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Universality of supersaturation in protein-fiber formation

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Abstract

The thermodynamics and kinetics of the aggregation of sickle-cell hemoglobin into fibers have been studied in great detail under a wide range of solution conditions. The stability of the fiber is measured by the solubility; the kinetics is characterized by a delay before the appearance of fibers. A review of data in the literature shows that there is no correlation of the delay time with fiber stability and only a weak correlation with the initial protein concentration. There is, however, a striking collapse of all the data onto a single universal curve when the delay time is plotted versus the supersaturation, which is the ratio of the initial protein concentration to the solubility, expressed as activities. Collapse onto the same universal curve is also obtained when using delay times calculated from the double-nucleation theoretical model.

For many chemical reactions, there is a linear relation between activation and equilibrium free energies or, equivalently, a power-law dependence of rate coefficients on equilibrium constants. Linear free-energy relations have been particularly important in protein kinetics, in which they have been used to obtain structural information on transition states for ligand binding¹, conformational changes² and protein folding^{3,4}, and also to greatly simplify the mathematical formulation of complex kinetic models^{5–7}.

The analogous relation for protein-fiber formation is quite different. In this case, the parameter that measures thermodynamic stability is the solubility, the concentration of protein monomers in equilibrium with the fiber. From studies on protein crystallization reactions⁸, the rate of fiber formation is expected to scale with the supersaturation, the ratio of the total protein concentration to the solubility. This ratio determines how far the system initially is from equilibrium.

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AUTHOR CONTRIBUTIONS

T.C., F.A.F. and W.A.E. performed the calculations, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper

For the aggregation of sickle-cell hemoglobin (HbS) into fibers, the relation between rates and supersaturation can be investigated in detail because of the existence of an extensive set of kinetic and thermodynamic data. The kinetics of sickle-fiber formation, characterized by a marked delay period before the appearance of fibers, is known to exhibit an enormous concentration dependence (up to the 40th power), the highest concentration dependence ever observed for any chemical or physical process. A very early study of HbS fiber formation kinetics under a limited range of concentrations and solubilities has shown that the delay time varies as a high power (27th-40th) of the supersaturation⁹. These experiments were performed with a slow temperature jump method with ~1-min time resolution. Consequently, variations in solution conditions resulting in changes in delay times of less than 200-fold were investigated. Subsequently, a laser photolysis method was developed¹⁰ that allows measurement of delay times with millisecond time resolution; data have now been reported over a 10⁷-fold range of delay times measured for solution conditions corresponding to a much larger range of supersaturations. We therefore set out to investigate the relation between delay times and supersaturation with this larger data set.

Here we reviewed all the published data on HbS and found that its fiber-formation kinetics displays yet another remarkable property. Whereas the duration of the delay period shows no overall correlation with solubility and only a weak correlation with total concentration, the data strikingly collapse onto a single universal curve when the delay time is plotted versus the supersaturation. We further show that the double-nucleation model for sickle-fiber formation, which explains all of the unusual kinetic properties, predicts the same universal curve. These results not only aid in assessing the effects of potential therapeutic inhibitors of fiber formation in sickle-cell disease but also suggest that additional insight into the mechanism of aggregation and inhibitory effects for other fiber-forming systems, such as those associated with amyloid diseases, may be obtained by investigating the relation between rates and supersaturation.

RESULTS

Delay times versus supersaturation

We reviewed all of the published delay-time, total concentration and solubility data for HbS⁹⁻¹⁶ (Fig. 1 and plotted data in Supplementary Table 1). Delay times have been determined from the onset of light scattering after the induction of fiber formation by either a temperature jump or photodissociation of the sickle hemoglobin carbon monoxide complex, whereas solubilities have been determined from the concentration of hemoglobin in the supernatant after sedimentation of fibers in an ultracentrifuge. Because the solutions are extremely nonideal, owing to large excluded volume effects at the high protein concentrations used in the experiments, a phenomenon called ‘macromolecular crowding’, the thermodynamically effective concentrations are not the measured concentrations but instead are activities obtained by multiplying the concentration by the known activity coefficient¹⁷. In the concentration range of the experiments discussed here (from 0.16 g/ml to 0.4 g/ml), the corresponding activity coefficients are 4 to 200 (equation (1) in Online Methods); therefore, nonideality plays a very important part in determining the thermodynamic driving force for this reaction.

We found no overall correlation between delay time and solubility (Fig. 1a). Moreover, there is a spread of the delay times at a single solubility of up to nearly 7 orders of magnitude. There is a weak correlation between the delay time and total concentration, but the delay times nonetheless vary by up to 5 orders of magnitude at the same concentration (Fig. 1b). However, when we plotted the delay time versus the supersaturation (Fig. 1c), all of the data collapsed onto a single universal curve. This striking collapse to a universal curve in Figure 1c is theoretically predicted by the double-nucleation mechanism for sickle-fiber formation (Fig. 1d); thus, it represents yet another important experimental test of this model (description of the model and theoretical calculation of delay times in Online Methods).

