Concentration-Temperature Superposition of Helix Folding Rates in Gelatin

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Using optical rotation as the primary technique, we have characterized the kinetics of helix renaturation in water solutions of gelatin. By covering a wide range of solution concentrations we identify a universal exponential dependence of folding rate on concentration and quench temperature. We demonstrate a new concentration-temperature superposition of data at all temperatures and concentrations, and build the corresponding master curve. The normalized rate constant is consistent with helix lengthening. Nucleation of the triple helix occurs rapidly and contributes less to the helical onset than previously thought.

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Kinetics of protein folding is one of the cornerstone problems in understanding biologically active macromolecules [1,2]. A number of experimental techniques focus on a great variety of natural and synthetic polypeptides undergoing their globular collapse, or denaturation into the coil. Although one might want to think of such a transition in terms of individual macromolecules, there is much evidence that cooperative effects between different chains play a significant role, an effect often referred to as oligomerization [3,4]. This extreme variability has, to date, defied any attempt of universal description, beyond the classical Zimm-Bragg abstraction [5].

In this work we examine gelatin and discover that the complexity of its folding kinetics, strongly dependent on temperature and concentration in the solvent, can be dramatically simplified by scaling onto a single master curve using a new procedure we call ''concentration-temperature superposition.'' There is a remarkable analogy: the classical time-temperature superposition [6,7] has allowed master curves to describe the glass transition in a variety of thermal viscoelastic systems, serving a great purpose in rheology for the past 50 years. The recent discovery of time-concentration superposition [8,9] has allowed the universal description of dynamic glass transition, or jamming, in lyotropic systems such as colloid suspensions. In both cases the cooperativity of interparticle interaction is the key, and we shall argue that it is relevant for the associating folding kinetics as well.

Gelatin is dissolved in water by heating the solution to 40 °C. Above this temperature peptide chains are random coils, though there may be a significant number of β-turn structures [10,11]. On cooling, transparent gels form containing extended physical cross-links formed by partial reversion to ordered triple-helical segments. The gelatin chain consists of a repeating motif, Gly-Xaa-Yaa, with proline and hydroxyproline most frequently found in the Xaa and Yaa positions. The triple helix consists of three molecular strands that, due to the high proline content, are arranged in a left-handed helical conformation and coil around each other to form a right-handed superhelix [12]. The cross-links are separated along the chain contour by peptide residues still in the random coil configuration [13]. Unlike most biopolymers, the coil-helix transition is slow in gelatin [14]. This allows one to study its detailed kinetics relatively simply using optical rotation and differential scanning calorimetry (DSC).

In an early optical rotation study, Flory and Weaver observed first-order kinetics in very dilute gelatin solutions ($c < 4$ mg/ml) [15], i.e., that the renaturation rate is concentration independent. They postulated the coil-helix transition proceeds via an intermediate state formed by intramolecular rearrangement 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: $k_1 = B\exp(-A/kT\Delta T)$, where A and B are constants, T is the quench temperature, and $\Delta T = T_m - T$ is the degree of supercooling below the equilibrium melting temperature T_m.

In semidilute solutions concentration dependent kinetics are usually reported. Most recently, Guo *et al.* [11] studied initial renaturation rates of gelatin solutions at concentrations up to 0.12 g/ml and observed what appeared as a combination of first-order and second-order kinetics; i.e., the rate of growth of the normalized helix fraction had a linear concentration dependence. This seems unusual, in view of the triple nature of helix linkages in collagen. However, observations of second-order gelation kinetics, in particular, the elastic modulus proportional to c^2, have been made for over a century [16]. A two-step mechanism with the rate limiting step formation of a nucleus of two helices wrapped together, followed by rapid subsequent wrapping of another coil segment to form the triple helix, was proposed a long time ago to account for these findings [17,18]. However, we argue that there is a big difference between optical rotation and rheological studies. The dominant contribution to the measured optical rotation comes from the secondary helices which wrap together to form the tertiary triple helix [19]. Optical rotation can access much shorter time scales than rheological studies which return the viscoelastic response of the gel and thus

rely on the triple-helix linkages between different chains in the network.

In an attempt to link together past findings, we studied the helix-coil transition in gelatin (porcine skin, Bloom 175, from Sigma) over a much wider concentration range, up to a very dense 0.4 g/ml. To measure the secondary helix content we used a high-sensitivity differential optical rotation detector described in [20]. The raw measurement of the total angle of polarization plane rotation Ψ is divided by the (constant) sample thickness to produce the rotation rate. It is then normalized by the solution concentration to obtain a specific rotation $[\alpha] = (1/c)[d\Psi/dz]$, from which we subtract a bare value $[\alpha]_0$ corresponding to the average amino acid optical activity, separately measured in the coil state at higher temperatures. The difference, $[\alpha] - [\alpha]_0$, is linearly proportional to the concentration of secondary helices in the medium, and is only weakly affected by the triple-helix structures. Birefringence due to concentration or temperature gradients also has a negligible effect on the measured rotation [19]. The typical readings, for a range of concentrations, are shown in Fig. 1 for a solution quenched from 50 °C to $T = 11$ °C in this case. As a result of $[\alpha]$ normalization, the y axis in Fig. 1 is directly proportional to the helix fraction in the sample.

