Self-association of Collagen Triple Helix Peptides into Higher Order Structures*

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Interest in self-association of peptides and proteins is motivated by an interest in the mechanism of physiologically higher order assembly of proteins such as collagen as well as the mechanism of pathological aggregation such as β-amyloid formation. The triple helical form of (Pro-Hyp-Gly)₁₀, a peptide that has proved a useful model for molecular features of collagen, was found to self-associate, and its association properties are reported here. Turbidimetry experiments indicate that the triple helical peptide self-assembles at neutral pH via a nucleation-growth mechanism, with a critical concentration near 1 mM. The associated form is more stable than individual molecules by about 25 °C, and the association is reversible. The rate of self-association increases with temperature, supporting an entropically favored process. After self-association, (Pro-Hyp-Gly)₁₀ forms branched filamentous structures, in contrast with the highly ordered axially periodic structure of collagen fibrils. Yet a number of characteristics of triple helix assembly for the peptide resemble those of collagen fibril formation. These include promotion of fibril formation by neutral pH and increasing temperature; inhibition by sugars; and a requirement for hydroxyproline. It is suggested that these similar features for peptide and collagen self-association are based on common lateral underlying interactions between triple helical molecules mediated by hydrogen-bonded hydration networks involving hydroxyproline.

There is increasing interest in the ability of proteins and peptides to self-associate into aggregates, both in normal and pathological processes. Normal self-association processes include fibril formation of collagen and polymerization of actin (1, 2), whereas pathological aggregation of amyloids is implicated in neurodegenerative diseases (3, 4). Interest has focused on the nature of protein aggregation and the molecular and environmental determinants of the self-association process. The study of the ability of collagen-like peptides to aggregate offers an opportunity to characterize a unique system, which may relate to the physiological self-association of collagen molecules.

Collagen, the major structural protein in the extracellular matrix, has a characteristic triple helical conformation, consisting of three polyproline II-like chains that are supercoiled around a common axis (5–7). The close packing of the three chains near the central axis generates a requirement for Gly as every third residue, (Gly-X-Y)₃n, whereas the high content of imino acids Pro and hydroxyproline (Hyp) stabilizes the individual polyproline II-like helices. Although imino acids are highly favorable for the triple helix, the post-translational modification of Pro to Hyp in the Y position confers an additional stabilizing contribution. This further stabilization of Hyp is likely to result from steroelectronic promotion of the more favorable exo ring pucker for the Y position and Hyp involvement in solvent-mediated hydrogen bonding (8–10). The favorable enthalpy of collagen indicates that hydrogen bonding is a major contributor to stability. One direct interchain peptide hydrogen bond is formed for each Gly-X-Y unit, together with an extensive water-mediated hydrogen bonding network (10–12). The ordered water network seen in crystal structures links the available backbone carbonyls of the triple helix and Hyp groups.

The family of collagens consists of 28 distinct genetic types with a range of tissue distributions and diverse functions (13). For most collagens, the molecules self-associate to form a higher order structure, such as fibrils and networks, and the supermolecular structure is responsible for mechanical and binding properties critical to function. The most abundant collagens are types I, II, and III, which are found in characteristic fibrils with an axial D = 670 Å period in tendon, skin, bone, cartilage, and other tissues. The process of association of type I collagen molecules into fibrils has been characterized from thermodynamic and kinetic approaches (1, 14–18). In addition, the assembly of type IV collagen into networks in basement membranes and the organization of type VII collagen association in anchoring fibrils of skin are beginning to be defined (19–22).

