peptide isomerization expected to be insensitive to the polarity of the environment. Recently, a "prolyl isomerase" has been found to produce modest enhancements in the rate of cis–trans isomerization of proline imidic peptide bonds in oligopeptides and proteins (Lang et al., 1987). In the transition state for peptide isomerization, there is presumably a major loss of resonance stabilization and susceptibility to polar interactions. Indeed, Drakenberg et al. (1972) have demonstrated that peptide isomerization becomes much more rapid in nonpolar environments than in water. It seems likely that the catalytic effects of prolyl isomerase might be produced, at least in part, by withdrawal of the susceptible peptide bond from water into an active site of lower polarity.

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REFERENCES

Binkley, J. S., Frisch, M. J., DeFrees, D. J., Rahavachari, K., Whiteside, R. A., Schlegel, H. B., Fulder, E. M., & Pople, J. A. (1986) copyright, Carnegie Mellon University, Pittsburgh, PA.

Corey, R. B., & Donohue, J. (1950) *J. Am. Chem. Soc. 72*, 2899.

Drakenberg, T., Dahlqvist, K.-I., & Forsén, S. (1972) *J. Phys. Chem. 76*, 2178.

Fogarasi, G., Pulay, P., Torok, F., & Boggs, J. E. (1979) *J. Mol. Struct. 57*, 259.

Graham, L. L., & Chang, C. Y. (1971) *J. Phys. Chem. 75*, 776.

Jorgensen, W., & Swenson, C. J. (1985) *J. Am. Chem. Soc. 107*, 1489.

Knecht, L. A. (1971) *Pure Appl. Chem. 27(1–2)*, 281.

Liler, M. (1972) *J. Chem. Soc., Perkin Trans. 2*, 720.

Nakanishi, H., & Roberts, J. D. (1981) *Org. Magn. Reson. 15*, 7.

Radzicka, A., & Wolfenden, R. (1988) *Biochemistry 27*, 1664–1670.

Ramachandran, G. N., & Mitra, A. K. (1976) *J. Mol. Biol. 107*, 85.

Rees, D. C., Lewis, M., Honzatko, R. B., Lipscomb, W. N., & Hardman, K. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3048.

Stewart, W. E., & Siddall, T. H., III (1970) *Chem. Rev. 70*, 517.

Wolfenden, R. (1976) *J. Am. Chem. Soc. 98*, 1987.

Wolfenden, R. (1978) *Biochemistry 17*, 201.

Solubility of Different Folding Conformers of Bovine Growth Hormone

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ABSTRACT: Previous folding studies have shown that equilibrium denaturation of bovine growth hormone (bGH) is a multistate process with stable intermediates. The native and unfolded species are monomeric, but intermediates are both monomeric and associated. In this study, the relative insolubility of an associated intermediate is used to distinguish its presence in equilibrium denaturation and during kinetic refolding. To study the role of the associated intermediate in the refolding pathway, a two-step procedure for its detection was developed. The first step of this procedure is used to populate the associated intermediate, and the second step involves dilution to solvent conditions in which only the associated intermediate precipitates. The amount of precipitate is quantitated either directly by formation of turbidity or indirectly by quantitation of the remaining soluble protein. The results show that an associated species is transiently populated during folding, it is incorrectly folded, and it occurs due to specific interactions of monomeric folding intermediates at moderate to high protein concentrations. This association of intermediates is a competing reaction that decreases the folding rate. The location of this competing reaction in the refolding pathway occurs after the formation of an early framework-type intermediate that contains considerable secondary structure but prior to the rate-limiting formation of the native tertiary structure. When refolding occurs in solutions that solubilize the associated intermediate, then native protein is obtained quantitatively. However, if refolding occurs in solutions that do not solubilize the associated intermediate, then most of the product results in an insoluble protein aggregate. The formation of precipitate that occurs upon refolding is inhibited by addition of fragments 96–133 or 109–133 that are derived from bGH. It is suggested that these fragments prevent precipitation by binding to the framework-type intermediate in a manner that prevents it from participating in the association reaction. The relationship of these results to general pathways of protein precipitation is discussed.

The solubility of proteins in aqueous solutions is a physical property of fundamental biological importance. Solubility occurs when solute (protein) to solvent (water) interactions are more favorable than solute to solute interactions. Insolubility occurs when the solute to solute interactions are more favorable than the solvent interactions. Protein insolubility at the molecular level is poorly understood and frequently causes experimental difficulties for protein studies in general.

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In particular, the insolubility of denatured proteins in aqueous solutions is a common feature.

Bovine growth hormone (bGH) is a protein of 191 amino acids that is secreted by the anterior pituitary gland. The three-dimensional structure of porcine growth hormone has recently been determined (Abdel-Meguid et al., 1987). It consists mainly of four antiparallel α-helices arranged in a left-twisted helical bundle.

