The Kinetics of Bovine Growth Hormone Folding Are Consistent with a Framework Model*

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The framework model of protein folding requires the hydrogen-bonded secondary structure to be formed early in folding (i.e., the formation of secondary structure precedes the tertiary structure) (Kim, P. S., and Baldwin, R. L. (1982) Annu. Rev. Biochem. 51, 459-489). To test this framework model directly, the kinetics of bovine growth hormone (bGH) folding were compared utilizing two methods of detection, one that measures the secondary structure (far ultraviolet circular dichroism) and another that measures the tertiary structure (near ultraviolet absorbance). The results demonstrate that, under identical folding conditions, the kinetics observed by far ultraviolet circular dichroism are faster than those observed by ultraviolet absorbance. The faster kinetics observed by circular dichroism indicate the existence of a helix-containing intermediate which is consistent with the framework model.

The effect of protein concentration and denaturant concentration on the kinetics of refolding were studied. The rate of refolding measured by absorbance and circular dichroism was dependent on protein concentration. The protein concentration dependence on refolding is due to the transient formation of an associated intermediate. The concentration dependence of folding is taken as evidence that folding is a sequential process with partially folded monomers responsible for the observed association effect. At dilute protein concentrations the refolding can be studied independent of the association phenomena.

The growth hormones utilized in this study were derived from Escherichia coli through recombinant DNA technology and from bovine pituitaries. The pituitary-derived bGH has been shown to be heterogeneous at the NH2 terminus (Lorenson, M. F., and Ellis, S. (1975) Endocrinology 96, 833-838), whereas the recombinant DNA-derived bGH contains a single NH2 terminus. No differences in the folding kinetics between the recombinant DNA and pituitary derived-bGH were observed. It is concluded that the heterogeneity of the NH2 terminus of growth hormone obtained from bovine pituitaries does not affect the observed in vitro folding kinetics.

Bovine growth hormone (bGH)† is a member of the class of large pituitary polypeptide hormones. It has 191 amino acids and is globular in conformation.Empirical methods indicate that it is approximately 50% α-helical (6). Crystals suitable for x-ray structure determination to at least 3.2-Å resolution have been obtained and are currently being studied (7). The genomic sequence coding for bGH has been cloned and expressed in Escherichia coli (8).

The equilibrium denaturation of bGH has been studied by a variety of physicochemical methods (2-4, 9, 10). The results are unusual in that they do not conform to a two-state process. Equilibrium intermediates have been postulated to account for these results. Both monomeric and multimeric intermediates have been identified (9, 10). At least one multimeric species has been identified and is populated only under partially denaturing conditions (9). Intermolecular association between helices of partially denatured bGH was shown to play a critical role in the formation of the multimeric intermediate (10). For monomeric unfolding the tertiary structure has been shown to be less stable to denaturation than the secondary structure.

Many models have been proposed to describe protein folding. One working model that retains popularity is the framework model. It states that folding is a sequential process with the hydrogen-bonded secondary structure forming early (1, 11). The chief criticism of this model is that generally the secondary structure of a protein is not stable in the absence of long range tertiary interactions. For bGH the existence of equilibrium intermediates that retain secondary structure in the absence of tertiary structure is supportive of the framework model.

In order to test the framework model more directly the kinetic ratio test has been applied to the folding of bGH (12). In the kinetic ratio test the folding reaction is measured by two or more different probes. If different kinetics are obtained, then a structural intermediate must be present if all unfolded species appear alike when measured by each probe. For this study the kinetics have been measured by absorbance at 290 nm, which reflects the integrity of the tertiary structure, and circular dichroism at 222 nm, which monitors the secondary structure (1-4).

**EXPERIMENTAL PROCEDURES**

Materials—Pituitary growth hormone (lot 8500) was obtained from bovine pituitaries by A. F. Pariow, Harbour Medical Center, University of California at Los Angeles. Recombinant DNA-derived bGH was obtained from a strain of E. coli, Am 7, carrying a temperature-sensitive runaway plasmid into which the appropriate gene sequence, along with a tryptophan promoter system, had been inserted (13). The procedure of Olsen (14) was used to isolate the bGH from the fermentation culture medium. Guanidine hydrochloride (GdnHCl) was ultrapure from Schwarz/Mann. Other reagents were analytical-grade.

