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Equilibrium Denaturation of Pituitary- and Recombinant-Derived Bovine Growth Hormone

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ABSTRACT: Holladay and co-workers [Holladay, L. A., Hammonds, R. G., & Puett, D. (1974) *Biochemistry 13*, 1653-1661] reported the presence of an equilibrium intermediate in the guanidine hydrochloride (GdnHCl) induced denaturation of pituitary-derived bovine growth hormone (p-bGH). Since then, numerous reports have appeared demonstrating the inherent heterogeneity in p-bGH. In this report we show that a standard preparation of p-bGH can be separated into two components of almost equal abundance differing in molecular weight by approximately 1000. Each of these two components could give rise to different denaturation transitions which would be interpreted as evidence for equilibrium intermediates. We report here the equilibrium denaturation of bGH produced by *Escherichia coli* through recombinant DNA technology. The recombinant-derived bGH (r-bGH) is more homogeneous than that derived from pituitary sources and is greater than 95% a single polypeptide entity. Nevertheless, the GdnHCl-induced denaturation profiles of both recombinant bGH and pituitary bGH are very similar. The presence of equilibrium intermediates is verified by the asymmetry of the denaturation transition as measured by size-exclusion high-performance liquid chromatography and by noncoincidence of the denaturation transitions as observed by ultraviolet absorbance, fluorescence intensity, and circular dichroism. These findings conclusively show that the secondary structure of bovine growth hormone is more stable than the tertiary structure and is consistent with a framework model of protein folding.

A fundamental problem remaining in biology today is that of protein folding; that is, how does a random polypeptide chain fold to a highly ordered conformation? Answers to the protein folding problem would help in determining the code by which the amino acid sequence of a protein specifies its tertiary structure. This would aid in the engineering of changes in protein structure through the use of recombinant DNA techniques. Studies of reversible denaturation reactions of

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proteins constitute a valuable approach to this problem, particularly if one can determine the pathway of folding. In order to do this, it is necessary to be able to detect and characterize intermediates in structure between the native and unfolded states. Most denaturation processes are known to be so highly cooperative that equilibrium measurements are represented by a simple two-state approximation and give little information about the folding pathway (Lumry et al., 1966; Privalov & Khechinashvili, 1974; Pace, 1975; Baldwin, 1975). However, in the past few years, several examples of proteins with populated equilibrium intermediates have been reported (Kim & Baldwin, 1982) and have been considered rather exceptional. The study of such equilibrium intermediates provides a useful system to probe the relationship between chemical structure and conformational stability. One such exception to the two-state denaturation process is that of bovine growth hormone (bGH) (Burger et al., 1966; Holladay et al., 1974). The guanidine hydrochloride (GdnHCl) unfolding transition of bGH shows changes in tertiary structure which occur at significantly lower GdnHCl concentrations than do changes in secondary structure. Pituitary preparations of growth hormone have been shown to consist of a class of growth hormone related polypeptides. Naturally occurring variants differing in charge, mass, and heterogeneity at the N-terminus and at residue 127 are well documented (Lorenson & Ellis, 1975; Wallis, 1980; Chawla et al., 1983). Therefore, the possibility exists that equilibrium intermediates which have been reported for pituitary bGH (Holladay et al., 1974) are a consequence of the inherent microheterogeneity of the preparations. We report here equilibrium denaturation studies of recombinant-derived bGH (r-bGH) and demonstrate that while r-bGH is principally a single entity, it unfolds in a multistate process.

At the present time, little is known about the three-dimensional structure of the pituitary hormones. However, bGH (Bell et al., 1985) and the closely related placental hormone human chorionic somatomammotropin [HCS, also known as human placental lactogen (Moffat, 1980; Hunt et al., 1981, 1983)] have been crystallized in forms that are suitable for high-resolution X-ray structure determination. It is anticipated that the X-ray structure determination of bGH and HCS will aid in further understanding the nature of the multistate denaturation of bGH.

Materials and Methods

Materials

Recombinant-derived bGH was obtained from the Amgen Co. of Thousand Oaks, CA. Pituitary-derived bGH (p-bGH) was obtained from A. F. Parlow, lot 7899C, from the Harbour Medical Center of the University of California at Los Angeles. GdnHCl was ultrapure from Schwarz/Mann. Other reagents were of analytical grade. High-performance liquid chromatography (HPLC) size-exclusion columns were TSK-2000 from Toyo Soda Manufacturing Co. and Zorbax GF-250 from Du Pont. Proteins utilized as molecular weight markers for HPLC or sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Sigma.

