Evidence for a Self-Associating Equilibrium Intermediate during Folding of Human Growth Hormone

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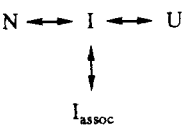
ABSTRACT: It has been previously shown, by equilibrium denaturation, that human growth hormone (hGH) folds by a cooperative two-state process. This is in contrast to the folding pathways of other nonhuman growth hormones that contain stable monomeric and multimeric equilibrium intermediates. We have reinvestigated the equilibrium denaturation of hGH at higher protein concentrations and found smooth transitions from the native to denatured state, but the calculated free energy for unfolding, ΔG, decreases with increasing protein concentration. The effect of protein concentration on the ΔG of unfolding is due to the presence of folding intermediates that have a tendency to self-associate. A correlation was found between the equilibrium denaturation data and the observation of precipitation that occurs upon refolding, suggesting that the presence of self-associated folding intermediates leads to precipitation. Direct evidence for the existence of a soluble, associated intermediate was obtained by dynamic light scattering (DLS) and equilibrium analytical ultracentrifugation. Peptide fragments from the third helix of either hGH or bovine growth hormone (bGH) were capable of inhibiting the formation of this aggregated species and prevent precipitation during refolding. The data show that the folding pathway of hGH is similar to that of nonhuman growth hormones except for differences in the tendency for intermediates to self-associate. These findings are relevant to the design and interpretation of equilibrium folding experiments, and may be important to understanding mechanistic details of protein folding and aggregation in vivo.

It is becoming more apparent that aggregation of partially folded or misfolded proteins is a potential side reaction in protein folding in vivo and may have important physiological implications. Such aggregation phenomena appear to be the underlying cause for the formation of insoluble plaques characteristic of a number of diseases termed amyloidosis (Glenner, 1980a,b), of which Alzheimer's disease is an example (Glenner, 1988; Selkoe, 1990; Goate et al., 1991; Spencer et al., 1991; Tomski & Murphy, 1992). Aggregation of misfolded protein also occurs in the endoplasmic reticulum and can affect protein transport (Hurtley & Helenius, 1989; Klausner & Sitia, 1990; Marquardt & Helenius, 1992). Finally, the formation of proteinaceous aggregates, referred to as inclusion bodies, results when foreign proteins are overexpressed in host cells (Mitraki & King, 1989; Bowden et al., 1991).

Recent evidence suggests (Fisher & Schmid, 1990; Lubben et al., 1990; Goloubinoff et al., 1991; Nilsson & Anderson, 1991; Creighton, 1991; Martin et al., 1991; Gething & Sambrook, 1992; Lorimer, 1992) that nature has designed accessory proteins to assist nascent proteins in achieving their final native conformations and in avoiding the unproductive side reaction of aggregation. Molecular chaperones, peptidylprolyl *cis-trans*-isomerase, and protein disulfide-isomerase are all examples of such proteins. The existence of accessory proteins only emphasizes the complexity of in vivo protein folding pathways. Consequently, the task of unraveling the mechanistic details of these processes is difficult.

The in vitro folding pathways elucidated for bovine growth hormone (bGH)[1] (Brems et al., 1986; Havel et al., 1986), as well as for other proteins (King et al., 1987; Cleland & Wang, 1990, 1992; Tandon & Horowitz, 1989), all contain folding intermediates that tend to aggregate. These proteins serve as model systems for understanding the relationship between folding and aggregation in vivo (Jaenicke, 1991; Kiefhaber et al., 1991; Schmid, 1992; Seckler & Jaenicke, 1992). In the case of bGH, the mechanism (Scheme I) contains a monomeric folding intermediate that, under certain conditions, self-associates where N is the native conformation, U is the fully denatured state, and I and I_{assoc} are the monomeric folding intermediate and the self-associated intermediate, respectively. The monomeric intermediate shares many structural similarities to a "molten globule state" (Brems & Havel, 1989). The formation of the self-associated intermediate can result in low folding yields due to precipitation.

Scheme I



In contrast, the folding pathway of recombinant-derived human growth hormone (hGH) was shown to be a cooperative two-state process (Brems et al., 1990). hGH and bGH are structurally very similar, sharing approximately 70% amino acid sequence identity. Both are single-domain proteins and contain 191 amino acids and 2 intramolecular disulfide bonds. The crystal structure for hGH bound to its receptor has recently been published (de Vos et al., 1992). Although bound to two molecules of its receptor (Cunningham et al., 1991), hGH was still found to be a four-helix bundle protein similar to the X-ray crystal structure determined for porcine growth hormone (pGH) (Abdel-Meguid et al., 1987). The crystal structure for bGH has not been solved, but is presumed to be closely related to the other growth hormones.

We report a reexamination of the folding pathway of hGH using much higher protein concentrations. Equilibrium

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[1] Abbreviations: bGH, bovine growth hormone; bGH(109–128), peptide fragment from the third helix of bGH residues 109–128; CD, circular dichroism spectroscopy; DLS, dynamic light scattering; hGH, human growth hormone recombinant derived; hGH(109–128), peptide fragment from the third helix of hGH residues 109–128; GdnHCl, guanidine hydrochloride; pGH, porcine growth hormone; SEC, size-exclusion chromatography.

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denaturation has uncovered a concentration-dependent formation of at least one associated intermediate. Hydrodynamic techniques are used to confirm the existence of a self-associated intermediate that is highly populated under partially denaturing conditions. The formation of this intermediate is correlated with precipitation that occurs upon refolding of hGH at high protein concentrations. Finally, the potential molecular site responsible for aggregation is localized, and we show how this information can be exploited to inhibit the aggregation and precipitation. These results are compared to the bGH folding mechanism, and the possible physiological significance of our findings is discussed.

