Universal kinetics of helix-coil transition in gelatin

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By covering a much wider concentration range than previous studies we find a very unusual exponential dependence of the rate of helix formation on concentration of gelatin in water and ethylene glycol solutions. By applying a procedure of concentration-temperature superposition we build a master curve describing the initial renaturation rates in both solvents. The growth of the normalized helical fraction $\chi(t)$ is a first-order process, with a rate constant consistent with *cis-trans* isomerization, in most situations. We propose that association of three separate chains to form a triple helical nucleus occurs rapidly and contributes less to the helical onset than previously thought. The measured helix content is a result of lengthening of the triple helix after nucleation, by zipping from the associated nuclei.

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I. INTRODUCTION

Gelatin is a protein derived from collagen by hydrolytic degradation [1]. Studies of calorimetry and mechanical response (see [2] and references therein) give a detailed introduction describing the collagen structure and its helix-coil transition. In particular the important issue is that of the repetitive Gly-Xaa-Yaa motif with proline and hydroxyproline most frequently found in the Xaa and Yaa positions. This sequence motif allows for the formation of a highly regular triple helix, which consists of three molecular strands that, due to the high proline content are arranged in a left-handed polyproline-II-helical conformation, with a pitch of 0.9 nm. These helices coil around each other to form a right-handed superhelix with a pitch of 8.6 nm [3]. The collagen triple helix is stabilized by interchain hydrogen bonds which are perpendicular to the chain axis [4].

On cooling a gelatin solution from a denatured state a transition occurs and a transparent, thermoreversible gel containing extended physical cross links is formed, with the cross links formed by partial reversion to ordered triple-helical segments, separated along the chain contour by peptide residues still in the random coil configuration [5–7]. At very low concentrations the renaturation, or folding, process is completely intramolecular and proceeds by a back-folding of the single chains [8]. With increasing concentration the renaturation becomes increasingly intermolecular. Gelation occurs at concentrations above 5 mg/ml due to the formation of an elastic network in the gelatin solution [9,10].

The coil-helix renaturation is fast in most biopolymers, including polysaccharides and DNA, and resembles a true first-order phase transition [11]. Compared with the folding rates of other coiled-coil structures and globular proteins, the renaturation in gelatin is very slow. The initial helix growth stage lasts several minutes and subsequent growth of the helices proceeds even more slowly as the network strives to reach the equilibrium collagen state [12]. The slow kinetics of triple helix formation is attributed to the high activation energy ($\sim$72 kJ/mol) of *cis* to *trans* isomerization reactions at prolyl peptide bonds [13]. In the native collagen triple helix all Gly-Pro and Xaa-Hyp peptide bonds are in the *trans* conformation, whereas in the unfolded state a significant proportion are in the *cis* conformation. Due to the difficulty in accessing the equilibrium state, most investigations focus on isothermal renaturation. The slow coil-helix transition in gelatin allows to study the details of the transition with relatively simple techniques which could not access the short time scales of folding seen in other biopolymer systems.

As all proteins, gelatin is an optically active material in both the coil and helical states. Most kinetic studies have used optical rotation as the primary technique. Due to coherent chiral ordering helical domains rotate the plane of light polarization much more strongly than the individual chiral amino acids in the coil state. Thus, the coherent optical activity gives a direct indication of the fraction of the monomers in the secondary helical states. Flory and Weaver [14], proposed a simple first-order kinetic scheme based on observations that the rate of reversion in very dilute rat tail tendon collagen solutions ($c<4$ mg/ml) is concentration independent. They postulated the coil-helix transition proceeds via an intermediate state formed by intramolecular reversed rearrangements of a single chain. Assuming this state consists of a secondary helix segment, consideration of the minimum stable segment length leads to the Flory-Weaver expression for the rate constant for the renaturation after quenching the dilute solution,

$$k_1 = \kappa \exp\left(\frac{-A}{kT\Delta T}\right), \tag{1}$$

where A and κ are constants, T is the renaturation temperature, and $\Delta T = T_m - T$ is the degree of supercooling below the equilibrium melting temperature T_m.

Other observations of dilute gelatin solutions agree with this first-order kinetic analysis [8], however, in semidilute solutions the concentration-dependent kinetics is usually reported by optical rotation studies [8,15,16]. Several new mechanisms have been proposed for the triple helix renaturation to account for this. Some have involved three different polypeptide chains coming together to form a triple helical nucleus in the rate limiting step [8], or different segments of the same chain coming together [17].

Differential scanning calorimetry (DSC) studies of semidilute gelatin solutions, however, support first-order kinetic

ideas. Godard *et al.* [18], suggesting an analogy between renaturation and crystallization, modeled the kinetics in terms of Avrami exponents [19], $\chi=1-\exp(-k_1t^n)$, where χ is the helix fraction. They experimentally determined the exponent n close to unity, which in crystallography means one-dimensional growth from predetermined nuclei.