Methods

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed in a discontinuous slab gel system consisting of a 5% acrylamide stacking gel (with L-10 buffer) and a 14% acrylamide separating gel (with L-20 buffer). This is a nongradient gel which utilizes the reagents and buffers of the ISO-DALT system which has been developed by the Argonne National Laboratory (Anderson, N. G., & Anderson, 1978; Anderson, N. L., & Anderson, 1978; Tollaksen, 1981). The ISO-DALT system consists of a series of modifications of the original technique of O'Farrell (1975).

HPLC Size Exclusion. For size-exclusion chromatography of native growth hormone in nondenaturing solvents, two Zorbax GF-250 columns (each 9.4 mm × 25 cm) arranged in series were utilized and developed at 1 mL/min with 0.1 M sodium phosphate, pH 7.0.

For denaturation studies, the elution time of bGH from a TSK-2000 column was measured as a function of GdnHCl concentration. The column was preequilibrated at each GdnHCl concentration, containing 0.01 M tris(hydroxymethyl)aminomethane (Tris) pH 8.0, prior to application of the protein sample (1 mg/mL) and developed isocratically at a flow rate of 1 mL/min. Protein molecular weight calibration curves for the TSK-2000 column in 6.0 M GdnHCl were determined and utilized to calculate the Stokes radii of bGH in varying concentrations of GdnHCl as described by Corbett & Roche (1984).

Sample Preparation for Equilibrium Denaturation. Samples were prepared by appropriate dilutions from stock solutions of 8 M GdnHCl, 1.0 M NH_4HCO_3 (pH 8.0–8.5), and bGH in 50 mM NH_4HCO_3 (pH 8.0–8.5) at concentrations of 4–5 mg/mL. Samples were made to constant volume with H_2O and a final protein concentration of 0.4–0.5 mg/mL. All stock solutions were filtered through a 0.45-μm filter prior to mixing. Vigorous mixing of solutions containing bGH induced turbidity; thus, care was taken to minimize surface denaturation during mixing. All measurements of bGH solutions were taken at 23 °C and were stable for at least 24 h.

Fluorescence Measurements. Fluorescence intensities were measured on a Perkin-Elmer MPF-44B fluorescence spectrophotometer. The excitation wavelength was 283 nm, and emission was monitored at 340 nm with slit widths of 4 nm. All intensities are referenced to *N*-acetyltryptophanamide which has an extinction coefficient of 5600 M^{-1} cm^{-1} at 280 nm. Blanks containing GdnHCl and buffer were also measured but gave no significant fluorescent emission.

Absorbance Measurements. The GdnHCl-induced difference spectrum is characterized by a 290-nm maximum which was the wavelength chosen for monitoring equilibrium denaturation. Absorbance measurements were taken on a Cary 219 spectrophotometer. The bGH concentrations were determined by the absorbance at 278 nm using an extinction coefficient of 15.27×10^3 cm^{-1} M^{-1} (Burger et al., 1966).

Circular Dichroism Measurements. CD spectra were obtained with a Jasco J-40 spectrophotometer. The far-ultraviolet spectrum has a minimum at 222 nm that corresponds to helix and is the wavelength for which changes in helicity were monitored. The mean residue ellipticity, $[\theta]$ in degrees centimeter squared per decimole, was calculated by using a mean residue weight of 113.

Reversibility. All the denaturation transitions were completely reversible within the transition zone (2.0–6.0 M GdnHCl). When protein samples containing denaturing amounts of GdnHCl were diluted to less than 2.0 M GdnHCl, irreversible turbidity developed. However, if the denatured samples were diluted to 2.0 M GdnHCl and allowed to fold for several minutes and then diluted to lower GdnHCl concentration, turbidity did not appear.

Results

Recombinant-Derived bGH Is a Single Entity Whereas Pituitary-Derived bGH Is Heterogeneous. SDS–PAGE of p-bGH demonstrates the presence of at least two major components (Figure 1). Densitometer traces indicate that the two major polypeptide bands constitute more than 95% of the

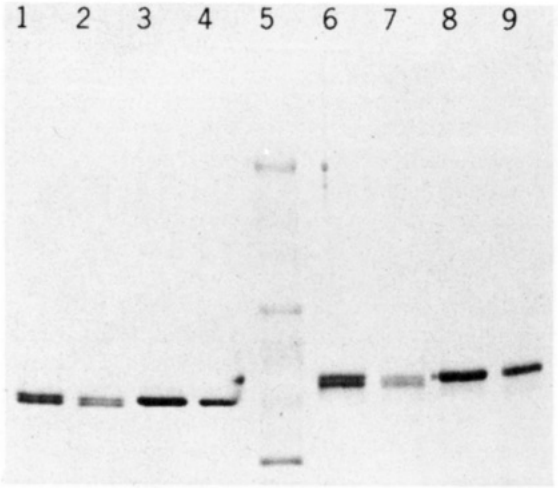


FIGURE 1: SDS–PAGE of bGH. Lanes 1 and 6 (20-μg sample application) and lanes 2 and 7 (10-μg sample application) are pituitary bGH. Lanes 3 and 8 (20-μg sample application) and lanes 4 and 9 (10-μg sample application) are recombinant bGH. Lane 5 is the molecular weight standards bovine serum albumin, carbonic anhydrase, and lysozyme, in descending order. Lanes 1–4 were subjected to electrophoresis under nonreducing conditions and lanes 5–9 to reducing conditions.

