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Reversible Self-Association of Bovine Growth Hormone during Equilibrium Unfolding

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Received February 7, 1986; Revised Manuscript Received May 9, 1986

ABSTRACT: Previous investigations have shown that bovine growth hormone (bGH, somatotropin) unfolds through a reversible multistate process with at least one stable equilibrium intermediate. In extending our knowledge of the folding process for bGH, we demonstrate that a self-associated form of partially denatured bGH is formed during equilibrium unfolding experiments. The self-associated species has been identified by hydrodynamic measurements (size exclusion high-performance liquid chromatography and static and dynamic light scattering) and by measurements of the bGH concentration dependence of aromatic amino acid spectral properties (fluorescence, second-derivative absorption, and circular dichroism). The apparent maximum concentration for self-association occurs when bGH is partially denatured, i.e., at 3.7 M guanidine hydrochloride or 8.5 M urea, and its formation is reversible. Some of the properties of the self-associated species include (a) quenched tryptophan fluorescence, (b) increased tryptophan circular dichroism intensity at 300 nm, (c) polar tryptophan environment, and (d) a weight-average radius of about 5 nm. The self-association of bGH is mediated by specific intermolecular interactions with little increase in molecular size occurring above the saturation level of 4 mg/mL bGH. These phenomena have important implications for the design and interpretation of folding experiments in vitro and may have physiological consequences.

One approach to studying the forces that stabilize the three-dimensional structure of native proteins is to use physicochemical methods to characterize the structural rearrangements of a protein as it folds or unfolds. We (Brems et al., 1985) and others (Holladay et al., 1974) have reported previously that bovine growth hormone (bGH, somatotropin)[1] unfolds in guanidine hydrochloride (Gdn-HCl) through a reversible multistate process with stable equilibrium intermediates. Interpretations of spectral data from acid-induced unfolding of bGH have also invoked equilibrium intermediates (Burger et al., 1966). The structural characteristics of these intermediates, however, have not been addressed and should provide insights into the relative stability of structural elements in native bGH and into the folding mechanism for bGH. In addition, it is important to establish whether the unfolding process is the same in urea (a nonionic chemical denaturant), in Gdn-HCl (an ionic chemical denaturant), and in acid.

In order to study the structural properties of intermediates which occur during bGH unfolding, we have used spectroscopic probes as indicators of the different chemical species which are present at different concentrations of denaturant. While conducting these studies, we became aware of a dependence on bGH concentration for the circular dichroism (CD) and fluorescence intensities under partially denaturing conditions. A detailed investigation of the causes of these phenomena has been undertaken by using optical spectroscopy and hydrodynamic measurements.

Applications of dynamic light scattering (photon correlation spectroscopy, PCS) to the study of protein denaturation have been reported previously (Rimai et al., 1970; Dubin et al., 1973; Nicoli & Benedek, 1976; Wang et al., 1980; Nystrom & Roots, 1982) and have provided measurements of the increase in molecular size upon denaturation. The experimental results for bGH provide these data and, in addition, indicate that the protein self-associates reversibly when partially denatured through specific protein–protein interactions. Our results emphasize the utility of monitoring molecular size in equilibrium denaturation studies and provide the impetus to determine the role played by the self-associated intermediate or intermediates in the kinetic pathway of folding. A partial characterization of the associated intermediate is discussed in the following paper in this issue (Brems et al., 1986).

Experimental Procedures

Materials

Pituitary-derived bGH was obtained from A. F. Parlow (UCLA, Los Angeles, CA); ultrapure Gdn-HCl and ultrapure urea were from Schwarz/Mann (Cleveland, OH). All other materials were analytical grade.

Methods

Buffer Conditions. All solutions were prepared in 50 mM ammonium bicarbonate buffer (pH 8.5). Denaturants were

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[1] Abbreviations: bGH, bovine growth hormone; Gdn-HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; PCS, photon correlation spectroscopy; R_θ, Rayleigh ratio.

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added before the pH was adjusted.

