Thermal Stability of Collagen Fibers in Ethylene Glycol

C. A. Miles* and T. V. Burjanadze[†]

*University of Bristol, Collagen Research Group, Department of Clinical Veterinary Science, Langford, Bristol BS40 5DU, United Kingdom; and [†]A. Natishvili Institute of Experimental Morphology, Georgian Academy of Science, 380059 Digomy, Tbilisi, Republic of Georgia

ABSTRACT   The mechanism that renders collagen molecules more stable when precipitated as fibers than the same molecules in solution is controversial. According to the polymer-melting mechanism the presence of a solvent depresses the melting point of the polymer due to a thermodynamic mechanism resembling the depression of the freezing point of a solvent due to the presence of a solute. On the other hand, according to the polymer-in-a-box mechanism, the change in configurational entropy of the collagen molecule on denaturation is reduced by its confinement by surrounding molecules in the fiber. Both mechanisms predict an approximately linear increase in the reciprocal of the denaturation temperature with the volume fraction (ϵ) of solvent, but the polymer-melting mechanism predicts that the slope is inversely proportional to the molecular mass of the solvent (M), whereas the polymer-in-a-box mechanism predicts a slope that is independent of M. Differential scanning calorimetry was used to measure the denaturation temperature of collagen in different concentrations of ethylene glycol ($M = 62$) and the slope found to be $(7.29 \pm 0.37) \times 10^{-4}$ K^{-1}, compared with $(7.31 \pm 0.42) \times 10^{-4}$ K^{-1} for water ($M = 18$). This behavior was consistent with the polymer-in-a-box mechanism but conflicts with the polymer-melting mechanism. Calorimetry showed that the enthalpy of denaturation of collagen fibers in ethylene glycol was high, varied only slowly within the glycol volume fraction range 0.2 to 1, and fell rapidly at low ϵ. That this was caused by the disruption of a network of hydrogen-bonded glycol molecules surrounding the collagen is the most likely explanation.

INTRODUCTION

Collagen fibers in vivo must be stable enough to withstand the disruptive influence of thermal agitation, but capable of assembly and disassembly of the component molecules. In solution, the unfolding temperatures of a wide range of fibrous collagens are within only a few degrees of the animal's body temperature (Burjanadze, 1982; Burjanadze and Veis, 1997), but when the molecules are aggregated to form fibers there is an increase in the transition temperature of ~27°C.

There are two alternative explanations of this effect. The first is due to Flory and Garrett (1958). They suggested that the helix-coil transition of collagen is a melting process, in which the melting point of the polymer is depressed by the presence of a solvent: the greater the concentration of solvent the greater the depression of the melting point. In the fiber, the local concentration of solvent is low relative to that in dilute solution and the melting point is higher than that in solution. On the basis of equilibrium thermodynamics, Flory (1953) derived an equation for the melting point depression of a polymer in the presence of a solvent. This equation is closely related to the freezing point depression of a solvent due to the presence of a solute:

$$\frac{1}{T_{\mathrm{m}}} - \frac{1}{T_{\mathrm{m}}^0} = \frac{R\rho_1}{\rho_2 M_1 \Delta h}(\epsilon - \chi_1 \epsilon^2) \tag{1}$$

In Eq. 1, ϵ represents the volume fraction of solvent; T_{m}^0 and T_{m} are the melting points of the polymer in the pure state and in the presence of solvent, respectively; R is the gas constant; Δh is the heat of fusion per unit mass of polymer; ρ_1 and ρ_2 are the densities of solvent and polymer, respectively; χ_1 is an interaction parameter, and M_1 is the molecular mass of the solvent.