DISCUSSION

In linear free-energy relations, $k \sim K^a$, where k is a rate coefficient, K is an equilibrium constant, and the exponent a ($0 < a < 1$) is a measure of the position of the transition state¹⁸, the top of the free-energy barrier along the reaction coordinate from reactants to products. The reaction coordinate for small-molecule reactions may be an interatomic distance or a dihedral angle, whereas for macromolecular reactions, order parameters are often used as reaction coordinates. In protein folding, for example, the fraction of inter-residue contacts that are present in the folded structure serves as an excellent reaction coordinate^{19,20}. A natural reaction coordinate for protein-fiber formation is the number of associated molecules. In this case, the exponent β in the power-law relation ($1/\text{delay time} \sim S^\beta$) can be much greater than 1 and is related to the number at the top of the free-energy barrier, i.e., the size of the critical nucleus. As the supersaturation (S) increases, the exponent β decreases (Fig. 1c), because nuclei become smaller as the thermodynamic driving force becomes larger, in a manner analogous to the Hammond postulate in chemical kinetics, in which a decreases as K increases.

The availability of a universal curve (Fig. 1c) has important applications in sickle-cell research because it allows determination of the delay time for any ratio of initial concentrations and solubilities. The importance of knowing the delay time is that the clinical severity of the disease is determined by the delay time relative to the transit time through the microcirculation (Fig. 2 and refs. 21–23). Therefore, increasing the delay time to allow more cells to escape the small vessels of the tissues before fibers form and cause vaso-occlusion is a goal for therapy. The universal curve shows that the sensitivity of the delay time varies considerably with supersaturation, from approximately the first power at the highest measured supersaturations to the tenth power at the lowest measured supersaturations. Consequently, the universal curve allows evaluation of delay-time increases measured at any supersaturation^{15,22,24,25}. This evaluation is extremely useful for comparing inhibitory effects and assessing therapeutic potential in drug screening assays performed at various supersaturations (Q. Li, E. Dunkelberger, B. Metaferia, T.C. and W.A.E., unpublished data). A notable example is the induction of fetal hemoglobin (HbF) synthesis as a therapy for sickle-cell disease, a subject being studied intensely in the hematology community²⁶, in which replacing 30% of the HbS with HbF produces a ten-fold increase in the delay time at the high supersaturations found in red cells¹⁵ and a 10,000-fold increase at low supersaturations, at which extensive measurements have been made^{12,25}.

Finally, the kinetics and thermodynamics of HbS fiber formation have served as a paradigm for biophysical studies of amyloid-fiber formation²⁷. Supersaturation has recently been suggested to be an important determinant of neurodegenerative disorders²⁸. It will therefore be interesting to consider the kinetics of these aggregation reactions and to determine whether universal supersaturation curves are observed and can be explained by theoretical models, as we have done here.

ONLINE METHODS

Activities and calculation of solubilities of hemoglobin mixtures

Solubilities are the total hemoglobin concentration of the supernatant (c_s) after sedimentation of the fibers. All of the solubilities reported in Supplementary Table 1 are the experimentally observed values, except for the mixtures containing hemoglobin F (HbF), hemoglobin A (HbA), and hemoglobin C (HbC). The activities were obtained by multiplying the measured total concentrations and solubilities by the activity coefficient, γ , which is independent of temperature and is given by:

$$\gamma = \exp(8Vc + 15V^2c^2 + 24.5V^3c^3 + 35.3V^4c^4 + 47.4V^5c^5 + 65.9V^6c^6) \quad (1)$$

where c is the total hemoglobin concentration (either the initial concentration or measured solubility) in g/ml, and $V = 0.79$ ml/g is the volume of the equivalent hard sphere^{17,24}. The importance of nonideality was not yet appreciated in the very early supersaturation studies, so activity coefficients were not used⁹. For the mixtures of HbS with HbF, HbA, or HbC, the solubilities were not measured for most of the experiments in which the delay times were measured. We therefore calculated the solubilities from theoretical expressions that have been shown to reproduce the measured solubilities well in five different laboratories²⁴ and therefore may be regarded as empirical expressions. In HbS-HbF mixtures, there is a binomial distribution of the three tetramers ($\alpha_2\beta^S\beta^S$, $\alpha_2\gamma\beta^S$ and $\alpha_2\gamma_2$ (ref. 31)), but only $\alpha_2\beta^S\beta^S$ enters the fiber. In the case of HbS-HbA mixtures, the homotetramer $\alpha_2\beta^A\beta^A$ does not enter the fiber, but the hybrid tetramer $\alpha_2\beta^A\beta^S$ does. The solubilities were calculated from polymerization theory and mass conservation from ref. 12.

