Polymer-in-a-Box Mechanism for the Thermal Stabilization of Collagen Molecules in Fibers

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ABSTRACT Collagen molecules in solution unfold close to the maximum body temperature of the species of animal from which the molecules are extracted. It is therefore vital that collagen is stabilized during fiber formation. In this paper, our concept that the collagen molecule is thermally stabilized by loss of configurational entropy of the molecule in the fiber lattice, is refined by examining the process theoretically. Combining an equation for the entropy of a polymer-in-a-box with our previously published rate theory analysis of collagen denaturation, we have derived a hyperbolic relationship between the denaturation temperature, T_m, and the volume fraction, ϵ, of water in the fiber. DSC data were consistent with the model for water volume fractions greater than 0.2. At a water volume fraction of about 0.2, there was an abrupt change in the slope of the linear relationship between $1/T_m$ and ϵ. This may have been caused by a collapse of the gap-overlap fiber structure at low hydrations. At more than 6 moles water per tripeptide, the enthalpy of denaturation on a dry tendon basis was independent of hydration at 58.55 ± 0.59 J g^{-1}. Between about 6 and 1 moles water per tripeptide, dehydration caused a substantial loss of enthalpy of denaturation, caused by a loss of water bridges from the hydration network surrounding the triple helix. At very low hydrations (less than 1 mole of water per tripeptide), where there was not enough water to form bridges and only sufficient to hydrogen bond to primary binding sites on the peptide chains, the enthalpy was approximately constant at 11.6 ± 0.69 J g^{-1}. This was assigned mainly to the breaking of the direct hydrogen bonds between the alpha chains.

INTRODUCTION

Collagen molecules embedded within the lattice of a fiber are substantially more thermally stable than the same molecules in dilute solution (see, e.g., Na, 1989; Tiktopulo and Kajava, 1998).

One explanation of this behavior is the mechanism proposed by Flory and Garrett (1958). They suggested that denaturation is a melting process, a proposal that earned such wide acceptance that the word "melting" has since commonly been used synonymously with denaturation or unfolding to denote the helix-coil transition of collagen. In the model of Flory and Garrett, the chemical potential of the polymer repeating unit is equal in the solid and liquid phases at the equilibrium melting temperature (Flory, 1953). Accordingly, at the melting point of the polymer in the presence of solvent, the chemical potential of the pure solid polymer is in equilibrium with a liquid phase comprising a solution of liquid polymer and solvent. The presence of solvent therefore depresses the melting point by a mechanism that is analogous to the way in which a solute depresses the freezing point of a solvent. Flory used a thermodynamic analysis to establish that

$$\frac{1}{T_m} - \frac{1}{T_m^0} = \frac{R}{\Delta H_u}\left(\frac{V_u}{V_1}\right)(\epsilon_1 - \chi_1 \epsilon_1^2) \qquad (1)$$

where ϵ_1 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; ΔH_u is the heat of fusion per polymer repeating unit; (V_u/V_1) is the ratio of the molar volumes of this unit and the solvent; and χ_1 is an interaction parameter that equals zero for an ideal solution. In the work of Flory and Garrett, ethylene glycol was used as the solvent ($\chi_1 = 0.3$), and the data were shown to conform to the quasi-hyperbolic form of Eq. 1 so well that Flory and Garrett stated that "the observations conclusively show that the transformation is properly represented as a phase transition involving the melting of crystalline regions, rather than as a rate process."

We have reexamined the Flory-Garrett mechanism because our recent work has shown that the temperature and shape of the collagen denaturation endotherm in fibers and in basement membranes are governed by an irreversible rate process (Miles, 1993; Miles et al., 1995) and not by equilibrium thermodynamics, as previously supposed. In that work, we developed a kinetic model of the thermal unfolding process in which the collagen molecule differs in thermal stability along its length. In the process of thermal activation, which involves the partial uncoupling of the α-chains forming the collagen triple helix, a region of the molecule called the *thermally labile domain* unfolds first. Once the three α-chains in this region are uncoupled, the whole structure becomes unstable and unzips. Using scanning calorimetry to compare the activation enthalpy with the enthalpy of unfolding, we identified this thermally labile domain in type I collagen as a hydroxyproline-free sequence 65 residues long near the C-terminus of the molecule (Miles et al., 1995). Calorimetric measurement of the activation entropy in the fiber and in a quasi-solution state showed that

Received for publication 29 September 1998 and in final form 4 March 1999.

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0006-3495/99/06/3243/10 $2.00

stabilization in the fiber is brought about mainly by a reduction in the entropy of activation. We speculated that this is caused by a loss of configurational entropy induced by cross-linking and by spatial confinement of the molecule within the lattice of the fiber, as illustrated in Fig. 1.

However, we have not previously attempted to analyze this mechanism quantitatively. In this paper we will present an analysis of collagen denaturation in terms of the interaxial spacing of the collagen molecules in the fiber. Reducing the lateral dimensions of the lattice (by dehydration, for example) will reduce the configurational entropy of the thermally labile domain governing the rate of denaturation and thereby increase the temperature of denaturation. In collagen fibers that have been dehydrated to different levels, the interaxial spacing is directly related to the volume fraction of water, which can be measured gravimetrically.

There have been several previous phenomenological studies of the effect of hydration on the thermal stability of dehydrated collagen fibers (Finch and Ledward, 1972; Luescher, 1974; Kopp et al., 1990), but those papers have not attempted to explain the data in terms of a theoretical model. This paper reexamines experimentally the effect of solvent concentration on the denaturation endotherm of collagen in fibers and proposes an explanation of the observations in terms of a polymer-in-a-box model. This model combines the previously proposed equations relating the temperature and shape of the endotherm to the magnitudes of the activation enthalpy and entropy of the underlying rate process (Miles, 1993; Miles et al., 1995) with previously published equations for the entropy of a polymer in a box (Doi and Edwards, 1986).

