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Thermodynamic characterization of an intermediate state of human growth hormone

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Abstract

The thermal denaturation of recombinant human growth hormone (rhGH) was studied by differential scanning calorimetry and circular dichroism spectroscopy (CD). The thermal unfolding is reversible only below pH 3.5, and under these conditions a single two-state transition was observed between 0 and 100 °C. The magnitudes of the ΔH and ΔC_p of this transition indicate that it corresponds to a partial unfolding of rhGH. This is also supported by CD data, which show that significant secondary structure remains after the unfolding. Above pH 3.5 the thermal denaturation is irreversible due to the aggregation of rhGH upon unfolding. This aggregation is prevented in aqueous solutions of alcohols such as *n*-propanol, 2-propanol, or 1,2-propanediol (propylene glycol), which suggests that the self-association of rhGH is caused by hydrophobic interactions. In addition, it was found that the native state of rhGH is stable in relatively high concentrations of propylene glycol (up to 45% v/v at pH 7–8 or 30% at pH 3) and that under these conditions the thermal unfolding is cooperative and corresponds to a transition from the native state to a partially folded state, as observed at acidic pH in the absence of alcohols. In higher concentrations of propylene glycol, the tertiary structure of rhGH is disrupted and the cooperativity of the unfolding decreases. Moreover, the CD and DSC data indicate that a partially folded intermediate with essentially native secondary structure and disordered tertiary structure becomes significantly populated in 70–80% propylene glycol.

Keywords: circular dichroism; differential scanning calorimetry; folding intermediates; human growth hormone; propylene glycol; protein folding

Recombinant human growth hormone (rhGH) is a single-chain protein of 191 amino acids. Its tertiary structure is characterized by a four-helix bundle with two long loops (de Vos et al., 1992; Ultsch et al., 1994). The native state is stable and does not undergo significant conformational changes between pH 2–11 (Turner et al., 1983; Kauffman et al., 1989; Abildgaard et al., 1992). Slight differences observed in the near-UV CD spectra below pH 4 have been shown to stem from local adjustments in the tertiary structure after the protonation of carboxyl groups, and not from major tertiary structure alterations (Kauffman et al., 1989; DeFelippis et al., 1995).

The guanidine hydrochloride (GuHCl) denaturation of rhGH has been extensively studied (Brems et al., 1990; DeFelippis et al., 1993, 1995; Bam et al., 1996). rhGH unfolds at about 4.5 M GuHCl, and it is fully unfolded above 5 M GuHCl. The unfolding transition does not conform to a two-state process, and it was found that the Gibbs free energy of unfolding is protein concentration dependent due to the self-association of an intermediate that becomes populated during the unfolding. A similar behavior has been observed also for the GuHCl denaturation of porcine and bovine growth hormones (Havel et al., 1986; Bastiras & Wallace, 1992). Surfactants and protein fragments capable of forming amphiphilic helices have been shown to reduce the self-association of this rhGH intermediate (DeFelippis et al., 1993; Bam et al., 1996); however, the thermodynamic characterization of this partially folded state has remained elusive.

We have studied the thermal denaturation of rhGH using differential scanning calorimetry (DSC) and circular dichroism spectroscopy (CD). Our results show that the thermal denaturation is reversible only below pH 3.5, and that under these conditions the temperature-induced unfolding corresponds to a two-state transition from the native state to a partially folded state. No further unfolding of this intermediate was observed within the temperature range studied, 0–100 °C. Nevertheless, because of the absence of aggregation under the conditions of our experiments, we have been able to characterize the energetics of this partially folded state of rhGH.

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Abbreviations: CD, circular dichroism; ΔC_p, heat capacity change; ΔG, Gibbs free energy change; ΔH, enthalpy change; ΔH_m, enthalpy change at the unfolding temperature; DSC, differential scanning calorimetry; T_m, temperature of the maximum in the heat capacity function; rhGH, recombinant human growth hormone; $\Delta H_{vh}/\Delta H_{cal}$, van't Hoff to calorimetric enthalpy ratio.