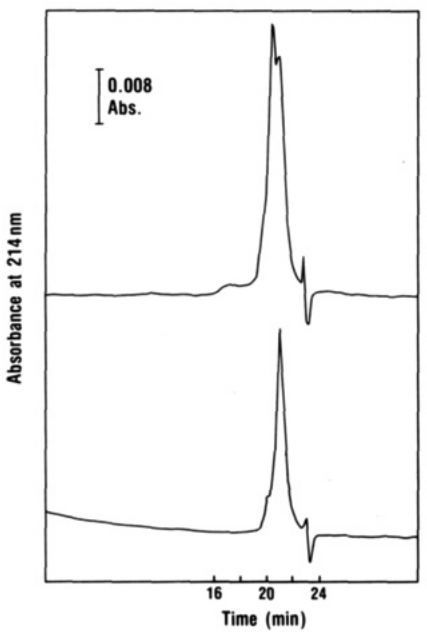


FIGURE 2: HPLC size exclusion of bGH in nondenaturing solvents. The upper trace represents the elution profile of pituitary bGH and the lower trace that of recombinant bGH. The protein sample concentration was 1.25 mg/mL.

stainable bands and have apparent molecular weights of 19 500 and 18 500 in nonreducing conditions and 22 500 and 21 000 in reducing conditions. This p-bGH is of the highest purity that is commercially available and has a specific activity of 1.9 IU/mg (World Health Organization bGH standard is 1.0 IU/mg) as determined by the conventional weight gain assay in the hypophysectomized rat (Evans et al., 1933). In comparison, the bGH used by Holladay et al. (1974) in their studies had a specific activity of 0.92 IU/mg. The detailed nature of the two components of pituitary-derived bGH has not been determined. A likely possibility is the documented heterogeneity at the N-terminus (Wallis, 1973). Both components are present in all p-bGH preparations that we have screened utilizing several different preparation procedures from the current literature. It is probable that these two components

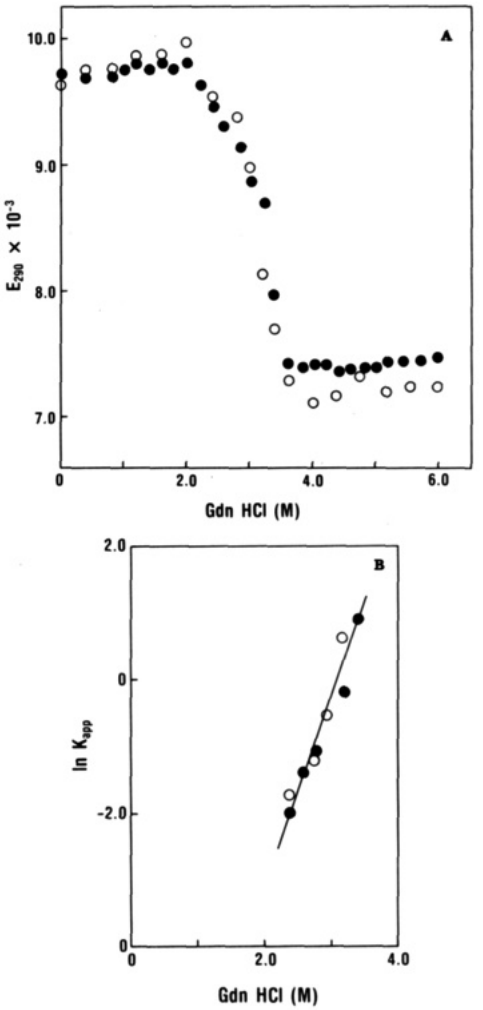


FIGURE 3: Equilibrium denaturation of bGH as monitored by UV absorbance. The open circles represent pituitary bGH, and the closed circles represent recombinant bGH. The protein sample concentration was 0.4–0.5 mg/mL. (A) Molar extinction coefficient at 290 nm vs. GdnHCl concentration. (B) Natural logarithm of the apparent equilibrium constant for unfolding as a function of GdnHCl concentration. The slope of the line drawn is 2.6 mol^{-1}, and the GdnHCl concentration required to generate equivalent concentrations of the assumed two states is 3.1 M.

are present in all current and past pituitary preparations. SDS–PAGE demonstrates that r-bGH consists of a single polypeptide band with an apparent molecular weight of 19 500 in nonreducing conditions and 22 500 in reducing conditions and represents more than 95% of the stainable bands. Reverse-phase HPLC utilizing a linear gradient of acetonitrile in 0.1% trifluoroacetic acid indicates that both p-bGH and r-bGH are greater than 95% pure. Recombinant-derived bGH has a specific activity of 2.5 IU/mg.