Size-Exclusion High-Performance Liquid Chromatography (HPLC). The HPLC methods used have been described previously (Brems et al., 1985). A DuPont Zorbax GF-250 column was used with a mobile phase obtained by dynamic mixing of two reservoirs: one containing 6 M Gdn-HCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), and 1 mM ethylenediaminetetraacetic acid (EDTA) with the pH adjusted to 8.0 with concentrated HCl and the other containing the same constituents without Gdn-HCl. The resulting mobile phase was pumped at 1 mL/min with a Perkin-Elmer Series 4 pump, and the eluent was detected by using the UV absorbance at 220 nm.

Circular Dichroism. CD spectra were measured on a Jasco J-500C spectropolarimeter at ambient temperature (about 23 °C). Calculations of mean residue ellipticity were made by using a mean residue weight of 113 g/mol. Calibration was performed with *d*-camphorsulfonic acid using a molar ellipticity of 7821 deg cm^2/dmol at 290 nm (Schippers & Dekkers, 1981).

Light Scattering. Dust-free samples for light-scattering measurements were prepared either by filtering a concentrated stock solution with a 0.1-μm polycarbonate filter (Nucleopore Corp.) and diluting with ultrafiltered solutions or by filtering each solution through a 0.1-μm filter before analysis.

Scattered intensity and PCS measurements were made with a Lexel 95 argon ion laser at 514.5 nm, a Brookhaven BI-200SM goniometer, and a Brookhaven BI-2030 autocorrelator with 256 linearly spaced channels and 4 delay channels. All data were collected at 25.0 ± 0.1 °C and 90° scattering angle. The PCS measurements involved collecting multiple correlation functions, sorting to eliminate those contaminated by contributions from dust particles, and summing into a final function using a Digital MINC/23 microcomputer. Weight-average Stokes' radius calculations were done by using the program CONTIN (Provencher et al., 1978; Provencher, 1979, 1983) on a Digital VAX/11-780 mainframe computer. Viscosity measurements were made by using an Ubbelohde viscometer at 25.0 ± 0.1 °C, and solution refractive indexes were measured with an Abbe refractometer.

Fluorescence. Fluorescence measurements were done with a Perkin-Elmer MPF-44B fluorometer at ambient temperature (about 23 °C). Emission from tryptophan alone was assured by using an excitation wavelength of 295 nm and monitoring at 350 nm with 5-nm slits. Where necessary, a correction for the inner filter effect was employed by measuring absorbances at 295 and 350 nm.

Absorbance. A Hewlett-Packard 8450A spectrometer was used for measuring absorbances at 290 nm and recording second-derivative spectra (without smoothing) at ambient temperature (about 23 °C). Measurements were made with a buffer solution as reference.

RESULTS

Denaturant Concentration Effects. The equilibrium denaturation of bGH in urea at pH 8.5 was monitored by UV absorption (290 nm), tryptophan fluorescence, and circular dichroism (222 nm) in the same manner as described previously for Gdn-HCl denaturation (Brems et al., 1985). Similar experiments have been carried out by other investigators (Edelhoch & Burger, 1966). The three structural probes monitored transitions which were distinct from one another, as was observed with Gdn-HCl, providing evidence that at least one intermediate is populated during equilibrium denaturation. However, even at 10 M urea some native bGH structure must be present as none of the curves had reached a plateau.

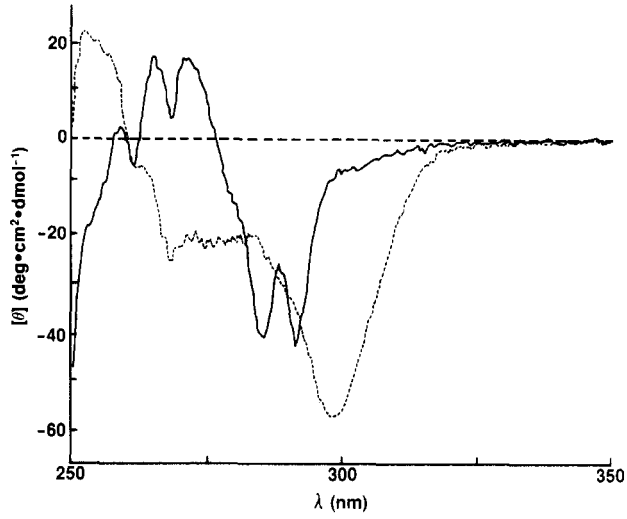

FIGURE 1: Near-UV circular dichroism spectra for 1 mg/mL bGH with (···) and without (—) 3.7 M Gdn-HCl.

