Reoxidation of Reduced Bovine Growth Hormone from a Stable Secondary Structure

Thomas F. Holzman,* David N. Brems, and John J. Dougherty, Jr.
Control Division, The Upjohn Company, Kalamazoo, Michigan 49001
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ABSTRACT: In order to determine solution conditions appropriate for reoxidizing reduced bovine growth hormone (bGH), we have examined the possibility of using a particular denaturant concentration to poise the secondary and tertiary structure of the reduced protein in a stable, nativelike state. It was envisioned that the structure of the reduced molecule would differ from that of the final oxidized molecule solely by the absence of disulfide bonds. Dilution of concentrated samples of reduced and unfolded protein from 6.0 M guanidine into 4.5 M urea followed by air oxidation indicated it was possible to induce refolding and reoxidation to an oxidized monomeric species in high yield (~90%). The choice of solution conditions was based on comparison of urea equilibrium denaturation data for native oxidized protein to those for completely reduced protein and to protein in which sulfhydryl groups had been either partially or completely reduced and subjected to modification with iodoacetamide or methyl methanethiolsulfonate. The denaturation behavior of these species supports the existence of equilibrium folding intermediates for bovine growth hormone and demonstrates that chemical modification of the protein is capable of inducing differences in the denaturation behavior of these intermediates. The changes in the protein absorption spectrum and helix-related circular dichroism signal, along with direct titration of protein sulfhydryl groups, indicated that the refolding/reoxidation of bGH is a multistate process. The ordered nature of the kinetic changes in these probes during reoxidation indicates that disulfide formation is a sequential process, with little mispairing in 4.5 M urea, and that it proceeds through one or more obligatory kinetic folding events. The equilibrium denaturation behavior of the oxidized molecule and the various chemically modified forms, together with the reoxidation data, indicated that the protein maintains a high degree of secondary structure without intrachain disulfide bonds. The formation of these disulfide bonds is a discrete process which occurs after a framework of protein secondary structure is established.

An understanding of the processes by which proteins fold to attain native conformations is one of the fundamental goals of the study of protein structure. As initially proposed by Anfinsen (1961), it is now accepted that the amino acid sequence of a protein, together with bulk solvent interactions, dictates folding of the polypeptide chain into a preferred three-dimensional structure. This conformation of the native molecule is maintained by a dynamic balance of forces including hydrogen bonding and hydrophobic and ionic interactions, which act in concert with covalent disulfide bridges. Fully active molecules can only form from protein in which the correct disulfide bonds are formed.

Bovine growth hormone (bGH)[1] (somatotropin) is a single polypeptide chain of M_r ~22 000 which is secreted from the pituitary gland and is isolated in a folded, biologically active, state (Paladini et al., 1983). The molecule contains two disulfide bridges between cysteine residues at positions 53–164 (large loop) and 181–189 (short loop). The aromatic region of the ultraviolet absorption spectrum of bGH is dominated by contributions from 1 tryptophan residue, 6 tyrosine residues, and 14 phenylalanine residues (Chen & Sonnenberg, 1977). Bewley (1974) has demonstrated disulfide contributions to the protein CD spectrum from ~240 to ~290 nm. In a related protein, human growth hormone, Bewley and Li (1984) have proposed assignments for the Tyr, Phe, and Trp absorbance bands. Although there are no crystal structures available for bGH or related molecules, the predicted secondary structure of bGH has been reported (Chen & Sonnenberg, 1977). The residues between the 53–164 disulfide bridge are proposed to include 2 helical segments, 4 sheet regions, and 10 turns. The C-terminal disulfide bridge (181–189) is predicted to encompass the shortest helical segment in the molecule (Chen & Sonnenberg, 1977). The conformation, structure, and a number of physical properties of purified pituitary protein and isolated fragments have been examined (Graf & Li, 1974; Holladay et al., 1974; Chen & Sonnenberg, 1977).

On the basis of the observations of Holladay et al. (1974), which suggested the existence of a folding intermediate(s), Brems et al. (1985) examined the equilibrium denaturation of pituitary and recombinant DNA derived hormones and verified the existence of an intermediate(s). These authors propose, based on their observations and earlier observations by others, that the folding behavior of bGH is consistent with a "framework" model of protein folding. In this model, the secondary structure of the protein is sufficiently stable in the absence of tertiary structure to provide a framework for the subsequent folding of the molecule.

We have chosen to examine the process of refolding and reoxidation of reduced bovine growth hormone with the naturally occurring pituitary protein because unlike the recombinant protein, which is found in inclusion bodies (Hart et al.,

* Correspondence should be addressed to this author.

[1] Abbreviations: bGH, bovine growth hormone; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DTE, dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); RCAM-1 bGH, reduced and carboxamidomethylated bGH at cysteines-181 and -189; HPSEC, high-pressure size-exclusion (SE) liquid chromatography; TSCAM bGH, tetra-*S*-carboxamidomethyl-bGH; TSSM bGH, tetra-*S*-*S*-methyl-bGH; HPLC, high-pressure liquid chromatography; PTH, phenylthiohydantoin; MMTS, methyl methanethiosulfonate; FPLC, fast protein liquid chromatography.

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1984), it allows us to start with a naturally oxidized, biologically active, material. In this work, we examine the spectral and chemical events associated with the refolding/reoxidation of reduced bovine growth hormone.

Experimental Procedures

Materials

Bovine pituitary growth hormone was obtained from A. F. Parlow, lot 8500 from the Harbor Medical Center of the University of California at Los Angeles. Unless specifically noted otherwise, all experiments were performed with pituitary hormone; for certain experiments, recombinant derived bGH from the Amgen Co. of Thousand Oaks, CA, was used. Ultrapure urea was obtained from Schwarz/Mann. The reducing agent, DTE, was "gold label" grade from Calbiochem. Size-exclusion HPLC was performed by using a Dupont Zorbax GF-250 column (Stodola et al., 1986). Protein molecular weight markers for HPLC were purchased from Sigma.

Methods

Absorbance Measurements and Reoxidation. UV absorbance measurements were performed with a Hewlett Packard 8451A diode array spectrophotometer equipped with a thermostated multiple cell transport and an HP 9821D microfloppy disk drive. Typically, a 1-s integration period was used to accurately record UV spectra over the range of 200–500 nm with a nominal resolution of 1 nm; all samples were equilibrated to 20 °C (±0.1 °C). Protein concentrations of native oxidized bGH were estimated from the 278-nm absorbance by using a molar extinction coefficient of 15 270 (Burger et al., 1966). The urea-induced UV difference spectrum is characterized by a 290-nm maximum which was the wavelength chosen for monitoring equilibrium denaturation and the kinetics associated with refolding/reoxidation of the reduced protein. The kinetics of refolding and reoxidation were initiated by diluting samples of reduced protein, free of reducing agent (prepared as described below) from the denaturing solvent conditions of sample storage, into a cuvette containing air-saturated 4.5 M urea, as specified in the figure legends. The concentration dependence of the UV reoxidation kinetics was examined in 4.5 M urea over the range from ~5 to ~35 μM reduced protein. Over this range, the apparent $t_{1/2}$ for reoxidation exhibited a linear increase of ~20% from a value of ~40 min to a value of ~50 min (see below). The slight increase in $t_{1/2}$ in 4.5 M urea was judged to have little effect, over the range of reduced protein concentration actually used, and so a detailed study of its effects was omitted.

Kinetics of Tyrosine Burial. It has been shown by Servillo et al. (1982) and Ragone et al. (1984) that the relative degree of tyrosine exposure for proteins with certain ratios of tyrosine to tryptophan can be estimated from the second-derivative spectrum of the protein in solvents of various polarities. The ratio of Tyr to Trp in bGH suggested [see Rangone et al. (1984) for criteria] that it would be possible to utilize this method to follow the time dependence of the sequestering (or "burial") of the protein Tyr residues from bulk solvent during the process of refolding/reoxidation. The extent of Tyr burial at various times was calculated from the ratio of peak to trough values from the second-derivative spectrum of protein absorbance, i.e., the peak to trough distance between the maximum at 288 nm and the minimum at 284 nm divided by the peak to trough distance between the maximum at 294 nm and the minimum at 291 nm.