Data obtained by HPLC size exclusion are similar to the SDS–PAGE results in that p-bGH elutes as two distinguishable peaks whereas r-bGH elutes as a single sharp peak (Figure 2). The elution peak of r-bGH shows a small shoulder on the leading edge which represents less than 5% of the total bGH peak area.

Equilibrium Denaturation of Pituitary- and Recombinant-Derived bGH Is Similar. (A) Absorbance. The GdnHCl-induced difference spectrum is characterized by a 290-nm maximum (Holladay et al., 1974). Figure 3A shows that, as a function of GdnHCl concentration, the absorbance at 290 nm for pituitary- and recombinant-derived bGH is indistinguishable. The Gibbs free energy of stabilization of bGH can be estimated by assuming a two-state analysis of the

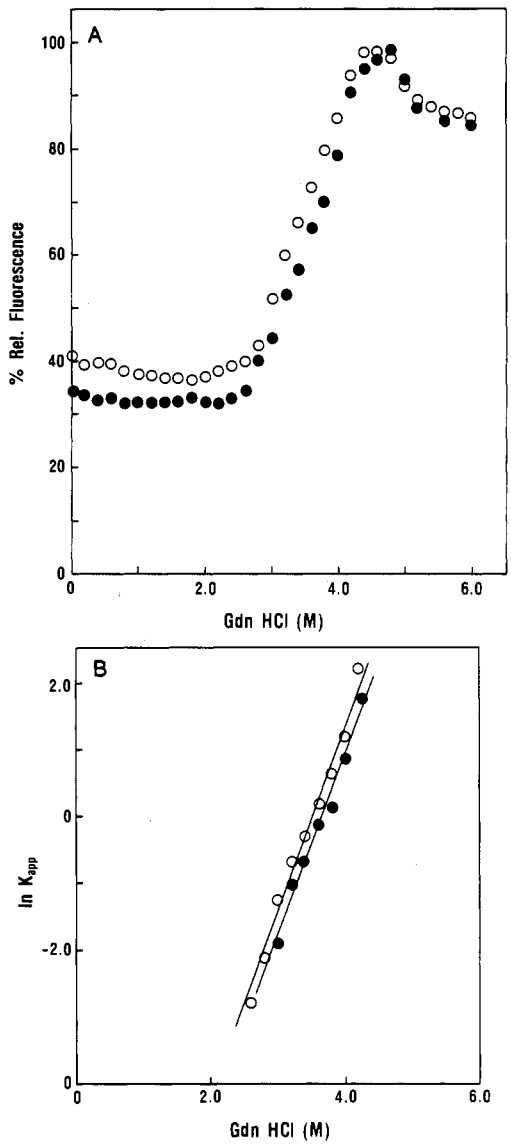


FIGURE 4: Equilibrium denaturation of bGH as monitored by fluorescence. The open circles indicate pituitary bGH, and the closed circles indicate recombinant bGH. The protein sample concentration was 0.4–0.5 mg/mL. (A) The percent fluorescence is relative to that of an equimolar concentration of *N*-acetyltryptophanamide in the same solvent. (B) Natural logarithm of the apparent equilibrium constant for unfolding as a function of GdnHCl concentration. The slope of the lines drawn is 2.6 mol^{-1}, and the GdnHCl concentrations required to generate equivalent concentrations of the assumed two states are 3.5 M for pituitary bGH and 3.67 M for recombinant bGH.

data presented in Figure 3A. A two-state treatment of equilibrium unfolding transitions is justified on empirical grounds (Lapanje, 1978). We have used the method of Schellman (1978) for analyzing solvent denaturation processes to obtain the free energy of unfolding in the absence of denaturant. Figure 3B shows that the relationship between the natural logarithm of the apparent equilibrium constant for unfolding and GdnHCl molarity is linear and gives an apparent free energy of unfolding of 4.7 kcal/mol for both pituitary and recombinant bGH.

(*B*) *Fluorescence.* The fluorescence of native bGH has an emission maximum at 325 nm at an excitation wavelength of 283 nm. Upon unfolding, the fluorescence emission undergoes a red-shift of 15 nm and an increase of intensity, and the band splits into two components. Figure 4A shows the similarities in equilibrium denaturation for pituitary- and recombinant-derived bGH as monitored by fluorescence. Analysis of this transition results in an apparent free energy of unfolding of 5.3 and 5.6 kcal/mol for pituitary and recombinant bGH, respectively (Figure 4B).

