pH-Induced Conformational States of Bovine Growth Hormone

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ABSTRACT: The folding behavior of bovine growth hormone (bGH) is examined by chemical and pH denaturation using several spectroscopic probes of protein secondary and tertiary structure. Partially denaturing concentrations of urea eliminate the native-state quenching of intrinsic tryptophan fluorescence, from the single protein tryptophan, but the fluorescence emission spectrum is not red-shifted like the unfolded state, and the protein retains substantial secondary structure. A neutral-to-acid pH shift also eliminates tryptophan quenching; however, the loss of quenching is not accompanied by an emission red-shift. In addition, the protein undergoes a pH-dependent UV absorbance transition; the changes in absorptivity have the same midpoint as the transition associated with the change in intrinsic tryptophan fluorescence. The magnitude of the absorption transition is similar to that observed previously for urea denaturation of the protein. In a similar fashion, a pH-dependent CD transition is also observed; however, the transition occurs at a higher pH. The behavior of the various optical probes indicates that the pH-induced conformational transition produces a highly populated species in which the microenvironment surrounding the single protein tryptophan residue resembles that observed during the urea-induced unfolding/refolding transition. The pH-induced changes in tertiary structure occur at a lower pH than the changes associated with a portion of the secondary structure. Proton NMR of the low-pH intermediate indicates that the three His and six Tyr resonances are indistinguishable from the unfolded state. The intermediate(s) observed by either chemical or pH-induced denaturation resemble(s) a molten globule state which contains significant secondary structure. The residual secondary structure present in the intermediate could be nonnative. However, the pH transition is reversible, which implies that even if the secondary structure is not along a major folding pathway it still represents a bonafide intermediate state. The state has unique spectral characteristics which include the disruption of certain aromatic hydrophobic interactions, unquenched but non-red-shifted tryptophan fluorescence, a reduced 290-nm extinction and blue-shifted absorbance, ~50% of the folded-state 222-nm CD signal, and proton NMR His and Tyr resonances which are characteristic of an unfolded state.

Bovine growth hormone (somatotropin) contains two disulfide bonds that bridge positions 53–164 (large loop) and 181–189 (short loop) of the primary structure (Santome et al., 1973; Wallis, 1973). The crystal structure of a closely related protein, porcine growth hormone, has recently been reported at 2.8-Å resolution (Abdel-Meguid et al., 1987). Analysis of the X-ray crystal structure revealed that the protein is comprised of a left-twisted bundle of four antiparallel α-helices. The helical regions encompass residues 8–34, 75–96, 106–128, and 153–183 (Abdel-Meguid et al., 1987). Bovine growth hormone contains a single tryptophan residue at position 86 of the sequence (Santome et al., 1973; Wallis, 1973). In the three-dimensional structure of the porcine growth hormone, the tryptophan is located in the second helix at position 86 (Abdel-Meguid et al., 1987). The absorption and fluorescence properties of this amino acid make it useful as an intrinsic probe for the study of bGH tertiary structure (Burger et al., 1966; Edelhoch & Burger, 1966). The fluorescence quantum yield, the wavelength of maximum emission of the indole fluorophore, and the molar absorptivity are sensitive to various forms of perturbation [see Lakowicz (1983) and references cited therein]. Changes in tryptophan absorption and fluorescence can provide information about the solution environment surrounding the indole ring. Proton nuclear magnetic resonance (NMR)[1] is another useful tool for studying protein structure and conformation. NMR studies of the reversible denaturation of proteins indicate the presence of intermediates in their unfolding processes [for example, see Kim and Baldwin (1982) and references cited therein]. In the absence of knowledge of the resonances of specific residues in a protein sequence, it is still possible to examine the behavior of classes of residues and the degree to which they are perturbed in response to changes in solvent and environmental conditions (Baum et al., 1989). The only reported ^{1}H NMR study of bovine growth hormone to date involved the assignment of the resonances of the three histidyl residues (MacKenzie et al., 1989). Thus, it was of some interest to explore the extent of which NMR could prove useful in examining any solvent-dependent conformational equilibria of bGH.

It has been demonstrated that scission of one or both disulfide bonds give rise to species that retain substantial ordered structure at low denaturant concentrations (~4 to ~7 M urea) (Holzman et al., 1986). The guanidine- or urea-induced

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[1] Abbreviations: bGH, bovine growth hormone; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NATA, *N*-acetyltryptophanamide; His, histidine; NMR, nuclear magnetic resonance.

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folding transition of the protein is consistent with the existence of folding intermediates and a molten globule structure (Holladay et al., 1974; Brems et al., 1985, 1987a; Holzman et al., 1986; Brems & Havel, 1989). Examination of the equilibrium denaturation behavior of native and S-modified forms of bGH using both CD and UV spectroscopy indicated that denaturant (urea) could be used to solubilize and poise the reduced molecule for reoxidation from a retained secondary structure to form native monomer in high yield (Holzman et al., 1986). In order to extend our understanding of the nature of the equilibrium folding events in bGH and the various signals associated with the folding processes, we have examined the pH-induced equilibrium folding/unfolding of bGH.