We envisage that a collagen molecule in a fiber is constrained by adjacent molecules in the lattice as if it were confined within a box, and this provides a quantitative explanation for the effect of dehydration on the denaturation temperature and predicts a sudden stabilization when collagen molecules form fibers from solution. The model also provides the potential for predicting effects such as fiber shrinkage and lateral swelling, an explanation for the retarding influence of fiber tension, and a means of differentiating between possible molecular packing arrangements.

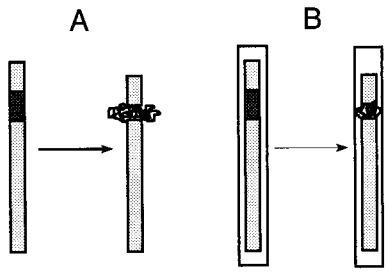

FIGURE 1 Simplified longitudinal cross section illustrating the thermal activation of the collagen molecule in solution (*A*) and in a fiber (*B*). The native triple helix is represented as a rectangle, and the thermally labile region is highlighted. In the fiber the number of possible configurations in the activated state is constrained by the presence of adjacent molecules within the lattice (represented by a *box*); this reduces the configurational entropy of activation and thereby increases the Gibbs free energy of activation, consequently stabilizing the structure.

THEORY: POLYMER-IN-A-BOX

Doi and Edwards (1986) relate the entropy of a polymer within a box to the box dimensions. They show that, if the polymer is small in relation to the size of the box, the effect of the box on the entropy is negligible, but if the size of the polymer is comparable to the dimensions of the box, the entropy of the polymer is reduced. Their equation was derived for a single-chain polymer whose ends are attached to opposite ends of a box of length L and cross-sectional area a_0^2. Using that equation (their Eq. 6.68), we may write the following for the activation entropy of a single molecule of collagen in a box:

$$\Delta s^{\Updownarrow} = \Delta s_0^{\Updownarrow} - 3k\left(\frac{3L^2}{2Nb^2} + \alpha_0 \frac{Nb^2}{a_0^2}\right) \tag{2}$$

The factor 3 multiplied by k in Eq. 2 arises because the activated state consists of three uncoupled α-chains, and each of these contributes equally to the entropy. $\Delta s_0^{\Updownarrow}$ represents the entropy of activation of the thermally labile domain in the absence of the box (i.e., in dilute solution) or in the presence of a box whose dimensions are very large (such as immature fibers swollen in acetic acid). We have previously identified the process of thermal activation of type I collagen with the uncoupling of the three α-chains in the thermally labile domain near the C-terminus of the molecule (Miles et al., 1995). Because this labile domain is situated in the gap zone, where it has space to expand laterally on thermal activation, the differences in thermal stability along the length of the molecule that exist in the isolated molecule are accentuated when the molecule is embedded in a fiber. It is therefore likely that the thermally labile domain of the molecule in dilute solution is the same region of the molecule in the fiber. However, this is not necessarily the case and is not required by the analysis. The process of thermal activation could also involve the uncoupling of α-chains in any region of the molecule; specifically, the analysis does not require that this region be the thermally labile domain that we have previously identified. The width of the box is a_0, and $\boldsymbol{\alpha}_0$ is a numerical factor dependent on the lateral cross section; for example, if it is a square of side a_0, $\boldsymbol{\alpha}_0 = \pi^2/3$. b denotes the effective bond length of the polymer, which consists of N links and is related to the actual bond length, b_0, via the equation (see Doi and Edwards, 1986; Flory, 1967; Richards, 1980)

$$b^2 = C_N b_0^2 \tag{3}$$

where, for a freely jointed chain, the coefficient, C_N, is unity for all values of N. For a stiff chain, C_N depends on N but asymptotically attains the value C_∞ when the polymer is sufficiently long. C_∞ is referred to as the "stiffness" of the polymer by Doi and Edwards and as the "characteristic ratio" by Flory (1967) and Richards (1980). For real poly-

mers, C_∞ is typically greater than unity (see Flory, 1967; Richards, 1980). Flory's (1967) statistical mechanics analysis of proteins considers the polypeptide chain to be made up of N virtual bonds, which are imagined to connect the α-carbon atoms of adjacent peptides in the chain.

Inserting Eq. 3 in Eq. 2 and allowing for the possibility that the activation process involves only a fraction, f, of the molecules in a mole, we may write Eq. 2 on a molar basis:

$$\Delta S^{\Updownarrow} = f\left[N_0 \Delta s_0^{\Updownarrow} - 3R\left(\frac{3L^2}{2C_N N b_0^2} + \alpha_0 \frac{C_N N b_0^2}{a_0^2}\right)\right] \quad (4)$$

Here N_0 represents Avogadro's number, and S represents the entropy per mole of the thermally labile domain.

To estimate the cross-sectional area of the box, it is necessary to know precisely how the collagen molecules are arranged in the fiber. In fact, this has not been unequivocally established (Wess et al., 1998). Fraser et al. (1987), on the basis of x-ray diffraction data, have suggested various possible molecular packing arrangements. In these structures the thermally labile domain, which occurs just below level 4 in the Fraser diagrams, is either surrounded in a quasi-hexagonal array by five molecules and one gap, or by four molecules and two gaps. The areas of cross section of the box in these arrangements are therefore approximately $2d_0^2$, and $3d_0^2$, respectively, where d_0 is the interaxial spacing of collagen molecules in the fiber.

In addition, we consider the case in which the gap region under extreme levels of dehydration collapses to give no gaps, and the area of cross section is simply d_0^2. Thus in general we may write

$$a_0^2 = n d_0^2 \quad (5)$$

Possible values for n and f for the different packing arrangements are given in Fig. 2 and Table 1. In reality, collagen packing is more complicated. The x-ray diffraction patterns contain a considerable amount of diffuse scatter (e.g., Wess et al., 1998), indicating substantial disorder in the fiber. Hulmes et al. (1995) suggest that the puzzling equatorial diffraction pattern of collagen fibers is best explained by a radially packed cylinder model in which liquid-like disorder is introduced into the packing structure. Thus a mixture of packing arrangements of the sort indicated in Table 1 and Fig. 2 may be more appropriate than any single arrangement and may contribute to the real values of f and n.

