Self-assembly of protein at aqueous solution surface in correlation to protein crystallization

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The assembly of lysozyme (hen egg white) at the surface of aqueous solution follows the same behaviors as amphiphilic molecules. The critical assembly concentration appearing in the protein solutions is found to coincide with the equilibrium concentration of protein crystals under given conditions. The crystallization of protein regarded as a typical case of protein self-assembly in three dimensions has been discussed. The result reveals also the correlation between protein crystallization and the two-dimensional self-assembly at the surface of substrates. It follows that the protein crystallization condition can be determined without protein crystals. © *2005 American Institute of Physics*. [DOI: 10.1063/1.1846153]

Protein crystallization has attracted much attention and has remained a hot topic in recent years, due to its wide application in drug delivery and protein structure determination.[1–6] The slow dissolution rate enables a protein crystal to function as an effective drug delivery material, so as to achieve a sustained release of medications.[7] However, protein structure data are needed, for instance, for rational drug design, or for the understanding of genome-structure-function correlations.[8] Among the two main methods (NMR and x-ray crystallography) available for the determination of protein structure, x-ray crystallography has the advantage of a much higher resolution and is more cost effective than NMR.[9] In order to take advantage of x-ray crystallography, sufficiently large and high quality single crystals are essential. The key problem associated with this is that in many cases, proteins will aggregate into an amorphous phase instead of the crystalline phase.[10,11] The above-mentioned issues have become a bottleneck for the determination of protein structures.

In order to grow protein crystals, the conditions for crystallization should be identified in all circumstances. Among different parameters for the control of protein crystallization, the equilibrium concentration of the protein for a given system is one of the key parameters. To precisely determine the crystallization condition is often a time consuming and difficult task. In particular, when protein crystals are not available, to measure the equilibrium point of protein crystallization is almost impossible.[12,13]

In this Letter, we will examine the protein assembly at the air/water interface, and establish the correlation between the crystallization condition and the surface assembly of the protein in order to identify protein crystallization conditions.

In our experiment, hen egg white lysozyme (six times purified by crystallization, purchased from Seikagaku) and concanavalin A (highly purified, purchased from Sigma) were used as model proteins. Tris-Ac, sodium acetate, sodium chloride, and ammonium sulfate, all of analytical grade, were purchased from Merck. High-purity deionized water (~18.2 MΩ) produced by a Millipore Milli-Q system (0.22 μm) was used for preparing 50 mM sodium acetate buffer at pH 4.5 and 50 mM Tris-Ac buffer at pH 6.5. The buffers were used as solvent for protein and salt. The protein solutions with an expected protein and salt concentration were kept at temperature $T=23$ °C without any disturbance for 24 h to allow the surface assembly to stabilize. The isotherm of the protein assembly at the air/water surface was studied by the Wilhelmy plate method using a K14 Krüss tensiometer. A circulating water bath was connected to the tensiometer maintaining the temperature at 23 ± 0.5 °C. The experimental sample chamber was saturated with pure water vapor to maintain a constant humidity environment. All the containers used in this experiment were cleaned by chromic acid to eliminate potential organic contaminations. The equilibrium surface tension is calculated by averaging 10 data points collected at the end of each run until the standard deviation became less than 0.01 mN/m.

As amphiphilic molecules, proteins will tend to assemble at the surface. Based on the Gibbs equation,[14,15]

$$\Gamma = -\frac{1}{RT}\frac{d\gamma}{d\ln a}, \tag{1}$$

where Γ is the surface excess of protein at the interface, a the protein activity which can be regarded as the protein concentration in a dilute solution, R the gas constant, and T the absolute temperature; the adsorption or assembly of protein at the air/water surface is directly related to the surface activity of the protein.