Studies of model peptides have contributed much to the understanding of the structure, stability, conformation, and dynamics of the collagen triple helix (7, 8, 23–25). The sequence

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Gly-Pro-Hyp is the most stabilizing and common triplet in collagen and the peptide (Pro-Hyp-Gly)₁₀ forms a highly stable triple helix (26, 27). The molecular structure of (Pro-Hyp-Gly)₁₀, determined to high resolution by x-ray crystallography, confirms the basic triple helical structure and shows an extensive hydration network (28, 29). Recently, there have been several reports of collagen-like peptides that self-associate to give irreversible samples of higher molecular weight structures, including studies on poly(Pro-Hyp-Gly)₁₀ (30), staggered arrangements of (Pro-Hyp-Gly)₁₀ chains (31), and liquid crystalline arrays of triple helical peptides (32–34). Cross-linked, high molecular weight forms of (Pro-Hyp-Gly)₁₀, but not the (Pro-Hyp-Gly)₁₀ molecule itself, have previously been shown to function as strong inducers of platelet aggregation (35). Thus, peptide studies may increase our understanding of the formation and biological activity of the polymeric collagen structure as well as giving information at the molecular level.

Here, self-assembly of the collagen model peptide (Pro-Hyp-Gly)₁₀ is characterized by a range of biophysical techniques, including turbidity, CD spectroscopy, differential scanning calorimetry (DSC), dynamic light scattering (DLS), and electron microscopy. The peptide is shown to self-associate with increasing temperature, following a nucleation-growth mechanism, from a simple trimer to a higher molecular weight form. This process is promoted by high concentration, neutral pH, increasing peptide length, and increasing temperature and is a reversible process. The self-association properties of this triple helical peptide are discussed in the context of collagen fibril formation.

MATERIALS AND METHODS

The peptide (Pro-Hyp-Gly)₁₀ was obtained from Peptides International (Louisville, KY). The peptides (Pro-Hyp-Gly)₇, (Pro-Hyp-Gly)₉, and (Pro-Hyp-Gly)₁₂ were synthesized by the Tufts University Core Facility (Boston, MA) and were purified on a reverse-phase high pressure liquid chromatography system (Shimadzu) with a C-18 column. Purity of all peptides was ensured by mass spectrometry using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF). Peptide concentrations of starting solutions were measured by monitoring the absorbance at 214 nm using ε²¹⁴ = 2200 cm⁻¹M⁻¹ per peptide bond. D-Glucose and D-fructose were obtained from Merck. Buffers used included 20 mM PBS (10 mM NaH₂PO₄, 10 mM Na₂HPO₄, 150 mM NaCl) for pH 7; acetate buffer (20 mM sodium acetate, 150 mM NaCl) for pH 3.0–4.5; and phosphate buffers (20 mM Na₂HPO₄, 150 mM NaCl) for pH 9–11.

Turbidity Measurements—Turbidity curves were obtained by monitoring the optical density at 313 nm as a function of time using a Beckman DU 640 spectrophotometer with attached Peltier temperature controller. A sample of 600 μl of the peptide solution was placed in a 2-mm cell, which was sealed to avoid evaporation, and then kept at the desired constant temperature. All turbidity measurements were repeated a minimum of three times.

The turbidity curves obtained at different temperatures were used to calculate the activation energy of self-assembly using the Arrhenius equation, $k = A e^{-E_a/RT}$, where $k$ is the rate constant obtained from the slope, $dA_{313\text{ nm}}/dt$, of the linear growth phase of each turbidity curve.

At the end of the plateau phase of the turbidity, the sample was centrifuged, and the supernatant was analyzed by the mean residue ellipticity at 225 nm in the CD spectrum to determine the percentage of peptide that remained in solution. Because some of the turbidity studies were carried out near the $T_m$ of the peptide, each supernatant was incubated for 4 °C for 2 days to allow complete formation of native triple helix of all peptide in solution prior to CD measurements.

For seeding experiments, 5% (v/v) aliquots of turbid sample containing aggregates of (Pro-Hyp-Gly)₁₀ were added to a fresh solution of the peptide prior to incubation. The rate of self-assembly in the absence and presence of seeding was monitored by the turbidity rise at 45 °C. For a comparison with DSC scans (see below), turbidity was also measured as a function of temperature at a heating rate of 1 °C/min (10–90 °C), with concentration and pH values identical to the DSC experiments.