In a fiber in which the diameter of the collagen molecule is taken as d, the volume fraction of collagen is ϵ and the volume fraction of water is ϵ_w:

$$\frac{\textit{Volume of collagen}}{\textit{Volume of fiber}} = \epsilon = 1 - \epsilon_w = \Psi \frac{d^2}{d_0^2} \quad (6)$$

where Ψ is a numerical coefficient that in general is a slowly varying function of $(d/d_0)^2$. For two noninteracting components, Ψ is constant. Thus at intermediate and high water contents, when the collagen molecule is fully hydrated, Ψ is approximately constant. Flory and Garrett (1958) effectively equated Ψ to 1 when they estimated the volume fraction of collagen as the square of the ratio of the swollen and unswollen fiber diameters. For a hypothetically hexagonally packed quarter stagger fiber, $\Psi = 0.88$ (i.e., 4.4/5). Inserting Eq. 5 and Eq. 6 in Eq. 4 yields

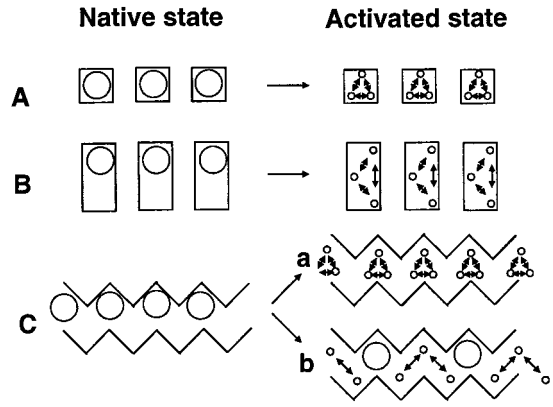

FIGURE 2 A diagram schematically illustrating some possible transverse cross sections through the thermally labile domain of collagen enclosed within the "box" formed by adjacent molecules. The drawings on the left-hand side of the figure refer to the native state; those on the right indicate the activated state in which the α-chains of the thermally labile domain are uncoupled. The large circles represent the triple helix; the three small circles with arrows represent the uncoupled α-chains, which are free to explore the "box." Three basic packing arrangements (discussed in the text) are envisaged, depending on the number of gaps surrounding the thermally labile domain in the quasi-hexagonal packing of molecules in a fiber. (*A*) No gaps, a collapsed structure, possibly relevant to low hydration levels. Here $f = 1$, $n = 1$. (B) One gap, corresponding to structures labeled 3A and 3B by Fraser et al. (1987). Here $f = 1$, $n = 2$. (*C*) Two gaps, corresponding to structures labeled 2A and 2B by Fraser et al. (1987). Two activation scenarios are envisaged. In *a* all molecules are activated simultaneously and $f = 1$, $n = 2$. In *b* half of the molecules only are activated and $f = ½$, $n = 3$. The meanings of f and n are given in the text.

$$\Delta S^{\Updownarrow} = f\left[N_0 \Delta s_0^{\Updownarrow} - 3R\left(\frac{3L^2}{2C_N N b_0^2} + \alpha_0 \frac{C_N N b_0^2 (1 - \epsilon_w)}{\Psi n d^2}\right)\right] \quad (7)$$

Equation 7 is the required expression that relates the activation entropy of the collagen molecule in a fiber to the volume fraction of water.

We have previously shown (Miles, 1993; Miles et al., 1995) that the temperature, T_m, at which the denaturation endotherm is a maximum depends on the rate, r, at which the temperature is scanned, the rate constant, $k(T_m)$, and its activation enthalpy, $\Delta H^{\Updownarrow}$, according to the equation

$$\frac{r}{T_m k(T_m)}\left(\frac{\Delta H^{\Updownarrow}}{RT_m} + 1\right) = 1 \quad (8)$$

where

$$k(T_m) = \frac{\kappa T_m}{h} \exp\left(\frac{-\Delta H^{\Updownarrow}}{RT_m}\right) \exp\left(\frac{\Delta S^{\Updownarrow}}{R}\right) \quad (9)$$

Inserting Eq. 9 into Eq. 8 and taking logarithms, we obtain

$$\Delta S^{\Updownarrow} = R\left[\ln\left(\frac{rh}{\kappa T_m^2}\left(\frac{\Delta H^{\Updownarrow}}{RT_m} + 1\right)\right) + \frac{\Delta H^{\Updownarrow}}{RT_m}\right] \quad (10)$$

TABLE 1 Example values of *f* and *n* for different packing arrangements of the collagen molecule in the fiber

Structure	No. of gaps surrounding each thermally labile domain	Diagram no. (Fraser et al., 1987)	*n*	*f*	Slope* K^{-1}
Collapsed structure at very low hydrations	0		1	1	$1.24 \times 10^{-4}\ \alpha_0 C_N/\Psi$
Intermediate and high hydrations	1	(3A, 3B)	2	1	$6.22 \times 10^{-5}\ \alpha_0 C_N/\Psi$
	2	(2A, 2B)	2	1	$6.22 \times 10^{-5}\ \alpha_0 C_N/\Psi$
	2	(2A, 2B)	3	½	$2.07 \times 10^{-5}\ \alpha_0 C_N/\Psi$

These values are used to predict the slope of the $1/T$ versus ϵ_w line in terms of the unknown stiffness coefficient C_N, with the aid of Eq. 13.
*$3\alpha_0 f C_N$ ($Nb_0^2/0.88\ nd^2$) ($R/\Delta H^{\Updownarrow}$). See Eq. 13. Calculations were based on the values of *f* and *n* in the table. Other parameters from the literature were as follows: $R = 8.314$ J mol^{-1} K^{-1}; $b_0 = 0.38 \times 10^{-9}$ nm; $d = 1.2 \times 10^{-9}$ nm; $\Delta H^* = 1.306 \times 10^6$ J mol^{-1}; $N = 65$.