The implication of Gibbs equation (1) is that the adsorption of protein on the surface leads to a lowering of the surface tension. Figure 1(a) shows that the surface tension decreases with the logarithm of the protein concentration in 1 M sodium chloride solution. At $C \geqslant C^*$(=4 mg/ml), the surface tension reaches a constant value. The concentration

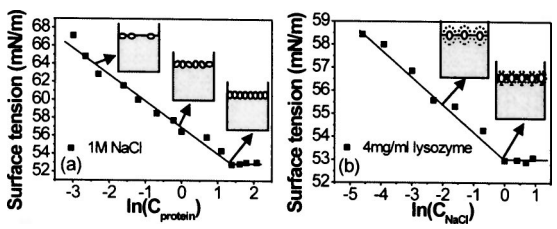

FIG. 1. Dependence of surface tension as a function of (a) protein concentration; (b) sodium chloride concentration.

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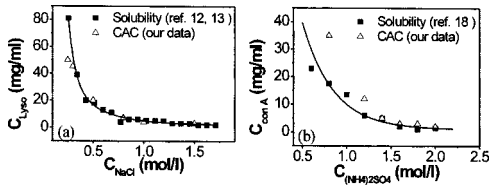

FIG. 2. Comparison between CAC and the solubility of protein (a) lysozyme in sodium chloride; (b) concanavalin A in ammonium sulfate.

C^* is defined as the critical aggregation or critical assembly concentration (CAC).[16] It corresponds to the concentration at which the protein has been optimally assembled at the surface, and any further addition of protein molecules would lead to the aggregation or assembly of protein in the bulk phase. Similarly to ionic surfactants, the surface activity of a protein also depends on the concentration of the electrolyte. Figure 1(b) shows that the CAC also occurs when changing the salt concentration. This may be attributed to the electrical double layer around the protein molecules.[15,17] Adding salt ions in the solution will shrink the effective size of the protein and thus can make the protein packing more compact at the surface.

The striking point in this study is the intrinsic relationship between CAC and the protein crystallization condition. As CAC is the critical point from which the protein begins to assemble or aggregate in the bulk solution, no additional protein can dissolve above CAC. Compared with the solubility of lysozyme in the $C_{\mathrm{lyso}} \sim C_{\mathrm{NaCl}}$ phase diagram at pH 4.5,[12,13] it is found that CAC falls on the solubility line precisely as shown in Fig. 2(a). To confirm the relationship between protein solubility and CAC, concanavalin A (con A) was also examined. Figure 2(b) shows that CAC of con *A* in ammonium sulfate at pH 6.5 is very near the solubility obtained by other groups.[18] Con A exists as a dimmer in aqueous solution at pH below 5 and tetramer at pH above 7.[19] At the air/water interface, the dimmer and tetramer may dissociate, which may cause some slight deviation of the CAC from the solubility. In this sense, if a severe dissociation or unfolding of proteins occurs at the surface, one may expect a notable deviation between the CAC and the solubility. Nevertheless, in the case where the dissociation and unfolding of protein is not severe upon the surface adsorption, CAC can give a good prediction of the solubility of protein in aqueous solution.

This discovery is not very surprising; however, it provides us with a precise method to measure the solubility of protein in aqueous solution. In particular, no protein crystal is needed to measure the crystallization condition of the protein. On the contrary, we can define the protein crystallization conditions before obtaining any crystals. The classical method to measure the protein crystallization condition is to dissolve the protein crystal until it reaches the equilibrium point. But it is impossible to measure the equilibrium concentration when the protein crystal is not available. Owing to our method, the protein crystallization condition can be determined without the protein crystal. The knowledge of the crystallization condition of the protein solution will definitely narrow the screening range of protein crystallization.

Because of the interactions between the charged and hydrophobic portions on the surface, the assembly of the molecules at the air/water interface changes the packing energy at the surface. At $C > C^*$, the constancy of the surface tension implies that C^* corresponds to the stable assembly state, which has the lowest packing energy. As is well known, the lowest packing energy corresponds to the crystal structure at supersaturation. Therefore, the assembly at the surface would approach the crystal structure and act as a self-template for nucleation if protein crystallization were to take place.

