Equilibrium Denaturation of Human Growth Hormone and Its Cysteine-modified Forms*

(Received for publication, July 13, 1989)

David N. Brems‡, Patricia L. Brown‡, and Gerald W. Becker‡

From the ‡Department of Pharmaceutical Research and •Department of Biochemistry, Eli Lilly & Co., Indianapolis, Indiana 46285

The equilibrium denaturation of human growth hormone (hGH) derived from heterologous gene expression in Escherichia coli was studied. Denaturation was measured by ultraviolet absorbance, intrinsic fluorescence, far ultraviolet circular dichroism, and size exclusion chromatography. The denaturation transitions obtained from each method of detection were coincident, indicating a two-state denaturation mechanism. The denaturation transitions were independent of the concentration of protein. The Gibbs free energy of unfolding was 14.5 ± 1 kcal/mol.

Human growth hormone contains two disulfide bridges between residues 53-165 (large loop) and 182-189 (small loop). The small loop was selectively reduced and cysteines alkylated with iodoacetic acid or iodoacetamide. The tetra-S-carbamidomethylated and tetra-S-carboxymethylated derivatives were also prepared. All S-alkylated hGH forms were indistinguishable from the native conformations in the absence of denaturant by far ultraviolet circular dichroism. The circular dichroism-detected equilibrium denaturation of each derivative was determined and the Gibbs free energy of unfolding of the tetra-S-modified forms was 5.3 ± 0.5 kcal/mol and of the di-S-alkylated derivatives was 11.2 ± 0.8 kcal/mol.

These results for hGH are different than previously obtained results for bovine, ovine, and rat growth hormones. Stable equilibrium intermediates have been identified for these non-human species of growth hormone. Reduction and alkylation caused hGH to denature similar to the nonhuman species of growth hormone. The stable intermediates observed in the denaturation of reduced, alkylated hGH or nonhuman growth hormones are similar and characterized as compact, helical, lacking native-like tertiary structure, and having a tendency to aggregate.

The apparent absence of intermediates in the folding of oxidized hGH is due to the relative instability of intermediates compared with their native structures. The hGH conformation is at least 3 kcal/mol more stable than the growth hormones from other species. Reduction and alkylation of the disulfide bridges of hGH diminish the stability differences between the native and intermediate states, such that the denaturation behavior is similar to the nonhuman growth hormones with well-populated intermediates. Most proteins do not demonstrate equilibrium folding intermediates presumably because intermediates are only marginally stable in conditions that disrupt the native state. The folding results with hGH and alkylated hGH substantiate this.

Growth hormones are polypeptides of approximately 190 amino acids that are best known for stimulating growth and numerous metabolic effects (1, 2). Growth hormones have been isolated from a wide variety of mammals and from some nonmammalian vertebrates. There is considerable variation in the amino acid sequences of growth hormones from the different species (3). In all cases, their primary structures contain two disulfide bridges, one of which joins distant parts of the molecule (large loop) and the other forms a small loop near the COOH terminus. All species contain approximately 50–60% α-helix (4, 5). Despite the sequence heterogeneity between the species their three-dimensional structures are thought to be very similar. The three-dimensional structure of porcine growth hormone, as determined by x-ray diffraction (6), shows a single-domain protein consisting of four antiparallel α-helices arranged in a left-twisted helical bundle. Crystals suitable for x-ray diffraction have also been obtained from human (7) and bovine (8) species, and their structures are currently being determined. Human growth hormone (hGH) is a pharmaceutical that is used to treat hypopituitary dwarfism. Growth hormones from other species are currently being developed as agents for stimulating growth and lactation in animals.

The folding of nonhuman growth hormones has been studied (9-18). Equilibrium and kinetic folding studies of bovine growth hormone (bGH) have been shown to be consistent with the mechanism of Scheme I

\[
N \rightarrow \cdots \rightarrow U \\
\downarrow \\
I_{assoc}.
\]

Scheme I

where N is native, I is intermediate, U is unfolded, and I_{assoc} is associated intermediate.

Intermediate I is populated at equilibrium (11) in denaturation studies and transiently during kinetic folding (14) and is characterized as having a compact structure, mostly helical.