Circular Dichroism Measurements. CD spectra were obtained by using a Jasco J-500C spectropolarimeter equipped with a DP-501N data acquisition and display system. Measurements were typically made at 20 °C. The far-ultraviolet spectrum of bGH has a minimum at 222 nm which is assigned to helix (Holladay et al., 1974) and is the wavelength at which changes in helicity were monitored during refolding/reoxidation and during equilibrium denaturation measurements. Estimates of protein secondary structure based on the protein CD spectra were made by using the CD data fitting package in the CONTIN program (distributed by the European Molecular Biology Laboratory of Heidelberg, FRG) developed by Provencher (1982a,b) and Provencher and Glockner (1981). The program uses a basis set of proteins of known structure and special algorithms to estimate the contributions to protein secondary structure from its observed CD spectrum (Provencher & Glockner, 1981). Computations were performed on a Digital Equipment Corp. VAX 11/780 mainframe computer.

Characterization of Reoxidized bGH. The best yields of reoxidized monomeric bGH were found at low denaturant concentrations, 4.0–5.0 M urea at pH 9.1. Reoxidized monomeric bGH from several reoxidation experiments in 4.5 M urea, pH 9.1, was isolated by FPLC in 6.0 M guanidine and 0.20 M phosphate, pH 7.0, on a Pharmacia Superose 12 size-exclusion column. The isolated monomer was then exhaustively dialyzed against water and lyophilized. The equilibrium denaturation behavior of the reoxidized protein was examined by both UV and CD spectroscopy. Tryptic mapping of the isolated monomer was performed by the method of Hartman et al. (1986). The tryptic fragment containing the large-loop disulfide was identified by sequence analysis using an Applied Biosystems 470A protein sequencer with version 2.0 software. Quantitative separation of PTH-amino acids was performed on a Hewlett-Packard 1090A HPLC system equipped with a diode array detector and a multichannel integrator using the method of Schuster (1983).

Preparation and Properties of Reduced bGH. A typical sample of reduced bGH was prepared as follows: 300 mg of native oxidized protein was added to 2.0 mL of buffered denaturant (6.0 M guanidine hydrochloride and 20 mM CHES, pH 9.1). The protein was slowly dissolved over a ~2-h period at room temperature (21 °C) with periodic gentle agitation. The sample was then treated with 2-mercaptoethanol (~0.5 M final concentration) for 1 h at room temperature and then heated to ~50 °C for 30 min, after which the solution was noticeably less viscous. Examination of protein treated with reducing agent, with or without heat treatment, by SE-HPLC revealed that the reducing agent in the presence of 6.0 M guanidine was sufficient to cause the complete reduction of the protein at room temperature. The reduction of the protein was evidenced by a shift in retention time. Once reduced, the protein was subjected to chromatography on Sephadex G-25 (1.5 × 30 cm column) in argon-sparged guanidine buffer (above) to remove reducing agent. The reduced protein from the excluded volume of the G-25 column was pooled and stored at −20 °C. The protein sample in the guanidine buffer remained in solution at this temperature; no guanidine was seen to precipitate from solution. The concentration of reduced protein was estimated from its absorption at 290 nm in the buffered denaturant using a molar absorptivity of 7500 (Brems et al., 1985). Reduced bGH prepared by this method was assayed spectrophotometrically for free sulfhydryl groups by using Ellman's reagent (Ellman, 1958) and was found to contain the requisite 4 mol of sulfhydryl/mol of protein. Samples of reduced protein were stored under argon at −20 °C to prevent atmospheric oxygen from contributing to protein reoxidation; reduced protein stored in this manner remained

reduced for at least 1 month. In contrast to the oxidized protein, the fully reduced protein was found to be insoluble in a variety of buffers from pH 4.5 to 9.5. It was found to be readily soluble in 4.5–10 M urea.

Preparation of Chemically Modified Growth Hormone. The tetra-S-S-methylated growth hormone was prepared as follows. A sample of protein (200 mg) was dissolved in 5.0 mL of 6.0 M guanidine hydrochloride and 20 mM CHES, pH 9.1, with gentle agitation over a period of 1 h. Exhaustive reduction of the protein was initiated by addition of a 0.50-mL aliquot of 2-mercaptoethanol (0.98 M final concentration); the mixture was allowed to incubate overnight in a sealed container at room temperature (21 °C). To the mixture was subsequently added 0.750 mL (1.2 M final concentration) of methyl methanethiolsulfonate (MMTS, Aldrich Chemical Co.); the MMTS was observed to rapidly disperse in the sample upon gentle agitation. The mixture was allowed to incubate for 2 h at room temperature. Subsequent testing for free sulfhydryl groups using Ellman's reagent (Ellman, 1958) indicated that mixed disulfide formation was complete. The mixture was then exhaustively dialyzed against 10 changes of 2 L each of distilled water over a 4-day period at 4 °C. The dialyzed protein was then lyophilized and stored at −20 °C. The tetra-S-S-methylated protein prepared in this fashion was referred to as TSSM bGH. Partially alkylated bGH (RCAM-1), in which the C-terminal disulfide (residues 181 and 189) is reduced and then alkylated with iodoacetamide, was prepared according to the method of Graf et al. (1975). Tetra-S-alkylated bGH (referred to as TSCAM bGH) was prepared with iodoacetamide according to the method of Crestfield et al. (1962).

Sample Preparation for Equilibrium Denaturation. Stock solutions of native oxidized bGH, RCAM-1 bGH, TSCAM bGH, and TSSM bGH were prepared by dissolving ~10 mg of protein in 1.0 mL of 8 M guanidine, 40 mM CHES, and 4 mM glycine, pH 9.1, followed by centrifugation at 15000*g* for 15 min at 4 °C. Reduced protein was prepared as described above.

For measurement of UV and CD signals associated with denaturation of the various protein samples, small aliquots of the stock solutions (10 μL) were diluted into varying urea concentrations. UV and CD equilibrium denaturation measurements were made at 20 °C. The final protein concentrations of the diluted samples were established by using the 290-nm absorbance of an equivalent dilution of each sample in 7.2 M guanidine, 20 mM CHES, and 1 mM glycine, pH 9.1, based on a molar absorptivity of the denatured protein of 7500 (Brems et al., 1985). To preclude interferences from signals associated with reoxidation, CD measurements of reduced bGH were made within 1 min of dilution. Denaturation transitions were completely reversible across the range of urea concentrations used. However, if samples of TSSM, TSCAM, RCAM-1, and reduced bGH were diluted to less than ~4.0 M urea, turbidity developed.

Results

Equilibrium Denaturation. The denaturation behavior of various chemically modified forms of bGH is examined in Figure 1. Solution conditions were desired in which the reduced protein was both soluble and yet would maintain a high degree of native structure. The data indicate differences in the denaturation behavior of the various forms of bGH in comparison to each other and to oxidized bGH. Differences exist in the extent of denaturation, in the apparent midpoints of the denaturation curves (Table I), and in the cooperativity of the denaturation process. Consistent with the framework