The most recent studies of helix renaturation in semidilute gelatin solutions report that a combination of first-order and second-order processes dominate in the early stages of renaturation [15,16]. The latter case means that the raw initial renaturation rate is dependent on a quadratic function of concentration c. This seems unusual, in view of the triple nature of tertiary helix linkages in collagen. However, the observations of second-order gelation kinetics, in particular the elastic modulus proportional to c^2, have been made for over a century [1]. Guo *et al.* proposed that the rate limiting step is formation of critical nucleus composed of two helical strands wrapped around each other, either from separate chains or by reverse folding of a single chain, followed by subsequent rapid wrapping of another coil segment to form the triple helix [15]. Such two-step mechanisms have been proposed for many years to account for similar findings [9,20]. In the early stages of renaturation, however, optical rotation and rheological studies do not necessarily monitor the same types of events. The latter return the viscoelastic response of the gel and thus rely on the triple-helix linkages between different chains in the percolating network. The dominant contribution to the optical rotation signal comes from the secondary helices and not the tertiary structure (which contributes negligibly at optical wavelengths of light) [21].

By examining a much wider concentration range than in previous studies, and combining optical rotation measurements with DSC, we find that the complexity of its folding kinetics, strongly dependent on temperature and concentration in the solvent, can be dramatically simplified. The kinetics of triple helix renaturation can be scaled onto a single master curve by a procedure we call "concentration-temperature superposition" [22]. However, in contrast to [22], here we use a slightly different, additive rather than multiplicative superposition procedure. There is still some analogy with the classical time-temperature superposition often used to produce master curves to describe the glass transition in a variety of thermal viscoelastic systems [23,24]. The effects of time and temperature on a system have equal effects in such a way that a simple shifting of the response function superimposes data taken from different temperatures. A much more recent discovery of time-concentration superposition [25,26] has allowed the universal description of dynamic glass transition, or jamming, in lyotropic systems such as colloid suspensions. We shall argue that the cooperativity of interparticle interaction is the key for the associating folding kinetics.

Most of the experimental work reported here is conducted using the method of differential detection of optical rotation, described in detail in [2,27]. Gelatin from porcine skin (Bloom ~175 from Sigma-Aldrich, St. Louis MO) was used, with the mean $Mw\sim 43$ kDa and broad polydispersity. The measured angle of rotation of the plane polarization of light passing through the sample, Ψ, was divided by the (constant) sample thickness to produce the rotation rate $\theta=d\Psi/dz$. It was then normalized by the solution concentration to obtain a specific rotation $[\alpha]=(1/c)(d\Psi/dz)$, from which we subtracted a value $[\alpha]_0$ corresponding to the amino acid optical activity, separately measured in the coil state at high temperatures. The resulting difference is directly proportional to the concentration of correlated secondary helices in the medium. Optical rotation measurements are mainly sensitive to the left-handed helical conformation of single chains; association to form triple helices has little effect on the optical rotation [12]. This is expected because of the different length scale of the tertiary winding and its weaker interaction with optical-wavelength light.

An important aspect of our work is the speed of quenching, which should be faster than the folding rate to allow us to examine the kinetics. Temperature control was achieved using a custom-built cuvette holder with temperature continuously measured in the holder with very high thermal conductivity and inside the cuvette (in the gelatin itself, a few mm away from the laser beam path). The temperature lag between the two measurements was very small, consistent with the thermal equilibration rate of ~10–20 s across a gap of several mm and the diffusion constant 10^{-7} m^2/s. The device provided a temperature ramp of ≥ 25 °C/min, with the final temperature stabilized within ± 0.1 °C.

At the end of the paper we shall compare the main results on the universality of the concentration dependence of folding rates with a separate experiment on the so-called gelation time (Fig. 11). This time tells how long it takes for the gelatin network to reach percolation after a rapid quench. In this case the quenching was achieved by pouring the hot solutions directly onto the rheometer plates held at the desired quench temperature. With a coefficient of thermal diffusion $D\sim 10^{-7}$ m^2/s, the equilibration time of a ~0.1-mm-thick sample is ~0.1 s. The (much longer) gelation time has been approximately determined as the first emergence of a storage modulus G' in a continuous rheometer reading.

