

Aggregation of Globular Proteins

LINDA R. DE YOUNG,^{*,†} ANTHONY L. FINK,[‡] AND KEN A. DILL[§]

Department of Pharmaceutical Research and Development, Genentech, 460 Point San Bruno Boulevard, South San Francisco, California 94080, Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143

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Introduction

Protein aggregation is of major importance in biomedicine, yet it is not well understood. Protein precipitates are the cause, or an associated symptom, of disease pathology in Down's syndrome, Alzheimer's disease,^{1,2} and cataracts.³ Cataracts appear to result from a shift in a liquid-liquid phase boundary (liquid phases consisting of concentrated and dilute solutions of γ -crystallins) from low temperature to ambient temperature, resulting in the light-scattering pathology of cataracts.^{3,4} Aggregation is a major impediment to the development of therapeutic protein products. For example, recombinant proteins often form precipitates, called inclusion bodies, in the host cell.⁵ Protein pharmaceuticals can aggregate, resulting in immunogenicity,⁶ decreased drug activity,⁷ and clogging of implantable delivery devices.⁸ Examples of protein aggregates include crystals, linear chains of hemoglobin-S,⁹ inclusion bodies, amorphous precipitates, fractal aggregates of BSA,¹⁰ small aggregates with specific structures such as the octamer formed by β -lactoglobulin,¹¹ and disulfide-cross-linked gels formed by ovalbumin.¹² Here, our focus is primarily on amorphous or noncrystalline precipitation, rather than crystallization.

We survey some of the physical principles that we believe underlie the aggregation and precipitation processes of globular proteins. First, proteins are colloids. Colloids are particles or molecules with diameters between 1 and 1000 nm that are dispersed in solution. We discuss theoretical and experimental models of colloidal aggregation that we believe can guide the understanding of protein aggregation, including DLVO and Smoluchowski theories and phase diagrams.

Linda De Young, born on November 29, 1958, in Fairbanks, AK, received her Ph.D. with Ken Dill at the University of California, San Francisco. She recently completed a postdoctoral fellowship in the laboratory of Professor Anthony Fink, where she studied protein aggregation. She is currently an Associate Scientist in the Department of Pharmaceutical Research and Development at Genentech, with research interests focused on the thermodynamics and kinetics of protein aggregation.

Anthony Fink, born on January 25, 1943, Hertford, England, received his B.Sc. in engineering chemistry and Ph.D. in chemistry (1968) from Queen's University. After postdoctoral work with Myron Bender (enzyme mechanisms) at Northwestern University, he began his professional career at the University of California, Santa Cruz, in the fall of 1969. He currently is a Professor in the Department of Chemistry, with major research interests in mechanisms of catalysis and inhibition of enzymes and mechanisms of protein folding, with emphasis on intermediate states.

Ken A. Dill, born on December 11, 1947, in Oklahoma City, OK, obtained his Ph.D. with Bruno H. Zimm at the University of California, San Diego, and did postdoctoral work with Paul J. Flory at Stanford from 1978 to 1980. After two years on the faculty at the University of Florida, he has been at the University of California, San Francisco, since 1983, where he is currently Professor in the Department of Pharmaceutical Chemistry. His research interests are in the statistical mechanics of biopolymers and proteins.

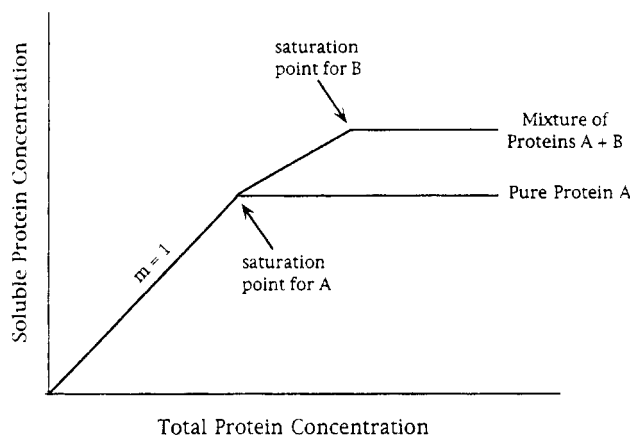


Figure 1. Solubility curves for a pure protein (A) and a mixture of two proteins (A and B) according to Gibb's phase rule. For a solution containing a single protein, the solubility is defined at the point where the slope breaks from 1 to 0. For a mixture of proteins with independent solubilities, a break in the curve will be observed at the solubility of each protein.

