Aggregation and Denaturation of Apomyoglobin in Aqueous Urea Solutions†

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ABSTRACT: The effects of urea on apomyoglobin solubility have been investigated. Apomyoglobin precipitation was found to be a thermodynamically reversible process independent of the pathway of aggregation. A liquid–solid phase diagram was constructed for the precipitation of apomyoglobin as a function of urea and protein concentration. Apomyoglobin solubility decreases by an order of magnitude between 0 and 1.5 M urea, reaching a minimum near 2.4 M urea and increasing at higher urea concentrations (the denaturation midpoint is at approximately 2.6 M urea). This decrease in protein solubility is opposite to that expected based on amino acid solubilities, since both polar and nonpolar molecules become more soluble with increasing urea concentration. Solubility minima for proteins have been rationalized in terms of folding intermediates. However, our structural studies show no evidence for folding intermediates in apomyoglobin under the experimental conditions, apart from small predenaturation changes. Our data are consistent with an alternative hypothesis, namely, that the primary aggregating species are denatured protein molecules, rather than intermediate states. Consistent with recent thermodynamic and statistical mechanical models, the solubility minimum may be described as the result of two competing effects of urea: (1) urea denatures the protein, and (2) urea makes the solvent more favorable for the native and any denatured state. At low urea concentration, solubility decreases with increasing urea concentration due to the domination of the solubility behavior by the increase in the population of aggregation–competent (denatured) protein molecules. However, at high urea concentration, the increasingly favorable nature of the solvent dominates, resulting in increasing solubility with urea concentration. The phase diagram provides guidance for the best experimental conditions (pathway) to use to avoid aggregation during the refolding of denaturant-unfolded protein.

Protein precipitation is a major biomedical problem. These precipitates are the cause, or an associated symptom, of several disease pathologies: Down's syndrome, Alzheimer's disease, malignant myeloma (Massry & Glassock, 1983; Benditt et al., 1980), and crush injury (Better, 1990) are examples. Protein aggregation is also an important biotechnological problem. During the production of recombinant proteins, precipitates of protein, called inclusion bodies, often build up within the host cell (Marston, 1986). The characterization, purification, large-scale production, stability, and delivery of protein drugs are also often complicated by aggregation processes. Protein drugs currently on the market continue to have aggregation problems: for example, implantable and portable delivery systems for insulin are commonly clogged by insulin precipitates (Lougheed et al., 1980). Despite the importance of protein precipitation, little is currently understood about the physical forces leading to aggregation, the secondary or tertiary structures of the protein in the aggregate, or the organization of protein monomers in the aggregated state.

The fundamental description of a reversible aggregation process is a phase diagram. Widely used in small-molecule, polymer, and colloid studies, few phase diagrams have been reported for proteins. In addition to providing a map of the accessible phases versus the external variables, these diagrams serve as valuable tests for thermodynamic and microscopic models of protein association processes (Dervichian, 1954; Thomson et al., 1987; Berland et al., 1992). Phase diagrams for protein precipitation can be constructed by measuring protein solubility as the driving force for aggregation is varied. The driving force for aggregation can be altered by adjusting the concentration of urea. Urea has been shown to increase the solubility of both polar compounds (e.g., uric acid, acetyltetraglycine ethyl ester, tyrosine) and nonpolar compounds (e.g., *n*-alkanes, naphthalene) (Nandi & Robinson, 1984; Nozaki & Tanford, 1963; Robinson & Jencks, 1965; Roseman & Jencks, 1975; Wetlaufer et al., 1964). Therefore, both native and denatured proteins would be expected to become more soluble in urea, based on the solvation properties of amino acids alone.

Even considering just globular proteins, there are many different types of phase diagrams. For example, liquid–liquid phase separations and protein crystallization have been described in phase diagrams. Crystallization phase diagrams have been determined for lysozyme as functions of pH, temperature, and salt type and concentration (Ries-Kautt & Ducruix, 1989; Ataka & Asai, 1988; Howard et al., 1988) and for canavalin and γ-crystallin as a function of temperature. The present work studies a different physical process, namely, the liquid–solid phase equilibria for apomyoglobin precipitation as a function of urea concentration. In this case, the solid phase will not have the regular order of a crystalline phase. We believe the precipitated solid phase may involve protein conformations that are considerably more disordered, perhaps highly entangled, and may vary from one molecule to the next in the precipitate. Moreover, whereas in crystals the native protein conformation will be relatively independent of external conditions, the conformations of precipitated apomyoglobin are likely to be highly dependent on external conditions.

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Apomyoglobin is a small globular protein (MW 17 000) which folds reversibly (Griko et al., 1988) and contains no cysteine residues. Myoglobin, the holoprotein, is about 70% helical (Kendrew et al., 1960), containing eight helices designated A through H. The heme pocket is located primarily between the E and F helices. The horse apomyoglobin used in these studies has two tryptophan residues, both located in the A helix. Apomyoglobulin contains approximately 60% α-helix (Harrison & Blout, 1965). The structure of the apoprotein is believed to be similar to the holoprotein: it is compact (Griko et al., 1988; Crumptom & Polson, 1965) and has a hydrophobic core (Griko et al., 1988), and its amide protons are protected from exchange in a manner similar to that in the holoprotein (Hughson et al., 1990).

MATERIALS AND METHODS

Horse heart myoglobin was purchased from Sigma, ultrapure urea from Boehringer Mannheim, and 8-anilino-1-naphthalenesulfonic acid (ANS)[1] from Eastman. Apomyoglobin was prepared using a modified Teale cold-butanone extraction (Teale, 1959; Rothgeb & Gurd, 1978) with dialysis of the aqueous apomyoglobin phase against 50 mg/L bicarbonate, 10^{-4} M EDTA, and then 10 mM Tris, pH 8.3. To remove residual heme and myoglobin, the apomyoglobin solution was purified by ion-exchange on a DEAE-cellulose column at pH 8.3 in 10 mM Tris (Wright & Boxer, 1981). The solution was then dialyzed against water, lyophilized, and stored at −20 °C. The lyophilized apomyoglobin was not completely soluble in buffer or at low urea concentrations. Solids were separated from these apomyoglobin solutions by brief centrifugation. All experiments were done at pH 6.7 in 50 mM potassium phosphate buffer. The p*I* of horse heart apomyoglobin was determined to be 7.3 by Phast gel electrophoresis (Pharmacia). On dissolution in buffer, a saturated solution of apomyoglobin had a pH of approximately 7.3; on adjustment of the pH to 6.7, protein precipitation was observed. Except in very dilute salt solutions, the pH at which the minimum protein solubility is observed is generally different than the true isoelectric point of the protein (Cohn & Edsall, 1943). Protein concentrations were determined in 8 M urea at pH 6.7 using the UV absorbance at 280 nm. Using an extinction coefficient in buffer (pH 7.2) of 15 700 cm^{-1} M^{-1} (Wilf & Minton, 1981), an extinction coefficient in 8 M urea (pH 6.7) of 14 800 cm^{-1} M^{-1} was determined by measuring the absorbance at 280 nm of equimolar protein solutions in 8 M urea and in buffer. Fresh urea solutions were prepared for each experiment. Urea concentrations were determined by refractive index measurements (Pace et al., 1989).