Folding studies of bGH have identified equilibrium and kinetic intermediates (Burger et al., 1966; Holladay et al., 1974; Brems et al., 1985, 1986, 1987a; Havel et al., 1986). At least one equilibrium intermediate was shown to be associated. Unique spectral signals related to the environment of the sole tryptophan occur as a result of this association. One of these signals is a negative circular dichroic band with a minimum at 300 nm (Havel et al., 1986; Brems et al., 1986). The equilibrium denaturation transition of bGH is completely reversible within the zone of 2–6 M guanidine hydrochloride (Gdn-HCl) (the transition region occurs between 2.8 and 5 M Gdn-HCl). However, bGH samples that contain denaturing amounts of Gdn-HCl that are diluted to less than 2 M Gdn-HCl result in irreversible turbidity. Yet, if the denatured samples were diluted to 2 M Gdn-HCl and allowed to fold for several minutes, before dilution to lower Gdn-HCl concentrations, turbidity did not appear (Brems et al., 1987).

In this study, the relative solubility of different folding conformers of bGH was investigated. The conformers investigated were native, unfolded, monomeric intermediate(s), and associated intermediates. The different folding conformers were populated either at equilibrium or for a short time by a folding reaction. Once populated, their solubility was determined by dilution to aqueous solvent conditions that induce precipitation. An associated intermediate species was shown to be responsible for the precipitation that occurs upon refolding.

Experimental Procedures

Materials

Gdn-HCl was ultrapure from Schwarz/Mann. Other reagents were of analytical grade. The bGH was obtained from a strain of *Escherichia coli*, Am 7, through recombinant DNA technology in which the appropriate gene sequence, along with a tryptophan promoter system, had been inserted into a temperature-sensitive runaway plasmid (Souza et al., 1984). The procedure of Olsen (1985) was used to isolate the bGH from the fermentation culture medium. Fragment 96–133 was obtained by limited trypsin digestion as described by Graf and Li (1974). Fragments 109–133 and 96–112 were obtained as described by Brems et al. (1987b).

Methods

The bGH concentrations were determined by the absorbance at 278 nm using an extinction coefficient of 15 270 M^{-1} cm^{-1}. All solutions and spectral cells were maintained at 3.0 ± 0.1 °C or 23.0 ± 0.1 °C with refrigerated circulating baths. Absorbance measurements were taken on an IBM 9420 spectrophotometer. Circular dichroism measurements were obtained with a Jasco J-500 C spectropolarimeter. All solutions contained 50 mM ammonium bicarbonate, pH 8.5, and were prefiltered through a 0.45-μm filter.

Two-Step Procedure for the Determination of Insoluble Species. All folding conformers of bGH are soluble in solutions of Gdn-HCl greater than 2 M; 2–3 M Gdn-HCl concentrations are nondenaturing and are solubilizing for all bGH conformers. If refolding occurs in less than 2 M Gdn-HCl, then precipitation of bGH results. In this study, 0.8 M Gdn-HCl and 0.18 mg/mL bGH were used as the solvent conditions for precipitation. The first step of the procedure is used to populate different bGH conformers. This was accomplished by varying the Gdn-HCl concentration or protein concentration or by interrupting a kinetic folding experiment. In the second step of the procedure, the various conformers were introduced by a simple dilution into solvent conditions that induce precipitation. After 30–60 min following the second step, the amount of precipitate was quantitated. The precipitate was quantitated in either of two ways. In one way, the turbidity at 450 nm was measured in a spectrophotometer. In the other way, the precipitate was sedimented by centrifugation, and the soluble fraction was filtered through a 0.45-μm filter, and the remaining soluble protein content was determined by the ultraviolet absorbance at 278 nm. The amount of precipitate was deduced by subtracting the remaining soluble protein from the total expected protein.

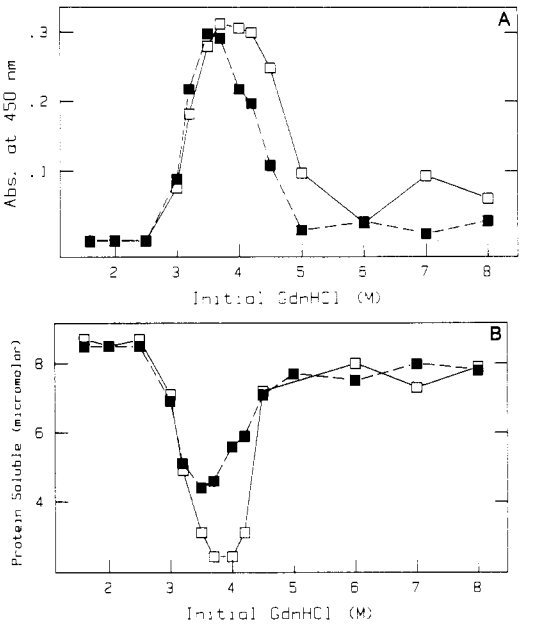


FIGURE 1: Precipitation following dilution from differing initial Gdn-HCl concentrations. The results were obtained by using the two-step procedure described under Experimental Procedures. In the first step, bGH (2 mg/mL) was incubated in varying concentrations of Gdn-HCl for at least 30 min. In the second step, the samples were diluted to 0.8 M Gdn-HCl and 0.2 mg/mL bGH in order to induce precipitation. The amount of precipitate was determined by the absorbance at 450 nm (A) or by measurement of the remaining soluble protein (B). The open symbols represent the results at 23 °C, and the closed symbols represent results at 3 °C.