1 The abbreviations used are: hGH, human growth hormone; bGH, bovine growth hormone; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HPLC, high performance liquid chromatography; GdnHCl, guanidine hydrochloride; I_{assoc}, associated folding intermediate; 1-RCOM hGH, di-S-carboxymethylated human growth hormone modified at cysteines 182 and 189; 2-RCOM hGH, tetra-S-carboxymethylated human growth hormone modified at cysteines 53, 165, 182, and 189; 1-RCAM hGH, di-S-carbamidomethylated human growth hormone modified at cysteines 182 and 189; 2-RCAM hGH, tetra-S-carbamidomethylated human growth hormone modified at cysteines 53, 165, 182, and 189.

5504
flexible, and a denatured-like tertiary structure as determined by UV absorbance (17). The structure of I has been likened to a "molten globule state" that may be a common folding intermediate for many types of proteins (17). The associated intermediate, $I_{assoc}$, is stable only at higher concentrations of protein, has an association constant of $\sim 10^7$ M$^{-1}$, and a weight-average radius of 50 Å (12). The third helix of bGH is made up of residues 107-128 (6, 18), is amphipathic, and has been shown to be critical to association. The associated intermediate is stabilized in part by the intermolecular packing between hydrophobic faces of the third helix (15, 16). Formation of $I_{assoc}$ results in a different environment surrounding the single tryptophan at position 86 that is manifested by a negative ellipticity at 300 nm in the circular dichroism (CD) spectrum (12). The presence of $I_{assoc}$ also alters the shape of the equilibrium denaturation transition as detected by CD at 222 nm and size-exclusion HPLC (12, 13).

In contrast, little has been reported concerning the folding of hGH. hGH has the property of being fully active in stimulating growth even if the two disulfides are reduced and alkylated with iodoacetamide (19) or if cysteine 165 (a member of the large loop) is mutated to alanine (20). Alkylation of both disulfides with iodoacetic acid rendered it inactive to growth stimulation, but it retained some in vitro receptor and lactation activity (19). The carboxymethylated derivative was 2 times more susceptible to in vitro trypsin proteolysis than the carbamidomethylated derivative (19).

In this report the equilibrium denaturation of hGH and its cysteine-alkylated forms are investigated. The folding of hGH is modulated by GdnHCl and the extent of folding is detected by multiple methods. High pressure gel filtration chromatography is used to determine the hydrodynamic volume, far UV CD to analyze the $\alpha$-helix content, UV absorption spectroscopy to assess the solvent environment of the aromatic amino acids, and intrinsic fluorescence to determine the solvent environment of the single tryptophan.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Fluorescence-detected Denaturation**—The intrinsic tryptophan fluorescence emission spectra of hGH in the absence of GdnHCl are illustrated in Fig. 1A. Native hGH is not quenched compared with its unfolded state and undergoes a 15-nm red-shift upon denaturation. The same is observed with 2-RCOM hGH (data not shown). The red-shift observed upon denaturation indicates an increase in the hydrophilic environment of the tryptophan side chain. The fluorescence of hGH undergoes a similar red-shift upon denaturation but is quite different in that the native state is quenched (21). The effect of GdnHCl on the emission spectra of hGH and 2-RCOM hGH as monitored by fluorescence. The protein concentration in A and B was 0.1 mg/ml in 20 mM HEPES, pH 7.5. The fluorescence intensity at 370 nm is in arbitrary units.

**UV Absorbance-detected Denaturation**—The aromatic spectrum of hGH results from 13 phenylalanines, 8 tyrosines, and 1 tryptophan. The absorbance spectra of native (- - -) and GdnHCl denatured (-----) hGH, B, equilibrium denaturation of hGH (●) and 2-RCOM hGH (○) as monitored by fluorescence. The protein concentration in A and B was 0.1 mg/ml in 20 mM HEPES, pH 7.5. The fluorescence intensity at 370 nm is in arbitrary units.