Circular Dichroism Spectroscopy—Circular dichroism (CD) measurements were carried out using an Aviv Model 62DS spectrophotometer (Aviv Biomedical, Inc.), and the characteristic triple helix CD maximum at 225 nm was used to monitor thermal transitions, refolding curves, and determination of the amount of triple helix peptide remaining in the supernatant following aggregation.

Rotary Shadowing Electron Microscopy—Electron microscopy was carried out on rotary-shadowed samples of the peptide to visualize the morphology of the aggregated structures. At various time points during the monitoring of turbidity at a fixed temperature, a small aliquot of the suspension was placed on a carbon-coated 400-mesh copper grid. Samples were allowed to adsorb for 2–3 min and then were washed with ethanol at different dilutions (20–100% v/v) and air-dried. Afterward samples were rotary shadow cast with tungsten using a JEOL (JEE-400) vacuum evaporator and examined by transmission electron microscopy (Phillips 420 TEM). The micrographs were taken at a magnification ranging from ×40,000 to 80,000.

Dynamic Light Scattering—DLS measurements are performed using a DynaPro Titan (Wyatt Technology Corp., Santa Barbara, CA) equipped with a temperature controller using a 12-μl quartz cuvette. All samples were centrifuged and filtered through 0.1-μm Whatman Anotop filters before measurements. To obtain the hydrodynamic radii ($R_h$), the intensity autocorrelation functions were analyzed by Dynamics software (Wyatt Technology Corp.). For data analysis, a viscosity value of $\eta_{20^\circ C} = 1.019$ centipoise was used for PBS.

Differential Scanning Calorimetry—DSC measurements were performed on a Nano-DSC II, model 6100, scanning calorimeter from Calorimetry Sciences Corp. (Lindon, UT). All DSC profiles were obtained at a scan rate of 1 °C/min, and each curve was base-line subtracted before data analysis. The concentration of the peptide (Pro-Hyp-Gly)₁₀ was 7 mg/ml, and prior to all measurements, peptide solutions were dialyzed. Calorimetric enthalpy values $\Delta H_{\text{cal}}$ were obtained by integrating the excess heat capacity curve.

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6 The abbreviations used are: DSC, differential scanning calorimetry; DLS, dynamic light scattering; PBS, phosphate-buffered saline.
RESULTS

Analysis of Self-association at a Constant Temperature by Turbidity

The peptide \((\text{Pro-Hyp-Gly})_{10}\) forms a stable triple helical structure, with a \(T_m\) of \(60^\circ\)C, and its thermodynamic and kinetic properties have been well characterized (26, 27, 36). Studies in our laboratory indicated that at higher concentrations and neutral pH, the peptide \((\text{Pro-Hyp-Gly})_{10}\) comes out of solution upon heating, and the conditions for this aggregation were investigated. The self-association of the peptide \((\text{Pro-Hyp-Gly})_{10}\) can be monitored by turbidity, following the change in absorbance at 313 nm as a function of time. Rapid formation of aggregates was observed at a concentration of 7 mg/ml at \(58^\circ\)C and pH 7. The turbidity curves of the self-assembly process consist of three stages: an initial lag phase with negligible absorbance; a rapid growth phase; and a plateau phase at a maximum absorbance level. After some time, the absorbance may drop because of the settling of aggregated material. The process of self-association of \((\text{Pro-Hyp-Gly})_{10}\) is found to be influenced by peptide concentration, temperature, the pH of the solution, and the length of the peptide. The effect of each of these factors, as well as the consequence of the addition of sugars, is described.

Concentration Dependence—At \(58^\circ\)C, pH 7, no turbidity was observed at 2 mg/ml within 4 h, suggesting a minimum requirement of concentration for \((\text{Pro-Hyp-Gly})_{10}\) to undergo self-association. At concentrations greater than 2 mg/ml, turbidity curves indicative of self-association were observed. Increasing concentration results in a decrease in the lag time and an increase in the rate of the growth phase (Fig. 1).