We then review evidence that some protein aggregation involves thermodynamic rather than kinetic control and discuss models for amorphous aggregation.

In the 1920s, proteins were shown to obey Gibb's phase rule,^{13,14} leading to widespread use of the phase rule for determining protein purity.^{13,15} The experimental test for protein purity is shown in Figure 1. The amount of dissolved protein increases linearly until the

* Author to whom correspondence should be addressed.

† Genentech.

‡ University of California, Santa Cruz.

§ University of California, San Francisco.

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solution becomes saturated (protein A in Figure 1) and the protein precipitates. Further addition of protein increases the volume of precipitated protein, but not the concentration of protein in solution or in the precipitated phase. For a mixture of two proteins with different solubilities, the curve will show two breaks, one at the solubility limit of each protein (see mixture of proteins A and B in Figure 1). Differences in solubility have been used extensively as a means of separating and purifying proteins. For example, salt fractionation ("salting out") was first used as a separation technique in the early 1850s.¹⁶ The effects of salts on protein solubility have been reviewed elsewhere.¹⁵⁻²¹

Colloid Theory and Protein Aggregation

Colloid chemistry has much to contribute to the understanding of protein aggregation. For example, DLVO (Deryagin-Landau-Verwey-Overbeek) theory²²⁻²⁴ provides a simple framework for describing the thermodynamics and kinetics of colloidal stability and aggregation. DLVO theory accounts for steric and electrostatic repulsions and van der Waals (vdW) attractions between particles in solution (Figure 2). vdW attractions act over a longer range for colloids than for small molecules mainly because colloids are larger and their vdW interactions are approximately additive over the atoms of each colloidal particle.²³

Figure 2 shows the main predictions of DLVO theory. The salt concentration determines whether particle association is governed by kinetic barriers or by equilibria. At high salt concentrations, the electrostatic charges on colloidal particles are shielded from each other and the vdW forces dominate, resulting in a strong attraction of two particles into a deep "primary" well of free energy (Figure 2, curve c). The rate of aggregation depends on the height of the barrier of electrostatic free energy. At high salt concentrations, DLVO theory predicts that aggregation will be rapid since there is no free energy barrier. This aggregation is often called "irreversible" because the primary free energy well is so deep ($>5-10$ kT) that redissolution by separation of the particles is exceedingly unfavorable. Since this situation involves no kinetic barrier, the aggregate is in equilibrium with the dissociated state. In contrast, at low salt concentrations, there is strong electrostatic repulsion between the colloid molecules, resulting in a large free barrier to aggregation (Figure 2, curve a). These solutions are referred to as kinetically stabilized²⁴ because, with barrier heights greater than 10-20 kT, the particles are very slow to aggregate. At intermediate salt concentrations, electrostatic and vdW forces become more balanced (Figure 2, curve b), and small amounts of added salt can substantially decrease the

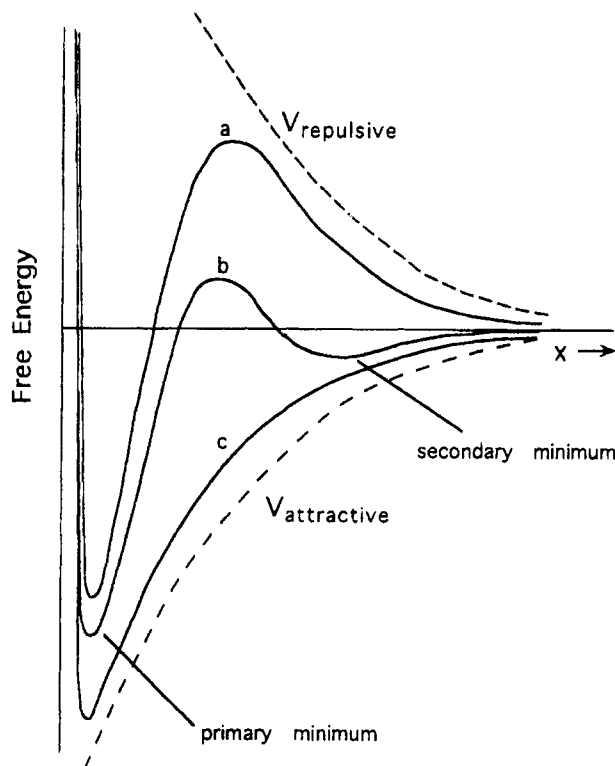


Figure 2. Summary of DLVO theory. Attractive van der Waals ($V_{\text{attractive}}$) and repulsive electrostatic forces ($V_{\text{repulsive}}$) are plotted as a function of intermolecular separation (x). The balance of these two forces at (a) low, (b) medium, and (c) high salt concentrations determines whether a colloidal solution will be stable or will aggregate in the primary or secondary minimum.

barrier height (decreasing the activation energy for association), causing large increases in the aggregation rate. For barrier heights that are not too large, the solution and aggregate can coexist at equilibrium.