**FIG. 1. Intrinsic fluorescence of hGH and 2-RCOM hGH. A,** fluorescence spectra of native (- - -) and GdnHCl denatured (-----) hGH. B, equilibrium denaturation of hGH (●) and 2-RCOM hGH (○) as monitored by fluorescence. The protein concentration in A and B was 0.1 mg/ml in 20 mM HEPES, pH 7.5. The fluorescence intensity at 370 nm is in arbitrary units.

**CD detected Denaturation**—The far UV CD spectra of hGH and its cysteine-alkylated forms are very similar (20). The far UV CD reflects the extent of secondary structure and is dominated by the large amount (50%) of $\alpha$-helix in hGH. The CD-detected transition of hGH in Fig. 3A is completely reversible and independent of the hGH concentration from 0.01 to 2 mg/ml. Also shown in Fig. 3A is the CD-detected denaturation of short-loop carboxymethylated (1-RCOM) hGH, short-loop carbamidomethylated (1-RCAM) hGH, and short-loop plus large-loop carboxymethylated (2-RCOM) hGH, and short-loop plus large-loop carbamidomethylated (2-RCAM) hGH. The results of Fig. 3A were obtained at a protein concentration of 0.01 mg/ml. As illustrated in Fig. 3A, the denaturation transitions are sensitive to which disulfide is modified and if both disulfides are modified the transitions are slightly sensitive to the type of modifying group. Fig. 3A also demonstrates that complete modification of the disulfides
does not result in significant alteration in the secondary structure in native-like solution conditions (<2.5 M GdnHCl). The intactness of the secondary structure of alkylated hGH has been reported previously (19).

The Gibbs free energy (ΔG) of stabilization of hGH can be estimated by assuming a two-state analysis of the data in Fig. 3A. We have used the method of Schellman (22) as described by Pace et al. (23) for analyzing solvent denaturation processes to obtain the free energy of unfolding in the absence of denaturant. Fig. 3B shows the linear relationship between the natural logarithm of the apparent equilibrium constant for unfolding and the GdnHCl molarity for hGH and the various cysteine-modified forms. The midpoints and ΔGs from the results in Fig. 3A and B are contained in Table I. hGH shows the greatest free energy of stabilization > l-RCAM hGH ≈ 1-RCOM hGH > 2-RCOM hGH ≈ 2-RCAM hGH. Assuming that each disulfide bond is independent (no conformational interaction between them) the small-loop disulfide contributes 3.3 kcal/mol (determined by subtracting the average ΔG of 1-RCOM hGH and 1-RCAM hGH from the ΔG for hGH) and the large-loop disulfide contributes 5.9 kcal/mol (determined by subtracting the average ΔG of 2-RCOM hGH and 2-RCAM hGH from the average ΔG of 1-RCOM hGH and 1-RCAM hGH) to the free energy of stabilization.

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Midpoint (M)</th>
<th>ΔG (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>4.59</td>
<td>14.45</td>
</tr>
<tr>
<td>1-RCAM hGH</td>
<td>4.16</td>
<td>10.40</td>
</tr>
<tr>
<td>1-RCOM hGH</td>
<td>4.13</td>
<td>11.91</td>
</tr>
<tr>
<td>2-RCOM hGH</td>
<td>3.17</td>
<td>5.20</td>
</tr>
<tr>
<td>2-RCAM hGH</td>
<td>3.44</td>
<td>5.34</td>
</tr>
</tbody>
</table>

* Calculated from the CD-detected denaturation results of Fig. 3 according to Pace et al. (23).

4A. hGH undergoes very little change in Stokes radius from 0 to 4 M GdnHCl, however, in the range of 4.0–5.5 M the radius increases due to unfolding. From 5.5 to 6.5 M GdnHCl,
Conformational Stability in the Absence of Disulfide Bonds

Complete denaturing conditions and its amount (Fig. 4B) and Stokes radius (Fig. 4A) vary depending on the concentration of denaturant. Fig. 4B shows that in the transition zone both species 1 and 3 are present as separate peaks indicating that the interconversion between them is slow compared with the chromatography time scale. In contrast, only a single peak is seen in the elution pattern for species 3. This can be explained by a conformational equilibrium for species 3 that is rapid, relative to the time scale of the elution process, with an elution time that is the weighted average of the forms in equilibrium.