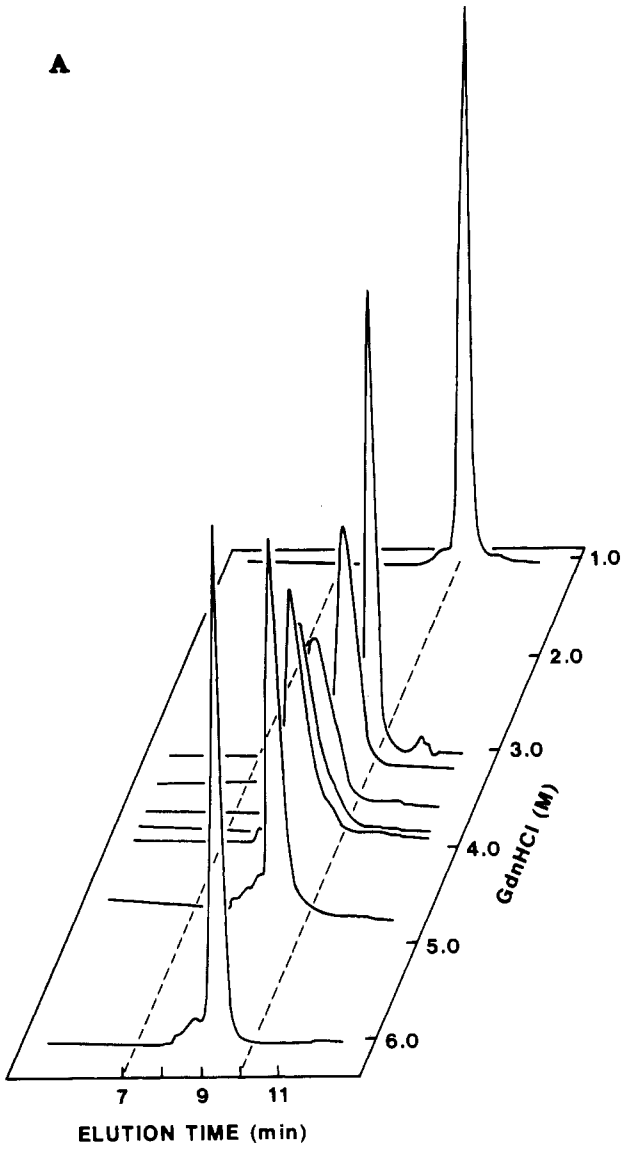
(*C*) *HPLC Size Exclusion.* HPLC size exclusion has recently been developed and applied to the measurement of molecular volume changes of denaturing proteins (Saito & Wada, 1983a,b; Corbett & Roche, 1984). Figure 5A illustrates the effect of GdnHCl denaturation on the elution of bGH from HPLC size exclusion. The GdnHCl-induced changes in the hydrodynamic exclusion volume for pituitary- and recombinant-derived bGH are alike. Within the denaturation transition region, the bGH elution peak shifts to smaller elution volumes and broadens (Figure 5B). This is expected if the conversion rate between the native conformation and any unfolded species is faster than the rate of gel permeation. It was not possible to measure the hydrodynamic exclusion volume of bGH in low levels of GdnHCl because bGH did not elute from the TSK-2000 column when the mobile phase contained less than 1.0 M GdnHCl. A calibration curve was obtained with 6 M GdnHCl by utilizing the molecular weight (M_r) standards ovalbumin, carbonic anhydrase, lysozyme, and aprotinin. In 6 M GdnHCl, bGH corresponds to a molecular weight of 22 000, which is in good agreement with that observed in SDS–PAGE. In nondenaturing concentrations of GdnHCl, bGH elutes with an apparent molecular weight of 9000 when extrapolated from the molecular weight calibration curve obtained in 6 M GdnHCl. Figure 5B illustrates that equilibrium unfolding of bGH, as monitored by HPLC size exclusion, is a multistate process and cannot be estimated by a two-state analysis.

(*D*) *Circular Dichroism.* The far-ultraviolet CD spectrum of native bGH indicates the presence of 45–50% helix content with minima at 222 and 208 nm (Holladay et al., 1974). Figure 6A shows the relative ellipticity at 222 nm for both pituitary and recombinant bGH as a function of GdnHCl concentration. The values obtained for the apparent free energy of unfolding for this transition are 5.9 and 6.7 kcal/mol for p-bGH and r-bGH, respectively (Figure 6B).