The second mechanism is based on the observation that the temperature and shape of the collagen denaturation endotherm in fibers and in basement membranes is governed by an irreversible rate process (Miles, 1993; Miles et al., 1995) and not by equilibrium thermodynamics as previously supposed (Privalov, 1982). In the process of thermal activation, which precedes the unfolding transition, a region of the molecule called the thermally labile unit unfolds first. Once the α-chains in this region are uncoupled, the whole molecule becomes unstable, rapidly unzips, and collapses to random coils. According to this model, stabilization of the collagen molecule in a fiber is brought about mainly by a reduction in the entropy of activation of the rate process. This could be caused by the spatial confinement of the molecule within the lattice of the fiber. Using rate theory and the polymer-in-a-box analysis of Doi and Edwards (1986) a different relation was derived between T_{m} temperature of unfolding and the volume fraction of diluent, ϵ (Miles and Ghelashvili, 1999):

$$\frac{1}{T_{\mathrm{m}}} - \frac{1}{T_0} = \frac{3fR\alpha_0 C_{\mathrm{N}} b_0^2}{0.88\, nd^2 \Delta H_{\mathrm{res}}^*}(\epsilon - \epsilon_0) \tag{2}$$

Here T_0 is the temperature of unfolding at a particular volume fraction ϵ_0; b_0 is the virtual bond length between adjacent C_α atoms in a collagen α-chain; d is the diameter of the collagen molecule; n is an integer, either 1, 2, or 3,

representing the number of gaps $+1$ around the thermally labile unit of the molecule when packed in a fiber; ΔH_{res}^* is the activation enthalpy of collagen unfolding per mole of residue; C_N is a numerical factor, which for collagen equals 9, but is unity for a freely jointed chain and referred to as the stiffness of the polymer by Doi and Edwards (1986) or the characteristic ratio by Flory (1967) and Richards (1980); α_0 is another numerical factor that depends on the shape of the cross section of the box.

According to Eq. 2, there should be a hyperbolic relation between the temperature of denaturation, T_m, and the volume fraction of diluent in the fiber, ϵ.

Eqs. 1 and 2 both fit the hyperbolic form of the experimental data very well (Flory and Garrett, 1958; Miles and Ghelashvili, 1999), and it is not possible to distinguish between the two models on the basis of the precision of the predictions. However, the models do differ markedly in the predicted effect of solvents of different molecular mass. In the Flory model, the unfolding temperature, being determined by a melting point depression, depends on the molar concentration of the solvent, and the molecular mass of the solvent therefore appears in Eq. 1. Other things being equal, according to the Flory and Garrett model, the slope of the relation between $1/T_m$ and ϵ varies as the reciprocal of the solvent's molecular mass. By contrast, the slope of the relationship between $1/T_m$ and ϵ predicted by the polymer-in-a-box model shows no dependence on the solvent's molecular mass, because the determining factor is the dimensions of the box in which the polymer is confined (i.e., the dimensions of the fiber lattice). It is this space (or lack of it) that affects the number of configurations available to the polymer in the activated state. A comparison of the unfolding temperatures of collagen fibers swollen in two solvents differing markedly in molecular mass therefore provides a sensitive means of differentiating between the two models.

This paper records an experiment that investigates calorimetrically the effect on the collagen helix-coil transition of swelling collagen fibers in ethylene glycol. The data allow comparison with previously published data obtained with water and consequently possible differentiation between the two models of collagen stabilization.

MATERIALS AND METHODS

Preparation of dried tendon

Tails were excised from the carcasses of 5- to 8-week-old rats and frozen at $-20°C$ until required. On removal from the freezer, tails were thawed, and the tendons removed, cleaned of all visible contaminants, and washed in distilled water. The tendons were placed in methanol and progressively dried in a vacuum oven by increasing the temperature in stages: 55°C for 2–3 h, 70°C for 2–3 h, 90°C for 2–3 h, and 100°C for 2–3 h. No correction was made to allow for any residual water that may have remained in the sample after this treatment.

Addition of ethylene glycol to dry tendon

To add small amounts, fiber bundles of the dried tendon were weighed, m_d, covered with an inverted beaker and placed on a sheet of gauze over a dish of ethylene glycol (Sigma Chemical Co., St. Louis, MO). This assembly was enclosed in a vacuum oven at 55°C and the air pumped out. The valve to the pump was then closed and the glycol vapor was allowed to adsorb on the fibers for different lengths of time. The mass of adsorbed glycol, m_g, was determined by reweighing and the volume fraction of glycol determined by applying the following relation:

$$\epsilon = \frac{\dfrac{m_g}{\rho_g}}{\dfrac{m_g}{\rho_g} + \dfrac{m_d}{\rho_d}}, \qquad (3)$$

where ρ_g and ρ_d, the densities of glycol and dry tendon were taken as $1.11 \times 10^3 \text{ kg m}^{-3}$ and $1.38 \times 10^3 \text{ kg m}^{-3}$, respectively.

