Prediction of protein crystallization based on interfacial and diffusion kinetics

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The interfacial kinetics of protein crystallization was studied via the kinetics of protein two-dimensional self-assembly. The competition between the protein volume transport and surface integration determines whether single crystals or amorphous aggregation will occur. A kinetic coefficient was found to provide an effective and reliable criterion to predict protein crystallization conditions. This criterion has been applied to lysozyme, concanavalin A and BSA crystallization, and it turned out to be very successful and more reliable than the second virial coefficient criterion. © *2005 American Institute of Physics*. [DOI: 10.1063/1.2040006]

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] Protein structural data are necessary to explore, e.g., biofunctionalities, drug design, and disease treatment.[7,8] Between the two main methods [nuclear magnetic resonance (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] Nevertheless, in order to take advantage of x-ray crystallography, sufficiently large and high quality single crystals must be available. The key problem associated with this is that in many cases, proteins aggregate into an amorphous phase instead of the crystalline phase.[10,11] This issue has become a bottleneck for the determination of protein structure. To overcome this obstacle, large scale screening is conducted in order to search for the crystallization conditions. This can be extremely costly and time consuming.

In order to predict protein crystallization conditions without resorting to screening experiments, the second virial coefficient, B_{22}, has been widely adopted.[12–15] The value of the second virial coefficient depends on the effective interaction between a pair of macromolecules in solution—a positive value reflecting predominantly repulsive interactions and a negative value indicating attractive interactions. A necessary condition for protein crystallization is that B_{22} lies in a crystallization window, $-8\times10^{-4}<B_{22}<-2\times10^{-4}$ ml mol/g^2. However, for macromolecules with complicated shape and conformation, the crystallization of protein involves a nucleation and a growth process. These two processes are determined to a large extent by the kinetics of incorporating protein molecules into the "embryo" surface. Therefore, the kinetics of incorporating protein molecules into the kink site at the surface of protein crystal embryos may not be a negligible process. On the other hand, the crystallization window provided by the second virial coefficient disregards kinetic and other factors, and leads to many failures in the prediction.

In our recent study,[16] it was found that the protein self-assembly at the surface of aqueous solution has the same structure as in the crystal structure. This two-dimensional assembly can act as a self-template for protein three-dimensional crystallization. Based on this study, it is possible to use the protein two-dimensional assembly kinetics as a probe to study the surface kinetics of protein crystallization.

In this letter, we will study the kinetics of the surface assembly of lysozyme as a model protein system in order to obtain a new understanding of the relationship between the kinetics and protein crystallization conditions. We wish to establish a completely new criterion for the prediction of protein crystallization based on kinetics.

Hen egg white lysozyme (purified six times by crystallization) was purchased from Seikagaku. BSA and concanavalin A (con A) were purchased from Sigma. All these proteins were used without further purification. 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 buffers, which were used as solvents for proteins and salts. The *p*H was adjusted by the corresponding acid and hydroxide within the accuracy of ±0.1. The stock solutions of proteins and salts were prepared and refrigerated at 4 °C for further use.

The kinetics of protein adsorption at the air-water interface was studied by the Wilhelmy plate method using a K14 Krüss tensiometer at 23±0.5 °C. The solutions were mixed with protein and salt stock solutions right before the measurement. The change of the interfacial tension was recorded at 1 min intervals after mixing the solution in order to reach the equilibrium surface tension. The equilibrium surface tension is calculated by averaging a certain number of data points collected at the end of each run until the standard deviation became less than 0.01 mN/m. The experimental sample chamber was saturated with pure water vapor to keep a consistently humid environment. All the containers used in this experiment were cleaned by chromic acid to eliminate potential organic contaminations.

The analysis of the kinetics of protein surface self-assembly is based on Ward and Tordai's work.[17] In their work, the effects of diffusion from the bulk liquid to the surface and the energy barrier associated with the molecular assembly at the surface are taken into account. The rate of reduction of the surface tension is determined by three consecutive or concurrent processes. At the very beginning of

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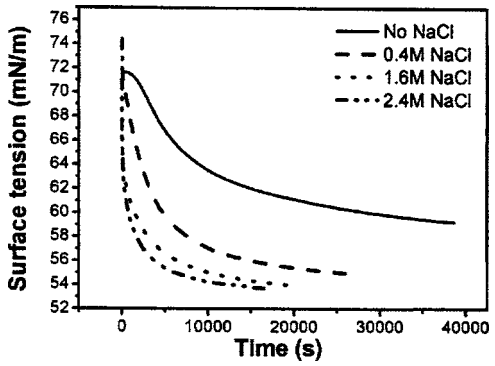

FIG. 1. Time dependence of surface tension at the air/water interface for 1 mg/ml lysozyme solutions at different sodium chloride concentrations.