Results

Precipitation during Refolding Depends on the Initial Unfolding Conditions and Occurs from a Partially Denatured Associated Form. In this experiment, a two-step procedure was used to investigate the solubility of different folding conformers of bGH. The initial concentrations of Gdn-HCl were varied from 1.5 to 8 M in the presence of bGH and equilibrated prior to dilution to the precipitation conditions as described for the second step of the two-step procedure. Figure 1 shows the results for two different temperatures. The results demonstrate that the native and denatured forms of bGH are soluble or gave rise to products that were soluble. However, if bGH was diluted from partially denaturing conditions (3–5 M Gdn-HCl), then precipitation resulted. It is concluded that only partially denatured forms are responsible for the precipitation. Figure 1 also shows that the extent of precipitation depends on temperature, with less precipitation

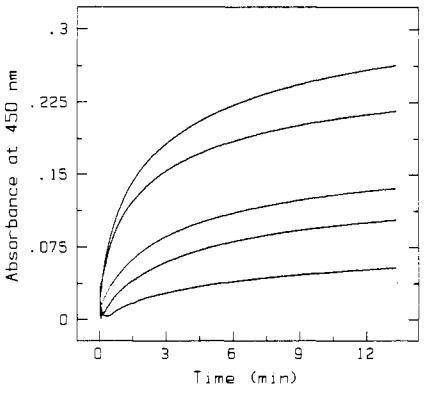


FIGURE 2: Inhibition of precipitation that occurs during folding by peptide 96–133. Folding was initiated by diluting a solution of bGH at 1.75 mg/mL and 3.5 M Gdn-HCl to refolding conditions that induce precipitation (0.8 M Gdn-HCl and 0.18 mg/mL bGH). Formation of turbidity was continuously monitored by the absorbance at 450 nm. All solutions were maintained at 3 °C. The top curve represents the time of turbidity formation in the absence of fragment 96–133. In descending order from the top curve, the fragment was present at a 1-, 3-, 5-, and 7-fold molar excess to bGH, respectively. The fragment was preincubated with bGH in the initial 3.5 M Gdn-HCl solution.

occurring at lower temperatures. The formation of turbidity in the two-step procedure was linearly dependent on the initial protein concentration in the first step (data not shown). The concentration dependence in the first step of the procedure indicates that intermolecular interactions between folding intermediates are a prerequisite for precipitation. Thus, the observed insolubility depends on the presence of an associated folding intermediate(s), and its concentration is related to the extent of turbidity. At 2 mg/mL and 3.5 M Gdn-HCl, it can be deduced from Figure 1B that at 3 °C approximately 50% and at room temperature 75% of the population is involved in the reaction that leads to insolubility.

Extraneous Fragments 96–133 or 109–133 Inhibit Formation of Precipitate. Figure 2 shows the kinetics of precipitation following dilution from 3.5 M Gdn-HCl to the conditions of the second step of the two-step procedure. A stable precipitate was formed that required several minutes to reach equilibrium. Figure 2 also illustrates the effect of fragment 96–133 on turbidity formation. The fragment was incubated with bGH in the initial 3.5 M Gdn-HCl solutions. Inhibition was not as effective if the fragment was included in only the final second-step solution conditions (data not shown). Preincubation of a 10-fold molar excess of the fragment to bGH in 3.5 M Gdn-HCl was effective in complete inhibition of precipitate. The truncated peptides 96–112 and 109–133 were also tested as inhibitors of the precipitation. Fragment 109–133 was effective in preventing precipitation, but 96–112 was not (data not shown). Peptide 109–133 has a half-maximal inhibition at a 3-fold molar excess to bGH. These results show that the C-terminal half of 96–133 is responsible for preventing precipitation and suggest that specific events are responsible for the formation of precipitate.