Comparison of Denaturation by Different Detection Methods—The results of hGH and 2-RCOM hGH denaturation as measured by the different detection methods are shown in Fig. 5. To obtain the results of Fig. 5, the predenaturation and postdenaturation base lines of each transition were determined by a least squares fit to the data as described by Pace et al. (23). The values for the fraction unfolded within the transition regions were obtained by extrapolation from the pre- and posttransitions regions into the transition region. As illustrated in Fig. 5, the denaturation transitions for hGH as detected by fluorescence, UV absorbance, CD, and size exclusion are all coincident (the slight differences of the size-exclusion results are not considered significant). The results for hGH are consistent with a two-state mechanism for unfolding with the two states consisting of native and denatured hGH. Denaturation of 2-RCOM hGH shows that the absorbance and fluorescence-detected transitions are similar but different than the CD-detected transitions with the latter showing a greater stability toward denaturant (Fig. 5). The results for 2-RCOM hGH denaturation are consistent with a multiple state mechanism with at least one populated intermediate.

Effect of Protein Concentration—The effect of protein concentration on the denaturation of 2-RCOM hGH was inves-

Fig. 4. Size-exclusion-detected equilibrium denaturation of hGH and 2-RCOM hGH. A. represents the Stokes radius versus GdnHCl, and, B, the peak percent versus GdnHCl. The concentration of hGH injected was 0.1 mg/ml and 2-RCOM hGH was 0.04 mg/ml. Symbols represent the following results: hGH (○), species 1 of 2-RCOM hGH (□), species 2 of 2-RCOM hGH (■), and species 3 of 2-RCOM hGH (●).

the Stokes radius remains constant and reflects completely unfolded hGH. The results for hGH in Fig. 4A are independent of the protein concentrations injected (0.01-1.0 mg/ml), are reversible, and for each chromatogram only one peak exists.

The effect of GdnHCl on the Stokes radius of 2-RCOM hGH is also illustrated in Fig. 4A. Depending on the concentration of GdnHCl as many as three different peaks were observed. One peak, referred to as species 1 (Fig. 4A) is the native species and represents 95% of the total in nondenaturing concentrations of GdnHCl (Fig. 4B). Species 2 has a larger apparent hydrodynamic volume than native and is present in all nondenaturing conditions but only accounts for 5% of the total (Fig. 4B). Species 2 may represent a trapped product of an alternative folding pathway. Species 3 is an altered conformational state because it is a separate peak from the native peak and has a different Stokes radius than the denatured state. Species 3 is present in partially and
protein cause the CD-detected transitions to occur at increased by the concentration of protein injected for comparative purposes.

The signal response for each chromatogram was divided. The signal response for each chromatogram was divided by the concentration of protein injected for comparative purposes.

Fig. 6. Effect of 2-RCOM hGH concentration on the size-exclusion chromatography. The solvent was 3.4 M GdnHCl and the chromatograms represent, from top to bottom, 0.04, 0.4, 2.7, and 8.0 mg/ml injected. The signal response for each chromatogram was divided by the concentration of protein injected for comparative purposes.

The filled symbols are the results for 0.01 mg/ml, and the open symbols are for 1.8 mg/ml. The slope of the transition for l-RCOM hGH and l-RCAM hGH is similar to hGH but displaced to lower GdnHCl. These values assume independence of the two disulfide bonds to the free energy of folding stability to GdnHCl.

The presence of intermediates can decrease the cooperativity of completely alkylated hGH. The results obtained using alkylated hGH are only valid so intermediate: (a) a biphasic transition as measured by a single detection method, and (b) noncoincident transitions as measured by different detection methods. Either one of these observations is sufficient evidence for an equilibrium intermediate (26). With this in mind the equilibrium denaturation of hGH was studied by intrinsic fluorescence (Fig. 1B), near UV absorbance (Fig. 2B), far UV CD (Fig. 3A), and molecular sieve chromatography (Fig. 4). The normalized denaturation transitions of hGH (Fig. 5) obtained from the different methods of detection are all monotonic and coincident. These results do not provide any evidence for the presence of equilibrium intermediates and are most consistent with a simple denaturation mechanism composed of two-states, native and denatured.

