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Research Paper

Non-native Intermediate Conformational States of Human Growth Hormone in the Presence of Organic Solvents

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Purpose. Manufacturing processes expose protein pharmaceuticals to organic solvents that may perturb the native folded state, increasing the potential for irreversible aggregation or surface adsorption. The aim of this study was to characterize the conformational states of human growth hormone (hGH) in aqueous ethanolic solutions.
Methods. The higher order structure of hGH was investigated using far- and near-UV circular dichroism (CD) and fluorescence spectroscopy as orthogonal techniques, and the hydrodynamic size was monitored using dynamic light scattering.
Results. CD data suggested that the secondary structure of hGH remained unchanged up to 50% (v/v) ethanol, but the tertiary structure was perturbed at >20% ethanol. Fluorescence anisotropy, however, showed that the mobility of the buried Trp residue was restricted even at 30% ethanol, suggesting a differently packed structural core in 30% ethanol relative to the native structure. Consistent with this result, thermal unfolding of hGH in 30% ethanol was more facile compared to that in 0% and 20% ethanol. At >40% ethanol, fluorescence data were consistent with increased solvent exposure of the tryptophan.
Conclusions. The results point to progressive unfolding of hGH that increases solvent exposure of the hydrophobic core as a function of ethanol concentration and suggest that non-native intermediate states are populated in 30–60% ethanol.

KEY WORDS: circular dichroism; fluorescence anisotropy; light scattering; protein folding; protein structure; solvent effects.

INTRODUCTION

The potential of protein pharmaceuticals to undergo irreversible, noncovalent aggregation and the strategies to minimize such degradation are important considerations during development and manufacture of protein pharmaceuticals (1,2). It is generally believed that these aggregation processes are driven by non-native conformational states with an increased surface exposure of hydrophobic groups, which also increases their propensity to adsorb to hydrophobic surfaces (3–5). Water-miscible organic solvents such as ethanol, acetonitrile, and *n*-propanol are often used during purification or subsequent manufacturing operations, and the exposure of pharmaceutical proteins to these solvents has the potential to perturb the native folded state of the molecule and stabilize non-native conformational states that are more prone to aggregation and surface adsorption (6–8). Manufacturing operations under conditions that promote non-native states may impact yield, quality, or both and may also impact long-term stability or immunogenicity (9) of a biotherapeutic. The use of organic solvents in protein pharmaceutical manufacture is not exclusive to purification processes. For example, microsphere technologies for depot drug delivery expose protein therapeutics to methylene chloride and ethyl acetate (10). Additionally, benzyl alcohol, a commonly used antimicrobial agent in biopharmaceuticals, has also been shown to promote protein aggregation in some cases (11–13).

As part of an on-going effort to understand the impact of organic solvents on structural properties of therapeutic proteins, and how these conformational changes relate to their chemical and physical stability, we have examined human growth hormone (hGH) in aqueous ethanolic solutions. hGH is an endogenous protein that stimulates cell growth and other metabolic processes and several injectable formulations of this protein are approved for a variety of growth promotion and replacement therapy indications (14). A predominantly α-helical single-domain globular protein with a molecular weight of ~22 kDa, the major structural motif of hGH is a four-helix bundle with an unusual up-up-down-down topology, as determined by X-ray crystallography data of the hormone complexed with its extracellular receptor (Fig. 1A) (15). There are two disulfide bonds, one connecting the long loop between helices 1 and 2 to helix 4 (C53-C165) and the second forming a much smaller loop at the C-terminus (C182-C189). A single tryptophan buried within the hydrophobic core and almost completely solvent-shielded (Fig. 1B) serves as a convenient intrinsic fluorescence probe for structural studies.

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ABBREVIATIONS: CD, circular dichroism; hGH, human growth hormone; TFE, trifluoroethanol; UV, ultraviolet.