Hence combining Eq. 7 and Eq. 10 yields

$$\ln\left(\frac{rh}{\kappa T_m^2}\left(\frac{\Delta H^{\Updownarrow}}{RT_m}+1\right)\right)+\frac{\Delta H^{\Updownarrow}}{RT_m} = f\left[\frac{N_0\Delta s_0^{\Updownarrow}}{R}-3\left(\frac{3L^2}{2C_N Nb_0^2}+\alpha_0\frac{C_N Nb_0^2(1-\epsilon_w)}{\Psi nd^2}\right)\right] \quad (11)$$

As this paper is concerned mainly with the effect of changes in the lateral dimensions of the box, it is convenient to eliminate L from the analysis. However, it should be noted that L will increase with tension, and consequently $\Delta S^{\Updownarrow}$ will decline with tension. The hypothesis that is developed in this paper therefore immediately provides a potential explanation for the retarding influence of tension on the thermal denaturation of cross-linked collagen fibers. We will refer to this again in the Discussion, but for the moment this is a side issue and is not directly involved in the logic of this analysis.

Let the temperature of the peak be T_0 at a particular volume fraction of water, ϵ_{w0}. The relation between T_0 and ϵ_{w0} is given simply by replacing ϵ_w with ϵ_{w0} and T_m with T_0 in Eq. 11. Subtracting this result from Eq. 11 and using the fact that $(\Delta H^{\Updownarrow}/RT) \gg 1$ yields the approximation

$$3\ln\left(\frac{T_0}{T_m}\right)+\frac{\Delta H^{\Updownarrow}}{R}\left(\frac{1}{T_m}-\frac{1}{T_0}\right)=3\alpha_0 f\frac{C_N Nb_0^2}{\Psi nd^2}(\epsilon_w-\epsilon_{w0}) \quad (12)$$

As the first term on the left-hand side of Eq. 14 is very much smaller than the second, we can also write the simpler approximation

$$\frac{\Delta H^{\Updownarrow}}{R}\left(\frac{1}{T_m}-\frac{1}{T_0}\right)=3\alpha_0 f\frac{C_N Nb_0^2}{\Psi nd^2}(\epsilon_w-\epsilon_{w0}) \quad (13)$$

According to Eq. 13, there should be a hyperbolic relation between the temperature of denaturation, T_m, and the volume fraction of water in the fiber, ϵ_w. Interestingly, the slope of the relation between $1/T_m$ and ϵ_w involves the ratio $(\Delta H^{\Updownarrow}/3N)$, which is the enthalpy of activation per mol residue. This is not strongly dependent on the size of the activated state.

It is emphasized that this analysis is concerned with the water that is loosely held between the collagen molecules (i.e., it is applicable to intermediate hydration levels). We are not concerned with water molecules, such as the water-bridge molecules, which are tightly bound to the collagen molecule (i.e., those remaining at low hydration levels) or the extrafibrillar water, which occurs as a separate phase at high hydrations.

MATERIALS AND METHODS

The methods are intended to change the lateral spacing of the collagen molecules within the native fiber lattice simply by adding water or taking it away. Collagen denaturation in fully hydrated native rat tail tendon fibrils bathed in water was previously shown to be governed by a rate process (Miles et al., 1995), and that system was therefore suitable for these experiments. The method of preparing the tendons followed the earlier work, and no pH buffer was used in any of the procedures, nor were enzyme inhibitors added.

Tendon preparation

Tails were excised from the carcasses of 5–8-week-old rats and frozen at −20°C until required. On removal from the freezer, tails were thawed, and the tendons were removed, cleaned of all visible contaminants, and washed in distilled water. The tendons were left in distilled water at 5°C until calorimetric measurements were made on weighed samples placed in Perkin-Elmer "volatile sample pans." Measurements at high hydrations were carried out on samples of tendon suspended in different volumes of water. To produce dehydrated samples, samples of tendon were placed in the pans and dried to different levels by exposure to a vacuum, or by freeze drying. The samples were then stored in a desiccator for different lengths of time with a desiccant that was silica gel, P_2O_5, $Mg(ClO_4)_2$, or BaO. Leaving the samples in these conditions or exposing samples to moist air for different lengths of time yielded samples with very low, low, or intermediate hydrations. Samples were sealed in the Perkin-Elmer sample pans and left to equilibrate before calorimetry.

Calorimetry

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 from 5°C to a specified temperature T, using an empty pan as a reference. Preliminary experiments showed, as expected for a rate process (Miles et al., 1995), that there were linear increases in T_{max} with log (scanning rate), whereas the enthalpy of denaturation was independent of scanning rate. All of the quoted data were measured at a scanning rate of 10°C/min. After calorimetry, the sample pans were pierced and dried over silica gel in an oven at 105°C for the calculation of dry matter. Preliminary experiments indicated that this was more effective than freeze drying, but

no correction was made to allow for any residual water that may have remained in the sample.

RESULTS

The calorimetric study generated data for collagen denaturation in fibers that ranged in their level of hydration over four orders of magnitude, and it is therefore convenient to display the results with hydration on a logarithmic scale (Fig. 3). Fig. 3 gives an overview of how the temperature, enthalpy, and width of the collagen denaturation were affected by the level of hydration.

