mx.ibaraki.ac.jp

Abstract. A crystallization phase diagram defining the meta-stable region of bovine β-lactoglobulin A (β-Lg) was firstly determined by a dialysis method. We have succeeded in growing a large single crystal of β-Lg by selecting a crystal grown in this “meta-stable region” method described in the present paper. The quality of protein crystals was characterized quantitatively via rapid X-ray data collections, followed by the use of Wilson plots to analyze their resulting average B-factors.

1. Introduction
β-lactoglobulin A (β-Lg) is one of the model proteins for the study of amyloid fibril formation. [1] It is well-known that property changes of these proteins are closely related to a change of the hydrogen bonding between β-strands, and therefore it is important to know the distinctive features of H-bonds of such proteins for a better understanding the mechanism of amyloid fibril formation.

Neutron protein crystallography, in principle, provides the information of all the hydrogen atoms and the hydration structure around a macromolecule, and it is one of the most powerful techniques to investigate H-bonds in which the positions of hydrogen atoms are explicitly included. [2] However, one of the main disadvantages of neutron protein crystallography is that large crystals are needed: currently, the volume required for single-crystal work is usually on the order of 1 mm3. In this paper the crystallization phase diagram of β-Lg will be reported and a method of growing a large single crystal of β-Lg on the basis of this phase diagram will be demonstrated. Finally, the qualities of several crystals grown under various conditions in the phase diagram will be described.

2. Experiments, results and discussion

2.1. Sample preparation
Bovine β-lactoglobulin A (β-Lg) was purchased from Sigma Chemical Co. (L7880), and protein solution was prepared into Tris/HCl (pH 7.9) buffer without further purification. Ammonium sulfate was selected to be the crystallization (precipitating) agent. [3]

4 Author to whom any correspondence should be addressed.
2.2.  The crystallization phase diagram

A typical and general crystallization phase diagram is shown in Figure 1, where Cp and Cc are the concentrations of the protein and the crystallization (precipitating) agent, respectively. The phase diagram is classified into three regions: unsaturated, meta-stable, and nucleation region. The particular crystallization phase diagram for β-Lg (shown in Figure 2) was determined by a dialysis method. A Lucite dialysis button, in which the volume of protein solution is 5 μl, was used. The molecular weight cutoff of the dialysis membrane was 10,000. Five protein solutions, with Cp of 4, 11, 15, 19 and 36 mg/ml, respectively, were prepared. For each concentration, four solutions were prepared in order to monitor reproducibility. The button containing the protein was immersed in reservoir solution, consisting of the crystallization agent and the buffer. Since protein solutions Cp of β-Lg higher than 40mg/ml become cloudy upon addition of ammonium sulfate, the maximum Cp was limited to 36 mg/ml in our experiments. Moreover it must be pointed out that the protein solution became translucent beyond about 1.5 M of Cc.

Firstly, the nucleation borderline between meta-stable region and nucleation region (dotted blue line in Figure 2) was determined as follows: The Cc was increased step by step. Each incremental step was 0.05 M and the protein solution in the button was kept for 1 week at T= 20°C, upon which it was noted whether crystallization had occurred or not.

Secondly, the solubility curve (solid red line in Figure 2) was determined by dissolving crystals in the button as follows: The Cc was decreased step by step, and the protein solution was kept in this condition for one day and it was observed whether crystals had completely dissolved or not. When the crystals were completely dissolved, a point (Cc, Cp) was marked in the phase diagram, whereas if crystals still remained, the Cc was decreased by one more step, -0.05M. It was experimentally confirmed that the solubility curve above 2.2 M of Cc comes very close to the low concentration limit of Cp (dotted red line in Figure 2). Crystals were put into protein-free solutions of ammonium sulfate between 2.2 M and 2.7 M of Cc, and kept there for two weeks. It was observed that only a tiny amount of dissolution of these crystals occurred under those high-precipitant concentrations, and when measured by UV ($A_{280}^{\text{nm}} = 9.6$) the protein concentrations turned out to have values of 0.018 mg/ml and 0.021 mg/ml for 2.2 M and 2.7 M of ammonium sulphate, respectively. The resulting crystallization phase diagram for bovine β-lactoglobulin A is shown in Figure 2. The meta-stable region has been clearly determined. The crystallization phase diagram of β-Lg, (in which the protein concentration Cp was kept below 40 mg/ml) was first determined by the dialysis method in order to define meta-stable region. It was found that this meta-stable region was rather wide, a feature which is normally conducive to the growth of a large single crystal. We succeeded in growing a large single crystal in the meta-stable region as described in the next subsection.