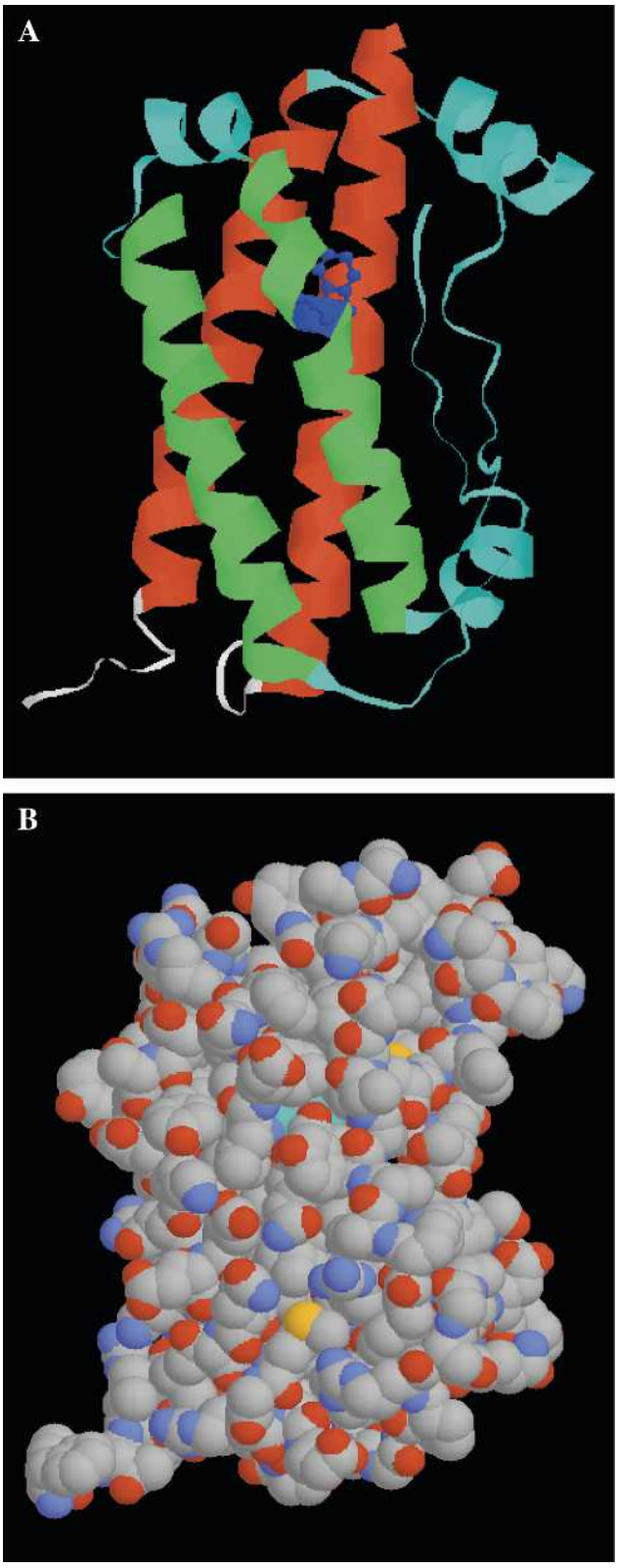

Fig. 1. (A) An illustration of hGH folding motif, based on the crystal structure (15) depicting the arrangement of helices. Helices 1 and 4 are in red, helices 2 and 3 are in green, and the connecting loops are shown in cyan. The side chain of the single Trp residue in the hydrophobic core of the protein is shown in blue. (B) Space filling representation based on the same structure. The single Trp residue within the hydrophobic core is shown in cyan. The figures were generated using *Rasmol* Version 2.6.

The folding pathway of both bovine and human growth hormones has been shown to contain intermediate states that are prone to aggregation (3,16–20). Such intermediate states can result in irreversible precipitation when hGH is refolded at high protein concentration. Thus, hGH represents a pharmaceutically relevant model to evaluate the impact of processing and solution conditions on higher order structure, to examine the extent to which intermediate conformational states are populated in the presence of organic solvents, and to characterize these species. To understand the retention behavior of hGH in reversed-phase chromatography, the effect of *n*-propanol and acetonitrile on the conformation of hGH in aqueous solutions have been studied previously (6). These studies have concluded that at >30% (v/v) *n*-propanol or >40% acetonitrile, a molten globule state retaining much of the secondary structure but perturbed tertiary structure is produced. These observations are consistent with the tendency of hGH to retain its secondary structure under a variety of denaturing conditions (3,18).

The current study aims to characterize the conformational states of hGH in the presence of increasing concentrations of ethanol and in particular highlights the population of conformational states with a differently packed hydrophobic core and a progressive unfolding of hGH that increases solvent exposure of the hydrophobic core as the concentration of ethanol is increased. The conformational properties of hGH were evaluated in aqueous solutions as a function of ethanol concentration (10–60% v/v), using far- and near-UV circular dichroism to probe secondary and tertiary structure, respectively. In addition, steady-state fluorescence emission and fluorescence anisotropy were used as orthogonal probes of the microenvironment of the single Trp residue in the hydrophobic core. To further characterize the stability of conformational states populated under these conditions, temperature dependent fluorescence emission was used to monitor thermal unfolding of the molecule as a function of ethanol concentration. The relatively high sensitivity of fluorescence measurement allowed the thermal unfolding experiments to be carried out at low concentrations (0.05 mg/ml), thus avoiding any complications due to temperature dependent aggregation of the protein at higher concentrations. The hydrodynamic radius of hGH under these solvent conditions was monitored by dynamic light scattering.