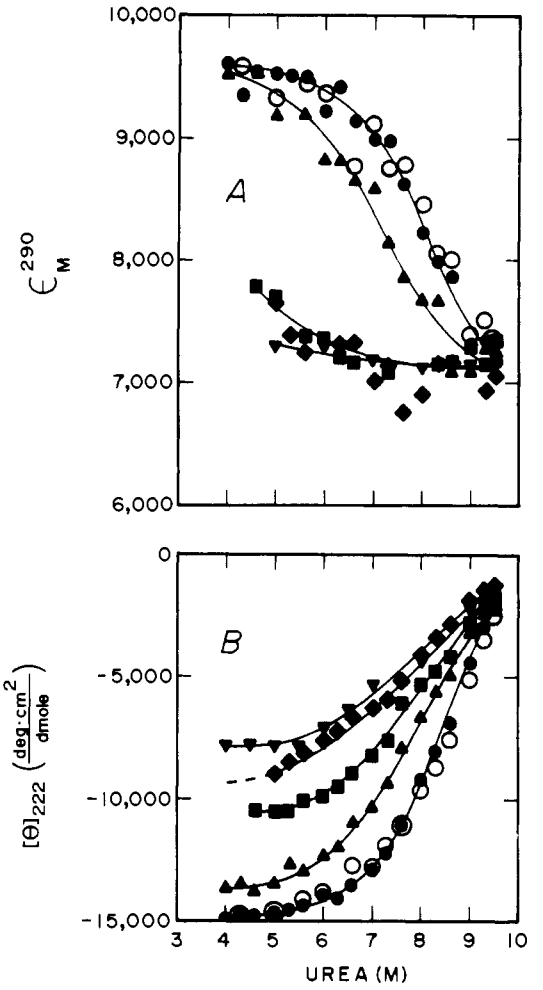


FIGURE 1: Equilibrium denaturation of various forms of bGH in the urea concentrations specified, 40 mM CHES, and 4 mM glycine, pH 9.1. Samples were prepared as described under Methods. (A) Measurement of protein absorbance at 290 nm; (B) measurement of protein CD signal at 222 nm. (●) Native oxidized bGH; (○) reoxidized bGH; (▲) RCAM-1 bGH; (♦) tetra-S-carboxamidomethyl-bGH; (■) tetra-S-S-methyl-bGH; (▼) reduced bGH. Absorbance measurements below 4.3 M urea for TSCAM bGH and 5.0 M urea for TSSM bGH exhibited the effects of turbidity and were judged unreliable. Final protein concentrations were as follows: native oxidized bGH, 9.2 μM; reoxidized bGH, 6.3 μM; RCAM-1 bGH, 7.9 μM; TSCAM bGH, 8.7 μM; TSSM bGH, 9.8 μM; reduced bGH UV data, 22 μM; reduced bGH CD data, 14 μM.

Table I: Estimated Urea Denaturation Curve Midpoints[a]

species	UV midpoint (M)	CD midpoint (M)	CD − UV (M)
reduced bGH		7.8	
ox bGH	7.9	8.3	0.4
reox bGH	7.9	8.3	0.4
RCAM-1	7.0	7.9	0.9
TSCAM bGH		8.0	
TSSM bGH		7.9	

[a] Estimates assume that CD signals for all species will give rise to $[\theta]$ at 222 nm ≃0 when the amino acid chain is in a completely random coil.

model, the helical structure is more stable in each protein species than the structure associated with the UV signal. The existence of noncoincident denaturation curves, when comparing UV to CD signals, for each protein species supports the existence of folding intermediates. It is evident that even at the highest concentrations of urea the unfolding transition of the oxidized molecule is incomplete as measured by either spectral technique and the reoxidized protein (described below)

exhibits similar unfolding transitions. In denaturation studies with guanidine (Holladay et al., 1974; Brems et al., 1985), the oxidized molecule can be fully unfolded and gives changes in absorptivity at 290 nm of ~2000–2500 M^{-1} cm^{-1} and $[\theta]$ at 222 nm of ~14000 deg·cm^2/dmol. Additionally, Bewley and Li (1984) have demonstrated that thermolysin digestion of human growth hormone results in a change in absorptivity of ~2500 M^{-1} cm^{-1} at 290 nm. We observe values similar to these for the denaturation of bGH (Figure 1). The extent of change in absorptivity at 290 nm for the denaturation transition of RCAM-1 bGH resembles that of oxidized bGH with the midpoint shifted to lower urea concentrations (Figure 1A, Table I). It is evident that the CD denaturation transition of RCAM-1 bGH was both shifted to lower urea and was slightly less in total extent of change in comparison to oxidized bGH (Figure 1B, Table I). On the basis of the observations of Bewley (1977) on the contributions of the bGH short-loop disulfide to the protein CD spectrum and the CD spectra and estimated contributions to secondary structure described below, it is unlikely that this reduction in helicity is due directly to spectral contributions of the short-loop disulfide itself. Rather, breaking the short-loop disulfide results in a small loss in helicity and a decrease in stability to denaturant. Edelhoch and Burger (1966) have observed the effects of urea on bGH denaturation and the enhancement of protein fluorescence, and Holladay et al. (1974) have demonstrated the apparent variability of tryptophan environment as indicated by CD measurements for various growth hormones. At this time, we do not have sufficient data to conclude that the UV absorption changes observed upon breaking the short-loop disulfide, or attaching alkyl side chains to the sulfhydryl groups, are due solely to changes in the environment of the tryptophan at position 86. Indeed, it is difficult to envision how the events associated with breaking the short-loop disulfide could be so specific as to alter only the environment of the single tryptophan; it would be expected that the other aromatic residues would also be affected. The complexity of the spectral behavior is underscored by a recent examination of the Raman spectra of various lyophilized samples of bGH, including RCAM-1. It indicates that breaking the protein C-terminal disulfide changes the protein tyrosine resonances (T. Thaman, unpublished observation). This suggests that the environments of one or more tyrosine residues are altered in RCAM-1.

The reduced bGH, TSSM bGH, and TSCAM bGH show other differences in stability. At urea concentrations above 6.0 M, these modified forms appear to be completely unfolded as judged by differences in the UV signals. Comparison of the UV signals to the CD signals for these species indicates that the transition in protein structure (unfolding) associated with the UV signal is complete (~6.0 M urea) prior to loss of the initial CD signal. The difference in the extent and midpoints of the CD denaturation transitions between, collectively, reduced bGH, TSSM bGH, TSCAM bGH, and both RCAM-1 and oxidized bGH indicates that there is a gain in protein secondary structure (~30–40%) and an increase in stability (Table I) afforded by the disulfide bridge between cysteine residues 53 and 164. In contrast, comparison of the UV signals of oxidized and RCAM-1 bGH to the CD signals of the other modified forms indicates that when the large-loop disulfide (53–164) is intact the protein structure associated with the UV signal is much more stable (Figure 1A and Table I). Thus, the 53–164 disulfide plays a major role in maintaining the environment of the tryptophan at position 86.

Examination and comparison of the CD denaturation transition for reduced bGH, TSCAM bGH, and TSSM bGH demonstrate that each of these species exhibits slightly different denaturation behavior. TSCAM bGH and reduced bGH denaturation transitions appear to have the least cooperativity in the CD. Comparison of the CD denaturation transitions for TSSM bGH and TSCAM bGH indicates that TSSM bGH contains more helical structure than TSCAM bGH (Figure 1). The inability to detect complete UV denaturation transitions for the reduced bGH and the chemically modified forms was unexpected since there were clearly observable CD transitions for these proteins. This indicates that the difference in stability of the structures responsible for the CD and UV signals is even greater in the fully modified forms than in native bGH or RCAM-1. For the reduced bGH, the absence of a 290-nm signal difference may be related to the disruption of a portion of the protein structure by intramolecular charge repulsions between ionized sulfhydryl groups. At this stage in our studies, we are hesitant to ascribe a specific cause for the behavior of the reduced bGH and the two modified forms; the data do suggest that there may be some distinct differences in the three-dimensional packing of the various species which, in turn, lead to altered unfolding behavior.

Stabilizing Effects of Protein Disulfides. The apparent Gibbs free energy of stabilization of bGH can be estimated by a two-state analysis of the CD data in Figure 1B using the method of Schellman (1978). The apparent free energy changes were estimated from a plot of log K_{eq} vs. urea concentration (Schellman, 1978); the end point of the CD transition in high urea concentration for oxidized bGH was assumed to be similar to that previously observed in guanidine (Brems et al., 1985). An apparent free energy of unfolding of −6.8 kcal/mol is obtained for oxidized bGH, which is similar to the value −5.9 kcal/mol reported by Brems et al. (1985) utilizing guanidine as the denaturant. Due to the differing final states attained in the equilibrium denaturation of the reduced and modified forms of bGH and the absence of complete denaturation transitions, quantitative comparison of the reduced and modified forms is precluded. However, qualitatively, two conclusions can be made. First, the large-loop disulfide provides more conformational stability than the small-loop disulfide (Figure 1), and this conformational stability is restored in reoxidized bGH. This conclusion is consistent with disulfide stabilization of the native state due to a reduction of the entropy of the unfolded protein; a shorter polypeptide chain between disulfide bridges results in a smaller entropy reduction (Schulz & Schirmer, 1978). Second, the nativelike conformation of bGH is only partially dependent on disulfide bonds; i.e., the CD spectra at 222 nm of reduced bGH and chemically modified bGH demonstrate considerable nativelike structure. In general, proteins containing disulfide bonds need a fraction of these bonds for stability. However, several proteins are known to retain partial activity or structure following complete reduction; examples include amylase (Straub, 1967) and lysozyme (Yutani et al., 1968; Saxena & Wetlaufer, 1970).