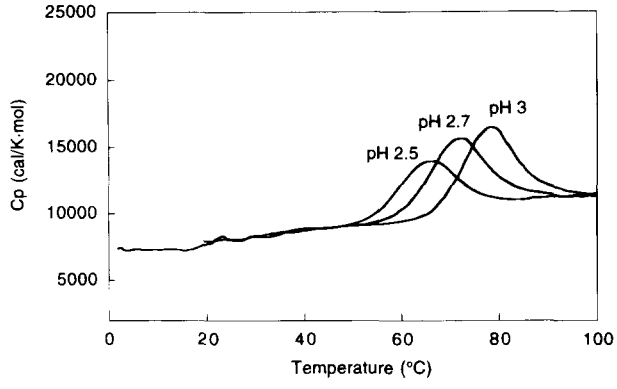

Fig. 1. Partial molar heat capacity of rhGH at different pH values.

Results and discussion

Thermal unfolding at different pH values

Figure 1 shows the partial molar heat capacity of rhGH as a function of temperature at different pH values. The reversibility of the unfolding was checked by performing repeated DSC scans of each sample, and it was found that the thermal denaturation of rhGH is reversible only below pH 3.5. Between pH 2–3.5, the thermal transition is independent of the rhGH concentration in the range 0.2–2 mg/mL, which indicates the absence of aggregation upon unfolding. In addition, under these conditions the thermal unfolding is essentially a two-state process ($\Delta H_{vh}/\Delta H_{cal} \sim 1$). Between pH 1–1.5, rhGH unfolds at about 42 °C, but the data from these DSC scans were not analyzed because significant protein hydrolysis was observed on samples heated to as low as 40 °C.

As shown in Figure 1, the thermal transition is highly sensitive to pH changes, which suggests that the unfolding is coupled to the protonation of carboxyl groups. Furthermore, the fact that the T_m and ΔH_m vary with pH allowed the calculation of the heat capacity of unfolding from the plot of ΔH_m versus T_m (Fig. 2). The ΔC_p of 1.13 kcal/K·mol determined from the linear fit of this plot compares well with the value of 0.92 kcal/K·mol obtained by subtracting the partial molar heat capacity of the native state from that of the unfolded state. The Gibbs free energy of unfolding was then calculated using Equation 1, with ΔC_p = 1.13 kcal/K·mol. ΔG (25 °C) varies from 3.01 kcal/mol at pH 2 to 6.61 kcal/mol at pH 3.5.

$$\Delta G(T) = \Delta H_m \cdot (1 - T/T_m) + \Delta C_p \cdot (T - T_m - T \cdot \ln T/T_m). \quad (1)$$

The thermal unfolding was also studied by CD. The unfolding profiles obtained by monitoring the changes in ellipticity in the far- and near-UV regions are shown in Figure 3. Both the far- and near-UV transitions were coincident at each pH studied between 2 and 3, and the T_m and ΔH_m calculated from these CD measurements agreed with the values determined by DSC. Because the far-

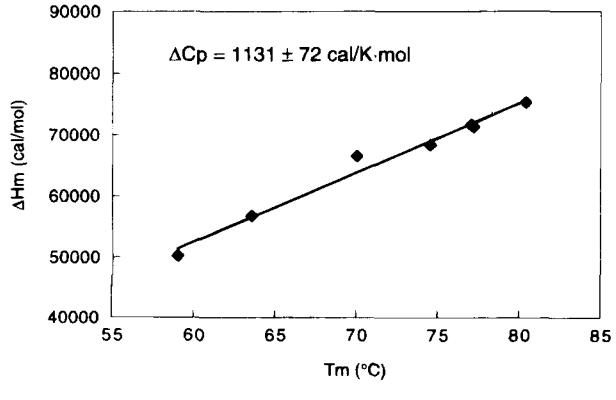

Fig. 2. Unfolding enthalpies versus the unfolding temperatures determined at different pHs.