Equilibrium Denaturation of bGH Is a Multistate Process. There are two applicable tests for an equilibrium intermediate: (a) a biphasic transition as measured by a single probe and (b) noncoincident transitions as measured by different probes. Either one of these observations is sufficient evidence for an equilibrium intermediate. The equilibrium unfolding of bGH, as monitored by size-exclusion chromatography (Figure 5B), is asymmetrical and is not consistent with a two-state phenomenon. Figure 7 is a plot of the comparative equilibrium denaturation transitions of r-bGH as measured by three different probes. The noncoincidence of the transitions also demonstrates that the equilibrium unfolding of r-bGH is a multistate process, with at least two well-populated equilibrium intermediates. Furthermore, Figures 3A, 4A, and 6A show that p-bGH, which consists of at least two major components, has the same unfolding characteristics as r-bGH which is predominantly a single entity. Therefore, we conclude that bGH unfolding is a legitimate exception to the highly cooperative two-state unfolding transition observed for most small globular proteins.

Discussion

The GdnHCl denaturation of bovine growth hormone is completely reversible and is not consistent with a two-state transition. When different observables are used to monitor denaturation, a noncoincidence of the transitions is observed (Figure 7). The analysis of denaturation curves in terms of a two-state approximation is useful even when denaturation



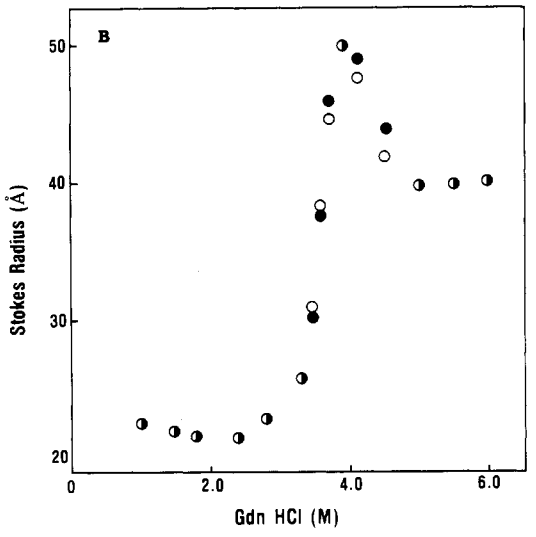


FIGURE 5: Equilibrium denaturation of bGH as measured by HPLC size exclusion. The protein sample concentration upon injection was 1.0 mg/mL. (A) Chromatographic elution profile of recombinant-derived bGH as a function of GdnHCl concentration. (B) Stokes radii of pituitary (open symbols) and recombinant (closed symbols) bGH as a function of GdnHCl concentration.

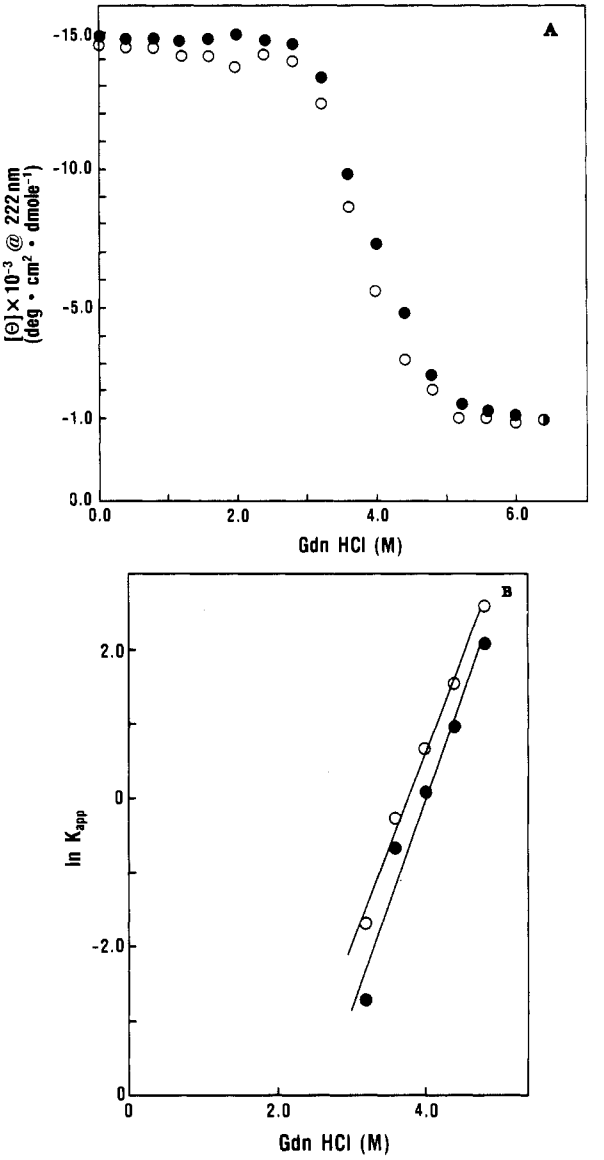


FIGURE 6: Equilibrium denaturation of bGH as monitored by CD. The open circles represent pituitary bGH, and the closed circles represent recombinant bGH. The protein sample concentration was 0.4–0.5 mg/mL. (A) Mean residue ellipticity of 222 nm vs. GdnHCl concentration. (B) Natural logarithm of the apparent equilibrium constant for unfolding as a function of GdnHCl concentration. The slopes of the lines drawn are 2.67 mol^{-1} for pituitary bGH and 2.85 mol^{-1} for recombinant bGH, and the GdnHCl concentrations required to generate equivalent concentrations of the assumed two states are 3.76 M for pituitary bGH and 4.0 M for recombinant bGH.