Spectral Changes Observed upon Reduction and Reoxidation. Studies of the behavior of bGH upon reduction and reoxidation were performed under conditions chosen to ensure that reoxidation would take place from solution conditions in which the protein structure resembled, as closely as was experimentally possible, folded oxidized bGH. Low urea concentrations (4.5 M) were chosen where oxidized protein was not denatured and where the reduced protein and the two chemically modified forms were soluble and had the greatest amount of secondary structure. Dilute protein concentrations were chosen (~10 μM) to minimize the opportunity for in-

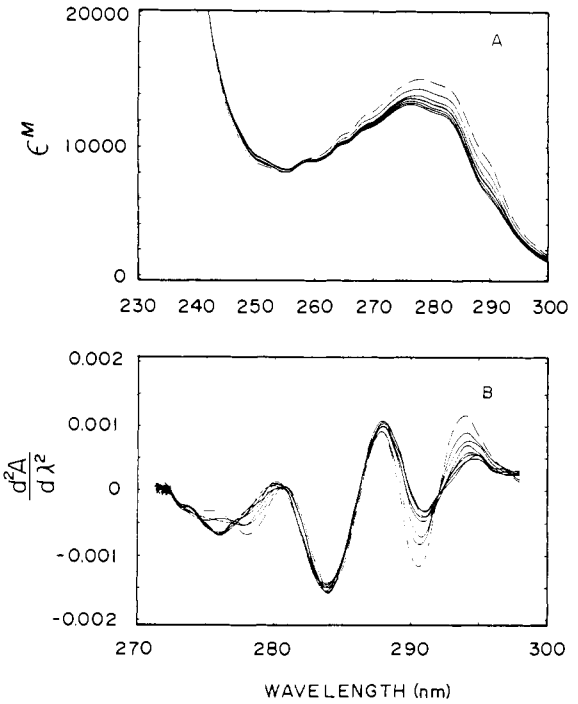


FIGURE 2: Changes in UV absorbance spectra (A) and second-derivative spectra (B) for the reduction of oxidized bGH (11 μM) in argon-sparged 4.5 M urea, 10 mM CHES, and 1 mM glycine, pH 9.1, under argon. Reduction was initiated by addition of 15 μL of 0.144 M DTE, in the same buffered urea, to 1.0 mL of sample. Spectra were measured at ~3.2-min intervals over ~6 h beginning at ~15 s (dashed curve) after addition of DTE to the sample. The spectra are plotted at 48-min intervals and represent ~5 h of absorbance data. Absorbance spectra were observed to decrease upon reduction. To obtain accurate data during reduction, measurements were made in partitioned cuvettes (~4.5 mm per cell chamber) as difference spectra between the DTE-treated sample and a reference without DTE; the data are plotted as sample absorbance vs. buffered urea with reducing agent.

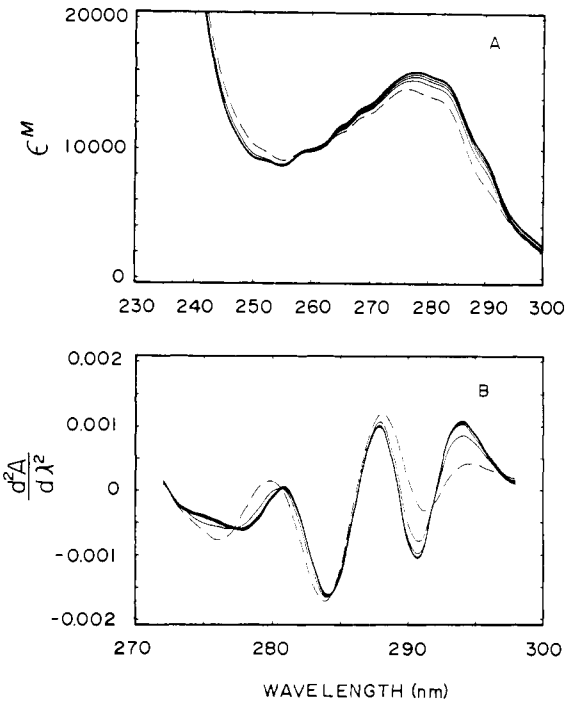


FIGURE 3: Changes in protein UV absorbance spectra (A) and second-derivative spectra (B) as a function of time for refolding/reoxidation of 10.3 μM reduced bGH in 4.5 M urea, 10 mM CHES, and 1 mM glycine, pH 9.1. Reoxidation and measurements were initiated on addition of 13 μL of a concentrated stock solution of reduced bGH to 1.0 mL of air-saturated buffered urea; a sample of buffered urea served as reference. The initial time is indicated by the dashed curve. Measurements were made at 10.7-min intervals. The time interval between plotted curves is 42.6 min. Absorbance spectra were observed to increase during reoxidation.

termolecular reoxidation. Additionally, at low urea concentrations, any problems associated with reoxidation from different equilibrium folding intermediates were expected to be small, since the intermediate state(s) was (were) unlikely to be populated to any degree. Complete reduction of the protein was accomplished by treatment with a 230-fold molar excess of reducing agent. Following addition of reducing agent, a time-dependent decrease in aromatic absorption was observed (Figure 2A). Size-exclusion HPLC confirmed the complete conversion of oxidized protein to reduced protein (see below). This conversion resulted in a decrease in the molar extinction coefficient of ~2600 M^{-1} cm^{-1} at 290 nm. The data also indicated an isosbestic point at ~258 nm for the conversion of oxidized to reduced protein and are consistent with the interconversion of two species, oxidized and reduced protein. The second-derivative spectra associated with reduction of oxidized bGH are presented in Figure 2B. The major changes appear to be associated with the tryptophan absorption; there is a decrease in the 291-nm trough and 294-nm peak.

In Figure 3A, the UV spectral changes associated with the reoxidation of reduced bGH are presented. Size-exclusion HPLC performed after the reaction confirmed the conversion of reduced bGH to oxidized bGH (see below). This conversion gives an isosbestic band between ~253 and 260 nm and an increase in the molar extinction coefficient of ~2400 M^{-1} cm^{-1} at 290 nm. The broad band is consistent with an interconversion of reduced protein to oxidized protein which involves more than two species. The changes in the protein second-derivative spectra upon reoxidation are presented in Figure 3B. The data demonstrate the relative change in the tyrosine and tryptophan bands. As with reduction of oxidized protein, reoxidation of reduced bGH results in major changes in the second-derivative band at 291 and 294 nm. Upon reoxidation, the changes, as expected, are opposite in direction to the absorbance changes associated with reduction.

The hypochromic shift upon reduction (Figure 2A) indicates that, on the average, the aromatic chromophores become more exposed to solvent, and the hyperchromic shift upon reoxidation (Figure 3A) indicates that the reoxidation diminishes the degree of exposure to solvent. The reduced protein maintains a majority of its helical structure (Figure 1B). The loss of helical structure upon reduction appears to coincide with alteration of the environment of the aromatic chromophores, primarily tryptophan (Figure 2A). This is consistent with the denaturation behavior of reduced bGH, TSCAM bGH, and TSSM bGH (Figure 1) in which most of the 290-nm signal change is observed before the helical structure is lost at high urea concentrations.

