Equilibrium Denaturation of Recombinant Porcine Growth Hormone†

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ABSTRACT: Equilibrium denaturation of recombinant porcine growth hormone (pGH) derived from *Escherichia coli* using the denaturant guanidine hydrochloride (GuHCl) was followed by ultraviolet absorption spectroscopy, intrinsic fluorescence, far-ultraviolet circular dichroism, and size-exclusion chromatography. The normalized denaturation transition curves for each of the above methods were not coincident; denaturation resulted in an initial disruption of the tertiary structure, whereas secondary structure and degree of compactness were disrupted at higher concentrations of denaturant. Size-exclusion chromatography also detected an associated form of pGH at intermediate GuHCl concentrations. These findings conclusively show that pGH does not follow a simple two-state folding mechanism but are consistent with the framework model of folding. Stable intermediates observed were similar to those seen in other nonhuman growth hormones and are characterized as compact and largely α-helical yet lacking nativelike tertiary structure.

Porcine growth hormone (pGH)[1] is a member of the class of large pituitary polypeptide hormones. It is a single-chain polypeptide containing 191 amino acids arranged to form an antiparallel four-α-helix bundle (Abdel-Meguid et al., 1987). Several structural features of pGH are conserved among the growth hormones of other species; the primary structure contains two disulfide bridges, one forming a large loop connecting distant parts of the primary sequence, and the second forming a short loop near the C-terminus. A single conserved tryptophan residue located in the second helix occupies a hydrophobic pocket formed by the hydrophobic faces of the four-helix bundle (Abdel-Meguid et al., 1987; Carlacci et al., 1991). The absorption and fluorescence properties of this amino acid make it useful as an intrinsic probe for the study of pGH tertiary structure. A recent report of the crystal structure of human growth hormone (hGH) complexed to its receptor (de Vos et al., 1992) has shown that the topography of hGH is similar to that described for pGH. In view of the high degree of conservation of their primary structures, it would be quite reasonable to assume that all members of the growth hormone family have similar three-dimensional structures.

Earlier studies on the equilibrium denaturation of several members of the GH family have shown that stable intermediates have been detected for bovine, ovine, and rat growth hormones (Burger et al., 1966; Holladay et al., 1974). More recently, the equilibrium denaturation of bovine growth hormone (bGH) has been shown to be a multistate process in which at least four species have been identified: native, a monomeric folding intermediate, an associated folding intermediate, and unfolded protein (Brems et al., 1985, 1986; Havel et al., 1986, 1988). The monomeric folding intermediate has the characteristics of a molten globule: is largely α-helical, retains a compact hydrodynamic radius, and has packing of the aromatic side chains that is similar to the unfolded state (Brems & Havel, 1989). The associated intermediate is present at protein concentrations greater than 10 μM. In addition, a bGH region that includes amino acid residues 109–133 appears to be directly involved in the association process (Brems et al., 1986). Unlike the nonhuman species of growth hormone examined thus far, denaturation of hGH follows a two-state mechanism, with no detectable intermediates (Brems et al., 1990).

Little has been reported concerning the folding of pGH, a protein that shares 91% and 68% primary sequence identity with bGH and hGH, respectively. This report presents the equilibrium denaturation of pGH derived from heterologous gene expression in *Escherichia coli*. Denaturation with guanidine hydrochloride (GuHCl) was followed by UV absorption spectroscopy, intrinsic fluorescence, far-UV circular dichroism, and size-exclusion chromatography. The denaturation transitions obtained from each method of detection were noncoincident, indicating a deviation from a two-state denaturation mechanism.

EXPERIMENTAL PROCEDURES

Materials. Recombinant-derived methionyl-pGH was produced at Bresatec Ltd., Adelaide, Australia. N-Terminal sequence analysis (Applied Biosystems, Inc., Model 470A protein sequencer) verified a homogeneous product with the N-terminal sequence Met-Phe-Pro-Ala. GuHCl was ultrapure grade from Schwarz/Mann. All other reagents were of analytical grade. Superose-12 high-performance liquid chromatography (HPLC) size-exclusion columns were from Pharmacia. Proteins used for molecular weight calibration of the Superose-12 column were purchased from Sigma.