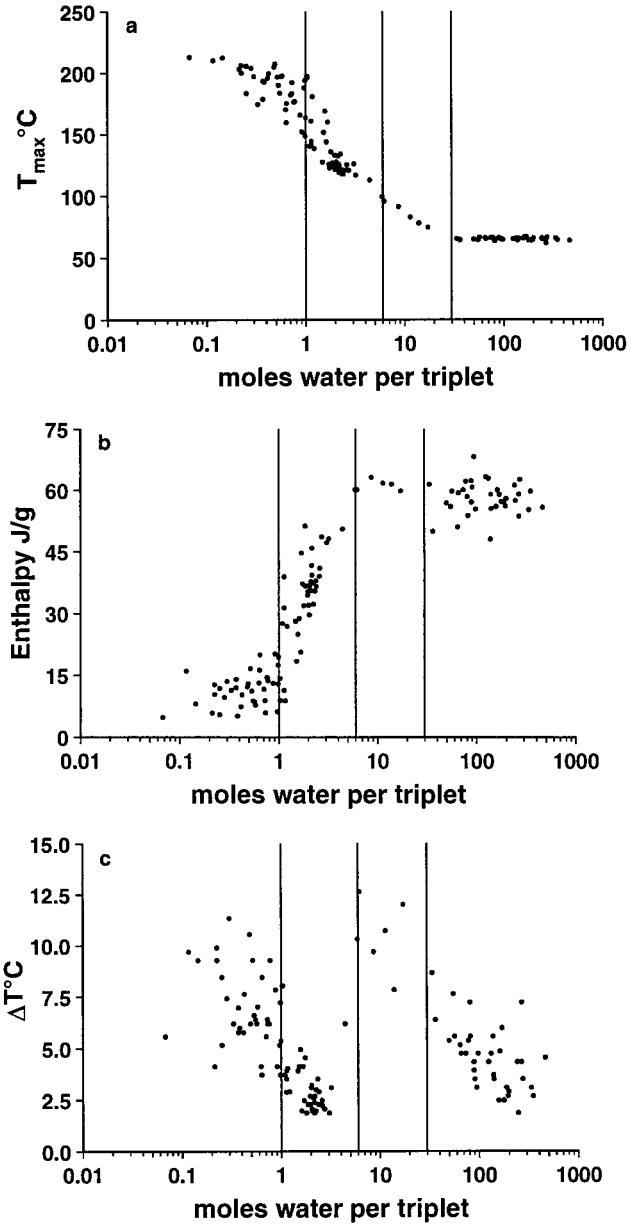

FIGURE 3 The effect of hydration on the thermal denaturation characteristics of collagen in native fibers. All data were determined at a scanning rate of 10°C/min. (*a*) Temperature of denaturation. (*b*) Enthalpy of denaturation. (*c*) The width of the endotherm at half-height. The vertical lines at 1, 6, and 30 moles water per tripeptide represent divisions between the hydration ranges discussed in the text: very low (Region IV), low (Region III), intermediate (Region II), and high (Region I).

Regions of hydration

Four regions can be identified.

Region I

At high hydrations, above 30 molecules of water per three-residue (Gly-X-Y) unit, the temperature and enthalpy of the endotherm were constant (see Fig. 3 and Table 2). In this region the fiber had swollen to its maximum extent in water, and the water existed in two phases (intrafibrillar water and external water). The addition of water merely increased the quantity of water in the external pool and had no affect on the intrafibrillar water or the interaxial molecular spacing of the collagen molecules in the fiber lattice.

Region II

At intermediate hydration levels, between six and 30 molecules of water per Gly-X-Y unit, the temperature of the denaturation increased with dehydration, yet the enthalpy of denaturation remained constant (see Fig. 3, Table 2).

Region III

At low hydrations, between about one and six molecules of water per Gly-X-Y unit, the enthalpy of denaturation rapidly declined with decreasing hydration, while the temperature of denaturation continued to rise. In this region the width of the peak showed a strong minimum at about three water molecules per triplet (see Fig. 3).

Region IV

At very low hydrations, below about one molecule of water per triplet, the enthalpy of denaturation was approximately constant (see Fig. 3 and Table 2), while the temperature of denaturation continued to rise with decreasing hydration.

In regions II, III, and IV, the volume fraction of water in the sample can be equated to the volume fraction of water in the fiber, ϵ_w.

Examples of the DSC thermograms of samples taken from each of these regions of hydration are shown in Fig. 4. Notice the single narrow peak in region I, the broad multicomponent peak of region II, the substantial narrowing of the peak and reduction in enthalpy in region III, and the broader very small peak in region IV.

Identification of the denaturation endotherm

This work provides the first observations of the collagen denaturation at very low levels of hydration (region IV). The following observations confirmed that the endotherm in this region was caused by collagen denaturation:

TABLE 2 Summary of how the collagen endotherm characteristics changed with increasing hydration

Hydration range (moles H_2O per triplet)	T_{max} (°C)	Enthalpy (J/g dry matter)	ΔT (°C)
0.07–1	Fell with hydration*	11.6 (37, 0.69)	Fell with hydration#
1–6	Fell with hydration*	Increased with hydration#	Exhibited a minimum at three moles water per triplet#
6–30	Fell with hydration*		Fell with hydration#
30–460	65.1 (36, 0.18)	58.55 (42, 0.59)	4.55 (36, 0.26)

Over certain ranges of hydration, some endotherm traits were constant and mean values are quoted. Data in parentheses represent the number of samples measured and the standard error.
*See Table 3.
#See Fig. 3.

(a) When a sample was scanned to a temperature just below the start of the endotherm, immediately cooled, and rescanned in the presence of excess water, a peak typical of collagen denaturation was observed (Fig. 5). This showed that the absence of an endotherm reliably indicated the absence of denaturation. When a similar sample was scanned to just above the completion of the endotherm, immediately cooled, and rescanned in the presence of excess water, no collagen denaturation endotherm was apparent. This showed that the endotherm was coincident with the disappearance of native triple helices.

(b) When a sample pan was opened and the fiber examined visually, after scanning to just above completion of the endotherm, the fiber had shrunk longitudinally, indicating a helix-to-random coil transition. Another sample pan opened just before the endotherm peak showed no evidence of shrinkage. The endotherm was thus coincident with longitudinal shrinkage, confirming that it was caused by a helix-coil transition.