An Associated Equilibrium Intermediate Is Populated Transiently during Kinetic Refolding. Figure 3 represents equilibrium denaturation of bGH as measured by the absorbance at 290 nm at two different protein concentrations. The absorbance at 290 nm is reflective of the solvent environment of the aromatic chromophores and has been shown to be sensitive to the native tertiary structure (Burger et al., 1986; Holladay et al., 1974; Brems et al., 1985). The equilibrium results of Figure 3 were independent of the protein concentration. Figure 4 illustrates the effect of protein concentration

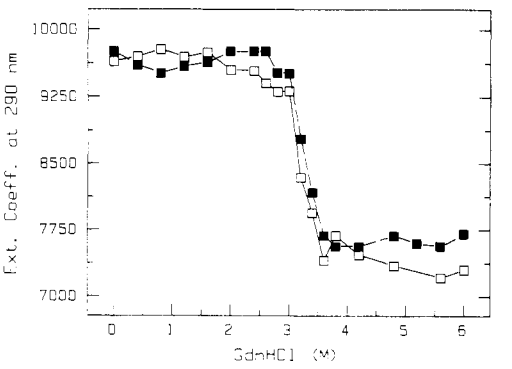


FIGURE 3: Equilibrium denaturation of bGH as detected by the UV absorbance at 290 nm at two protein concentrations. Open squares represent the results at 2 mg/mL and closed squares 0.2 mg/mL bGH.

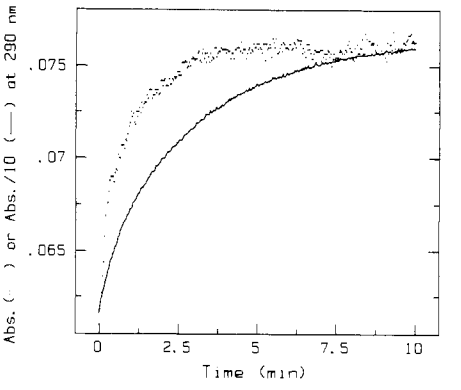


FIGURE 4: Refolding of bGH monitored by the UV absorbance at 290 nm at two protein concentrations. Denatured bGH in 6 M Gdn-HCl was diluted to 2.2 M Gdn-HCl and either 1.75 mg/mL (—) or 0.175 mg/mL (···) bGH. All solutions were maintained at 3 °C. Zero time on the abscissa corresponds to 10 s after mixing due to a program delay before data accumulation in order to allow mixing to occur.

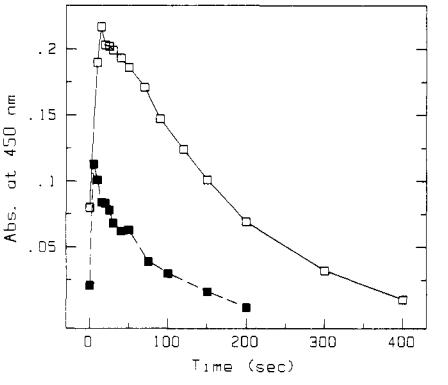


FIGURE 5: Two-step procedure for the detection of precipitable species during refolding. In the first step, unfolded bGH in 6 M Gdn-HCl was diluted to 2.2 M Gdn-HCl and either 1.75 mg/mL (□) or 0.5 mg/mL (■) bGH. At varying times, samples were removed and diluted to the precipitating conditions of the second step of the procedure. After complete precipitation occurred, turbidity was measured by the absorbance at 450 nm. All solutions were maintained at 3 °C.

on the kinetics of refolding as measured by the absorbance at 290 nm. Refolding was initiated by dilution of denatured bGH in 6 M Gdn-HCl to 2.2 M Gdn-HCl. Under these conditions, all the folding conformers were soluble, and the native conformation was attained. Increasing the concentration of protein decreased the kinetic folding rate. Semilog plots of the kinetic data indicate the existence of two phases with only the slower phase showing a protein concentration effect.

Figure 5 represents data for which fully denatured bGH (in 6 M Gdn-HCl) was diluted to 2.2 M Gdn-HCl in the first step

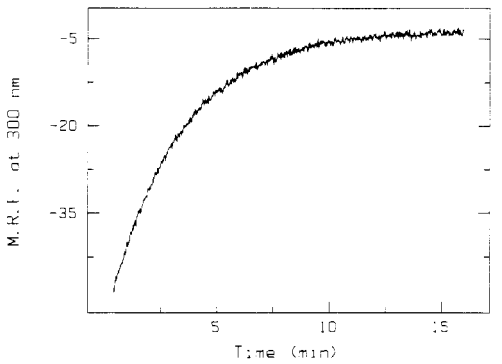


FIGURE 6: Refolding in 2.2 M Gdn-HCl as measured by circular dichroism at 300 nm. In order to induce refolding, bGH in 6 M Gdn-HCl was diluted to 2.2 M Gdn-HCl, 1.75 mg/mL, and 3 °C. The abbreviation M.R.E. refers to mean residue ellipticity in degrees centimeter squared per decimole.

of a two-step procedure. In the second step of the procedure, portions of the refolding solution were removed at varying times and diluted to the solution conditions that induce precipitation of the associated intermediate. As depicted in Figure 5, an intermediate that resulted in precipitation in the second step of the assay accumulated rapidly and disappeared with time. Two different protein concentrations in the first step of the assay were explored. The amount of precipitated protein present during folding was dependent on the protein concentration. The presence of a transient species during folding that is protein concentration dependent and that precipitates as a result of the second step of the procedure demonstrates that an associated intermediate is populated during kinetic refolding. From the amount of turbidity formed, it can be estimated from Figure 1 that at the higher protein concentration approximately 33% of the total protein was involved in the associated form at early times in the kinetic pathway.