Methods. (*a*) *Equilibrium Denaturation Experiments.* All equilibrium denaturation solutions were prepared by mixing the appropriate stock solutions of pGH dissolved in 25 mM borate (pH 9.1) (buffer A) with 6.6 M GuHCl/buffer A. In preliminary experiments, the reversibility of the denaturation was ensured by mixing two stock solutions of pGH at equal concentrations, one in buffer containing no GuHCl and the other in buffer containing 6 M GuHCl, in the appropriate proportions, to arrive at each final GuHCl concentration. This method of sample preparation gave results identical to the

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[1] Abbreviations: bGH, bovine growth hormone; CD, circular dichroism; GuHCl, guanidine hydrochloride; hGH, human growth hormone; HPLC, high-performance liquid chromatography; λ_{max}, fluorescence emission maximum; pGH, porcine growth hormone.

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method described above. Following incubation for a minimum of 2 h to ensure equilibrium had been reached, the samples were centrifuged prior to analysis. All measurements were performed at 25 °C.

(*b*) *Size-Exclusion Chromatography.* Samples were prepared by incubating pGH (0.66 mg/mL) at each GuHCl concentration in buffer A. A 100-μL sample was injected onto a Superose-12 column preequilibrated in the same concentration of GuHCl in buffer A. A flow rate of 0.5 mL/min was maintained by a P-500 pump (Pharmacia). Protein molecular weight calibration curves for the Superose-12 column in 6 M GuHCl were determined and utilized to calculate the Stokes radii of pGH at each concentration of GuHCl as described by Corbett and Roche (1984).

(*c*) *Intrinsic Fluorescence Measurements.* Intrinsic fluorescence measurements were performed on a Perkin-Elmer LS-50 fluorescence spectrophotometer. The single tryptophan of pGH was selectively excited using an excitation wavelength of 295 nm. Emission spectra were scanned from 305 to 400 nm and corrected for buffer contributions. The slit widths were 5 nm, and the scan rate was 60 nm/min. Samples were prepared by incubating pGH at each concentration of GuHCl in buffer A at a final protein concentration of 0.08 mg/mL.

(*d*) *Absorbance Measurements.* Absorbance measurements were performed on a Cary 3 spectrophotometer using a single matched pair of 1-cm cuvettes. The absorbance at 290 and 278 nm was recorded for each GuHCl concentration in the denaturation curve. The concentration of pGH used was 0.22 mg/mL. pGH concentrations were determined by measuring the absorbance at 278 nm using an extinction coefficient of 15 714 M^{-1} cm^{-1} previously determined using the method of Bewley (1982).

(*e*) *Circular Dichroism Measurements.* CD spectra were recorded on an Aviv 62DS model spectropolarimeter. The α-helix content of pGH at each concentration of GuHCl was determined by measuring the mean residue ellipticity at 222 nm, $[\theta]_{222}$, in deg·cm^2·$dmol^{-1}$, using a mean residue weight of 115. In the far-ultraviolet region, a pGH concentration of 0.16 mg/mL was used in a 1-mm cell. A time constant of 3 s was used. Reported values represent the average of two measurements.