Polymer in a box

As predicted by Eq. 13, plotting $1/T_m$ against the volume fraction of water in the sample (see Fig. 6) yielded a straight line with positive slope in the intermediate hydration range $0.2 \leq \epsilon \leq 0.8$. Numerical details of the regression line are given in Table 3. The observed gradient yielded reasonable estimates for C_N (Table 1), particularly for the case where $f = ½$, $n = 3$ (viz. $C_N = 9.4 \pm 0.5$, on the basis that $\alpha_0 = \pi^2/3$ and $\Psi = 0.88$). At higher water contents, the volume fraction of water in the fiber was constant, and therefore, as expected, the denaturation temperature was constant. Therefore the data were consistent with the polymer-in-a-box hypothesis over the range $\epsilon \geq 0.2$. At lower hydrations, $\epsilon < 0.2$, the gradient was much steeper, indicating a rearranged structure with estimates of C_N, made as above, on the basis of the slope at very low hydrations ranging from 6.1, if $n = 1$ and $f = 1$, to 12.2, if $n = 2$ and $f = 1$ (compare Tables 1 and 3).

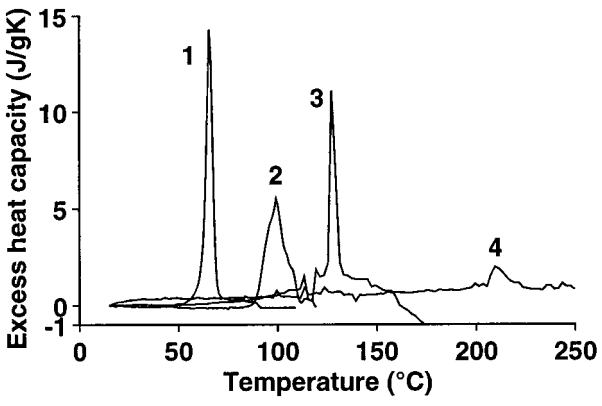

FIGURE 4 Differential scanning calorimetry thermograms of collagen in fibers at different hydrations. All scans were made at a rate of 10°C/min. (1) In excess water (88.2% v/v water), Region I. (2) 48.5% water v/v, Region II. (3) 14.3% water v/v, Region III. (4) 0.9% water v/v, Region IV.

DISCUSSION

In the first part of this discussion, the measurements recorded in this work will be compared with similar data recorded in the published literature. This provides support

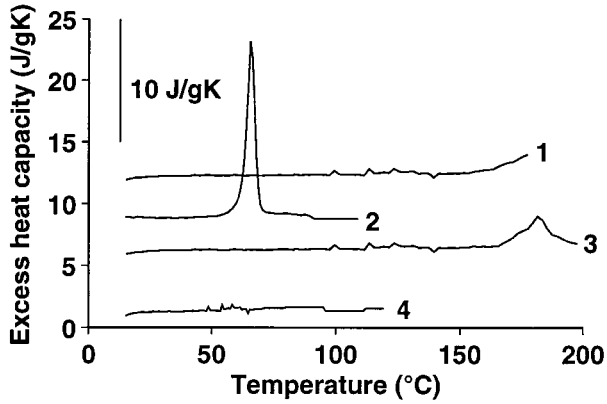

FIGURE 5 DSC evidence to show that the small peak observed at very low hydrations (Region IV) was caused by collagen denaturation. All scans were made at a rate of 10°C/min. (1) Dry collagen fiber scanned to the leading edge of the denaturation endotherm. (2) When the previous sample (number 1) was rapidly cooled, the pan was opened, and the sample was resealed with excess water in a new pan, the endotherm characteristic of fully hydrated native collagen was observed. (3) Collagen fiber scanned to the end of the denaturation endotherm. (4) When the previous sample (number 3) was rehydrated in excess water and rescanned, there was no evidence of the endotherm for native collagen.

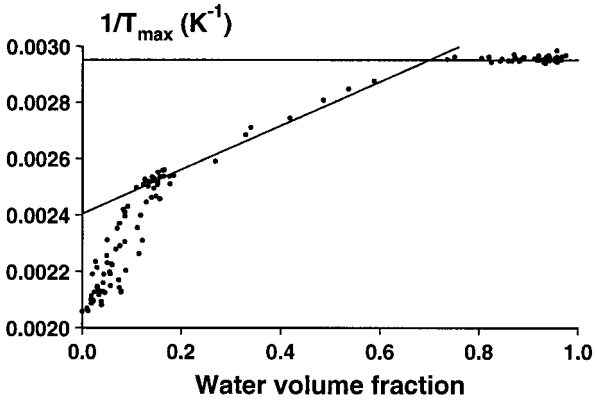

FIGURE 6 Relation between the temperature T_m and the concentration of water in the sample, expressed as a volume fraction. A linear relation between $1/T_m$ and the volume fraction of water in the fiber is predicted by the polymer-in-a-box model (Eq. 13). All data were obtained at a scanning rate of 10°C/min.

for the reliability of the present data. The rest of the discussion analyzes the extent to which the data agree with predictions based on the polymer-in-a-box hypothesis, which the experiment was designed to test, and suggests reasons for the deviations observed at very low hydrations.

Comparisons with previous work

The substantial increase in the thermal stability of collagen induced by dehydration, which has been recorded in this study, is consistent with previous reports by others (e.g., Flory and Garrett, 1958; Monaselidze and Bakradze, 1969; Finch and Ledward, 1972; Luescher, 1974; Kopp et al., 1990; Bigi et al., 1987), but the present work has extended the hydration range beyond that recorded previously. Finch and Ledward (1972), for example, merely reported that the T_{max} in collagen fibers containing 13% water by mass was 112°C, compared with 66°C for fully hydrated fibers at neutral pH. These values may be compared with the results recorded here for single fibers with similar water contents: at 11.5%, 119°C, and at 15%, 96°C, and the mean value for fully hydrated fibers (Table 2) of 65.5°C. The effect of hydration on the thermal stability of collagen fibers was more extensively studied by Luescher (1974) and Kopp et al. (1990), and the data of those studies show an approximately hyperbolic relation between temperature and hydration, as confirmed in the present work, but those data were discussed only qualitatively, and no mathematical relation was proposed or fitted. Furthermore, the earlier work did not extend the dehydration levels to the low values studied here (region IV, corresponding to water concentrations of one molecule or less per Gly-X-Y unit), and consequently the present work is the only study showing denaturations in the temperature range 150–210°C.

