Characterization of Triple Helical Structures of Synthetic Collagen Analogs by Reverse-Phase High-Performance Liquid Chromatography

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ABSTRACT: Reverse-phase high-performance liquid chromatography (RP-HPLC) is used to characterize triple helical structures of synthetic collagen analogs. This technique is based on the different retention times exhibited by the triple helical conformations and the non triple helical structures formed by the same collagen analog. The difference in retention times is mainly caused by the change of hydrophobicity after triple helix formation. For synthetic collagen analogs composed of tripeptide sequences which do not possess hydrophilic groups in their side chains, such as the Gly−Pro−Pro and Gly−Pro−Nleu (Nleu represents *N*-isobutylglycine residue) sequences, the triple helical conformations possess longer retention times than the non triple helical structures. On the other hand, for collagen analogs composed of Gly−Pro−Hyp sequences which have hydrophilic groups in the side chains, the triple helical conformations elute at shorter retention times than the non triple helical structures. In reverse-phase HPLC profiles, the template-assembled analog, KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ (KTA represents *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid), exhibits one single peak which arises from the triple helical conformation. The HPLC profile of Ac−(Gly−Pro−Nleu)$_9$−NH$_2$ is compared to that of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ to establish its triple helical HPLC peak. Both peaks from triple helical and non triple helical structures are observed in the HPLC profiles of single-chain collagen analogs Ac−(Gly−Pro−Nleu)$_9$−NH$_2$, (Gly−Pro−Pro)$_{10}$, and Ac−(Gly−Pro−Hyp)$_9$−NH$_2$. Under appropriate conditions, we demonstrate that the peaks associated with the triple helical structures are interconvertable with the peaks from the non triple helical structures.

Introduction

The structural domain of collagen is a triple helix. The triple helical structure contains three extended left-handed polyproline-II-like chains which are intertwined with a one residue register shift to form a right-handed super helix.[1−4] The primary structures of these polyproline-II-like chains are characterized by repeating Gly−X−Y trimers, where X and Y can be any amino acid residues. The formation of a triple helical conformation is favored by the presence of natural imino acid residues proline (Pro) and 4-*trans*-hydroxyproline (Hyp) in the X or Y positions. The restricted rotation around the ϕ and ψ angles of these imino acid residues can help the folding of each chain into a polyproline-II-like helix.[5] Therefore, many single-chain and template-assembled collagen analogs composed of Gly−Pro−Y or Gly−X−Pro sequences have been prepared to mimic the collagen structures.[6−20] These studies have helped elucidate the special triple helical conformation, discover the sequences and residues that favor the triple helical structure, and provide insight toward the preparation of novel collagen-like biomaterials.

Many experimental techniques have been employed to characterize the triple helical conformation of synthetic collagen analogs. Circular dichroism (CD) spectroscopy is the most frequently used technique. The natural collagen triple helix exhibits a unique CD spectrum with a small positive peak around 220 nm, a crossover at 213 nm, and a large negative peak around 197 nm.[9,10,21] In addition, a CD peak intensity related parameter Rpn, which denotes the ratio of positive intensity over negative peak intensity, has been introduced in our laboratory[12,13,22] and utilized in other laboratories.[23] Rpn values can be used to distinguish triple helical conformations from polyproline-II-like structures. Melting curve measurements are often used together with the CD spectra to confirm the presence of triple helical conformations in solution. Similar to other ordered structures such as α-helices and β-sheets, triple helical structures exhibit cooperative melting curves during denaturation. NMR spectroscopy is a technique which has been used in recent years to detect triple helical structures.[21,24−26] One or all of the following NMR signals can be observed when a triple helical conformation is formed in solution: a new set of resonances in 1-D NMR spectra, which is absent in the non triple helical structures; amide NH proton signals (in D_2O), which indicate a slow proton exchange because of triple helix formation; interchain NOEs in 2-D NMR spectra, which are unique to the triple helical packing; splitting of peaks arising from template or spacer groups in template-assembled collagen analogs arising from the anisotropy of a triple helical structure. In addition to the above commonly used techniques, X-ray crystallography,[19,27] equilibration sedimentation,[21] and vibration spectroscopy[7] have also been utilized to characterize triple helical conformations.