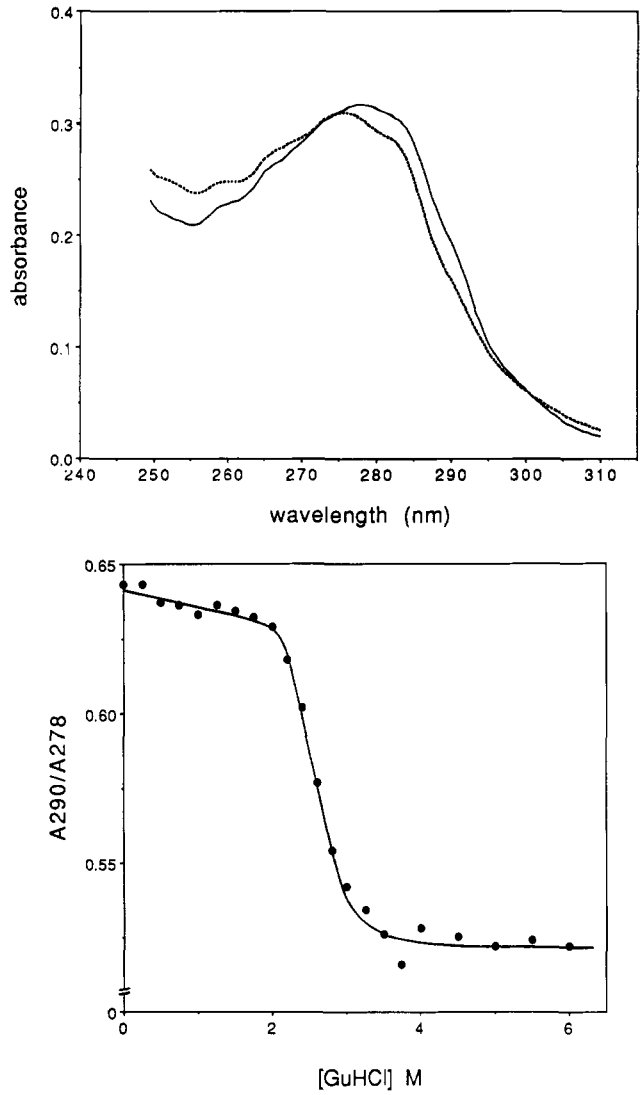


FIGURE 1: Equilibrium denaturation of pGH as monitored by UV absorbance. The protein concentration was 0.22 mg/mL in buffers containing 25 mM borate (pH 9.1). (A) Ultraviolet absorbance spectra of native (—) and GuHCl-denatured (– – –) pGH. (B) Effect of GuHCl concentration on the absorbance at 290 nm relative to 278 nm under equilibrium conditions.

RESULTS

UV Absorbance. The UV absorbance spectrum of native pGH displays an absorption maximum at 278.2 ± 0.1 nm. The prominent shoulder centered near 290 nm is due mainly to the absorption of the single tryptophan residue (Figure 1A). Denaturation of pGH in 6 M GuHCl results in a considerable loss in extinction (hypochromicity), with a general blue-shifting of the spectrum toward lower wavelengths (Figure 1A). The GuHCl-induced difference in absorbance at 290 nm is indicative of the exposure of the single internalized tryptophan to the solvent exterior upon unfolding. Figure 1B shows that the loss of extinction at 290 nm relative to 278 nm (a region of the spectrum where little change occurs), as a function of GuHCl concentration, follows a sigmoidal plot with a midpoint centered at 2.7 M GuHCl.

Fluorescence. The fluorescence emission maximum (λ_{max}) of tryptophan residues is highly sensitive to the polarity of the environment surrounding the tryptophan. An increase in polarity may be a result of a direct interaction of the tryptophan with the aqueous solvent or with a charged internal residue. Using an excitation wavelength of 295 nm, the corrected fluorescence emission spectrum of native pGH displays a broad single band with a maximum at 338 ± 0.5 nm (Figure 2A). Previous studies have reported values of λ_{max} for native pGH near 330 nm (Seely & Hollis, 1986; Kauffman et al., 1989), suggestive of a more nonpolar tryptophan environment. The discrepancy in the figures could reflect differences in recombinant pGH preparations. Native bGH has a λ_{max} of 335 nm, similar to that for pGH reported here, and is indicative of a moderately nonpolar tryptophan environment (Havel et al., 1988). When the GuHCl concentration is increased, the fluorescence emission undergoes an increase of intensity, maximal at 3.25 M GuHCl, followed by a 15% decrease at higher concentrations of denaturant (Figure 2A,C). A linear increase in λ_{max} accompanied the unfolding, suggestive of a gradual removal of the tryptophan from the interior of the protein to the solvent exterior (Figure 2B). Equilibrium denaturation of pGH as monitored by relative fluorescence at 350 nm follows a nonsigmoidal profile with the midpoint of the major transition at 2.7 M GuHCl (Figure 2C). This profile is almost identical to that reported for bGH (Havel et al., 1988), except that the pGH curve is shifted to lower concentrations of GuHCl. As with bGH, the native-state tryptophan residue in pGH is located close to an