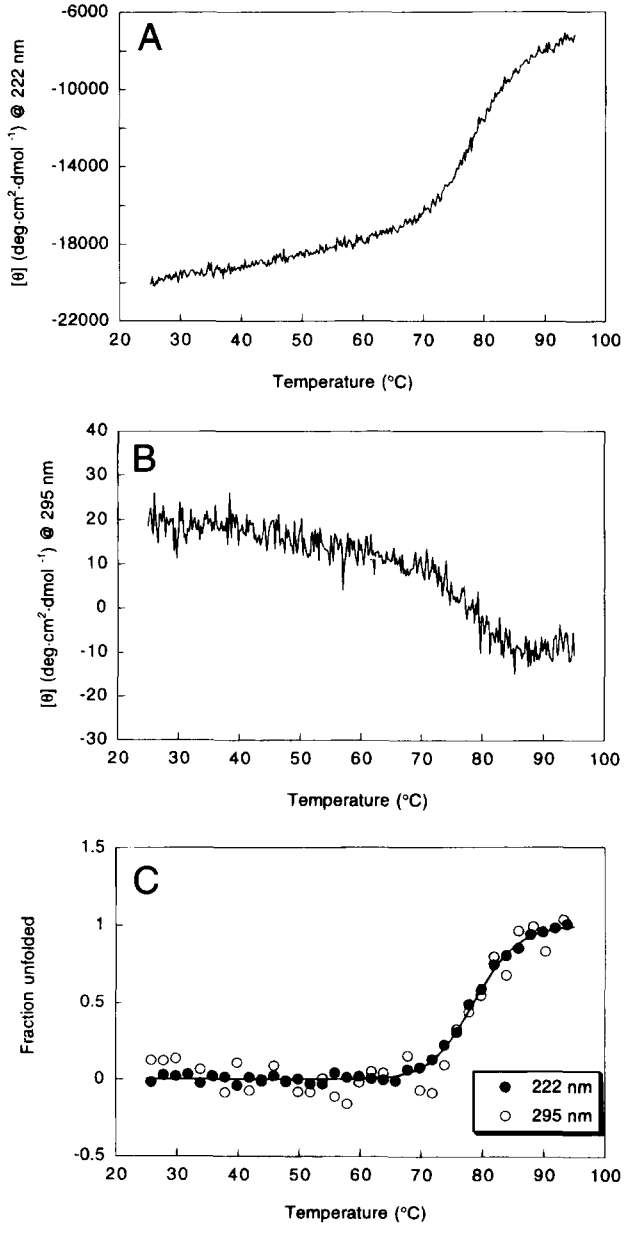

Fig. 3. Thermal unfolding of rhGH at pH 3 monitored by CD at (**A**) 222 nm and (**B**) 295 nm. The measurements were performed on aliquots of the same sample because the unfolding is sensitive to slight pH changes (see Fig. 1). (**C**) Fraction of unfolded protein as a function of temperature, calculated from the unfolding measurements at 222 and 295 nm.

Table 1. *Experimental and calculated enthalpy and heat capacity of rhGH unfolding*[a]

	ΔH (60 °C) (kcal/mol)	ΔC_p (kcal/K·mol)
Experimental	52.5	1.1
Calculated[b]	118.8	2.7 at 25 °C 2.0 at 70 °C
Calculated[c]	149.5	2.2

[a]The calculations were done using the coordinates from the Brookhaven Protein Data Bank file 1huw. Similar values were obtained using the coordinates from files 1hgu or 3hhr.
[b]Values calculated using the parameterization developed by Murphy and Freire (1992), Xie and Freire (1994), and Gomez et al. (1995).
[c]Calculated using the model proposed by Spolar et al. (1992).

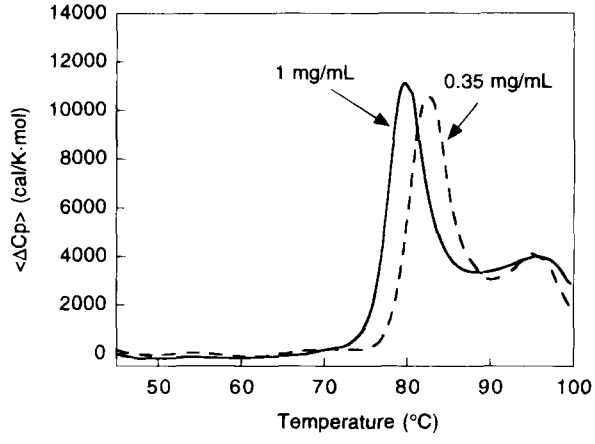

Fig. 5. Excess heat capacity of rhGH at pH 7.5.

and near-UV ellipticity report changes in the secondary and tertiary structure, respectively, the coincidence of the unfolding profiles confirms that the thermal unfolding of rhGH is essentially a two-state process, as suggested by the $\Delta H_{vh}/\Delta H_{cal} \sim 1$ obtained from the DSC measurements.