EXPERIMENTAL PROCEDURES

Materials

Human growth hormone was produced by recombinant DNA techniques at Eli Lilly and Co. and had the naturally occurring amino terminus. GdnHCl was ultrapure from ICN Biochemicals (Cleveland, OH). All other reagents were analytical grade and were obtained from standard sources. Buffers were prepared using water purified by a Millipore Milli-Q Plus system.

Methods

Equilibrium Denaturation. The procedure followed for these experiments was identical to that described in Brems et al. (1990) with the only exception being that the hGH concentration was varied. Measurements were performed at 222 nm using an Aviv 62DS circular dichroism spectrometer. Protein concentrations for these and all other experiments were determined using an Aviv 14DS or Hewlett Packard 8452A diode array spectrophotometer and the extinction coefficient of 18 890 M^{-1} cm^{-1} at 278 nm reported in the literature (Brems et al., 1990).

Precipitation of hGH during Refolding. Quantitation of precipitation formed during equilibrium refolding was performed as previously described (Brems, 1988). For determination of the protein concentration dependence, varying amounts of hGH were incubated for 2 h at room temperature in 6 M GdnHCl containing 0.02 M HEPES at pH 7.5. Each solution was then diluted by a constant value to induce folding. After 1 h, the precipitate was removed by centrifugation and/or filtration through a 0.2-μm low-protein binding filter. The amount of hGH remaining soluble at each concentration was determined by an absorbance measurement at 278 nm. The percent precipitated was calculated by comparing the concentration soluble to the concentration expected had no precipitation occurred.

To determine the GdnHCl dependence on precipitation, solutions were prepared containing either 2 or 20 mg/mL hGH dissolved in variable amounts of GdnHCl and 0.02 M HEPES, pH 7.5. The solutions were incubated at room temperature and then diluted to a final GdnHCl concentration of 0.8 M to induce folding. The final hGH concentration was 0.3 mg/mL in both cases. After 1 h, the precipitate was removed by filtration, and the percent precipitation was determined as before.

Dynamic Light Scattering. DLS experiments were performed with a Brookhaven Instruments 2030AT autocorrelator (136 total channels, sample times from 0.7 to 1 ms/channel) and goniometer. All measurements were made with a 400-μm pinhole at 90° scattering angle using a scattering source at 514.5 nm that was provided by a Lexel 3500 argon ion laser. Sample temperature was maintained at 25.0 °C by a Neslab RTE-110 temperature bath. Brookhaven Instruments software, written to use CONTIN (Provencher, 1982a,b), was used to calculate the weight-average diffusion coefficients of the scattering species from the measured autocorrelation function. Diffusion coefficients were converted to mean diameters using the Stokes–Einstein relationship. Solution viscosities were measured with an Ubbelohde capillary viscometer.

Equilibrium Analytical Ultracentrifugation. Sedimentation equilibrium experiments were done at 22 °C in a Spinco Model E analytical ultracentrifuge equipped with a 6-place titanium rotor and photoelectric scanning optical system. Overspeeding was used to decrease the time needed to reach equilibrium. Scans of the cells performed after 28 and 50 h were identical within experimental error, indicating that equilibrium had been reached at the earlier time. Standard 12-mm and custom-made 3-mm charcoal-filled epon centerpieces were used in the centrifuge cells. Protein samples were prepared in solutions containing varying concentrations of GdnHCl and 0.1 M HEPES adjusted to pH 7.5. The loading concentration of hGH was 1–5 mg/mL. Scans were performed at the near-UV absorbance maximum for hGH (monochromator setting was 278 nm).

Apparent weight-average molecular weights were calculated using the expression:

$$M_{w,app} = \frac{RT}{(1-\bar{v}\rho)\omega^2}\frac{1}{rC}\frac{dC}{dr} \quad (1)$$

where R is the gas constant, T is the absolute temperature, ω is the rotor speed, $\bar{v}$ is the partial specific volume, ρ is the solvent density, r is the radius, and C is the total protein concentration in milligrams per milliliter. A partial specific volume of 0.732 mL/g was calculated from the amino acid sequence (Schachman, 1957) of hGH and assumed to be independent of the GdnHCl concentration. Densities of the solvents were measured at 22 °C in a 25-mL Weld pycnometer.

The internal calibration factors of the scanner were used to convert data on the strip chart recordings to optical densities as a function of radius. Data points were selected at equally spaced radial positions so that least-squares smoothing procedures could be used in calculating the results (Savitzky & Golay, 1964).

Peptide Fragments. The third helix peptide fragments hGH(109–128) and bGH(109–128) were purchased from Bachem (Torrance, CA) in >99% purity and were used without further purification. A series of solutions were prepared containing 5 mg/mL hGH in 4.5 M GdnHCl, 0.02 M HEPES, pH 7.5, and an increasing molar excess (1–10-fold) of either the hGH(109–128) or the bGH(109–128) peptide fragment. Peptide concentrations were determined using calculated extinction coefficients at 278 nm for a 1 mg/mL solution in a 1-cm cell of 0.553 and 0.540 for the hGH(109–128) and bGH(109–128) fragments, respectively. After 1 h at room temperature, the solutions were diluted to a final GdnHCl concentration of 0.8 M. Precipitated material was removed by filtration, and the filtrates were analyzed by HPLC. Alternatively, the amount of precipitation was determined by an absorbance measurement at 350 nm. HPLC analyses were performed on a Hewlett Packard 1090M series II chromatograph equipped with a diode array detector. A Du Pont Zorbax GF-250 "Special" size-exclusion column maintained at ambient temperature was used for the chromatography. The mobile phase consisted of 0.05 M ammonium bicarbonate having a measured pH of 8, and the flow rate was 0.6 mL/min. The sample volume injected was 20 μL.

GdnHCl Concentration Determinations. All GdnHCl concentrations were determined by refractometry as described

in Nozaki (1972).