All previous studies in which the effect has been examined have shown a decline in the enthalpy of denaturation of collagen with dehydration below a given level (see Luescher, 1974; Kopp et al., 1990), agreeing with the observations made here (see Fig. 3). However, measurements have not previously been made at very low hydration levels. Specifically, our region IV, corresponding to water concentrations of one molecule or less water per Gly-X-Y unit, has not previously been studied. The results of the present work show that in this region collagen has a low but approximately constant enthalpy, independent of water content (see Table 2, Fig. 3). The low enthalpy is ~1/5 of the enthalpy of the fully hydrated triple helix, which implies that, compared with the fully hydrated state, the triple helix at very low hydrations is held together by ~1/5 of its original number of hydrogen bonds. We propose that the identity of the very low hydration bond is the direct hydrogen bond connecting the H-N group of the glycine residue on one chain to a C═O group on an adjacent chain. Furthermore, our data show that this very low hydration triple helix is native and can be reversibly converted to the fully hydrated triple helix by the addition of water. We therefore propose that it is effectively the non-water-bridge structure proposed by Rich and Crick (1961). Previous studies have estimated the enthalpy at zero water as zero simply by extrapolating the enthalpy data measured at low concentrations; this implies a random-coil state at zero hydration, which conflicts with our new measurements.

There is only one previous study that examined the effect of hydration on the half-width of the collagen denaturation endotherm (Monaselidze and Bakradze, 1969), and it is not strictly comparable; it studied the effect on dehydrated solutions of collagen, not collagen fibers. However, interestingly, that work showed a substantial increase in width with dehydration up to a maximum of ~15°C in a 70% collagen gel, and this was followed by a rapid fall to ~2°C at 95% collagen (the maximum dehydration recorded). This behavior follows that recorded here in fibers (see Fig. 3). The present data also show that fibers exhibit an increase in width at lower hydrations, and it would be interesting to find out whether collagen gels show the same effect.

Finally, to conclude the first part of the discussion, it should be noted that the values of the enthalpy of collagen denaturation and T_{max} that are recorded here at high hydrations (see Fig. 2 and Table 2) are consistent with previous calorimetric measurements (some early work is conveniently summarized by Lim, 1976; see also Miles et al., 1995).

Having confirmed the reliability of the data obtained in these experiments by comparison with previous work, we now analyze the extent to which the data agree with predictions based on the polymer-in-a-box hypothesis, which the present experiment was performed to test.

Polymer-in-a-box hypothesis

Considering first the data at intermediate hydrations, it is interesting to calculate the intercepts at $\epsilon_w = 0$ and 1. Extrapolation to $\epsilon_w = 1$ yields a T_{max} of 41.1 ± 2.2°C. This

TABLE 3 Regression line characteristics of $1/T_m$ against volume fraction of water in the fiber

Hydration range (moles H_2O per triplet)	Slope (standard error) (K^{-1})	Intercept (standard error) (K^{-1})	Residual standard deviation (K^{-1})
0.07–1.0	0.00283 (0.00048)	0.002053 (0.000022)	0.000058
1–6	0.00208 (0.00033)	0.002168 (0.000048)	0.000082
6–30	0.000731 (0.000042)	0.002451 (0.000019)	0.0000098
30–460*	0	0.002956 (0.0000015)	0.0000093

*In the range 30–460 moles water per triplet, the regression was not significant, and the mean, standard error, and standard deviation are quoted.

represents the denaturation temperature in a hypothetical state in which the collagen molecules are in a fiber with infinite interaxial spacing. This is a quasi-solution state, and it is significant that this temperature is close to the temperature observed when collagen is in dilute solution or when in a grossly swollen fiber. The intersection of the regression line with the T_{max} value for the fully hydrated fiber (region I) occurs at $\epsilon_w = 0.69$. That is, the results show that the fully hydrated fiber in water contained 69% water by volume. Extrapolation of the regression line to $\epsilon = 0$ yields a T_{max} of 134.8 ± 2.9°C. Fig. 6 shows that this is much lower than the denaturation temperature of collagen in the fully dehydrated fiber; it represents the denaturation temperature of a hypothetical condition in which the hydrated polymer is surrounded by similar hydrated molecules in a quarter-stagger arrangement in the absence of water, i.e., touching one another laterally. This value is similar to the intercept values recorded by Flory and Garrett (1958), Luescher (1974), and Kopp et al. (1990). In reality, a regular quarter-stagger arrangement of the molecules is unlikely to be maintained at all hydrations, and we propose that at low hydrations the molecules rearrange into a collapsed structure to compensate for the loss of water from the gaps. Equation 13 shows that the slope of the relation between $1/T_m$ and ϵ_w depends on the packing of the molecules in the fiber, as represented by the parameters f and n. Table 1 considers the effect on the slope of various possible packing arrangements, two of which ($f = 1$, $n = 1$ and $f = 1$, $n = 2$) straddle the observed value (as revealed by the estimates of C_N; see Results). These are more tightly packed structures and therefore are better approximations of the proposed collapsed state. Thus a change in packing arrangement could account for the substantially increased gradient of the $1/T_m$ versus ϵ_w locus at low hydrations (see Fig. 6).