MATERIALS AND METHODS

Materials

Recombinant human growth hormone was from Eli Lilly and Company (Indianapolis, IN, USA). Sodium phosphate, ammonium bicarbonate, and ethanol were obtained from standard sources. *N*-Acetyl tryptophan-amide was obtained from Sigma Chemical Company (St. Louis, MO, USA). Deionized water was used in making all solutions.

Circular Dichroism Measurements

Far- and near-UV CD spectra were measured on an Aviv Circular Dichroism Spectrometer Model 62A DS (Lakewood, NJ, USA). The measurements were made in an aqueous buffer (pH 7 ± 0.5) containing 0.24 mg/ml sodium phosphate, 15 mg/ml ammonium bicarbonate, and varying concentrations

of ethanol (10–60% v/v). The final concentrations of hGH were 0.2 mg/ml and 1 mg/ml, for far- and near-UV measurements, respectively. The protein concentrations were determined by measuring the absorbance at 278 nm and using an extinction coefficient of 0.86 $mg^{-1}ml\ cm^{-1}$ (17). Far- and near-UV CD spectra were collected over the wavelength ranges 198–250 and 250–350 nm, respectively. The spectra were averaged over 3 scans, and the data at each wavelength was averaged for 3 s. The CD data were converted to mean residue ellipticity (MRE) units, using a mean residue weight of 116 (18).

Fluorescence Emission and Anisotropy Measurements

Fluorescence measurements were made on a Jobin Yvon Horiba Fluorolog-3 Fluorometer (Instruments S. A. Inc., Edison, NJ, USA), with a Peltier temperature control unit. The solution conditions were similar to those of CD measurements with the exception of hGH concentration, which was 0.05–0.1 mg/ml for fluorescence measurements. Emission spectra were collected in the wavelength range 305–450 nm, with an excitation wavelength of 295 nm. Data were collected at 1-nm increments, with an integration time of 0.1 s and a slit width of 2 nm. Fluorescence anisotropy data were collected in the wavelength range 300–350 nm at 5-nm increments, with an integration time of 1 s and a slit width of 5 nm. For thermal unfolding experiments, emission spectra were collected in 2°C increments from 20°C to 90°C, at a protein concentration of 0.05 mg/ml and ethanol concentration of 0%, 20%, and 30% (v/v). The samples were allowed to equilibrate for 2 min at each temperature.

Dynamic Light Scattering

The hydrodynamic diameter of hGH (1 mg/ml) in aqueous solutions as a function of ethanol concentration was determined at 25°C by measuring the autocorrelation function at 90° scattering angle on a Brookhaven Instruments Corporation (Holtsville, NY, USA) laser light scattering instrument. Samples were filtered through 0.22-μm, 25-mm low protein binding Millex GV durapore membrane filters. The mean hydrodynamic diameter based on weight distribution, assuming equivalent density for all species, was computed from the autocorrelation function using non-negatively constrained least square analysis. Four separate measurements were made to derive average and standard deviation results. The data were corrected for changes in the solvent viscosity as a function of ethanol concentration. The viscosities of water-ethanol mixtures were obtained from the *Handbook of Chemistry and Physics* (21) and calculated, where necessary, by linear interpolation.

RESULTS AND DISCUSSION

Secondary and Tertiary Structure of hGH as a Function of Ethanol Concentration: CD Spectroscopy

The secondary structure of hGH as a function of ethanol concentration was probed by far-UV CD. The circular dichroism spectra were characterized by two bands with strong negative ellipticity at 222 and 208 nm, as expected for a predominantly α-helical protein. The spectra were virtually independent of ethanol concentration up to 50% ethanol (Fig. 2A), indicating that the secondary structure was not significantly disrupted by ethanol. In contrast, the tertiary structure of hGH as reflected by the near-UV CD signature was significantly modified at ethanol concentrations >20%. Figure 2B compares the spectra at 0% and 50% ethanol. The most significant changes in the ellipticity occurred at ~295 nm, attributable to the Trp residue (22). Other regions of the spectra were characterized by minimal changes as a function of ethanol concentration. The changes in the ellipticity at 295 nm as a function of ethanol concentration are shown in the inset of Fig. 2B. It can be seen that significant changes in the ellipticity at 295 nm begin to occur at >20% (v/v) ethanol. The far- and near-UV CD data together suggest that addition of ethanol to aqueous solutions of hGH causes perturbations to the tertiary structure without significantly altering the secondary structure. The conformational states present at ethanol concentrations >20% could therefore be described as molten globules (23).