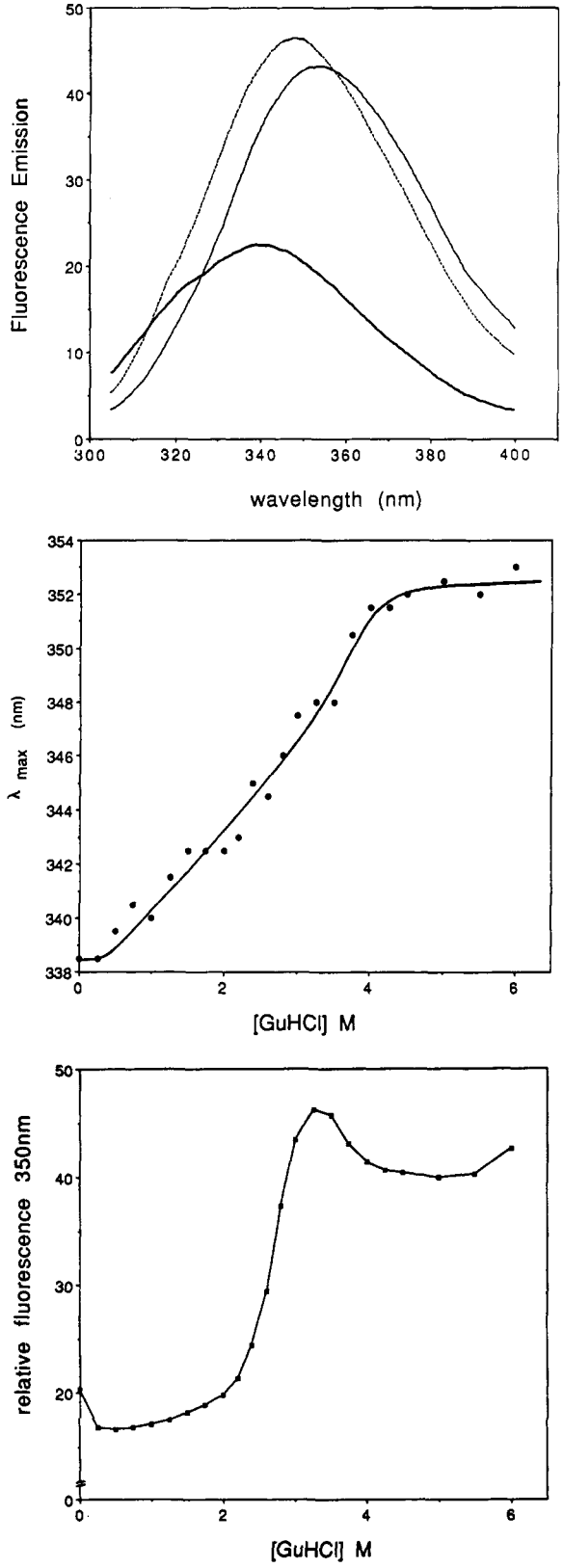


FIGURE 2: Equilibrium denaturation of pGH as monitored by intrinsic fluorescence. The protein concentration was 0.08 mg/mL in buffers containing 25 mM borate (pH 9.1). (A) Emission spectra for native (—), intermediate at 3.25 M GuHCl (– – –), and unfolded in 6 M GuHCl (—) pGH. Excitation was at 295 nm. (B) Effect of GuHCl concentration on the fluorescence emission maximum (λ_{max}) of pGH. (C) Denaturation curve for pGH as monitored by tryptophan fluorescence emission at 350 nm.

intramolecular quenching group. Upon unfolding, the interaction with the quenching group is diminished and the intensity of fluorescence increases to a maximum level at 3.25 M GuHCl. Increasing the denaturant concentration further results in the complete unfolding of the molecule with the concomitant quenching of the tryptophan by the bulk solvent resulting in the slight decrease in intensity.

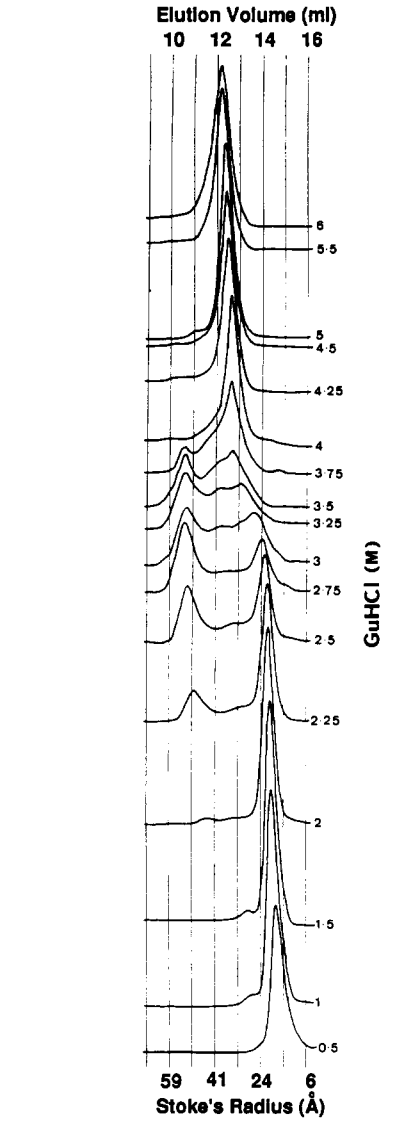
Size-Exclusion Chromatography. The GuHCl-induced changes in the hydrodynamic radius of pGH are shown in Figure 3A. The hydrodynamic radius of pGH increases from approximately 20 Å in the folded state to approximately 40 Å in the unfolded state. However, between 2.25 and 3.75 M GuHCl, as many as three different peaks were observed. Two of these peaks coincide with those for the folded and unfolded protein, while the third, early-eluting peak, appears to be an associated form of pGH with a radius of approximately 52 Å. The proportion of the associated form is at its highest between 2.75 and 3.25 M GuHCl. The rate of equilibration of partially unfolded monomeric, unfolded, and associated forms is slow on the time scale of the chromatographic experiment, as the three forms are well separated on the column in the transition region. A plot of the pGH monomer radius as a function of GuHCl concentration results in a sigmoidal curve with a midpoint at 3.1 M GuHCl (Figure 3B).