Nature of the Associated Species That Gives Rise to Precipitation. Previous equilibrium denaturation studies of bGH have identified the existence of a stable intermediate that is associated and that has a unique circular dichroism signal at 300 nm (Havel et al., 1986; Brems et al., 1986). This intermediate will be referred to as I_{assoc_1}. In order to test if it is populated during kinetic refolding, the presence of a transient circular dichroism signal at 300 nm was investigated. Figure 6 shows that following dilution of bGH from 6 to 2.2 M Gdn-HCl there was a rapid formation followed by a time-dependent loss of negative ellipticity at 300 nm with an apparent $t_{1/2}$ of 150 s. Since the native and completely denatured species have negligible ellipticity at 300 nm, it is concluded that I_{assoc_1} is rapidly populated during kinetic refolding and then diminishes as the folding reaction matures.

The rate of formation in 3.5 M Gdn-HCl of an intermediate form that gives rise to precipitate was determined by using the two-step procedure. In the first step, the native protein was mixed with Gdn-HCl to give 3.5 M Gdn-HCl and 2 mg/mL protein. At varying times, the solution was diluted in a second step to induce precipitation. The results are shown in Figure 7. An initial lag of 30 s was required before any species was formed that gave rise to precipitation. After the lag period, a precipitable species was formed in a time-dependent fashion as shown in Figure 7.

Is I_{assoc_1} the same species that leads to insolubility? In order to address this question, the formation of negative ellipticity at 300 nm under identical conditions as used in the first step in Figure 7 was ascertained. Figure 8 contains the results and demonstrates that formation of I_{assoc_1} (apparent $t_{1/2}$ of 25 s) occurred principally during the lag period of Figure 7 in which

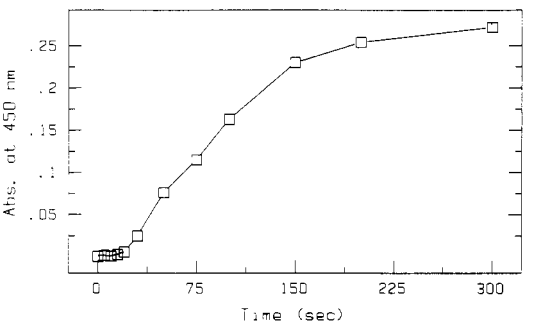


FIGURE 7: Two-step procedure for the detection of precipitable species during partial unfolding. In the first step, native protein was partially denatured in 3.5 M Gdn-HCl and 1.75 mg/mL bGH. At varying times, samples were withdrawn and diluted to the precipitating conditions of the second step of the procedure. After complete precipitation occurred, the turbidity was monitored by the absorbance at 450 nm. All solutions were maintained at 3 °C.

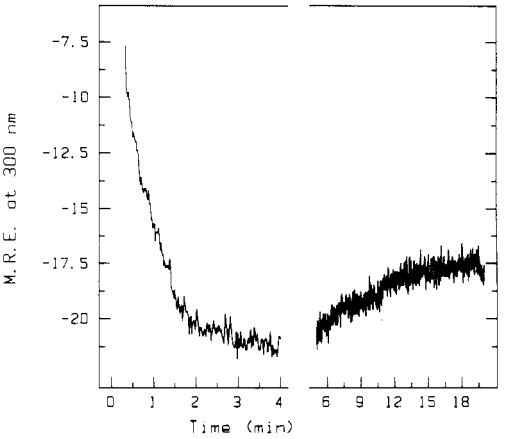


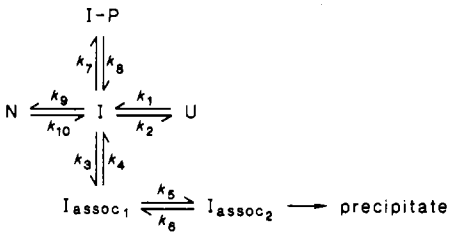
FIGURE 8: Partial unfolding as detected by circular dichroism at 300 nm. Native bGH was mixed with an equal volume of 7 M Gdn-HCl to produce the partial unfolding conditions of 3.5 M Gdn-HCl, 1.75 mg/mL, and 3 °C. The abbreviation M.R.E. refers to mean residue ellipticity in degrees centimeter squared per decimole.

no precipitable species was formed. Figure 8 also shows that after maximal formation of I_{assoc_1}, a slower event occurred that lead to its diminution. Monitoring the unfolding reaction in 3.5 M Gdn-HCl by circular dichroism at 300 nm demonstrates that at least two events occur. In the first event, I_{assoc_1} is formed rapidly. In the second event, another species is formed with a more positive ellipticity at 300 nm. Since the species which leads to precipitation does not form during the time course of the first event, it is concluded that I_{assoc_1} does not directly lead to precipitation. At least one additional associated intermediate is responsible for the observed insolubility.