Urea Denaturation of Apomyoglobin. Several techniques were used to monitor the denaturation of apomyoglobin by urea at 25 °C. Horse heart apomyoglobin contains two tryptophan and two tyrosine residues. The fluorescence intensity at 330 nm and the wavelength of maximum fluorescence intensity of these residues were monitored using an excitation wavelength of 280 nm. Far-UV circular dichroism (CD) spectra were collected on an AVIV Model 60DS spectrometer at 25 °C using a 1-mm path-length cell. Spectra were background-corrected, and molar ellipticities at 222 nm were calculated using a molecular weight of 17 000 for apomyoglobin. For fluorescence and far-UV CD studies, aqueous urea solutions containing 0.2–0.4 mg/mL apomyoglobin were filtered through a 0.45-μm filter and equilibrated for at least 3 h prior to measurement. The unfolding of apomyoglobin in urea is relatively rapid; the fluorescence signal reached its equilibrium value within the sample preparation time. The binding of ANS to apomyoglobin was also studied at 25 °C as a function of urea concentration. ANS binds specifically to the heme pocket of apomyoglobin with an increase in quantum yield of approximately 200× (Stryer, 1965). Fluorescence excitation was at 390 nm, and emission was monitored at 460 nm. The ANS concentration was 20 μM, and the apomyoglobin concentration was 19 μM.

Procedures for Aggregation Experiments. Saturated solutions of apomyoglobin were prepared by precipitation of the protein from the soluble state by the addition of urea. Solutions of 50 mM potassium phosphate (buffer), 8 M urea in 50 mM potassium phosphate (8 M urea), and apomyoglobin in either buffer or 8 M urea were prepared. All solutions were adjusted to pH 6.7 and filtered through a 0.45-μm filter. The stock protein concentration varied from 35 to 54 mg/mL in buffer and from 40 to 80 mg/mL in 8 M urea. The experiments were initiated by placing aliquots of urea, buffer, and protein into silanized Eppendorf tubes to obtain final urea concentrations ranging from 0.25 to 7 M urea. The solutions were vortexed for approximately 0.5 s and incubated in a water bath at 25 °C. Samples were not stirred as this increased the amount of protein precipitated probably due to shear forces. After incubation, the samples were centrifuged at 14K rpm for 3 s to pellet the precipitate. Longer centrifugation times resulted in significant heating of the sample. In samples where clear supernatants were obtained, centrifugation was shown not to alter experimental results. An aliquot of the soluble phase was then diluted into 8 M urea for protein concentration determination by the UV absorbance at 280 nm. The precipitate (concentrated phase) was not characterized in these experiments; only the dilute arm of the phase diagram was determined. The solubility of apomyoglobin in buffer was studied by two different procedures. Supersaturated apomyoglobin solutions were prepared by incremental addition of solid protein, at a constant pH of 6.7, with mixing by gentle rotation. Protein precipitated from these samples during the subsequent 3-day equilibration at 25 °C. In the second method, a saturated solution of apomyoglobin was prepared at pH 7.3 and the pH then dropped to 6.7, where the protein is less soluble.

Aggregation Kinetics. To study the dependence of the aggregation kinetics on protein and urea concentrations, aggregation was monitored by the turbidity at 340 nm. No absorbing species are present at this wavelength, so the light beam is attenuated by scattering from the aggregating protein particles. Samples were prepared as above and transferred to a cuvette, and the OD at 340 mm was monitored at 25 °C.

Reversibility of Aggregation. Precipitates of apomyoglobin formed in urea do not dissolve on dilution with buffer (pathway A in Figure 1). A particular dissolution pathway may be kinetically unavailable on the time scale of the experiment, but other pathways of equilibrium steps may be taken to ascertain thermodynamic reversibility. Here, the precipitates were dissolved in 8 M urea for 5 h. The samples were then diluted 1 to 20 with buffer and allowed to refold for approximately 16 h at 25 °C (pathway B in Figure 1). On buffer dilution of protein solutions in 6 M urea, we found refolding to occur within 30 s. Refolding of protein taken from the supernatant in equilibrium with the precipitate was also investigated by diluting an aliquot of the supernatant 1 to 20 with buffer. Using the fluorescence peak wavelength, we monitored the tertiary structure resulting from refolding

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; far-UV CD, far-ultraviolet circular dichroism; Gdn·HCl, guanidine hydrochloride.

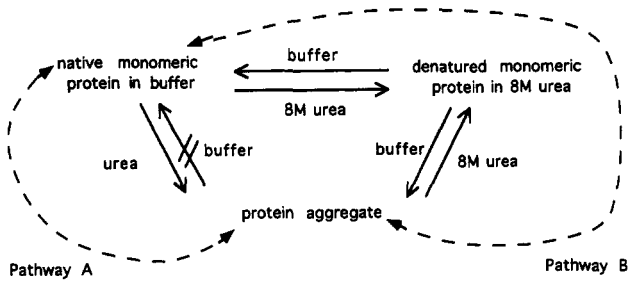

FIGURE 1: Aggregation, dissolution, and refolding pathways used in apomyoglobin aggregation. See text for explanation of pathways A and B.

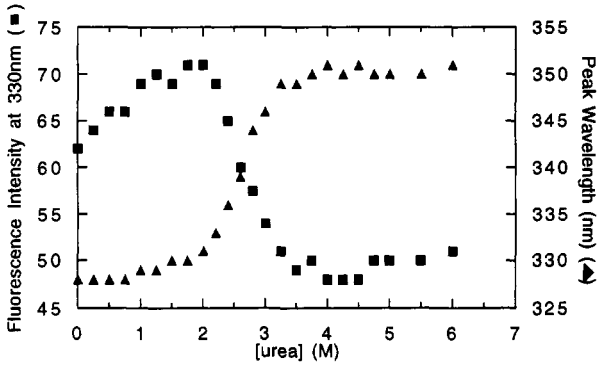

FIGURE 2: Urea denaturation of apomyoglobin at 25 °C. Fluorescence peak wavelength (▲) and fluorescence intensity at 330 nm (■). Excitation wavelength was 280 nm, and apomyoglobin concentration was 0.3 mg/mL.

of protein in supernatants and precipitates which were formed by incubation for 2 days at 25 °C in 0.5–4 M urea. The protein secondary structure formed on refolding of the protein from aggregates and supernatants formed in 1–3.6 M urea was also monitored by far-UV CD. For fluorescence studies, the final urea concentration during refolding was between 0 and 0.6 M, and the final protein concentration ranged from 0.1 to 0.7 mg/mL. For CD studies, the final urea concentration was between 0 and 0.3 M and the protein concentration ranged from 0.08 to 0.6 mg/mL. There was no correlation between the recovery of native protein and the final urea and protein concentrations in these ranges.

Path Independence of the Phase Boundary. To establish whether the position of the liquid–solid phase boundary was independent of the experimental path taken, aggregation experiments were initiated with the protein both in buffer and in 8 M urea. In another set of experiments, the phase boundary was approached from different directions in a stepwise manner. For example, at 2.6 M urea, the protein concentration was increased from 2 to 4 to 6 to 8 to 10 mg/mL, with a 1-day equilibration at 25 °C at each protein concentration, and 3 days of equilibration after the final protein concentration increment. To maintain the urea concentration, urea was added at each protein increment. Two other pathways were taken: incrementing the urea concentration at a constant protein concentration and incrementing both the protein and urea concentrations simultaneously. Results were independent of the order of protein and urea addition to the solution.