deviates substantially from a two-state mechanism. For a two-state approximation, the fraction of protein denatured is defined as f_D where $f_D = (Y - Y_N)/(Y_D - Y_N)$ and Y_N, Y_D, and Y are the values of the observable for native, denatured, and at intermediate points in the transition, respectively. When intermediates are present, as is the case for bGH, the apparent equilibrium constant and apparent free energy of unfolding are used rather than K_D and ΔG_D. Tanford (1968) has shown that for stable intermediates characterized by the property Y_i and concentration f_i, then the observed extent of unfolding $f_{obsd} = f_D + \sum_i f_i Z_i$ where $Z_i = (Y_i - Y_N)(Y_D - Y_N)$. Thus, f_{obsd} will differ from f_D by an amount that depends on the concentration of the intermediates weighted by their Z_i values. For some observables such as circular dichroism and hydrodynamic exclusion volume, the Z_i value will be roughly proportional to the extent to which the intermediate is unfolded. For other observables, like UV absorbance and fluorescence,

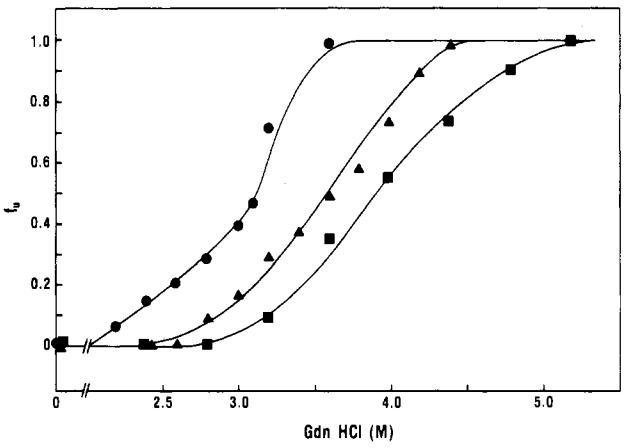


FIGURE 7: Fraction of unfolded recombinant bGH as a function of GdnHCl concentration. f_u ($=Y-Y_N)/(Y_D-Y_N$) was calculated from UV absorbance at 290 nm (●), fluorescence (▲), and CD at 222 nm (■).

whose measurements are dependent on only a small number of residues, there may be little correlation between Z_i and the extent of unfolding. The relationship between K_{app} [$=f_{obsd}/(1-f_{obsd})$] and K_D ($=f_D/f_N$) is

$$K_{app} = K_D \frac{1 + \sum Z_i K_i / K_D}{1 + \sum (1 - Z_i) K_i}$$

where $K_i = f_i/f_N$. For reasonable values of Z_i (0.1–0.9), K_D and K_{app} will be equal somewhere near the midpoint of the transition ($K_{app} = 1$), but K_D will be less than K_{app} below the midpoint and greater than K_{app} above the midpoint of the transition. Thus, the presence of intermediates will cause the slope of plots such as those in Figures 3B, 4B, and 6B to be less than the slope would be for a plot of a strictly two-state denaturation. This means that estimates of the free energy of unfolding derived from two-state analysis of a system with stable intermediates will at least serve as lower limits for the true value of ΔG. A two-state approximation of the denaturation curves in Figure 7 results in different free energies of unfolding for each of the observables. It is curious that two observables, which predominantly reflect changes in the environment around the tryptophan (absorbance at 290 nm and fluorescence), are noncoincident. In the native conformation, the fluorescence intensity of the tryptophan is significantly quenched (Figure 2A). The quenching of fluorescence is thought to be due to the nearby presence of an electron acceptor (Burger et al., 1966). We have obtained preliminary evidence that lysine is related to fluorescence quenching. This is suggested by unpublished work in which the lysine residues of bGH have been chemically acylated with citraconic anhydride with retention of native structure as monitored by UV absorbance, CD, and hydrodynamic exclusion volume but with anomalous tryptophan fluorescence.

