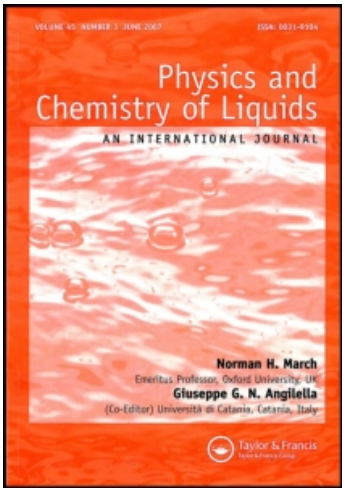

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James K. Baird[a]; John C. Clunie[a]
[a] Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL

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KINETICS OF LYSOZYME CRYSTALLIZATION FROM SOLUTION

JAMES K. BAIRD* and JOHN C. CLUNIE

Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL 35899

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We suggest that the growth of molecular aggregates is the rate-controlling step in the crystallization of lysozyme from pH buffered aqueous solutions of strong electrolytes. We propose that the aggregation reaction passes through a charged transition state whose rate of formation is accelerated by Debye-Huckel screening and whose charge is stabilized by ion exchange with the solution. Applying the theory of the "primary kinetic salt effect", we predict that the half-life for decay of the lysozyme concentration in solution in contact with a growing crystal should decrease linearly with the square root of the ionic strength. This prediction is confirmed experimentally in the case of lysozyme crystals precipitating at 4°C from pH buffered aqueous solutions of sodium chloride.

Keywords: Lysozyme crystallization; solution; pH; ionic strength

INTRODUCTION

A complete understanding of the biological function of proteins requires a knowledge of the arrangement of the atoms in the molecule. Modern methods of X-ray diffraction are capable of revealing this structural information in cases where high quality single crystals of the protein are available. Nature has conspired to make crystallization of proteins a difficult process, however, due to the deleterious effects that this event would otherwise cause in living organisms.

In the laboratory, proteins are crystallized from aqueous solution. The crystallization recipes ordinarily call for a pH buffered solution of the protein supported by the addition of strong electrolyte. In the aqueous phase, the protein molecules exist as macro-ions. The pH

*Corresponding author.

determines the average charge on the macro-ion, [1] while the pH together with the strong electrolyte determines the equilibrium thermodynamic solubility of the crystal [2].

By contrast, the effect of pH and added salt on the kinetics of crystallization of proteins is less well understood. Below, we explore this question from the point of view of chemical kinetics.

Measurements of the rate of dialysis of solutions of lysozyme [3] as well as measurements of the rate of growth of lysozyme crystals, [4] themselves, suggest that lysozyme crystallizes by a mechanism involving pre-existing oligimers. These species with specific molecular contacts having crystal-like symmetry are to be distinguished from the intermolecular chains, which have been described by Feher and Kam, [5] and which are the precursors to the formation of amorphous precipitates.

Ataka and Asai suggested that the rate of crystallization of lysozyme is controlled by the coalescence of n protein molecules to form an n-mer aggregate followed by the reaction of the n-mer with an additional molecule of protein [6]. They selected rate equations, which they solved for the integral rate law governing the time dependence of the protein concentration in the solution. As a test of their mechanism, they grew lysozyme crystals and made measurements of the time dependence of the protein concentration in the supernatant solution held at 35°C, pH = 4.6, and 3% w/v NaCl. In agreement with their integrated rate law, they found a straight line plot of log $t_{1/2}$ *vs.* log c_o, where $t_{1/2}$ is the half-life for decay of the lysozyme concentration, while c_o is its initial value. Comparing the slope of this plot with their rate law, they concluded that $n = 4$.

We suggest that the rate equations proposed by Ataka and Asai [6] fail to conserve mass when compared with their two-step chemical mechanism. Although their half-life formula is in quantitative accord with their measurements of log $t_{1/2}$ *vs.* log c_o, we shall demonstrate that this relationship can also be understood by invoking a one-step mechanism and a rate equation which conserves mass.