There are delicate issues of the slow drift of gelatin towards the native collagen structure [14], reflected in the small deviation of the long-time data from the simple exponential fit of each data set (shown by the lines in Fig. 1). Rheological studies [18] are focused on this regime, where the tertiary structure (and the gel elasticity) are being consolidated and the elastic modulus increases dramatically. We, however, are concerned with the initial rates of secondary helix renaturation, essentially the slopes $R_0(c, T) \equiv d[\alpha]/dt = A/\tau$ at $t \to 0$, from the fitted functions $A[1 - \exp(-t/\tau)]$ in Fig. 1. For each quench temperature, these slopes depend on the solution concentration as shown in Fig. 2. The highly nonlinear concentration dependence of initial renaturation rates is apparent from the exponential fits of all data sets. The highest solution concentration quantitatively studied to date was $c \sim 0.12$ g/ml, by Guo *et al.* [11] who claimed renaturation is a second-order process. We arrive at a different conclusion—the reversion has an exponential character. The proposed second-order dependence is the result of studying too narrow a concentration range.

Guided by the experience in time-temperature and time-concentration superposition, we notice that the data sets for the initial renaturation rates in Fig. 2 can be shifted along the concentration axis by an amount that is a function of quench temperature. Selecting a reference temperature (we arbitrarily choose $T_{\text{ref}} = 21$ °C) one scales the concentration c for each data set such that $\tilde{c} = \beta c$, with the coefficient (the shift factor) a function of the quench temperature for each data set, $\beta = \beta(T)$. The fact that these sets do superpose means there is a universal underlying expression for the initial renaturation rate $R_0[\beta(T)c]$. The resulting master curve, Fig. 3(a), is an indication of such a physical process that controls the helix nucleation and growth in all regions of the (T, c)-phase diagram. It is important to establish how the shift factors β depend on temperature. Figure 3(b) plots the set of $\beta(T)$ required to produce the master curve. It follows an obvious linear function, fitted by $\beta = 0.1(T_c - T)$, with the "critical temperature" $T_c \approx 30$ °C (more precisely, 302.5 K) defined as the point at which $\beta = 0$.

Figure 4(a) shows a sequence of DSC traces of the helix-coil transition, obtained on reheating gelatin solutions of different concentration after holding for 30 min at 7 °C. Figure 4(b) shows the corresponding transition line $T_m(c)$. Since the transition temperature is strongly dependent on gelation time and temperature a range of values is reported

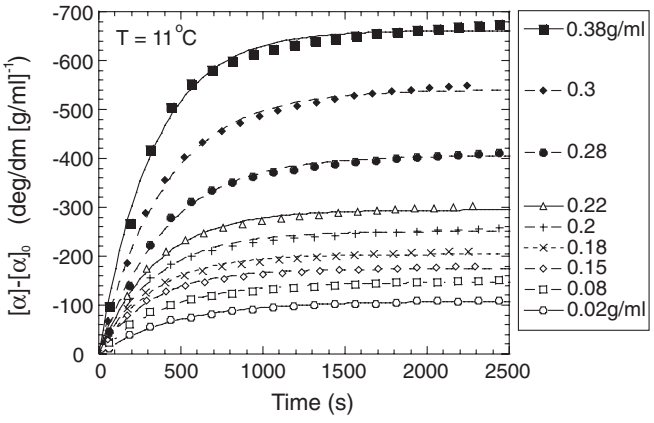

FIG. 1. Typical traces of normalized optical rotation, directly proportional to the secondary helix density after quenching (at a fixed rate of 25 °C/ min) the denatured collagen solution to $T = 11$ °C. Solid lines and dashed lines are fits with $A(1 - \exp[-t/\tau])$.