To obtain intermediate glycol concentrations, single fibers were hung from buttons over open cuvettes using fisherman's shot to keep the fibers straight. The cuvette was then filled with glycol and the fiber allowed to swell. The diameter of the fiber was measured using a vernier microscope in two perpendicular directions (a and b) in the dry state (d) and in the presence of glycol (g), and the volume fraction of ethylene fiber was calculated from

$$\epsilon = 1 - \frac{a_d b_d}{a_g b_g}. \qquad (4)$$

Finally, measurements were also made of weighed dried fibers to which weighed ethylene glycol was added in excess.

Preparation of solutions of collagen in ethylene glycol

To prepare freeze-dried collagen, the skin of a rat was cut into small pieces, washed in water, and then stirred in 0.5 M acetic acid for 48 h at 8°C. Insoluble components were removed by centrifugation at $15,000 \times g$ for 30 min. Fat was extracted by shaking with chloroform, in the volume ratio 1:1 with the supernatant. To remove any polysaccharides, the solution was centrifuged at $5000 \times g$ for 2 h. A 20% NaCl solution was added to the collagen solution to obtain a final concentration of 5% salt, and this mixture was stirred overnight in the fridge. The centrifuged pellet was dissolved again in 0.05 M acetic acid and dialyzed against 0.01 M acetic acid at 8°C. After exhaustive dialysis, the solution was lyophilized and stored at 8°C in a desiccator in the presence of silica gel.

Collagen solutions (1 mg/ml) were made up either in ethylene glycol or aqueous acetic acid solution (0.05 M) for scanning calorimetry and polarimetry. Suspensions of freeze-dried collagen were also mixed in the Perkin-Elmer (Norwalk, CT) differential scanning calorimetry (DSC) sample pans themselves and allowed to equilibrate overnight at 8°C for calorimetric tests the next day.

Calorimetry

Tendons and collagen suspensions were scanned in a computer-controlled Perkin-Elmer DSC-7, fitted with an Intracooler, and running software supplied by the manufacturer (1991 revision) was used for the calorimetric measurements. Weighed samples (±0.01 mg) were heated at either 2°C or 10°C per minute from 5°C to an appropriate specified temperature using an empty pan as a reference.

Solutions were scanned in a DASM-4 (manufactured in Puschino, Moscow region, Russia) scanning microcalorimeter with a 0.75-ml work-
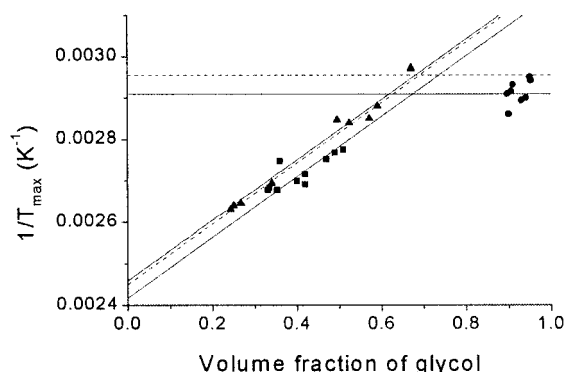
FIGURE 1 Thermal stability of rat tail tendon fibers swollen to different levels in ethylene glycol. Data obtained by DSC at different scanning rates: ■, 10°C/min; ▲, 2°C/min; ●, 10°C/min, obtained with ethylene glycol in excess. Solid lines are least-squares regression lines; dotted lines are corresponding lines for water at 10° C/min, taken from Miles and Ghelashvili (1999).

ing volume using a scanning rate of 0.25°C/min (Privalov and Plotnikov, 1989). All calorimetric experiments were carried out at a heating rate of 0.25 K/min and a protein concentration of 1 mg/ml. The partial specific heat capacity of collagen was determined (Privalov and Potekhin, 1986) using a partial specific volume of 0.686 ml/g (Christensen and Cassel, 1967).