II. HELIX NUCLEATION

Typical measurements of the specific optical rotation of gelatin in water and ethylene glycol (EG) solutions, as a function of time after quenching from 50 °C to the desired renaturation temperature, are shown in Figs. 1 and 2, respectively. The curves are characterized by a rapid increase in specific rotation as soon as the renaturation temperature is reached, followed by an increase at a much reduced rate. As $[\alpha]$ is normalized by the solution concentration, the y axis is directly proportional to the helical fraction in the sample. The specific rotation is negative because gelatin is laevorotatory. The specific rotation has a strong concentration dependence, at a given renaturation temperature, in water solutions, Fig. 1(a). There are significant differences in EG solutions, the level of helix renaturation is reduced and, for concentrations greater than 0.2 g/ml, the curves are no longer dependent on concentration Fig. 2(a). The closer the renaturation temperature is to the helix-coil transition temperature the slower the helix renaturation rate in both solvents, Figs. 1(b) and 2(b).

At all concentrations and renaturation temperatures tested the helical fraction, as determined by optical rotation, can

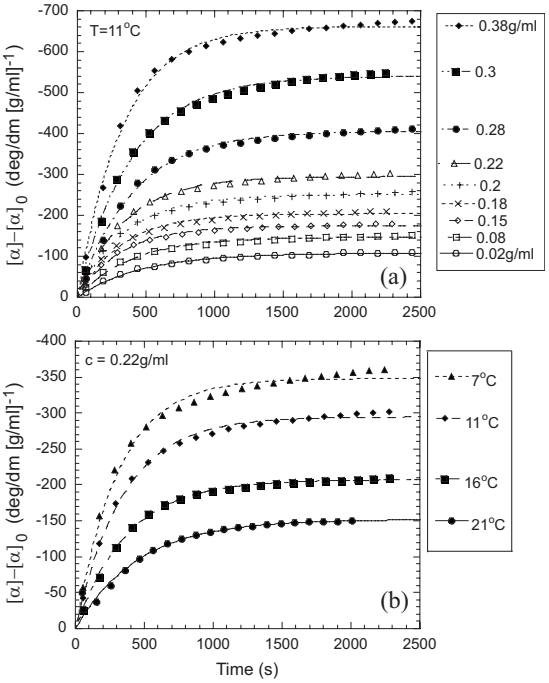

FIG. 1. Typical traces of specific rotation, directly proportional to the helix density, as function of time after quenching the denatured collagen in water solution. (a) Quenching to T_Q=11 °C, different data sets correspond to concentrations labeled on the graph. (b) c=0.22 g/ml, for a range of renaturation temperatures labeled on the graph. Solid or dashed lines are fits with $A[1-\exp(-t/\tau)]$.

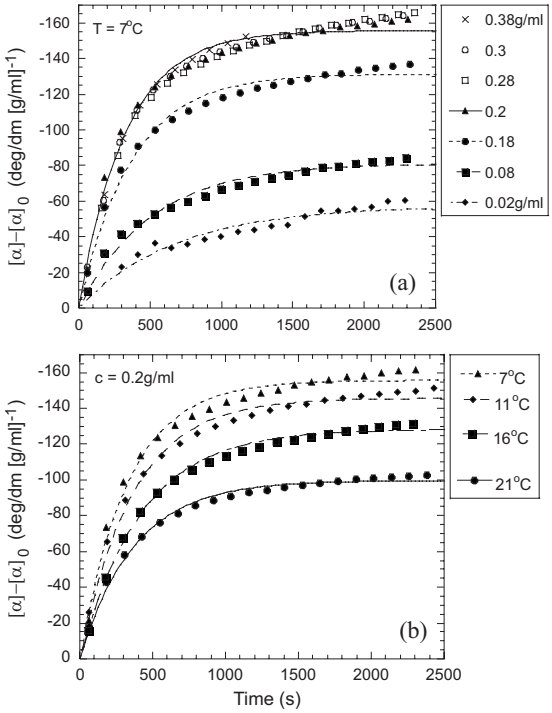

FIG. 2. Traces of specific rotation as function of time after quenching the denatured collagen in ethylene glycol solution. (a) T_Q=7 °C, (b) c=0.2 g/ml. Solid or dashed lines are fits with $A[1-\exp(-t/\tau)]$.

be fit by a single exponential function of time, $A[1-\exp(-t/\tau)]$ (shown by the lines in Figs. 1 and 2). The simple exponential fit of each data set is not surprising: for many proteins both the folding and unfolding processes are single exponential functions of time [28]. The long time data shows a small deviation from the simple exponential fit. This is due to the slow drift of the collagen toward its native state which proceeds via lengthening and reorganization of the triple helices, which proceeds at a logarithmic rate [10,12]. This process is faster at higher renaturation temperatures and is more pronounced in EG solutions. The proportion of the final helical content which results from lengthening is greater in these conditions. The lengthening and reorganization regime is the domain of rheological studies, addressed in [2]. The tertiary structure is consolidated and the gel elasticity shows a significant increase [9,29]. Our opinion [27] is that the properly measured optical rotation signal is determined by secondary helices and not the tertiary structure. Optical rotation measurements can access much shorter time scales before the tertiary structure is consolidated.