The CD spectrum of a protein in the near-UV region (250–350 nm) provides information on the chiral nature of the environment in the vicinity of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The near-UV CD spectrum of bGH has been reported previously (Edelhoch & Lippoldt, 1970; Sonenberg & Beychok, 1971; Holladay et al., 1974). Shown in Figure 1 are spectra which are obtained for 1 mg/mL bGH in buffer plus 3.7 M Gdn-HCl. When bGH is completely denatured, i.e., in 6 M Gdn-HCl, the near-UV CD spectrum is featureless except for some weak bands from 250 to 275 nm. The large CD band which appears at about 300 nm for partially denatured bGH has been assigned previously to the single tryptophan in bGH, and CD spectral changes near 269 and 280 nm may also be due to tryptophan transitions (Holladay et al., 1974). The 300-nm band cannot be assigned to a disulfide transition since reduced, alkylated bGH is found to display the 300-nm band (data not shown). The change in intensity (molar ellipticity) of the CD band at 300 nm as the denaturant concentration is increased is shown in Figure 2 and has a minimum, i.e., has the most optical activity, where bGH is partially denatured (at about 3.7 M Gdn-HCl or 8.5 M urea). All of these data have been shown to be reversible by mixing bGH samples at high and low denaturant concentration; samples prepared in this way have molar ellipticities at 300 nm which are not significantly different than those obtained by direct solubilization of bGH at the corresponding denaturant concentrations.

Size-exclusion HPLC provides a measurement of the mean hydrodynamic or Stokes' radius of a protein as measured by its retention time when a sample is eluted from a chromatographic column that has been calibrated with protein standards. By varying the Gdn-HCl concentration in the mobile phase, it is thus possible to measure the molecular size changes which occur as a protein undergoes equilibrium unfolding. The results for injecting a 1 mg/mL bGH sample are shown in Figure 3A and show a maximum size of 4 nm at about 4 M Gdn-HCl; under both native (buffer only) and denaturing (6 M Gdn-HCl) conditions, the mean bGH radius is smaller than under partially denaturing conditions. These results are corroborated by PCS results at 1 mg/mL bGH (Figure 3C) which also provide a measurement of mean hydrodynamic size. The agreement between the two methods indicates that the HPLC results have not been biased by the chromatographic process, e.g., by sample dilution, binding of protein to the resin, etc.; the lack of quantitative agreement may be due to the