In lysozyme crystal growth experiments carried out in unbuffered growth solutions made acid with HCl, Elgersma *et al.* [7] reported a drop in Cl^- concentration in the supernatant as well as a less noticeable decrease in pH as crystallization proceeded. They suggested that in the process of growth, the lysozyme crystals were absorbing

Cl^- from solution while weakly rejecting H^+ to it. Subsequent experiments have also detected ion exchange effects between the solution and the solid phase [8, 9].

Besides their potential role in ion exchange, Na^+ and Cl^- in the growth solution also produce a physical (Debye-Huckel) screening of the charges on reacting ions. The screening accelerates the rate of reaction between ions of like sign and retards the rate of reactions between ions of unlike sign. The signature of this phenomenon, called the "primary kinetic salt effect", is a linear dependence of the logarithm of the specific rate on $\sqrt{I}$, where I is the ionic strength [10]. Using data from some preliminary experiments carried out at fixed lysozyme concentration, we have found that ln $t_{1/2}$ is a decreasing linear function of $\sqrt{I}$. The negative slope implies that lysozyme nucleates by agglomeration of ions of like sign.

EXPERIMENT

Lysozyme was crystallized from aqueous NaCl solutions held at 4°C and buffered to pH = 4.15 using sodium acetate/acetic acid buffer. The NaCl concentrations ranged between 0.257 M and 0.685 M. Since the buffer was dilute, and NaCl is a 1-1 electrolyte, the solution ionic strength values and the NaCl concentrations were identical. Under these conditions, the solubility of lysozyme [2, 11] was independent of salt concentration and was equal to about 3 mg/mL. For each experiment, the initial lysozyme concentration was $c_o = 27$ mg/mL.

As the crystals appeared, aliquots of the supernatant solution were collected at various times using a filter tip syringe. The tip kept crystals that were suspended in the solution from entering the syringe. The aliquots were then diluted, and their original lysozyme concentration determined by optical density measurements at 280 nm.

If c is the lysozyme concentration measured at time, t, the ratio, c/c_o, was computed and plotted on the ordinate as a function of t on the abscissa. The shapes of the curves obtained were similar to those reported in Figure 1 of Ataka and Asai [6]. However, our time scale (at 4°C) on the abscissa was minutes, whereas theirs (at 35°C) was days. Indeed, we found the time scale to be a strong function of temperature,

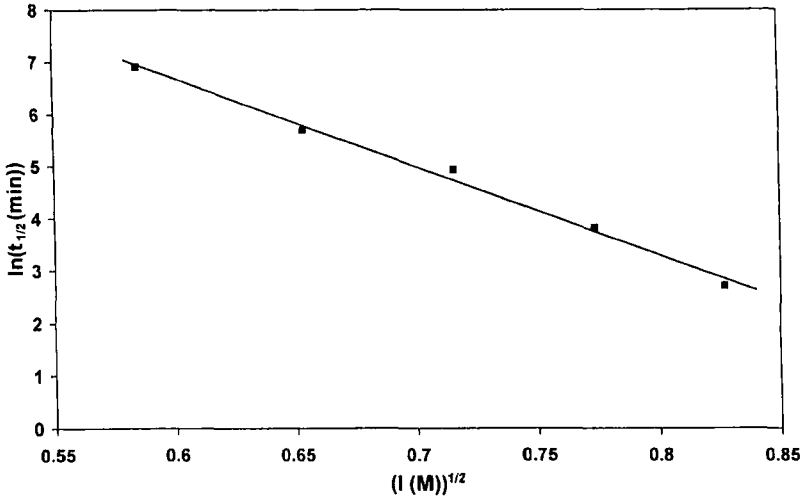

FIGURE 1 Half-life, $t_{1/2}$, for decay of the lysozyme concentration in a crystallizing solution held at 4°C and pH = 4.15 as a function of ionic strength, I. The sodium acetate/acetic acid buffer was sufficiently dilute that I was identical with the concentration of the added NaCl.

and chose 4°C to make the rate of crystallization convenient for determination by the aliquot method.

From each of our curves, the value of the time, $t_{1/2}$, where $c/c_o = 0.5$ was determined. As shown in our Figure 1, a plot of these half-lives in the form of ln $t_{1/2}$ *vs.* $\sqrt{I}$ resulted in a straight line of negative slope. In the next section, after first discussing the two-step mechanism of Ataka and Asai [6], we introduce an alternative one-step mechanism, which provides an interpretation for that slope.