Samples were centrifuged after the plateau phase was obtained, and the CD signal at 225 nm was used to determine the concentration of peptide in the supernatant, which indicates the amount of peptide in the precipitate. Higher concentrations led to a greater fraction of peptide in the aggregated state (Fig. 1, inset). Regardless of the initial concentration (3–7 mg/ml), the concentrations of all supernatants were in the range of 2.6–3.4 mg/ml (\(<1\) mm), suggesting that this is the critical concentration.

Temperature Dependence—The self-association process is strongly dependent on temperature (Fig. 2a). At or below 35 °C, no turbidity was seen within 4 h for peptide solutions of concentration 7 mg/ml. At temperatures higher than 35 °C, turbidity was observed within 1 h. With increasing temperature, the lag time decreased and the rate of self-association increased, as measured by the slope of the linear rise \((dA_{313}/dt)\) in the growth phase of each curve (Fig. 2a). The maximum rates occur for temperatures in the range of 55 to 62 °C. The rates observed at different temperatures fit a linear Arrhenius plot with an activation energy of 137 ± 10 kJ/mol (Fig. 2b).

Effect of pH, Sugars, and Peptide Length—Self-association was studied as a function of pH at a constant temperature (58 °C) and concentration (7 mg/ml). Turbidity was seen between pH 3.8–9.8, with no aggregation detectable at lower or higher pH values. The rate of self-association was highest and the lag time was the least near neutral pH (see supplemental Fig. 8).

Because sugars were previously found to inhibit protein aggregation and collagen fibril formation (37), the effect of glucose and fructose were investigated for this peptide system. The addition of D-glucose (1 M) or D-fructose (1 M) to a solution of \((\text{Pro-Hyp-Gly})_{10}\) at 7 mg/ml, pH 7, resulted in complete inhibition of self-association at 58 °C as monitored by turbidity measurements (see supplemental Fig. 9).

Reliable CD melting curves could not be obtained for \((\text{Pro-Hyp-Gly})_{10}\) at concentrations greater than 3 mg/ml in PBS at neutral pH because of aggregation. Because aggregation of \((\text{Pro-Hyp-Gly})_{10}\) is not seen at any temperature under acid conditions or in the presence of sugars, CD melting curves under these conditions represent a two-state trimer-monomer process. Thermal transitions with \(T_m\) value at 65 °C were observed at pH 3 or in the presence of glucose or fructose (data not shown).
To investigate the effect of peptide length on self-association, isothermal turbidity studies were carried out on (Pro-Hyp-Gly)_n, where n = 7, 8, 10, and 12. These peptides all formed stable triple helical structures with melting temperature values of T_m = 25 °C (n = 7), 47 °C (n = 8), 60 °C (n = 10), and 70 °C (n = 12) under standard conditions (38). Peptide (Pro-Hyp-Gly)_7 showed no indication of self-association at 7 mg/ml at any of the temperatures studied, whereas n = 8 showed a small rise in turbidity at 7 mg/ml at 48 °C. The peptide with n = 10 showed the substantial increase reported here at various temperatures. The longest peptide, (Pro-Hyp-Gly)_12 showed the highest propensity for aggregation, with self-association at lower concentrations and lower temperatures than seen for (Pro-Hyp-Gly)_10 (see supplemental Fig. 10).

Effect of Seeding on Self-assembly—To analyze the effect of seeding, a very small amount (5% v/v) of aggregated sample was added to a fresh solution of the peptide, and then the kinetics of self-association were monitored by turbidity measurements.

Without seeding, (Pro-Hyp-Gly)_10 exhibited a lag phase of ~12 min when incubated at 45 °C (at 7 mg/ml, pH 7). With seeding, no lag phase was observed, and the maximum turbidity was obtained much earlier but with a similar growth rate (Fig. 3). The effect of seeding is consistent with the nucleation propagation mechanism of the process of self-assembly.