RESULTS

Equilibrium Denaturation Uncovers a Concentration-Dependent Formation of Folding Intermediates. In a previous report (Brems et al., 1990), equilibrium denaturation of hGH was performed using a variety of detection techniques. All of the denaturation transitions were coincident, indicating a two-state denaturation mechanism. The protein concentration used for these studies was 0.1 mg/mL. We have reexamined the CD-detected denaturation of hGH as a function of protein concentration up to 11 mg/mL. For each protein concentration tested, the denaturation curves displayed apparent monophasic transitions. However, as the concentration of hGH was increased, the transition region showed broadening (Figure 1A). This observation was attributed to the presence of equilibrium intermediates that are not resolved to any great extent, but whose presence, nonetheless, affected the appearance of the denaturation curves. Equilibrium denaturation experiments using UV absorbance (295 nm) detection were coincident with the CD results for the concentrations tested (data not shown). Since the denaturation data showed no obvious biphasic behavior and there was coincidence using different detection techniques, the curves were analyzed assuming simple two-state behavior as previously described (Pace et al., 1987) to obtain estimates for the Gibbs free energy of unfolding (ΔG). Figure 1B shows the protein concentration dependence for the ΔG values, indicating the effect caused by the broadening in the transition region.[2] There was no apparent effect on the estimated midpoints, and an average value of 4.5 was obtained. These results suggest that the folding pathway of hGH contains intermediates that display a concentration-dependent association.

Formation of Associated Intermediates Is Related to the Precipitation of hGH during Refolding. Equilibrium refolding reactions were performed by initially unfolding different concentrations of hGH in 6 M GdnHCl and then diluting to renaturing conditions of 0.8 M GdnHCl. In most cases, the refolding step caused precipitation of protein (Figure 1B), and the insolubility was quantitated as previously described (Brems, 1988). The amount of precipitation formed during refolding increased with increasing protein concentration, reaching a plateau level of approximately 70% insoluble material under the conditions examined. There is a correlation between this protein concentration-dependent precipitation and the equilibrium denaturation results (Figure 1B). As the population of the intermediate(s) increases (indicated by the lowering of ΔG values), the amount of precipitation increases concomitantly.

To examine the solubility characteristics of different folding conformers of hGH, the refolding reaction was studied as a function of initial GdnHCl concentration using protein concentrations of 2 and 20 mg/mL. The results obtained for the two protein concentrations are summarized in Figure 2. For the low protein concentration, the amount of hGH lost as a result of precipitation is minimal and constant, within experimental error, throughout the entire range of GdnHCl concentrations tested. Because hGH was found to be insoluble at 20 mg/mL, only solutions containing initial GdnHCl concentrations above 3 M could be studied. However, at this protein concentration, the precipitation increases above 3.5

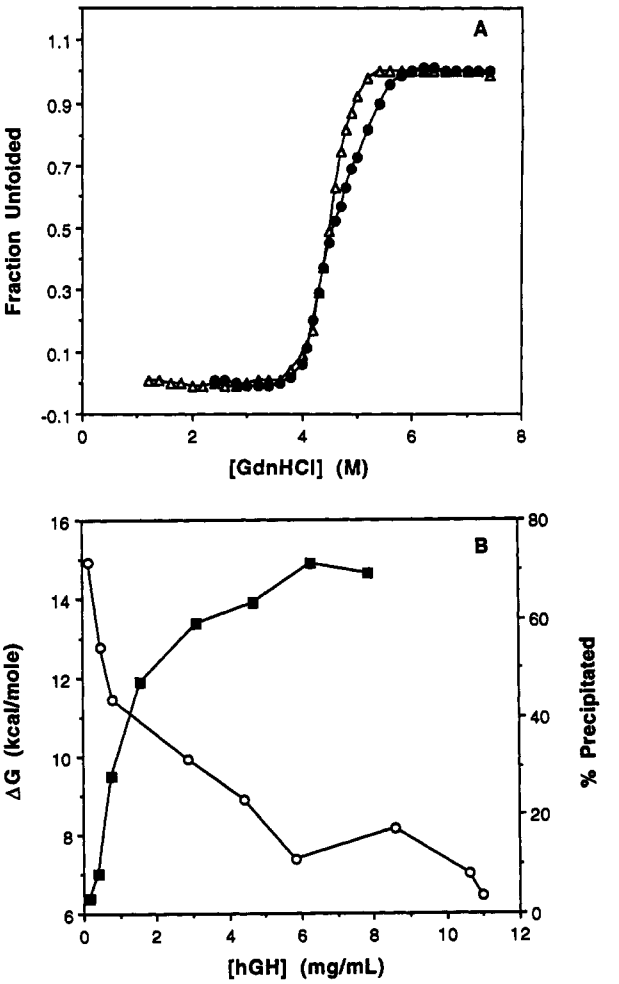

FIGURE 1: Concentration dependence of the Gibbs free energy of unfolding, ΔG, and of the precipitation due to refolding. (A) CD-detected (222 nm) equilibrium denaturation curves obtained using hGH concentrations of 0.1 mg/mL (▲) and 8.0 mg/mL (●) showing the broadening in the transition region at high protein concentration. (B) (○) Individual equilibrium denaturation experiments were performed with different concentrations of hGH and analyzed by assuming a two-state mechanism and using the linear extrapolation method to obtain values for ΔG. The ΔG values are plotted against the concentration of hGH used in each experiment. (■) Precipitation of hGH during refolding was induced by incubating various concentrations of hGH in 6 M GdnHCl/0.02 M HEPES, pH 7.5, at room temperature. Refolding was initiated by a constant dilution to 0.8 M GdnHCl. Precipitated protein was removed, and the hGH remaining soluble was quantitated by an absorbance measurement at 278 nm. Values for the fraction remaining soluble were determined by dividing the calculated amount of soluble hGH by the concentration expected assuming no precipitation and then multiplying by 100. These values were subtracted from 100 to obtain the percent precipitated that are plotted versus the expected final hGH concentration after refolding.