Fluorescence Emission and Anisotropy of hGH as a Function of Ethanol Concentration

As an orthogonal approach to CD, the tertiary environment of the buried tryptophan was probed by following fluo-

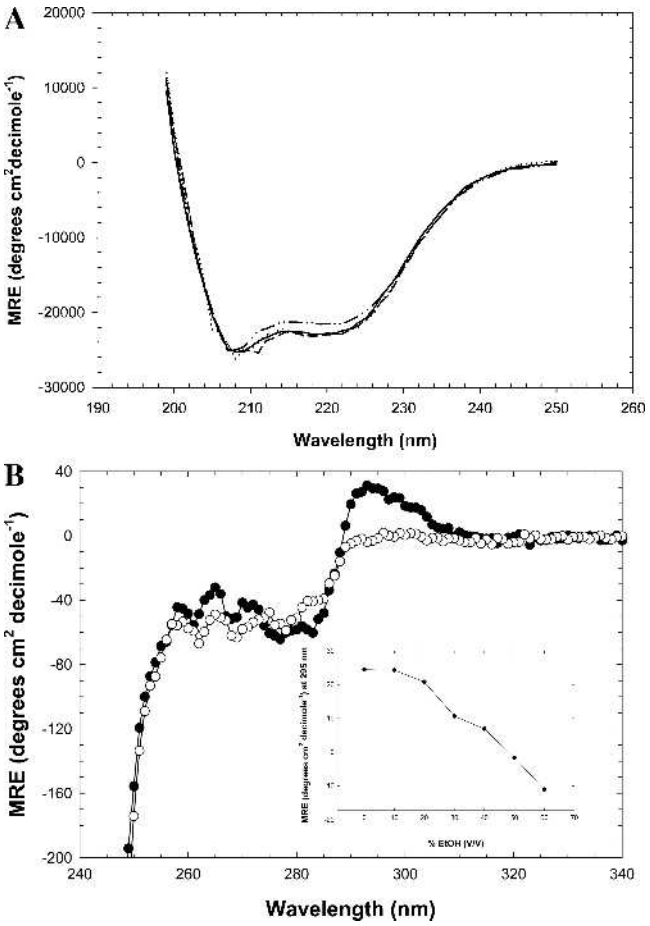

Fig. 2. (A) Far-UV CD spectra of hGH in 0% (—), 20% (····), 30% (– –), and 50% (– ·· –) ethanol. (B) Near-UV CD spectra of hGH in 0% (●) and 50% (○) ethanol. The changes in the near-UV ellipticity of hGH at 295 nm as a function of ethanol concentration are shown as an inset in (B).

rescence emission characteristics and fluorescence anisotropy of hGH as a function of ethanol concentration. The fluorescence emission maxima of hGH as a function of ethanol concentration are plotted in Fig. 3. As a control for a fully solvent exposed Trp, the emission maxima for *N*-acetyl tryptophanamide under corresponding conditions were also examined. In pure aqueous solutions, the emission maximum for hGH is significantly blue-shifted relative to *N*-acetyl tryptophanamide, which is consistent with a solvent-shielded environment for the single Trp residue in hGH. As the ethanol concentration was increased, minimal changes were observed in the emission maximum up to 30% ethanol. Above 30% ethanol, the emission maximum was red shifted in the direction of a fully exposed Trp. These data suggest that at >30% ethanol, the molecule begins to unfold to increase the solvent accessibility of the buried Trp residue. Comparisons with *N*-acetyltryptophan-amide also helped establish that the changes in Trp emission were not a simple consequence of changes in solvent polarity, but reflect changes in the solvent exposure of the Trp residue.

The fluorescence anisotropy of hGH as a function of ethanol concentration is plotted in Fig. 4A, along with the changes in emission maxima, for comparison. Consistent with fluorescence emission data, a significant drop in fluorescence anisotropy begins to occur at ethanol concentrations greater than 30% (v/v), indicative of changes in fluorescence decay time and/or effective rotational correlation time of the Trp residue. It is important to note, however, that the solvent viscosity is expected to change as a function of ethanol concentration. These changes in viscosity may impact the rotational correlation time of the entire molecule, and therefore the observed anisotropy may not represent changes in the mobility of the Trp side-chain. To take into account the changes in solvent viscosity, the reciprocal of anisotropy was plotted as a function T/η (Fig. 4B). According to the Perrin equation (22),

$$r_0/r = (1 + \tau/\phi) \quad (1)$$

the steady-state anisotropy r is a function of r_0, which is the limiting or intrinsic anisotropy, the rotational correlation time ϕ, and the fluorescence lifetime τ. The rotational correlation time of a sphere may be described by Stokes-Einstein equation as