2.3.  Growth of a large single crystal

The phase diagram which is useful for growing a large single crystal of a protein is illustrated by following the gray arrows of Figure 1. A crystal starts growing in the nucleation region. When Cc is decreased into the meta-stable region, this crystal becomes a seed crystal and continues to grow in the meta-stable region (left gray arrow). As the crystal grows, the protein concentration remaining in solution decreases simultaneously (downwards gray arrow) and reaches the solubility curve (solid curved line) and crystal growth stops. Then Cc is increased to the right boundary (right gray arrow) of the meta-stable region and the crystal growth resumes. When this procedure is repeated, the protein molecules corresponding to the concentration differences between the initial and the final conditions are concentrated into the one crystal and a large crystal is expected to grow (Figure 3). This method has been applied to β-Lg on the basis of the initially determined crystallization phase diagram as shown schematically in Figure 2. The procedure of the Cc change and the photographs of the actual grown crystal are shown in Figure 3. The numerical Cc values and the actual period of days at each precipitant level are indicated in Table 1. In this experiment, 4mg/ml concentration of β-Lg was selected, because low concentration of Cp is easy to control nucleation in comparison with high
concentration of Cp. The initial size of the crystal (0.1 mm x 0.1 mm x 0.1 mm) was grown to 1.0 mm x 0.5 mm x 0.4 mm in size eventually, and the crystal was irradiated by using a neutron diffractometer, BIX-4 at JRR-3 in JAEA, and data to 3.0Å resolution was obtained. It is worthy to note that during such a very long crystal-growing period no new nucleation had occurred, and only a single seeded crystal was observed to grow within the nucleation boundaries of the meta-stable region of the phase diagram, and it is proved that the method of growing a large single crystal is promising.

3. Conclusion
1) The crystallization phase diagram of bovine β-lactoglobulin A has been determined by the dialysis method.
2) In the meta-stable region, the large crystal was grown. The broadness of the meta-stable region might help to grow a large crystal, and it is important that in meta-stable region no nucleation occurs and only the seed introduced grows.

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Figure 1. A typical and general crystallization phase diagram. The principle of crystallization of a large crystal is illustrated by the sequence of gray arrows.

Figure 2. The obtained crystallization phase diagram of bovine β-lactoglobulin A. The meta-stable region is defined by the area between the dotted blue line and the red solid line, the latter of which is often referred to as the “solubility curve”. The solubility curve at low Cp concentration has been determined by dissolution of crystals at defined Cc and shown in dotted red line.
**Figure 3.** Photographs of a crystal grown stepwise in a systematic way by changing Cc, as indicated by the series of red arrows (also see Table 1 in the text).

**Table 1.** A period of days at each precipitant concentration level (Cc) for the crystal shown in Figure 3.

| Cc (M) | 2.50 | 2.53 | 2.54 | 2.55 | 2.56 | 2.57 | 2.58 | 2.59 | 2.60 | 2.61 | 2.62 |
|--------|------|------|------|------|------|------|------|------|------|------|------|
| Duration (Days) | 92 | 10 | 3 | 11 | 11 | 9 | 5 | 6 | 7 | 29 | 14 |

**References**

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