Chromatographic Changes upon Reduction and Reoxidation. Reduction of oxidized bGH was accompanied by a significant and reproducible increase in the protein hydrodynamic volume, as evidenced by an altered retention time measured by SE-HPLC with a denaturing mobile phase of 6.0 M guanidine (Figure 4). Chromatography of reduced and oxidized bGH demonstrated that it was possible to resolve these species. Chromatographic analysis of a sample of the completely reoxidized protein from the experiment in Figure 3 demonstrated that the reoxidized protein formed material with the same retention time as the native monomer (Figure 4). Integration of the peaks from this sample indicated that reoxidation resulted in the formation of 90% monomer and 10% high molecular weight forms. Under the reoxidation

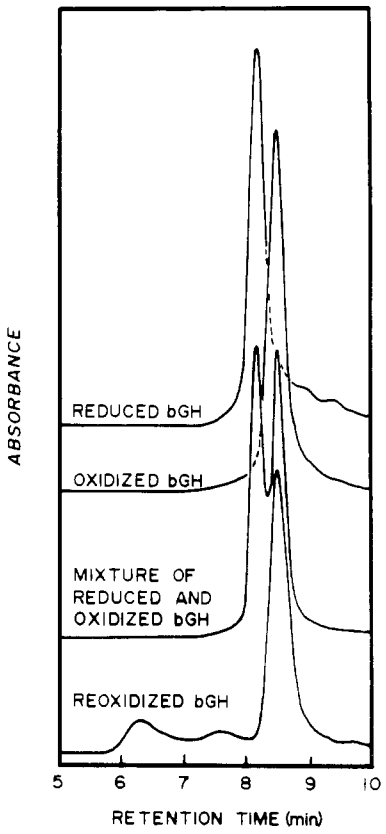


FIGURE 4: Size-exclusion HPLC profiles of bGH at 220 nm in 6.0 M guanidine and 0.20 M phosphate, pH 7.0, at 1.5 mL/min on the DuPont Zorbax GF-250 column. Reduced bGH was analyzed after reduction (see Figure 2) by injection of 100 μL of sample (at 11 μM). Comparison to oxidized bGH (100-μL injection of 10 μM solution) confirmed the conversion of oxidized bGH to reduced bGH as evidenced by the change in retention time. A mixture of reduced and oxidized bGH (~5 μM each) was analyzed by injection of 100 μL of sample. Resolution of the two components demonstrates the shift in retention time observed upon reduction of oxidized bGH. Reoxidized bGH (see Figure 3) was analyzed by injection of 100 μL of sample at 10.3 μM. Integration of the peaks from the reoxidized sample indicated ~90% monomer and ~10% higher molecular weight forms.

conditions outlined in Figure 3, these ratios of monomer to total high molecular weight forms were typical. Analysis of isolated reoxidized monomer for free sulfhydryls using DTNB (Ellman, 1958) indicated it contained <0.02 mol of free SH/mol of protein and thus was >99% reoxidized.

Samples of reduced bGH and oxidized bGH were also chromatographed by SE-HPLC in a nondenaturing mobile phase of 4.5 M urea and 50 mM CHES, pH 9.1, on the DuPont Zorbax GF-250 column as a test of the chromatographic system to resolve *folded* protein (data not shown). In this solvent, the reduced protein exhibited the same retention time as the oxidized protein. Thus, the differences in the retention times of reduced and oxidized protein observed under denaturing conditions (Figure 4) are due directly to the presence (or absence) of protein disulfide bonds. Furthermore, the conformational differences between reduced protein and oxidized protein in the urea mobile phase are sufficiently subtle as to leave the protein hydrodynamic volumes of the two species indistinguishable.

Secondary Structures of Native, Reoxidized, and Sulfhydryl-Modified Growth Hormones. To determine the extent to which the overall secondary structure of the various protein species was perturbed, an analysis of the contributions to secondary structure was undertaken. The analysis was based on the far-UV CD spectra of samples of each protein (Figure 5); the calculated values of secondary structure are presented in Table II. The data demonstrate that native and reoxidized bGH have similar CD spectra. These spectra lead to estimated values of secondary structure which are similar. These data indicate that, as judged by the respective CD data, there are little if any differences in the various contributions to secondary structure between native and reoxidized bGH. The CD spectra of RCAM-1 bGH and its estimated secondary structure differ slightly from those of native or reoxidized bGH. The reduction and alkylation of the protein short loop appear to mainly affect the stability of RCAM-1 bGH to unfolding by denaturants (Figure 1). The major deviations from native structure are observed for TSCAM bGH, TSSM bGH, and reduced bGH. These molecules have less helix content and an enhanced

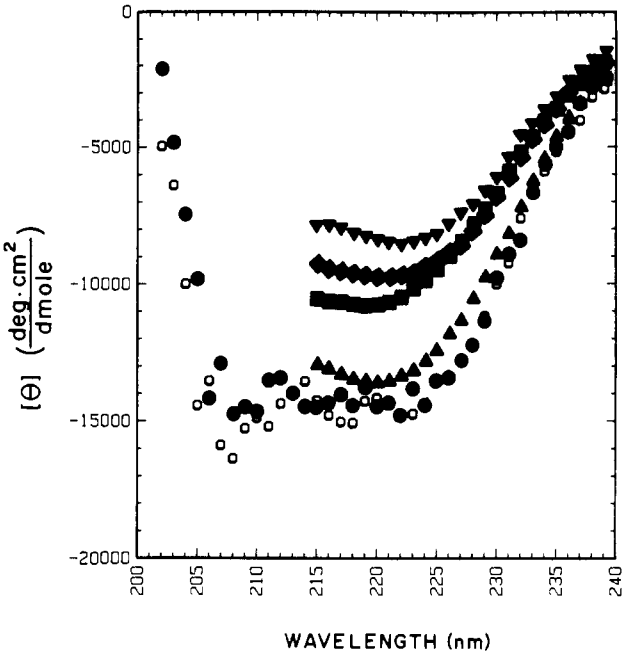


FIGURE 5: CD spectra of native, reoxidized, and sulfhydryl-modified bGH. Protein concentrations of each sample were as follows: (●) native bGH, 4.9 μM in 50 mM CHES, pH 9.1; (○) reoxidized bGH, 3.5 μM in 50 mM CHES, pH 9.1; (▲) RCAM-1 bGH, 3.9 μM in 4.5 M urea with 50 mM CHES, pH 9.1; (♦) TSCAM bGH, 4.7 μM in 4.5 M urea with 50 mM CHES, pH 9.1; (■) TSSM bGH, 3.8 μM in 4.5 M urea with 50 mM CHES, pH 9.1; (▼) reduced bGH, 5.0 μM in 4.5 M urea with 50 mM CHES, pH 9.1. Spectral measurements of samples in urea were judged to contain too much noise below 215 nm due to the high background absorbance of the urea. The individual ellipticity values from each spectrum were used in conjunction with the CONTIN program (see Methods) to generate the predicted contributions to protein secondary structure (see Table II).

Table II: Estimated Secondary Structure Contributions to Native, Reoxidized, and Sulfhydryl-Modified bGH[a]

species	% contributions		
	helix	sheet	random
native bGH[b]	46 ± 4	20 ± 6	34 ± 5
reox bGH[b]	45 ± 1	17 ± 1	38 ± 1
RCAM-1[c]	41 ± 3	17 ± 3	42 ± 3
TSCAM bGH[c]	25 ± 2	23 ± 3	52 ± 3
TSSM bGH[c]	33 ± 2	23 ± 3	44 ± 3
reduced bGH[c]	31 ± 2	27 ± 2	42 ± 2

[a] By use of the CONTIN program described under Methods, CD data from Figure 5 were used to calculate estimated secondary structure values; fitting uncertainties are expressed as standard error limits. [b] Sample in 50 mM CHES, pH 9.1; ellipticity values for fitting were taken between 197 and 240 nm. [c] Sample in 4.5 M urea and 50 mM CHES, pH 9.1; due to high background absorbance from urea at wavelengths less than ~210 nm, ellipticity values for fitting were taken between 215 and 240 nm.

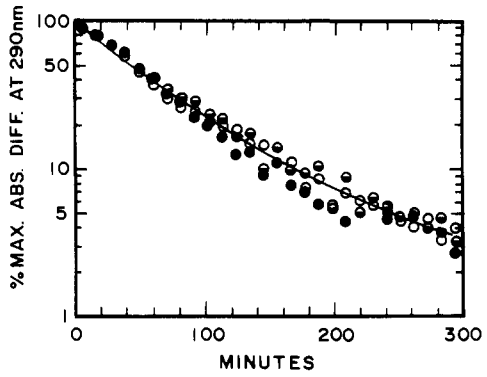


FIGURE 6: Time dependence of the absorbance change upon reoxidation/refolding of bGH measured at 290 nm. (○) Reduced protein at 10 μM; (◒) reduced protein at 22.5 μM; (●) reduced protein at 32.5 μM. The data are plotted as the percent decrease in the 290-nm signal; see Figure 3A for accompanying change in protein absorption spectrum at 10 μM reduced protein. The theoretical curve through the data for reduced protein at 10 μM represents the sum of two exponentials and was calculated from the equation $y = 0.5e^{(-\ln 2/30)t} + 0.5e^{(-\ln 2/75)t}$.