Figure 7 demonstrates that the probes of tertiary structure, such as UV absorbance and fluorescence, reflect denaturation at lower concentrations of GdnHCl than does a probe of secondary structure such as circular dichroism. The relative stability of the secondary structure with respect to the tertiary structure is consistent with the framework model of protein folding (Ptitsyn & Finkelstein, 1980; Kim & Baldwin, 1982) in which the hydrogen-bonded secondary structure is formed early and precedes tertiary interactions. Kinetic folding studies of ribonucleases A and S have demonstrated that secondary structural changes as monitored by CD and hydrogen exchange occur more rapidly than tertiary structural changes as reflected by burial of aromatic residues or regain of catalytic activity (Schmid & Baldwin, 1979; Kim & Baldwin, 1980; Brems & Baldwin, 1985; Labhardt, 1985). Other folding studies which support the framework model are equilibrium and kinetic measurements of carbonic anhydrase (McCoy et al., 1980; Wong & Tanford, 1973; Ko et al., 1977), equilibrium studies on penicillinase (Robson & Pain, 1973), and equilibrium and kinetic studies of α-lactalbumin (Kuwajima et al., 1976; Kuwajima, 1977). Thus, evidence has accumulated in a variety of proteins in support of a framework model.

Our observations are generally consistent with that previously reported by Burger et al. (1976) and Holladay et al. (1974). Burger et al. reported that for pituitary bGH, a pH-induced unfolding intermediate exists that contains considerable helical structure but has a UV absorption spectrum of denatured protein. Holladay et al. demonstrated the presence of a similar intermediate induced by GdnHCl as observed by the noncoincidence of the UV absorbance and circular dichroic detected transitions. We report here a more complex denaturation process as evidenced by the existence of multiple equilibrium unfolding intermediates. Moreover, the standard purification of pituitary-derived growth hormone does not provide a homogeneous product, but rather a mixture of native and modified forms and fragments of growth hormone, each of which may have different denaturation transitions. For proteins which demonstrate such natural variance, preparations from bacteria using recombinant DNA technology provide a model source of a single protein. Using a homogeneous preparation of recombinant bGH, we have demonstrated the existence of equilibrium intermediates in the denaturation of bGH. At the present time, it is not certain if any of these equilibrium intermediates are kinetic intermediates on the pathway of folding. We are currently pursuing kinetic folding studies to determine if any of these equilibrium intermediates have kinetic significance.

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Studies of 6-Fluoropyridoxal and 6-Fluoropyridoxamine 5′-Phosphates in Cytosolic Aspartate Aminotransferase†

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ABSTRACT: The chemical and spectroscopic properties of 6-fluoropyridoxal 5′-phosphate, of its Schiff base with valine, and of 6-fluoropyridoxamine 5′-phosphate have been investigated. The modified coenzymes have also been combined with the apo form of cytosolic aspartate aminotransferase, and the properties of the resulting enzymes and of their complexes with substrates and inhibitors have been recorded. Although the presence of the 6-fluoro substituent reduces the basicity of the ring nitrogen over 10000-fold, the modified coenzymes bind predominately in their dipolar ionic ring forms as do the natural coenzymes. Enzyme containing the modified coenzymes binds substrates and dicarboxylate inhibitors normally and has about 42% of the catalytic activity of the native enzyme. The fluorine nucleus provides a convenient NMR probe that is sensitive to changes in the state of protonation of both the ring nitrogen and the imine or the -OH group of free enzyme and of complexes with substrates or inhibitors. The NMR measurements show that the ring nitrogen of bound 6-fluoropyridoxamine phosphate is protonated at pH 7 or below but becomes deprotonated at high pH around a pK_a of 8.2. The bound 6-fluoropyridoxal phosphate, which exists as a Schiff base with a dipolar ionic ring at high pH, becomes protonated with a pK_a of ~7.1, corresponding to the pK_a of ~6.4 in the native enzyme. Below this pK_a a single ^{19}F resonance is seen, but there are two light absorption bands corresponding to ketoenamine and enolimine tautomers of the Schiff base. The tautomeric ratio is altered markedly upon binding of dicarboxylate inhibitors. From the chemical shift values, we conclude that during the rapid tautomerization a proton is synchronously moved from the ring nitrogen (in the ketoenamine) onto the aspartate-222 carboxylate (in the enolimine). The possible implications for catalysis are discussed.

The function of pyridoxal 5′-phosphate (PLP)[1] in its catalysis of reactions of amino acids is to provide an electron-accepting center that permits cleavage of C–H or C–C bonds within the substrate. This function makes use of the powerful electron-withdrawing action of the N-protonated pyridine ring (**1**). However, this electron withdrawal is modulated by the donation of electrons into the ring from the unprotonated phenolic

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; 6-FPLP, 6-fluoropyridoxal 5′-phosphate; 6-FPMP, 6-fluoropyridoxamine 5′-phosphate; E-FPLP and E-FPMP, complexes of cytosolic apo-aspartate aminotransferase with 6-FPLP and 6-FPMP; Tris, tris(hydroxymethyl)aminomethane.

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