Circular Dichroism. The far-UV CD spectrum of pGH shown in Figure 4A, displays minima at 221 and 209 nm, which is characteristic of the high α-helix content seen in other members of the GH family of proteins (Holladay et al., 1974; Bewley & Yang, 1980). The loss of α-helix upon denaturation, followed by measurement of the relative ellipticity at 222 nm, results in a broad sigmoidal plot with a midpoint centered at 3.05 M GuHCl (Figure 4B).

Analysis of Denaturation Curves. The equilibrium unfolding transitions of most small, globular proteins are highly cooperative, and the two-state approximation (N ↔ U) is usually applicable in the analysis. There are two tests for an equilibrium intermediate based on the use of probes: (1) a biphasic transition as measured by a single probe and (2) noncoincident transitions as measured by different probes. Either one of these observations is sufficient evidence for an equilibrium intermediate (Kim & Baldwin, 1982). A two-state approximation was assumed in the analysis of pGH denaturation, even though the denaturation deviates from a two-state mechanism. The changes in the observed parameters of pGH were compared by normalizing each transition curve to the apparent fraction of the unfolded form, F_{app}:

$$F_{app} = (Y_{obs} - Y_{nat})/(Y_{unf} - Y_{nat})$$

where Y_{obs} is the observed A_{290nm}/A_{278nm}, fluorescence intensity, molar ellipticity, or Stokes' radius at a given GuHCl concentration and Y_{nat} and Y_{unf} are the observed values for the native and unfolded forms, respectively, at the same denaturant concentration (Pace, 1986). A plot of the apparent fraction of unfolded protein, F_{app}, for each of the detection methods used is shown in Figure 5. The midpoints of the denaturation transitions were independent of the protein concentration. Denaturation of pGH results in absorbance and fluorescence transitions which are coincident, but the CD- and hydrodynamic radius-detected transitions occur at higher concentrations of denaturant, indicative of a greater stability toward denaturation. These results are consistent with a multiple-state mechanism with at least one populated intermediate. This intermediate is compact, retains about 50% of its nativelike secondary structure, yet has a tertiary structure similar to that of the unfolded state.



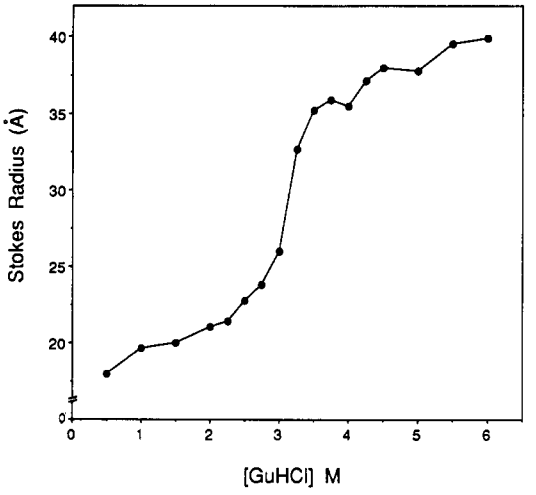
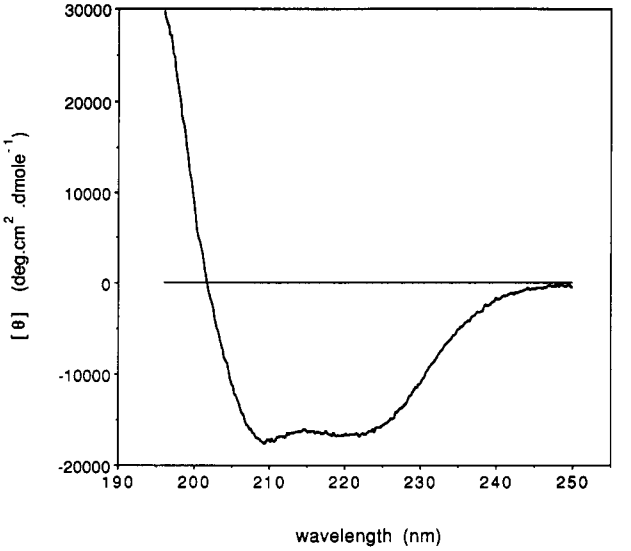