The slope of the specific rotation vs time curve is a direct measure of the helix renaturation rate. The initial rates of helix renaturation are the slopes $R_0(c,T) \equiv d[\alpha]/dt = A/\tau$ at $t \to 0$, determined from the fitted functions $A[1-\exp(-t/\tau)]$ shown in Figs. 1 and 2. For each renaturation temperature, these slopes depend on the solution concentration. The summary of this dependence is given in Figs. 3 and 4 for water and EG solutions, respectively. We note that the highest solution concentration studied previously using optical rotation was $c \sim 0.12$ g/ml, by Guo *et al.* [15] If renaturation was a second-order process we would expect a linear dependence of R_0 on concentration. By covering a much wider concentration range we are able to see that the concentration dependence is highly nonlinear, as is demonstrated by the exponential fits of the data sets from each renaturation temperature. The fits only differ by the preexponential factor, having the same $Y \approx 6.4$ ml/g.

In EG solutions a similar exponential dependence is found for concentrations less than 0.2 g/ml (Fig. 4), with the same value for Y. The initial helix renaturation rates are compa-

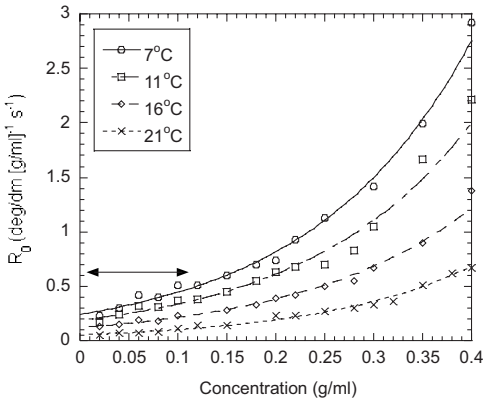

FIG. 3. Initial rates of helix renaturation in gelatin in water solutions, R_0 at $t \to 0$, as functions of concentration, for a range of renaturation temperatures labeled on the plot. Solid or dashed lines are fits with exponential $B\exp(Yc)$. The arrow indicates the greatest concentration range of earlier studies [15].

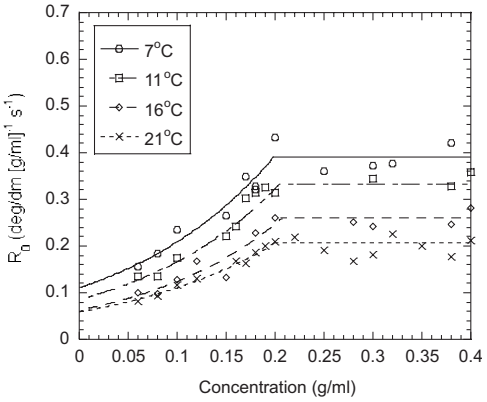

FIG. 4. Initial rates of helical nuclei growth in gelatin in EG, for a range of renaturation temperatures labeled on the plot. Solid or dashed lines are fits with $B\exp(Yc)$ below 0.2 g/ml and are guides to the eye to indicate the flat dependence of the growth rate on concentration above 0.2 g/ml.

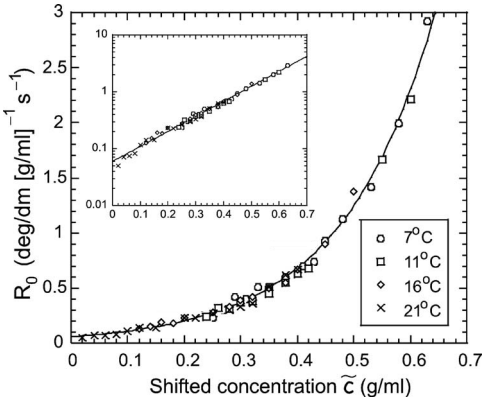

FIG. 5. The master curve of initial renaturation rate vs concentration, $R_0(\tilde{c})$, for gelatin in water solutions obtained by superposing the results for different renaturation temperatures (labeled on the plot). The solid line is a universal fit with $B\exp(Y\tilde{c})$. The logarithm-linear representation of the same plot (shown in the inset) demonstrates the exponential nature of the master curve.

rable to those seen in water solutions at the highest renaturation temperature studied, 21 °C. At lower temperatures the renaturation rate is considerably reduced. In contrast to the water solutions, where an exponential dependence is found over the entire concentration range studied, for concentrations greater than 0.2 g/ml the initial renaturation rates are independent of concentration, as seen in Fig. 2(a).