In this paper, we report the characterization of triple helical structures of synthetic collagen analogs by a chromatography technique. This technique is based on reverse-phase high-performance liquid chromatography (RP-HPLC). For a collagen analog, the triple helical and non triple helical structures have different molecular weights, shapes, and hydrophilicities. Therefore, these structures exhibit distinct HPLC peaks at different retention times in their chromatography profiles.

Experimental Section

Materials. Ethylene glycol (EG, reagent grade), CH_3CN, and H_2O (HPLC grade) were purchased from Fisher Scientific and used without further purification. Trifluoroacetic acid

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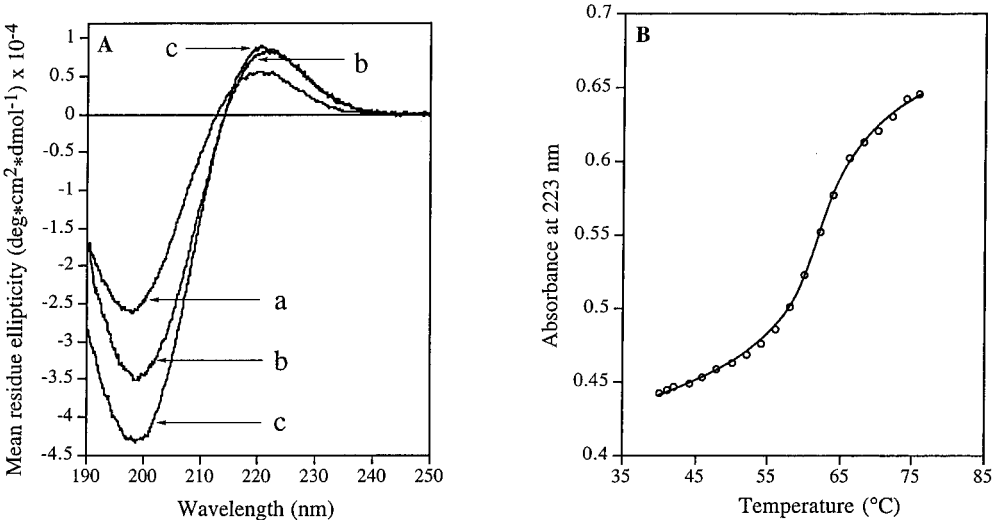

Figure 1. A: CD spectra at 20 °C at a concentration of 0.2 mg/mL for Ac−(Gly−Pro−Nleu)$_9$NH$_2$ in H_2O (a) and in EG/H_2O (v/v, 2:1) (b) and KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ in H_2O (c). B: Thermal melting curve of Ac−(Gly−Pro−Nleu)$_9$−NH$_2$ in EG/H_2O (v/v, 2:1, 0.04 mg/mL) obtained by UV absorbance measurements.

(TFA, HPLC grade) was purchased from Chem-Impex International. The 30-residue peptide, (Gly−Pro−Pro)$_{10}$, was purchased from Peninsula Laboratory and used without further purification. All other synthetic collagen analogs, Ac−(Gly−Pro−Hyp)$_9$−NH$_2$, Ac−(Gly−Pro−Nleu)$_9$−NH$_2$, and KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ (KTA represents *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, also known as the Kemp triacid) were prepared as previously reported.[12,13]

Sample Preparation. The collagen analogs were dissolved in either H_2O or EG/H_2O (v/v, 2:1) at a concentration of 0.2 mg/mL at a pH of 3−6. In order to reach equilibrium for triple helix formation, the solutions for both chromatography experiments and chiroptical studies were incubated at 4 °C for at least 2 weeks for collagen analogs composed of Gly−Pro−Pro and Gly−Pro−Hyp sequences, and for at least 6 months for collagen mimetics composed of Gly−Pro−Nleu sequences.