Experimental Procedures

Materials

Recombinant DNA derived growth hormone was purified from *Escherichia coli* as previously described (Evans & Knuth, 1985). Ultrapure urea was purchased from Schwarz/Mann. Glycine-d_4 (99 atom %) was from Cambridge Isotopes, and deuterium oxide (99.9 atom %) was from Merck, Sharp & Dohme. All other reagents and solvents were analytical reagent grade or better.

Methods

Sample Preparation for Urea Equilibrium Denaturation Measured by Fluorescence and UV Absorbance. Stock solutions were prepared by dissolving 10 mg of protein in 1.0 mL of 8.0 M guanidine hydrochloride, 40 mM CHES, and 4 mM glycine, pH 9.1 (0.44 mM final [bGH]), followed by centrifugation at 15000*g* for 15 min at 4 °C. Samples for fluorescence measurements were prepared by diluting the protein stock solutions to a final concentration of 10 μM in varying urea concentrations containing 40 mM CHES/4 mM glycine, pH 9.1. The protein concentrations of the diluted samples were determined from an equivalent dilution into 7.2 M guanidine, 20 mM CHES, and 1 mM glycine, pH 9.1, using the molar absorptivity of the unfolded protein of 7500 at 290 nm (Brems et al., 1985). Diluted samples were allowed to come to equilibrium by incubation for ~1 h at room temperature prior to measurement. Unfolding transitions, across the urea concentrations employed, were completely reversible.

Sample Preparation for pH Titration Measured by Fluorescence, UV Absorbance, and Circular Dichroism. Stock solutions were prepared and the diluted sample concentrations were determined as described above. Samples were prepared by diluting the protein to a concentration of 10 μM into solutions buffered at various pH values containing nondenaturing concentrations of urea (4.5 M) to permit direct comparison to previous observations (Holzman et al., 1986). The final solution composition was 4.5 M urea and 20 mM in each of the following buffers: sodium acetate, MES, Tris-HCl, and sodium bicarbonate. The diluted samples were allowed to equilibrate for ~1 h at room temperature before fluorescence measurements were performed. The observed pH-induced transitions were found to be reversible over the pH range examined. The pH titration behavior was found to exhibit a small but measurable sensitivity to ionic strength, in a fashion similar to that previously reported (Burger et al., 1966).

Fluorescence, UV Absorbance, and Circular Dichroism Measurements. Fluorescence measurements were made at 25 °C using an SLM 8000 spectrofluorometer equipped with an IBM XT computer. In order to observe the protein tryptophan fluorescence, an excitation wavelength of 295 nm with an 8-nm band-pass was chosen. All fluorescence data presented are technical spectra plotted as the relative fluorescence and were obtained with identical instrument settings to permit direct comparison of both main figures and insets in the data presented. UV absorbance measurements were performed as previously described (Holzman et al., 1986) using a Hewlett Packard 8451A diode array spectrophotometer; all samples were equilibrated to 25 °C (±0.1 °C). Circular dichroism measurements were performed with a Jasco J-500C spectropolarimeter as previously described (Holzman et al., 1986).

Sample Preparation for Proton NMR Measurements. Fully deuterated urea (urea-d_4) was prepared by dissolving urea (Schwarz/Mann Ultra-pure) in deuterium oxide (Merck) followed by freeze–drying. This cycle was repeated 3 times. Protein samples for NMR analysis were prepared by dissolving 20 mg of protein in 1.0 mL of 4.5 M urea-d_4 in D_2O with 10 mM glycine-d_4, pD 9.5. For direct measurement of the pK values using NMR, it has been shown that the effect of deuterium on the glass electrode is offset by an isotope effect acting in the reverse direction on the ionizable group itself (Kalinichenko, 1976; Wüthrich & Bundi, 1979), making corrections for isotope effects on the glass electrode unnecessary. Under the conditions above, exchangeable protons (amino, amido, hydroxyl, carboxyl) were fully exchanged with deuterium within a few minutes. The solution pD was adjusted with small quantities of dilute DCl or NaOD, and pD readings were taken before and after data acquisition. The spectrum was accepted only if the deviation of the pD was less than 0.04 unit. Protein samples were incubated for at least 30 min at 25 °C before NMR analysis.

NMR Spectroscopy. ^{1}H NMR spectra were recorded at 25 °C on a Bruker AM-400 spectrometer operating at 399.96 MHz for ^{1}H nuclei in the Fourier-transform mode. A total of 500 transients were averaged for each spectrum; each transient was obtained by quadrature detection, with a spectral width of 4 kHz over 16000 data points and an acquisition time of 2.0 s. The free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz prior to Fourier transformation. Chemical shifts (ppm) are expressed relative to internal dioxane (3.740 ppm) downfield from 4,4-dimethyl-4-silapentane-1-sulfonate. Residual HOD was suppressed by presaturation (1 s) prior to data acquisition.