The effect of protein concentration on the CD-detected denaturation is illustrated in Fig. 3, A and B, and Table I. The selective reduction and alkylation of the short-loop destabilized the native structure by 3.3 kcal/mol and was independent of the chemical structure of the alkylating reagent. Reduction and modification of both disulfide loops decreased the free energy of folding by 5.9 kcal/mol and was slightly dependent on the structure of the alkylating reagent. These values assume independence of the two disulfide bridges (meaning no direct bonding interactions between them). The slope of the transition for 1-RCOM hGH and 1-RCAM hGH is similar to hGH but displaced to lower GdnHCl concentrations. Completely alkylated hGH shows less cooperative transitions with midpoints less than dialkylated hGH. The presence of intermediates can decrease the cooperativity of denaturation transitions (27) and are probably the cause of the decreased cooperativity of completely alkylated hGH. The results obtained using alkylated hGH are only valid so
far as the size and charge of the alkylating reagent itself does not seriously alter the conformation stability of the modified hGH. We feel that conformational stability of cysteine-modified hGH is only slightly affected by the size and charge of the modifying reagent. Fig. 3, A and B, and Table I show that charge of the alkylating group does not make a large difference. We have obtained results for hGH that were completely reduced with mercaptoethanol (where the modifying group was hydrogen, data not shown), and it has a similar denaturation transition as 2-RCOM hGH and 2-RCAM hGH.

Bewley et al. (19) have studied the effect of these cysteine modifications on hGH biological activity. They report that 2-RCAM hGH is fully active in the hypophysectomized rat and pigeon crop assays, whereas 2 RCOM hGH was only active in the pigeon crop assay. They also showed that 2-RCOM hGH was ~2 times more susceptible than 2-RCAM hGH to in vitro trypsin hydrolysis. It was suggested that the lack of growth promoting activity in the hypophysectomized rat of 2-RCOM hGH may be due to its rapid in vivo proteolytic degradation. Our equilibrium denaturation results show that the overall secondary structure of 2-RCOM hGH is no more stable than that of 2-RCAM hGH. It is possible that degradation by trypsin reflects a very small or local conformational difference between 2-RCOM hGH and 2-RCAM hGH that escapes detection by CD-detected equilibrium denaturation. This apparent difference may be better understood by studying equilibrium denaturation using other methods of detection. Alternatively, the differences in activity between 2-RCOM hGH and 2-RCAM hGH may be due to physical differences in solubility which affect the biological measurements differently. For instance the rat growth assay (10, 28) utilized 10 times more hormone than the pigeon crop assay (19, 29, 30). The biological assay results need to be interpreted carefully in light of this additional physical information.

**Evidence for a 2-RCOM hGH Folding Intermediate**—The results of Fig. 5 show that the equilibrium denaturation transitions of 2-RCOM hGH detected by different methods are not coincident and intermediate(s) must be present. In particular, denaturation measured by UV absorbance or fluorescence is quite similar but occurs at a lower concentration than the far UV CD-detected denaturation (Fig. 5). Fluorescence and UV absorbance chiefly represent the environment of the aromatic chromophores or the tertiary structure while the far UV CD reflects the secondary structure. Fig. 5 illustrates that the secondary structure is more stable to denaturation than is the tertiary structure.

The results from size-exclusion-detected denaturation are illustrated in Fig. 4 and provide a measure of the hydrodynamic volume of the protein during denaturation. The denaturation transition obtained by size exclusion has a midpoint similar to that of the CD-detected transition but is more cooperative (it occurs over a narrower range of denaturant) than the transitions observed by CD, absorbance, or fluorescence. The presence of stable intermediates decrease the cooperativity or steepness of the denaturation transition.

Comparison of the transitions show that the packing of the aromatic side chains is disrupted initially and the degree of compactness and secondary structure denature differently but at higher concentrations of denaturant. These findings are similar to those obtained for bGH and led to the disruption of folding intermediate I (Scheme I) as a molten globule which contains a high content of helical secondary structure, compact hydrodynamic radius, and packing of the aromatic side chains that are flexible and like the unfolded state (17).