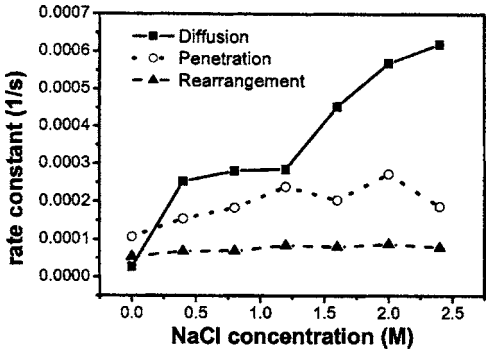

FIG. 2. Rate constants of diffusion k_d, penetration k_p, and rearrangement k_r as a function of sodium chloride concentration.

the surface assembly, there is no energy barrier associated with the molecular assembly. In this step, diffusion is the rate-determining factor. At higher surface coverage, there are energy barriers to the penetration of molecules into the adsorbed protein layer. The penetration of the molecules into the adsorbed layer is the rate-limiting step. When the surface is almost fully covered, the reduction of the surface tension will be determined by the molecular rearrangement in the film. For such a case, one has[18–20]

$$\ln\left(d\,\Pi\,(t)/dt\right) = \ln(\bar{k}C_p) - \Pi\,(t)\Delta A/kT, \tag{1}$$

where $\Pi(t)=\gamma_0-\gamma(t)$ is the surface pressure at time t, $\bar{k}$ a constant related to adsorption ability, and ΔA represents the mean area created in the film in order to adsorb a protein molecule. If ΔA is assumed to be a constant, then a plot of $\ln(d\Pi(t)/dt)$ vs Π should be linear with a slope giving ΔA. The values of ΔA, $\bar{k}$ and C_p can be considered to be constants within each rate-determining process, but these values will change when the rate-determining process is changed. Therefore, each successive linear part of $\ln(d\Pi(t)/dt)$ vs Π corresponds to each kinetic step.

The rates of these processes can be analyzed to first order by[19,20]

$$\ln\left(1-\Pi\,(t)/\Pi_e\right) = -t/\tau, \tag{2}$$

where Π_e is the surface pressure value at the equilibrium state and τ is the relaxation time. The rate constants, $k=1/\tau$, can be estimated from the slopes of $\ln(1-\Pi(t)/\Pi_e)$ vs t at each kinetic step.

Figure 1 shows the changes in surface tension of 1 mg/ml lysozyme with different concentrations of sodium chloride. It appears that the presence of sodium chloride resulted in a decrease of the time (t_e) that is necessary to reach a steady state as well as the equilibrium surface pressure. Figure 2 shows the relationship between the concentration of sodium chloride and the rate constants of diffusion k_d, penetration k_p, and rearrangement k_r. It appears that the rate constant of each step depends on the salt concentration. With the increase of sodium chloride, the diffusion rate increases dramatically. The changes in the rate constants of penetration and rearrangement are much less significant, although a slightly higher k_p value is obtained for the high salt sample.

In order to anticipate the probability of forming crystalline nuclei or amorphous aggregate nuclei, we introduced a kinetic coefficient, ξ_c, which is defined as the ratio between the diffusion rate and the surface integration rate

$$\xi_c = k_d/k_s, \tag{3}$$

where k_d is the rate constant of diffusion and k_s is the rate constant of surface integration, which involves the penetration and rearrangement of protein molecules in the assembled layer. A large ξ_c corresponds to fast diffusion compared with surface integration. If this happens, the packing of nuclei in a crystalline phase will be much slower than the formation of an amorphous phase.