Results and Discussion

Fluorescence Measurements of Urea-Induced Equilibrium Denaturation. Upon unfolding in urea, the relative fluorescence intensity of the tryptophan residue in the protein undergoes about a 3-fold increase (Figure 1). These data indicate that the intrinsic fluorescence of folded bGH is quenched relative to the unfolded protein formed in higher concentrations of urea. A comparison of these results to the behavior of the model compound, NATA, under identical solution conditions demonstrates that the change in the relative fluorescence intensity is due to urea-induced changes in the protein structure (Figure 1, inset). Examination of the spectra presented in Figure 1 reveals that the wavelength of the intensity maximum of the fluorescence emission does not measurably change until the protein is exposed to high concentrations of urea (>~9 M urea), an effect also observed in guanidine (H. Havel, unpublished observation). In the absence of a folding intermediate(s), a simple two-state model would apply to the equilibrium denaturation of a protein (Baldwin, 1975; Pace, 1975). For a two-state transition, a continuous red-shift in the wavelength of the fluorescence emission would be anticipated as the denaturant concentration is increased. Similarly, the observed wavelength of the emission maximum would be a function of the relative popu-

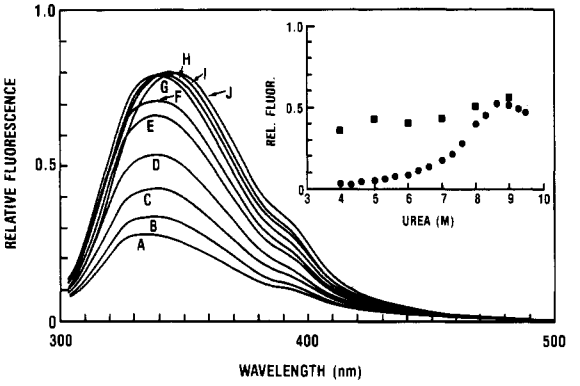

FIGURE 1: Urea-induced equilibrium denaturation of bGH as measured by intrinsic protein tryptophan fluorescence. All spectral data were obtained with identical instrument settings and protein concentrations in both the main figure and inset. Samples contained 10 μM bGH, 40 mM CHES, 4 mM glycine, pH 9.1, and varying concentrations of urea. The technical fluorescence spectra of bGH at various concentrations of urea were measured at 25 °C using an excitation wavelength of 295 nm. Spectra A–J were obtained in urea concentrations of 4.0, 6.0, 7.0, 7.6, 8.0, 8.3, 8.6, 9.0, 9.3, and 9.5 M, respectively. Inset: Equilibrium denaturation curve of bGH (circles). The relative fluorescence intensity data at 335 nm. The effect of urea concentration on the fluorescence of *N*-acetyltryptophanamide (NATA) is also provided (squares). The fluorescence emission maximum of NATA in the same buffer at 10 μM is constant at 350 nm across the range of urea concentrations examined.

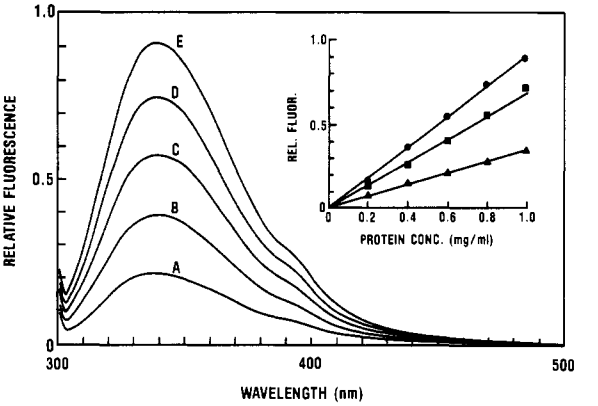

FIGURE 2: Effect of protein concentration on the urea-induced equilibrium denaturation of bGH. All spectral data were obtained with identical instrument settings and solvent conditions in both the main figure and inset. The fluorescence spectra of bGH (A–E) were recorded at protein concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL (10–45 μM) in 7.6 M urea, 40 mM CHES, and 4 mM glycine, pH 9.1. Fluorescence spectra were also obtained for bGH at identical protein concentrations in 9.0 M urea and 4.6 M urea (data not shown). Inset: A plot of the fluorescence intensity at 335 nm versus the protein concentration at 9.0 M urea (circles), 7.6 M urea (squares), and 4.6 M urea (triangles). The fluorescence intensities were taken directly from the digitized spectral data and are corrected for inner filter effects using the estimation described by Lakowicz (1983).

lations of native and fully unfolded protein. This red-shift would result from the exposure of the tryptophan residue to bulk solvent (Lakowicz, 1983). Since the shift in the emission wavelength maximum as a function of urea concentration does not coincide with the changes in tryptophan fluorescence quenching over the same denaturant concentration (Figure 1), the partially folded (unquenched) protein occupies an intermediate state between native (quenched) and unfolded (unquenched and red-shifted). Thus, the folding of the protein does not appear to fit a simple two-state process.