Chromatography. Two HPLC systems were used to analyze the elution characteristics of synthetic collagen analogs: a Waters system (with 510 pumps and a 484 detector), and a MILLENNIUM 2010 system consisting of a Waters 715 Ultra WISP sample processor, a Waters 996 photodiode array detector, and two Waters 510 pumps. Solvents used in HPLC included solvent A, H_2O with or without 0.1% TFA, and solvent B, CH_3CN with or without 0.1% TFA. The flow rate was 4 mL/min for the semipreparatory column (Vydac 218TP1010 column, C-18, 25 × 1.0 cm), and 1.0−1.2 mL/min for the analytical column (Vydac 218TP54 column, C-18, 25 × 0.46 cm). The pore size for both columns was 300 Å.

Chiroptical Studies. All circular dichroism (CD) measurements were carried out on a modified Cary-61 spectropolarimeter.[12] The spectra were obtained using either a 0.02 cm or a 0.05 cm path length cell by signal-averaging 10 scans from 190 to 300 nm at a scan speed of 1.0 nm/s. Both UV absorbance and optical rotation measurements were used to obtain the melting curves. The ultraviolet (UV) measurements were carried out on a Cary-1E UV Spectrometer equipped with a Cary temperature controller. To perform the melting experiments, the sample was equilibrated for 1 h at the initial temperature. Data were collected at 223 nm at a heating rate of 0.2 °C/min. Optical rotations were measured using a Perkin-Elmer 241 polarimeter equipped with a Model 900 Isotemp refrigerator circulator (Fisher Scientific). Data were collected at 365 nm (Hg) at a concentration of 0.2 mg/mL in a 10 cm pathlength cell. Before optical rotation was recorded, the sample was equilibrated for 1 h at the initial temperature. At each subsequent temperature point, the sample was allowed to equilibrate until the optical rotation was constant.

Results and Discussion

Collagen Analogs Composed of Gly−Pro−Nleu Sequences. The unnatural peptoid residue Nleu (*N*-isobutylglycine) was introduced in our laboratory into collagen-like sequences as a proline surrogate to enhance the biostability (against enzymatic attacks) of synthetic collagen analogs.[28] Circular dichroism spectroscopy and melting curve measurements demonstrate that collagen analogs containing the Nleu residue form triple helical conformations in H_2O and in EG/H_2O (v/v, 2:1) (0.2 mg/mL).[13] As shown in Figure 1A, the CD spectra of the selected peptide−peptoid collagen analogs closely resemble that of natural collagen which has a small positive peak around 220 nm, a crossover at 213 nm, and a large negative peak at 197 nm.[9,10,21] The presence of triple helical conformations for these analogs is further confirmed by our NMR experiments[26] and melting studies by optical rotation measurements.[13] In addition, as with natural collagen,[29] a hyperchromic effect was observed during thermal denaturation for these collagen analogs. In Figure 1B, it can be seen that the absorbance of the triple helical conformation of Ac−(Gly−Pro−Nleu)$_9$−NH$_2$ is lower than that of its denatured structures (unordered and/or polyproline-II-like structures).

The RP-HPLC profile of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ in H_2O (0.2 mg/mL) at room temperature (*ca.* 23 °C) exhibits one single peak with a retention time of 19 min (Figure 2a). The KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ is a template-assembled collagen analog with three 27-residue chains attached to the template. This molecule is in a triple helical conformation in solution as shown by its CD spectrum (Figure 1A). In addition, its melting temperature is 47 °C in H_2O,[13] much higher than the 23 °C at which the HPLC experiments are carried out. Therefore, there is no thermal denaturation of triple helices expected for KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ under the HPLC conditions. On the basis of these biophysical results, it can be concluded that the HPLC peak in Figure 2a arises from the triple helical conformation of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$. This peak is thus denoted as peak **t** where **t** represents triple helix.