The size-exclusion-detected denaturation (Fig. 4) also demonstrates conclusively the existence of intermediates. In the transition zone, two separate peaks exist, one is the native state and the other is a combination of intermediate and denatured forms. The native form is in slow exchange with the peak containing the intermediate and unfolded forms, whereas the intermediate and unfolded forms are in fast exchange. The elution time of the peak composed of intermediate and denatured states depends on the relative proportions of each. The intermediate must be nearly as compact as the native state since in the initial part of the transition zone where the intermediate is highly populated it has a similar elution time to the native peak. Fig. 4 shows that the loss of the native peak correlates directly to the gain of the peak composed of intermediate and denatured forms. The results of Fig. 4 were obtained at very low protein concentration (0.04 mg/ml injected) to eliminate the complication of aggregation. A peak corresponding to <5% of the total was consistently observed from 2 to 3.4 M GdnHCl and has an elution time between the native and denatured peaks. The nature of this species is not understood at the present time. The size-exclusion-detected denaturation results are consistent with Scheme I worked out for bGH except that the kinetics of the step N → I are slow compared with the time required for chromatography, while for bGH these forms are in fast exchange.

**Evidence for a 2-RCOM hGH-associated Folding Intermediate**—Another property related to intermediate I of bGH is that the hydrophobic surface of the third helix (residues 106–127) forms an interactive surface that stabilizes association through intermolecular hydrophobic interactions to give rise to I_{agg}. (13, 15, 16). This association was manifested by increased hydrodynamic volume as determined by size exclusion and dynamic light scattering (12), altered far UV CD denaturation transition (13), unique near UV CD band (12), and a lower solubility than other conformations (15). The altered far UV CD transition of I_{agg} is due to the helices packed in an intermolecular arrangement (13). The near UV CD band at 300 nm results from the altered environment of the tryptophan in the intermolecular aggregate (12). The low solubility of I_{agg} was first noticed by the hysteresis of the equilibrium denaturation transitions (11). The denaturation transitions were completely reversible providing the GdnHCl concentration remained >2 M. However, if denatured bGH was diluted to <2 M GdnHCl, significant precipitation of the protein resulted. Yet if the denatured samples were diluted to 2 M GdnHCl and allowed to fold for several minutes, before dilution to lower GdnHCl concentrations, turbidity did not appear. I_{agg} was shown to be responsible for the precipitation that occurs upon refolding (15).

The tendency for 2-RCOM hGH to aggregate in partially denaturing solutions is demonstrated in Figs. 6 and 7. Fig. 6 shows that in 3.4 M GdnHCl, increasing concentrations of protein result in a decrease in elution time. At high protein concentration (>2 mg/ml injected), the majority of 2-RCOM hGH elutes as higher molecular weight aggregates. These aggregates are highly stable but not covalently linked, since if they are isolated and re.injected at the same concentration of denaturant they elute with the same "aggregate-type" elution time, but if re-injected in completely denaturing conditions they elute as monomeric unfolded protein. A dimeric form of hGH that has been identified as a contaminant in all biosynthetic and pituitary hGH production lots (31) may be related to these higher molecular weight forms of 2-RCOM hGH. Further characterization of these aggregates is under current investigation.

Fig. 7 shows the effect of protein concentration on the far UV CD-detected denaturation. At higher protein concentrations (>2 mg/ml), the denaturation transi-
tion is shifted to higher concentrations of denaturant and appears more stable. This indicates that the helices of monomeric I are less stable than the helices of I_{soc}. This difference represents the stability provided by the intermolecular interactions. The solubility of 2-RCOM hGH is similar to bGH (11, 15) in that denatured samples of 2-RCOM hGH, if diluted to <2 M GdnHCl, precipitate. Solutions of refolded 2-RCOM hGH can be obtained by an intermediate dilution of the GdnHCl to 2 M as described for bGH. Such precipitation as a result of refolding was not observed for hGH, presumably due to the absence of I_{soc} in the refolding pathway. The absorbance-detected denaturation transition of 2-RCOM hGH (Fig. 2B) is not dependent on the protein concentration and can be explained by Scheme I, if the UV absorbance change is limited to the N → I step as is the case for bGH (15). Unlike bGH (12), 2-RCOM hGH I_{soc} does not demonstrate the negative ellipticity band at 300 nm. We presume this is due to a different environment for the sole tryptophan at position 86 for hGH. This difference is evident in the fluorescence of hGH compared with bGH. Fig. 1, A and B shows that for 2-RCOM hGH (and hGH) the native state is not quenched relative to the denatured state, and the wavelength maximum occurs at 335 nm. In contrast, for bGH the tryptophan is quenched in the native state and has a wavelength maximum at 353 nm. Similar differences in the tryptophan environment between human and nonhuman growth hormones have been previously demonstrated by second-derivative absorbance spectroscopy (32) and resonance Raman spectroscopy (33).