A plot of the fluorescence intensity (335 nm) as a function of urea concentration gives rise to a characteristic curve for bGH (Figure 1, inset). Specifically, the curve is an index of the change in solvent exposure during unfolding. Although the extent of the transition is limited by the solubility of urea at the temperature of the experiment, the midpoint is estimated to be 7.7–7.8 M urea. By comparison, the UV absorption and circular dichroism transition midpoints for the equilibrium unfolding of bGH in urea are respectively 7.9 and 8.3 M (Holzman et al., 1986) under identical solution conditions. The transition midpoints determined in urea for the 290-nm absorbance and the 335-nm fluorescence are similar. However, the absence of coincident transition midpoints for the 222-nm CD signal in comparison to these two probes is consistent with the presence of an equilibrium folding intermediate(s) (Baldwin, 1975; Pace, 1975) and is in agreement with previous reports (Holladay et al., 1974; Burger et al., 1976; Brems et al., 1985).

Havel et al. (1986) have observed a small degree (~5–15%) of quenching of tryptophan fluorescence at high protein concentrations (~1 mg/mL) for partially folded protein during guanidine-induced unfolding and have suggested a relationship to association of monomers. This report (Havel et al., 1986) indicates a small increase in quenching in guanidine; however, we observe a decrease in quenching in urea. The increased quenching reported by Havel et al. only appears to occur at high concentrations of the partially folded protein. In order to ascertain the behavior in urea, we examined the dependence of the intrinsic tryptophan fluorescence and wavelength of emission on protein concentration across both the urea-induced folding transition (Figure 2) and the pH-induced transition (described below). The intrinsic tryptophan fluorescence (Figure 2) exhibited a linear dependence on protein concentration, and the wavelength of emission was independent of protein concentration on either side of the urea-induced unfolding transition. Therefore, the changes in fluorescence quenching are independent of protein concentration under the conditions examined. The denaturant-induced unfolding of the protein observed here, as measured by the intrinsic fluorescence, supports earlier observations. That is, the native three-dimensional structure of the protein results in an interaction(s) giving rise to quenched tryptophan fluorescence, relative to the more unfolded form (Edelhoch & Burger, 1966; Burger et al., 1966). Taken together, these data are consistent with the proposal that bGH can undergo a urea- or guanidine-induced alteration in protein conformation which modifies the environment of the tryptophan residue.

Fluorescence Measurements of pH-Induced Equilibrium Denaturation. The fluorescence emission spectra of bGH at various pH values are presented in Figure 3. At neutral-to-basic pH, the intrinsic tryptophan emission is essentially constant. Under the pH conditions examined, the fluorescence intensity at 335 nm (Figure 3, inset) exhibits an increase at acid pH and no measurable change in the wavelength of the emission. A comparison of this result with the behavior of NATA under identical conditions (Figure 3) indicates that the acid-induced changes in the protein fluorescence result from structural alterations in the protein. The lack of change in the wavelength of the emission maximum, upon completion of the acid transition, indicates that an increase in the hydrogen ion concentration induces a conformational change in the protein, but not complete unfolding. The conformational change is produced by the protonation of a protein side chain(s) with an apparent midpoint of pH ~4.7. Although direct comparison is difficult, this result is similar to that previously observed for bGH by Edelhoch and Burger (1966) in urea. Inspection of the data (Figure 3, inset) reveals that the acid-induced transition in the protein fluorescence occurs over a narrow pH range (~1 pH unit) and thus is highly

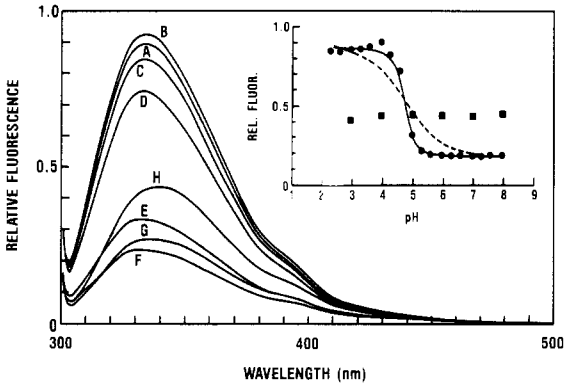

FIGURE 3: Dependence of the intrinsic tryptophan fluorescence of bGH on pH. All spectral data were obtained with identical instrument settings and protein concentrations in both the main figure and inset. Samples contained 10 μM bGH and 4.5 M urea and were 20 mM in each of the following buffers: sodium acetate, MES, Tris, and sodium bicarbonate. The technical fluorescence spectra of bGH at various pH values were measured at 25 °C using an excitation wavelength of 295 nm. Spectra A–H were obtained at pH values of 3.6, 4.0, 4.3, 4.6, 5.0, 5.3, 10.0, and 11.0, respectively. As the pH is changed from acid to neutral, the intrinsic tryptophan fluorescence is quenched. Inset: A plot of the fluorescence intensity at 335 nm of bGH as a function of pH (circles). The pH dependence of the fluorescence intensity of NATA (10 μM) at 350 nm (the wavelength of maximum emission across the pH range examined) is also provided (squares). The dashed line represents the curve predicted for a single-proton equilibria; the solid line represents the curve predicted for a cooperative two-proton equilibria using a model for highly cooperative binding described by Adair's equation (Van Holde, 1971).