FIGURE 3: Equilibrium denaturation of pGH as measured by size-exclusion chromatography. The protein concentration upon injection was 0.66 mg/mL. (A) Chromatographic elution profile of pGH as a function of GuHCl concentration. (B) Effect of GuHCl concentration on the Stokes radius of the monomeric species of pGH.

DISCUSSION

Earlier equilibrium denaturation studies of several members of the GH family detected the presence of stable intermediates in the bovine, ovine, and rat growth hormones (Burger et al., 1966; Holladay et al., 1974). More recently, Brems and coworkers' thorough equilibrium and kinetic examination has shown that denaturation of bGH is a multistate process in which at least two folding intermediates have been identified. However, hGH has been shown to follow a two-state denaturation mechanism with no detectable intermediates (Brems et al., 1990). A comparison of the unfolding transition midpoints for these two proteins and pGH is shown in Table I. hGH is more resistant to chemical denaturation than bGH or pGH and folds cooperatively with all transition midpoints at 4.6 M GuHCl. The tertiary structure of bGH is less stable than the secondary structure, as evidenced by the lower denaturation midpoints detected by UV and fluorescence, compared to that for CD. The denaturation of pGH follows a pattern similar to that for bGH, except that the transitions are shifted to lower concentrations of GuHCl and they occur over a narrower range of denaturant concentration. The fact that the denaturation midpoints of pGH are shifted to lower concentrations of GuHCl does not necessarily mean that pGH is less stable than bGH. The conformational stability [$\Delta G(H_2O)$] of a protein is dependent on the steepness of the denaturation transition (the m value) as well as the denaturation midpoint (Pace, 1986). Assuming a two-state



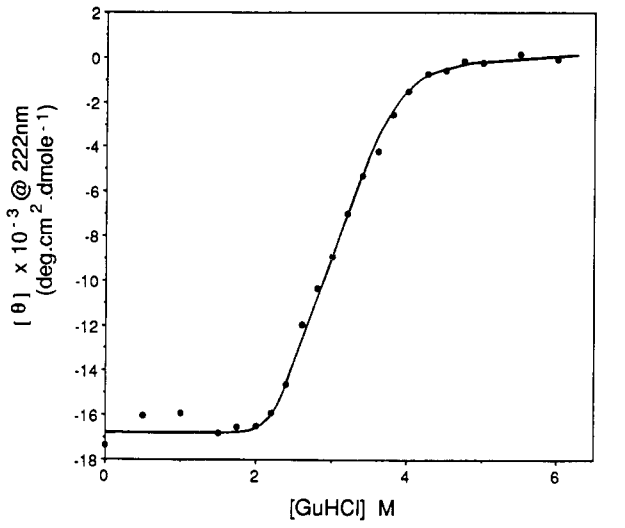


FIGURE 4: Equilibrium denaturation of pGH as monitored by far-UV circular dichroism. (A) Far-UV CD spectrum of native pGH. The protein concentration was 0.16 mg/mL in 25 mM borate (pH 9.1). (B) Effect of GuHCl concentration on the mean residue ellipticity at 222 nm.