Methods—All solutions contained 50 mM ammonium bicarbonate, pH 8.5, and were prefitered through a 0.45-μm filter. Solutions were made to the desired bGH concentration by dilution from a concentrated stock solution. The bGH concentrations were determined by

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†The abbreviations used are: bGH, bovine growth hormone; GdnHCl, guanidine hydrochloride.
the absorbance at 278 nm using an extinction coefficient of 15,270 M$^{-1}$cm$^{-1}$ (3). The solutions and spectral cells were maintained at 3 °C with refrigerated circulating baths. Absorbance measurements were taken on an IBM 9420 spectrophotometer. Circular dichroism measurements were obtained with a Jasco J-500C spectropolarimeter. The stopped-flow measurements were made using a DIONex D-117 stopped-flow spectrophotometer. The kinetic data were analyzed in one of two ways: either first-order plots or the observed changes were constructed and the multiphasic reactions were deconvoluted by one of two ways: either first-order plots or the data analyzed by an On-Line Instrument Systems (OLIS, Jefferson, GA) manual peel-back procedure, or the data were analyzed by an On-Line Instrument Systems (OLIS, Jefferson, GA) manual peel-back procedure, or the data were analyzed by an On-Line Instrument Systems (OLIS, Jefferson, GA) manual peel-back procedure, or the data were analyzed by an On-Line Instrument Systems (OLIS, Jefferson, GA) manual peel-back procedure, or the data were analyzed by an On-Line Instrument Systems (OLIS, Jefferson, GA) manual peel-back procedure. The kinetic data were presented as relaxation times or time constants ($\tau$) and are defined as rate$^{-1}$. The NH$_2$-terminal sequence analysis was performed by gas-phase sequencing (Applied Biosystems, Inc. Model 470-A Protein Sequencer). The first cycle from Edman degradation was subjected to separation and quantitation using a Hewlett-Packard 1090 liquid chromatograph and the Applied Biosystems method for the separation of the phenylthiohydantoin (15).

**RESULTS**

Equilibrium Denaturation Demonstrates That the Secondary Structure Is More Stable than Is the Tertiary Structure—Ample evidence exists that bovine growth hormone unfolds in a multistep process (2-4, 9, 10). Fig. 1 illustrates the equilibrium unfolding transitions monitored by ultraviolet absorbance and far ultraviolet circular dichroism at 3 °C. The noncoincidence of the transitions observed at 3 °C in Fig. 1 demonstrates that secondary structure is more stable than the tertiary structure and is similar to that previously reported at room temperature (4). The equilibrium results at 3 °C served to establish conditions for the kinetic studies which follow. It was shown previously that the unfolding transition monitored by CD is dependent on the protein concentration (10). However, at 3 °C and the concentration utilized in Fig. 1 (0.2 mg/ml), any contribution due to association is minimal.

Effect of Protein Concentration on the Kinetics of Folding—In order to interpret kinetic folding studies, monomeric and multimeric effects must be clearly delineated. Table I shows the effect of protein concentration on the ultraviolet absorbance-detected kinetics of refolding. The kinetics of refolding measured by ultraviolet absorbance can be fit to a first-order kinetic equation containing two exponential terms. All of the expected extinction change at 290 nm observed from the equilibrium denaturation studies can be accounted for by a concentration-independent phase with a relaxation time of 17 ± 4 s and a slower concentration-dependent phase. For unfolding, only 50–60% of the reaction is observable, even with stopped-flow techniques. We assume the unobserved extinction changes occur more rapidly than 5 ms, which is the dead-time of the stopped-flow spectrophotometer. The data in Table I demonstrate that the relaxation times and amplitudes of the unfolding reactions that are observable by absorbance at 290 nm are independent of protein concentration.

Only a fraction of the unfolding and refolding reactions are observed by manual mixing techniques when monitored by far ultraviolet circular dichroism. Both unfolding and refolding as observed by circular dichroism are dependent on protein concentration (Table I). For refolding, whether measured by absorbance or circular dichroism at concentrations of less than 0.2 mg/ml, the results are independent of concentration and interpreted as monomeric folding (free of association contributions). The effect of protein concentration on equilibrium denaturation was previously reported (9, 10); the absorbance-detected equilibrium transitions were shown to be independent of concentration and the helix-monitored transitions were dependent. The presence of a reversible association intermediate lacking native-like absorbance but with considerable helix structure was demonstrated and explained the concentration effects on the equilibrium results. We therefore explored the possibility that the protein concentration effect observed in the kinetic results was due to the tendency of a bGH folding intermediate(s) to associate.

![Figure 1](https://via.placeholder.com/150)

**FIG. 1.** Equilibrium denaturation for pituitary bGH as measured by absorbance at 290 nm (○) and circular dichroism at 222 nm (▲) at 3 °C and 0.2 mg/ml.