$$\phi = V\eta/RT \quad (2)$$

where V is the hydrated molecular volume, η is the viscosity of the solution, R is the gas constant, and T is the absolute temperature. Based on Eqs. (1) and (2), a plot of 1/r vs. T/η is expected to be linear. A nonlinear plot indicates the presence of more than one rotational mode. It is clearly seen from Fig. 4B, that the viscosity-accounted anisotropy is fairly constant up to 30% ethanol begins to decrease between 30% and 40% ethanol and is significantly affected above 40% ethanol. The linearity of the plot between 0% and 30% also indicates that the anisotropy represents a single rotational mode, reflecting the rotational correlation time of the entire molecule. Nonlinear behavior beyond 30% ethanol likely reflects the significant local mobility of the Trp side chain, consistent with the increased solvent exposure shown by the fluorescence emission data. The overall rotational correlation time of hGH is expected to be largely unchanged as a function of ethanol concentration, based on hydrodynamic measurements using dynamic light scattering (see later discussion). Therefore, the changes in the fluorescence anisotropy likely reflect the local mobility of the Trp side-chain.

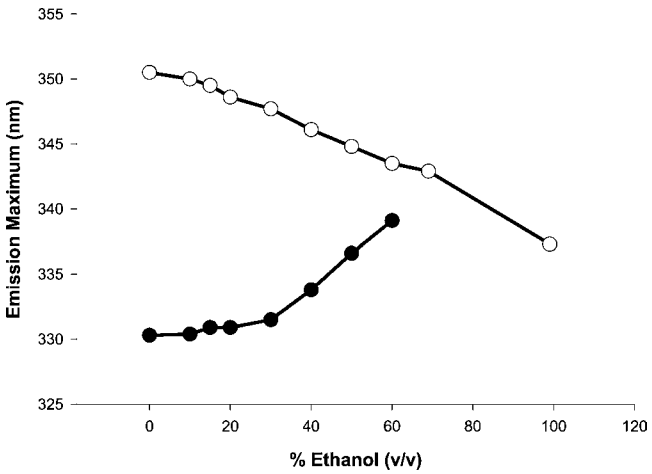

Fig. 3. Fluorescence emission maxima of hGH as a function of ethanol concentration (●). The fluorescence emission maxima for *N*-acetyl-tryptophan-amide are also plotted (○) as a control for a fully solvent exposed Trp residue.

Conformational State of hGH in 30% Ethanol

Comparing CD and fluorescence data reveals interesting characteristics of the conformation of hGH in 30% ethanol. At this concentration of ethanol, although near-UV CD was characterized by a significantly lower ellipticity at 295 nm, suggesting an altered chiral environment of the buried tryptophan, fluorescence anisotropy indicated that the mobility of the single tryptophan within the hydrophobic core was significantly restricted. Fluorescence emission data under these conditions further suggested that the Trp residue was also significantly solvent shielded. Thus, the conformation of hGH in 30% ethanol likely represents a differently packed hydrophobic core, relative to the native structure.

To further investigate the differences in the structure of hGH in 0%, 20%, and 30% ethanol, thermal unfolding of the molecule was followed under these conditions. The denaturation temperature for hGH at neutral pH has been shown to be concentration-dependent and thermal unfolding has been shown to be reversible only at low (0.2 mg/ml) concentrations (3,20). At this concentration, hGH has a denaturation temperature of ~84°C. In this study, thermal unfolding was followed at very low (0.05 mg/ml) concentrations, taking advantage of the inherently high sensitivity of fluorescence emission. In studying thermal unfolding by fluorescence, it is important to use a parameter that is linearly related to the fraction conversion from native to denatured state, and fluorescence intensity at a fixed wavelength is one such parameter (24). Significant temperature dependence of intrinsic fluorescence due to collisional quenching makes accurate measurement of denaturation temperatures difficult. In this study, the temperature dependence of fluorescence intensity at λ_{max} was used to discern qualitative differences in thermal unfolding at 0%, 20%, and 30% ethanol. The fluorescence emission spectra were collected as a function of temperature from 20°C to 80°C and the spectra at 20°C and 80°C are shown in Fig. 5