The kinetic coefficient, ξ_c, associated with crystallization and amorphous aggregation conditions[11] is shown in Fig. 3 for lysozyme with sodium chloride. Comparing the crystallization conditions with the kinetic coefficient, it turned out that for crystallization, ξ_c lay in a certain range, $1<\xi_c<8$. The reason may be that when $\xi_c<1$, the volume diffusion rate is less than the surface integration rate. The diffusion rate is so slow that it is hard for the molecules to collide. Therefore, the chance for the embryo to grow to the critical size and overcome the nucleation barrier is also low. On the other hand, when $\xi_c>8$, volume diffusion is faster than surface integration by almost an order of magnitude. The molecules aggregate so fast that they grow and overcome the nucleation barrier before they can find their optimal orientation and conformation. In this case, amorphous aggregation is more favorable than crystallization. Only in the range $1<\xi_c<8$ are the diffusion and surface integration comparable. The formation of single crystals is kinetically favorable. This is called the "kinetic crystallization window", providing us with a new criterion in predicting protein crystallization conditions.

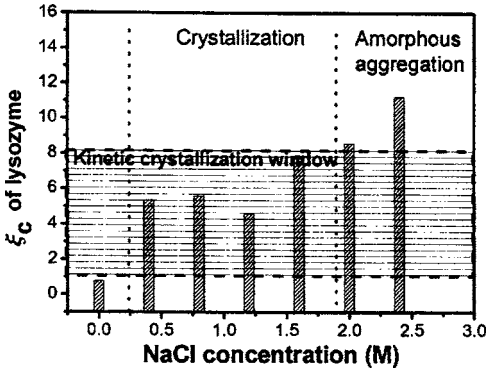

FIG. 3. The crystallization coefficient, ξ_c, of lysozyme in a sodium chloride solution at different concentrations. The crystallization and amorphous aggregation in the salt conditions are also shown as a reference. A kinetic crystallization window is determined by comparison with the crystallization results.

TABLE I. The kinetic coefficient, ξ_c, as a prediction criterion for various proteins in crystallization and aggregation conditions at 20 °C compared with that of B_{22}.

Protein	Salt	*p*H	ξ_c	Crystallization results	B_{22} prediction	Surface tension prediction
Lysozyme	NaCl (<0.3 M)	4.5	0.8	S[a]	S[a]	S[a]
Lysozyme	NaCl (0.3–2 M)	4.5	4–7	C[b]	C[b]	C[b]
Lysozyme	NaCl (>2 M)	4.5	8–12	A[c]	A[c]	A[c]
Lysozyme	$(NH_4)_2SO_4$ (2 M)	4.5	9.5	A[c]	C[b] (incorrect)	A[c]
Con A	$(NH_4)_2SO_4$ (1 M)	7.0	7.6	C[b]	···	C[b]
BSA	$(NH_4)_2SO_4$ (52%)	6.2	24	C[b]	···	A[c] (incorrect)

[a]S: solution
[b]C: crystallization
[c]A: aggregation.

To further test the applicability of this kinetic criterion on the prediction of protein crystallization, the crystallization and amorphous aggregation conditions obtained for lysozyme were applied to different solvents and different proteins, such as BSA and con A. The results are shown in Table I. It can be seen that in most cases, the criterion is valid.

Compared with the B_{22} criterion, the surface tension prediction is more reliable and physically more sound. This can be seen in the case of lysozyme in ammonium sulfate. Crystallizing lysozyme from ammonium sulfate is generally considered impossible.[21] As shown in Table I, our prediction of amorphous aggregation is correct, while the B_{22} prediction is wrong. Furthermore, the method of measuring the surface tension is easier to perform than light scattering to obtain B_{22} and requires only a small amount of protein.

We noticed that in some cases, such as BSA with 52% saturated ammonium sulfate, our prediction also gave rise to an incorrect outcome. This may be attributed to the unfolding of BSA molecules at the air/water interface.[22] The protein unfolding changes the surface assembly kinetics, which results in a deviation from the genuine crystallization condition relevant to the bulk phase. Therefore, if severe unfolding of protein occurs at the air/water interface, the prediction method of the kinetic crystallization window based on the surface tension becomes invalid. In general, in cases where the dissociation and unfolding of protein at the surface is not severe, the kinetic crystallization window offers a reliable and sound tool for predicting protein crystallization from solution. This criterion provides an easy and reliable way to crystallize new proteins based on a series of simple surface tension measurements.

In summary, we investigated the kinetics of protein two-dimensional self-assembly at the interface using surface tension measurements. The tendency of protein to aggregate amorphously can be illustrated by the kinetic coefficient, ξ_c, which is the ratio between the volume diffusion rate and surface integration rate. From the study of protein two-dimensional assembly at the air/water interface, a kinetic crystallization window was determined, which lies in the range $1<\xi_c<8$. This kinetic crystallization window turns out to be more reliable and sound than the criterion based on the second virial coefficient.

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