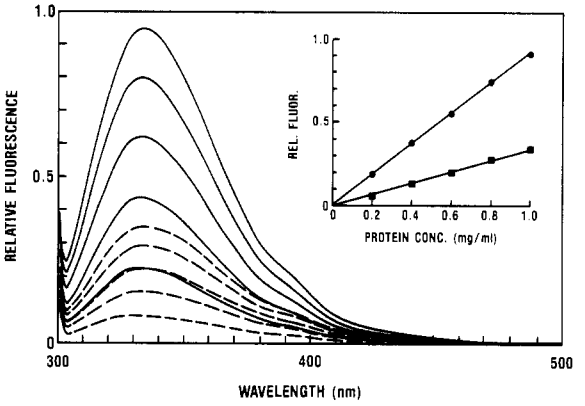

FIGURE 4: Effect of protein concentration on the acid-induced equilibrium denaturation of bGH. All spectral data were obtained with identical instrument settings and solvent conditions in both the main figure and inset. The fluorescence spectra were recorded at protein concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL (10–45 μM) in 4.5 M urea/20 mM buffer (see Methods) at pH 4.0 (solid lines) and at pH 6.0 (dashed lines). In each case, increasing concentrations correspond to increasing fluorescence intensity. Inset: A plot of the fluorescence intensity at 335 nm versus the protein concentration at pH 4.0 (circles) and at pH 6.0 (squares). The slope of the plot at pH 4.0 is greater than the slope at pH 6.0 because at pH 4.0 the protein tryptophan fluorescence is not quenched and the protein has a greater intrinsic fluorescence emission. The fluorescence intensities were taken directly from the digitized spectral data and are corrected for inner filter effects using the estimation described by Lakowicz (1983).

cooperative. It is evident that the pH shift disrupts an interaction(s) which quench(es) the fluorescence of the native protein. The extent of cooperativity can be gauged by comparing the observed data to the titration curves predicted for a single-proton equilibrium and a cooperative two-proton equilibrium (Figure 3, inset). The observed values clearly deviate from a simple, single-proton, equilibrium and more closely resemble a cooperative event involving two protons. It should be noted that these data do not distinguish between an increase in protein fluorescence due to a direct interaction between the protonating group(s) and the fluorophore or an increase resulting from a remote protonation event followed by a conformational alteration which reduces quenching. Like that described above for fluorescence changes induced by urea, the interpretation of the pH-dependent fluorescence quenching data would be altered if, under the chosen experimental conditions, the fluorescence emission properties exhibited a nonlinear dependence on protein concentration. In order to examine the effects of protein concentration on the pH-dependent transition, the quenching behavior was examined at a series of protein concentrations across the pH-induced transition (Figure 4). It is evident that under the conditions examined, 10 μM protein (0.22 mg/mL), and for protein concentrations ~5-fold higher, there is a simple linear relationship between protein concentration and the intrinsic tryptophan fluorescence across the pH transition. Similarly, the wavelength of emission was independent of protein concentration.

UV Absorbance Measurements of pH-Induced Equilibrium Denaturation. Burger et al. (1966) have previously demonstrated a UV difference trough at 290 nm for bGH between pH 5 and pH 3. We have performed a similar analysis (data not shown) and found our results in agreement with this observation. The pH-dependent 290-nm absorbance changes of bGH are presented in Figure 5. The midpoint of the transition is at pH ~4.7. There are several observations concerning this curve. Across this acid transition, the protein undergoes a total change in absorptivity (~2000 M^{-1} cm^{-1}) which is equivalent

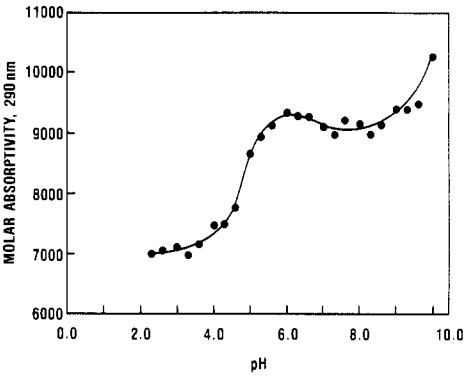

FIGURE 5: Dependence of the 290-nm protein absorbance on pH. Samples were prepared as described in Figure 3.