The different parameterizations proposed to estimate the ΔH and ΔC_p of unfolding from the changes in solvent-accessible surface areas (Murphy & Freire, 1992; Spolar et al., 1992; Xie & Freire, 1994; Gomez et al., 1995) were used to calculate the thermodynamic parameters corresponding to the complete unfolding of rhGH. The calculated values are compared to our experimental data in Table 1. It can be seen that the calculated ΔH and ΔC_p of unfolding are significantly higher than the experimental values, which suggests that the thermal transition observed by DSC and CD corresponds to a partial unfolding of rhGH. This interpretation is also supported by the CD spectra of rhGH at temperatures above the T_m, which indicate that significant secondary structure remains after the unfolding (Fig. 4). Thus, both the CD and the thermodynamic data lead to the conclusion that the thermal unfolding of rhGH corresponds to a transition from the native state (N) to a partially folded state (I),

$$N \rightleftharpoons I. \quad (2)$$

As shown in Figures 1 and 3, the unfolding temperature is relatively high, and no additional unfolding transitions were observed at temperatures up to 100 °C. It is possible that further unfolding of this partially folded rhGH occurs in a noncooperative way as has been observed for other protein intermediates (Jeng & Englander, 1991; Griko et al., 1994). Some of these intermediates have shown increased unfolding cooperativity in high ionic strength solutions (Potekhin & Pfeil, 1989; Kuroda et al., 1992; Xie et al., 1995), but, unfortunately, the effect of the ionic strength on the unfolding of rhGH could not be investigated because the protein precipitates at acidic pH in NaCl solutions above 80 mM.

Above pH 3.5, the thermal unfolding of rhGH is irreversible, as indicated by the lack of a transition in a repeated DSC scan of the same sample. The $\Delta H_{vh}/\Delta H_{cal}$ is about 4, and the T_m decreases with increasing concentrations of rhGH. These results suggest that the irreversibility is caused by aggregation upon unfolding. Although no precipitate or turbidity was observed in the samples after the temperature scans, the presence of aggregates in solution was confirmed by light scattering (data not shown).

Between pH 7–8 the heat capacity function of rhGH shows two transitions: the first at about 80 °C, and the second at about 95 °C. No transition was observed in a repeated scan of the same sample. As the rhGH concentration was increased (0.2–1.5 mg/mL), the T_m of the first transition decreased and the T_m of the second transition increased (Fig. 5). This behavior suggests that the first transition involves the self-association of rhGH upon unfolding and that the second transition is coupled to oligomer dissociation. At protein concentrations above 1.5 mg/mL, the second transition was no longer observed, suggesting that the aggregation becomes more extensive and irreversible with increasing protein concentrations.

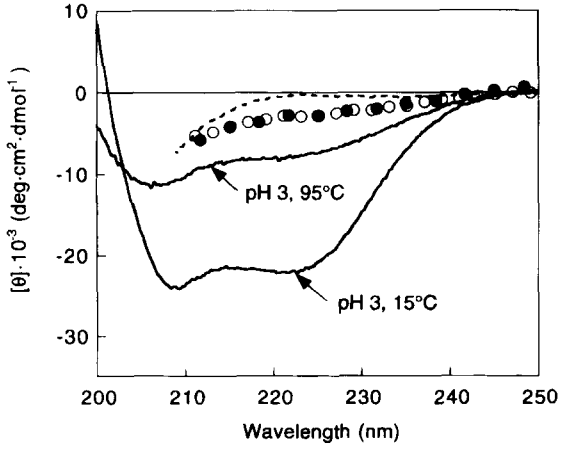

Fig. 4. Far-UV CD spectra of rhGH at pH 3 below (15 °C) and above (95 °C) the T_m. For comparison, the CD spectra of rhGH unfolded in 6 M GuHCl are also plotted: pH 7.5 and 6 M GuHCl at 15 °C (dashed line); pH 3 and 6 M GuHCl at 90 °C (open circles); pH 7.5 and 6 M GuHCl at 90 °C (solid circles).

Effect of alcohols on the thermal unfolding

In aqueous solutions of either 10% v/v *n*-propanol, 10% 2-propanol, or 20% 1,2-propanediol (propylene glycol), the thermal denaturation of rhGH at pH 7–8 is characterized by a single, reversible

transition with a $\Delta H_{vh}/\Delta H_{cal} \sim 1$. Under these conditions, the thermal unfolding is independent of the protein concentration, indicating the absence of aggregation. At temperatures below the T_m, the far- and near-UV CD spectra of rhGH in these alcohol solutions are superimposable on the spectra obtained in the absence of alcohols, which suggests that the native conformation is not affected by these solvent conditions. Above the T_m, the CD spectra of rhGH in these water/alcohol mixtures are similar to the CD spectrum of the intermediate obtained upon unfolding at acidic pH (Fig. 4). Therefore, the thermal unfolding in these mixtures may correspond to a partial unfolding as observed at acidic pH.