III. *T-c* SUPERPOSITION AND MASTER CURVES

The sets of data for the initial renaturation rates in Figs. 3 and 4 can be shifted along the concentration axis by an amount that is a function of renaturation temperature to produce a master curve. Motivated by the same exponent factor Y in the fits above, we arbitrarily choose $T_{\mathrm{ref}}=21$ °C to be the reference temperature and shift the concentration c for each data set such that $Y\tilde{c}=Yc+\ln\beta(T)$. This superposition procedure is different from the classical time-temperature superposition, where one scales the variable by a multiplicative factor [22]. For our exponential concentration dependence curves the addition in the exponent is equivalent to the multiplication by a preexponential factor $\beta(T)$, which is a function of the renaturation temperature for each data set. The resulting superposed master curves for water solutions are shown in Fig. 5. Remarkably, the master curve in EG is identical to that found in water. This is emphasized by the solid line in Fig. 6 which is the master curve for water from Fig. 5 plotted over the shifted data points for EG. The fact that these sets do superpose means that there is a universal underlying expression for the initial renaturation rate $R_0[T,c]$. The resulting master curves are an indication of such a universal physical process, independent of solvent, that controls the helical nucleation and growth in all regions of the (T,c)-phase diagram below T_m. We would like to emphasize that "universality" in this context does not imply that all proteins should fold in the same way—merely that this gelatin folds in the same way at all concentrations and temperatures.

Figure 7 shows the set of shift factors, $\beta(T)$, required to produce the superposed master curves in Figs. 5 and 6 for the initial rate of helix formation. The shift factors for both EG and water solutions follow an obvious linear dependence on temperature, $\beta=C(T^*-T)$. The critical temperature T^* is defined as the point at which $\beta=0$ and the fitting gives $T^*\approx 25$ °C in water solutions and 37 °C EG solutions. These are not accidental values.

Earlier literature [2,29] investigated the helix-coil transition in our gelatin solutions in some detail. Exact phase diagrams are difficult to obtain because of the strong kinetic and concentration dependence. However, it is clear from Fig. 8 that the critical temperatures T^* emerging from the concentration-temperature superposition are closely related to the thermal transition temperatures. We therefore conclude that, within the errors of our experiment and analysis (which combine and compare several very different techniques), the critical temperature T^* is in fact the true line of the helix-coil transition.

With this assumption, the initial renaturation rate, as obtained from the master curves in Figs. 5 and 6, follows the equation

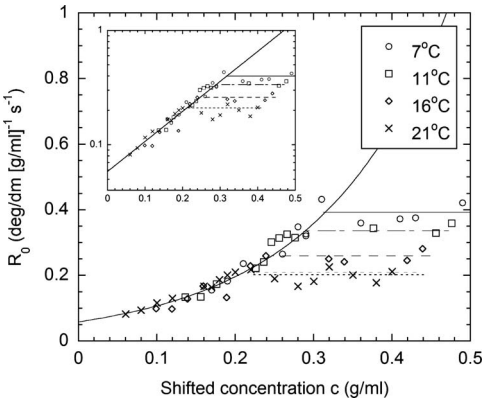

FIG. 6. The corresponding master curve of initial renaturation rate vs concentration, $R_0(\tilde{c})$, for EG solutions. The solid line is the universal fit of the superposed data for water solutions, determined in Fig. 5, which fits the EG data as well. The flat lines indicate the plateaus for higher (shifted) concentrations, cf. Fig. 4.

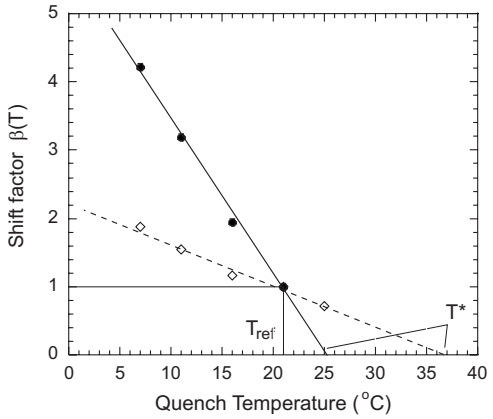

FIG. 7. Dependence of the shift factor β on renaturation temperature in water (●) and EG (◇) solutions. The reference temperature chosen at 21 °C corresponds to the raw, nonshifted data, or $\ln \beta = 0$. The solid or dashed lines are linear fits with the function $\beta = C(T^* - T)$.

$$R_0 = \left.\frac{d[\alpha]}{dt}\right|_{t\to 0} = b(T^* - T)\exp(Yc) \tag{2}$$

with fixed parameters b and Y. For water solutions, we determine $b \approx 0.012$ deg/dm(ml/g)(K s)$^{-1}$, while in EG $b \approx 0.004$ deg/dm(ml/g)(K s)$^{-1}$. The exponent Y is the same in water and EG solutions: $Y \approx 6.4$ (g/ml)$^{-1}$.