A secondary minimum in free energy may appear at intermediate salt concentrations. For particle diameters greater than about 100 Å the secondary minimum is deep enough to result in another stable state.²² Aggregation in the secondary minimum is generally called "reversible" since the well depths are small (<5 kT) and there is a measurable equilibrium between aggregated and dissolved phases.

How well do DLVO and related theories describe the aggregation of globular proteins? Not much is yet known. We mention here a few recent efforts. DLVO theories of protein aggregation generally assume that the native protein is a hard sphere which repels other proteins through a uniform distribution of surface charge and attracts other proteins through van der Waals interactions. Haynes et al.²⁵ have added dipole and induced dipole terms to DLVO theory, and they find that the added contributions are small. DLVO theory treats explicitly only the interactions between the colloid particles, and it otherwise models the simple electrolyte and solvent as a continuum. Vlachy and Prausnitz²⁶ have gone further and modeled the simple salt as charged hard spheres using an integral equation method. They find that the simpler DLVO theory predicts well the osmotic pressures of bovine serum albumin solutions, but that it underestimates the extent to which added salt leads to attractions between

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proteins. Perturbation and random-phase approximation theories have also been used to treat the effects of simple electrolytes and polymers on the precipitation of hard-sphere native proteins.²⁷⁻²⁹ DLVO theory appears to be a useful starting point for modeling protein aggregation. To date, little effort has been devoted to understanding the aggregation of charged non-native proteins, the molecular details of intervening water structure, the temperature dependence of the hydrophobic interactions, or the hydrogen bonding or other specific interactions that appear to be important, for example, in protein crystallization.

Colloidal Aggregation Kinetics

The structures of colloidal aggregates depend on the kinetics of their formation. The kinetics of colloidal aggregation is often treated using Smoluchowski theory, which is based on a generalized diffusion equation containing a flux term, in this case for the flux of particles into an aggregate. According to the Smoluchowski theory of diffusion-limited aggregation, the kinetics of aggregation depends on the diffusion coefficient, particle number, and particle radius.^{22,30,31} Smoluchowski theory has been used successfully to model protein aggregation.³²⁻³⁴ In diffusion-limited aggregation (DLA) particles stick so strongly to the aggregate that the rate-limiting step is diffusion of the particles to the aggregate. Diffusion-limited aggregation processes can be divided into several subclasses.^{24,35,36} In the simplest case, referred to as particle-cluster aggregation, growth of the aggregate proceeds by addition of monomers (resembling free-radical polymerization of polymers, in which monomers are added one at a time). In cluster-cluster aggregation (DLCCA), growth occurs by the collision and association of clusters of any size (resembling condensation polymerization, in which two polymer chains bond together).³⁶ In another mechanism, referred to as reaction-limited aggregation (RLA), the association process rather than the diffusion process is rate limiting. An example is the case of the large free energy barriers at low salt concentration (Figure 2a). In this case association is weak, or requires activation, and a large number of particle collisions must occur before some pairs stick. If only the denatured conformations of a protein can aggregate, then denaturation of the protein would be an example of "activation".¹⁰

Whereas the distinction between "diffusion-limited" and "reaction-limited" involves the different mechanisms for how a monomer or cluster attaches to the aggregate, another important distinction is how *one monomer attachment influences the next*, and whether