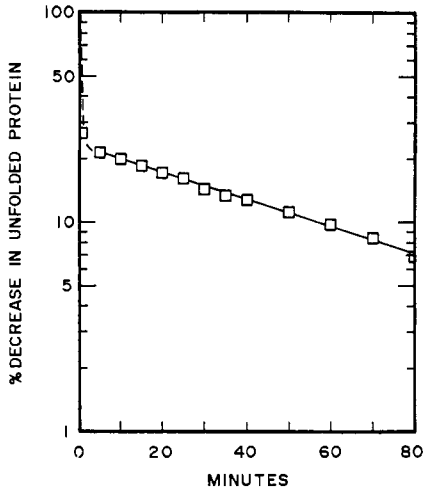


FIGURE 7: Time-dependent changes in the loss of denatured (unfolded) protein during the reoxidation of 20 μM bGH in air-saturated 4.5 M urea, 10 mM CHES, and 1 mM glycine, pH 9.1, 20 °C, as measured by the change in the helix-related CD signal. The CD data are plotted in this fashion to examine the kinetic order of reaction and to estimate the relative contributions from the different time-dependent processes. The decrease in unfolded protein corresponds to an increase in the helix-related CD signal, i.e., an increase in folded protein.

random structure relative to native or reoxidized bGH. Additionally, the CD signals (values of $[\theta]$) of all of the protein species examined were not dependent on protein concentration from ~2 to ~30 μM (data not shown), indicating the observed values of secondary structure are not attributable to any protein–protein interactions. These data indicate that reduction of the protein large-loop disulfide leads to a diminution of helical structure. Conversely, reoxidation of the protein leads to a recovery of helical structure. Taken together, these data have a number of implications. First, under the conditions examined, 4.5 M urea at pH 9.1, reduction and alkylation of the C-terminal short loop of the protein do little to alter the secondary structure of bGH, as expected given the observations of Bewley (1977). Second, reduction and modification of all sulfhydryls do not lead to complete loss of secondary structure. Third, the large loop of bGH plays an important, but not exclusive, role in maintaining the protein secondary structure. Finally, the data strongly suggest that failure of the large loop to re-form during reoxidation might be expected to substantially alter the helical content of the reoxidized molecule.

Kinetics of UV and CD Signal Changes, Tyrosine Burial, and Sulfhydryl Reoxidation. The time-dependent change in the 290-nm signal during reoxidation is presented in Figure 6. When the signal is plotted to represent the decrease in the initial state (reduced bGH), it is not log-linear. Only small changes in the kinetic time course are apparent for protein concentrations ranging from ~10 to ~30 μM. The curve for 10 μM protein was chosen for analysis and is approximated by the sum of two exponentials over about 95% of the total signal change. The fitted curve indicates two processes of equal amplitude with half-lives of about 30 min for the "fast" reaction and about 75 min for the "slow" reaction. An exact interpretation of the different rates and equal amplitudes is not evident at this time. However, in addition to the UV isosbestic band observed upon reoxidation (Figure 3A), the fact that the signal changes associated with refolding/reoxidation at 290 nm can be approximated by the sum of two first-order reactions rather than a single reaction suggests that, under the conditions of this experiment, there is more than one species involved in the time-dependent changes observed in the 290-nm signal.

The change in CD signal at 222 nm upon refolding and reoxidation is examined in Figure 7. Measurement was initiated ~15 s after mixing. The signal had two phases; the first phase, which represented ~65–75% of the signal change and was coincident with dilution from 6.0 M guanidine to 4.5 M urea, occurred too quickly for accurate measurement and was essentially complete in less than 2 min. The second phase was log-linear; it represented ~25–35% of the signal change and had a $t_{1/2}$ of ~48 min. We find this kinetic behavior to be representative of the CD signals observed during reoxidation of reduced protein across the range from ~10 to ~30 μM protein; no concentration-dependent change in the kinetics is observed. The kinetic behavior of the slow phase is characteristic of a first-order process. The slow phase in the CD signal was faster than reoxidation (Figure 8) and about equal in rate to the overall 290-nm signal change (Figure 6).

The degree of tyrosine exposure, expressed as a percentage of exposure at zero time, is plotted in Figure 8 as a function of time after the initiation of refolding/reoxidation. The kinetics of sequestering of the tyrosines away from bulk solvent or burial into the folded protein structure are complete in ~100 min. The decrease in exposure is not log-linear and can be approximated by the sum of two exponential processes. In a similar fashion to that observed for the 290-nm UV signal, there are only small changes in the kinetics of the reaction for protein concentrations ranging from ~10 to ~30 μM. Fitting to two exponentials for 10 μM protein indicated that the fast phase represented ~70% of the signal change with a half-life of ~8 min while the slow phase represented ~30% of the signal change with a half-life of ~24 min. The calculated curve coincided with the observed data over about 98% of the total signal change.

As with the raw absorbance data, it is not clear why the signal associated with sequestering the tyrosine residues from bulk solvent should be separable into two phases. The fact that there are only small changes in the kinetic behavior at different protein concentrations for both the UV and tyrosine exposure data suggests that the apparent multiexponential kinetics observed for each individual signal do not result from association events. It is likely that the 290-nm signal changes upon reoxidation, and tyrosine burial involve species with different conformational states.

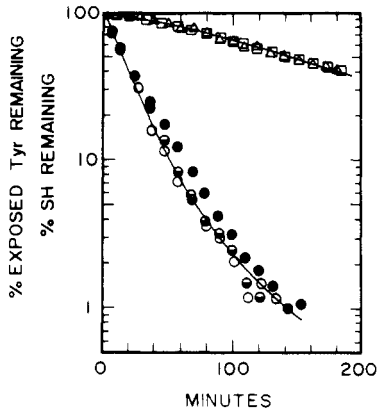


FIGURE 8: Time-dependent changes in the extent of tyrosine exposure and free sulfhydryl content during the reoxidation of bGH in air-saturated 4.5 M urea, 10 mM CHES, and 1 mM glycine, pH 9.1, 20 °C. (○) Reduced protein at 10 μM; (◒) reduced protein at 22.5 μM; (●) reduced protein at 32.5 μM. The tyrosine burial data were calculated as described in the text based on second-derivative data (see Figure 3B for representative data at 10 μM protein). The solid curve for Tyr burial is calculated for protein at 10 μM and represents the sum of to exponentials; the equation $y = 0.7e^{(-\ln 2/8)t} + 0.3e^{(-\ln 2/24)t}$ was used to generate the theoretical curve. Changes in free SH groups were determined by using 15 μM reduced pituitary bGH (□) or 15 μM reduced recombinant bGH (△). At each time point, an aliquot of sample was withdrawn and mixed with DTNB (0.5 mM final concentration) in buffered 4.5 M urea (see text) and allowed to stand for 25–30 min before measurement of the free thionitrobenzoate anion vs. a protein-free blank incubated for the same period of time.

The rate of disulfide formation, measured as free sulfhydryl content by DTNB titration (Ellman, 1958), during reoxidation is presented in Figure 8 for both pituitary and recombinant bGH. It is apparent that the reoxidation process for both is not log-linear and that it has an initial slow phase and a faster secondary phase. Investigation of the effects of protein concentration on the sulfhydryl titration kinetics of both the pituitary and recombinant proteins, from ~10 to ~30 μM protein, indicates only small effects, about a 10% reduction in the apparent reaction half-life with no change in the ordering of the slow and fast reaction phases. The initial slow phase and faster secondary phase indicate that there is a rate-limiting process(es) which must occur prior to complete reoxidation of the molecule. The biphasic kinetics indicate that there must be at least one kinetic intermediate present during the reoxidation process. The minimum sequential kinetic scheme required is

$$P_{red} \xrightarrow{slow} P' \xrightarrow{fast} P_{ox}$$

where P_{red} is the fully reduced species, P′ is the intermediate, and P_{ox} is the fully oxidized molecule. One obvious choice for an intermediate is a molecule with one disulfide formed and two free sulfhydryls. In order for the behavior of such an intermediate species to account for the kinetics, the two disulfides must be formed in a preferential order at different rates. That is, for reoxidation to correctly oxidized monomer, the reoxidation of one pair of sulfhydryls is obligatory prior to the reoxidation of the second pair. Additionally, the fact that the apparent half-life for the reoxidation process is longer than any of those of the spectral probes suggests that events leading to the spectral changes occur prior to the reoxidation of even the first pair of sulfhydryls.