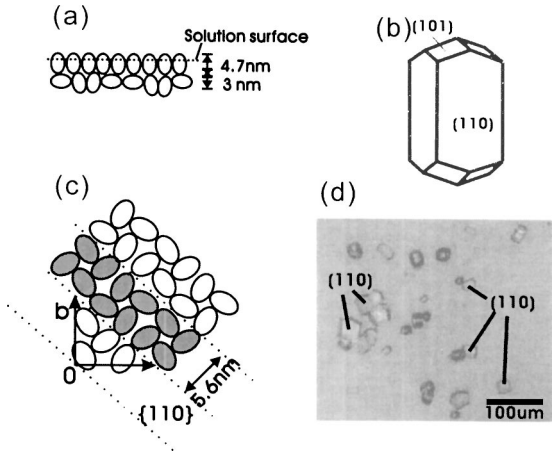

FIG. 3. (a) Protein 2D assembly on the water surface. (b) Lysozyme crystal morphology. Only two faces show up in the experiment. (c) Lysozyme crystal projected on (001). (d) Lysozyme crystals floating on the water surface.

Let us examine the correlation between the protein surface assembly and protein crystallization. The growth of protein crystals is normally governed by a layer-by-layer mechanism.[20–24] This implies that the growth of defect-free three-dimensional (3D) protein crystals is accomplished by a sequence of two-dimensional (2D) birth and spread events on an existing crystal surface.[23] If protein crystallization is considered as a protein molecular assembly having 3D long-range order, the 2D assembly of the protein molecules at the surface can be then considered as a process analogous to 2D protein crystal growth. In this connection, we can probe the protein crystallization in terms of protein assembly at a surface/interface.

For a protein assembly at the surface, according to the Gibbs equation (1), the surface excess can be obtained from the slope of γ versus $\ln a$. Based on the monolayer model of adsorption, the area per molecule (APM) at the surface can be obtained from[25]

$$\mathrm{APM} = \frac{10^{20}}{N_a \Gamma}\ (\text{Å}^2/\text{molecule}), \tag{2}$$

where N_a is the Avogadro constant, and Γ is the surface excess. Assuming that the protein assembly on the surface is a monolayer just before the onset of CAC, a calculation of the APM for the lysozyme adsorption in solutions with different sodium chloride concentrations gives 100 Å^2, which means that the average size of the protein is about 1 nm. This is much smaller than the size of lysozyme, which has an ellipsoidal shape with a short axis about 3 nm and a long axis about 4.5 nm.[26] Therefore, the adsorption must be multilayer, which has a certain structure having the lowest free energy. The assembly of lysozyme at the air/water surface has been studied by Lu's group using a small-angle neutron-scattering technique.[27,28] According to their results, when the lysozyme concentration increases, the adsorption of lysozyme on the surface also increases. This increase results in a two-layer assembly as shown in Fig. 3(a). The

upper layer is about 4.7 nm while the following layer is a loosely packed layer about 3 nm.

As for the lysozyme crystal structure, lysozyme crystallizes in the tetragonal system ($a=b=78.54$ Å, $c=37.77$ Å, and space group $P4_32_12$) at 23 °C.[26] In the observed morphology of the lysozyme crystal, only two faces show up: (110) and (101), as shown in Fig. 3(b).[29] The interplanar distances for these two faces are 5.6 and 3.4 nm, respectively. The molecular assembly of protein on the (110) face projected on (001) is shown in Fig. 3(c). The molecules assemble compactly and smoothly in this face. Noting that the sublayer of protein 2D assembly on the surface [Fig. 3(a)] also has such a structure, this 2D assembly would act as a self-template for the protein 3D crystallization. Experimentally, it is often observed that when lysozyme crystals occur at the surface of aqueous solutions, the {110} faces are often in parallel to the surface, as shown in Fig. 3(d). This can be regarded as a piece of indirect evidence for the above argument.

In conclusion, we have developed a method to determine the protein crystallization condition without the need to first grow protein crystals. The protein 2D assembly on the water surface acts as a self-template for protein 3D crystallization. Based on this study, it should be possible to use the protein 2D assembly kinetics as a probe to study the kinetics of protein crystallization, which has thus far been largely impossible. The understanding of the kinetics of protein crystallization would benefit the advances in drug design and drug delivery, as well as the determination of the protein structure.

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