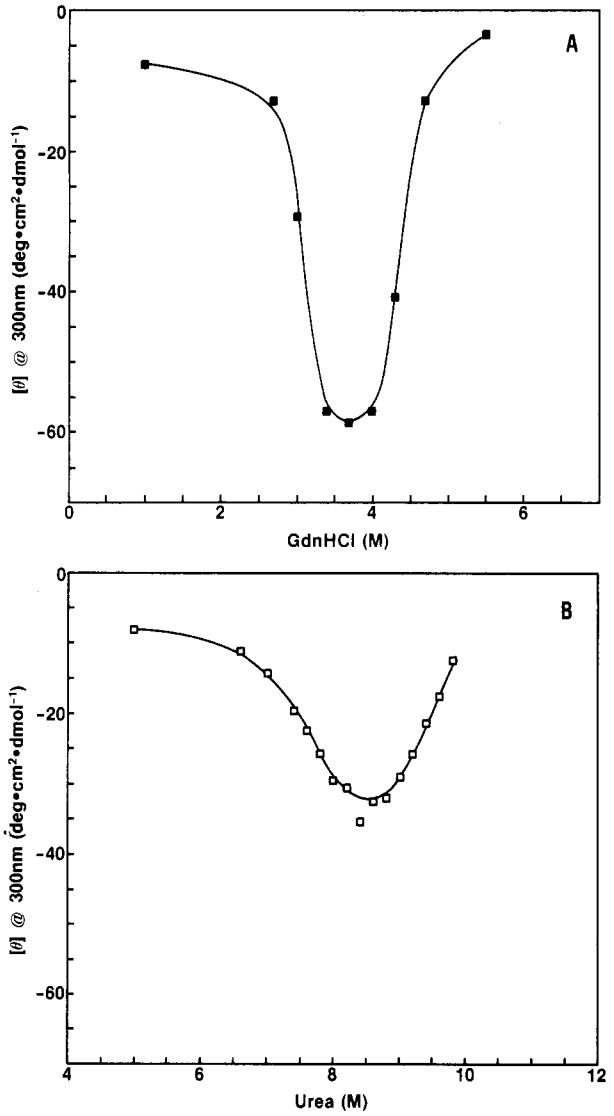

FIGURE 2: Molar ellipticity at 300 nm for bGH unfolding in Gdn-HCl (■, A) and urea (□, B) at 1 mg/mL bGH.

different mean size measured with the two techniques. Further corroborating evidence for these results comes from measurements of the scattered light intensity (Rayleigh ratio, R_θ) as the Gdn-HCl concentration is varied (data not shown). An increase in R_θ is observed from 2 to 4 M Gdn-HCl and a decrease from 4 to 6 M Gdn-HCl as would be expected from the data in Figure 3C.

Second-derivative absorption spectroscopy in the near-UV region has been shown to provide useful structural information about the aromatic amino acids which absorb in this spectral range (Servillo et al., 1982; Terada et al., 1984; Ragone et al., 1984). In particular, changes in the second-derivative spectra of proteins upon denaturation in 6 M Gdn-HCl have been shown to indicate changes in the polarity of the environment of tryptophan and tyrosine residues (Terada et al., 1984). Shown in Figure 4 are the second-derivative spectra for 0.3 mg/mL bGH dissolved in 2, 3.7, and 6 M Gdn-HCl; similar spectra have been obtained for denaturation in urea. Although the spectra are complicated by overlap of absorption bands from tryptophan and tyrosine (but not from phenylalanine), examination of the spectral changes from 291 to 295 nm (due principally to tryptophan) and from 276 to 278 nm (due principally to tyrosine) provides indications of their environment polarity. The spectral changes observed here are not due to changes in solvent but due to changes in protein structure