The folding results of 2-RCOM hGH are consistent with the results obtained for the combined reoxidation/refolding of reduced bGH (34). For reduced bGH, the secondary structure was formed early and prior to the reoxidation of the disulfide bonds. The main difference is that reduced hGH is soluble in water and reduced bGH is not. We interpret this physical difference to be due to the relative differences in the stabilities of intermediates. Reduced bGH is not soluble in <4 M urea or <2 M GdnHCl presumably because this represents partially denaturing conditions and I_{soc} is highly populated. Because of the low solubility of I_{soc}, reduced bGH precipitates in aqueous solution containing <2 M GdnHCl. However, the partially denaturing conditions for reduced hGH occur from 2.5 to 4.5 M GdnHCl, which are conditions that solubilize I_{soc}. In <2 M GdnHCl reduced hGH is in a native-like conformation and consequently soluble.

Relevance to Other Proteins—There are several points of general interest to protein folding that may be concluded from these studies. 1) The folding mechanisms for oxidized and reduced growth hormones are very similar. The in vitro refolding of reduced, denatured proteins is sometimes considered more relevant to in vivo folding than refolding of oxidized denatured proteins. The similar mechanism observed for the refolding of bGH and 2-RCOM hGH suggests that noncovalent interactions dominate and that refolding of oxidized denatured proteins is relevant. 2) The general insolvability often observed for reduced proteins may be due to the tendency for folding intermediates to agglomerate and thus precipitate in nondenaturing solutions rather than the often concluded reason of inherent insolubility of the unfolded protein. If true, this suggests that the stabilizing intermolecular interactions could be specific and result from folded portions of an intermediate and may be readily altered by extrinsic reagents or small changes in the amino acid sequence as in the case for bGH (16). 3) Most often, the equilibrium denaturation of proteins is a cooperative event explained by a two-state process (absence of intermediates) (35). It has been suggested that this is due to the relative instability of intermediate structures compared with the native state (26). Denaturing conditions sufficient to disrupt the native state are probably unfavorable for population of intermediate states. The folding of hGH and reduced alkylated hGH provides a definitive example of this suggestion.

REFERENCES

Conformational Stability in the Absence of Disulfide Bonds

EXPERIMENTAL PROCEDURES

Materials

Chemicals: Glutamic acid (Glu), cysteine, and cysteine hydrochloride dihydrate (GSH) were obtained from Sigma Chemical Co. All other reagents were of reagent grade.

Methods

Equilibrium Titration

The samples were titrated in 0.1 M sodium phosphate buffer (pH 7.0) at 25 °C using a pH stat (Radiometer CPM 733). The pH of the buffer was recorded (pH 7.0) and the sample volume was adjusted to 1.5 ml. The samples were maintained at 25 °C by immersing the sample cuvettes in a water bath.

Results

Table 1: Table of amino acid analysis for GSH, pyruvate, and samples modified forms

Table 2: Table of conformational stability analysis for GSH, pyruvate, and samples modified forms

Fig. 1: Figure showing the conformational stability of GSH and cysteine derivatives. A) shows the conformational stability of GSH and cysteine derivatives. B) shows the conformational stability of GSH and cysteine derivatives.
Equilibrium denaturation of human growth hormone and its cysteine-modified forms.
D N Brems, P L Brown and G W Becker


Access the most updated version of this article at http://www.jbc.org/content/265/10/5504

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/10/5504.full.html#ref-list-1