Self-association Studies at a Constant Heating Rate: Differential Scanning Calorimetry and Turbidity Studies of (Pro-Hyp-Gly)_10

To further characterize the process of self-association, DSC measurements at a constant heating rate were carried out to monitor the temperature dependence of the heat capacity. Under non-aggregating conditions (at pH 3 or in the presence of sugars), the DSC of the peptide shows only one transition at 65 °C, representing the native trimer to unfolded monomer transition (data not shown). Under aggregating conditions, the DSC of (Pro-Hyp-Gly)_10 at pH 7 (7 mg/ml) shows two transitions upon heating, with a first peak at 65 °C and a second peak at 92 °C (Fig. 4). The first peak represents the unfolding of indi-
individual triple helices, whereas the second peak represents the melting of the aggregated form of the peptide. The CD spectrum taken at 95 °C shows that only unfolded single chains are present at very high temperatures (see supplemental Fig. 11), indicating that the second DSC peak represents the melting of an associated form into unfolded monomers. The appearance of a second peak higher than the triple helix melting temperature suggests that interactions between triple helices within an aggregate contributes extra stability, raising the transition temperature by almost 25 °C.

To examine the reversibility of the processes, cooling experiments were carried out at the same scan rate (1 °C/min) from 95 to 0 °C (See supplemental Fig. 12). Upon cooling from a high temperature, the DSC curve showed a dominant transition at 65 °C, reflecting the refolding of the monomers to triple helices under these conditions, as confirmed by CD studies. The observation that both cooling and heating resulted in a peak at the $T_m$ value of 65 °C indicates that the monomer-trimer association/dissociation process is close to equilibrium and is reversible. The second DSC transition at 92 °C seen upon heating was not observed upon cooling. This suggests that aggregation does not arise directly from monomers but is dependent on the presence of native triple helices. As the temperature was cooled below the $T_m$ value, the turbidity began to increase, indicating that refolded triple helices are capable of self-association within the aggregation-prone temperature range of 37–60 °C. With continued cooling below 30 °C, the turbidity fell, and a small negative DSC peak was observed near 20 °C, which may reflect the dissociation of some aggregate into native triple helices at low temperature. Reheating again led to the same two DSC peaks observed upon initial heating, confirming the overall reversibility of the process.

**Dynamic Light Scattering**

DLS studies were carried out on (Pro-Hyp-Gly)$_{10}$ to monitor peptide size, homogeneity, and aggregation (Fig. 5). The translational diffusion coefficient of the sample derived from DLS was processed to derive a size distribution of the molecular assemblies in terms of hydrodynamic radius ($R_h$). The size distribution profile of (Pro-Hyp-Gly)$_{10}$ at 7 mg/ml in PBS, pH 7, was determined at low temperatures, where there is no aggregation. At 4 °C the initial peptide sample gave an apparent hydrodynamic radius ($R_h$) of 1.9 nm, consistent with a single population of native triple helices (Fig. 5a). When heated to 58 °C, rapid peptide aggregation occurred and saturated the system, preventing the recording of DLS data. However, when the 58 °C aggregated sample was cooled back to 4 °C, it immediately became completely soluble. The DLS again showed a single population with $R_h = 1.9$ nm (Fig. 5b), and the CD spectra showed that the peptide was fully triple helical (supplemental Fig. 11). This indicates that the aggregation process is fully reversible.

To characterize the presence of any smaller aggregates within the range of DLS capability, a sample of (Pro-Hyp-Gly)$_{10}$ at 7 mg/ml was monitored at 37 °C, where the lag time was very long (>1 h). DLS measurements taken at 37 °C at different time points showed a single population with $R_h \approx 2$ nm up to 60 min (Fig. 5c). After 75 min, a second population with higher $R_h$ values (60–75 nm) was also observed, which constituted ~20% of the total mass (Fig. 5d). The presence of a higher molecular weight DLS peak indicates the formation of smaller associated structures prior to the formation of larger aggregates.