M GdnHCl, becomes maximal at a denaturant concentration of 4.5 M (the denaturation midpoint), and then remains constant. These results are consistent with the interpretation that partially denatured forms of hGH are responsible for the precipitation observed upon refolding. The data also indicate that refolding from the fully unfolded form of the protein gives rise to insoluble products.

Direct Evidence for a Self-Associated Intermediate. After identifying the existence of equilibrium intermediates and their role in the aggregation of hGH, we next considered experimentation aimed at obtaining a direct observation of the associated species. A number of spectroscopic and hydrodynamic techniques were applied to identify an associated intermediate in the folding pathway of bGH (Havel et al., 1986); we have applied many of these same approaches to

[2] It is emphasized that the broadening in the transition region, due to the presence of intermediates, affected the slopes of the lines obtained from the linear extrapolation method of data analysis. These changes in slope are reflected in the calculated ΔG values (Pace, 1975).

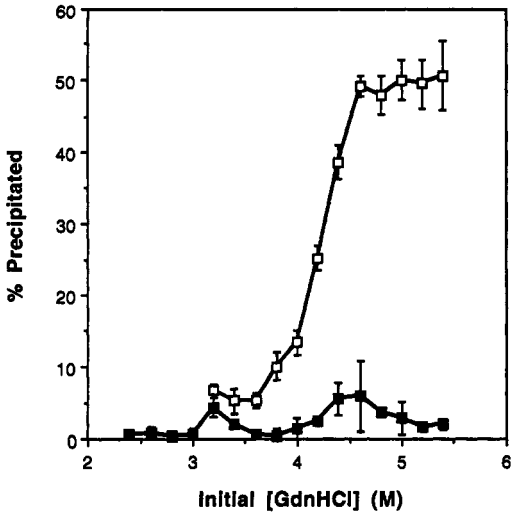

FIGURE 2: Precipitation of hGH during refolding from varying concentrations of GdnHCl. Solutions contained either 2 (■) or 20 (□) mg/mL hGH dissolved in variable amounts of GdnHCl and 0.02 M HEPES, pH 7.5. Precipitation was induced by diluting to refolding conditions of 0.8 M GdnHCl. The final protein concentration in each case was 0.3 mg/mL. Insoluble hGH was removed, and the soluble fraction was quantitated by an absorbance measurement at 278 nm. The percent precipitated was calculated as described in the caption of Figure 1B. The error bars represent the variability of multiple refolding procedures.

study the folding pathway of hGH.

Havel et al. (1986) showed that near-UV CD spectroscopy was capable of detecting the bGH-associated intermediate. Using the CD band at 300 nm, they demonstrated that the maximal optical activity occurred under partially denaturing conditions and proposed that this change reflected the self-association of a monomeric bGH intermediate. The 300-nm band was previously assigned to transitions involving the single tryptophan in the protein (Holladay et al., 1974). We were unable to detect a near-UV CD signal indicative of any associated intermediates for hGH (data not shown). This confirms the observations made by Lehrman et al. (1991), who showed that there is no change in the 300-nm band for solutions of hGH at concentrations as high as 10 mg/mL prepared in 4.6 M GdnHCl. Since spectroscopic techniques could not be successfully applied to directly demonstrate the presence of associated intermediates, we then turned to the hydrodynamic techniques of DLS and equilibrium analytical ultracentrifugation.

DLS has been used to identify associated equilibrium intermediates in the folding pathways of other proteins (Havel et al., 1986; Cleland & Wang, 1990, 1992). This technique has an advantage over SEC because potential stationary-phase interactions or sample dilution does not occur and there are no pressure effects. It has been previously reported that the mean diameter of hGH, as measured by DLS, showed minimal changes as it is unfolded with GdnHCl (Lehrman et al., 1991). On the basis of our work, this would be expected at the low protein (0.5 mg/mL) concentration used in that study. We have examined the molecular size of hGH as it unfolds using a protein concentration of 7 mg/mL, and the results are summarized in Figure 3A. The mean hydrodynamic diameter is about 4 nm at GdnHCl concentrations that are nondenaturing (<3.5 M). As the protein is unfolded, the molecular size increases and becomes maximal at the midpoint concentration for hGH denaturation (4.5–5.0 M GdnHCl). It is concluded that this size increase is the result of the aggregation of at least one intermediate. Finally, the mean hydrodynamic diameter decreases at high denaturant concentrations, indicating the dissociation of the aggregated species. The small