Polarimetry

Denaturation transitions of collagen in solution were observed using a Polamat A polarimeter (Carl Zeiss, Jena, Germany) with an angle resolution of 0.005° at 546 nm. Measurements were made over a 5- or 20-cm path length in a cuvette surrounded by a thermostatically controlled jacket that was maintained at specified temperatures with a precision of 0.1 K. The temperature of the sample was monitored by a thermocouple wound round the cuvette, and temperature scanning was performed at the same rate as solution calorimetry, 0.25 K/min.

RESULTS

When $1/T_{max}$ was plotted against the volume fraction of diluent, the data for ethylene glycol followed closely data for water (see Fig. 1 and Table 1).

At high ethylene glycol concentrations, samples exhibited two phases: swollen fibers and excess ethylene glycol. In this region, changes in the proportions of ethylene glycol merely changed the quantity of excess glycol; the fibers were fully swollen and the dimensions of the fiber lattice

remained unchanged. The values of T_{max} recorded in this concentration range (Table 2) were therefore of the fully swollen fiber and were used with the data of Table 1 to estimate the volume fraction of glycol in the fully swollen fiber as 0.68 ± 0.05. This is very similar to the measured volume fraction of solvent in fully hydrated collagen fibers ($\epsilon = 0.69$; Miles and Ghelashvili, 1999), presumably because the swelling is limited to the same extent in both solvents by the presence of intermolecular cross-links.

The enthalpy of denaturation in fibers did not vary significantly ($p > 0.05$) within the glycol volume fraction range 0.2 to 1, but fell rapidly at low volume fractions (see Table 2 and Fig. 2).

The calorimetric measurements of solutions of collagen in both solvents (Fig. 3 and Table 3) showed the highly energetic and sharp denaturation endotherm that is characteristic of the triple helix. Polarimetric measurements (Fig. 4) proved that the transitions were also accompanied by a sharp and substantial loss of optical activity, confirming that the molecule at low temperatures existed as a triple-helical structure in both solvents. When the linear portion of Fig. 1 was extrapolated to $\epsilon = 1$, T_{max} occurred at 44.5 ± 1.8°C in ethylene glycol and 41.1 ± 1.8°C in acetic acid (both measured at a scanning rate of 10°C min^{-1}). These temperatures are comparable with those measured in dilute solution despite the protonation state of the molecule in acetic acid differing from that in water.

DISCUSSION

This section is in two parts. The first discusses collagen stabilization in fibers in terms of the polymer-in a-box and melting-point-depression mechanisms. The second part considers the intrinsic stability of the molecule itself, especially the source of the high enthalpy of unfolding in both water and glycol.

Polymer-in-a-box

This experiment (Fig. 1 and Table 1) clearly showed that the polymer-in-a-box mechanism for thermal stabilization of collagen in fibers is compatible with the observed effect on the denaturation temperature of swelling fibers in either ethylene glycol or water, but the polymer melting mecha-

TABLE 1 Regression line characteristics of $1/T_m$ against volume fraction of diluent in the fiber

Diluent	Scanning rate (°C min^{-1})	Slope (SE) (K^{-1})	Intercept (SE) (K^{-1})	Residual SD (K^{-1})
	2		0.002460 (0.000018)	
Ethylene glycol*	10	0.000729 (0.000037)	0.002419 (0.000018)	0.000023
Water†	10	0.000731 (0.000042)	0.002451 (0.000019)	0.0000098

*Data at two scanning rates fit regression lines with a common slope but different intercepts.
†Data for water taken from Miles and Ghelashvili (1999).

nism is not. On the basis of Eq. 1 we would expect, if everything else was equal, that the slope of the regression would be inversely proportional to the molecular mass of the solvent and that therefore the slope in water should be ~62/18 times that in ethylene glycol. We found that the slopes were very similar (see Table 1) as predicted by Eq. 2. This behavior is therefore consistent with Eq. 2 but conflicts with Eq. 1. It is stressed that we do not deduce that the polymer-in-a-box mechanism is the only possible explanation of these data or that other factors do not contribute to the stability of the collagen molecule in fibers. Furthermore, the present data for native fibers were obtained at high scanning rates. On the presented data we cannot exclude other mechanisms being important at slower scanning rates. However, other data confirm that the DSC endotherm of type I collagen is controlled by a rate process when in the form of native fibers (Miles et al., 1995) or in dilute solution (C. A. Miles and A. J. Bailey, unpublished) at scanning rates ranging down to isothermal denaturation.