to that previously observed for unfolding in urea (Holzman et al., 1986) and in guanidine (Brems et al., 1985) and to that reported by Burger et al. (1966) for the pH transition (once corrected to the actual value of the protein molecular weight). In addition, absorbance spectra taken across the acid transition exhibit a gradual blue shift of ~4 nm upon transfer of the protein to an acidic environment (data not shown) as previously observed (Burger et al., 1966). Comparison of the midpoints of the fluorescence and UV data and comparison of the cooperativity of the acid absorbance transitions to those observed by fluorescence (Figure 3) indicate that both of these transitions are of the same form and occur over the same pH range. The pH-induced absorbance and fluorescence transitions suggest that pH-induced conformational changes of bGH give rise to changes in these structural probes which may be related to those observed for the chemically induced unfolding process. The concentration dependence of the UV absorption was examined across the pH-induced transition (data not shown) in a similar fashion to that described above for the fluorescence transition. The UV extinction changes undergone by the protein exhibited a linear dependence on protein concentration from 0.22 to 1.0 mg/mL across the pH transition. Therefore,

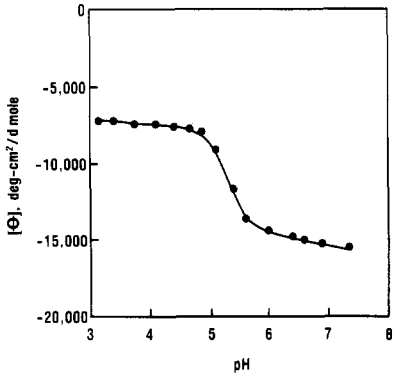

FIGURE 6: Dependence of the 22-nm helix-related CD signal on pH. Samples were prepared as described under Methods.

both absorption and fluorescence are sensitive to the same structural event(s) induced by the protonation equilibria established during the pH-induced conformational transition.

Circular Dichroism Measurements of pH-Induced Equilibrium Denaturation. The pH-dependent changes in the helix contribution to secondary structure of bGH, monitored at 222 nm, are presented in Figure 6. The data indicate that the helix contribution to secondary structure is dependent on pH and exhibits a transition at pH ~5.3, a value higher than that for the coincident fluorescence and UV transitions. At pH values below the transition, the protein exhibits a molar ellipticity of ~−7000 to ~−8000 deg/(cm²·dmol). This corresponds to about 50% of the native protein helix-related CD signal observed at pH values above the transition. On the basis of optical rotation measurements through the acid transition, Burger et al. (1966) and Edelhoch and Burger (1966) proposed that it was possible to selectively alter tertiary structure without altering protein secondary structure. The CD observations we report show that in 4.5 M urea changes in pH disrupt only a portion of the helical content of the protein while completely disrupting the UV and fluorescence probes of protein tertiary structure. The helix-dependent 222-nm CD signal was examined for concentration dependence (data not shown) across the pH transition up to a protein concentration of 1.0 mg/mL. No evidence of concentration dependence was observed.

Examination of pH-Induced Equilibrium Denaturation by Proton NMR. The broad resonances observed in the ^{1}H NMR spectra of bGH are typical for proteins in the molecular mass range of 20000. Figure 7 is composed of a series of spectra, restricted to the aromatic region of the protein, at various pD values. Figure 7A is the ^{1}H NMR spectrum of the protein recorded at pD 2.15, where a limited number of the amino acid side-chain resonances are relatively well resolved. For example, resonances I and II (Figure 7A) are assignable to the 2′,6′ and the 3′,5′ aromatic protons, respectively, of the six tyrosine residues of the protein. The average chemical shifts of 7.12 and 6.79 ppm, respectively, are almost identical with those for tyrosine in small peptides (Bundi & Wüthrich, 1979). Similarly, resonance III (Figure 7A) can be assigned to the C2H imidazole ring protons of the three histidine residues of the protein. The chemical shifts of 8.64, 8.64, and 8.62 ppm are consistent with those of fully protonated histidine residues both in the isolated amino acid (Wüthrich, 1976) and in small peptides (Markley, 1975). The small difference in the chemical shifts above ~8 Hz is due to primary sequence variation in the region of these residues. Increasing the pD (Figure 7A–F) causes perturbations in the chemical shifts of the aromatic resonances. These can be ascribed to a combination of factors including direct ionization and proximal electrostatic interactions together with the environmental changes of various

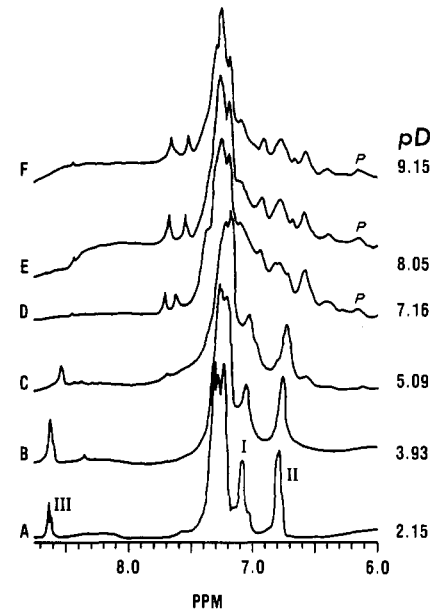

FIGURE 7: Aromatic proton NMR spectra at various pD values in 4.5 M urea. Samples were prepared as described under Methods. I and II correspond to aromatic protons of protein Tyr residues (see text) and III to the protein C2H imidazole ring protons (see text).