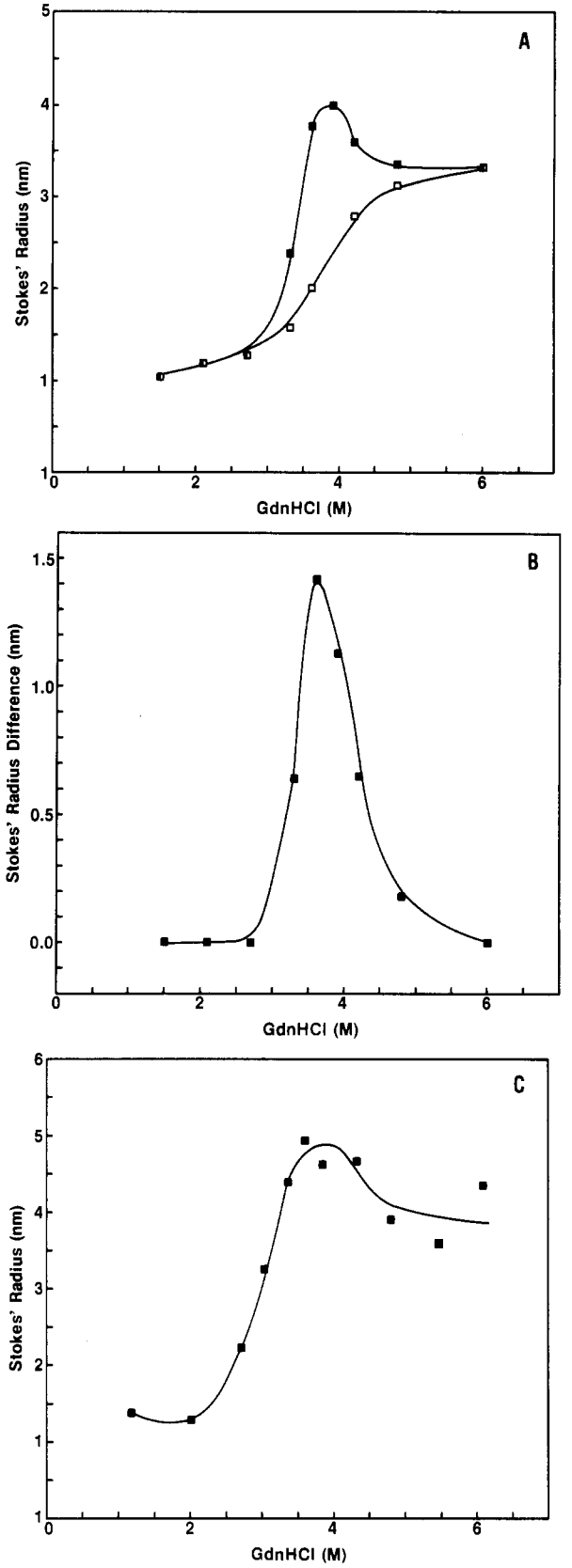

FIGURE 3: (A) Stokes' radius for bGH unfolding in Gdn-HCl as measured by size-exclusion HPLC at 1 (■) and 0.1 mg/mL bGH (□). (B) Difference in Stokes' radius between high (1 mg/mL) and low (0.01 mg/mL) bGH concentrations as measured by size-exclusion HPLC. (C) Weight-average Stokes' radius for bGH unfolding in Gdn-HCl as measured by PCS at 1 mg/mL bGH.

as bGH denatures. This conclusion is based on the limited effect of solvent (from buffer to 6 M Gdn-HCl) on the second-derivative absorption spectra of model compounds for

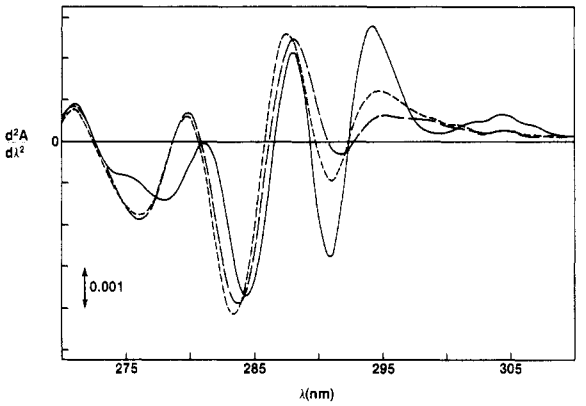

FIGURE 4: Second-derivative absorption spectra for 0.3 mg/mL bGH at different Gdn-HCl concentrations: 2 M (—), 3.7 M (– –), and 6 M (- - -).

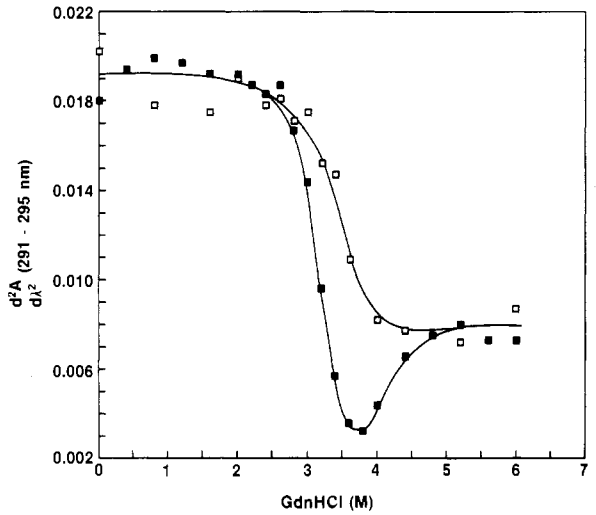

FIGURE 5: Second-derivative peak to trough distance (291–295 nm) for bGH unfolding in Gdn-HCl at 0.05 (□) and 0.3 mg/mL (■) bGH.

tryptophan (*N*-acetyltryptophanamide) and tyrosine (*N*-acetyltyrosinamide).

In the tryptophan region (291–295 nm), there is a large reduction in intensity (increase in effective solvent polarity) from 2 to 3.7 M Gdn-HCl followed by an increased intensity (decrease in polarity) from 3.7 to 6 M Gdn-HCl (Figure 4). The behavior throughout the denaturation transition is shown in Figure 5 for low (0.05 mg/mL) and high (0.3 mg/mL) bGH concentrations by measuring the peak (295 nm) to trough (291 nm) intensity for the second-derivative bands. As bGH unfolds at low protein concentration, the peak to trough intensity changes smoothly as the Gdn-HCl concentration is varied: the tryptophan environment changes smoothly from nonpolar to polar as expected. At high bGH concentrations, unfolding causes the peak to trough intensity to go through a minimum near 3.7 M Gdn-HCl, indicating a highly polar environment under these partially denaturing conditions.