The present results cannot simply be explained by acknowledging that the rate process controlling the shape and position of the denaturation endotherm is controlled by the polymer-in-a-box mechanism, while maintaining that the difference between the Gibbs free energies of the helix and coil states is controlled by a melting process. The observa-
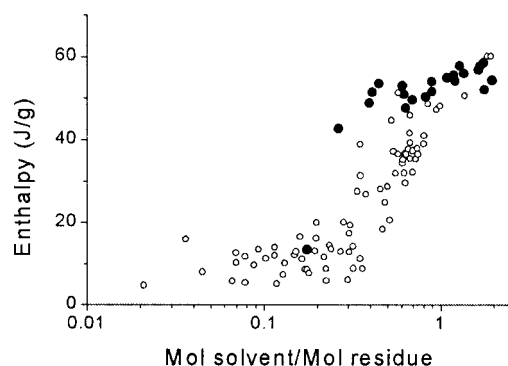

FIGURE 2 The effect of solvent concentration on the enthalpy of denaturation of collagen fibers. Comparison between data for ethylene glycol (●) of this study and those for water (○) taken from Miles and Ghelashvili (1999).
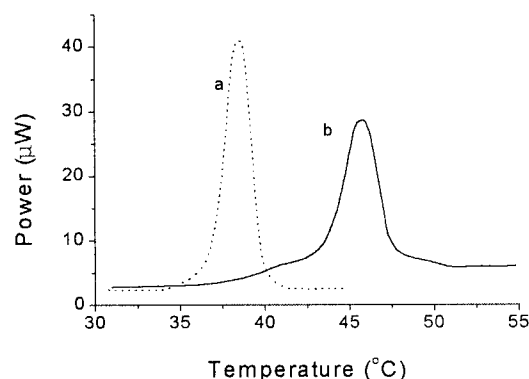

FIGURE 3. Examples of DSC thermograms of rat tail tendon collagen dissolved in different solvents: · · ·, 0.5 M acetic acid; ——, ethylene glycol.

tions were made over too wide a T_m range for this to be likely. We suggest, therefore, that the difference between the Gibbs free energies of the helix and coil must also be determined by the volume fraction of the diluent rather than by its mole fraction.

Free energy expressions (Eqs. 1 and 2) for equilibrium between final states or for a rate difference between ground and transition states should be fundamentally the same, but neither analysis quantifies explicitly all the thermodynamic terms contributing to the denaturation temperature. In broad terms, the Flory expression includes terms for the increase in the entropy of the solvent with polymer melting and for polymer-solvent interactions, but does not specifically quantify the increased entropy of the polymer. The polymer-in-a-box model excludes predictions in the low-solvent-concentration range ($\epsilon < 0.2$), does not explicitly quantify the solvent terms, but does include terms for the increase in polymer entropy of activation. The two models therefore differ fundamentally in what physical phenomena are considered to be important. At high and intermediate solvent concentrations, the results of this study indicate that polymer entropy effects are more important than solvent effects in determining differences between collagen stability in fibers of different solvent content. The result is notable in that it shows that the collagen molecule is stabilized by a mechanism that differs profoundly from the solid/melt equilibrium proposed by Flory and Garrett (1958).

It is therefore interesting to calculate the entropy of denaturation at each T_{max}, although it is stressed that this is not the difference in the entropies of two states in equilibrium. These data are shown in Fig. 5. At volume fractions above 0.2, the collagen molecule is fully saturated with hydrogen-bonded glycol and the entropy increases linearly with volume fraction as predicted by the polymer-in-a box model. At $\epsilon = 1$, the box is so large that the molecule is effectively unconfined and the entropy corresponds to the entropy of collagen in dilute solution. Collagen molecules are unlikely to be monomeric in this state but oligomeric.