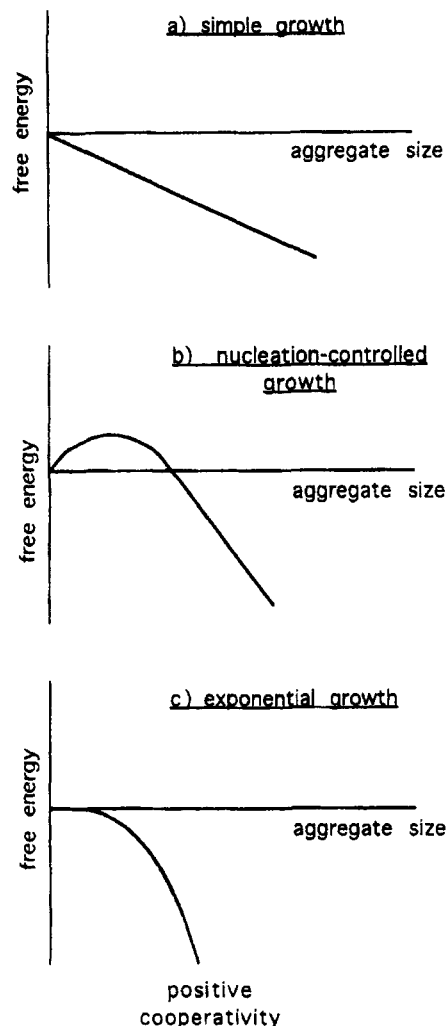


Figure 3. Free energy dependence on aggregate size for (a) simple growth, (b) nucleation-controlled growth, and (c) exponential growth. Positive cooperativity is an example of exponential growth. See text for details.

growth is (a) simple, (b) nucleation controlled,³⁷ or (c) exponential (Figure 3). Simple growth occurs when each monomer addition is favorable to about the same degree, independent of the size of the growing aggregate. Aggregation begins when a monomer forms a dimer, then adds another monomer to become a trimer, etc., ultimately to become a macroscopic phase (as in diffusion-limited particle-cluster aggregation). The free energy per added monomer is negative (favorable) and is about the same for each monomer (Figure 3a). In this case, larger aggregates are favored but smaller ones are also populated according to their Boltzmann factors, particularly at early times. In nucleation-controlled growth, for example protein crystallization,³⁷⁻³⁹ small aggregates are unfavorable and larger ones are favorable. The free energy of adding monomers is positive when the aggregate is small, and negative when the aggregate is larger; the peak of free energy identifies the size of the critical nucleus (Figure 3b). In this case, structures smaller than the critical nucleus will be unstable and not observed. In exponential growth, an example of positive cooperativity, the addition of each unit is more favorable than for the preceding unit (Figure 3). For example, if monomers

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have four sticky sites each, then a dimer will have six sticky sites, a trimer will have eight, etc., so larger aggregates are stickier than small ones (as in DLCCA). Exponential growth is an example of "positive cooperativity". Some aggregation processes can involve multiple growth mechanisms. For example, the Ca^{2+} -induced aggregation of α -casein occurs via exponential aggregation kinetics followed by Smoluchowski aggregation.³²

Different growth mechanisms lead to different aggregate structures and different time evolution histories of the distributions of particle sizes. Light scattering has been used to find solution conditions that lead to amorphous versus crystalline protein aggregates.^{37,39-41} In amorphous aggregation there is an approximately linear increase in particle size with protein concentration. In contrast, crystallization is a cooperative process in which the protein remains monomeric until nucleation, and then growth occurs upon supersaturation.^{37,39} Amorphous colloidal aggregates often have highly disordered and fractal structures. In fractal aggregates, the structure of the aggregate is invariant with a change in scale. Consequently, the mass of the aggregate, M , scales with its radius, R , as $M \propto R^d$, where d is the fractal dimension. Different types of aggregation mechanisms have different fractal dimensions.^{10,36,42} The fractal dimension can be measured by neutron scattering, by light scattering, and by electron microscopy for larger particles.³⁵ Theoretical studies have predicted fractal dimensions of 2.5,⁴³ 1.75,⁴⁴⁻⁴⁶ and 2.1,⁴⁷ for particle-cluster DLA, DLCCA, and RLA, respectively.^{10,36} The fractal dimensionality appears to depend on the type of association process, and not on the structural details of the material; for example, colloidal gold, silica, and polystyrene latex all have the same fractal dimensionality for DLA and RLA.⁴² Protein aggregates also show characteristic fractal dimensionalities. For the aggregation of native BSA (pH 5.6), $d = 1.75$, the same as for polystyrene in water, indicating that aggregation occurs through DLCCA.¹⁰ However, BSA denatured at pH 3 or 10.9 has a fractal dimensionality of 2.6.¹⁰