**Electron Microscopy of (Pro-Hyp-Gly)$_{10}$ Aggregates**

To visualize the structures formed as result of self-association of (Pro-Hyp-Gly)$_{10}$, samples were observed by electron microscopy after rotary shadowing (Fig. 6). The micrographs show a filamentous, branching structure. Many branches had

![FIGURE 5. Dynamic light scattering analysis of the hydrodynamic radius ($R_h$) of molecular species for (Pro-Hyp-Gly)$_{10}$, 7 mg/ml PBS, pH 7. a, original sample at 4 °C before aggregation; b, sample returned to 4 °C after aggregation at 58 °C; c, sample kept at 37 °C for 60 min (end of lag phase); d, sample kept at 37 °C for 80 min (beginning of growth phase).](image)

![FIGURE 6. Electron micrograph of the self-assembled structure formed by (Pro-Hyp-Gly)$_{10}$ peptide after rotary shadowing. The peptide (Pro-Hyp-Gly)$_{10}$ (7 mg/ml, pH 7) incubated at 58 °C for 8 min (in the rapid growth region of the turbidity rise) was collected for microscopy.](image)
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an average diameter of ~20 nm, but because of the metal deposition, it was difficult to estimate values accurately. Observations made at later time points showed more branched and larger assembled structures. The electron microscope structure of aggregates of the peptide (Pro-Hyp-Gly)\textsubscript{10} was found to be similar to that of (Pro-Hyp-Gly)\textsubscript{10}.

Comparison of (Pro-Pro-Gly)\textsubscript{10} with (Pro-Hyp-Gly)\textsubscript{10}

Similar studies were carried out on (Pro-Pro-Gly)\textsubscript{10} to investigate the role of Hyp in self-association. The peptide (Pro-Pro-Gly)\textsubscript{10} forms a triple helix that is much less stable than that of (Pro-Hyp-Gly)\textsubscript{10} ($T_m = 30$ °C compared with $T_m = 65$ °C) (26). Turbidity studies were carried out on the peptide (Pro-Pro-Gly)\textsubscript{10} but no aggregation was seen at concentrations up to 14 mg/ml and over a wide range of temperatures (10–70 °C).

DISCUSSION

The triple helical peptide (Pro-Hyp-Gly)\textsubscript{10} is reported here to self-associate under defined conditions of concentration, temperature, and pH. The turbidity curve monitoring self-association is similar to that seen for collagen fibril formation and the polymerization of other proteins, in having a lag or nucleation phase followed by a rapid growth phase and a plateau (1, 2, 16, 39). A nucleation-growth mechanism was confirmed by seeding experiments, which eliminated the lag phase. Consistent with this mechanism is the concentration dependence of the aggregation of (Pro-Hyp-Gly)\textsubscript{10}, which shows a critical concentration of about 1 mM.

The lag phase decreases, whereas the rate of fibril growth is enhanced with increasing temperature, with a maximum rate occurring somewhat below the $T_m$ of (Pro-Hyp-Gly)\textsubscript{10}. The effect of temperature on the rate of association gave a linear Arrhenius plot with a negative slope. The promotion of self-association with increasing temperature suggests the energetically unfavorable formation of a nucleus during the lag phase is entropically driven. Increasing temperature could loosen the triple helix and its associated water, which may promote self-association. A schematic diagram of the self-association process for (Pro-Hyp-Gly)\textsubscript{10} is shown in Fig. 7, suggesting an intermediate loosened state of the triple helix, which has a propensity for association. This is reminiscent of the temperature dependence of assembly of collagen into fibrils (14, 15, 40), which has been analyzed in terms of forces (41). Leikin et al. (41) showed this “temperature favored” process of collagen fibril formation is largely because of hydrophilic interactions, which could relate to release of structured water or increasing mobility of side chains. In the case of (Pro-Hyp-Gly)\textsubscript{10}, it is possible that a small increase in mobility of the rigid imino acids could play a role, but it is more likely that loosening of the ordered hydration shell due to increasing temperature is an important factor. Leikin’s laboratory has shown that sugars inhibit fibril formation of collagen by disrupting water mediated hydrogen bonds between triple helices (37), and the inhibitory effect of sugars on the self-association process of (Pro-Hyp-Gly)\textsubscript{10} seen in our present work indicates a similar importance of hydration related hydrogen bonding for the peptide association.