TABLE 3 Endotherm characteristics of the denaturation of collagen in aqueous and ethylene glycol solutions

Solvent	Δh (J g^{-1})	ΔH (kJ mol^{-1} residues)	ΔS (J mol^{-1} K^{-1} residues)	T_d (K)	ΔT (K)	ΔC_P (J g^{-1} K^{-1})
0.5 M acetic acid in water	58.2 ± 0.5	5.41 ± 0.05	17.38	311.14	2.0 ± 0.1	0.15
Ethylene glycol	58.55 ± 0.5	5.445 ± 0.05	17.08	318.74	2.8 ± 0.1	1.6

Data were obtained at a scanning rate of 0.25 K min^{-1} and are mean values of six measurements in each solvent.

However, the intrinsic effect of collagen cross-links on the temperature of collagen molecules in dilute solution is relatively small, as shown for example by the work of Na (1989) who observed very similar denaturation temperatures in solutions of monomeric collagen and collagen oligomers. Cross-linking of fibers and basement membranes also has a rather small effect, typically a few degrees or less (Bailey et al., 1993), and some of this change may be due to the reduction in intermolecular distances induced by the cross-links themselves, rather than an intrinsic entropic effect due to the polymer cross-linking.

At the other extreme, extrapolating the line to $\epsilon = 0$, yields the entropy of a hypothetical state in which the collagen molecule, fully hydrogen bonded with glycol, is surrounded and touching similar molecules in a quarter stagger arrangement, in the absence of any glycol. Actually, below $\epsilon = 0.2$, the entropy falls abruptly with reducing ϵ as the hydrogen-bonded glycols are stripped away from the collagen. The difference between the actual measurements and the linear extrapolation is caused by the large contribution of the hydrogen-bonded solvent. This is expected to be large as the hydrogen-bonded network of glycol molecules around the native triple helix will be disrupted around the random coils, and glycol molecules, immobilized as glycol bridges, will be released into the liquid state.

Although the polymer-in-a-box mechanism predicts that the slopes of the $1/T_m$ versus ϵ loci of collagen fibers in ethylene glycol and water should be similar, it does not predict that they should be identical, as observed in Table 1.

Some of the parameters determining the slope are clearly independent of solvent (the gas constant and b_0 the virtual bond length between adjacent C_α atoms in a collagen α-chain, for example); others are unlikely to be affected by the solvent provided the triple-helical structure is maintained (the diameter of the collagen molecule, d, and the characteristic ratio, C_N), and yet others are unlikely to be affected provided the molecular packing in the fiber remains the same (e.g., the numerical factors n, f, and α_0). However, ΔH_{res}^*, the activation enthalpy of collagen unfolding per mole of residue, could be affected by solvent, and the fact that the slope in water is the same (within the quoted uncertainties) as that in glycol indicates that ΔH_{res}^* is the same in both solvents. Supporting this conclusion is our observation that ΔH in ethylene glycol is remarkably similar to that in water, when the collagen was measured either in solution or in fibers. We suppose that the activation process consists of the uncoupling of a short section of the collagen molecule and ΔH_{res}^* is approximately the same as ΔH_{res} (see Miles et al., 1995).

The derivation of Eq. 2 required the use of an explicit function for the rate constant for collagen denaturation (Miles and Ghelashvili, 1999). We showed previously that the shape and position of the collagen denaturation endotherm are relatively insensitive to the precise form of the rate constant function (Miles, 1993; Miles et al., 1995). Although we chose the absolute rate theory equation for the polymer-in-a-box calculations, which does not allow for the effect of variations in viscosity on $k(T)$, we could equally
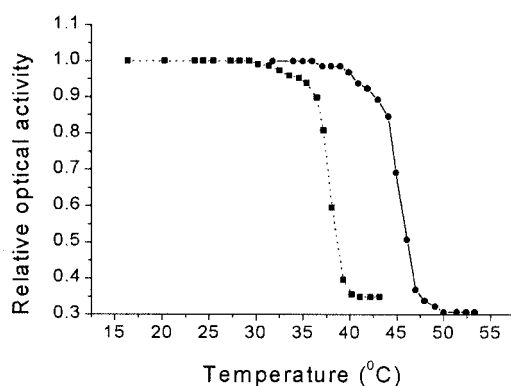