**Table 1**

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<th>Fast phase</th>
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Protein Concentration Effects Are Due to the Association of Transient Intermediates—In a previous report (9) the formation of an associated equilibrium intermediate was shown to occur from solution conditions that induced partial denaturation and this association gave rise to a CD band centered at 300 nm. The kinetics of appearance and disappearance of the associated intermediate was investigated by monitoring the CD at 300 nm, and the data are shown in Table II. The kinetics of appearance of the associated intermediate were measured by starting with bGH either fully native or denatured and altering the GdnHCl concentration to 3.8 M, which is partially denaturing and induces association. The disappearance of the associated intermediate was studied by starting with bGH in partially denaturing conditions (3.8 M GdnHCl) and inducing dissociating by altering the GdnHCl solution to fully native or denatured conditions. The kinetics for the reaction of associated intermediate dissociating to the native state (Table II), is similar to that for the slower kinetic phase of refolding that is observed by absorbance at higher protein concentrations (Table I). A rapid formation of the associated species from the unfolded state and subsequent slow conversion to the native state could explain the concentration dependence on refolding if dissociation is rate-limiting. In order to demonstrate unequivocally that the association effects observed for refolding are due to association of an intermediate species as opposed to the folded or unfolded states, the following experiments were performed. To demonstrate that association does not arise from the unfolded state, refolding studies were conducted at varying protein concentrations in the starting unfolded state but with a constant protein concentration in the final folding solution. The results are shown in Table III and are independent of the protein concentration in the starting solution. To test the possible effect of association in the folded state, varying concentrations of native protein were included in the final folding solutions and, as demonstrated in Table III, there was no effect. Therefore we conclude that the concentration dependence observed in refolding is a result of association from a partially folded intermediate.

In contrast to that observed by absorbance, the unfolding monitored by circular dichroism is protein concentration-dependent (Table I). The concentration dependence observed in the kinetics of unfolding by circular dichroism but not by absorbance can be explained by a sequential pathway for unfolding in which association occurs from an intermediate that is denatured with regard to ultraviolet absorbance but partially native-like with respect to helix structure. In this manner the absorbance-detected unfolding is silent to association yet the circular dichroism-detected unfolding is sensitive to association.

Effect of Final GdnHCl Concentration on Unfolding and Refolding—Fig. 2A depicts the effect of final denaturant concentration on the kinetics of folding and unfolding of bGH. They were obtained at a low protein concentration (0.2 mg/ml) in order to be free from contributions of association. Refolding could not be studied in final GdnHCl concentrations of less than 2 M because of precipitation. This is believed to be due to the general insolubility of any populated denatured species or associated intermediates in less than 2 M GdnHCl (9). The observed refolding kinetics are best explained by two kinetic phases, both of which show little or no dependence on GdnHCl. Unfolding in GdnHCl concentrations that are fully denaturing, as measured by absorbance at 290 nm, results in one kinetic phase that is strongly dependent on the final GdnHCl. Unfolding in GdnHCl concentrations that are partially denaturing results in two kinetic phases. The unexpected lack of coincidence for unfolding and refolding when measured at identical final GdnHCl conditions remains unexplained. We are pursuing further studies in order to better understand this observation.

The amplitudes for the absorbance-detected kinetics at varying final GdnHCl concentrations are depicted in Fig. 2B. For refolding experiments all of the expected extinction changes from the equilibrium measurements can be observed. For unfolding in final GdnHCl concentrations that are completely denaturing according to absorbance, only one kinetic phase is observed that accounts for approximately 50% of the expected extinction change. The unobserved reaction was too rapid to detect by stopped-flow techniques. Unfolding in final GdnHCl concentrations that are partially denaturing results in two observed kinetic phases that account for all of the expected extinction change.

The effect of GdnHCl on the kinetics as monitored by circular dichroism is illustrated in Fig. 3A. For refolding under completely native conditions the observed kinetics are explained by a single phase that has little or no dependence on GdnHCl. For unfolding and refolding in partially denaturing conditions two phases are required to explain the kinetics. Unfolding in completely denaturing solutions results in a single observed phase that is dependent on GdnHCl.
FIG. 2. Effect of final GdnHCl concentration on folding and unfolding as measured by absorbance at 280 nm. A shows the relaxation times and B the amplitudes. Folding (open symbols) was initiated from 6.0 M GdnHCl and unfolding (closed symbols) was initiated from 0 M GdnHCl. The circles represent the slower reaction phase and triangles the faster phase when two kinetic phases were observed. The squares and diamonds represent the results when a single observable kinetic phase is obtained by manual and stopped-flow mixing methods, respectively. Temperature was 3°C and the protein concentration was 0.2 mg/ml.