RESULTS AND DISCUSSION

Urea Denaturation of Apomyoglobin. The denaturation of apomyoglobin by urea is shown in Figures 2 and 3. With increasing urea concentration, the wavelength of maximum fluorescence intensity increases from 328 to 350 nm (see Figure 2). This shift to a higher wavelength indicates that the tryptophan residues are exposed to a more polar environment on denaturation. Figure 2 also shows the fluorescence intensity at 330 nm as a function of urea concentration. The fluorescence intensity increases between 0 and approximately 1.5 M urea and then decreases above 1.5 M urea. The decrease in fluorescence intensity above 1.5 M coincides with the denaturation transition seen in Figure 2 by the peak fluorescence wavelength. A predenaturational transition occurs between 0 and 1.5 M urea as evidenced by fluorescence intensity measurements; no significant change in the fluorescence peak wavelength is observed in this range of urea concentration. For the fluorescence intensity at 330 nm, the evaluation of the midpoint of the denaturation transition by extrapolation of the base lines is complicated due to the presence of the predenaturation transition. Using a peak wavelength in the predenaturation region that is independent of urea concentration, we find a transition midpoint (C_m) of 2.6 M urea for the data in Figure 2. In three runs, the C_m, as monitored by the peak wavelength, was 2.8 ± 0.2 M urea. By inspection, the fluorescence intensity at 330 nm has a similar denaturation midpoint.

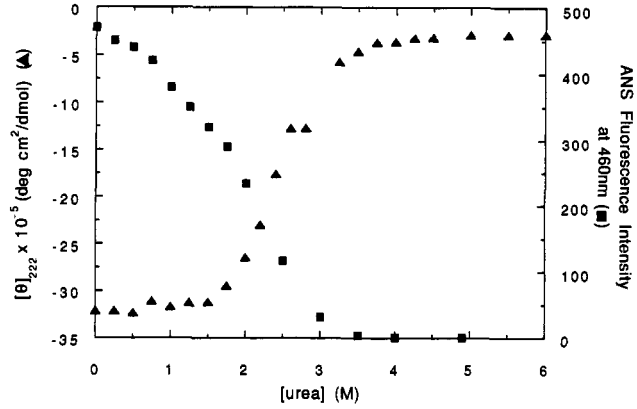

FIGURE 3: Denaturation of apomyoglobin by urea as monitored by far-UV CD and ANS binding. Far-UV CD molar ellipticity at 222 nm (▲). Apomyoglobin concentration was 0.3 mg/mL. ANS fluorescence intensity at 460 nm (■). Excitation was at 390 nm. ANS and protein concentrations were 20 and 19 μM, respectively.

The protein denaturation by urea was also monitored by far-UV CD at 25 °C. No significant changes in the molar ellipticity at 222 nm were observed in the predenaturation region as shown in Figure 3. Using extrapolated base lines, the midpoints of the CD transition in two experiments were 2.4 and 2.5 M urea. The difference in the C_m values obtained by CD and fluorescence is likely due to uncertainties in baseline extrapolation since the curves shown are almost superimposable in the denaturation region.

We also studied the binding of ANS to apomyoglobin. Previous studies have shown the binding of ANS to apomyoglobin to be affected by low concentrations of Gdn·HCl (Colonna et al., 1982; Balestrieri et al., 1976). To determine whether urea had a similar effect, the binding of ANS to apomyoglobin at various urea concentrations was monitored by the ANS fluorescence at 460 nm, as shown in Figure 3. These data were collected after a 1.5-h equilibration of ANS and apomyoglobin at 22 °C. The ANS fluorescence intensity in buffer and urea solutions was negligible in comparison to that in the presence of apomyoglobin. ANS fluorescence intensities at 1.5 and 16 h were similar; three runs gave similar trends in ANS fluorescence with urea concentration. As shown in Figure 3, in the presence of apomyoglobin the ANS fluorescence intensity decreases 32% between 0 and 1.5 M

urea. This decrease in ANS binding could result either from a conformational change in the apomyoglobin heme pocket, which alters ANS binding, or from a decreased protein/solution partition coefficient due to increased affinity of ANS for the aqueous urea solvent.

Irace, Colonna, and co-workers have investigated the intrinsic fluorescence and ANS binding properties of apomyoglobulin from various species as a function of guanidine hydrochloride (Gdn·HCl) concentration (Colonna et al., 1982; Irace et al., 1981; Balestrieri et al., 1976). They conclude that two conformational transitions occur on titration with Gdn·HCl. Between approximately 0 and 1 M Gdn·HCl, the conformation of the heme pocket is disrupted, and between 1 and 2 M Gdn·HCl, further conformational changes occur resulting in fluorescence signals characteristic of the denatured state. They believe the increase in fluorescence intensity at low Gdn·HCl concentrations is due to disruption of the heme pocket which moves Lys-79 away from Trp-7, increasing the Trp-7 quantum yield. Their ANS binding studies support this hypothesis as the ability to bind ANS is lost by the time the Gdn·HCl concentration reaches 1 M (Colonna et al., 1982; Balestrieri et al., 1976). Our fluorescence results using urea are similar; the urea concentrations required to bring about similar spectral changes are about twice the required Gdn·HCl concentrations. These results and the data in Figures 2 and 3 suggest that the urea-induced predenaturation conformational change observed here by intrinsic fluorescence intensity and ANS binding is small and local since no spectroscopic changes are observed by far-UV CD or fluorescence peak wavelength.

Kinetics of Aggregation Monitored by Turbidity. The formation of protein aggregates was monitored by the turbidity at 340 nm to study how the kinetics of aggregation depend on protein and urea concentration. The rate of formation of scattering particles increases dramatically with protein concentration (1 M urea, Figure 4A, and 2.4 M urea, Figure 4B); similar behavior has been observed for other proteins (Przybycien & Bailey, 1989; Parker & Dalgleish, 1977; Zettlmeissl et al., 1979). The turbidity curves are not highly reproducible, as shown by the three curves for 6 mg/mL at 2.4 M urea. This is probably due to differences in sample mixing (Przybycien & Bailey, 1989; Walton, 1967). An increase in urea concentration from 1 to 2.4 M also increases the rate of formation of aggregates. This would be puzzling if the only effect of urea was to make the solvation of polar and nonpolar groups more favorable. However, urea also increases the population of denatured protein molecules. The data in Figure 4 are consistent with a model in which denatured protein is the aggregating species; the rate of aggregation is related to the concentration of denatured protein. The population of denatured molecules can be increased either by increasing the total protein concentration or by increasing the urea concentration. The presence of a time lag before significant scattering occurs, as monitored by turbidity, has been interpreted as indicating that aggregation occurs by a nucleation and growth phenomenon. However, the time lag and curvature occurring at low turbidities could also result from the particle size dependence of scattered light. Light scattering is proportional to the square of the particle volume. Therefore, if the particle size increases linearly with time, curvature similar to that seen in Figure 4 would be expected.

Dependence of Apomyoglobin Solubility on Total Protein Concentration. As solid protein is added to a solution, the concentration of soluble protein should equal the total protein concentration until the solubility limit of the protein is reached.