The structure and concentration of a protein in the solid state is as important as its structure in solution for determining protein solubility. For example, aldolase can form bipyramid or needle crystals which have different solubilities and heats of solution of opposite sign.¹⁹ With the exception of protein crystals, and specific aggregate structures such as those of hemoglobin-S, little is known about the conformations of aggregated proteins. Non-native aggregates are believed to be distinguished from denatured or partially unfolded aggregates (such as those induced by thermal or urea unfolding) by their reversibility.^{48,49} Przybycien

and Bailey^{48,50} found an increase in the amount of β -sheet in lyophilized precipitates produced by chaotropic (structure-breaking) salts. Raman, FTIR, and CD studies have generally found significant secondary structure in protein aggregates, where an increase in β -structure is often observed.⁵⁰⁻⁵⁴

Some Protein Aggregation Is Thermodynamically Reversible

According to DLVO theory, colloidal aggregation may be under either kinetic or thermodynamic control on a typical laboratory time scale. Protein aggregation may or may not involve kinetic barriers. Distinguishing kinetic from thermodynamic control can be challenging. For a protein at a pH near its isoelectric point (pI) and/or in high-salt solutions, DLVO theory would predict small kinetic barriers from electrostatic repulsions. Consistent with this prediction is the reversibility of ammonium sulfate precipitation of many proteins. For some protein solutions, the aggregation rate is found to increase with protein concentration, particularly in studies of protein refolding from the denatured state.⁵⁴⁻⁵⁷ Although this may indicate that aggregation is under kinetic control, a similar increase in aggregation rate with protein concentration has been observed for the reversible aggregation of apomyoglobin.⁵⁸

Thermal Phase Diagrams for Protein Aggregation

Aggregation and solubilization equilibria are described by phase diagrams. Despite their widespread use in polymer, colloid, and small molecule studies, few phase diagrams have been reported for proteins. Recently, however, liquid-solid phase diagrams have been reported for equilibrium protein crystallization⁵⁹⁻⁶³ and for equilibrium noncrystalline or amorphous precipitation.^{58,64} It has been suggested, for noncrystalline precipitates, that liquid-liquid phase diagrams more accurately represent the inhomogeneous "solid" than do liquid-solid phase diagrams.^{27,61} In addition to providing a map of the accessible phases versus the external variables, these diagrams are useful for testing microscopic models of protein association processes.^{3,58,61,63,65}

Before protein phase diagrams are described, it is helpful to review classical binary liquid-liquid phase

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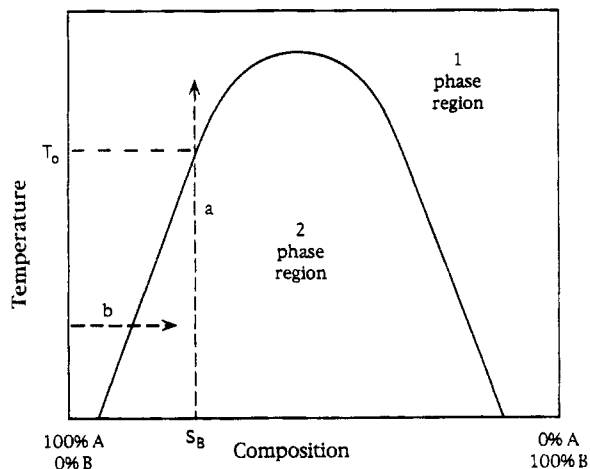


Figure 4. Classical liquid-liquid phase diagram for the mixing of A and B molecules driven by temperature.

equilibria of a mixture of two simple components, A and B, as functions of temperature and composition (see Figure 4). For simple systems governed by van der Waals forces, enthalpies of A-A attractions and B-B attractions are generally more favorable than for A-B attractions. Enthalpies dominate the free energy at low temperatures, and the system separates into A-rich and B-rich phases. As the temperature is increased at a fixed composition (see line a in Figure 4), a point is reached at which the favorable translational entropy of mixing prevails, and A and B will mix to become a single-phase solution. Alternatively, at fixed temperature, as the concentration of B in A increases (along line b), the mixing enthalpy becomes increasingly unfavorable while the mixing entropy becomes more favorable. The solubility limit of B in solvent A (S_B), at temperature T_0 , is defined as the concentration of B corresponding to the point on the phase boundary at which the second phase just begins to appear. Figure 4 shows the classical result that, as temperature increases, the solubility of B in A increases. In general, the phase boundary defines a balance, $T = \Delta H/\Delta S$, between enthalpic tendencies of A and B species to self-associate and their entropic tendencies to mix. A large two-phase region implies that enthalpic self-attraction is strong. In this way, phase diagrams provide information about molecular interactions.