Near the transition temperature T^* we find no critical or singular behavior as would be suggested by the Flory-Weaver expression for the rate constant, Eq. (1), or the case in classical phase transitions. However, our model equation suggests that the initial renaturation rate R_0 turns to zero at $T = T^*$, and becomes negative above T^*. This could be interpreted as denaturation of existing helices, as opposed to the folding of new ones below T^*.

IV. NUCLEATION KINETICS

Previous kinetic studies have focused on the normalized helical fraction, which in our notation is defined as

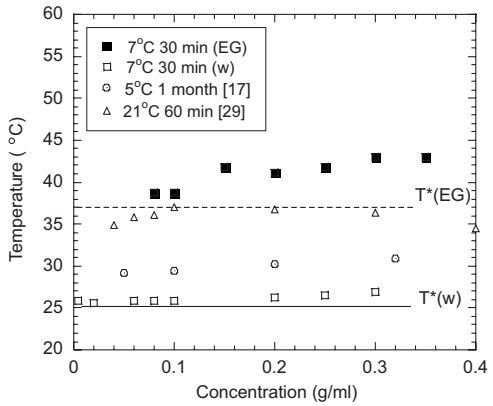

FIG. 8. Phase diagrams of helix-coil transition, $T_m(c)$, obtained here from the DSC data for water (⊙) and EG (△), with results for different preparation methods in the literature (⊡ [18], ■ [30]). The values of T^* are obtained from the concentration-temperature superposition analysis, Fig. 7.

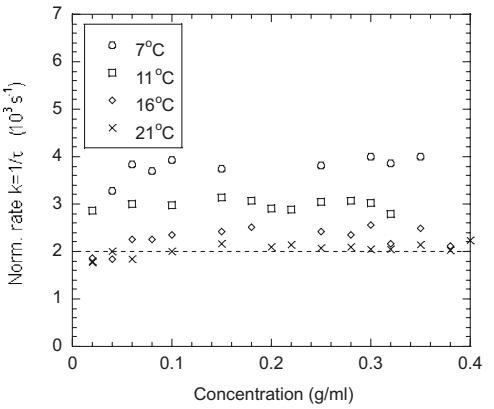

FIG. 9. Growth rate of the normalized helical fraction, k, as functions of concentration at different renaturation temperatures (labeled on the plot) for gelatin in water solutions. The dashed line is a guide for the eye. A flat dependence at all renaturation temperatures indicates a first-order folding process.

$$\chi(t) = \frac{[\alpha] - [\alpha]_0}{A(c,T)} = 1 - \exp(-t/\tau), \tag{3}$$

according to Figs. 1 and 2, and earlier work [18]. Note that it is common in the literature to normalize the measured $[\alpha] - [\alpha]_0$ by the specific rotation of the native collagen structure. This might give different results for the perceived normalized helical fraction $\tilde{\chi}(t)$. Our choice of normalization in Eq. (3) is dictated by the measured exponential growth law of the early renaturation (Figs. 1 and 2) and thus gives the pure rate constant $k = 1/\tau$ at short times.

Figures 9 and 10 show our results for this rate constant k for the growth of the normalized helical fraction, $\chi(t)$, for gelatin in water and EG solutions, respectively. The large noise in the $k(c)$ data is due to problems with fitting the data using a single exponential which arise because of the deviation from the fit at long times due to helix lengthening and reorganization of the network. The qualitative picture sug-

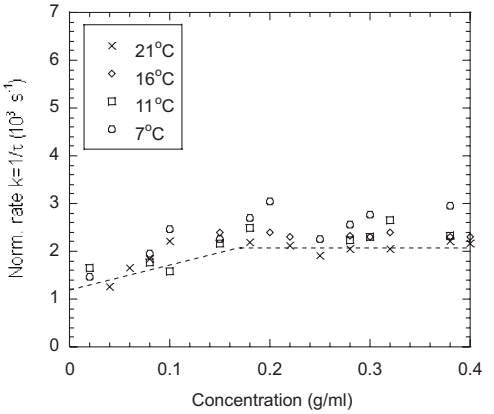

FIG. 10. Dependence of k on concentration for gelatin-EG solutions at different renaturation temperatures. At concentrations greater than 0.2 g/ml a first-order process dominates at all renaturation temperatures. Below this concentration the dependence is approximately linear.

gests that in water solutions, across the entire concentration range studied, the true rate constant k is independent of concentration. In EG solutions at concentrations greater than 0.2 g/ml, there is no relevant concentration dependence of k either. However, a possibly linear increase is seen at concentrations less than 0.2 g/ml in EG solutions. The origin of the unusual exponential concentration dependence of the initial renaturation rate, Eq. (2), is in the constant $A(c)$, while $k = R_0(c,t)/A(c)|_{t\to 0}$ remains a temperature-dependent constant.