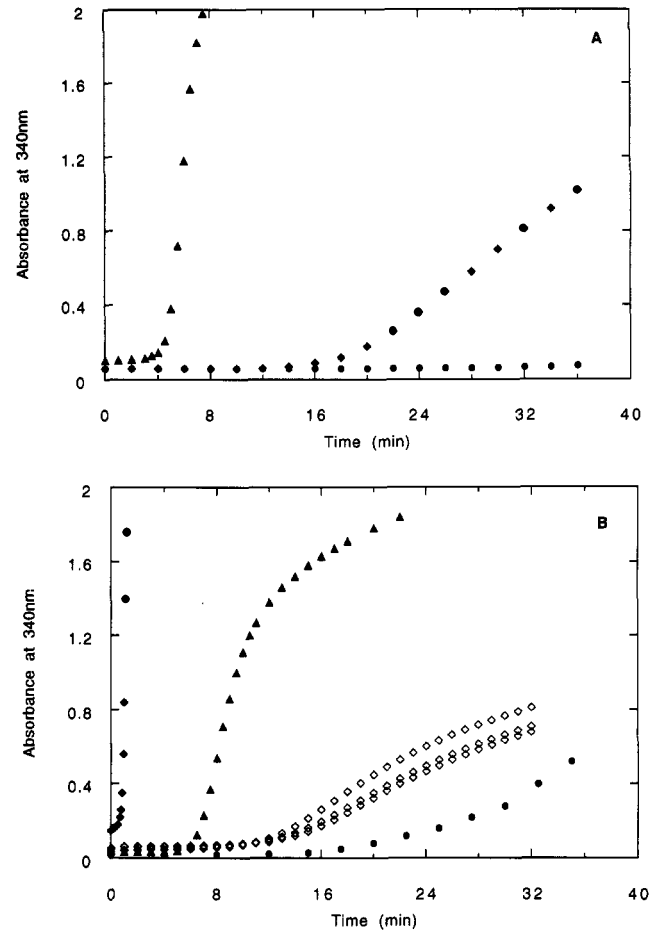

FIGURE 4: Effect of protein and urea concentration on the rate of formation of scattering particles as monitored by the absorbance at 340 nm at 25 °C. Panel A: aggregation at 1 M urea and apomyoglobin concentrations of (●) 17, (♦) 21, and (▲) 25 mg/mL. Panel B: aggregation at 2.4 M urea and apomyoglobin concentrations of (●) 4, (◊) 6, (▲) 8, and 12 (♦) mg/mL. Three separate experiments were done at 6 mg/mL to show the experimental error in this measurement.

From classical phase behavior, it is expected that above the solubility limit, at fixed urea concentration, the protein concentration in the dilute phase should then remain independent of the total protein concentration (Falconer & Taylor, 1946; Tombs, 1957; Arakawa & Timasheff, 1985). In a plot of soluble protein versus total protein concentration, a linear increase with a slope of 1 would be observed below the solubility limit, and at the solubility limit, the curve would break to a horizontal line (see Figure 5A). The protein concentration dependence of apomyoglobin solubility was studied to determine the position of the dilute arm of the liquid–solid phase boundary (the liquidus) which separates the single-phase soluble-protein region from the two-phase region and to study the solubility behavior at protein concentrations in the two-phase region. The concentration of protein in the precipitate was not characterized in these experiments, and therefore only the left half of the phase diagram is shown.

The soluble protein concentration was determined as a function of the total protein concentration at each urea concentration used to construct the phase diagram. Representative behavior is shown in Figure 5 for apomyoglobin in 1, 1.6, 2.4, and 3.6 M urea. Samples were equilibrated for 2 days at 25 °C, and the stock protein solution was prepared in buffer. [The concentration dependence was also studied for protein initially in 8 M urea. These data were sufficient to determine the point of saturation (data not shown), but

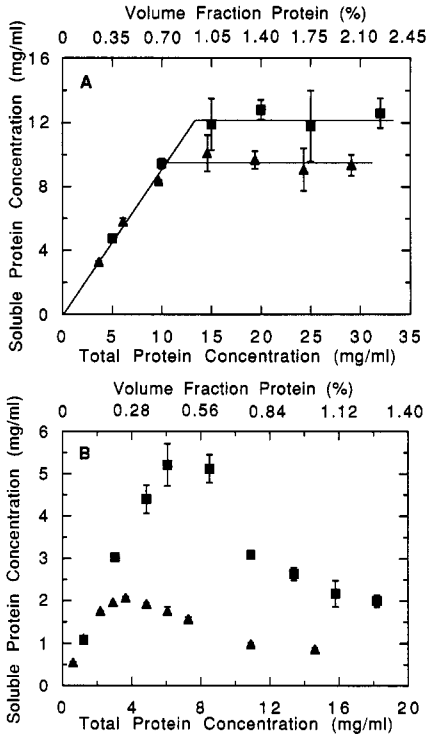

FIGURE 5: Dependence of the soluble protein concentration on the total protein concentration at various urea concentrations. Samples equilibrated for 2 days at 25 °C. Panel A: 1 (■) and 3.6 M (▲) urea. Panel B: 1.6 (■) and 2.4 M (▲) urea. Error bars are standard deviations within a single run. Protein volume fractions calculated using a specific volume for apomyoglobin equal to that of lysozyme, 0.70 mL/g (Sophianoopoulas et al., 1962). Lines are drawn in (A) to illustrate classical solubility behavior of a pure protein. The protein solubility is equal to the protein concentration at the break in the curve.

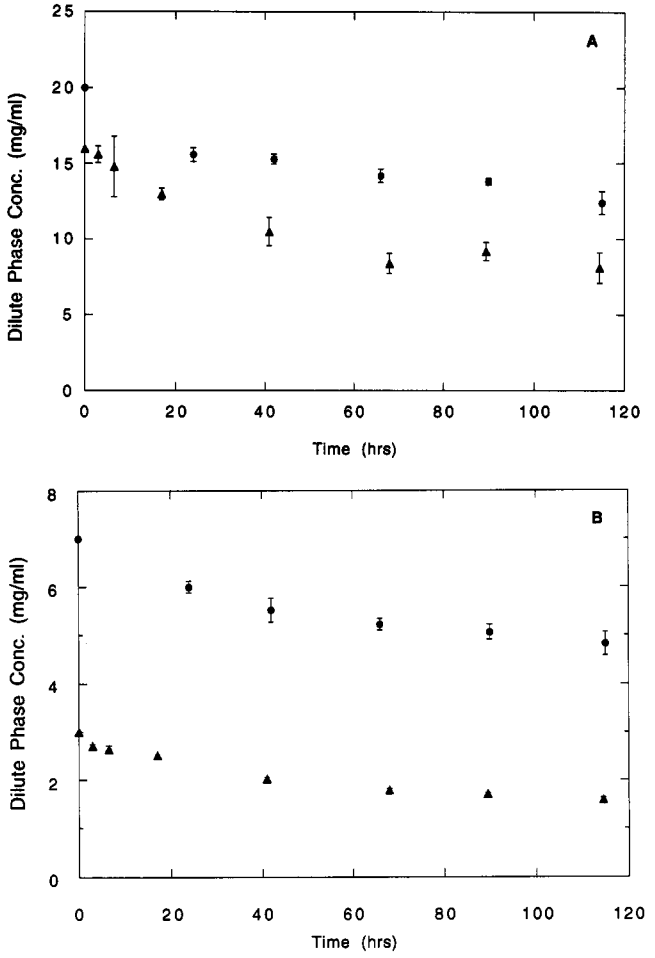

FIGURE 6: Time dependence of the soluble protein concentration in a precipitating protein solution. Panel A: (●) 1 M urea and 20 mg/mL protein; (▲) 3.6 M urea and 16 mg/mL protein. Panel B: (●) 1.6 M urea and 7 mg/mL protein; (▲) 2.4 M urea and 3 mg/mL protein. Error bars are standard deviations within a single run.

trends at higher protein concentration were not studied.] At 1 and 3.6 M urea, concentrations in the pre- and postdenaturation regions, respectively, the classic behavior described above is observed, with the soluble protein concentration being independent of the total protein concentration in the two-phase region (see Figure 5A). In contrast, at urea concentrations in the denaturation transition region (1.6 and 2.4 M urea), the soluble protein concentration decreases with increasing protein concentration in the two-phase region (shown in Figure 5B). Two scales are given for the abscissa: the lower scale in milligrams per milliliter units and the upper scale in volume fraction units. The volume fraction of protein is the volume of protein in the solution divided by the total volume of the solution. According to Flory–Huggins theory, the appropriate concentration variable for chemical potentials in polymeric solutions is volume fraction (Flory, 1953). The volume of protein in the dilute phase was calculated from the mass of protein using a specific volume for apomyoglobin equivalent to that for lysozyme of 0.70 mL/g (Sophianoocpoulos et al., 1962). Standard deviations represent errors within a single experiment. The data were reproducible. The slope in the one-phase region (below the solubility limit) is less than 1, probably due to protein surface adsorption. The origin of the solubility dependence on protein concentration observed at 1.6 and 2.4 M urea in Figure 5B is not clear. It may result from a thermodynamic or kinetic coupling of the denaturation and aggregation processes. Further studies are being conducted to gain a better understanding of this concentration dependence.