Partial phase diagrams have been constructed for γ -crystallin and lysozyme liquid-liquid phase separation into dilute and concentrated protein phases^{3,66} and the crystallization of lysozyme and γ -crystallin.^{61,67,68} The phase diagrams for the crystallization of γ IIIb- and γ II-crystallin, from Berland et al.,⁶¹ are shown in Figure 5. They have developed a thermodynamic model for liquid-solid (crystal) phase separation which fits their experimental data for γ -crystallin (see Figure 5).⁶¹ Other proteins show very different phase behavior. For example, crystalline canavalin shows a decrease in solubility with an increase in temperature.⁶⁰ In addition, an increase in temperature often results in conformational changes in the protein and a decrease in protein solubility.^{69,70}

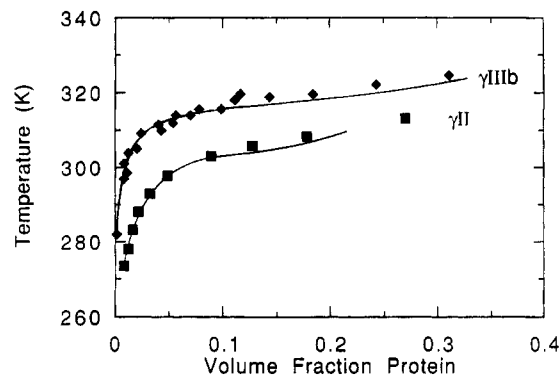


Figure 5. Liquid-solid phase diagram for γ II-crystallin and γ IIIb-crystallin crystallization. Replotted, with permission, from Berland et al.⁶¹ Curves are theoretical predictions of Berland et al.⁶¹

Denaturants Change Protein Solubility

Denaturants, such as urea and guanidine hydrochloride (GdnHCl), also alter solubilities of proteins. Urea and GdnHCl increase the solubilities of polar and nonpolar molecules in aqueous solutions^{20,71,72} and are therefore expected to increase the solubilities of both native and denatured proteins.^{71,73} The effect of urea and GdnHCl on polar group solubility has been attributed to hydrogen bonding.^{71,74} The effect on nonpolar group solubility has been attributed to alterations in the structure of water.⁷¹

How do denaturants affect the solubilities of proteins? Consider n molecules of a protein (P), in a soluble phase, in equilibrium with a phase of insoluble aggregate (A):



If urea and GdnHCl weaken the polar and hydrophobic attractions that drive proteins to aggregate, then increasing urea or GdnHCl concentration will shift the equilibrium in eq 1 to the left. Thus, protein solubilities should increase with denaturant concentration, just as the solubility of B in A increases with temperature in simple systems (Figure 4).

The solubilities of some proteins, however, behave very differently than the solubilities of amino acids in water. For example, Figure 6A shows the equilibrium solubility of apomyoglobin as a function of urea, and Figure 6B shows the corresponding equilibrium denaturation profile.⁵⁸ Two main conclusions are evident from this figure: (1) the solubility of apomyoglobin at low urea concentrations decreases with an increase in urea concentration, and has a *minimum* as a function of urea, rather than increasing monotonically as predicted from the simple model above; and (2) the concentration of urea at which the protein is least soluble is near the denaturation midpoint. For some proteins the concentration of protein in coexisting soluble and precipitated phases may depend on total protein concentration, requiring the determination of equilibrium constants rather than solubilities.⁶⁴ A solubility minimum has also been observed for rhodanese, a multidomain protein, in GdnHCl.⁷⁵

Two alternative models, described below, can account for the behavior shown in Figure 6: (1) aggregation

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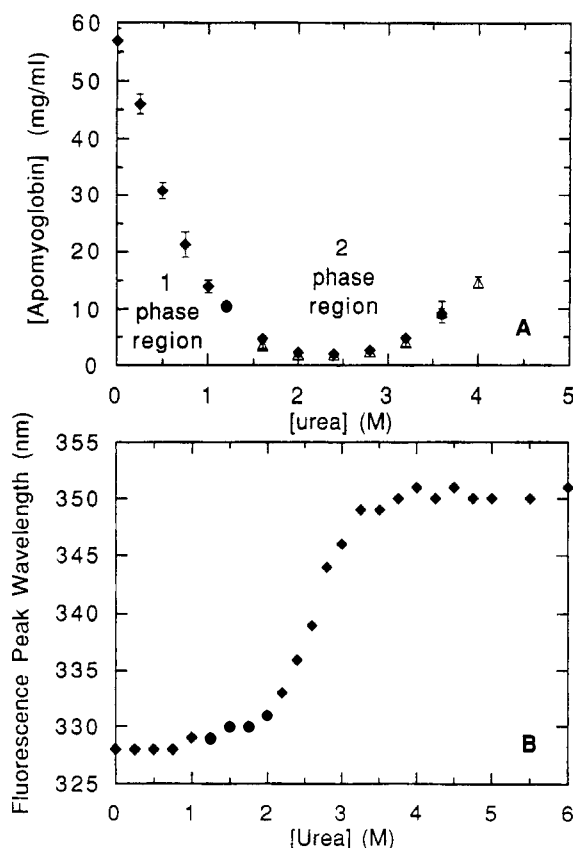
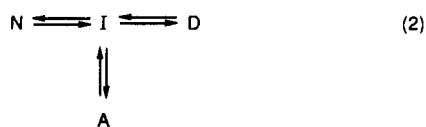