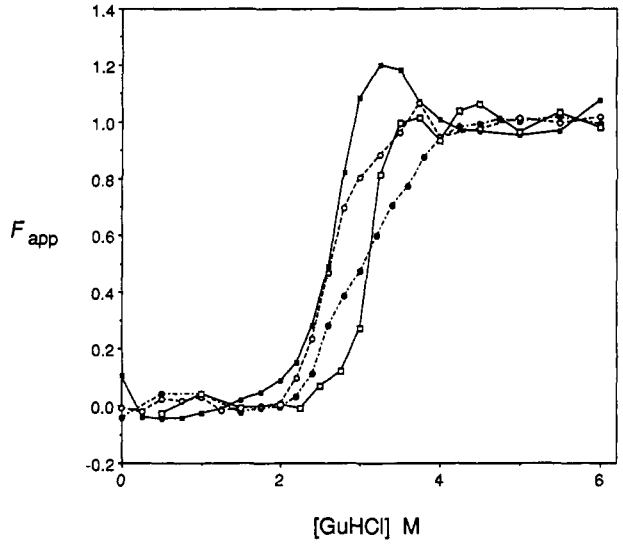


FIGURE 5: Apparent fraction of unfolded pGH as a function of GuHCl concentration. $F_{app} = (Y_{obs} - Y_{nat})/(Y_{unf} - Y_{nat})$, where Y_{obs} is the value of the measured observable and Y_{nat} and Y_{unf} are the observed values for the native and unfolded forms, respectively. Results are represented by the following symbols: fluorescence (■), UV absorbance (○), size exclusion (□), and CD (●).

Table I: GuHCl Denaturation Midpoints of pGH, bGH, and hGH As Determined by Different Spectral/Physicochemical Methods[a]

proteins	far-UV circular dichroism	fluorescence	size-exclusn chromatogr	UV absorbance
pGH	3.1	2.7	3.1	2.7
bGH[b]	3.8	3.3	3.7	3.1
hGH[c]	4.6	4.6	4.6	4.6

[a] Units are in molar. [b] Results for bGH were obtained from Brems and Havel (1989) and Lehrman et al. (1991). [c] Results for hGH were obtained from Brems et al. (1990).

analysis, the UV absorbance-detected transition of pGH (Figure 1B) results in a $\Delta G(H_2O)$ of about 7 kcal/mol. Published values of the $\Delta G(H_2O)$ for bGH vary between 5 and 7 kcal/mol (Brems et al., 1985). Even though these values may be underestimates as a consequence of the two-state assumption in the analysis, it would be reasonable to say that pGH and bGH have comparable stabilities but are much less stable than hGH, which has a $\Delta G(H_2O)$ value of 14.5 kcal/mol (Brems et al., 1990). Comparison of the pGH transitions shows that the tertiary structure is disrupted initially (as evidenced by UV and fluorescence probes) whereas secondary structure and degree of compactness are disrupted at higher concentrations of denaturant (Figure 5). The gradual loss of helix as evidenced by CD is suggestive of a biphasic transition; the initial loss of helix accompanies the tertiary structure disruption.

The similarities in the equilibrium denaturation of pGH and bGH are echoed in the similar fluorescence and size-exclusion chromatography results. Both proteins possess a single tryptophan residue located in a moderately nonpolar environment with a quenched native-state fluorescence. As with bGH, size-exclusion chromatography experiments of pGH have detected the presence of a partially denatured self-associated species at intermediate denaturant concentrations. For bGH, the properties of this species include increased near-UV circular dichroism at 300 nm, a polar tryptophan environment (second-derivative spectroscopy), and a propensity to precipitate when diluted to nondenaturing concentrations of denaturant (Havel et al., 1986; Brems, 1988). Preliminary experiments with pGH under conditions favorable for formation of the associated state have detected (1) a similar intense near-UV CD band near 300 nm, (2) an asymmetric second-derivative 291–295-nm UV absorbance transition, and (3) precipitation upon subsequent dilution to nondenaturing concentrations of GuHCl (unpublished observations). However, unlike bGH, size-exclusion chromatography of pGH at GuHCl concentrations between 2.25 and 3.75 M resulted in chromatograms displaying multiple peaks (Figure 3A). This multiple-peak phenomenon during size-exclusion chromatography had previously been reported in the equilibrium denaturation of a mutant form of bGH and an alkylated form of hGH (Brems et al., 1988, 1990). A lysine-112 to leucine mutation in the third helix of bGH resulted in a protein which refolds 30-fold slower than wild-type bGH. The mutant protein also has a propensity to precipitate upon refolding due to stabilization of an associated folding intermediate via interactions involving the third helix. Size-exclusion chromatography of wild-type bGH demonstrates the presence of an associated species that has a larger Stokes radius than the unfolded protein, but only a single peak is observed at each GuHCl concentration, indicating that the various species are in fast equilibrium compared to the chromatography time scale. The associated form is only detected at bGH concentrations of more than 10 μM (Brems et al., 1985). The experiments reported here were conducted at pGH concentrations of 30 μM. The most favorable condition for self-association of pGH was in buffers containing 3–3.25 M GuHCl (Figure 3A).

