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## Short Communication

# Folding of Bovine Growth Hormone Is Consistent With the Molten Globule Hypothesis

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***ABSTRACT*** **Previous results from equilibrium and kinetic studies of the folding of bovine growth hormone (bGH) have demonstrated that bGH does not follow a simple two-step folding mechanism. These results are summarized and interpreted according to the "molten globule" model. The molten globule state of bGH is characterized as a folding intermediate which is largely α-helical, retains a compact hydrodynamic radius, has packing of the aromatic side chains that is similar to the unfolded state, and possesses a solvent-exposed hydrophobic surface along helix 106–127 that readily leads to association.**

**Key words: folding intermediate, molten globule state, protein folding**

## INTRODUCTION

Several models have been proposed to explain how proteins fold. One model that is empirically consistent is the framework model.[1] In this model, folding is described as a sequential process that begins with the formation of secondary structure followed by collapse to native tertiary structure. Recently, Ptitsyn[2] has added to the framework model and suggested that the experimental evidence points to the existence of a "molten globule" state, which is a compact intermediate state with a high degree of secondary structure but contains fluctuating tertiary structure. The molten globule state appears to be a state that can be populated kinetically and possesses sufficient stability to exist at equilibrium. The folding of carbonic anhydrase B[3] and α-lactalbumin[4,5] have been shown to be consistent with the molten globule hypothesis. In this communication, we discuss results from previously published folding studies of bovine growth hormone (bGH) in light of the molten globule model.

## MATERIALS AND METHODS

### Materials

Recombinant-DNA derived bGH was obtained from *Escherichia coli* carrying a temperature-sensitive runaway plasmid into which had been inserted the bGH gene sequence and a tryptophan promoter system.[6] The fermentation, isolation, and purification of bGH was done using the procedure described by Evans and Knuth.[7] Guanidine hydrochloride (Gdn HCl) was ultrapure from Schwarz/Mann. Other reagents were analytical grade.

### Methods

#### *Sample preparation*

Solutions for UV absorbance, fluorescence quenching, and circular dichroism were prepared in 50 mM ammonium bicarbonate buffer (pH 8.5). For size-exclusion HPLC, the buffer was 10 mM tris(hydroxymethyl)-aminomethane (Tris) (pH 8.0). All measurements were made at room temperature (≈23°C).

#### *Spectral measurements*

Absorbance measurements were taken at 290 nm using a Cary 219 spectrometer,[8] CD measurements were taken at 222 nm using a Jasco J-500C spectropolarimeter,[9] and fluorescence intensity measurements were made with an SLM 8000C spectrofluorometer using an excitation wavelength of 295 nm and an emission wavelength of 350 nm.[10]

#### *Size-exclusion HPLC*

As previously described,[9] a DuPont Zorbax GF-250 column was used with a mobile phase obtained by dynamic mixing of two reservoirs: one containing 6 M Gdn HCl, 10 mM Tris, and 1 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0) and the other containing the same constituents without Gdn HCl. The mobile phase was pumped at 1 ml/min with a Perkin-Elmer series 4 pump and the eluent was detected using UV absorbance at 220 nm.

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**TABLE I. Comparison of Native, Intermediate, and Unfolded States of Bovine Growth Hormone**

| | Native* | Intermediate† | Unfolded‡ | Units | Reference |
|---|---|---|---|---|---|
| $\epsilon_{290\ nm}$ | 9.8 | 7.4 | 7.4 | $10^3\ M^{-1} \cdot cm^{-1}$ | 3 |
| $[\theta]_{222\ nm}$ | −15.0 | −7.5 | −1.0 | $10^3\ deg \cdot cm^2 \cdot dmol^{-1}$ | 3,6 |
| Molecular radius | 1.8 | 2.6 | 3.7 | nm | 3,6 |
| $k_q$ (acrylamide) | 1.1 | 1.4 | 2.4 | $10^9\ M^{-1} \cdot sec^{-1}$ | 8 |

*Buffer.
†3.7 M Gdn HCl.
‡6.0 M Gdn HCl.