A strong length dependence is observed for peptide association. An increase in peptide length could either lead to a larger number of cooperative interactions or promote a molecular crowding effect because of the larger hydrodynamic radius of a longer rod-like molecule. Consistent with molecular crowding, it has been reported that aggregation of several collagen-like peptides can be induced by very high concentrations (>10 mM) (32–34). Higher molecular weight peptides and cross-linked trimers of staggered chains with the sequence Gly-Pro-Hyp form aggregates with a morphology similar to that reported here (30, 31).

Self-association also depends on pH, with an optimum near neutral pH and no aggregation at low and high pH values. The pH dependence of the self-association could be related to water activity, or association could be promoted by lower thermal stability and a resultant loosening of the triple helix, because the melting temperature of (Pro-Hyp-Gly)\textsubscript{10} shows a small decrease near neutral pH (42).

The observation that a homologous triple helical peptide (Pro-Pro-Gly)\textsubscript{10} does not come out of solution under the conditions used in this study suggests that all triple helical peptides do not have the same propensity for self-association. The differences observed between (Pro-Pro-Gly)\textsubscript{10} and (Pro-Hyp-Gly)\textsubscript{10} suggest a role for Hyp in promoting self-assembly, which is supported by the observation that recombinant collagen without Hyp does not undergo fibril formation under physiological conditions (43). The role of Hyp in triple helix association could relate to its involvement in the hydration network or direct intermolecular hydrogen-bonding interactions between triple helices as observed in the crystals of collagen-like peptides (29, 44, 45) or as proposed by Gustavson in the 1950s (46). Preliminary observations in our laboratory on various triple helical peptides with diverse sequences sandwiched
Hyp-Gly)10. Collagen fibrillogenesis is faster at higher temperatures, with a maximum rate somewhat below the $T_m$ of collagen (15, 41); a similar temperature dependence pattern was observed for (Pro-Hyp-Gly)10 association. The formation of reconstituted collagen fibrils from soluble collagen shows two DSC transitions (49), which correspond to the rise and fall of turbidity, resembling the DSC curves reported here for (Pro-Hyp-Gly)10. Similar to the peptide association, collagen self-association into fibrils is reversible unless aldehyde-mediated covalent cross-links are formed (50). We suggest that the lateral self-assembly of triple helices may exhibit similar properties because of their common Hyp-mediated hydration networks. These hydrogen-bonding hydration networks involving Hyp are inherent in the molecular structure of the triple helix and serve to provide regular hydrogen bonding with the two available backbone carbonyl groups within each Gly-X-Y unit. Such hydration forces have been implicated as critical to the lateral fibril organization (41).

Physiological interactions often depend on collagen being present in fibrillar or other higher order structures. For example, platelets are activated by collagen via glycoprotein VI; this activation can be mimicked by a cross-linked higher molecular form of (Gly-Pro-Hyp)10 but not by the triple helical (Gly-Pro-Hyp)10 molecule (35). Similarly, binding to certain integrins may involve collagens in a fibrillar form (51). The ability to study the association of triple helical peptides may provide a tool for investigating interactions that require higher order structure as well as clarifying the principles of triple helix self-association. Elucidation of the principles of triple helix self-association may also be important in the design of biomaterials and tissue engineering scaffolds.