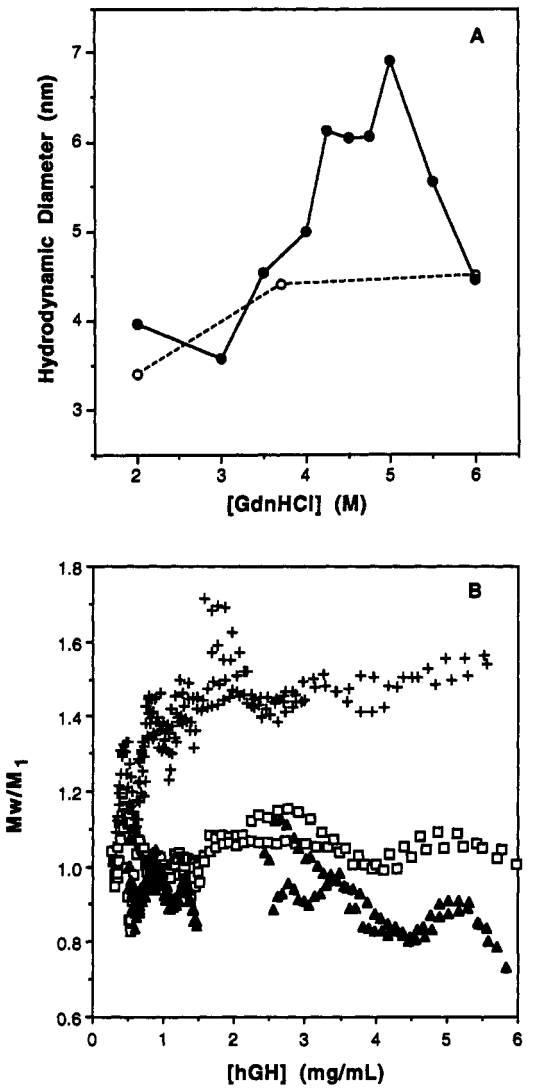

FIGURE 3: Dynamic light scattering and sedimentation equilibrium as a function of GdnHCl concentration. (A) DLS experiments were performed at 25 °C as described under Experimental Procedures using solutions containing 7 mg/mL hGH, variable concentrations of GdnHCl, and 0.02 M HEPES, pH 7.5: 7 mg/mL (●); 0.5 mg/mL (○) [from Lehrman et al. (1991)]. The estimated precision for both data sets is ±10%. (B) Equilibrium analytical ultracentrifugation was performed at 22 °C as described under Experimental Procedures. Protein samples were prepared in 0.1 M HEPES and 0 (□), 4.5 (+), or 6 (▲) M GdnHCl. The loading concentration of hGH was 1–5 mg/mL. The ratio M_w/M_1 is the weight-average molecular weight as determined by ultracentrifugation divided by the monomer molecular weight of hGH.

increase in the mean diameter observed upon unfolding is expected due to the extended conformation of the unfolded protein (Nicoli & Benedek, 1976).

The hydrodynamic properties of hGH in various folded states were also studied by equilibrium sedimentation (Figure 3B). Others (Jaenicke & Lehle, 1991) have applied this technique in protein folding experiments despite potential problems associated with the presence of high concentrations of denaturants (Schachman & Edelstein, 1966; Munk & Cox, 1972). For nondenaturing solution conditions (0 M GdnHCl), the analysis indicates that the apparent weight-average molecular weight is independent of protein concentration and the protein sediments as a monomer. Essentially the same results were obtained when the protein was unfolded in 6 M GdnHCl. However, because the excluded volume of unfolded hGH would be substantially larger than that of native hGH, one could expect to observe nonideality in the sedimentation behavior of the protein in 6.0 M GdnHCl (Munk & Cox,

1972; Van Holde, 1985). The ratio of the apparent weight-average molecular weight to the true weight-average molecular weight would decrease as the protein concentration increased. This behavior is indeed observed experimentally. The apparent weight-average molecular weight of hGH is close to the monomer molecular weight at low protein concentrations, but it is only about 80% of the monomer molecular weight at a protein concentration of 5 mg/mL. These ultracentrifugation results indicate that hGH does not associate in the native or denatured state. The DLS results, as well as the fact that we could not demonstrate a concentration-dependent association of hGH up to a concentration of 100 mg/mL using SEC-HPLC and a mobile phase of 6 M GdnHCl (data not shown), support our interpretation of these sedimentation equilibrium data.

In 4.5 M GdnHCl, where hGH is partially unfolded, the ultracentrifugation data indicate that a concentration-dependent self-association takes place. The apparent weight-average molecular weight is approaching that of a dimer. However, for the same reasons given above for the case of 6 M GdnHCl, the observed apparent weight-average molecular weight is probably lower than its actual value. Since the 4.5 M GdnHCl solution likely contains a variety of different monomer conformations (compact as well as unfolded) along with several aggregated species, sedimentation behavior is expected to be complex. For this reason, attempting to fit the data to a particular association model (e.g., monomer–dimer mechanism) to obtain a value for the equilibrium constant may be inappropriate because it is not expected to accurately describe the true association behavior. However, for comparative purposes, we used a previously described fitting procedure (Pekar & Frank, 1972) and assumed a monomer–dimer mechanism to determine an equilibrium constant of 7×10^3 M^{-1}. This compares with an equilibrium constant of 1.6×10^5 M^{-1} for the aggregation of bGH determined from near-UV CD data (Brems et al., 1988). Note that the equilibrium constants were obtained under different experimental conditions: bGH association was studied in 3.7 M GdnHCl and 0.05 M ammonium bicarbonate, pH 8.5.

Peptide Fragments from the Third Helix of Growth Hormones Can Inhibit Aggregation. A peptide fragment containing the third helix of bGH (residues 96–133) was shown to inhibit aggregation-induced precipitation in the folding pathway of this growth hormone, presumably by preventing the formation of an associated intermediate (Brems et al., 1986; Brems, 1988). It was proposed that this region of the protein, in its partially unfolded state, was the site of the molecular interactions resulting in aggregation. The hydrophobic face of this amphipathic helical region is apparently exposed in the partially unfolded conformer. The interaction of this fragment with the folding intermediate was shown to be rather specific in that truncated forms of the peptide and other sequences did not show the same inhibitory effects.