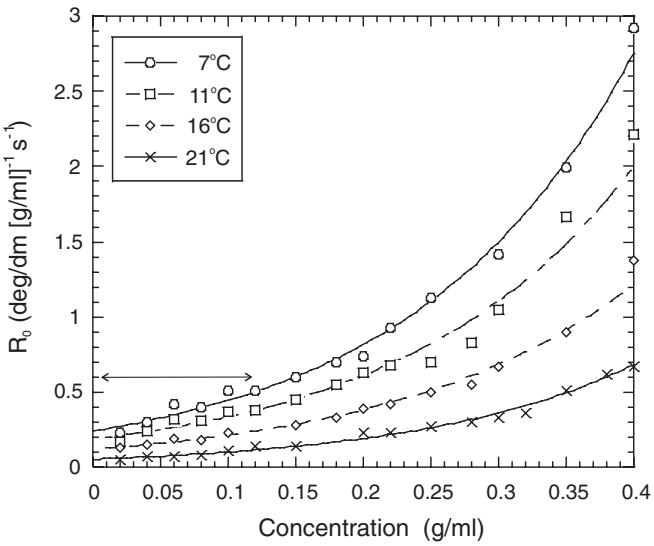

FIG. 2. Initial rates of helical growth R_0 as functions of solution concentration, for a range of quench temperatures. Solid lines and dashed lines are fits with exponential $B\exp[Yc]$. The arrow indicates the concentration range of earlier studies [11].

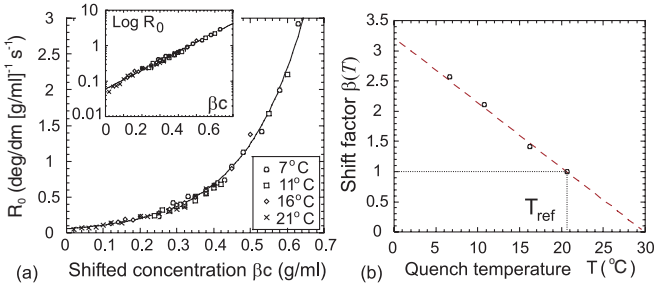

FIG. 3 (color online). (a) The master curve of growth rate versus shifted concentration, $R_0(\tilde{c})$, superposing the results for different quench temperatures. The solid line is a universal fit with $B\exp[Y\tilde{c}]$. The same plot inset is in log-linear representation. (b) Dependence of the shift factor β on quench temperature T. The linear fit crosses the $\beta = 0$ line at $T_c \sim 30\,^{\circ}\mathrm{C}$.

in the literature. Note that the gelation phase diagram, obtained by rheological methods, would show a drop of the gel point $T_g(c)$ below 5 mg/ml, the concentration above which gelation occurs [21].

The critical temperature T_c is labeled in the gelatin phase diagram and the sequence of DSC scans, Fig. 4(a). Exact phase diagrams are difficult to obtain because of the strong kinetic and concentration dependence; however, it is clear that T_c is closely related to the thermal transition temperatures. We conclude that (within errors of our experiment and analysis) this critical temperature is indeed the line of the helix-coil transition, $T_c = T_m(c)$. With this assumption, the renaturation rate, as obtained from the master curve in Fig. 3(a), follows the equation

$$R_0 = \frac{d[\alpha]}{dt}\bigg|_{t\to 0} = B\exp[b(T_m - T)c] \qquad (1)$$

with fixed parameters $B \approx 0.06$ deg /dm $(\mathrm{g/ml})^{-1}\,\mathrm{s}^{-1}$ and $b \approx 0.6$ (ml/g) K. Note we find no critical or singular temperature behavior near the transition. The initial renaturation rate becomes exponentially small at $T > T_m$. In retrospect, it is not surprising that we did not obtain a critical vanishing of the renaturation rate, as would be the case with classical phase transitions, or was suggested in [15]. Our present finding supports the idea of a diffuse helix-coil transition in a single chain, with chain fluctuations capable of creating a nonvanishing (but exponentially small) helical fraction above $T_m(c)$, and equally partially disrupt thermodynamically equilibrium helices formed below $T_m(c)$. In other words, there is no sharp phase boundary $T_m(c)$ for the helix-coil transition. In equilibrium models of such transitions, T_m is identified as a point of steepest gradient in helical fraction $\chi(T)$. In our study of kinetics, we obtain roughly the same value at a point when $R_0(c, T)$ changes from the decaying to the growing exponential function $\exp[Yc]$.

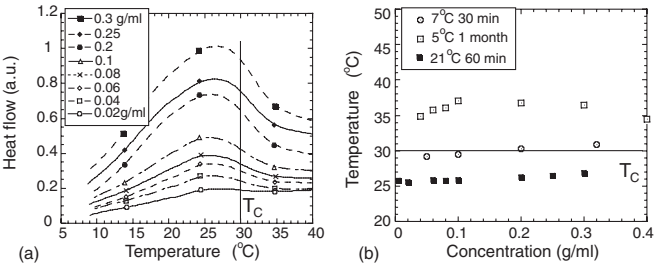

FIG. 4. (a) DSC traces of the helix-coil transition in gelatin-water solutions of different concentrations. (b) A thermal phase diagram of this transition, $T_m(c)$, obtained from the DSC data (⊙), with results for different preparation methods from the literature [⊡: [22]; ■: [25]].