The absence of aggregation in these alcohol solutions suggests that the self-association is caused by hydrophobic interactions between apolar surfaces of rhGH that become exposed during the unfolding. These interactions are weaker in low polarity solvents such as water/alcohol mixtures, and, therefore, the aggregation is inhibited. Similarly, Bam et al. (1996) have shown that the self-association of rhGH in GuHCl decreases in the presence of surfactants, an indication that this aggregation also stems from nonpolar interactions.

Between pH 3.5 and 7, however, the aggregation upon unfolding could not be prevented completely, even in the presence of relatively high alcohol concentrations (40–50%). It is possible that the aggregation becomes more extensive as the pH approaches the isoelectric point of rhGH (pH ~ 5.3) (Pearlman & Bewley, 1993).

Between pH 7–8, the CD spectra indicate that rhGH remains essentially native in propylene glycol concentrations up to 45%, and, surprisingly, rhGH unfolds in a cooperative, two-state fashion ($\Delta H_{vh}/\Delta H_{cal} \sim 1$) in 20–45% propylene glycol within this pH range. To be able to compare the thermal unfolding in the presence of propylene glycol with the unfolding in pure aqueous solution, the effect of propylene glycol on rhGH was also studied at pH 3, where the unfolding is reversible and uncoupled to aggregation in the absence of alcohols. At this pH, the CD spectra indicate that the native state is stable in propylene glycol concentrations up to 30%, and it was found that the thermal unfolding is reversible and cooperative ($\Delta H_{vh}/\Delta H_{cal} \sim 1$) in 0–30% propylene glycol. The magnitudes of the unfolding enthalpies and the CD spectra of rhGH above the T_m indicate that under these conditions the thermal transition corresponds to partial unfolding of rhGH. Thus, at pH 3 the unfolding process is essentially the same in the presence and in the absence of propylene glycol, and the cooperativity of the unfolding is not affected by propylene glycol concentrations below 30%.

Figure 6 shows the variation of the T_m and ΔH_m with the propylene glycol concentration at pH 3 and pH 7.5. ΔH_m initially increases and then decreases with increasing propylene glycol concentrations. A similar behavior has been reported for the ΔH_m of hen egg white lysozyme and cytochrome *c* in different water/alcohol mixtures (Velicelebi & Sturtevant, 1979; Fu & Freire, 1992). The T_m varies linearly with the propylene glycol concentration. At pH 3, the *y*-intercept from the linear fit of the T_m values is in agreement with the experimental T_m in the absence of propylene glycol. At pH 7.5, the *y*-intercept from the linear fit of the T_m data is 91.3 °C, which presumably corresponds to the unfolding temperature without propylene glycol in the absence of aggregation. The unfolding enthalpy corresponding to this T_m was calculated using the ΔC_p and ΔH determined for the partial unfolding at acidic pH (Fig. 2). The value obtained, 86.8 kcal/mol, compares well with the ΔH_m values determined from the DSC scans at different propylene glycol concentrations (Fig. 6B), which suggests that in the absence of aggregation the thermal unfolding at pH 7.5 would correspond to a partial unfolding as at acidic pH.

In propylene glycol concentrations above 45% at pH 7–8 or 30% at pH 3, the near-UV CD spectra of rhGH show differences from the spectra in the absence of propylene glycol. These differences are more pronounced in 70–80% propylene glycol and are mainly observed in the region of the spectrum that is assigned to tryptophan residues (288–310 nm). The ellipticity in the 255–285 nm region, which is attributed to the optical activity of tyrosines and phenylalanines (Bewley, 1979) appears less altered (Fig. 7A). These changes in the near-UV ellipticity reflect alterations in the local environment and rotational freedom of the aromatic side chains. rhGH has a single tryptophan (Trp 86), which is mostly buried in the hydrophobic core of the protein (de Vos et al., 1992; Ultsch et al., 1994). The absence of ellipticity in the tryptophan region at 80% propylene glycol indicates that this residue has gained rotational freedom and that its local environment has changed, and, therefore, that the tertiary structure of rhGH is somehow disrupted. Most phenylalanines and tyrosines, on the other hand, are located on the surface of rhGH; thus, it is not