Discussion

Previous studies have shown that the UV absorbance detected kinetics of bGH refolding can be described by a first-order kinetic equation that contains two exponential terms (Brems et al., 1987a). One of these exponential terms (corresponding to the slower phase) was dependent on protein concentration while the other (corresponding to the faster phase) was not. The slower kinetic phase, that corresponded to the concentration-dependent term, accounted for approximately 70% of the total reaction. If refolding was monitored by circular dichroism at 222 nm, a first-order equation with a single-exponential term could account for the results, but only a fraction (30%) of the total reaction was observed. The remaining reaction occurred too rapidly to be detected by manual mixing techniques. The kinetic folding reaction observed by circular dichroism was dependent on protein con-

Scheme I: Folding Mechanism of the Slow Kinetic Reaction[a]



[a] $K_1 = k_1/k_2$; $K_2 = k_3/k_4$; $K_3 = k_5/k_6$; $K_4 = k_7/k_8$; $K_5 = k_9/k_{10}$; U = unfolded; I = intermediate; P = peptide 96–133; N = native.

centration and was similar in rate to the slower kinetic phase detected by UV absorbance. It is this slower, concentration-dependent phase that is described by the results reported here. Scheme I is consistent with the folding data obtained for the slower kinetic phase of bGH folding.

Reaction K_1. This reaction reflects the major change of secondary structure. For the conditions used in refolding, K_1 is large ($k_1 \gg k_2$), and the observed reaction equals k_1. The time constant of k_1 has not been measured, but it is more rapid than 10 s. I has no nativelike tertiary structure as measured by UV absorbance at 290 nm. I is likely present in equilibrium denaturation measurements, but its connection to an equilibrium intermediate still remains unproven. Scheme I does not distinguish between multiple forms of U that may differ by proline isomerization. The exact role of proline isomerization in bGH folding is under current investigation.

Reaction K_2. I_{assoc_1} is characterized by negative ellipticity at 300 nm in the near-UV circular dichroism spectrum. The reaction for the formation of I_{assoc_1} is represented by the initial decrease in the ellipticity at 300 nm in Figure 8. In 3.5 M Gdn-HCl, it has an apparent $t_{1/2}$ of 25 s. The reaction for the disappearance of I_{assoc_1} is shown in Figure 6 and has an apparent $t_{1/2}$ of 150 s in 2.2 M Gdn-HCl. Figure 7 shows that for 30 s after dilution from native to conditions that favor population of I_{assoc_1}, no precipitate was obtained, yet during this time the majority of I_{assoc_1} was formed. This result demonstrates that I_{assoc_1} is soluble and that an additional intermediate must be responsible for the observed precipitation. Comparison of the amount of I_{assoc_1} that was transiently obtained during kinetic refolding (Figure 6) to that at equilibrium (Figure 8) indicates approximately a 2-fold greater concentration in the former. This would indicate that for the conditions of kinetic folding (2.2 M Gdn-HCl) I_{assoc_1} is more stable than for the equilibrium conditions of 3.5 M Gdn-HCl.

Reaction K_3. I_{assoc_2} is the putative species that directly leads to precipitation during refolding. In Scheme I, I_{assoc_1} is indicated as a precursor to I_{assoc_2} due to the lag period that preceded formation of I_{assoc_2} (Figure 7), wherein the lag period represents the time required for the rate-limiting formation of I_{assoc_1}. Figures 5 and 6 show that during refolding the rates of disappearance of I_{assoc_1} and I_{assoc_2} are similar. These results are consistent with the precursor relationship of I_{assoc_1} to I_{assoc_2} if during folding the reaction k_4 is rate limiting. Figure 8 demonstrates that partial unfolding of bGH in 3.5 M Gdn-HCl as detected by circular dichroism at 300 nm resulted in an initial increase in negative ellipticity that decreased with time. This can be explained if I_{assoc_1} is a precursor to I_{assoc_2} and if I_{assoc_2} has more positive ellipticity at 300 nm. The precipitation that occurred upon folding in solutions containing less than 2 M Gdn-HCl is explainable if I_{assoc_2} is insoluble in less than 2 M Gdn-HCl. If refolding was conducted in solutions containing 2–3 M Gdn-HCl, all of the folding species were soluble, and the reaction eventually lead to the native state. However, in these conditions, the reaction rate k_9 is decreased due to the rate-limiting participation of reactions K_2 and K_3. Further details of the molecular nature of I_{assoc_2} are not known at the present time.