THEORY

If we suppose that isolated lysozyme macro-ions, $(1)^{Z_1}$, having valence, Z_1, serve as monomers for the formation of n-mer aggregates, $(n)^{Z_n}$, having valence, Z_n, then the mechanism proposed by Ataka and Asai [6] can be written

$$n\,(1)^{Z_1} \rightarrow (n)^{Z_n} \qquad (1)$$

$$(1)^{Z_1} + (n)^{Z_n} \rightarrow (n+1)^{Z_{n+1}} \tag{2}$$

Denoting the rate constants for the reactions in Eqs. (1) and (2) as k_1 and k_2, respectively, they introduced the rate laws

$$\frac{dm}{dt} = k_1 c^n \tag{3}$$

$$\frac{dc}{dt} = -k_2 mc \tag{4}$$

where c is the concentration of $(1)^{Z_1}$, while m is the concentration of $(n)^{Z_n}$.

The rate laws in Eqs. (3) and (4), however, fail to reflect the mass balance implied by the chemical reactions in Eqs. (1) and (2). Indeed, since $(n)^{Z_n}$ is consumed through Eq. (2), there should be added to the right hand side of Eq. (3) the term, $-k_2mc$, so that the mass of $(n)^{Z_n}$ is conserved. Likewise, since $(1)^{Z_1}$ is consumed in Eq. (1), there should be added to the right hand side of Eq. (4) the term, $-nk_1c^n$, so that the mass of $(1)^{Z_1}$ is conserved. Although under special conditions, one term may be larger than the other, it is impossible simultaneously for k_1c^n to dominate Eq. (3) while $-k_2mc$ dominates Eq. (4), as Ataka and Asai assumed [6].

One can, however, propose a simple mechanism that is not only consistent with the principle of mass balance but also takes into account the available kinetic data. Due to electrostatic repulsion, reactions between like-charged, polyvalent macro-ions are slow. We suggest that under crystallizing conditions, rapid ion exchange between the solution and the protein macro-ions occurs, and this serves to reduce their charge. We let Z_1' be this partially neutralized valence. Within the framework of classical chemical kinetics, these partially neutralized macro-ions can agglomerate according to

$$n(1)^{Z_1'} \rightarrow (n)^{nZ_1'} \rightarrow \text{crystals} \tag{5}$$

where $(n)^{nZ_1'}$ is the transition state whose rate of production controls the subsequent rate of nucleation.

Taking k to be the rate constant, the rate of formation of nuclei by this forward reaction is kc^n. When the concentration of free protein has decayed to its solubility limit, c_s, this rate is kc_s^n. As this defines

the point of equilibrium where the rates of formation and dissolution of nuclei are equal, the rate of the reverse of the reaction in Eq. (5) must also be kc_s^n. This argument is based upon the principle of microscopic reversibility. Away from equilibrium, where the forward and back reactions no longer compensate, the net rate of formation of nuclei is thus $kc^n - kc_s^n = kc_s^n[(c/c_s)^n - 1]$.

In the case of our experiments, the solubility, c_s, was just 3 mg/mL, whereas c varied over the course of two half-lives between 27 mg/mL and about 6 mg/mL; consequently, for the duration of a run, we could expect $(c/c_s)^n \gg 1$ for sufficiently large n. In this limit, the net rate of formation of nuclei could be adequately approximated by kc^n. Thus, the rate equation, which satisfies mass balance, and is consistent with Eq. (5) could be written

$$\frac{dc}{dt} = -nkc^n \tag{6}$$

Eq. (6) has the integral

$$(c_o/c)^{n-1} - 1 = n(n-1)\,c_o^{n-1}\,kt \tag{7}$$

By setting $t = t_{1/2}$ and $c_o/c = 2$, one finds

$$t_{1/2} = \frac{(2^{n-1} - 1)}{n(n-1)\,k\,c_o^{n-1}} \tag{8}$$

After taking the log of both sides, the half-life formula,

$$\log t_{1/2} = \log\left(\frac{2^{n-1} - 1}{n(n-1)\,k}\right) - (n-1)\log c_o \tag{9}$$

is obtained.