FIGURE 4   Relative optical activity of rat skin collagen dissolved in two different solvents: ■, 0.5 M aqueous acetic acid solution; ●, ethylene glycol. To convert relative optical activity to specific optical rotation (see, for example, Hauschka and Harrington (1970) for definition), multiply the acetic acid data by 411 and the ethylene glycol data by 283.
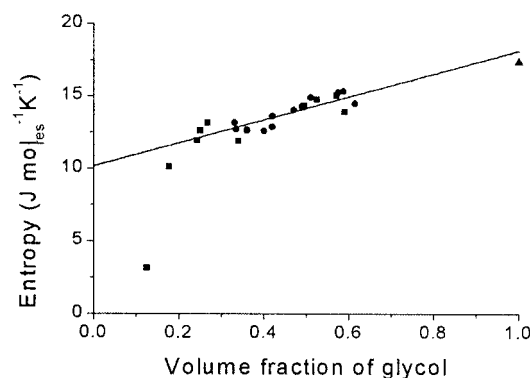

FIGURE 5   The effect of solvent concentration on the entropy of denaturation of collagen fibers. The line is the least-squares regression line for the data with volume fractions above 0.2. A datum for a dilute solution collagen in glycol is superimposed to show its coincidence with the extrapolated line.

have chosen an equation such as the modified Kramers relation (Frauenfelder and Marden, 1989), which does. The application of this and several other equations to collagen denaturation is discussed by Miles et al. (1995). If the Frauenfelder and Marden (1989) relation had been selected for the derivation, instead of the absolute rate theory equation, we would expect the final form of Eq. 2 to be largely unaffected. This is because changes in the viscosity term, which appears linearly in the rate equation, would be small in comparison with changes in the Gibbs free energy terms, which occur exponentially.

Reasons for concentrating on an entropic term for the stabilization are fully discussed by Miles et al. (1995). Briefly, measurements of the rate of denaturation showed that the Arrhenius parameter a was reduced by a factor of 10^{138} in fibers compared with solutions (Miles et al., 1995). The most likely explanation of this very large number was that it was caused by an exponential term and was therefore entropic in origin. Other factors such as component viscosities, which undoubtedly could affect the rate of denaturation by several orders of magnitude, were negligible in comparison with a factor of 10^{138}. The Arrhenius activation energy was also reduced in fibers, so the change in the enthalpy of activation in fact caused a destabilization, not a stabilization at all. Thus, changes in the enthalpy, induced by changes in solvation or pressure/volume terms, are unlikely to be the prime cause of the stabilization.

At solvent concentrations above $\epsilon = 0.2$ the enthalpies of denaturation in ethylene glycol and in water are approximately constant and Eq. 2 is obeyed. Below $\epsilon = 0.2$, the enthalpy of denaturation falls, and the effect of solvent on the enthalpy becomes apparent. Although this region is outside the limits of applicability of the polymer-in-a-box formulation, which the experiment was designed to test, these data are important as they probe the source of collagen's high enthalpy of denaturation.

Source of the high enthalpy of unfolding

The high enthalpy of unfolding of collagen in water is thought to derive mainly from the breaking of the hydrogen bonds forming the hydration network around the collagen molecule (Privalov, 1982; Burjanadze, 1992). These hydrogen bonds comprise intrachain, interchain, and intermolecular water bridges (Bella et al., 1995; Kramer et al., 1999). The observation of a similar large enthalpy when collagen was swollen in ethylene glycol implies that there must be a hydrogen-bonded network of glycol molecules around the collagen. The existence of an ethylene glycol network has been proposed by others (Kuznetsova et al., 1997) on the basis of an observed exponential increase in force as two molecules approach one another. Hence the calorimetric data presented here supports the suggestion that a network of hydrogen-bonded ethylene glycol molecules forms around a framework of hydrogen bonding sites formed by the collagen triple helix.