The amplitudes for the circular dichroism-detected kinetics obtained at varying final GdnHCl concentrations are depicted in Fig. 3B. For refolding in completely native conditions the single kinetic phase represents only 30% of the expected reaction, with the remaining unobserved portion occurring too rapidly to measure. For unfolding in completely denaturing solutions approximately 50% of the reaction is observable. Unfolding and refolding in GdnHCl concentrations that are partially denaturing result in 80% of the expected circular dichroism change. We estimate that, by using manual mixing techniques, reactions with time constants greater than 5 s can be detected.

Kinetics Obtained by Circular Dichroism Are More Rapid than Those Obtained by Absorbance—Fig. 4 compares refolding results obtained by absorbance and circular dichroism under identical conditions. It may be concluded that, at any time in refolding, a greater portion of helix signal is regained prior to the absorbance signal. Hence in refolding of bGH the regain of secondary structure precedes the tertiary structure.

Bovine Growth Hormone Derived from E. coli by Recombinant DNA Technology Does Not Contain the NH₂-terminal Heterogeneity Observed for Pituitary-derived Hormone—Pituitary bGH has been shown to be heterogeneous at the NH₂ terminus (5). Edman degradation of pituitary bGH demonstrates three NH₂-terminal residues: alanine (33%), ß-phenylalanine (33%), and methionine (33%). This heterogeneity is thought to result from post-translation processing. Fig. 5 illustrates that only alanine is identified from the first cycle of Edman degradation for recombinant DNA-produced bGH.

Kinetic folding studies were conducted on pituitary and recombinant DNA-derived bGH. No difference between the two was observed. We conclude that the NH₂-terminal heterogeneity of pituitary bGH does not affect the folding results herein reported.

DISCUSSION

These studies on the folding kinetics of bGH have been measured by two different probes of conformation. One of
Folding of bGH as measured by absorbance at 290 nm (○) and circular dichroism at 222 nm (●). Folding was initiated by dilution of the GdnHCl concentration from 6.0 to 2.2 M. The protein concentration was 0.2 mg/ml.

The equilibrium denaturation results of Fig. 1 demonstrate that, for bGH, some secondary structure is stable in the absence of the long-range tertiary structure. From thermodynamic considerations it is envisioned that, during kinetic folding, the short-range interactions occur with greater probability and consequently precede the long-range interactions. Therefore the intermediate(s) obtained in equilibrium could reflect an early intermediate(s) populated in the kinetic pathway of folding.

Evidence that bGH folding occurs through a sequential pathway with populated intermediates comes from the observed dependence on protein concentration. The results of Table III show that the concentration effect on refolding kinetics is not derived from the starting nor final states but from association of transient intermediates. The time dependence of the concentration effects on folding are the same as for the formation of a previously identified associated equilibrium intermediate. The associated equilibrium intermediate was shown to arise from partially folded monomers. Helix to helix intermolecular interactions were further shown to play a critical role in the association. These findings suggest that the concentration dependence observed on kinetics of folding arises from the association of an early folding intermediate that is similar in nature to the associated intermediate populated in equilibrium measurements.

To investigate further the pathway of folding we compared the kinetics measured by circular dichroism at 222 nm and absorbance at 290 nm under dilute protein conditions in order to avoid contributions of association. As can be seen in Fig. 4, the majority of the circular dichroism signal returns too rapidly to observe by manual mixing methods. Whereas all of the refolding absorbance signal at 290 nm can be observed by

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These probes are the circular dichroism at 220 nm. The far ultraviolet circular dichroism spectra of bGH indicates 50% α-helix, 10–20% β-sheet, and 30% random coil (3). This estimation of secondary structure from circular dichroism is reaffirmed by other methods such as infrared and Raman spectroscopy. The circular dichroism at 222 nm represents a local minimum in the spectra and reflects predominantly the α-helix. The other probe utilized for these studies is the ultraviolet absorbance at 290 nm. The aromatic ultraviolet spectrum of bGH is a composite of the single tryptophan, 6 tyrosines, and 13 phenylalanines. Upon denaturation the aromatic UV spectrum blue shifts representing the exposure of the aromatic chromophores from an apolar to polar solvent. The maximum difference between the native and denatured state occurs at 290 nm (3). The absorbance signal at 290 nm represents the opening of the globular structure or unfolding of the tertiary structure. This is reconfirmed by other physicochemical methods that are sensitive to the Stokes radius such as size-exclusion chromatography and photon correlation spectroscopy (10).