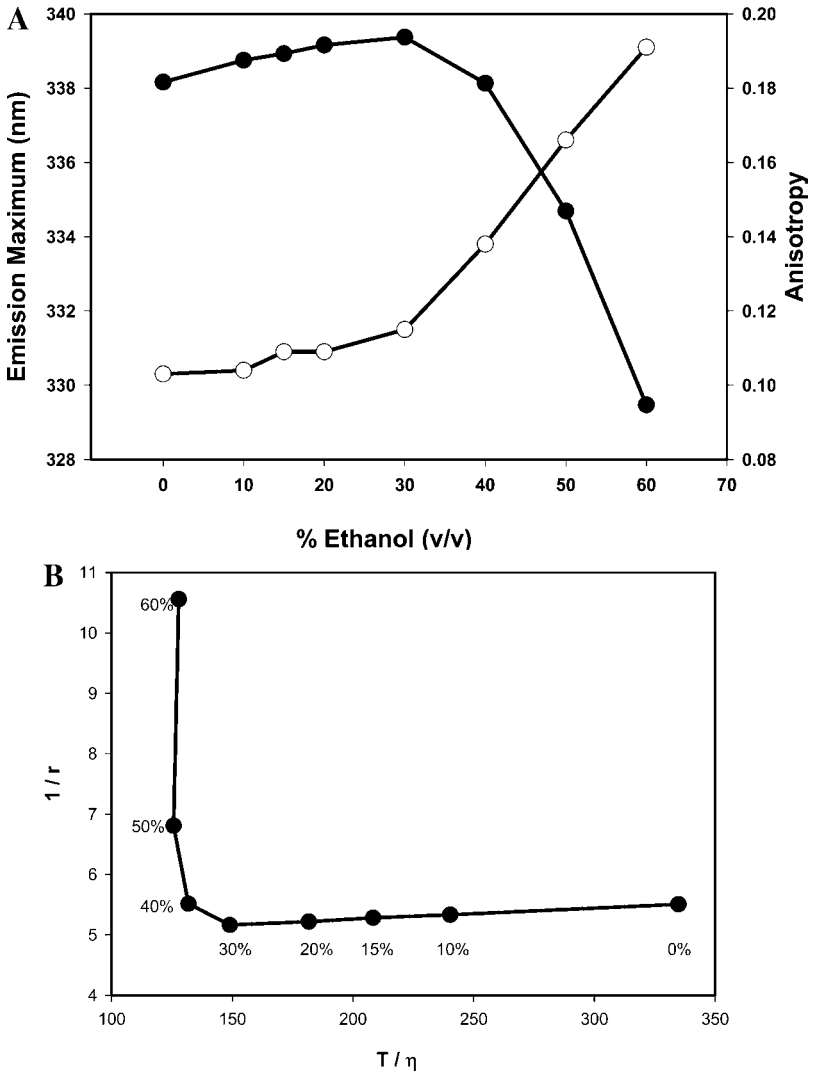

Fig. 4. (A) Steady-state fluorescence anisotropy of hGH as a function of ethanol concentration (●). The fluorescence emission data (○) is also shown for comparison. (B) Reciprocal of steady-state fluorescence anisotropy plotted as a function of (T/η), where T is absolute temperature and η is solvent viscosity.

(A–C). In all cases, a significant decrease in fluorescence intensity was seen due to the combined effects of thermal unfolding and collisional quenching. At 0% and 20% ethanol, there was no significant change in the emission maximum (λ_{max}) as a function of temperature. At 30% ethanol, there was a red shift from 331 nm at 20°C to 340 nm at 80°C, consistent with thermally induced unfolding that increased solvent exposure of the tryptophan. The relative fluorescence intensity at the λ_{max}, defined as Fluorescence Intensity at T°C/Fluorescence Intensity at 20°C, is plotted as a function of temperature in Fig. 6. A distinct unfolding transition was not seen at 0% or 20% ethanol, presumably because the T_m was at the edge of the temperature range examined and also because the changes due to denaturation are smaller compared to the steep temperature dependence. However, it is apparent that while the temperature-dependence of fluorescence was virtually superimposable at 0% and 20% ethanol, in 30% ethanol, the fluorescence emission decreased more steeply above approximately 55°C. These data are consistent with increased solvent exposure and solvent-induced quenching at greater than 55°C in 30% ethanol. The virtual superimposition of relative fluorescence intensity at 0%, 20%, and 30% ethanol up to ~55°C and a significantly different behavior at 30% ethanol at greater than 55°C, taken together with a red-shift in the λ_{max}, indicates that hGH unfolds more readily in 30% ethanol relative to 0% or 20% ethanol. Thus, while fluorescence anisotropy data and near-UV CD data together suggest a differently packed hydrophobic core in 30% ethanol, relative to that in 20% ethanol, the decreased thermal stability of hGH in 30% ethanol likely reflects suboptimal packing of the hydrophobic core in this medium. However, in principle, the differences in thermal unfolding could also be due to the differences in the unfolded state under these conditions. The apparent differences in the temperature dependent changes in the conformation of the molecule, as a function of solvent composition, may also be partially due to the indirect effects of changes in effective pH and ionic strength as a function of temperature.