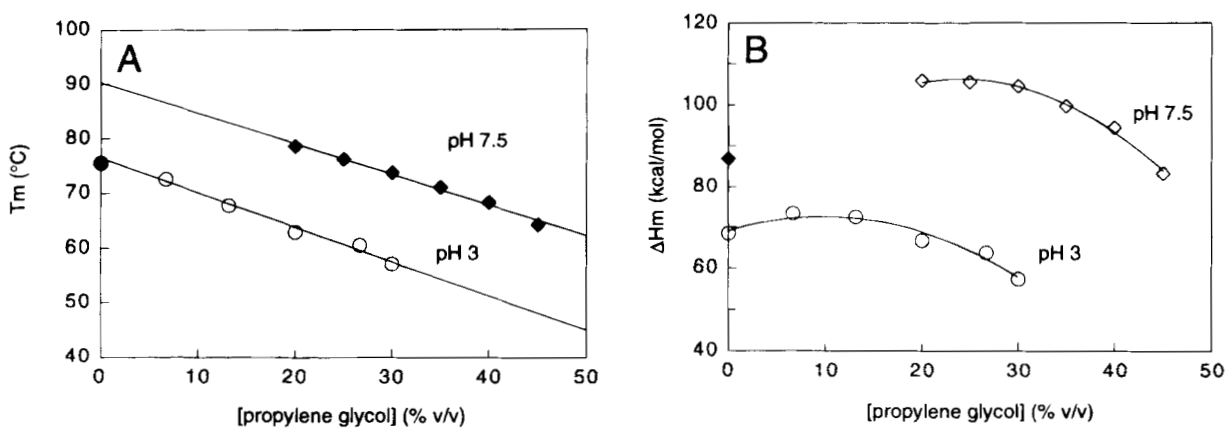

Fig. 6. A: Variation of the unfolding temperature with the propylene glycol concentration. The solid circle corresponds to the experimental T_m at pH 3 in the absence of propylene glycol. This value was not included in the linear fitting. **B:** Variation of the unfolding enthalpy with the propylene glycol concentration. The solid diamond represents the calculated ΔH_m at pH 7.5 in the absence of propylene glycol (see text).

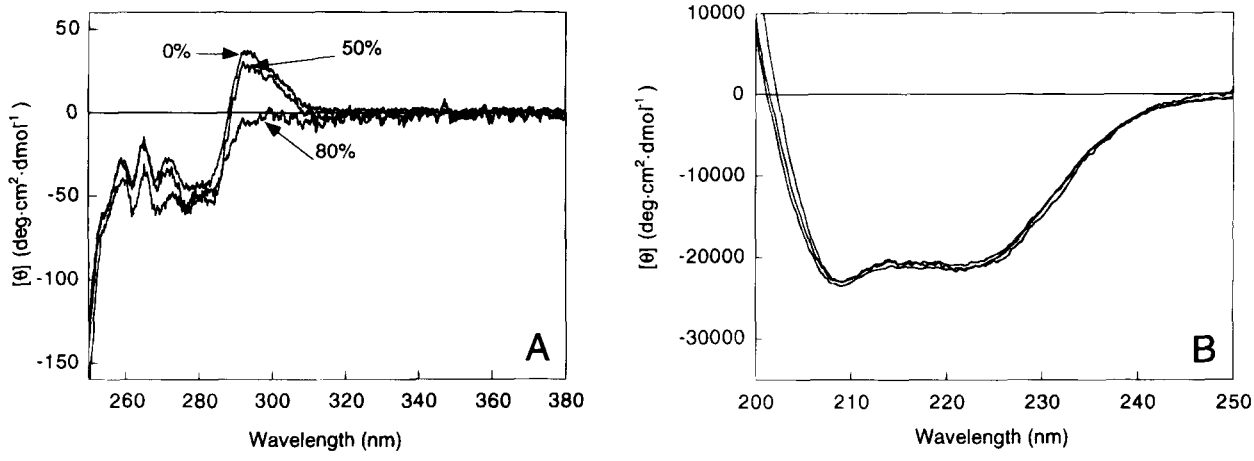

Fig. 7. (**A**) Near- and (**B**) far-UV CD spectra of rhGH in 0, 50, and 80% v/v propylene glycol at pH 7.5 and 15 °C.

surprising that tertiary structure alterations have a lower impact on the average ellipticity of these residues. No significant changes in the secondary structure were detected by CD, even in 80% propylene glycol (Fig. 7B).