Reaction K_4. Previous studies have shown that peptide 96–133 partially inhibited formation of I_{assoc_1} in 3.5 M Gdn-HCl. It was proposed that the region 110–127 of bGH participated in this intermolecular self-association (Brems et al., 1986). The reaction K_4 depicts a direct interaction between peptide and I. By complexing the monomeric I with peptide, the folding reaction is prevented from participating in the K_2 and K_3 reactions. Direct experimental evidence for binding of 96–133 to I has not been obtained. Isolated fragment 96–133 is helical in aqueous solutions and has some helicity even in 3.5 M Gdn-HCl (Brems et al., 1986). Modeling of this helix strongly suggests that it is amphiphilic (Brems et al., 1987b). It is proposed that the complex I–P results from interactions between the two hydrophobic surfaces of I and the peptide. Species I contains little nativelike tertiary structure but considerable helical structure. If I has formed the amphiphilic helix 110–127 with an exposed hydrophobic face, then in a complementary fashion the corresponding hydrophobic face of the helix of the fragment might specifically interact and prevent further self-association. Other soluble fragments from bGH were tested for their ability to inhibit precipitation. Fragment 109–133 was equally effective as 96–133 in inhibiting precipitation whereas fragment 96–112 was not. This result demonstrates that the C-terminal half of 96–133 is sufficient to inhibit precipitation. Conformational studies of fragment 109–133 (Brems et al., 1987b) have shown that it is helical in aqueous solutions and argue that helix-to-helix interactions between the fragment and I give rise to the complex I–P of Scheme I. Fragment 125–149 showed only minimal inhibition of precipitation at a 20-fold molar excess. Fragment 180–191 did not inhibit precipitation even at a 20-fold molar excess. The results of Figure 2 were obtained for preincubation of the peptide with partially unfolded bGH prior to dilution to the precipitating conditions. The peptides were not as effective in preventing precipitation if they were only included in the final precipitating conditions. These results indicate that the peptides inhibit precipitation by interacting with a species that is present in the initial conditions of the first step of the assay.

Reaction K_5. This reaction is monitored by the UV absorbance change at 290 nm that is observed during folding. It represents the regain of nativelike tertiary structure which reflects the burial of the aromatic amino acid chromophores into a more hydrophobic environment. Also concurrent with this reaction is a change in 30% of the total helical signal as determined by circular dichroism at 222 nm.

Relationship to Other bGH Folding Studies. Scheme I is consistent with other results on bGH folding. Equilibrium denaturation as determined by circular dichroism was shown to be dependent on the protein concentration (Brems et al., 1986). This can be explained by a change in the concentrations of I_{assoc_1} and I_{assoc_2} at different protein concentrations since reactions K_2 and/or K_3 result in direct changes in helicity. The results of Figure 3 show that equilibrium denaturation as detected by UV absorbance at 290 nm was not dependent on protein concentration. This is consistent if the concentration-dependent reactions K_2 and K_3 do not result in any observable UV absorbance change at 290 nm.

Previous results showed that the UV absorbance detected unfolding kinetics were independent of protein concentration (Brems et al., 1987), yet Figure 4 shows that refolding was dependent. These results are understandable since for un-

folding the concentration-dependent reactions K_2 and K_3 occur subsequent to the UV absorbance change and the apparent kinetics of unfolding would not be affected by protein concentration. For refolding, if reactions K_2 and/or K_3 are rate limiting, then the observed kinetics of reaction K_5 would be affected by protein concentration.

The refolding reaction observed by circular dichroism at 222 nm only represented 30% of the total change expected from the equilibrium denaturation results (the remainder occurred too rapidly to measure). The circular dichroism detected kinetics of refolding were dependent on protein concentration (Brems et al., 1987a). This result would be anticipated from Scheme I if the observed folding kinetics detected by circular dichroism and reaction K_5 are concurrent. This seems reasonable since the rates for the slow folding reaction determined by UV absorbance and circular dichroism are the same.

The rate of the unfolding reaction detected by circular dichroism was observed to increase with increasing protein concentrations (Brems et al., 1987a). The reaction(s) K_2 and/or K_3 induce(s) increased helix stability due to intermolecular helix-to-helix interactions (Brems et al., 1986). By the law of mass action, increasing protein concentration should increase the rate(s) of k_3 and/or k_5 and consequently increase the helix-detected rate of unfolding. If bGH folding is conducted at low protein concentrations (<0.2 mg/mL), the reactions K_2 and K_3 do not appreciably occur, and the refolding rates reflect the monomeric folding pathway described by reactions K_1 and K_5.

Scheme I is consistent with the results of reoxidation of reduced bGH (Holzman et al., 1986). Reoxidation studies indicated that disulfide formation is a discrete process that occurs along a sequential pathway after a framework of protein helical structure has been established. This framework intermediate likely resembles the I species described here. Unreported results of bGH reoxidation in partially denaturing conditions resulted in a high population of specific oligomers of limited size (T. F. Holzman, unpublished results). This result would be consistent with the predisposition of reduced bGH in the I_{assoc_1} or I_{assoc_2} forms. Indeed, the reduced protein is like I_{assoc_2} in that they are both insoluble in Gdn-HCl solutions <2 or 4.5 M urea.