Time To Reach Equilibrium. The concentration of apomyoglobin in the soluble phase, in equilibrium with precipitate, was monitored to determine the time required to reach equilibrium solubility values. For apomyoglobin concentrations just above saturation, the dilute phase protein concentration was followed with time at 1, 1.6, 2.4, and 3.6 M urea (protein concentrations of 20, 7, 3, and 16 mg/mL, respectively) as shown in Figure 6. Precipitation will be relatively slow at these protein concentrations (see Figure 4, 1 and 2.4 M urea). Within the first day, the dilute phase protein concentration decreases substantially, as shown in Figure 6. Between 2 and 5 days, the dilute phase protein concentration decreases slowly, approximately 6% a day except at 3.6 M urea where it is constant within error. Similar time dependencies of the soluble protein concentration were obtained for protein initially in buffer or 8 M urea. The degradation of urea to form cyanates is significant in this time frame at 25 °C (Hagel et al., 1971). This can lead to covalent modification of the protein lysine groups (Stark, 1965). Other processes including adsorption of the soluble protein onto the silanized tubes, bacterial growth (Tombs et al., 1974), and time-dependent changes in the structure of the solid (Smithies, 1953) may contribute to the time dependence of solubility. To determine the extent to which this time dependence would affect our final conclusions, a partial "phase diagram" was constructed for solubilities determined at 2, 3, and 5 days. As shown in Figure 7, the shape of the liquid–solid phase boundary and the absolute values of the points on the phase boundary are altered to only a minor extent by the kinetic processes occurring beyond a 2-day equilibration time.

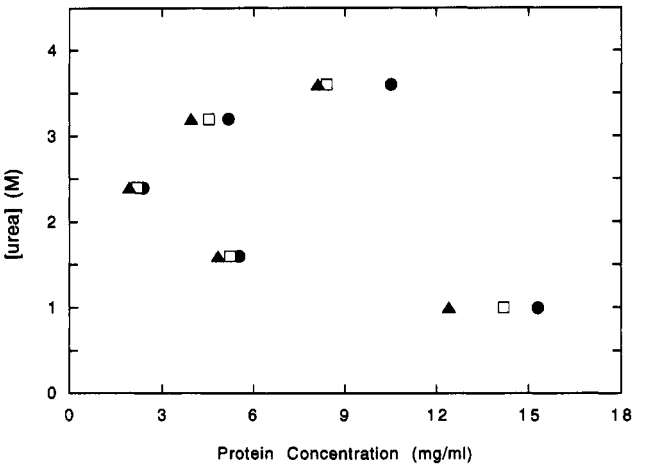

FIGURE 7: Effect of equilibration time on the solubility of apomyoglobin in various concentrations of urea. Equilibration at 25 °C for (●) 2 days, (□) 3 days, and (▲) 5 days. Initial protein concentrations were 20, 7, 3, 8, and 16 mg/mL at 1, 1.6, 2.4, 3.2, and 3.6 M urea, respectively.

The final phase diagram was constructed using a 3-day equilibration time.

Reversibility of Aggregation. The aggregation of apomyoglobin in aqueous urea is not reversible on dilution with buffer (pathway A in Figure 1). This may be due to the existence of a kinetic barrier to renaturation, possibly due to the entanglement of denatured or partially denatured chains. To ascertain whether apomyoglobin aggregation is reversible on an experimentally viable time scale, we take another pathway for renaturation, pathway B in Figure 1. If our system is at thermodynamic equilibrium, all pathways must be reversible (though some may be much slower than others), and our results should be independent of path. It has been reported for several proteins (Jaenicke, 1967; Acampora & Hermans, 1967) and polymers (Schultz & Flory, 1952) that aggregation is directly reversible, by returning the solution to the original solvent conditions, only if aggregation has not been allowed to go to completion. Here, precipitates formed after a 2-day incubation in urea were dissolved in 8 M urea and refolded by buffer dilution (pathway B in Figure 1). The refolded protein solution was monitored for secondary structure with far-UV CD and for tertiary structure with fluorescence spectroscopy. The reversibility of conformational changes for protein in solution was also studied. The proteins in the supernatants above the precipitates were refolded directly by buffer dilution. After dilution, the proteins in the supernatants which were formed in 0.5–4 M urea all gave native fluorescence peak wavelength signals and recovered an average of 92% of the native secondary structure (range of 89–94%). The precipitates formed in 0.5–4 M urea, after dissolution in 8 M urea and refolding by buffer dilution (pathway B in Figure 1), also gave native fluorescence peak wavelength signals, and an average of 89% of the native secondary structure (range of 84–100%) was recovered. Thus, precipitates formed by pathway A can be returned to the native state by pathway B. For apomyoglobin initially in 8 M urea and precipitated by the addition of buffer (pathway B in Figure 1), solubilities were approximately equal to those formed by pathway A at urea concentrations above 1.6 M. These studies show that the aggregation of apomyoglobin by urea is reversible.

In an equilibrium thermodynamic system, the position of the liquid–solid phase boundary should be independent of the path taken to the phase boundary. The reversibility study above considers two paths to the phase boundary. We now consider additional paths where incremental steps in urea, protein, or urea and protein concentrations were taken to the same fixed end point. Using the procedure describe above, the representative results listed in Table I were obtained. We find that the position of the phase boundary is independent of path. The paths taken through incremental addition of urea, protein, or urea and protein simultaneously, all give the same phase boundary as that determined if the protein initially in buffer or 8 M urea was equilibrated at the final protein and urea concentrations directly. One interesting exception to these results was noted; if a solution of 2 M urea/10 mg/mL protein is made directly, the protein solubility is reproducibly 1.2 mg/mL, lower than would be determined at 2 M urea/4 mg/mL protein (see Table I). This dependence of protein solubility on total protein concentration, in the denaturation transition region, is similar to that obtained at 2.4 M urea, as shown in Figure 5. However, if an incremental pathway is taken to the end point of 2 M urea/10 mg/mL protein, the soluble protein concentration is 2–2.4 mg/mL, the same as that determined at 2 M urea and 4 mg/mL protein, as shown in Table I. This may be due to the reduced driving force for aggregation in the incremental pathway.