Eq. (9) has the same form as Eq. (4) of Ataka and Asai [6], although the coefficients have meanings different than those proposed by them. By experiment, they found that $\log t_{1/2}$ *vs.* $\log c_o$ had slope equal to -2 (see their Fig. 2). On the basis of this value and Eq. (9), we conclude that $n = 3$, which implies that the formation of the aggregate of critical size is termolecular. According to Figure 2 of Ataka and Asai [6], when $c_o = 1$ wt%, $t_{1/2}$ equals 70 days. Using this convenient point and

Eq. (8) with $n = 3$, we find $k = 0.007$ (wt%)$^{-2}$(day)$^{-1}$, which is the specific rate of the termolecular reaction under the conditions of their experiment.

In the Appendix, we derive a formula (Eq. (A8)), that can be used to evaluate the ionic strength dependence of $t_{1/2}$. For this equation to apply to the n-molecular reaction in Eq. (5), we set $m = n$, $M = 1$, $Z_M = Z_1'$ and $q = 0$. The result is

$$k = k_o (c')^{1-n} \exp\left[n(n-1)(Z_1')^2 A\sqrt{I}\right] \tag{10}$$

Upon substitution of Eq. (10) into Eq. (8) and formation of the natural logarithm of both sides, we obtain

$$\ln t_{1/2} = \text{constant} - n(n-1)(Z_1')^2 A\sqrt{I} \tag{11}$$

which demonstrates, in agreement with the data in our Figure 1, that $\ln t_{1/2}$ should be a decreasing linear function of $\sqrt{I}$.

The magnitude of the slope in our Figure 1 is $S = 16.8$. According to Eq. (11), $S = n(n-1)(Z_1')^2 A$. After substitution of $T = 277$ K and setting $\varepsilon = 86.5$ appropriate at that temperature [12], we use Eq. (A.7) to compute $A = 1.13$. Letting $n = 3$, we find that under our conditions at pH = 4.15 and a temperature of 4°C, the average charge on a crystallizing lysozyme macro-ion to be $Z_1' = +1.6$. This also suggests that under these conditions, the average valence of the transition state is 3 (1.6) = + 4.8.

DISCUSSION

Within the scope of classical chemical kinetics, the lysozyme crystallization experiments of Ataka and Asai [6] imply that, at 35°C, pH = 4.6, and 3% w/v NaCl, the protein nucleates by a termolecular reaction. Termolecular reactions are uncommon but not unknown.

Our own experiments at 4°C and pH = 4.15 demonstrate that the rate of nucleation is accelerated by the addition of NaCl and that the logarithm of the nucleation half-life decreases linearly with the square-root of the ionic strength. According to the theory of primary kinetic salt effect, the value of the slope of this line implies that the average

charge of a nucleating lysozyme macro-ion is + 1.6 and that the nucleation process passes through a transition state having an average charge of + 4.8. These charges need not be integral, because Roxby and Tanford [1] show that fluctuations in ionization make the titration curve vary continuously with pH. At 25° C and pH = 4.15, they find the average charge on a lysozyme macro-ion to be about + 12. Because of the temperature difference, however, the magnitudes of charges determined in this equilibrium experiment and in our kinetics experiment cannot be directly compared. Should, on the other hand, temperature not be a decisive factor, then comparison implies that crystallizing lysozyme macro-ions undergo a charge reducing ion exchange with the solution before formation of the transition state. The titration curve also shows that the macro-ion average charge decreases with increasing pH, reaching zero at pH = pI = 11.1. According to Eq. (11), if Z_1' follows this same pattern, then the nucleation half-life should be independent of ionic strength at the pI.

So far, we have relied upon classical chemical kinetics to rationalize the role played by pH and ionic strength on the formation of crystal nuclei. This start, which emphasizes the charge states of the macro-ions and the nuclei, can be the basis, however, for adapting classical nucleation theory to the protein crystal growth problem. We outline the process below.

In nucleation theory, one finds that there is a nucleus of "critical" size that has maximum free energy of formation and a minimum representation in the population of nuclei. This nucleus is critical in the sense that its net rate of formation controls the rate of the whole nucleation process.