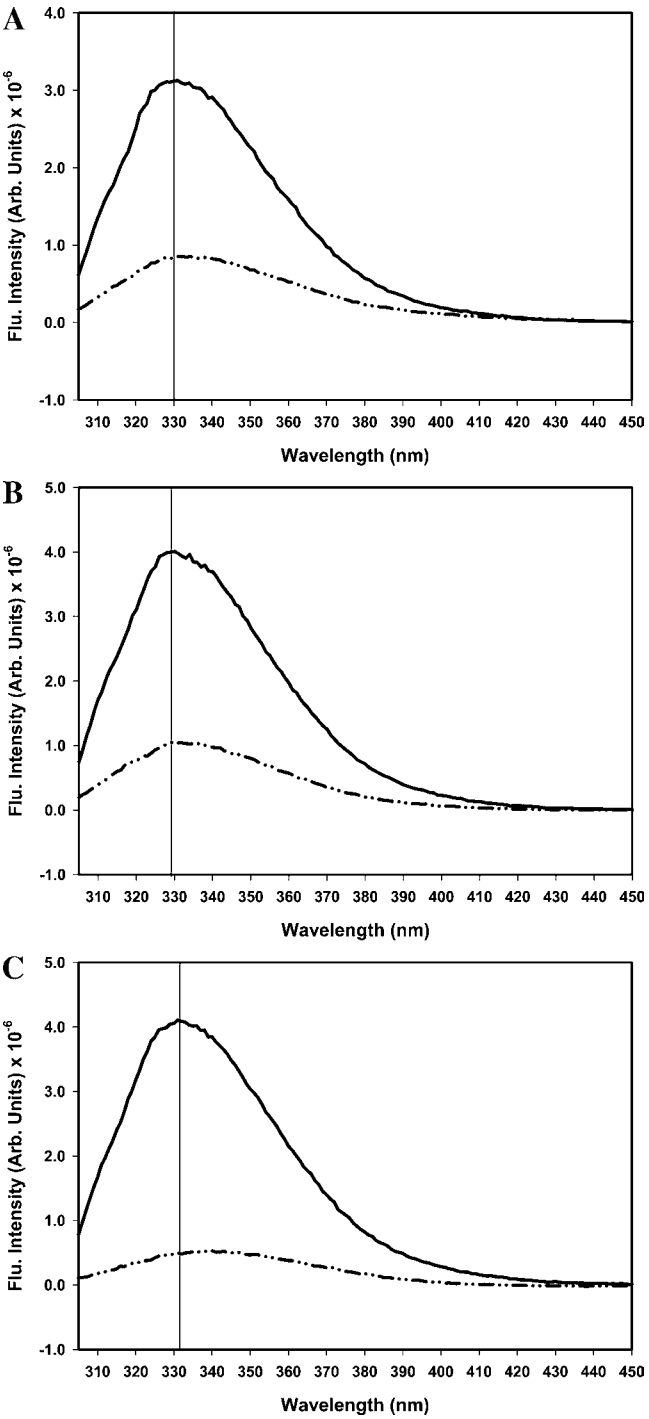

Fig. 5. Temperature dependence of fluorescence emission spectra of hGH in 0% (A), 20% (B), and 30% (C) ethanol. Spectra at 20°C (—) and 80°C (–···–) are shown. A vertical line is drawn through the λ_{max} at 20°C as a guide to follow temperature-dependent changes.

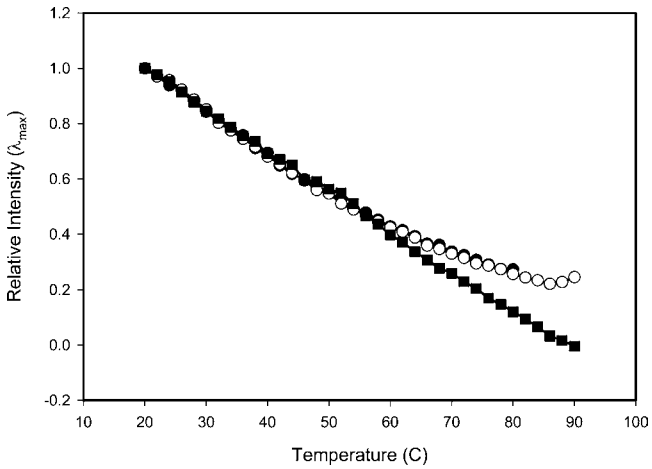

Fig. 6. The relative fluorescence intensity at λ_{max} plotted as a function of temperature for hGH in 0% (●), 20% (○), and 30% (■) ethanol.

Hydrodynamic Diameter of hGH as a Function of Ethanol Concentration

In order to characterize the changes in the hydrodynamic size that may accompany changes in the tertiary structure of hGH as a function of ethanol concentration, dynamic light scattering was used to measure effective hydrodynamic diameter. The data are summarized in Table I. No significant changes in the hydrodynamic size were observed between 0% and 40% ethanol. At >40% ethanol, the data may indicate a slight expansion of the molecule, but the magnitude of these changes is within the variability of the method (±1 nm). These data are also consistent with previous studies, which showed that partial or complete unfolding of hGH resulted in only minimal changes in the hydrodynamic diameter (18). Because of minimal changes in hGH hydrodynamic size up to 40% ethanol, the relatively high, native-like, fluorescence anisotropy at 30% ethanol was interpreted as arising from the restricted mobility of the Trp side-chain as opposed to changes in the overall rotational correlation time of the molecule.