Table I: Apomyoglobin Solubility Determined for Samples Where Protein and Urea Were Added to the Solution in Incremental Steps

[urea] (M)	[P] (mg/mL)	days equilibrated after final increment	$[P]_{soluble}$ (mg/mL)
0.5	39	3	31
0.5	10→20→30→35→40	2	33
0→0.25→0.5	40	2	34
1	20	3	14
1	5→10→15→20	3	15
1	5→10→15→20→25	2	17
0→0.5→0.7→1	25	2	17
2	4	3	2.3
2	10	4	1.2
2	2→4→6	3	2.2
2	2→4→6→8→10	4	2.0
0.5→1→1.5→2	10	3	2.4
0.5→1→1.5→2	0.8→1.6→2.2→3	3	2.2
2.6	10	3	2.0
2.6	2→6→10	3	2.0
1→2→2.6	10	3	1.9
1→1.6→2.1→2.6	1→1.5→2→3.25	3	1.9
3.2	8	3	4.8
3.2	2→5→9	4	4.6

Phase Diagram for Apomyoglobin Aggregation. The overall summary of our results is shown in the form of a liquid–solid phase diagram in Figure 8 (using a 3-day equilibration at 25 °C). Each point represents the protein solubility at that urea concentration or, equivalently, the dilute phase protein concentration in equilibrium with precipitate; data are listed in Table II. All protein in solution was considered part of the soluble phase; it was not determined whether soluble protein aggregates were present. Apomyoglobin solubilities for protein initially in buffer and 8 M urea were determined between 0–3.6 and 1.6–4 M urea, respectively. Error bars shown are standard deviations of the mean solubility value determined in a minimum of three experiments. Solubilities were determined at total protein concentrations just inside the two-phase region. For the aggregation experiment at 1.6 M urea where the protein was initially in 8 M urea, the protein has to refold at a relatively high protein concentration (compared to the experiment at 2.4 M urea). Under these conditions, kinetic processes probably interfere with the attainment of equilibrium (Jaenicke & Rudolph, 1977; Teipel & Koshland, 1971; Zettlmeissl et al., 1979).

Protein solubility in buffer was determined by two different approaches, as described above. Precipitation by pH adjust-

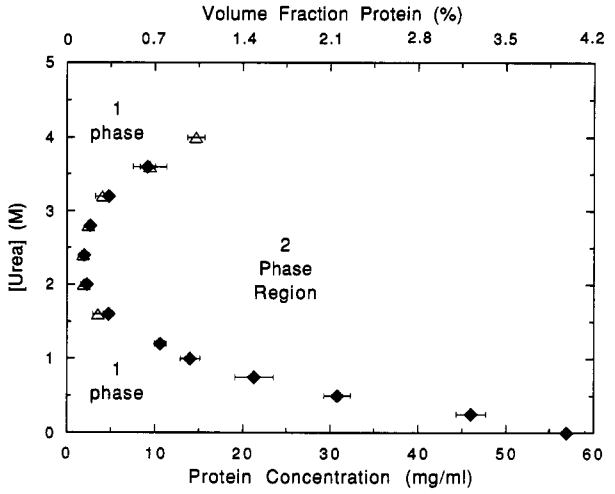

FIGURE 8: Phase diagram for apomyoglobin precipitation in aqueous urea solutions. Data taken from Table II. The dilute arm of the liquid–solid phase boundary was determined for protein initially in buffer (♦) and initially in 8 M urea (△). Error bars are the standard deviations of the mean solubility value determined in three or more experiments.

Table II: Apomyoglobin Solubility in Aqueous Urea Solutions

[urea] (M)	[apomyoglobin] (mg/mL)	apomyoglobin solubility (mg/mL) ± SD
	Protein Initially in Buffer	
0		56.9 ± 2.1
0.25	54	46.0 ± 1.7
0.5	39	30.8 ± 1.5
0.75	28	21.3 ± 2.2
1.0	20	14.0 ± 1.1
1.2	12	10.6 ± 0.70
1.6	7	4.72 ± 0.51
2.0	4	2.28 ± 0.18
2.4	3	1.98 ± 0.18
2.8	6	2.65 ± 0.05
3.2	8	4.78 ± 0.21
3.6	16	9.19 ± 0.86
	Protein Initially in 8 M Urea	
1.6	5	3.52 ± 0.61
2.0	4	1.93 ± 0.36
2.4	3	1.86 ± 0.15
2.8	5	2.44 ± 0.16
3.2	8	4.06 ± 0.81
3.6	16	9.46 ± 1.9
4.0	24	14.7 ± 1.0

ment and by supersaturation with solid protein gave similar solubility values. The method of supersaturation with solid protein was also used for 0.25 and 0.5 M urea samples. Soluble protein concentrations for these samples were similar to those obtained with the general method used.

As shown in Figure 8, the solubility of apomyoglobin decreases dramatically with small increases in urea concentration between approximately 0 and 1.5 M urea. The solubility is at a minimum near the denaturation midpoint and increases at higher urea concentrations. In preliminary studies above 4 M urea, the protein solutions appear to gel, with the gel-like character increasing with the protein concentration (progression from highly viscous to jello-like character). Greater protein concentrations are required to gel the protein at increasing urea concentrations. For example, at 5 and 7 M urea, gels formed at approximately 120 and 200 mg/mL apomyoglobin, respectively. Gels have previously been observed for proteins denatured by high urea concentrations (Ferry, 1948; Huggins et al., 1951; Frensdorf et al., 1953).

Aggregation by Intermediates or Denatured Molecules? We consider two hypotheses below: that aggregation takes place by association of protein molecules in intermediate states or by association of protein molecules in their denatured states.

What is the basis for the aggregation of proteins in *intermediate states*? The logic behind intermediate aggregation would be the following. If a protein aggregates most strongly under native conditions, then the main species that aggregates is native protein. If a protein aggregates most strongly under denaturing conditions, then the main species that aggregates would be denatured protein. However, if a protein aggregates most strongly under conditions that are intermediate between native and denatured conditions (for example, near the midpoint of the denaturation transition, as we observe here for apomyoglobin), then perhaps the aggregating species is an intermediate state. Here, an intermediate (I) has properties intermediate between those of the native and denatured states. However, by thermodynamic definition, a state is stable, and therefore has a minimum in free energy with a corresponding measurable population. To prove the existence of a thermodynamic intermediate state, the existence of a third population (or free energy minimum) in addition to the two stable native and denatured states must be demonstrated; there must be two barriers of free energy: N to I and I to D (Dill & Shortle, 1991).

It has been an experimental challenge to prove the existence of stable intermediate conformations that are distinguishable from N and D. Evidence for folding intermediates has generally included noncoincident unfolding transitions, ANS binding, low solubility, loss of tertiary structure with maintenance of secondary structure, and a hydrodynamic radius between the native and fully unfolded states (Kuwajima, 1989; Kim & Baldwin, 1990). However, all these properties also characterize compact denatured states, including noncoincident transitions (Dill & Shortle, 1991). Rather than invoking the existence of a third stable state, the intermediate, it is simpler and may often be more appropriate to recognize that the denatured states of proteins vary with solution conditions. As the denatured state varies from a compact structure in a poor solvent to a highly expanded conformation in a good solvent, the hydrodynamic radius, amount of secondary structure, and degree of hydrophobic exposure, for example, will vary (Dill & Shortle, 1991; Dill, 1985; Alonso et al., 1991; Stigter et al., 1991). Some proteins have been shown to form stable folding intermediates at low urea or Gdn·HCl concentrations: the single-domain proteins bovine carbonic anhydrase (Semisotnov et al., 1987; Doligkh et al., 1984) and β-lactamase (Pain & Robson, 1976; Ptitsyn et al., 1990) are examples.

For proteins consisting of a single domain, there are only a few cases where intermediates have conclusively been shown to be involved in the aggregation of the protein (Cleland & Wang, 1990; Havel et al., 1986). However, some investigators have concluded that intermediates are generally involved in aggregation (De Bernardez-Clark & Georgiou, 1991; Mitraki & King, 1989). Mitraki et al. (1991) have stated that aggregation is not a function of the solubility and stability properties of the native state, but those of the folding intermediates in relation to the environment they are folding in. Other investigators believe that intermediates, while possibly important for the aggregation of some proteins under certain solvent conditions, do not generally have to be invoked to explain protein aggregation (Strandberg & Enfors, 1991; Schein, 1989; Light, 1985).