$$[c_0(c_p - c_s)]X_F^2 + [2c_0(c_s - c_p)]X_F + \left[(c_p - c_0) \left(c_s - \frac{\gamma_s^0}{\gamma_s} c_s^0 \right) \right] = 0 \quad (2)$$

In this equation, c_0 is the initial total hemoglobin concentration; c_s is the measured solubility; c_s^0 is the solubility of pure HbS ($\alpha_2\beta^S\beta^S$); γ_s and γ_s^0 are the activity coefficients for the mixture and for pure HbS, respectively, calculated from equation (1); c_p (0.69 g/ml) is the concentration of hemoglobin in the fiber phase; and X_F is the mole fraction of HbF in the sample. The temperature dependence of the solubility of $\alpha_2\beta^S\beta^S$ is given by the empirical equation in ref. 24: c_s^0 (g/ml) = $0.319 - 0.00883 T + 0.000125 T^2$, with T in degrees centigrade. For mixtures of HbS with either HbA or HbC, the solubility was calculated from ref. 12:

$$\begin{aligned} & (B_1 + B_2 - (1 - X_{A,C})^2 B_3)(e_2 B_1 + B_2) - 2X_{A,C}(1 - X_{A,C})(B_1 + B_2)B_3 e_2 = 0 \\ & B_1 \equiv c_p(c_0 - c_s), \quad B_2 \equiv \frac{\gamma_s^0}{\gamma_s} c_s^0 (c_p - c_0), \quad B_3 \equiv c_0(c_p - c_s) \end{aligned} \quad (3)$$

where, $X_{A,C}$ is the mole fraction of HbA or HbC. The probability, e_2 , relative to the HbS tetramer, $\alpha_2\beta^S_2$, of the hybrid tetramers, $\alpha_2\beta^A\beta^S$ or $\alpha_2\beta^C\beta^S$, entering the fiber is 0.4 (ref. 24). Notably, for $e_2 = 0$, equation (3) becomes identical to equation (2). We have not included the HbS-F and HbA-S mixture data from Sunshine *et al.*¹², because of the ambiguity in determining accurate concentrations from contamination of the samples with an unknown percentage of carbon monoxide¹⁵.

Empirical universal curve

The function used for the least-squares fit to the observed delay times in Figure 1c is: $y = a \exp(-bx) + c$, where y is the $\log_{10}$ (delay time(s)); x is $\log_{10}$ (supersaturation); and $a = 11.2 \pm 0.5$, $b = 1.49 \pm 0.20$, and $c = -2.64 \pm 0.48$. For very low supersaturations, an additional term would be required to ensure that the delay time approaches infinity at a supersaturation of 1.0 ($x = 0$). The r.m.s. deviations of the experimental delay times (Fig. 1c) and the theoretical delay times (Fig. 1d) from the empirical universal curve are both 0.65.

Description of the double-nucleation model

This mechanism was initially invented to explain the exponential appearance of fibers that is responsible for the delay period as well as the high concentration dependence of the delay time. Simple nucleation-growth models, which have successfully been applied to actin and flagellin³², may explain the high initial concentration (c_0) dependence of the delay time ($1/c_0^{n/2}$) by invoking a large nucleus of size n (ref. 33). However, this model predicts a weak (t^2) time dependence that does not produce a lag phase, whereas breakage models produce autocatalysis, and therefore an exponential time dependence and a lag phase, but yield a very weak dependence on initial concentration ($c_0^{1/2}$ in the high breakage limit)³⁴. In the double-nucleation model^{14,24}, the first fiber in a given volume forms by homogeneous nucleation (i.e., a simple nucleation growth mechanism). Heterogeneous (secondary) nucleation then takes place on the surface of preexisting fibers. As fibers form, the increasing surface area provides additional surface for fibers to form by heterogeneous nucleation, thus resulting in an autocatalytic and therefore an initially exponential appearance of fibers. The large size of both homogeneous and heterogeneous nuclei and the strong dependence of activity coefficients on concentration together produce the high concentration dependence of the delay time. One of the striking predictions of the double-nucleation model is that the concentration dependence of the rate of homogeneous nucleation is approximately twice that of the delay time. This prediction has been confirmed by two different types of experiments. In the first³⁵, for solutions displaying an inverse ~25th-power concentration dependence of the delay time, a concentration dependence of the ~50th power for the homogeneous nucleation rate was determined from the average of the stochastically and exponentially distributed delay times in measurements on small volumes, in which the kinetics of formation of a single fiber molecule was monitored. Single-molecule kinetics could be observed because a single homogeneously nucleated fiber can trigger the formation of a

large number of heterogeneously nucleated fibers, which are detectable by light scattering^{36,37}. This scenario is conceptually analogous to patch-clamp experiments, in which the opening of a channel of a single molecule is detected from the flow of a large number of ions through the channel. The second experiment³⁸ was based on the finding that a single homogeneously nucleated fiber triggers the formation of a single large birefringent domain of fibers. Consequently, the homogeneous nucleation rate was obtained by simply counting the number of birefringent domains in an optical microscope image and dividing by the measured delay time. In these experiments, an ~80th-power concentration dependence for the rate of homogenous nucleation was found for solutions exhibiting an ~40th-power concentration dependence on the delay time³⁸.