The growth of the normalized helical fraction appears to be a first-order or unimolecular process. This may appear a surprising result since, with increasing concentration, intramolecular rearrangements of single chains become limited. Unimolecular kinetics are usually interpreted as arising from intramolecular reversed rearrangements of the chain to form the renatured triple helix, first suggested by Flory and Weaver [14]. Light scattering studies of very dilute gelatin solutions measure the radius of gyration, R_g, of the polypeptide chain to be ~ 50 nm at 35 °C [31]. This gives an overlap concentration ($c \sim Mw/R_g^3$) of the order of 1 mg/ml. The gelatin chains are highly overlapped across the concentration range studied and, therefore, reversed rearrangements of a single chain are unlikely. In addition, in order to form an elastic gel, different chains must in fact participate in the physical cross links. How can we find a compromise between these facts and an apparent unimolecular folding process?

Recently, the renaturation of type III collagens has been studied. In these, and other collagens, the exact register of the chains in the native structure is maintained in the denatured state by disulphide bonds between the chains at the C-terminus [13,32]. (*In vivo* these C-terminal domains are cleaved from the triple helix after renaturation hence they are not present in our gelatin samples [33].) A zipper mechanism of triple helix renaturation is thought to occur, when a helical nucleus forms at the C-terminal domain and then propagates from the nucleus to the N-terminus. The rate of renaturation is limited by *cis-trans* isomerization reactions at single Gly-Pro and Pro-Pro bonds. These isomerization reactions have rate constants of 2.9×10^{-3} and 0.6×10^{-3} s^{-1}, respectively, at 4 °C [34]. The measured values of k in Figs. 9 and 10 are consistent with *cis-trans* isomerization.

This leads us to speculate that nucleation of the triple helix is fast and exponentially dependent on concentration and/or temperature, but contributes little to the subsequently measured helical content. On quenching, all *trans* regions may act as nucleation sites for triple helix formation. The rate of helix formation in all *trans* gelatin chains is much faster and comparable to that found in globular proteins ($k_{trans} \sim 113 \pm 20$ s^{-1}) [13]. Of course, gelatin is a heterogeneous mixture of polypeptides and there are many possible candidates for nucleation sites. Association of three nucleation sites (whatever they may be) to form a triple helical intermediate will occur rapidly because of the high degree of chain overlap. Following nucleation, the helix content, measured in Figs. 1 and 2, results predominantly from lengthening of these nuclei via the zipping mechanism described above. This gives the observed unimolecular growth of the normalized helical fraction. A triple helix will result if it reaches the minimum stable helix length at the given renaturation temperature before it is disrupted by thermal fluctuations. The renatured triple helices will lengthen until the lack of registration between the collagen chains means no further lengthening can occur without rearrangements of the existing helices, which proceeds at a much slower rate.

In EG solutions, for concentrations less than 0.2 g/ml, we find a linear dependence of k on concentration. Clearly, in this regime chain association is rate limiting. The concentration above which association is no longer rate limiting is a measure of the overlap concentration for gelatin chains in EG. 0.2 g/ml gives a radius of gyration of ~ 20 nm. This means that the gelatin chains in EG explore only $\sim 10\%$ of the volume explored in water solutions. This fits well with the qualitative observation that the degree of swelling of gelatin powder is much smaller in EG compared to water. Addition of EG not only increases the viscosity of the solvent, but it can also affect the conformational stability of a protein. It has been shown to stabilize the molten globule state of other proteins, e.g., cytochrome C [35]. This is attributed to favoring of the peptide-peptide hydrogen bonds. It is reasonable to assume the same effect occurs in gelatin leading to a more compact chain conformation in EG solutions. Concentration-dependent kinetics is therefore seen in EG at concentrations where association is not rate limiting as in water solutions.

V. FOLDING AT HIGH CONCENTRATIONS

The exponential concentration-temperature dependence of the initial renaturation rate R_0 seen across the concentration range in water solutions and below 0.2 g/ml in EG is a striking result, contradicting most expectations. Virial arguments all lead to various power-law scalings, $R_0 \propto c^x$. We present a simple argument here to show how such an exponential dependence can arise in $A(c)$.