The similarity in the equilibrium denaturation of bGH and pGH is not surprising considering their sequence similarity. It has been shown previously that the third helix is directly involved in the association of partially unfolded bGH (Brems et al., 1986). Association is stabilized by the hydrophobic interactions resulting from the intermolecular packing of the lipophilic faces of partially exposed helices. The third helix of pGH is identical in sequence to that for bGH except for a Leu → Gln substitution at residue 121. It is quite possible that helix-3 of pGH may be involved in the self-association process. The association phenomenon in nonhuman growth hormones cannot be solely attributed to interactions between partially unfolded helix-3. Recently, a mutant bGH containing the helix-3 sequence of hGH substituted for the wild-type sequence was shown to exhibit a significant decrease in aggregation compared to wild-type bGH, when incubated under conditions favoring self-association (Lehrman et al., 1991). Aggregation was not completely abolished however, suggesting other partially exposed hydrophobic sites are involved. From equilibrium denaturation studies of hGH, it was concluded that hGH does not form an associated state because the relatively greater stability of native hGH compared to the nonhuman growth hormones precludes the observation of hGH folding intermediates (Brems et al., 1990). The general topography of hGH is very similar to that for pGH (Abdel-Meguid et al., 1987; de Vos et al., 1992), suggesting that the molecular interactions contributing to the presence of intermediates and subsequent self-association seen in nonhuman growth hormones are very specific. Specific residues present in the internal architecture of hGH, but absent in nonhuman growth hormones, may be involved in conferring greater stability to the native state. These residues could participate in maintaining a compact configuration of the four-α-helix bundle.

Carlacci and co-workers' (1991) heuristic approach to predicting the three-dimensional structure of bGH resulted

in a structure very similar to that of pGH. Using energy minimization models, they predicted that the four-α-helix bundle of bGH is stabilized considerably by the connecting loops between helices. This stabilization was principally due to hydrophobic and electrostatic interactions between the α-helices and loops. When the hGH/receptor complex X-ray structure was revealed (de Vos et al., 1992), several interesting differences between the porcine and human structures were observed. Firstly, two short helices (residues 38–47 and 64–70) present in the connecting loop between helix-1 and helix-2 of hGH are absent in pGH. This region of hGH is involved in receptor binding, and it is thought that these short helices may represent conformational changes upon receptor binding. In addition, another short helix (residues 94–100) is present in hGH in the short connection between helix-2 and helix-3 which has an Ω-loop conformation in pGH. This probably represents a true structural difference between the two hormones since this connection is not involved in receptor binding. It is possible that this short helix present in hGH bestows greater rigidity to the connection between helix-2 and helix-3 and, by stabilizing the interhelical interactions, prevents the exposure of the hydrophobic face of helix-3 to the solvent. Furthermore, considering that the greatest sequence divergence between the human and nonhuman GHs is in the connecting loops (Nicoll et al., 1986), these regions may hold the key to explaining why hGH is more stable. Reduction and alkylation of both the disulfide bridges of hGH greatly decreases its stability, such that the denaturation behavior is similar to the nonhuman GHs with well-populated intermediates (Brems et al., 1990). Reduction of the disulfide bonds removes the covalent links between the long connecting loop and helix-4 (disulfide bond 53–164), and the short C-terminal extension and helix-4, thus decreasing the loop–helix interactions. Obviously, oxidized pGH and bGH still contain these covalent bonds, but the fluidity of the helix-connecting loops in these hormones may be similar to that of the alkylated hGH. Tight packing of the connecting loops against the helices could be important in stabilizing the structure of the hGH molecule as a single entity. Interactions (hydrophobic and electrostatic) between specific helix residues and the connecting loops would contribute to this stabilization process. If increasing protein stability is the key to preventing intermediate formation in the nonhuman growth hormones, we will have to wait for a better understanding of the processes involved in conferring protein stability.

The framework model of protein folding describes folding as a sequential process beginning with the formation of secondary structure followed by collapse to the native tertiary structure (Kim & Baldwin, 1982). In conclusion, the equilibrium denaturation studies reported here have shown that pGH does not follow a simple two-step folding mechanism, but is consistent with the framework model. Stable intermediates observed are similar to those seen in other nonhuman growth hormones and are characterized as compact, helical yet lacking nativelike tertiary structure.

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