Tryptic mapping studies were performed, as described by Hartman et al. (1986) for pituitary and recombinant bGH, to compare the fragmentation patterns of the native oxidized molecule and the reoxidized monomer. The fragment containing the large-loop disulfide was isolated and identified by protein sequence analysis; the peptide fragments containing the short-loop disulfide were tentatively identified by their chromatographic behavior under oxidizing and reducing conditions (P. A. Hartman, J. J. Dougherty, J. D. Stodola, and T. F. Holzman, unpublished results). Examination of chromatographic profiles of tryptic digests of the reoxidized monomer gave no indication of appreciable amounts of incorrectly paired disulfide fragments.

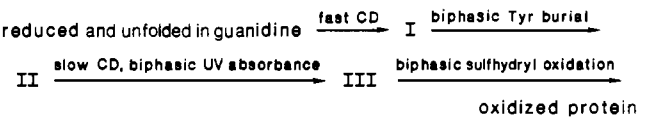
Finally, an examination was undertaken for other potential causes for the multiphasic reoxidation behavior of certain of the probes (UV, tyrosine burial, SH titration) and the differential time courses between each of the methods of probing the reoxidation kinetics. The kinetics of reoxidation of reduced pituitary and recombinant bGH were determined with and without the 50 °C heat-treatment step described under Methods. Samples without the heat-treatment step during reduction exhibited the same reoxidation kinetics when examined by each probe as samples subjected to heat treatment during reduction (data not shown). Thus, the multiphasic kinetic behavior of the UV probes and SH titration and the differential time response between the different probes did not appear to result from any artifacts of sample preparation.

Discussion

Folding of bGH as a Multistate Process. The equilibrium transition between folded and unfolded states has been studied extensively for a variety of proteins. In small proteins, like bGH, these transitions are most frequently characterized as being highly cooperative and two state in nature. The cooperativity of the transition and the two-state behavior at equilibrium are taken as evidence that intermediate folding states, should they exist, are unstable and not highly populated (Creighton, 1985). Alternatively, examination of folding behavior by spectral techniques and other methods including proteolytic clipping (Burgess et al., 1974) and antibody binding (Chavez & Sheraga, 1977) has implied that equilibrium folding intermediates may be a relatively common phenomena. However, on the basis of direct thermodynamic measurements, it has also been suggested that interpretation of the causal bases of such reports may be clouded by other phenomena, including the effects of preferential binding events in stabilizing folded or unfolded states (Creighton, 1985). Clear exceptions to the two-state folding process are exhibited by proteins with more than one domain which fold independently (Creighton, 1985).

Although there is no evidence that bGH contains more than one domain, we believe the current data, and previous data (Brems et al., 1985) strongly infer that the equilibrium folding behavior of the protein occurs via a multistate process. A more definitive argument can be made with direct calorimetric measurements of the protein, which are now under investigation. In a previous report (Brems et al., 1985), equilibrium denaturation data for bGH were used to propose a "framework" model for the folding of bGH. The data presented here for urea denaturation support this proposal. That is, the protein undergoes a concerted hierarchy of folding events which are characteristic of a framework process. In this process, the most stable structural elements are the regions of helical secondary structure. The secondary structure is the first to form during refolding and is the last to be lost during unfolding. The behavior of the chemically modified forms of the protein also indicates that alteration of the sulfhydryl groups can modify, in some cases dramatically, the character of the folding equilibria. Furthermore, the behavior of these modified proteins indicates that it is possible to alter selectively the denaturation process at one level of the folding hierarchy,

Scheme I



the structure responsible for the 290-nm signal, while leaving another level, the secondary structure responsible for the CD signal, relatively unaffected.

Kinetic Intermediates during Reoxidation of bGH. Within the time scale of the initial experimental observations, i.e., ~5 min after mixing at room temperature, all measurable events of protein folding are complete for the oxidized molecule (D. Brems, unpublished observation). Although the reduced molecule has not been characterized with respect to the fast-folding events, it is expected that these events are likely to represent the rapid formation of secondary structure, the protein framework. Thus, upon dilution from the high protein concentration and guanidine denaturant into a low or intermediate concentration of urea, the secondary structure of the diluted protein forms almost immediately. The formation of this structure is characterized by an immediate decrease (<1 min) in the 222-nm CD band (increase in helicity), which we estimate to be complete in less than 2 min (Figure 6).

After these initial very fast events lead to recovery of helical structure, the spectral and chemical probes reveal that there are at least three processes occurring during the slow phase of refolding/reoxidation. The first step involves the sequestering of protein Tyr groups. This process appears to occur first, prior to reoxidation and UV difference changes (alteration of Trp exposure), and is faster than the slow CD signal. Both Tyr burial and CD changes precede the actual chemical events of reoxidation (Figure 7). The ordering of the various probe signals is indicated in Scheme I.

The intermediate states listed are the minimum required to account for the signals observed from the various spectral and chemical probes.[2] It is an open question as to whether these probes are reflecting different aspects of processes involving discrete structural intermediates, for example, kinetic folding intermediates or an intermediate which leads to a species in which one pair of the sulfhydryls is reoxidized. In any case, the small effects of protein concentration cannot account for the large differences in the kinetic behavior of each of the different probes. The prime example of this is the kinetic time course of the UV absorbance vs. that for tyrosine burial; they exhibit substantially different kinetics and yet are calculated from exactly the same spectral data. The changes in each are sensitive to different kinetic events as the protein refolds and reoxidizes. The behavior of the signals associated with secondary structure (CD) and tertiary structure (disulfide formation) indicates that within the range of protein concentrations reported here there is little effect of protein concentration on the kinetics of reoxidation. Thus, the differential kinetics we observe upon comparing the different methods of measurement to each other cannot arise from small differences in protein concentrations between methods but rather reflect true differences in the kinetic responses for each method of measurement. We have also examined the reoxidation kinetics of reduced recombinant bGH, and data presented here (Figure 8) for the reoxidation as measured by SH titration are representative. The recombinant protein does not suffer from the reported heterogeneity of the pituitary protein and behaves like the pituitary protein during equilibrium denaturation (Brems et al., 1985). We find that reduced recombinant protein exhibits exactly the same kinetic behavior upon reoxidation, when examined by each spectral probe (T. F. Holzman and J. J. Dougherty, unpublished observations), as the pituitary protein described here. Therefore, it seems unlikely that the kinetic behavior observed for pituitary protein is related to sample inhomogeneity in the natural protein. Finally, the observed kinetic behavior did not appear to be an artifact of the heating step in the preparation of the reduced protein since the various probes of the kinetics of reoxidation behave the same whether or not the sample was heated during reduction.

The folding and reoxidation behavior of bGH present a unique opportunity to study the kinetically separated events of folding and reoxidation. It will be of general interest to establish the existence of any relationships between potential structural intermediates during the kinetic process of refolding/reoxidation and intermediates in the equilibrium folding of the molecule.

Poising the Conformation of bGH for Reoxidation. The extent and pathway of refolding of a protein will depend on the solvent conditions in which the refolding takes place. Creighton (1974a,b, 1977) has shown that for bovine pancreatic trypsin inhibitor, which has three disulfides in the native state, the conversion of reduced to oxidized protein proceeds through a series of intermediate species. Certain of these intermediate species are characterized by the existence of nonnative intramolecular pairing of protein sulfhydryl groups. Appropriate arrangement of solution conditions with mixtures of reducing and oxidizing agents leads to a kinetic pathway for sulfhydryl "shuffling" in which the molecules eventually attain sulfhydryl pairings identical with those in the native state. If the sulfhydryls of the reduced protein are prevented from undergoing a shuffling cascade by strongly oxidizing solvent conditions, reoxidation would be expected to give rise both to disulfide-paired intermolecular aggregates and to monomeric species in which the cysteines are partially or completely mispaired (Creighton, 1974a,b). On the basis of these observations, it would be expected that if a protein is locked into an incorrect tertiary structure, it should have little biological activity and would be expected to possess physical characteristics different from those of "correctly" oxidized protein.