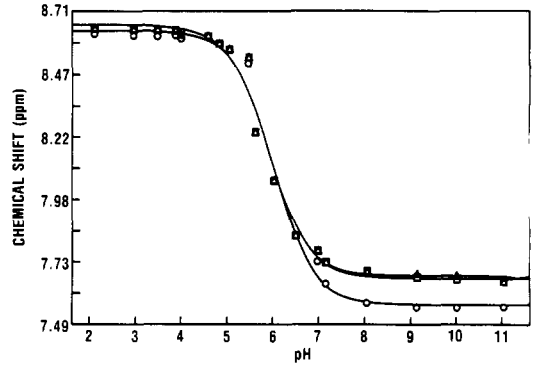

FIGURE 8: Plot of the chemical shift as a function of pD for the three protein histidyl groups in 4.5 M urea. Samples were prepared as described under Methods.

aromatic side chains due to the pH-induced folding of the protein.

Specifically, the magnitude of the chemical shift dispersion from folded to unfolded states (Figure 7B → D) suggests the juxtaposition of aromatic side chains and the subsequent mutual effects of their ring currents. Resonance P (Figure 7D → F), for example, is attributed to an aromatic proton of a phenylalanine residue due to its pH independence (high-pH titration data not shown) and triplet appearance in resolution-enhanced spectra (not shown). The chemical shift of resonance P is 6.10 ppm, and it is shifted upfield by more than 1 ppm from the random-coil value (Bundi & Wüthrich, 1979). Thus, aromatic hydrophobic interactions are an important structural feature of bGH under these solution conditions. This behavior is similar to, but not identical with, that described for bGH in the absence of chemical denaturant (MacKenzie et al., 1989; unpublished results). These data suggest that, in the absence or denaturant, the imidazole ring of either His-20 or His-22 is part of a hydrophobic core below the physiological pH range. A hydrophobic environment is consistent with both an abnormally low His pK_a value (of 4.67) and the chemical shift of the C2H proton. These characteristics are not displayed by any of the histidine residues in 4.5 M urea-d_4. Titration data for His residues 20, 22, and 170, characterized by resonance III (Figure 7A), were analyzed

by a three-parameter fit to the Henderson–Hasselbach equation using a nonlinear regression program. The results are shown in Figure 8. In 4.5 M urea-d_4, the pK_a values of His residues 20, 22, and 170 (without specific assignment) are 6.05, 5.92, and 5.99, respectively. From a chemical shift dispersion criterion, the transition between unfolded and folded occurs at pH 6 (Figure 7B → D). The similarity of this value to the His pK_a values described above is presently thought to be coincidental because in the absence of chemical denaturants the same transition occurs at approximately pH 4.7 (MacKenzie, unpublished results), which is the pK_a value for either His-20 or His-22 of bGH (MacKenzie et al., 1989).

The pathway to the formation of folded-state hydrophobic interactions is of interest. Previous studies and the results reported here show that the denaturant-induced folding of bGH does not follow a simple two-step mechanism. The folding has been interpreted in terms of an intermediate "molten globule" state (Brems & Havel, 1989). The NMR data allow a significant refinement to this model for bGH with regard to the susceptibility to pH-induced unfolding/refolding measured at equilibrium. In particular, the folded-state hydrophobic interactions are established at a pH higher than the fluorescence and UV signals characteristic of the folded protein. Therefore, these interactions are the most easily disrupted upon pH-induced unfolding.

Conclusions

pH-Induced Conformational Transition Is Not a Simple Two-State Process. A two-state model of protein folding will give rise to coincident transitions for multiple detection methods. The noncoincident conformational transitions detected by CD and UV absorbance or fluorescence demonstrate that pH-induced unfolding/refolding of bGH is a multistate process. The noncoincident pH unfolding transitions are due to the presence of an intermediate(s). The intermediate(s) formed at low pH exhibit(s) certain spectral characteristics: (1) aromatic hydrophobic interactions are disrupted as evidenced by proton NMR; (2) a partial loss of the 222-nm CD signal (~50%) is seen; (3) the tertiary structure related UV absorbance is disrupted as indicated by both a change in the molar extinction and an absorbance blue-shift; (4) the folded-state quenching is disrupted; and (5) the tryptophan fluorescence is not red-shifted.