We have performed similar experiments using peptide fragments from the third helices of either hGH or bGH to determine whether the precipitation occurring during refolding could be inhibited. The partially unfolded intermediates were populated by incubating hGH in solutions containing 4.5 M GdnHCl. Portions of this solution were mixed with an increasing molar excess of either the bGH(109–128) or the hGH(109–128) peptide fragment. Folding was then initiated by dilution to 0.8 M GdnHCl. After removal of the precipitated material, the filtrates were analyzed by SEC-HPLC. Some representative chromatograms are shown in Figure 4. When hGH is incubated at 5 mg/mL in a native buffer condition (i.e., 0.8 M GdnHCl and 0.02 M HEPES, pH 7.5), and then diluted to 0.9 mg/mL, the chromatogram shows a peak that elutes with a retention time corresponding to the monomeric species (approximately 14 min). The late eluting peaks correspond to the buffer components present in the sample. However, if the protein is refolded from 4.5 M GdnHCl to a final GdnHCl concentration of 0.8 M in the absence of any peptide fragment, a new peak appears, eluting earlier (approximately 12.8 min) than the monomer (Figure 4, shaded peak). This peak is substantially diminished when folding is accomplished in the presence of a molar excess of either peptide fragment. The results show the effect of a 10-fold molar excess of the hGH(109–128) peptide as an example. The high molecular weight peak was eliminated when solutions containing it were chromatographed using a denaturing mobile phase (0.05 M ammonium bicarbonate and 6 M GdnHCl; data not shown). This suggests that the peak is a noncovalent aggregate. Indeed, a noncovalent dimer of hGH has been previously isolated and characterized (Becker et al., 1987). Although treated as an impurity with reduced potency in biosynthetic hGH preparations, the exact cause for the formation of this species is not known.[3] The high molecular weight peak appearing in our chromatograms has not been characterized; however, on the basis of its retention time and apparent noncovalent nature, we speculate that it is a product of an off-pathway folding intermediate and is very similar to the species previously identified by Becker et al. (1987).

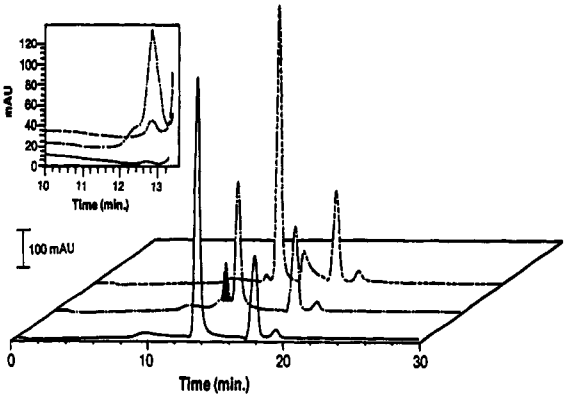

FIGURE 4: HPLC analysis of folded and refolded hGH. Solutions initially contained hGH at 5 mg/mL in 0.8 or 4.5 M GdnHCl and 0.02 M HEPES, pH 7.5. The solutions were diluted to 0.8 M GdnHCl and a final protein concentration of 0.9 mg/mL. Any precipitated protein was removed by filtration, and the filtrates were analyzed using the SEC method described under Experimental Procedures. (—) Folded hGH (sample was simply diluted while maintaining a constant GdnHCl concentration of 0.8 M); (– · –) hGH refolded from an initial partially denaturing GdnHCl concentration of 4.5 M (the shaded peak is the high molecular weight species that is formed as a result of refolding); (– – –) hGH refolded as before except in the presence of a 10-fold molar excess of the hGH(109–128) peptide fragment. Inset: enlargement of the high molecular weight region.

The integrated areas for the hGH monomer and high molecular weight peaks were plotted against an increasing mole ratio of peptide fragment to hGH (Figure 5A,B). It can be seen that as the mole ratio increases, there is an increase in the area of the hGH peak with a concomitant decrease in the amount of the high molecular weight peak. Both of these effects reach a saturation maximum at the highest levels of either peptide tested. There appear to be only small differ-

[3] The potential presence of a dimer species is not exclusive to biosynthetic hGH preparations, because material from other sources can contain higher molecular weight and/or dimeric forms that can be either covalent or noncovalent aggregates. It is not clear, however, whether the dimer species in all preparations are chemically identical. See Becker et al. (1987) and the references cited therein for further discussions.