CONCLUSIONS

The CD and fluorescence analysis of hGH in aqueous media presented here shows that this 4-helix bundle protein retains all of its secondary structure as the concentration of ethanol is increased up to 50% but undergoes subtle changes in the tertiary environment of the Trp residue within the hydrophobic core of the molecule and a progressive opening of the structure resulting in increased solvent exposure of the

Table I. Mean Hydrodynamic Diameter of Human Growth Hormone as a Function of Ethanol Concentration[a]

Ethanol concentration (% v/v)	Mean hydrodynamic diameter ± standard deviation (nm)
0	3.3[b]
10	3.9 ± 0.3
15	3.9 ± 0.8
20	4.1 ± 0.2
23	3.3 ± 1.0
26	3.6 ± 0.4
30	3.2 ± 1.0
40	3.6 ± 1.1
50	4.8 ± 0.1
60	4.8 ± 0.1

[a] Data represents composite of two separate studies. Standard deviations are based on 3–8 measurements.

[b] Average of two measurements, whose individual values were 3.2 and 3.4 nm.

buried Trp residue. Based on far- and near-UV CD data, no significant changes were observed in either secondary or tertiary structure of the molecule up to an ethanol concentration of 20%. At 30% ethanol, significant changes in the near-UV CD ellipticity indicated a perturbed tertiary structure, but interestingly these changes were not accompanied by a corresponding change in the fluorescence emission maximum or fluorescence anisotropy of the buried Trp residue, suggesting that the Trp side-chain is still solvent-shielded and its motion significantly restricted. These results are consistent with a differently packed hydrophobic core at 30% ethanol, relative to the native structure. Thermal unfolding followed by fluorescence measurements show that the conformational state/s populated at 30% ethanol unfolds more readily, relative to hGH unfolding at 0% or 20% ethanol. Taken together, these observations indicate that the conformational state in 30% ethanol represents a differently packed, but somewhat loose hydrophobic core, compared to the native state. At >40% ethanol, the fluorescence emission and anisotropy are consistent with increased solvent exposure and mobility of the Trp residue. However, as shown by far-UV CD, the molecule retains its secondary structure under these conditions, and measurements of hydrodynamic diameter indicated only a slight expansion, suggesting the molecule remains fairly compact. The studies presented here highlight the importance of applying complementary biophysical approaches to characterize the conformational transitions that a biotherapeutic can undergo when exposed to various processing conditions. Both far-UV CD and fluorescence anisotropy alone would have missed the subtle conformational changes that occur in 30% ethanol. It is important to note that while the available data do not allow quantitative estimation of the fraction of various conformational states, they allow a qualitative characterization of the changes in average conformational properties as a function of solvent composition. In assessing the potential impact of the conformational changes observed here on manufacturing operations, a key aspect to consider is reversibility. Although the tendency of hGH to undergo irreversible aggregation and precipitation has been documented (3), no precipitation has been observed under conditions of the current study, suggesting that the conformational changes observed here are likely reversible. However, the question of reversibility and the ultimate impact on the quality of the product needs to be examined in the context of specific manufacturing operations, taking into consideration such process variables as concentration, the length of time and the temperature of exposure.

The effects of ethanol on protein denaturation likely arise from changes in the solvation of exposed hydrophobic groups in the denatured state (7). Thermodynamic studies of lysozyme denaturation in alcohol-water mixtures have shown that both the temperature of denaturation and the changes in heat capacity upon unfolding decreased with increasing alcohol concentration (8). Our results for hGH are consistent with these earlier studies. In addition, the non-native conformational states of hGH observed here may have relevance to understanding protein folding. For example, the partially folded states of lysozyme stabilized in the presence of trifluoroethanol retain native-like secondary structure and have been shown to resemble early kinetic intermediates (25).

The propensity of hGH to form native-like intermediate states described here is consistent with earlier studies that demonstrated the presence of intermediate states in the equilibrium folding pathway of hGH (3,17,18,20). Furthermore, detailed calorimetric investigations of hGH (20) have shown that the free energy of these intermediate states is only 3 kcal/mol apart from the native state, suggesting that the conformational equilibrium for hGH can be a sensitive function of solvent composition and physical conditions. Future studies will focus on the reversibility of conformational changes observed here as a function of operational variables and a detailed characterization of the propensity of the non-native states to undergo chemical and physical degradation and aggregation. The four-helix bundle topology of hGH is shared by other growth factors and cytokines and it will be interesting to examine if the kind of conformational transitions noted here are general for this structural class.

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