Given the high helix content of growth hormone (Abdel-Meguid et al., 1987) and the unusual helical stability at low pH and in denaturants, it may be possible to find fragments of growth hormone that are helical in aqueous solutions. Such helices might represent regions that comprise retained secondary structure in the folding intermediate. These type of helical fragments have been demonstrated; fragments 96–133 or 109–133 from bovine growth hormone (Brems et al., 1987b; Gooley & MacKenzie, 1988; Gooley et al., 1988) and fragment 1–28 of human growth hormone (Roongta et al., 1988) contain helical structure in aqueous solutions. These fragments correspond to the residues of the first helical segment 7–34 and the third helical segment 106–127 of the four-helix bundle structure of growth hormones (Abdel-Meguid et al., 1987).

A recent study of the folding kinetics of bGH in guanidine indicates that, upon refolding, the signal associated with secondary structure (222-nm CD) is gained before the signal associated with tertiary structure (290-nm UV) (Brems et al., 1987a). These data support the proposal that in the presence of the ionic denaturant guanidine the helix-related secondary structure of the molecule forms before the tertiary structure associated with the microenvironment surrounding the single protein tryptophan residue. These results are consistent with a sequential folding process that begins with the formation of secondary structure and has been referred to as a framework model. A further refinement of the framework-type intermediate is the molten globule state (Ohgusha & Wada, 1983; Ptitsyn, 1987; Brems & Havel, 1989). The molten globule is a compact intermediate state with a high degree of secondary structure and a fluctuating tertiary structure. The state appears to be one which can be populated kinetically and possesses sufficient stability to exist at equilibrium. The intermediate state observed at low pH and in denaturant exhibits properties similar to those attributed to the molten globule: denatured-like tertiary structure and substantial secondary structure. The degree of compactness of the low-pH form has not been measured; however, the denaturant-induced intermediate has a similar compactness as the native state. The fluorescence of the bGH intermediate observed here indicates that the tryptophan has lost nativelike quenching but is not completely solvent-exposed as indicated by the absence of a red-shifted spectrum characteristic of the unfolded form. Fluorescence studies of other proteins that have a molten globule state, such as human or bovine α-lactalbumin (Kronman & Holmes, 1965; Gilmanshin et al., 1982) and bovine carbonic anhydrase B (Semisotnov et al., 1986), indicate that tryptophan residues are much less exposed to solvent than in their unfolded forms. These results led to the suggestion that aromatic residues in the molten globule do not loose their contacts with surrounding groups. However, their contacts are time-averaged in such a way that a nativelike rigid environment is not ensured, as manifested in their near-UV CD or UV absorbance spectra (Dolgikh et al., 1981, 1985; Gilmanshin et al., 1982; Ptitsyn et al., 1983). It should be noted that the data for the low-pH form of bGH do not necessarily imply the remaining structure is nativelike. It is possible that the residual secondary structure in the intermediate could represent a protein which was largely unfolded but which has a reorganized set of local structures in different places than in the native protein. However, the reversibility of the pH transition implies that even if the structure of the low-pH form is not directly along a pathway to native protein, it still represents a highly populated equilibrium intermediate.

Partially Folded Acid Form of bGH Resembles an Intermediate Observed during Chemical Denaturation. The pH-dependent and urea-induced changes in fluorescence observed for bGH are consistent with the intermediate(s) exhibiting an altered tryptophan environment. In human growth hormone, UV spectroscopy suggests that the single tryptophan residue is hydrogen bonded to a carboxylate residue (Bewley & Li, 1984). Although for bGH it is tempting to consider that the tryptophan quenching may be a direct effect involving a specific residue, it is difficult to reconcile this possibility with a pH-dependent emission titration curve which fits a two-proton event.

There are few well-documented examples of proteins in which it is possible to induce the formation of equilibrium folding intermediates (Ghelis & Yon, 1981; Kim & Baldwin, 1982). The data for bGH presented here and previously (Brems et al., 1985, 1987a; Holzman et al., 1986) indicate that equilibrium intermediates are populated. A shift from neutral to low pH produces a species with both unquenched fluorescence and a 290-nm absorbance characteristic of the unfolded state. The fluorescence and absorbance unfolding transitions exhibit similar midpoints, indicating the absorption and fluorescence properties of the single tryptophan change in concert with pH. These data, taken together with previous data on urea-induced unfolding (Holzman et al., 1986), suggest

that the tryptophan microenvironment of the conformer present at acid pH, with ~50% of its helix-related CD signal remaining, is similar to the conformer present after the high-urea (~8.5 M) fluorescence (Figure 1) and UV transitions (Holzman et al., 1986) are complete. Thus, at least one of the equilibrium folding intermediates observed in urea has UV, fluorescence, and CD properties similar to those observed for a conformer resulting from pH-induced unfolding. The data indicate that it is possible to define solution conditions (pH) in which the intermediate is a predominant molecular form of bGH.

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