Whereas an intermediate in folding has been identified for apomyoglobin between pH 4 and 5 (Griko et al., 1988;

Hughson et al., 1990) and at low pH on the addition of salt (Goto & Fink, 1990), no folding intermediates have been reported for apomyoglobin at low denaturant concentrations nor have we observed any here. We, therefore, have no evidence that folding intermediates are involved in apomyoglobin aggregation. The small changes shown in Figures 2 and 3 are probably most accurately called predenaturational changes, since by most of our conformational experiments these conformations are indistinguishable from native molecules. It is possible that the decrease in solubility of apomyoglobin with increasing urea concentration is associated with this small predenaturation conformational change. Hydrophobic surface may be exposed as a result of this conformational change, leading to a decrease in solubility.

The second hypothesis is that the aggregation of apomyoglobin involves the association of denatured molecules. This would be consistent with our lack of observation of intermediates. An explanation for how aggregation can be greatest at a urea concentration near the denaturation midpoint was first given by a thermodynamic model of Arakawa and Timasheff (Arakawa, 1987; Arakawa & Timasheff, 1985). Using the phase rule, the equilibrium between two conformations of a molecule as a function of perturbant, and the change in solubility of each of the conformations as a function of the perturbant, Arakawa and Timasheff generated phase diagrams for solubility. For some choices of parameters, e.g., the solubility of one conformation increasing and the other decreasing with an increase in perturbant concentration, phase diagrams with curvature similar to that in Figure 8 were calculated (Arakawa, 1987; Arkawa & Timasheff, 1985).

Statistical mechanical theories for the collapse and aggregation of polymer [for example, see Post and Zimm (1982)] and protein molecules (Fields et al., 1992) have also been developed. In these models, which are based on Flory–Huggins theory (Flory, 1953), the aggregate is envisioned as an entangled network of unfolded chains. With increasing polymer concentration, intermolecular polymer–polymer contacts become favored over intramolecular contacts due to the much greater conformational freedom of the chains in the aggregate. In the model of Fields et al. (1992), hydrophobic interactions drive the protein to aggregate as an entangled network of denatured chains. Stigter and Dill (1992) have recently extended this theory to include the folding equilibrium between native and denatured states. The calculated liquid–solid phase boundary, for apomyoglobin aggregation at its p*I*, has curvature qualitatively similar to that shown in Figure 8, with a negative slope at low urea concentrations.

The explanation for the observed solubility minimum is as follows. In a first approximation, only denatured molecules can aggregate. Two factors determine the solubility phase boundary: (i) Increasing urea concentrations make the solvent more favorable for native and denatured states, thereby weakening the tendency to aggregate. (ii) Increasing urea concentration denatures the protein, increasing the concentration of aggregation-competent molecules. The results of the statistical mechanical theory are that at low urea concentrations factor ii is most important and at high urea concentrations factor i dominates (Stigter & Dill, 1992). That is, at low urea concentrations, increasing the urea concentration leads to an increased population of denatured species. Since aggregation occurs only among denatured proteins, increasing the urea concentration leads to increased aggregation. On the other hand, at high urea concentrations, most of the protein is already denatured, so the dominant effect of increasing the urea concentration is the weakening of the tendency of proteins to aggregate. The result is a minimum in protein solubility at urea concentrations near the denaturation midpoint. In the Stigter and Dill theory (1992), the radius of the denatured species is also predicted to depend on urea and protein concentrations.

In more detail, the negative slope in Figure 8 arises as follows: Since the native and denatured molecules are in equilibrium, once the denatured protein reaches its solubility limit the chemical potential of the system is fixed and the concentration of native protein in solution also becomes fixed, never reaching its solubility limit. Further addition of protein results only in an increased volume of denatured aggregate. As the urea concentration increases, the population of denatured molecules increases, and therefore a lower total protein concentration is necessary to generate a precipitating concentration of denatured molecules. From the fluorescence peak wavelength data in Figure 2, the extrapolated free energy of unfolding, $\Delta G(H_2O)$, was determined using a two-state model (Pace et al., 1989). $\Delta G(H_2O)$ was found to equal 5.1 kcal/mol. Using the extrapolated ΔG at 0, 0.5, 1, and 1.5 M urea, the ratios of folded to unfolded molecules were calculated; these values were 5600/1, 1000/1, 200/1, and 37/1, respectively. The sensitivity of solubility to these populations of denatured molecules is shown by the large decrease in solubility which is observed even before spectroscopic techniques show the denaturation transition to have begun.

The hypothesis that aggregation involves denatured protein is consistent with our data. We find no spectroscopic evidence for the presence of equilibrium intermediates above 2 M urea; the CD, fluorescence intensity, and fluorescence peak wavelength dependencies on urea concentration are coincident. This does not preclude their existence, but suggests that any intermediates present would be at low concentration and/or be transient or that their spectroscopic properties are similar to those of the native or denatured states. The current data cannot eliminate the possibility that the predenaturation conformational change results in a decrease in protein solubility with increasing urea concentration.

Our hypothesis that apomyoglobin in urea aggregates in the denatured state cannot be generalized to all protein associations. Specific aggregates of nativelike molecules are known to occur, for example: protein crystallization, the linear aggregation of hemoglobin-S molecules, and the helical packing of monomers of the tobacco mosaic virus coat protein (Banaszak et al., 1981). However, we believe that the association of denatured protein might be a common mechanism of protein aggregation. The apomyoglobin data presented here suggest that the most populated conformation of the protein in solution is not necessarily reflected in the conformation of the protein in the aggregate. Native solvent conditions may result in denatured aggregates. One practical result from these experiments concerns the refolding of proteins from the denatured state. Protein refolding is often initiated by rapid dilution with buffer. For apomyoglobin, this dilution will lead to crossing of thermodynamic phase boundaries, which may result in aggregation. To obtain better yields in refolding, it may be advantageous to use refolding pathways which stay in the one-phase region, outside the liquid–solid phase boundary.

CONCLUSIONS

We observe that apomyoglobin precipitation is a thermodynamically reversible process, independent of aggregation

pathway at protein concentrations near the liquid–solid phase boundary. Apomyoglobin solubility is at a minimum near 2.4 M urea, close to the denaturation midpoint. At low urea concentrations, the solubility of apomyoglobin decreases with an increase in urea concentration; this is opposite to what would be expected from the increase in solubility of small polar and nonpolar molecules in urea. Two possible explanations are given for this observation: (i) at increasing urea concentrations, the population of denatured molecules will increase, reducing the total protein concentration necessary to precipitate the protein in a denatured aggregate: (ii) the observed urea-induced predenaturation conformational change may expose hydrophobic surface, resulting in reduced protein solubility. Recent thermodynamic theories have shown protein aggregation to result from hydrophobic association of entangled denatured protein chains. Our aggregation behavior at all urea concentrations is consistent with this model, where the protein is aggregating in the denatured state, even under solvent conditions which strongly favor the native state in solution. Folding intermediates are not required to explain the solubility dependence on urea concentration.