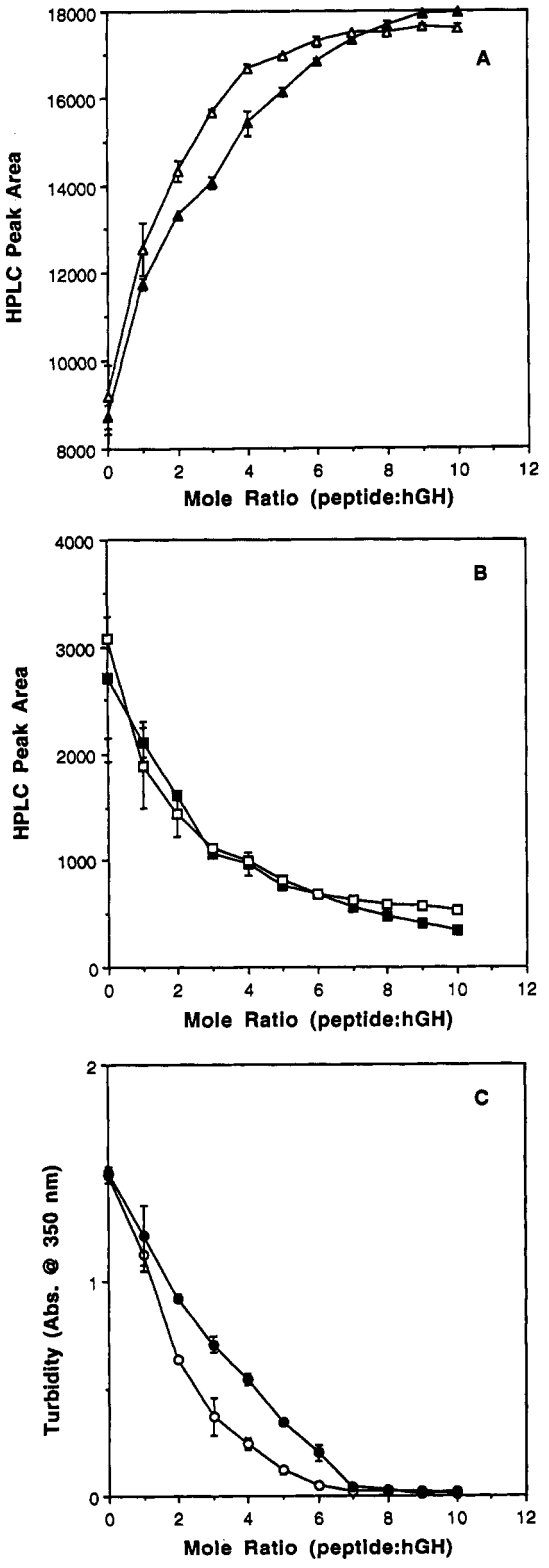

FIGURE 5: Analysis of the soluble and insoluble fractions produced during refolding of hGH solutions in the presence of hGH(109–128) and bGH(109–128). (A) HPLC peak areas for the monomer peak contained in the soluble fraction after refolding hGH from partially denaturing conditions (4.5 M GdnHCl). Refolding conditions were similar to those described in the caption of Figure 4 except that the mole ratio (peptide to hGH) was varied as indicated: (▲) hGH-(109–128); (△) bGH(109–128). (B) Conditions were identical to (A), except that the peak areas for the high molecular weight peak are plotted: (■) hGH(109–128); (□) bGH(109–128). (C) Determination of precipitated hGH in the insoluble fraction. Sample conditions were identical to those described in (A) except that the amount of turbidity was measured directly by an absorbance measurement at 350 nm: (●) hGH(109–128); (○) bGH(109–18). The error bars represent the variability of multiple refolding procedures.

ences, if any, between the two peptide fragments in their ability to inhibit the formation of the high molecular weight peak (Figure 5B). However, the bGH(109–128) peptide is slightly more potent in its ability to recover the hGH monomer peak (Figure 5A).

Turbidity was also used to examine the effect of each peptide on the precipitation of hGH that occurs during refolding (Figure 5C). The results indicate that the peptides not only diminish the amount of the soluble aggregate that forms but also inhibit the precipitation. There is a small difference between the two fragments in their ability to prevent insolubility of the folded protein. bGH(109–128) shows a stronger inhibitory effect at lower mole ratios.

We have not determined exactly how specific these peptide sequences are in inhibiting the precipitation of hGH during refolding. In the case of the bGH-associated intermediate, the specificity of the bGH third helix fragment (residues 96–133) has been demonstrated (Brems, 1988). The sequences used in this study are very similar. Because the bGH fragment also inhibits precipitation, we have no reason to suspect that the nature of the interaction for our peptides with the hGH-associated intermediate is any different than what has already been described for bGH. It is clear that amphiphilicity is an important characteristic, so it is reasonable to expect that other unrelated amphipathic peptides might also inhibit precipitation. Curiously, a peptide fragment (residues 96–134) of hGH did not show any significant inhibitory effect on the precipitation of aggregated bGH (Lehrman et al., 1991).

DISCUSSION

Evidence for an Associated Folding Intermediate of hGH. The equilibrium folding pathway of hGH was previously shown to be a cooperative, two-state process (Brems et al., 1990). However, we show that at higher protein concentrations than previously studied, the equilibrium denaturation of hGH is protein concentration dependent (Figure 1). For a unimolecular reaction, the calculated values of ΔG should not be affected by the protein concentration, and we propose that this observed concentration-dependent decrease indicates the presence of an associated intermediate. This indirect evidence for an associated intermediate was substantiated by DLS and equilibrium ultracentrifugation experiments. In both experiments, under partially denaturing conditions and high protein concentration, a species was detected with an apparent larger hydrodynamic diameter and higher weight-average molecular weight than any monomeric state of hGH (Figure 3). The formation of the associated intermediate is correlated with the precipitation of hGH that occurs during refolding. Refolding of hGH from partially denaturing conditions also resulted in the formation of an aggregate that eluted from SEC-HPLC as a soluble form in the mobile phase of 0.05 M NH_4HCO_3, pH 8 (Figure 4). It is not known whether the aggregated species observed by HPLC is identical to the one observed by DLS and equilibrium analytical ultracentrifugation. If these species are related, then it is likely that a portion of the self-associated intermediate, which forms under partially denaturing conditions, becomes trapped after refolding to native conditions.

How Do the Equilibrium Folding Pathways of hGH and bGH Compare? It has been demonstrated, by equilibrium denaturation, that the folding pathway of recombinant-derived bGH contains at least one stable monomeric intermediate (Brems et al., 1985). This intermediate has the propensity to self-associate, under certain conditions, giving rise to a stable, aggregated species (Havel et al., 1986; Brems et al., 1986) (Scheme I). Formation of the aggregated species is

responsible for precipitation of bGH during refolding. In contrast, we were unable to directly detect a monomeric intermediate under our experimental conditions, but propose that one must exist in the hGH folding pathway as well. This is likely, because the self-associated intermediate we observed only forms under partially denaturing conditions and must result from the assembly of a monomeric intermediate species. The existence of a self-associated intermediate in the folding pathway of hGH is analogous to the situation for bGH, although the self-associated intermediates are not necessarily structurally identical in every detail. For example, there is no near-UV CD band (300 nm) corresponding to the formation of the hGH self-associated intermediate as there is with bGH. Therefore, the environments around the tryptophan residues in the self-associated intermediates of hGH and bGH appear to be different. It should be noted that the tryptophan fluorescence properties of native hGH and bGH differ considerably (Havel et al., 1988; Kauffman et al., 1989; Brems et al., 1990). Similar to bGH, our data on hGH suggest that the interactions ultimately responsible for the self-association involve the third helix (Brems et al., 1986; Brems, 1988) (see later discussion).