Unfolded proteins are frequently insoluble in aqueous solutions. Common examples are the following: (1) formation of inclusion bodies in *E. coli* that have been engineered by recombinant DNA technology to overexpress heterologous proteins (Uren, 1984); (2) precipitation of heat-treated globular proteins (Privalov, 1979); and (3) irreversibility of protein renaturation due to aggregation (Light, 1985). Apparent irreversibility of in vitro refolding was reported for tryptophanase (London et al., 1974), β-galactosidase (Goldberg, 1972), catalytic subunit of aspartate transcarbamylase (Ghelis & Hervé, 1978), elastase (Zilber, 1979), the dehydrogenases (Jaenicke & Rudolph, 1983), rhodanese (Horowitz & Criscimagna, 1986), phosphorylase *b* (Price & Stevens, 1983), and antithrombin (Fish et al., 1985). For these proteins, the irreversibility of refolding was shown to occur only at critical concentrations of denaturant. Irreversibility was attributed to side reactions that occur with intermediates in the folding pathway and corresponds to incorrect refolding. In some cases, the irreversibility was related to the formation of aggregates. In vivo studies of the P22 tailspike endorhamnosidase have shown that aggregation forms from a specific intermediate in the folding and subunit assembly pathway (King, 1986). For bGH, it is now demonstrated that insolubility arises principally from association events that occur from monomeric folding intermediates and not from the unfolded species. This insolubility can be prevented by inclusion of an excess of fragment derived from bGH that is involved in the putative interaction site for association. Perhaps the insolubility of folding intermediates in general could account for much of protein insolubility.

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REFERENCES

Abdel-Meguid, S. S., Shieh, H.-S., Smith, W. W., Dayringer, H. E., Violand, B. N., & Bentle, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6434–6437.

Brems, D. N., Plaisted, S. M., Havel, H. A., Kauffman, E. W., Stodola, J. D., Eaton, L. C., & White, R. D. (1985) *Biochemistry 24*, 7662–7668.

Brems, D. N., Plaisted, S. M., Kauffman, E. W., & Havel, H. A. (1986) *Biochemistry 25*, 6539–6543.

Brems, D. N., Plaisted, S. M., Dougherty, J. J., Jr., & Holzman, T. F. (1987a) *J. Biol. Chem. 262*, 2590–2596.

Brems, D. N., Plaisted, S. M., Kauffman, E. W., Lund, M., & Lehrman, S. R. (1987b) *Biochemistry 26*, 7774–7778.

Burger, H. G., Edelhogh, H., & Condliffe, P. G. (1966) *J. Biol. Chem. 241*, 449–457.

Fish, W. W., Danielsson, A., Nordling, K., Miller, S. H., Zam, C. F., & Bjork, I. (1985) *Biochemistry 24*, 1510–1517.

Ghelis, C., & Hervé, G. (1978) *Int. Biophys. Congr., 6th*, Kyoto V-18.

Goldberg, M. E. (1972) in *Dynamic Aspects of Conformation Changes in Biological Macromolecules* (Sadron, C., Ed.) pp 57–65, Reidel, Dordrecht, The Netherlands.

Graf, L., & Li, C. H. (1974) *Biochemistry 13*, 5408–5415.

Havel, H. A., Kauffman, E. W., Plaisted, S. M., & Brems, D. N. (1986) *Biochemistry 25*, 6533–6538.

Holladay, L. A., Hammonds, R. G., & Puett, D. (1974) *Biochemistry 13*, 1653–1661.

Holzman, T. F., Brems, D. N., & Dougherty, J. J., Jr. (1986) *Biochemistry 25*, 6907–6917.

Horowitz, P., & Criscimagna, N. L. (1986) *J. Biol. Chem. 261*, 15652–15657.

Jaenicke, R., & Rudolph, R. (1983) in *Biological Oxidations* (Sund, H., & Ullrich, V., Eds.) pp 62–90 Springer-Verlag, Heidelberg.

King, J. (1986) *Bio/Technology 4*, 297–303.

Light, A. (1985) *Biotechniques 3*, 298–306.

London, J., Skrzynia, C., & Goldberg, M. E. (1974) *Eur. J. Biochem. 47*, 409–415.

Olsen, K. C. (1985) U.S. Patent 4 518 526.

Price, N. C., & Stevens, E. (1983) *Biochem. J. 213*, 595–602.

Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.

Souza, L. M., Boone, T. C., Murdock, D., Langley, K., Wypych, J., Fenton, D., Johnson, S., Lai, P. H., Everett, R., Hsu, R. Y., & Busselman, B. (1984) *J. Exp. Zool. 232*, 465–573.

Uren, J. (1984) *Am. Biotechnol. Lab. 2*, 51–54.

Zilber, E. (1979) Thèse de Spécialité, Université de Paris-Sud, Orsay, France.