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### TABLE 1

Comparison of properties of self-association of (Pro-Hyp-Gly)10 with fibril formation of type I collagen

| Properties                          | (Pro-Hyp-Gly)10 | Type I collagen |
|-------------------------------------|-----------------|----------------|
| Molecular $T_m$                      | 65 °C           | 38 °C          |
| Length (Gly-X-Y)                     | 10 triplets     | 338 triplets   |
| Amino acid content (%)               | 66              | 20             |

| Self-association properties | Acid, No | Acid, No |
|-----------------------------|----------|----------|
| pH                          | Neutral, Yes | Neutral, Yes |
| Temperature $T_m$            | $T \geq 35^\circ$ C, optimum | $T \geq 29^\circ$ C, optimum |
| Stability of associated form| $T_m + 25^\circ$ C | $T_m + 23^\circ$ C |
| Reversibility               | Yes      | Yes     |
| Activation energy            | 137 kJ/mol | 113 kJ/mol |
| Critical concentration       | 1 mm     | 50 μM    |
| Requirement for Hyp          | Yes      | Yes     |
| Inhibition by sugars         | Yes      | Yes     |
| Seeding effect               | Yes      | Yes     |
| Axial staggering             | No       | Yes     |
| Morphology                   | Branched | Linear  |

*At pH 3, 7 mg/ml.
* Ref. 49.
* Ref. 48.
* Ref. 15.
* Ref. 51.
* Ref. 50.
* Ref. 1.
* Ref. 1.
* Ref. 43.
* Ref. 37.
* Ref. 40.

between terminal Pro-Hyp-Gly repeats indicate that self-association is negatively influenced by the presence of charged residues, a Gly substitution, or an amino acid-deficient zone, in contrast to the positive influence of Hyp residues.

The aggregation of (Pro-Hyp-Gly)10 is reversible. The triple helical peptide comes out of solution as the temperature is increased, forming some supramolecular structure of triple helices. However, at temperatures higher than 80 °C, the aggregate fully dissolves into denatured monomeric chains. When cooling the unfolded monomers, the triple helix can re-form in a reversible manner, and while in the aggregation temperature range (37–60 °C) there is again aggregation of these refolded triple helices. Interestingly, both the monomer-trimer transition and the trimer-aggregate transition appear close to equilibrium.

The results reported here indicate that the triple helical (Pro-Hyp-Gly)10 molecules are self-associating to form a higher order structure, which is more stable than the individual triple helices and which is fully reversible. For the peptide, interacting triple helical molecules give rise to branched, fibrillar structures with no long-range order, which contrasts with the highly ordered axially periodic fibrils formed by type I collagen molecules (47). The short length of the model peptides and the uniformity of sequence are likely to contribute to its less ordered, branched structure and its high critical concentration value. Yet, a number of similarities between the peptide and collagen were observed in the nature of the self-association process and interactions (Table 1). Collagen fibril formation is optimum near neutral pH and does not occur at low pH (48), resembling the pH dependence observed for association of (Pro-Hyp-Gly)10. Collagen fibrillogenesis is faster at higher temperatures, with a maximum rate somewhat below the $T_m$ of collagen (15, 41); a similar temperature dependence pattern was observed for (Pro-Hyp-Gly)10 association. The formation of reconstituted collagen fibrils from soluble collagen shows two DSC transitions (49), which correspond to the rise and fall of turbidity, resembling the DSC curves reported here for (Pro-Hyp-Gly)10. Similar to the peptide association, collagen self-association into fibrils is reversible unless aldehyde-mediated covalent cross-links are formed (50). We suggest that the lateral self-assembly of triple helices may exhibit similar properties because of their common Hyp-mediated hydration networks. These hydrogen-bonding hydration networks involving Hyp are inherent in the molecular structure of the triple helix and serve to provide regular hydrogen bonding with the two available backbone carbonyl groups within each Gly-X-Y unit. Such hydration forces have been implicated as critical to the lateral fibril organization (41).
Higher Order Structure of a Collagen Peptide

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