The self-associated intermediate can also lead to irreversible precipitation of hGH during refolding. Precipitation occurs when refolding is performed with GdnHCl concentrations at and above the denaturation midpoint of hGH (Figure 2). In contrast, conditions were obtained for bGH in which precipitation only occurred when bGH was folded from partially denaturing GdnHCl concentrations (Brems, 1988). This difference could be explained by the decreased concentration of the hGH monomeric intermediate under partially denaturing conditions compared to the bGH monomeric intermediate. The concentrations of the monomeric intermediates are determined by the kinetic rate constants that control the on-pathway and off-pathway reaction[4] (Scheme I). The high protein concentrations used in our work drive the system toward the off-pathway reaction that results in self-association and precipitation (Kiefhaber et al., 1991). We have determined that dilution of a 20 mg/mL solution of bGH in 6 M GdnHCl and 0.05 M ammonium bicarbonate, pH 8.5, to refolding conditions will result in approximately 80% loss of protein due to precipitation. Therefore, the precipitation behavior of bGH, at 20 mg/mL, may be quite similar to what we have observed for hGH.

Brems et al. (1990) have previously proposed that the folding pathways of hGH and bGH differ because of the relative stabilities of intermediates compared to the native state. An equilibrium intermediate is observed for bGH because the native state of this protein is not highly stabilized relative to the intermediate. In the case of hGH, monomeric equilibrium intermediates are not observed because none are proportionately stabilized compared to the highly stabilized native state. However, we demonstrate that an intermediate can be populated during refolding using high concentrations of hGH. This intermediate appears to be only transiently populated, since it cannot be directly observed at equilibrium, and rapidly proceeds onto an off-pathway associated form that is more stable. Reduction and alkylation of the disulfide bonds of hGH result in the destabilization of the native state relative to the intermediate state as determined by the substantial population of intermediates present under partially denaturing conditions (Brems et al., 1990). Therefore, the folding pathways of bGH and hGH appear to be identical except for differences in the kinetic rate constants defining the folding (on-pathway) and off-pathway aggregation reactions. Kinetic folding experiments on bGH have already been reported (Brems et al., 1987a). We are currently pursuing experiments on the folding kinetics of hGH to obtain evidence for transient intermediates and rate constants for comparison to bGH.

The equilibrium denaturation of recombinant pGH has recently been reported (Bastiras & Wallace, 1992). The folding mechanism was found to be very similar to bGH; however, we note two significant observations of relevance to this work. First, it was demonstrated by SEC that an associated form of pGH was populated at intermediate GdnHCl concentrations. Second, it was mentioned that precipitation occurs upon dilution to nondenaturing GdnHCl conditions. These results suggest the presence of a self-associated pGH intermediate that can result in precipitation and are consistent with our findings and those previously reported for bGH. Therefore, the equilibrium folding pathways of hGH, bGH, and pGH are all very similar.

Aggregation of Folding Intermediates Is a Result of Specific Interactions. Partial denaturation of bGH exposes the hydrophobic surface of the third helix (109–128), and if the concentration of bGH is sufficiently high, the hydrophobic face of one amphipathic helix will interact with similar surfaces of adjacent molecules, resulting in the formation of the associated intermediate (Brems et al., 1986). An isolated fragment from the third helix was shown to inhibit the precipitation that occurs during folding of bGH (Brems et al., 1986; Brems, 1988). The fragment is helical in solution, and it was proposed that the hydrophobic surface of the fragment interacted with the exposed hydrophobic surface of partially denatured bGH to prevent self-association of bGH intermediates.

In an analogous way, we show that the precipitation resulting from refolding hGH can be inhibited. Furthermore, we have obtained direct evidence that the peptide fragments used in our work can reduce the amount of the soluble aggregate detected by SEC-HPLC that is formed as a result of folding. There are small differences in the potency of hGH(109–128) compared to bGH(109–128). The bGH(109–128) fragment recovers more of the hGH monomer and inhibits the precipitation at lower mole ratios. The hydrophobic surfaces of both helical fragments are similarly aligned along one face as shown by helix wheel diagrams [see Lehrman et al. (1991)]. It is known that the helix content of these peptides is strongly dependent on concentration [data not shown; see also Brems et al. (1987b)]. We propose that a plausible explanation for the differences in potency is the extent of helix structure for each peptide at a given concentration as previously argued (Lehrman et al., 1990).

Lehrman et al. (1991) have shown that a peptide fragment of hGH (residues 96–134) did not inhibit precipitation of bGH during folding. However, they also reported that another fragment, designated as 8H-bGH(96–133), at a 4:1 peptide: bGH mole ratio inhibited bGH precipitation by 36% under their experimental conditions. The amino acid sequences of the overlapping segments of 8H-bGH(96–133) and our peptide fragment, hGH(109–128), are identical. We conclude that the differences they observed between bGH(96–133) and 8H-bGH(96–133) are analogous to what we observe for bGH-(109–128) and hGH(109–128). It is also reasonable to suspect that the additional 18 amino acid residues (13 at the N-terminus and 5 at the C-terminus) present in the peptides used by Lehrman et al. (1991) might interfere with the interactions occurring with the bGH intermediate. Thus, the

[4] The expression "on-pathway" refers to the folding reaction that results in the production of native hGH, and the expression "off-pathway" refers to the side reaction that occurs when intermediates proceed onto higher order aggregates.

inhibitory effect on precipitation could be diminished to some extent.

Physiological Implications. Our results provide insight into in vivo protein folding processes. The fact that hGH displays a concentration-dependent aggregation during folding may be directly related to the mechanism of inclusion body formation when this protein is overproduced in bacteria. In addition, the aggregation and precipitation can be specifically inhibited by amphipathic peptides, and this might also have physiological relevance. It is known that molecular chaperones provide surfaces to stabilize partially folded intermediates, which precludes association with other protein molecules. We speculate that the peptide fragments used in this and previous studies may be acting in an analogous fashion to chaperones. Namely, the peptides bind specifically at the third-helical region of partially folded hGH or bGH and prevent self-association. The main difference is that ATP is not required for the process as it is with chaperones. The relatedness of these processes is supported by the demonstration that hGH inclusion body formation could be inhibited by co-overproduction of the DnaK chaperonin in vivo (Blum et al., 1992).

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