The triple helical characteristics of peak **t** in Figure 2a are also verified by triple helix (thermal) denaturation−refolding experiments. The H_2O solution of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH$_2$]$_3$ (0.2 mg/mL) was thermally denatured (80 °C for 3 h; the melting temperature is 47 °C in H_2O[13]) immediately before it was injected into the HPLC system. The thermal denaturation shows the presence of non triple helical structures

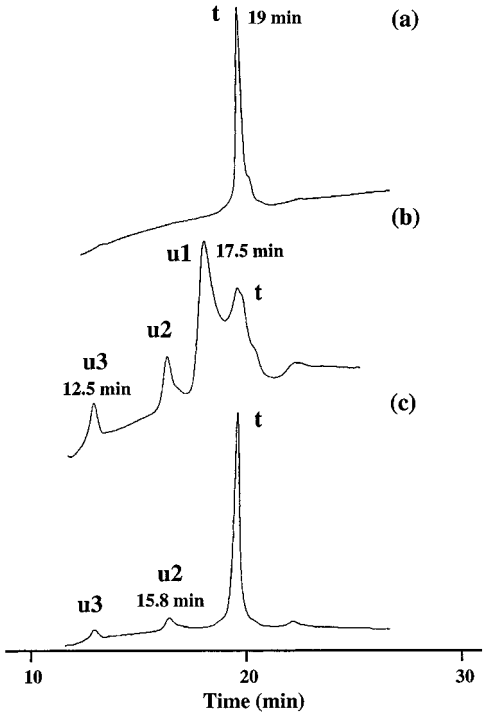

Figure 2. HPLC profiles of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ dissolved in H_2O (0.2 mg/mL) before and after thermal denaturation. Conditions: Vydac column, C-18, 25 × 0.46 cm. 30−90% B in 30 min, 1.0 mL/min, monitored at 215 nm. Solvent A: 0.1% TFA/H_2O. Solvent B: 0.15% TFA/CH_3CN. Key: (a) sample injected before denaturation; (b) sample injected immediately after denaturation (80 °C, 3 h); (c) sample injected after the denatured sample was kept at room temperature for 1 day.

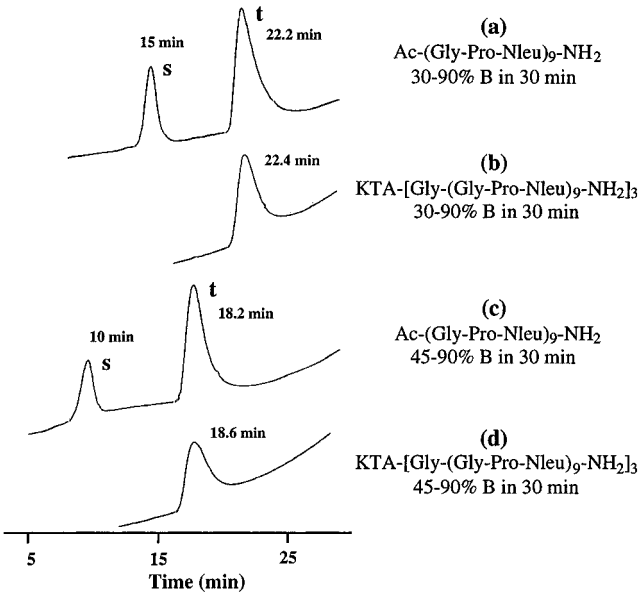

Figure 3. Comparisons of HPLC profiles of Ac−(Gly−Pro−Nleu)$_9$−NH_2 and KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ dissolved in EG/H_2O (v/v, 2:1) (0.2 mg/mL). Conditions: Vydac column, C-18, 25 × 0.46 cm, 1.2 mL/min, monitored at 215 nm. Solvent A: 0.1% TFA/H_2O. Solvent B: 0.1% TFA/CH_3-CN.

in solution when the sample is injected. Previous kinetic studies show that much more time is necessary for the triple helix folding of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ in H_2O.[30] Three more peaks are observed in Figure 2b with shorter retention times (RT = 12.5 min, 15.8 min, 17.5 min) when the sample was injected immediately after thermal denaturation. The intensity of peak **t** is significantly decreased. However, when the HPLC experiment was carried out after the denatured sample was stored for 1 day at room temperature, the HPLC (Figure 2c) exhibits the major peak **t**. The three new peaks in Figure 2b, which almost disappear in Figure 2c, originate from the unassembled structures. They are denoted as peak **u1**, peak **u2**, and peak **u3**, respectively (here **u** represents unassembled).