Figure 6. (A) Experimental phase diagram for apomyoglobin precipitation by urea:⁵⁸ (Δ) protein initially in 8 M urea and refolded to the final urea concentration; (\blacklozenge) protein initially in buffer and unfolded to the final urea concentration. (B) Denaturation of apomyoglobin by urea as monitored by the wavelength of the maximum fluorescence intensity. The fluorescence spectrum for apomyoglobin is dominated by the tryptophan (trp) residues which are buried in the protein in the native state. Trp in a hydrophobic environment (interior of the protein) has a maximum fluorescence intensity near 330 nm. In a polar environment (solvent exposed) the intensity maximum shifts to near 350 nm. The unfolding of the protein can therefore be followed by the change in trp environment as reported by the wavelength of maximum fluorescence intensity.

occurs via protein folding *intermediates*, or (2) aggregation occurs via *denatured states*.

Model 1: Aggregation via Intermediate States

Model 1 proposes that some state (I), involving conformations intermediate between those of the native (N) and highly unfolded (D) species, has the greatest tendency to aggregate. This equilibrium (in contrast to the commonly assumed two-state equilibrium) can be modeled by eq 2. The results in Figure 6 could be



explained if the intermediate state was most populated near the denaturation midpoint, and if the solubility of I was less than that of N or D. Such a model has been proposed for aggregation during the refolding of

proteins from the denatured state, and for the formation of inclusion bodies.^{76,77}

Model 2: Denatured-State Aggregation

In a second model, we assume that it is *denatured molecules* that aggregate, and that the denatured state is in folding equilibrium with the native state (eq 3).



This and other thermodynamic models have been explored by Arakawa and Timasheff.^{17,78}

The equilibrium process in model 2 has also been studied using statistical mechanical theory.^{79,80} The theory has two components. The N/D folding equilibrium is treated as a balance between hydrophobic interactions that favor chain collapse, and conformational entropies that oppose collapse.⁸¹⁻⁸³ The aggregation equilibrium is modeled as an entangled network of denatured chains. With increasing protein concentration, intermolecular hydrophobic protein-protein contacts become favored over intramolecular hydrophobic contacts due to the much greater conformational freedom of the chains in the aggregated state.⁷⁹ This theory predicts that the protein will have a minimum in solubility versus denaturant concentration.⁸⁰ This solubility minimum results from two denaturant effects. First, urea weakens the protein-protein attraction and thus increases protein solubility. Second, urea denatures the protein, driving the N/D equilibrium toward D, thus favoring aggregation. The balance of these two effects results in a minimum in protein solubility with denaturant.

What Are Folding Intermediates and How Do They Differ from Compact Denatured States?

To determine the relative merits of models 1 and 2, it is necessary to define more precisely the meaning of an "intermediate state". By thermodynamic convention, a stable state refers to a measurable population corresponding to a minimum in free energy (Figure 7). If the native state, N, and the denatured state, D, define stable states with identifiable populations, then a thermodynamically stable intermediate state, I, is a third population separated by free energy barriers from N and D.⁸⁴ Denatured states are complex conformational ensembles that can have radii dependent on solution conditions. The most well-studied denatured states are those in high concentrations of denaturants which are good solvents for proteins, resulting in relatively large hydrodynamic radii. In contrast, it is more difficult to observe the small population of