The environment around tyrosine residues can be monitored by the shift in the second-derivative spectrum from 278 to 276 nm with increasing Gdn-HCl concentration (Figure 4). This shift in the spectrum starts at 3 M Gdn-HCl and is complete at 3.4 M Gdn-HCl (data not shown), indicating that the effective polarity of the tyrosine environment has become more polar upon bGH unfolding and is unchanged from 3.4 to 6 M Gdn-HCl; it does not depend on bGH concentration.

Protein Concentration Effects. To further investigate the apparent formation of a large molecular weight component under partially denaturing conditions, the effect of bGH concentration was determined. The effect of increasing bGH concentration on the molar ellipticity at 300 nm is shown in Figure 6. The monotonic decrease in signal that occurs with both denaturants until about 4 mg/mL indicates an intermolecular interaction which becomes saturated above this protein concentration. At denaturant concentrations where the molar ellipticity at 300 nm is essentially zero, e.g., at 2 or 5.5 M Gdn-HCl in Figure 2, protein concentrations as high as 10 mg/mL still have molar ellipticities which are not significantly different from zero, indicating an absence of the associated equilibrium intermediate at these Gdn-HCl concentrations. A similar effect has been observed for the peak (295 nm) to trough (291 nm) intensity in second-derivative absorption spectra.

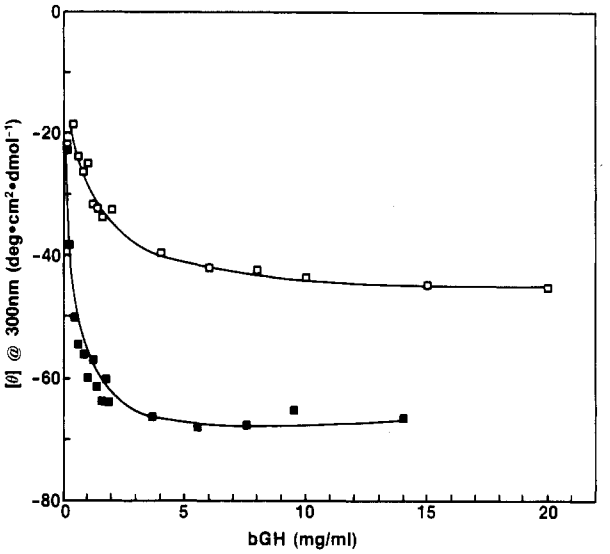

FIGURE 6: Effect of bGH concentration in 3.7 M Gdn-HCl (■) and 8.5 M urea (□) on molar ellipticity at 300 nm.

The effect of bGH concentration on the fluorescence emission of the single tryptophan depends on the concentration of denaturant. As shown in Figure 7, at high or low concentrations of denaturant (fully unfolded or native bGH, respectively), there is virtually no effect: the amount of fluorescence emitted is independent of bGH concentration. Near 4 M Gdn-HCl, however, increasing the bGH concentration lowers the molar fluorescence emission significantly due to a quenching of tryptophan fluorescence at this denaturant concentration. The molecular species present at high bGH concentration have less fluorescence intensity than at low bGH concentration (Figure 7A). The similarity of the curves in Figures 7B and 2 should be noted.

Size-exclusion HPLC and light-scattering measurements are also dependent on bGH concentration. Shown in Figure 3A are size-exclusion HPLC experiments at high (1 mg/mL injected) and low (0.01 mg/mL injected) bGH concentrations. At 0.01 mg/mL bGH injected, or lower, the denaturation transitions are independent of protein concentration and are interpreted as monomeric unfolding. In contrast, at higher bGH concentrations, the species populated under partially denaturing conditions (4 M Gdn-HCl) are larger than monomer bGH. The difference in Stokes' radius between the two protein concentrations is plotted vs. Gdn-HCl concentration in Figure 3B. The on-column dilution of the protein sample was determined to be 15-fold.