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REFERENCES

Acampora, G., & Hermans, J. (1967) *J. Am. Chem. Soc. 89*, 1543.
Alonso, D., Stigter, D., & Dill, K. (1991) *Biopolymers 31*, 1631.
Arakawa, T. (1987) *Biopolymers 26*, 45.
Arakawa, T., & Timasheff, S. (1985) *Methods Enzymol. 114*, 49.
Ataka, M., & Asai, M. (1988) *J. Cryst. Growth 90*, 86.
Balestrieri, C., Colonna, G., Giovane, A., Irace, G., & Servillo, L. (1976) *FEBS Lett. 66*, 60.
Banaszak, L., Birktoft, J., & Barry, C. (1981) in *Protein–Protein Interactions* (Frieden, C., & Nichol, L., Eds.) Wiley-Interscience, New York.
Benditt, E., Cohen, A., Costa, P., Franklin, E., Glenner, G., & Husby, G., (1980) *Int. Congr. Ser. No. 497*.
Berland, C., Thurston, G., Kondo, M., Broide, M., Pande, J., Ogun, O., & Benedek, G. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1214.
Better, O. (1990) *Nephron 55*, 97.
Brems, D. (1988) *Biochemistry 27*, 4541.
Cleland, J., & Wang, D. (1990) *Biochemistry 29*, 11072.
Cohn, E., Edsall, J. (1943) *Proteins, Amino Acids and Peptides*, Hafner Publishing Co., New York.
Colonna, G., Balestrieri, C., Bismuto, E., Servillo, L., & Irace, G. (1982) *Biochemistry 21*, 212.
Crumpton, M., & Polson, A. (1965) *J. Mol. Biol. 11*, 722.
De Bernardez-Clark, E., & Georgiou, G. (1991) in *Protein Refolding* (Georgiou, G., De Bernardez-Clark, E., Eds.) American Chemical Society, Washington, D.C.
DeMattei, R., & Feigelson, R. (1991) *J. Cryst. Growth 110*, 34.
Dervichian, D. (1954) *Discuss. Faraday Soc. 18*, 231.
Dill, K. (1985) *Biochemistry 24*, 1501.
Dill, K., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795.
Dolgikh, D., Kolomiets, A., Bolotina, I., & Ptitsyn, O. (1984) *FEBS Lett. 165*, 88.
Falconer, J., & Taylor, D. (1946) *Biochem. J. 40*, 835.
Ferry, J. (1948) *Adv. Protein Chem. 4*, 1.
Fields, G., Alonso, D., Stigter, D., & Dill, K. (1992) *J. Phys. Chem. 96*, 3874.
Flory, P. (1953) *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, NY.
Frensdorff, H., Watson, M., & Kauzmann, W. (1953) *J. Phys. Chem. 75*, 5157.
Goto, Y., & Fink, A. (1990) *J. Mol. Biol. 214*, 803.
Goto, Y., Calciano, L. J., and Fink, A. L. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577.
Goto, Y., Takahashi, N., & Fink, A. L. (1990b) *Biochemistry 29*, 3480–3488.
Griko, Y., Privalov, P., Venyaminov, S., & Kutyshenko, V. (1988) *J. Mol. Biol. 202*, 127.
Hagel, P., Gerding, J., Fieggen, W., & Bloemendal, H. (1971) *Biochim. Biophys. Acta 243*, 366.
Harrison, S., & Blout, E. (1965) *J. Biol. Chem. 240*, 299.
Havel, H., Kaufman, E., Plaisted, S., & Brems, D. (1986) *Biochemistry 25*, 6533.
Howard, S., Twigg, P., Baird, J., & Meehan, E. (1988) *J. Cryst. Growth 90*, 94.
Huggins, C., Tapley, D., & Jensen, E. (1951) *Nature 167*, 592.
Hughson, F., Wright, P., & Baldwin, R. (1990) *Science 249*, 1544.
Ikai, A., Tanaka, S., & Noda, A. (1978) *Arch. Biochem. Biophys. 190*, 39.
Ikeguchi, M., Kuwajima, K., Mitani, M., & Sugai S. (1986) *Biochemistry 25*, 6965.
Irace, G., Balestrieri, C., Parlato, G., Servillo, L., & Colonna, G. (1981) *Biochemistry 20*, 792.
Jaenicke, R. (1967) *J. Polym. Sci. 16*, 2143.
Jaenicke, R., & Rudolph, R. (1977) *FEBS–Symp. No. 49*, 351.
Kendrew, J., Dickerson, R., Strandberg, B., Hart, R., Davies, D., Phillips, D., & Shore, V. (1960) *Nature 185*, 442.
Kim, P., & Baldwin, R. (1990) *Annu. Rev. Biochem. 59*, 631.
Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87.
Light, A. (1985) *BioTechniques 3*, 298.
Lougheed, W., Woulfe-Flanagan, H., Clement, J., & Albisser, F. (1980) *Diabetologia 19*, 1.
Marston, F. (1986) *Biochem. J. 240*, 1.
Massry, S., & Glassock, R. (1983) *Textbook of Nephrology*, Williams & Wilkins, Baltimore, MD.
Mitraki, A., & King, J. (1989) *Bio/Technology 7*, 690.
Mitraki, A., Haase-Pettingell, C., & King, J. (1991) in *Protein Refolding* (Georgiou, G., & De Bernardez-Clark, E., Eds.) American Chemical Society, Washington, D.C.
Nandi, P., & Robinson, D. (1984) *Biochemistry 23*, 6661.
Nozaki, Y., & Tanford, C. (1963) *J. Biol. Chem. 238*, 4074.
Nozaka, M., Kuwajima, K., Nitta, D., & Sugai, S. (1978) *Biochemistry 17*, 3753.
Pace, C., Shirley, B., & Thomson, J. (1989) in *Protein Structure—A Practical Approach* (Creighton, T. E., Ed.) IRL Press at Oxford University Press, England.
Parker, T., & Dalgleish, D. (1977) *Biopolymers 16*, 2533.
Post, C., & Zimm, B. (1982) *Biopolymers 21*, 2123.
Przybycien, T., & Bailey, J. (1989) *AIChE J. 35*, 1779.
Ries-Kautt, M., & Ducruix, A. (1989) *J. Biol. Chem. 264*, 745.
Robinson, D., & Jencks, W. (1965) *J. Am. Chem. Soc. 87*, 2462.
Roseman, M., & Jencks, W. (1975) *J. Am. Chem. Soc. 97*, 631.
Rothgeb, T., & Gurd, F. (1978) *Methods Enzymol. 52*, 473.
Schein, C. (1989) *Bio/Technology 7*, 1141.
Schultz, A., & Flory, P. (1952) *Biophys. J. 74*, 4760.
Semisotnov, G., Rodionova, N., Kutyshenko, V., Ebert, B., Blanck, F., & Ptitsyn, O. (1987) *FEBS Lett. 224*, 9.
Smithies, O. (1953) *Biochem. J. 58*, 31.
Sophianoopoulos, A., Rhodes, C., Holcomb, D., & Van Holde, K. (1962) *J. Biol. Chem. 237*, 1107.
Stark, G. (1965) *Biochemistry 4*, 1030.
Stigter, D., & Dill, K. (1993) *Fluid Phase Equilib.* (in press).
Stigter, D., Alonso, D., & Dill, K. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4176.
Strandberg, L., & Enfors, S.-O. (1991) *Appl. Environ. Microbiol. 57*, 1669.

Stryer, L. (1965) *J. Mol. Biol. 13*, 482.
Teale, F. (1959) *Biochim. Biophys. Acta 35*, 543.
Teipel, J., & Koshland, D. (1971) *Biochemistry 10*, 792.
Thompson, J., Schurtenberger, P., Thurston, G., & Benedek, G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7079.
Tombs, M. (1957) *Biochem. J. 67*, 517.
Tombs, M., Newsom, B., & Wilding, P. (1974) *Int. J. Pept. Protein Res. 6*, 253.
Walton, A. (1967) *The Formation and Properties of Precipitates*, Interscience Publishers, New York.
Wetlaufer, D., Malik, S., Stoller, L., & Coffin, R. (1964) *J. Am. Chem. Soc. 86*, 508.
Wilf, J., & Minton, A. (1981) *Biochim. Biophys. Acta 670*, 316.
Wright, K., & Boxer, S. (1981) *Biochemistry 20*, 7546.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567.