Having established the master curve for renaturation rates $R_0(c, T)$, we must now make contact with other kinetic studies, which traditionally focus on the normalized helical fraction, defined as

$$\chi(t) = \frac{[\alpha] - [\alpha]_0}{A(c, T)} = 1 - \exp(-t/\tau), \qquad (2)$$

according to Fig. 1 and earlier work [22]. Figure 5 shows the rate constant for the growth of the normalized helical fraction, k $(= 1/\tau)$, over a concentration range much wider than in any previous work. There are delicate issues of fitting the data that include the slow long-time tail, which accounts for the large noise in the $k(c)$ data. However, the qualitative picture clearly suggests that there is no relevant concentration dependence. The origin of the exponential concentration dependence, Eq. (1), is in the constant $A(c)$, while k is equivalent to R_0/A.

Recently, the refolding of denatured collagen chains in which the associations between chains and registration of the chains are maintained by disulphide bonds has been studied [23]. These studies suggest that the rate of helix lengthening is limited by the rate of *cis-trans* isomerization of peptide bonds, randomly distributed along the chains, zipping into the triple helix originating from the associated nuclei. Our values of k are consistent with *cis-trans* isomerization reactions at single Gly-Pro and Pro-Pro bonds,

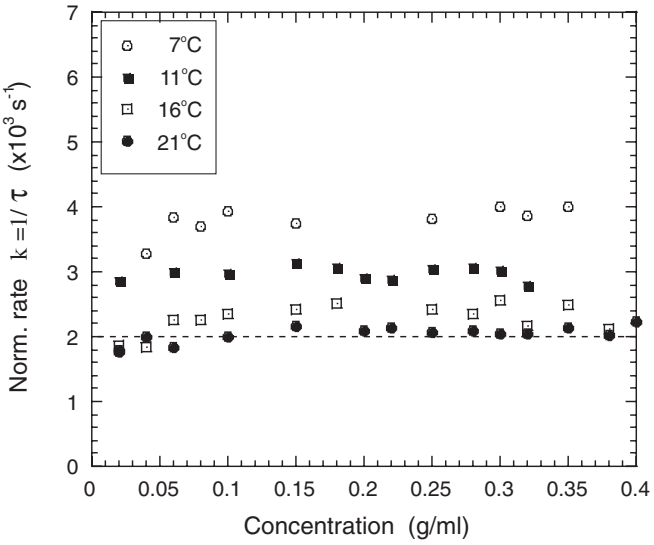

FIG. 5. Normalized folding rates k as functions of concentration at different quench temperatures (labeled on plot).

which have rate constants of 2.9×10^{-3} and 0.6×10^{-3} s^{-1} at 4 °C, respectively [23]. Combined with its concentration independence, this indicates that the normalized growth rate k is indeed characteristic of lengthening of the triple helix. This leads us to speculate that nucleation of the triple helices is fast and exponentially dependent on concentration or temperature, but contributes little to the measured helix content. Following rapid nucleation, the helix content in Fig. 1 results predominantly from linear growth of these nuclei. The triple helices will lengthen until the lack of registration between the chains means no further growth can occur. Further growth can then only proceed via rearrangements of the triple helices, at a much slower rate.

It remains to offer an explanation for how an exponential concentration or temperature dependence of the renaturation rate can arise in $A(c)$. It is a very unusual result, contradicting most expectations (based on the probability of three-particle collisions, or other virial arguments, all leading to various power-law scalings, $R_0 \propto c^x$). We can only suggest a qualitative idea outlined below.

Let us assume there are M gelatin chains in solution, each N residues long, with nucleation sites located a fixed distance of n residues apart along the chain. The concentration c of residues in the solution is given by $c = MN/V$, 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 triple helix will grow from it. 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. 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 attached to the existing network, with $h(s)$ junctions. The infinitesimal increase in the number of triple 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: $dh/ds = \alpha h$ hence $h(c) \propto e^{(\alpha/n)c}$.

In summary, by applying a new concentration-temperature superposition we found a universal master curve describing the initial folding rates R_0 of gelatin solutions over a broad range of concentrations and temperatures, spanning the nominal helix-coil transition point T_m. The exponential (c, T) dependence of R_0 arises from the increasing overall amount of helices in different systems. The normalized helical fraction $\chi(t)$ grows with a c-independent rate, characteristic of helix lengthening. This suggests that nucleation occurs rapidly and contributes less to the helical onset than previously thought. There is a hope, supported by another study of universality [24], that these results are in fact general. One needs to study other oligomerizing proteins to test the further universality of the discovered master curves.

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