The triple helical property of peak **t** is further supported by UV spectroscopy. Normalized UV spectra (not shown) demonstrate that peaks **u1**, **u2**, and **u3** have similar absorbance and they are all higher than that of peak **t** over the spectral range 210−250 nm (not shown). Since a hypochromic effect is always accompanied with triple helix formation,[29] these UV absorbance results are consistent with a triple helical conformation for peak **t**.

The longer retention time for peak **t** than for peaks **u1**, **u2**, and **u3** is consistent with the triple helical packing of collagen and collagen analogs. In the triple helical structure containing Gly−X−Y sequences, the hydrophilic carbonyl and amide groups project toward the interior of the ensemble while the side chains of residues X and Y project toward the outside of the triple helix core and are exposed to the solvent.[31] This unique triple helical packing is also confirmed by our molecular modeling and NMR studies for Gly−Pro−Nleu sequences.[26] Therefore, the triple helical conformation of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ is more hydrophobic than the unassembled structures since all the side chains are hydrophobic in this analog. In RP-HPLC measurements, the higher hydrophobicity for the triple helical conformations leads to longer retention times than for non triple helical structures of collagen analogs composed of sequences which do not possess hydrophilic groups in their side chains, such as the Gly−Pro−Nleu sequences and Gly−Pro−Pro sequences (see below).

For the corresponding single-chain collagen analog Ac−(Gly−Pro−Nleu)$_9$−NH_2, our biophysical studies indicate formation of stable triple helical conformations in both H_2O and EG/H_2O (v/v, 2:1) at a concentration of 0.2 mg/mL. Its CD spectra (Figure 1A-a,b) are consistent with a collagen-like triple helical structure. The 2D NMR spectra for this molecule exhibit unique interchain NOEs characteristic of triple helical packing.[26] More importantly, its melting temperature in H_2O (39 °C) is much higher than 23 °C at which the HPLC measurements are carried out. These biophysical properties suggest a triple helical peak is expected in the HPLC profile of Ac−(Gly−Pro−Nleu)$_9$−NH_2.

As shown in parts a and c of Figure 3, two HPLC peaks are obtained. A number of experimental observations indicate that the first peak arises from the non triple helical structures [denoted as peak **s** where **s** represents the single-chain structure] while the second peak originates from the triple helical conformations [denoted as peak **t**]. The most convicing evidence for the triple helical characteristics of peak **t** comes from the comparisons between HPLC profiles of Ac−(Gly−Pro−Nleu)$_9$−NH_2 and KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$. As demonstrated in Figure 3, the retention times and the shapes of peak **t** of Ac−(Gly−Pro−Nleu)$_9$−NH_2 are very similar to those of the HPLC peak of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ under two different chromatographic conditions. The HPLC peak of KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ has already been assigned to the triple helical conformation. The triple helical structure formed by Ac−(Gly−Pro−Nleu)$_9$−NH_2 should be similar in size and shape to that formed by KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$, since they possess identical chain lengths. Therefore, the RP-HPLC