Calculation of delay times from the double-nucleation model

Because fibers form from time zero, the delay time depends on the sensitivity of the method used to detect their appearance and is constructed from the intersection of the time axis with the maximum slope of the kinetic progress curve. The analytic counterpart calculated from the double-nucleation model, which differs insignificantly from the delay time for the present purposes, is the time at which the fiber formation reaction reaches one-tenth of its final value. Tenth times were computed as described in Bishop and Ferrone³⁹, from the equations for the double-nucleation model described in Ferrone *et al.*¹⁴, with two modifications. Theoretical calculation of delay times was performed for all delay-time measurements apart from those measured as a function of urea concentration or pH, for which a theory has not yet been developed. Nonideality was included for heterogeneous nucleation, and the final mass of polymerized hemoglobin was taken not as the initial concentration (c_0) minus the solubility (c_s) but as 2/3 of that quantity, because the reaction has been found to reach a metastable limit in kinetic experiments⁴⁰. Thus, the parameters used in all the calculations were taken from Ferrone *et al.*⁴¹ and Cao and Ferrone⁴². The calculation of the delay times for mixtures of HbS and HbF¹⁵ were made under the assumption that only the $\alpha_2\beta^S_2$ tetramer enters the fiber^{12,24}, and activity coefficients for the initial concentration of $\alpha_2\beta^S_2$ are determined by the total hemoglobin concentration (c_0), i.e., the sum of the concentrations of the polymerizing ($\alpha_2\beta^S_2$) and nonpolymerizing ($\alpha_2\gamma\beta^S$ and $\alpha_2\beta_2$) tetramers. The calculation of delay times for mixtures of HbS and HbA is more complicated because the hybrid tetramer ($\alpha_2\beta^A\beta^S$) copolymerizes, as described in Rotter *et al.*¹⁶. The delay times as a function of the fractional saturation with carbon monoxide were calculated by Yosmanovich *et al.*⁴³ with an extension of the Monod, Wyman, Changeux two-state allosteric model^{6,44,45}, which assumes that only the T quaternary structure can enter the fiber.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Relations among delay time and solubility, initial concentration and supersaturation as ratio of activities.

Symbol key for all panels is shown at top.

(a) Experimentally measured delay times as a function of solubility, as measured by the total hemoglobin concentration in the solution phase (the concentration in the supernatant after fiber sedimentation) multiplied by the activity coefficient calculated for that concentration. The solubility was varied by addition of carbon monoxide^{9,11}; pH changes⁹; addition of urea⁹; temperature changes^{10,14}; or partial replacement of hemoglobin S with hemoglobin F¹², hemoglobin A^{12,13} or hemoglobin C¹³.

(b) Experimentally measured delay times as a function of the total hemoglobin concentration, multiplied by the activity coefficient for that concentration.

(c) Experimentally measured delay times as a function of the supersaturation, i.e., the ratio of the activity before fiber formation to the activity of the solution phase at equilibrium. The continuous curve is a least-squares empirical fit to the data.

(d) Delay times as a function of supersaturation from the total hemoglobin concentration and composition of the solution for the points in c, as calculated with the double-nucleation model (Online Methods).

The curve through the data is the same as in c. Solution composition and conditions; total concentrations; solubilities and their corresponding activities; observed and calculated delay times; and references to the experiments for each point in the figure are given in Supplementary Table 1.

Figure 2. Kinetics of sickle-hemoglobin aggregation and connection to disease pathogenesis. **(a)** Schematic kinetic progress curve showing delay time before the detection of any fibers. **(b)** Cartoon of microcirculation showing the flow of a cell that contains no fibers, and therefore is easily deformable, through a muscled arteriole, a capillary and a venule. As shown by Mozzarelli *et al.*²⁹, the delay time for *in vivo* oxygen pressures is sufficiently long that the majority cells (>90%) escape the microcirculation before sickling has occurred. **(c)** Cartoon showing that if the delay time is sufficiently short or disappears because the intracellular HbS concentration is so high that fibers or seeds remain even after oxygenation in the lungs³⁰, then sickling can occur in the microcirculation, and the cell becomes much less flexible, thus introducing the possibility of vaso-occlusion by producing a 'log jam' of cells.