The dependence of R_θ on bGH concentration (from 0.1 to 14 mg/mL) in 3.7 M Gdn-HCl produces a nonlinear plot (data not shown) indicating that more than one component is present at high protein concentration (Tanford, 1961). These data

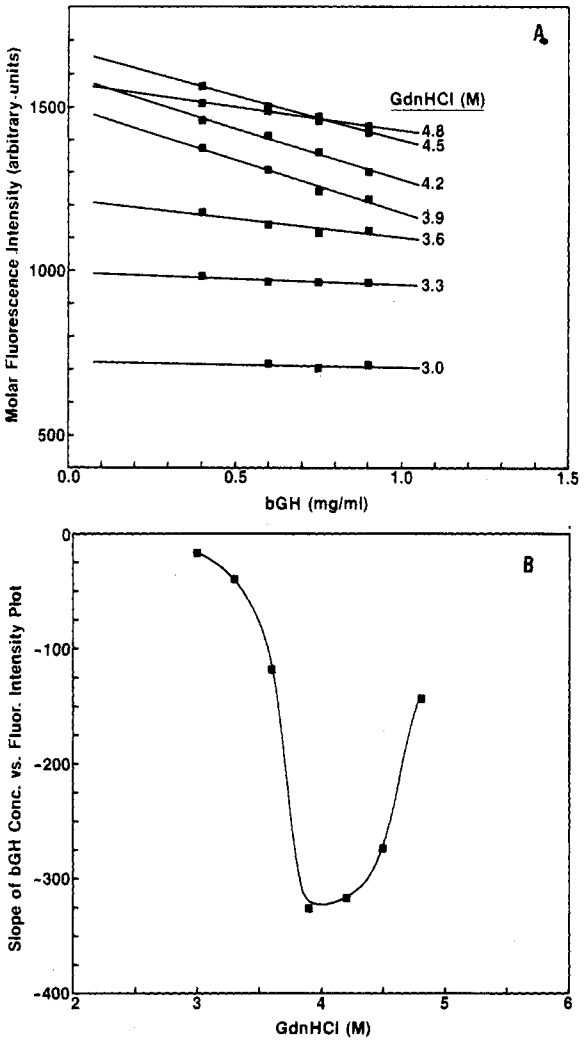

FIGURE 7: (A) Effect of bGH concentration on molar fluorescence intensity (emission corrected for bGH concentration) at several Gdn-HCl concentrations. (B) Dependence of the slopes of lines in Figure 7A on Gdn-HCl concentration.

are similar to those observed previously for the dimerization of insulin (Doty & Myers, 1953). When PCS is used to measured molecular size as a function of bGH concentration in 3.7 M Gdn-HCl, little change in size occurs over the range 1 to 10 mg/mL (data are not shown). Measurements below this concentration range were unreliable due to the relatively small amount of scattered light from the protein with respect to that from the solvent.

Discussion

Self-Association of Partially Unfolded bGH. The most compelling evidence for self-association of bGH comes from hydrodynamic measurements (light scattering and size-exclusion HPLC) as a function of denaturant concentration (Figure 3). These data indicate the existence of components under partially denaturing conditions (near 4 M Gdn-HCl) with large enough radii to give a mean radius at least twice that of monomer, native bGH (about 4.5 nm vs. about 2 nm), and also larger than monomer, denatured bGH (about 4.5 nm vs. about 4 nm). The fact that the mean size decreases as more denaturant is added rules out the possibility that the species at 4 M Gdn-HCl is a highly extended form of monomer bGH. A more likely explanation would be the disappearance of the self-associated form(s) as the population of a partially denatured monomeric intermediate (from which the self-associated species are derived) is depleted. According to Figure 6, the monomeric intermediate(s) must be fully saturated at concentrations above 4 mg/mL. Below this concentration, there is a mixture of the partially denatured monomer and its self-associated product(s).