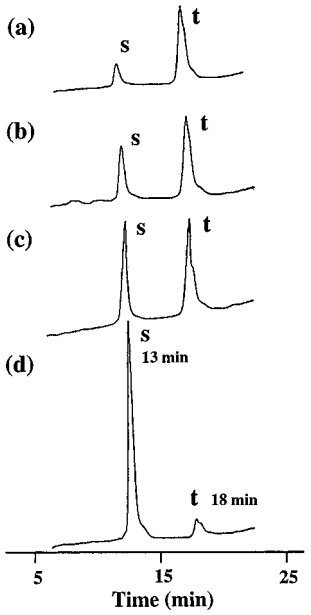

Figure 4. HPLC profiles following reinjection of the collected peak **t** of Ac−(Gly−Pro−Nleu)$_9$−NH_2 shown in Figure 3a. Conditions: Vydac column, C-18, 25 × 1.0 cm, 4 mL/min, monitored at 215 nm, 30−90% B in 25 min. Solvent A: 0.1% TFA/H_2O. Solvent B: 0.1% TFA/CH_3CN. Key: (a) sample reinjected 20 min after collection; (b) sample reinjected 1 h after collection; (c) sample reinjected 3.5 h after collection; (d) sample reinjected 24 h after collection.

peaks arising from the triple helical conformations of both Ac−(Gly−Pro−Nleu)$_9$−NH_2 and KTA−[Gly−(Gly−Pro−Nleu)$_9$−NH_2]$_3$ possess similar shapes and retention times. Results from UV spectroscopy also support the HPLC peak assignment of Ac−(Gly−Pro−Nleu)$_9$−NH_2. Figure 1B demonstrated that the absorbance of the triple helical structure of Ac−(Gly−Pro−Nleu)$_9$−NH_2 is lower than that of the non triple helical structure. Normalized UV spectra of peak **t** and peak **s** in Figure 3a were obtained by the PDA detector (photodiode detector) of the HPLC system. Results show that the absorbance of peak **t** is lower than that of peak **s** over the spectral range 210−250 nm (not shown), consistent with the result shown in Figure 1B.

Mass spectrometric analyses demonstrate that the materials collected from both peak **s** and peak **t** possess the same parent molecular weight (2465). Reinjection HPLC experiments confirm that peak **t** arises from the aggregation of the material in peak **s**. On a semipreparatory column (Vydac 218TP1010 column, 25 × 1.0 cm), the HPLC pattern is similar to that shown in Figure 3c for Ac−(Gly−Pro−Nleu)$_9$−NH_2. Peak **t** was collected. The collected solution was then reinjected onto the semipreparatory column to obtain HPLC profiles at different times. As demonstrated in Figure 4, the HPLC profiles of the collected elutions of peak **t** exhibited both peak **s** and peak **t**. In addition, the intensities of these two peaks are time dependent. The intensity of peak **s** increases with the storage time while that of peak **t** decreases with the storage time. After 24 h, peak **t** almost disappears. These results demonstrate that peak **s** and peak **t** are interconvertable. The time dependence of HPLC profiles in Figure 4 results from the denaturation of triple helical structures by the dilution after HPLC separation. The concentration of the collected elution from HPLC separation is much lower than that of the solution injected. Previous studies show that the triple helicity of Ac−(Gly−Pro−Nleu)$_9$−NH_2 is significantly concentration dependent when the concentration is lower than its critical triple helical concentration (*ca.* 1 mg/mL).[13] The lower the concentration, the lower the percentage of triple helical conformations in solution. The triple helix denaturation (or folding) of peptide−peptoid collagen mimetics containing the Nleu residue is slow in dilute solutions.[30] As a result, the HPLC profiles of the collected elution of peak **t** exhibit a (storage) time dependence such as that shown in Figure 4.

The RP-HPLC system (columns and elution solvents) has been reported to be capable of denaturing triple helical conformations of natural collagen and synthetic collagen analogs.[32,33] However, no denaturation for the triple helices of the Gly−Pro−Nleu sequences is observed during the time of chromatography (<30 min). As demonstrated in parts a and c of Figure 3, the ratios of intensities of peak **t** over peak **s** are the same under two different chromatographic conditions (Figure 3a, 30−90% B in 30 min; Figure 3c, 45−90% B in 30 min). If there is any denaturation by the HPLC system, the peak intensity would be dependent on the chromatographic conditions (retention times). The longer the retention time, the more significant the denaturation by the HPLC system (see discussions below for the HPLC results of collagen analogs composed of Gly−Pro−Hyp sequences). However, peak integration shows that the intensity ratios of peak **s** and peak **t** in parts a and c of Figure 3 are the same, indicating denaturation of the triple helices of Ac−(Gly−Pro−Nleu)$_9$−NH_2 by the HPLC system is negligible during the time of chromatography (<30 min).