The data show a rapid fall in enthalpy of denaturation at glycol concentrations below ~0.4 mol of glycol/mol of residue, although previous data for water show the same decline below ~0.8 mol of water/mol of residue (Fig. 2). Previous work also showed that a stable-state triple helix, of low enthalpy (11.6 J g^{-1}), existed below ~about 0.1 mol of water/mol of residue. This experiment did not investigate such low levels of glycol, but in the absence of data to the contrary, we presume this state also exists at extremely low levels of glycol. The solvent-associated increase in enthalpy is therefore similar in both solvents (~45 J/g, or 4.2 kJ/mol of residue). Expressing this on a mole-solvent basis yields, for glycol: 4.2/0.4 = 10.5 kJ/mol of glycol; and for water: 4.2/0.8 = 5.3 kJ/mol of water. These values are similar to the latent heats of fusion of the two solvents (respectively, 11.2 kJ/mol and 6 kJ/mol), providing further support for the notion that most of the bond energy stabilizing the triple helix derives from the hydrogen bonds of the network of solvent molecules surrounding the molecule both in glycol and aqueous environments.

It is stressed that this solvent effect is evident at low concentrations, i.e., where the solvent interacts directly with the collagen. The similarity of the enthalpies in each solvent implies that the energy of the hydrogen bonding may be dominated by the number and layout of the fixed hydrogen bonding sites on the collagen itself, e.g., C=O, N—H, and hydroxyl groups on hydroxyproline, that are exposed to the solvent and available for supporting hydrogen-bonded solvent bridges (Bella et al., 1995; Kramer et al., 1999).

However, the recent paper of Raines and co-workers (Holmgren et al., 1998) points in an opposite direction. Their studies on the substitution of the hydroxyl group of hydroxyproline by fluorine, which does not form hydrogen bonds, in polypeptides suggest that the hydroxyl group acts through an inductive effect to stabilize the triple helix (Panasik et al., 1994; Eberhardt et al., 1996; Holmgren et al., 1998). They suggest that the immobilization of water molecules as water bridges is too expensive entropically (Holmgren et al., 1998). Engel and Prockop (1998), following these ideas and their earlier work on the stability of collagen-like peptides in non-aqueous solvents (Engel et al., 1977), further suggested that the water molecules seen surrounding the collagen molecule in structures defined by x-ray diffraction (Bella et al., 1995; Kramer et al., 1998) may not provide a significant bonding energy.

There is no doubt that the enthalpy of denaturation of collagen is much higher than most other proteins and the fundamental question is: from where does this energy derive? Unfortunately, the work of the Raines group provides no answer to this central question, as their proposed inductive mechanism for the stabilization of collagen by hydroxyproline is entropic and does not depend on the formation of new bonds. It has been widely accepted that the enormously large enthalpy of unfolding is the result of extensive hydrogen bonding caused by a network of hydro-

gen-bonded water molecules surrounding the molecule, as cogently argued by Privalov (1982) and others, and this explanation accords with the present findings. Hydration networks of 3–6 mol of water/tripeptide have indeed been revealed in the recent x-ray diffraction data of collagen-like peptides (Bella et al., 1994, 1995; Kramer et al., 1998, 1999). These data demonstrate that the collagen molecule does indeed pay the high entropic cost of immobilizing the water in the form of water bridges.

The x-ray studies clearly show a substantial deficit of direct hydrogen bonds in the collagen structure compared with the α-helix and β-sheet (Beck and Brodsky, 1998), yet the latter structures have much smaller enthalpies than collagen. The collagen structures determined by diffraction show a molecule with its X and Y residues pointing away from the center of the molecule, available to interact with its environment and with extended α-chains open for solvent interactions with the backbone groups. Thus, in contrast to the α-helix and β-sheet, the triple-helix structure is designed so that it exposes the unused hydrogen bonding sites along the α-chains to the environmental fluid that is available to hydrogen bond to them. Brodsky and co-workers (Bella et al., 1995) recent detailed x-ray studies suggest a critical role for hydroxyproline by linking two molecules via hydrogen bonds to a carbonyl on the backbone of an adjacent chain, making a water bridge similar to that originally proposed by Ramachandran et al. (1973).

The notion that water molecules are not involved in stabilizing the structure does not explain collagen's unusually high enthalpy of unfolding, which is the core problem in understanding the stability of a structure so bereft of internal stabilizing bonds. That this is caused by the disruption of a network of hydrogen-bonded water molecules surrounding the molecule, as elegantly identified by Brodsky and co-workers (Bella et al., 1995), is currently the most likely explanation.

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