The free energy of formation of this nucleus has contributions from molecules in its interior making contacts with one another and molecules at its surface making contacts with the solution. Although in the case of lysozyme, the critical nucleus is charged, the Debye-Huckel screening may possibly have no effect on the nucleus size and population, because the net electrostatic contribution to the free energy caused by the adhesion of an additional lysozyme cation and its counter-ions is zero [13].

In nucelation theory, the critical nucleus is assembled by the step-wise addition of monomers. In the case of lysozyme, where the monomers are charged, we should expect that the kinetic net rate of

formation of nuclei of critical size will be affected by mutual electrostatic repulsion and will depend upon the ionic strength. Indeed, the kinetic factors should depend upon the square-root of the ionic strength, so it is not implausible that the results of nucleation theory will be consistent with the straight line decrease in ln $t_{1/2}$ with $\sqrt{I}$ which was found in our experiments. However, given the current state of nucleation theory [14] some substantial effort will be required to demonstrate this simple result.

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APPENDIX

The effect of Debye-Huckel ionic screening on the rates of ionic reactions (the "primary kinetic salt effect") is ordinarily considered only in the case of bimolecular reactions [10]. Here we extend the theory to apply to binary reactions of arbitrary molecularity.

Consider the charged species, M^{Z_M} and Q^{Z_Q}, reacting through the charged transition state, $(M_m Q_q)^{mZ_M + qZ_Q}$,

$$mM^{Z_M} + qQ^{Z_Q} \rightarrow (M_m Q_q)^{mZ_M + qZ_Q} \rightarrow \text{products} \tag{A.1}$$

According to transition state theory, the first reaction is a dynamic equilibrium. If K is the equilibrium constant, then

$$K = \frac{\gamma_{M_m Q_q} [M_m Q_q]/c'}{(\gamma_M [M]/c')^m (\gamma_Q [Q]/c')^q} \tag{A.2}$$

In Eq. (A.2), c' is the concentration in the thermodynamic standard state, $[M]$ and $[Q]$, are the concentrations of the ions, M^{Z_M} and Q^{Z_Q}, respectively, while $[M_m Q_q]$ is the concentration of their transition state. The respective activity coefficients are γ_M, γ_Q and $\gamma_{M_m Q_q}$.

If $[M_m Q_q]$ decays at the specific rate, k', the observed reaction rate constant, k, satisfies:

$$\frac{1}{m}\frac{d[M]}{dt} = \frac{1}{q}\frac{d[Q]}{dt} = -k[M]^m [Q]^q = -k'[M_m Q_q] \tag{A.3}$$

Using Eq. (A.2), we can solve the last equality in Eq. (A.3) for k:

$$k = k' K \frac{(\gamma_M)^M (\gamma_Q)^q}{\gamma_{M_m Q_q}} (c')^{1-(m+q)} \tag{A.4}$$

According to Debye-Huckel theory, the activity coefficient of an ionic species, j, having valene Z_j is given by

$$\ln \gamma_j = -Z_j^2 A \sqrt{I} \tag{A.5}$$

where

$$I = \frac{1}{2}\sum_i Z_i^2 [i] \tag{A.6}$$

is called the ionic strength and where the sum includes the concentrations, $[i]$, and charges, Z_i, of all ionic species in the solution. The Debye-Huckel Constant, A, has the units $L^{1/2}\text{mol}^{-1/2}$ and is defined by

$$A = \left(\frac{2\pi N e^6}{1000 (\varepsilon k T)^3} \right)^{1/2} \tag{A.7}$$

where N is Avagadro's number, e is the electron charge, k is Boltzmann's constant, T is the absolute temperature, and ε is the dielectric constant of the solvent [15].

Eq. (A.5) can be used to evaluate the activity coefficients in Eq. (A.4). The result is

$$\begin{aligned} k = k_o \exp \{ [& m(m-1) Z_M^2 + q(q-1) Z_Q^2, \\ & + 2mq Z_M Z_Q] A \sqrt{I} \} (c')^{1-(m+q)} \end{aligned} \tag{A.8}$$

where $k_o = k'K$ and $k_o (c')^{1-(m+q)}$ is the specific rate of reaction when $I = 0$.