The DSC scans performed at the propylene glycol concentrations in which the tertiary structure of rhGH appears to be altered (>45% at pH 7–8 or >30% at pH 3) show that the cooperativity of the unfolding decreases ($\Delta H_{vh}/\Delta H_{cal} < 1$) and the unfolding transition becomes broader and progressively less endothermic as the propylene glycol concentration is increased, until finally, at 80% propylene glycol, no transition was detected by DSC (Fig. 8A). At this propylene glycol concentration, the thermal unfolding monitored by CD at 222 nm is characterized by a gradual change in the molar ellipticity with temperature (Fig. 8B), indicating that the absence of a detectable transition in the DSC scan is due to the low cooperativity of the unfolding under these conditions. This low unfolding cooperativity suggests that the tertiary structure of rhGH is largely disordered at this propylene glycol concentration, in agreement with the near-UV CD data (Fig. 7A). These observations, thus, lead to the conclusion that a partially folded form of rhGH, with native-like secondary structure and disordered tertiary structure, is significantly populated in 80% propylene glycol.

Intermediate states of rhGH

Two partially folded states of rhGH have been characterized in this paper: (1) an intermediate with disordered tertiary structure but essentially native secondary structure that is stabilized in 80% propylene glycol at low temperatures (Fig. 7), and (2) an intermediate with a partially unfolded secondary structure that becomes populated at high temperatures and acidic pH (Fig. 4).

Propylene glycol and other alcohols are known to induce the partial unfolding of proteins through the disruption of tertiary structure interactions (Liu & Bolen, 1995; Shiraki et al., 1995; Dib et al., 1996; Kamatari et al., 1996; Schönbrunner et al., 1996; Hirota et al., 1997; Uversky et al., 1997). In general, alcohol-stabilized intermediates are characterized by having disordered tertiary structure and high alpha-helical content, and usually they contain non-native secondary structure elements because alcohols

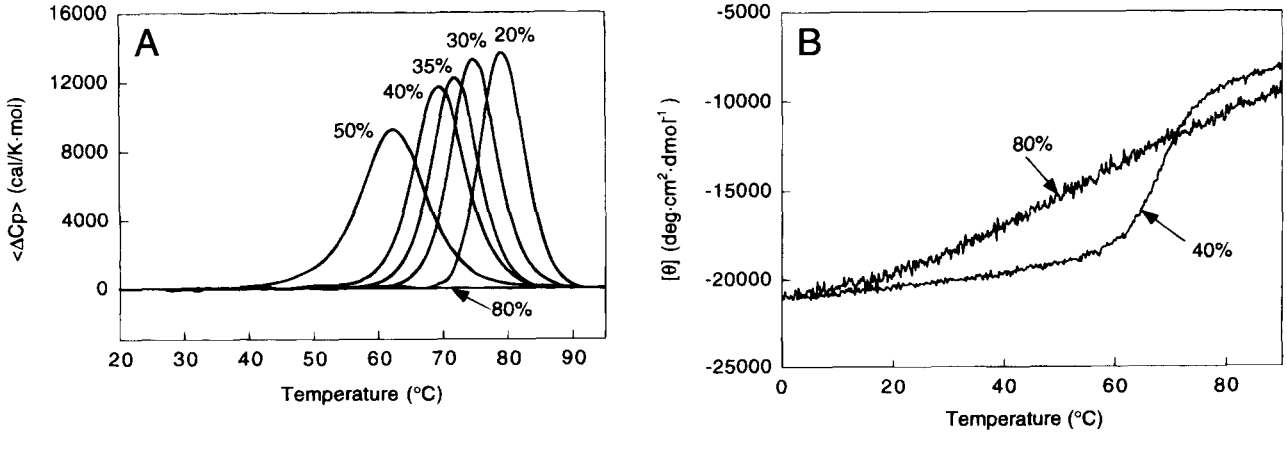

Fig. 8. **A:** Excess heat capacity of rhGH at pH 7.5 in different propylene glycol concentrations. **B:** Temperature-induced unfolding of rhGH at pH 7.5 in 40 and 80% propylene glycol monitored by CD at 222 nm.

induce the population of α-helical structure in regions of the protein that are non-helical in the native state. The rhGH intermediate populated in 80% propylene glycol has the structural characteristics of alcohol-stabilized intermediates, but the secondary structure of this intermediate is predominantly native because rhGH is mostly helical. Similar rhGH intermediates have been previously observed in aqueous solutions of 1-propanol and acetonitrile (Wicar et al., 1994). Alcohol-stabilized intermediates of various proteins have been shown to be involved in early folding events (Buck et al., 1995; Hamada et al., 1996) and in the translocation of proteins across membranes (Bychkova et al., 1996). Thus, rhGH intermediates analogous to the intermediate populated in propylene glycol could be biologically relevant or play a role in the folding mechanisms of rhGH.