Let us assume there are M gelatin chains in solution, each N residues long, with nucleation sites located at a fixed distance of n residues apart along the chain. There are many possible candidates for these nucleation sites, e.g., all *trans* regions of the chain, however, the nature of the nucleation sites is not crucial for this simple model. The concentration, c of residues in the solution is $c=MN/V$, where V is the volume of the solution, and the concentration of nucleation sites, s, is c/n. When three nucleation sites come together, a nucleus is formed and a triple helix will result if the nucleus is longer than the critical stable helix length at the given renaturation temperature. In the ideal-gas regime, as $s\to 0$, the probability to nucleate a triple helix must be proportional to s^3, the three-particle collision effect. However, at sufficiently large s, we can assume that a gel is forming, being held together by a number h of triple helices arising from the nucleation of h triplet nuclei. Once we have gelled $h(s)$, we can consider the effect of adding a small concentration of new chains and hence a small concentration of new nucleation sites $s\to s+ds$. At large enough N/n we may assume that all the new nucleation sites belong to the dangling chains already attached to the existing network with $h(s)$ junctions. The infinitesimal increase in the number of triple

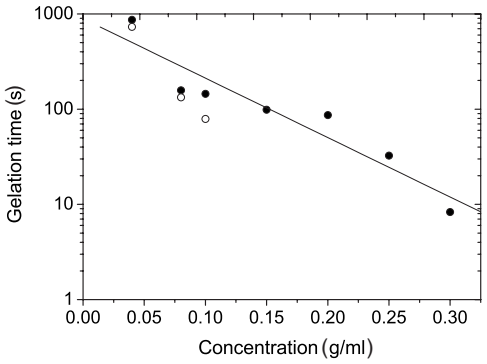

FIG. 11. The gelation time as a function of concentration for water (●) and EG (○) solutions quenched to 21 °C. The solid line is a fit to the water data with a function $e^{-(Yc)}$.

helices is then proportional to the number of these junction sources, $\Delta h \propto h\Delta s$. This construction leads to the exponential concentration dependence

$$dh/ds = \alpha h, \quad \text{hence} \quad h(c) \propto e^{(\alpha/n)c}.$$

Assuming all $h(c)$ triple helices are roughly the same length, the observable rotation rate will be $\propto h(c) \sim e^{Yc}$.

There are several reports of the universal value of helix concentration at the gel point, in a rapidly quenched gelatin solution [29]. This observation means that regardless of the gelatin concentration or temperature, a critical total helix length per unit volume must be achieved in order for a gel to form in the given solvent [2]. Here we proposed that nucleation of the triple helices happens rapidly and the number of nucleation sites is exponentially dependent on concentration, $e^{(Yc)}$, while the subsequent helix growth occurs by lengthening of these nuclei, the rate of which is limited by *cis-trans* isomerization. If this is the case, then the rate at which the individual helix lengths in the sample increase following nucleation is independent of concentration. Then the gelation time t_{gel}, i.e., the time it takes to reach percolation after a rapid quench, should be inversely proportional to the number of nucleation sites, that is, to $e^{(-Yc)}$. Figure 11 shows t_{gel} as a function of concentration for both solutions quenched to 21 °C. The data is noisy but it is clear from the exponential fit that $t_{\text{gel}} \propto e^{-(Yc)}$. The value of the constant Y emerging from this fit is 6.24 ml/g. This compares very favorably with the value obtained from the analysis of the rate of helix formation determined by optical rotation, in Figs. 3 and 4 where we had Y of 6.4 ml/g.

VI. CONCLUSIONS

We have characterized the helix-coil transition in gelatin solutions over a much wider concentration range than previously studied combining optical rotation measurements with differential scanning calorimetry. By applying a new concentration-temperature superposition we found a universal master curve describing the initial renaturation rates R_0 of gelatin solutions over a broad range of concentrations and temperatures, spanning the nominal helix-coil transition point T_m. The form of the master curve is the same in water and EG solutions, following the same exponential c dependence of R_0 in different systems.

The early growth of the normalized helical fraction $\chi(t)$ appears to be a first-order process, with its rate constant consistent with the rate of *cis-trans* isomerization. We proposed that nucleation of the triple helix happens rapidly and contributes less to the helical onset than previously thought. The measured helical content comes predominantly from lengthening of these triple helical nuclei, which is a one-dimensional growth process leading to the apparent first-order kinetics. Favoring of peptide-peptide hydrogen bonds in EG solutions leads to a more compact chain configuration in solution and a concentration-dependent normalized rate up to relatively high concentrations. In both water and EG solutions plots of G' vs rotation rate θ for solutions of different concentration are superimposed to form a master curve of their own [2,29]. The critical helix concentration is smaller in EG solutions because the minimum stable helix length is smaller in EG solutions at all renaturation temperatures. The dependence of G' on helix concentration and the fundamental concentration-temperature superposition of the helix folding rates has allowed other investigators to scale curves of G' vs time to form a master curve for concentrations and/or temperatures where gelation occurs before renaturation slows. One needs to study other oligomerizing proteins to test the further universality of the discovered master curves for renaturation rates.

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