Specific Intermolecular Interactions. The similarity of Figures 2 and 3B leads to the hypothesis that the molar ellipticity at 300 nm can be correlated with the increase in molecular size upon self-association of a monomeric intermediate of bGH. Furthermore, since (1) this CD band is due to a tryptophan transition, (2) the fluorescence of the tryptophan is quenched upon self-association (Figure 7), and (3) the tryptophan environment is more polar in the associated state than in either the folded or the unfolded monomer states (Figure 5), it is reasoned that the single tryptophan residue in bGH is a good reporter group for self-association and may be in the interface region between self-associating molecules. Such an interaction was observed previously for insulin self-association which involved an increase in the CD of tyrosine residues due to dipole–dipole coupling (Strickland & Mercola, 1976). The similarity between parts A and B of Figure 2 and the data in Figure 6 demonstrates that specific protein–denaturant interactions are not responsible for self-association as both an ionic (Gdn-HCl) and a nonionic (urea) denaturant give similar results. The slight differences in molar ellipticity for the two denaturants may reflect slight differences in the molecular structure of the self-associated form(s), or their relative population, in these two solvent systems. An increase in molar ellipticity of bGH at 300 nm under acidic conditions (pH 3) was observed previously (Edelhoch & Lippoldt, 1970) and confirmed by our own measurements (data not shown). These data and observation of an increased radius (4 nm) by PCS measurements at acidic pH suggest that self-association is also occurring when bGH is denatured by acid as well as with chemical denaturants, as was proposed earlier for a 0.1 M acetate solution (Burger et al., 1966).

Specific intermolecular interactions are also indicated by the bGH concentration dependence (at 3.7 M Gdn-HCl) of the molar ellipticity at 300 nm (Figure 6), the second-derivative trough to peak intensity (Figure 5), and the size as measured by PCS (data not shown). For these data, the measured quantity has reached a plateau at about 4 mg/mL bGH which is consistent with the formation of a specific self-associated structure. A nonspecific interaction between bGH molecules would be expected to continue to increase in size as the bGH concentration is increased.

An estimate of the number of bGH molecules involved in the self-associated species can be obtained from size-exclusion HPLC (Figure 3A): if the monomer expands to a 2.5-nm Stokes' radius at 3.7 M Gdn-HCl, then it will take two to three monomer units to achieve a 4-nm Stokes' radius for the associated species depending on the shape of the unfolded monomer units and their organization in the associated form. More accurate estimates of the number of associated monomers could be obtained by making scattered intensity measurements at lower bGH concentrations (less than the 0.1 mg/mL used here) and/or lower scattering angle (less than 90°), by sedimentation equilibrium experiments, or by chemical cross-linking of the self-associated species.

Biochemical Implications. The presence of self-associated species has important implications to any equilibrium or kinetic folding studies of bGH. As previously reported (Edelhoch & Berger, 1966; Burger et al., 1966; Holladay et al., 1974; Brems et al., 1985), bGH denatures in a multistate process with at least one equilibrium intermediate whereas most protein denaturation processes are highly cooperative and may be rep-

resented by a simple two-state approximation. Multistate denaturation processes provide useful systems for gaining information about folding pathways providing the monomeric intramolecular interactions are distinguishable from the multimeric intermolecular interactions. In this report, we have identified an associated species during equilibrium unfolding and determined denaturation conditions (dilute protein concentrations) for its selective absence. Under dilute protein concentrations where self-association does not occur, the denaturation process still occurs in a multistate manner with at least one well-populated monomeric intermediate as evidenced by the noncoincidence of denaturation transitions when monitored by UV absorbance at 290 nm (which is protein concentration independent), size-exclusion HPLC (Figure 3), and UV circular dichroism (Brems et al., 1986). The importance of the self-associated species in the folding pathway for bGH, i.e., the determination of whether this equilibrium intermediate is also a kinetic intermediate, is still under investigation; however, preliminary results indicate that self-association does occur during the folding process in vitro.

The physiological importance of intermolecular interactions is well documented; the significance of the intermolecular interactions responsible for an equilibrium-associated intermediate has not been determined, but might have relevance to physiological conditions were bGH is highly concentrated such as the ribosomal site of protein synthesis or in the storage secretory granules of the pituitary gland.

Acknowledgments

We acknowledge several helpful suggestions on data interpretation from Dr. R. D. White and comments on the manuscript by Dr. T. F. Holzman.

Registry No. Growth hormone, 9002-72-6.

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