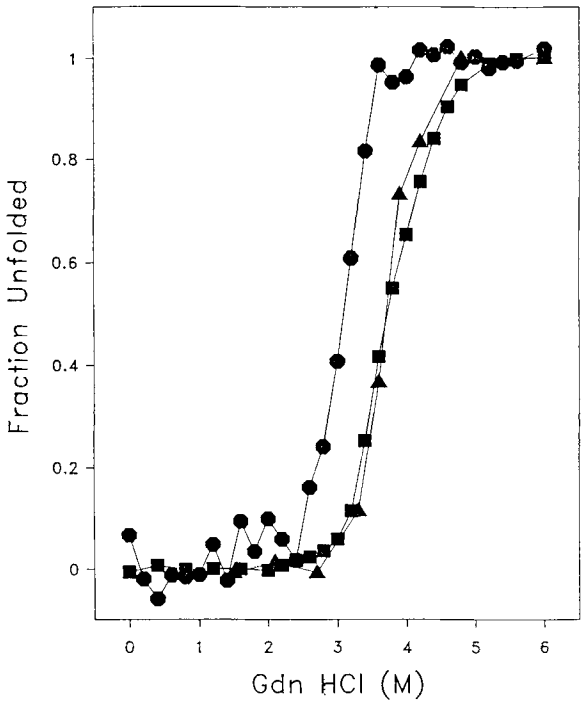


Fig. 1. Equilibrium unfolding curves for bGH in Gdn HCl monitored by absorption ($\epsilon_{290\ nm}$, ●), circular dichroism ($[\theta]_{222\ nm}$, ■), and molecular size (size-exclusion HPLC, ▲). Absorption results are independent of protein concentration and were obtained with 23 μM bGH solutions.[3] CD and HPLC results were obtained at dilute protein concentrations[6] where association is minimized: 1.8 and 4.5 μM bGH solutions, respectively.

## RESULTS AND DISCUSSION

Equilibrium folding studies of bGH have identified stable intermediates that are monomeric and self-associated.[8,9,11–13] The dissociation constant for the self-associated intermediate[12] is about 10 μM, and thus the monomeric intermediate can be studied at low protein concentration (less than 2 μM) where association is insignificant and the self-associated intermediate can be studied at higher protein concentrations where association is considerable. The equilibrium denaturation of bGH at low protein concentration has been studied by methods that measure a) the extent of compactness[9,12] (size-exclusion HPLC), b) secondary structure[8,9,11,12] (far-ultraviolet circular dichroism, far-UV CD), and c) solvent exposure or degree of packing of the aromatic side chains[8] (ultraviolet absorbance). The results of such measurements are illustrated in Figure 1 and demonstrate that the denaturation transitions detected by the three methods are not coincident which is evidence for the existence of a multistate folding process.[1] Comparison of the transitions shows that the packing of the aromatic side chains (1 tryptophan and 6 tyrosines) is disrupted initially and the degree of compactness and secondary structure denature similarly but at higher concentrations of denaturant.

Further investigations into the folding of bGH tertiary structure have been pursued by tryptophan fluorescence quenching measurements.[10] Different conformations of bGH were studied using various chemical quenchers (iodide, acrylamide, and trichloroethanol). The quenching of tryptophan fluorescence in the monomeric intermediate state by acrylamide (a polar but nonionic quencher) is slightly greater than the native state but less than the denatured state. These results are in agreement with results reported by Ptitsyn[2] for acrylamide quenching of bovine carbonic anhydrase B.

The equilibrium unfolding results for bGH (Fig. 1) are consistent with the existence of a stable intermediate that has a high content of α-helical secondary structure, a compact hydrodynamic radius similar to the native state, and a packing of the aromatic side chains that is more flexible than the native state, that is, more like the unfolded state. These properties of the bGH intermediate are those described[2] for a molten globule state. The molecular properties of the bGH molten globule state can be estimated by measuring the properties of bGH solutions containing 3.7 M Gdn HCl (Table I). Under these conditions, the molten globule state is highly populated, but the amount of native and unfolded protein present has not been determined so the results given in Table I for the molten globule state include contributions from all conformational states present in 3.7 M Gdn HCl solutions.

Kinetic refolding studies[14] at dilute protein concentration have shown that the rate detected by far-UV CD is faster than the rate detected by UV absorbance. The faster kinetics obtained by far-UV CD

indicate the existence of a kinetic folding intermediate with appreciable amounts of α-helix. These results are also consistent with the molten globule hypothesis.

The molten globule state has been characterized by Ptitsyn[2] as having a more hydrophobic surface than the native state and hence may be important for membrane penetration of proteins. The associated folding intermediate of bGH is an aggregated form of the molten globule state.[9,11–13] The hydrophobic surface of the third helix in bGH (residues 106–127) forms an interactive surface that stabilizes the association through intermolecular hydrophobic interactions.[12] Since this specific intermolecular interaction only exists in partially denaturing conditions, the hydrophobic surface of the third helix must be more exposed to solvent in the bGH molten globule state compared to its solvent exposure in native bGH.

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