In the case of a protein with sulfhydryl groups which do not require a shuffling cascade, it would be expected that correct folding and reoxidation would be favored by conditions in which the reduced protein has a structure resembling that of the oxidized protein. These conditions would poise the protein structure for reoxidation. The absence of fixed amounts of reducing and oxidizing agents should not present a problem as long as the solvent conditions lead to a poised tertiary structure. Ideally, the poised structure would differ from the structure of the oxidized molecule solely by the absence of correctly paired disulfide bonds. If solvent conditions are arranged so that reoxidation, at dilute protein concentrations, occurs from a poised state, the opportunity for correct disulfide pairing should be maximized.

In an attempt to provide conditions which would favor the production of correctly oxidized monomeric bGH, denaturant concentrations were chosen in which the reduced molecule was soluble and had a secondary structure close to that of the oxidized monomer. In other proteins, for example, ribonuclease (Hantgan et al., 1974; Galat et al., 1981) and bovine

[2] We wish the reader to note that states I–III are listed solely to distinguish the time courses of the various signals. These states are not meant to infer a specific number of kinetic folding intermediates.

pancreatic trypsin inhibitor (Creighton, 1974a,b), correct disulfide pairing occurs only if the reduced protein is incubated with certain mixtures of oxidizing and reducing agents. For ribonuclease at least, the secondary structure of the protein is relatively unstable after reduction and alkylation (Harrington & Schellman, 1956; Harrington & Sela, 1959). For these proteins, it is clear that in strongly oxidizing conditions, species with incorrect disulfide pairing will predominate (Creighton, 1974a,b; Hantgan et al., 1974; Galat et al., 1981). Thus, for these molecules, it is unlikely that a set of empirical solution conditions can be arrived at which selectively stabilize the secondary structure and permit poising the sulfhydryl groups for correct reoxidation. In other proteins, which either possess fewer disulfide pairs or have differentially stable secondary structures in the reduced state, it may be possible to poise the conformation for correct sulfhydryl reoxidation. Data presented here suggest that bGH fulfills these criteria. It appears to represent a case in which the framework secondary structure of the reduced protein can be preferentially formed so that the protein exists in a poised state which resembles that of the native oxidized protein.

An example of another protein which appears to have a framework secondary structure like bGH is found in α-lactalbumin. Folding studies by Kuwajima et al. (1975, 1976, 1977), Kita et al. (1976), and Nozaka et al. (1978) demonstrate that α-lactalbumin behaves similarly to bGH. The equilibrium unfolding transition shows noncoincident changes in the CD signal at several wavelengths; aromatic signals show an unfolding transition at lower denaturant concentration than that observed for unfolding of the protein secondary structure. A folding intermediate has been identified that exhibits a secondary structure similar to, though not identical with, native structure, but at the same time the intermediate does not exhibit tertiary structure. The intermediate is in rapid equilibrium with the unfolded state but in a much slower equilibrium with the native state. The intermediate is present even when the protein disulfides are reduced.

The conserved nature of growth hormone and prolactin protein sequences has been noted [for example, see Paladini et al. (1983)]. On the basis of the conserved sequences in these molecules, the folding behavior of bGH may be reflective of these other proteins, and the reoxidation behavior of bGH may be characteristic of mammalian growth hormones in particular.

We have suggested above that the absence of a 290-nm difference signal for reduced bGH and the small changes observed for the fully modified forms may be related to the ionization state of the sulfhydryls and the ability of the protein structure to fold with alkyl groups attached to the protein cysteine residues. Whether or not this is the case, it is clear that the environment of the protein Trp residue in reduced bGH, TSCAM bGH, and TSSM bGH is different than in RCAM-1 and oxidized bGH. The size-exclusion HPLC profile of the reoxidized protein (Figure 4) indicates that the yield of oxidized monomer is not drastically reduced by the formation of high concentrations of polymerized protein. Indeed, the higher molecular weight forms seen in the HPLC analysis correspond to material of *limited* size with molecular weights appropriate for limited oligomeric aggregates of bGH. This reoxidation to (primarily) monomer is consistent with the protein framework providing structural direction so that various molecular features of the reduced molecule are brought into juxtaposition to permit formation of the correct disulfide bonds. The effects of protein concentration on the formation of aggregated species and the pH dependence of these processes are under investigation.

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Model of Fibronectin Tertiary Structure Based on Studies of Interactions between Fragments†

Gene A. Homandberg*,‡ and John W. Erickson‡

Department of Medicine, Hemostasis Research Laboratory, University of Wisconsin Medical School, Milwaukee Clinical Campus, Mount Sinai Medical Center, Milwaukee, Wisconsin 53233

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ABSTRACT: Human plasma fibronectin aggregates in solution and is thought to form fibrils on cell surfaces, perhaps by self-associating and by interacting with other components such as proteoglycans. We have localized the self-association domains by testing the ability of various fragments of fibronectin to interact with each other. Complexation between fluorescamine-labeled fragments and unlabeled fragments or whole molecules was assessed by gel filtration high-performance liquid chromatography. The fragments studied included nonoverlapping fragments that are situated on the fibronectin polypeptide chain in the following order, beginning from the amino terminus: the 29-, 50-, 120-, 35-, and 25-kDa fragments, as well as multiple-domain fragments of 72 kDa containing the 29- and 50-kDa segments, a fragment of 150 kDa containing the 120- and 35-kDa segment, a fragment of 190 kDa containing the 120- and 35-kDa segments, a fragment of 190 kDa containing the 50-, 150-, and 25-kDa segments, and a 45-kDa fragment containing the 35-kDa segment. The amino-terminal 29-kDa fragment bound to the carboxyl-terminal heparin-binding (Hep II) 35-kDa fragment as well as the 150- and 190-kDa fragments that contain the 35-kDa segment. On the other hand, carboxyl-terminal 35- and 45-kDa Hep II containing fragments bound to each other as well as to amino-terminal 29- and 72-kDa fragments and to the 190-kDa fragment. Further, the 25-kDa carboxyl-terminal fibrin-binding fragment bound the 190-kDa fragment, the only fragment containing the 25-kDa segment. We conclude that fibronectin folds in such a way as to allow interaction of the amino-terminal 29-kDa region with the carboxyl-terminal 35-kDa Hep II domains in a fashion that may account for the polymerization of fibronectin. Also, interaction of the carboxyl-terminal 35-kDa heparin-binding and 25-kDa fibrin-binding segments with their like counterparts on adjacent subunits may account for the noncovalent monomer–monomer interactions in the native structure.

Fibronectin is an adhesive glycoprotein consisting of two subunits of 215–250 kDa, which binds a variety of ligands, including fibrin, fibrinogen, collagen, heparin, proteoglycans, actin, and DNA, and cells such as fibroblasts, monocytes, macrophages, and *Staphylococcus aureus* [see reviews by Mosesson and Amrani (1980), Ruoslahti et al. (1981), Hynes and Yamada, (1982), Furcht (1983), Yamada (1983), and Akiyama and Yamada (1983)]. The domains responsible for most of these activities have been identified as proteolytic fragments [see reviews by Mosesson and Amrani (1980), Ruoslahti et al. (1981), Hynes and Yamada (1982), Furcht (1983), Yamada (1983), and Akiyama and Yamada (1983)] and are situated on each subunit of fibronectin in the order shown in Figure 1. There are two sulfhydryls per subunit that are about 30 and 70 kD from the carboxyl terminus (Wagner & Hynes, 1980; Sekiguchi et al., 1981) and that have been located in the amino acid sequence of bovine fibronectin (Petersen & Skorstengaard, 1985).

Electron microscopy of fibronectin molecules deposited on mica from solutions containing 30–40% glycerol suggest an extended V-shaped structure (Engel et al., 1981; Erickson et al., 1981; Erickson & Carrell, 1983) or a rather compact or loosely folded molecule with dimensions varying from 16 × 9 nm to 51 × 32 nm [Koteliansky et al., 1980; Price et al.,

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* Author to whom correspondence should be addressed.
‡ Present address: Physical Biochemistry Lab, Abbott Laboratories, Abbott Park, IL 60064.

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