In addition, simultaneously with the rearrangement in the molecular packing of collagen in the fiber, water molecules that are connected via hydrogen bonds to the triple helix itself are gradually stripped away, as the fiber is further and further dehydrated. Some of these molecules are involved in water bridge structures that connect one α-chain to another. These hydrogen bonds would need to be broken on denaturation. Thus stripping away of water bridge molecules has several effects. First, it reduces the enthalpy of denaturation because fewer hydrogen bonds need to be broken to separate the α-chains and reduce them to more or less random coils. This is indeed observed in region III (see Results). The reduction in the number of water bridges would be expected to increase the size of the thermally labile domain, which is thought to be controlled in the fully hydrated state by a local deficiency of water bridges caused by the absence of the water-bridge-enhancing residue hydroxyproline (Miles et al., 1995, 1998). An increase in the size of the thermally labile domain would be seen thermally as a decrease in the width of the endotherm. A sudden reduction in the width is indeed observed in exactly the expected hydration range (see Fig. 3). As the size of the thermally labile domain affects only the intercept of the regression between $1/T_m$ and ϵ_w, and not its slope, the effect of the reduction in the number of water bridges (region III) is to increase the scatter of the data about a regression line.

In region IV, with one molecule or less of water per Gly-X-Y, the enthalpy was unaffected by changes in water concentration, indicating that this water is not involved in forming interchain water bridges. In this region, changes in water content should affect the dimensions of the thermally labile domain, and consequently, Eq. 13 should again apply exactly, with an appropriate choice of the packing parameters f and n. In this region, therefore, there should be less scatter of the data about the regression line, as is indeed indicated by the data in Table 3. Although highly thermally stable in the dehydrated fiber, the collagen molecule in this hydration range is intrinsically highly unstable, because it is devoid of all stabilizing water bridges. A measure of the instability may be obtained by extrapolating the regression line (Table 3) to $\epsilon_w = 1$, which yields a denaturation temperature, T_{max}, of −68.4 ± 18.3°C. We suggest that this very unstable structure of collagen is of a hypothetical, fully dehydrated collagen molecule that has been isolated from the stabilizing influence of adjacent collagen molecules. This may conflict with the suggestion of Holmgren et al. (1998) that water bridges do not contribute to the stability of the collagen molecule.

This work has concerned fibers that had been equilibrated to a given hydration for a long period of time and gives no information about the effect of time on the thermal stability. The polymer-in-a-box model requires that the thermal stability is determined by the box dimensions, and consequently one would expect that the stabilization that occurs on fiber formation would be instantaneous. The experiments of Na (1989) confirm that a substantial part of the thermal stabilization is coincident with, or practically coincident with, the formation of fibers. Na found that he could detect fibrillogenesis as a small endotherm at ~25°C when concentrated neutral solutions of collagen were scanned in a

DSC. On continuing scanning above 25°C, the denaturation endotherm characteristic of collagen in solution was absent and replaced by a new endotherm at ~55°C, representing denaturation of collagen in the fiber. His data clearly show that an initial substantial stabilization of collagen was practically complete 30 min after fiber formation and was not the result of some slow chemical change, such as the development of intermolecular cross-links. Thus the observations of Na are consistent with the polymer-in-a-box model.

Swelling the immature fiber by the addition of 0.5 M acetic acid produces a quasi-solution state with a denaturation temperature very close to that of a dilute solution (Miles et al., 1995). As far as one can tell, the processes of swelling and loss of thermal stability are coincident. It is as if the mere confinement within the fiber lattice is enough to thermally stabilize the collagen molecule, also consistent with the polymer-in-a-box model.

The equilibrium established between the water molecules forming water bridges around the triple helix and other water molecules present in the fiber is affected by water concentration. At total water levels of six molecules per Gly-X-Y and over, all possible water-bridges are formed, and the enthalpy of denaturation is therefore unaffected by an increase in water content (Fig. 3 *b*). Below six molecules, the equilibrium condition requires water bridge disruption, and at a total water content of about two water molecules per Gly-X-Y the reduction in enthalpy of denaturation indicates that about half the water bridge hydrogen bonds are broken (Fig. 3 *b*). We may therefore deduce from these data that the number of water bridge molecules per Gly-X-Y is less than six; kinetic models would be needed to establish the exact form of the equilibrium and hence the precise number of water bridges.

All of the experiments and analysis described in this paper have concerned the thermal denaturation of mechanically unrestrained tendon, but the polymer-in-a-box model could be generalized to include fibers in tension. Here the thermally labile domain in its activated, uncoupled, random-coil state would be longer and of reduced entropy compared with the mechanically unconstrained, zero-tension state. (The relation between tension and length is given analytically by the equation given by Doi and Edwards.) Consequently, the rate of denaturation would be reduced and the fiber stabilized.

Another consequence of the polymer-in-a-box model is that it will predict an elastic fiber when the collagen is denatured in the presence of substantial thermally stable cross-linking, and again the elasticity should be derivable analytically from the equations given by Doi and Edwards (1986).

Finally, as the random-coil state is confined by the dimensions of the fiber lattice, the polymer-in-a-box model predicts a radial, outward pressure on thermal denaturation, and the forms of these equations are also derivable from the equations given by Doi and Edwards (1986).

The denaturation temperature of collagen in native collagen fibers is 23–27°C higher than that of collagen in solution (see Bailey, 1968, for a summary of early measurements; also Burjanadze, 1982), and the difference in temperatures between fiber and solution was remarkably constant across animal phyla, hydroxyproline content, and denaturation temperature. That this stabilization should be approximately constant and independent of hydroxyproline content is consistent with the polymer-in-a-box hypothesis, which demands that the stabilization depend only on the dimensions of the box.

This study has shown that the confinement of collagen molecules within the fiber lattice reduces the configurational entropy of the activated, uncoupled, random-coil, thermally labile domain and thereby thermally stabilizes the collagen molecule. The proposed mechanism is fundamental and must apply not only to the fibrillar collagens in fibers, but also to other macromolecular assemblies. We therefore propose that there will be a polymer-in-a-box contribution to the thermal stability of all biological macromolecules in macromolecular assemblies, such as the Type IV collagen in the network structure of basement membranes, the RNA molecules in ribosomes, the myosin tail in the thick filament, and the nucleic acids in virus structures. As far as we are aware, this mechanism of thermal stabilization has not been proposed hitherto.

We are grateful to A. J. Bailey and T. V. Burjanadze for discussion.

This work was supported by the Wellcome Trust.

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