The NH₂-terminal analysis of recombinant DNA-derived bGH. The top chromatogram represents the elution profile from the first cycle of Edman degradation of recombinant DNA-derived bGH. The initial yield was 45%. The bottom chromatogram represents the elution profile of the standard phenylthiohydantoins using an Applied Biosystems method. N,N'-diphenylthiourea (DPTU) and N,N-di-methyl-N'-phenylthiourea (DMPTU) are by-products of the gas-phase sequencing. Also illustrated are the NH₂-terminal sequences present in pituitary bGH as determined by Edman degradation and the NH₂-terminal sequence of recombinant bGH. NLEU, norleucine.
Folding Kinetics of Bovine Growth Hormone

manual mixing methods with 30 and 70% of the reaction having relaxation times of 16 and 70 s, respectively, the remaining 30% of the circular dichroism signal shows a time constant similar to the slower phase detected by absorbance. Therefore, by the kinetic ratio test, intermediates are populated in refolding with the formation of helix structure forming earlier than the tertiary structure.

All of the unfolding results (Figs. 2 and 3) which end in final concentrations of guanidine that are partially denaturing result in the formation of the stable, associated intermediate. Previous results have shown that the associated intermediate is fully denatured with respect to the absorbance at 290 nm but partially native-like by far ultraviolet circular dichroism (9). Consequently the unfolding monitored by absorbance was expected and found to be concentration-independent (because association occurs subsequent to absorbance-detected denaturation), whereas the circular dichroism shows concentration dependence (because association results in a circular dichroism signal change). At low protein concentrations the unfolding kinetics measured by absorbance are very similar to those obtained by circular dichroism. These results at low protein concentration demonstrate that, for the observed kinetics, the unfolding process is cooperative. However, it is uncertain how the unfolding measured by absorbance and circular dichroism compares for the unobserved kinetics.

The refolding results are consistent and provide new support for the framework model of protein folding. Many studies of other proteins support the framework model of folding. The best defined are those of ribonuclease A, in which the rate of unfolding was compared by amide proton exchange and by ultraviolet absorbance (17-19). The studies on ribonuclease A demonstrate the presence of early intermediates in which the hydrogen bonding network (secondary structure) is formed prior to regaining the native-like absorbance signal (or tertiary structure). Studies from penicillinase (20-26), α-lactalbumin (27-35), and carbonic anhydrase (36-38) all support the notion of the secondary structure preceding the tertiary structure in folding. Reoxidation and refolding studies on bovine pancreatic trypsin inhibitor (39) clearly demonstrate the sequential nature of protein folding.

Our results on bGH are particularly intriguing because of the similarities between the kinetic intermediate(s) and the stable equilibrium intermediate(s). These similarities suggest that the equilibrium intermediate(s) is closely related to the kinetic intermediate(s). We have no data that would suggest otherwise. These observations warrant further effort toward better characterization of the equilibrium intermediate(s).

Folding and oxidation studies of reduced bGH have been conducted. Qualitatively the results are like those reported here. Because of the stability of the secondary structure in the absence of tertiary structure, the reduced molecule could be “poised” in a partial native-like character prior to reoxidation, which results in high yield (>90%) of the monomeric native state (16).

The folding studies reported here should be considered in light of the possibility that bGH is a multidomain protein. There is no evidence available that would indicate more than one domain. If multidomains exist the kinetic ratio test is still valid in demonstrating folding intermediates. The framework model states that secondary structure is formed prior to tertiary structure. In order to establish that a multidomain protein is consistent with a framework folding pattern, it is necessary for probes for each level of structure to exist in each domain. The data presented here indicate that a framework-type intermediate for multidomain folding is valid, unless more than 70% of the total helix is contained in one domain and that 100% of the absorbance change at 290 nm is contained in a different domain.

The similarities in the folding kinetics of pituitary and recombinant DNA-derived bGH demonstrate that the NH2-terminal heterogeneity of pituitary bGH does not alter the in vitro folding kinetics. These in vitro results suggest that the NH2-terminal heterogeneity does not affect the in vivo folding process.

Our future folding studies will be directed toward defining a detailed mechanism of bGH folding. Outstanding questions remain: what is the origin of the multiple phases? Which helical segments are formed first? Do the equilibrium intermediates represent kinetic intermediates that lie on the direct pathway of folding?

Acknowledgment—We are indebted to R. L. Garlick for the preparation of recombinant DNA-derived bGH.

REFERENCES

Folding Kinetics of Bovine Growth Hormone