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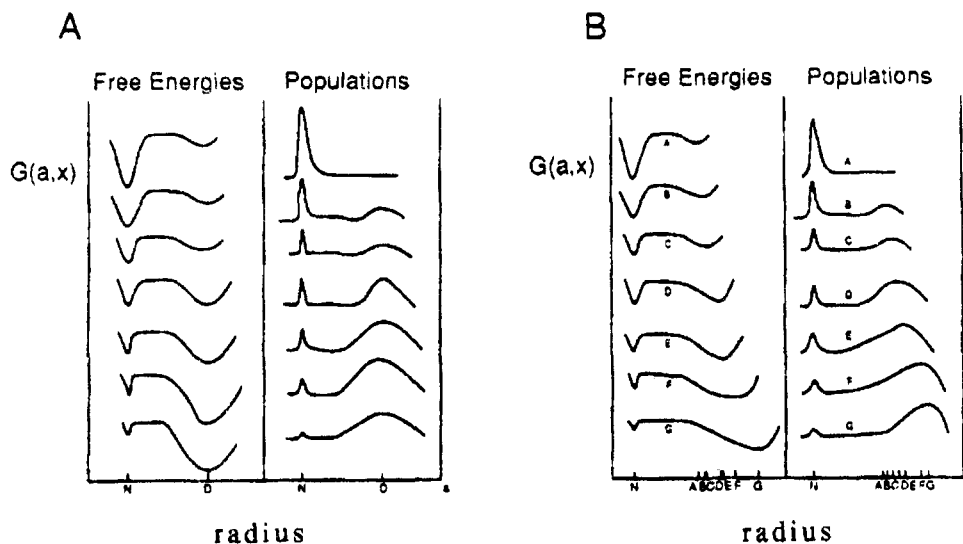


Figure 7. Free energy and population diagrams for protein denaturation as a function of protein radius. The deepest minimum in free energy (ΔG) corresponds to the stable state. Populations are given by the Boltzmann distribution law where the population is proportional to $\exp(-\Delta G/RT)$. The series of curves A–G represent increasing concentration of denaturant. (A) Two states, the native and the denatured state, are populated, and there is a single free energy barrier separating them. (B) Three stable states, N, I, and D, are in equilibrium, and two energy barriers are present, one separating N and I and the other between I and D. Reprinted with permission from Dill and Shortle.⁸⁴

compact denatured molecules present under solvent conditions favoring the native state. There is considerable evidence that compact denatured states can expand through a continuum of denatured conformations without a change of state.^{84–87} This is shown in Figure 7A, where the average size of the denatured state increases with denaturant concentration, without a new free energy minimum (a new state) appearing.⁸⁴

If there exists one stable non-native state (ensemble of conformations), whatever its average radius, we call it the denatured state. If there exists more than one stable non-native state, we refer to the most expanded one as denatured and more compact ones as intermediates. There is substantial evidence for the existence of compact non-native states;^{84,88–90} but these compact conformations may often be denatured states, by the above definition, rather than intermediates. Several experimental observations have been taken as evidence for folding intermediates, including noncoincident unfolding transitions, ANS (8-anilino-1-naphthalene-sulfonic acid) binding, low solubility, loss of tertiary structure with total or partial retention of secondary structure, and a hydrodynamic radius between those of the native and fully unfolded states,^{88,90} but these properties also characterize compact denatured states. To identify an intermediate state requires evidence of a third stable population in addition to the native and denatured states.

Only a few studies have shown that folding intermediates participate in the aggregation of single-domain

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globular proteins. An intermediate in the refolding of carbonic anhydrase from concentrated GdnHCl forms a dimer, which is proposed to be the nucleating species for further aggregation.⁹¹ Bovine growth hormone also forms an equilibrium intermediate which aggregates at moderate GdnHCl concentrations.⁹² When there is no evidence for the existence of a stable third “intermediate” state, we believe that the existence of solubility minima, such as shown in Figure 6, can be explained more simply as arising from aggregation of denatured molecules and coupling of the aggregation equilibrium to the folding equilibrium (model 2).

Summary

Proteins are both colloids and polymers. The association of colloidal particles, as described for example in DLVO theory and polymer phase behavior, has been described in a large body of experimental and theoretical data. These models promise to be a useful starting point for understanding protein aggregation. As more phase diagrams for equilibrium protein precipitation are reported, in crystalline and noncrystalline states, a better understanding of the balance of forces involved in protein aggregation will be developed. Greatly needed are light-scattering experiments on aggregation kinetics as well as Raman and FTIR measurements of the secondary and tertiary structures of protein in the aggregated state. Progress in understanding kinetic and equilibrium protein precipitation should lead to advances in protein crystallization, biotechnological processes, protein formulation development, and drug therapies.

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