The partially folded state stabilized at high temperatures and acidic pH, on the other hand, is an equilibrium folding intermediate characterized by having the secondary structure partially unfolded. Equilibrium and kinetic folding intermediates are often alike (Jennings & Wright, 1993; Balbach et al., 1995; Roder, 1995; Chamberlain et al., 1996); thus, it is possible that this intermediate shares structural similarities with the intermediates detected in studies of the folding kinetics of rhGH (Youngman et al., 1995) and the partially folded forms of rhGH (equilibrium intermediates) populated during the unfolding in GuHCl (DeFelippis et al., 1993, 1995; Bam et al., 1996). The structural and thermodynamic characterization of folding intermediates is of fundamental importance in understanding the mechanisms of protein folding and the interactions that determine the stability of native proteins. Until now the characterization of the folding intermediates of rhGH has been impeded by the aggregation of these intermediates under the conditions in which they become populated. We have identified conditions in which a partially folded form of rhGH is significantly populated without aggregation, and have characterized the energetics of this intermediate state. These conditions can now be used to characterize the structure of this partially folded state. A better understanding of the folding mechanisms of rhGH should be possible from the thermodynamic information reported in this paper combined with future structural characterizations of this intermediate state.

Materials and methods

Materials

1,2-Propanediol (propylene glycol) was obtained from Sigma. 2-Propanol and *n*-propanol were from Aldrich (Milwaukee, Wisconsin). Human growth hormone was obtained by recombinant DNA techniques. The primary structure of rhGH corresponds to that reported by Goeddel et al. (1979), and has Phe at the N-terminal. The purity of rhGH was confirmed by size exclusion and reversed-phase chromatography and mass spectrometry. The protein solutions were prepared by dialysis overnight, at 4 °C, against 4 L of the appropriate buffer. The rhGH concentration was determined spectrophotometrically, using an extinction coefficient of 18,890 M^{-1} cm^{-1} at 278 nm (Brems et al., 1990). The buffers used were 10 mM glycine/HCl (pH 1 to 3.5), 10 mM sodium acetate (pH 4 to 5), and 10 mM sodium phosphate (pH 6 to 8).

Differential scanning calorimetry (DSC)

A Nano Differential Scanning Calorimeter (Model 5100) (Calorimetry Sciences Corporation, Provo, Utah) was used for the calorimetric experiments. The temperature scans were performed at 1 °C/min on samples containing 0.2–2 mg/mL of rhGH. Deconvolution of the heat capacity function was performed with nonlinear least-squares fitting software developed by Ernesto Freire (The Johns Hopkins University) (Freire & Biltonen, 1978; Freire, 1994; Xie et al., 1995).

Circular dichroism (CD)

CD spectra were recorded on a Jasco J-715 spectropolarimeter. The instrument was calibrated with an aqueous solution of 0.06% w/v ammonium d-camphor-10 sulfonate. Each spectrum was obtained as the average of four consecutive wavelength scans. Round thermostatic cells of 1 cm and 0.02 cm path length were used for the near- and far-UV regions, respectively. The temperature was regulated with a Neslab RTE-111 temperature controller. Wavelength scans were performed on samples containing 0.5–1 mg/mL of rhGH.

The unfolding measurements were performed on samples containing 1 mg/mL rhGH. The samples were heated at a constant rate (1 °C/min). The unfolding curves were analyzed assuming a two-state behavior, and the fraction of unfolded protein (P_u) was calculated according to Equation 2,

$$P_u = \{Y - (Y_n + a \cdot T)\}/\{(Y_u + b \cdot T) - (Y_n + a \cdot T)\} \quad (3)$$

where Y represents the molar ellipticity at any temperature (T), and $(Y_u + b \cdot T)$ and $(Y_n + a \cdot T)$ are linear equations obtained from the least-squares fitting of the post-transition and pre-transition data, respectively.

Surface areas

Solvent accessible surface areas were calculated with the program ACCESS, developed by Lee and Richards (1971) and implemented by T. Richmond and S. Presnell, using the coordinates from the files 1huw, 1hgu, and 3hhr of the Brookhaven Protein Data Bank

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