Collagen Analogs Composed of Gly−Pro−Pro Sequences. In order to expand the application of the RP-HPLC technique in the characterization of triple helical conformations of synthetic collagen analogs, a standard compound, (Gly−Pro−Pro)$_{10}$, was used to compare HPLC profiles with Ac−(Gly−Pro−Nleu)$_9$−NH_2. The 30 residue peptide, (Gly−Pro−Pro)$_{10}$, has been demonstrated to form triple helical structures in H_2O in several laboratories.[17,27,34,35] As shown in Figure 5a, the melting temperature of this collagen analog in H_2O (0.2 mg/mL) is *ca.* 25 °C and the triple helices begin to denature at *ca.* 15 °C which is lower than 23 °C at which the HPLC experiments are carried out. To amplify the HPLC peak arising from the triple helical conformation, a sample of (Gly−Pro−Pro)$_{10}$ in H_2O (0.2 mg/mL) kept at 4 °C was subjected to HPLC analysis. As shown in Figure 5b, two major peaks are observed. Based on the retention times of these two peaks, the broad peak **t** is assigned to the triple helical conformation and the sharp peak **s** is a contribution from the non triple helical structures. The peak from the triple helical conformation of (Gly−Pro−Pro)$_{10}$ exhibits a longer retention time since the Gly−Pro−Pro sequence possesses only nonpolar side chains (see discussion for Ac−(Gly−Pro−Nleu)$_9$−NH_2).

The triple helical characteristics of the broad peak **t** are further supported by comparing the HPLC patterns of (Gly−Pro−Pro)$_{10}$ under different chromatography conditions (Figure 5b,c). The HPLC gradient is the same in both parts b and c of Figure 5. However, the sample [in Figure 5b] was maintained at 4 °C. Therefore, the triple helical peak **t** is the major observed peak. On the other hand, the sample [in Figure 5c] was warmed to room temperature (*ca.* 23 °C). As a result, the intensity of peak **t** decreases significantly, as shown in Figure 5c.

Collagen Analogs Composed of Gly−Pro−Hyp Sequences. Triple helical conformations formed by collagen analogs composed of Gly−Pro−Hyp sequences possess much higher thermal stability than analogs

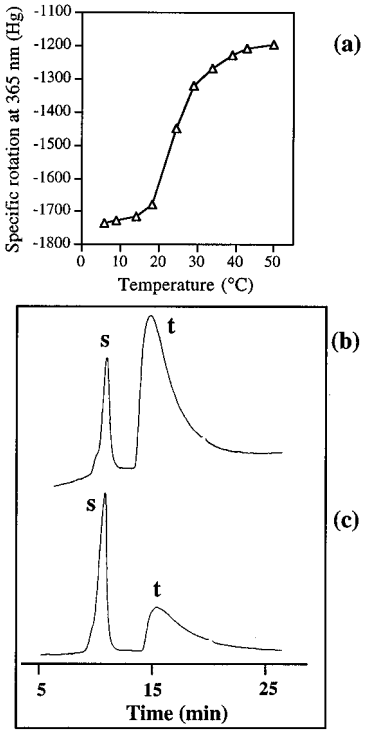

Figure 5. (a) Melting curve of $(Gly-Pro-Pro)_{10}$ in H_2O (0.2 mg/mL) obtained by optical rotation measurements. (b and c): HPLC profiles of $(Gly-Pro-Pro)_{10}$. Conditions: Vydac column, C-18, 25 × 0.46 cm, 1.2 mL/min, monitored at 215 nm, 15−70% B in 30 min. Solvent A: 0.1% TFA/H_2O. Solvent B: 0.1% TFA/CH_3CN. The sample was dissolved in H_2O (0.2 mg/mL). (b) The sample was injected at 4 °C. (c) The sample was injected at 23 °C.

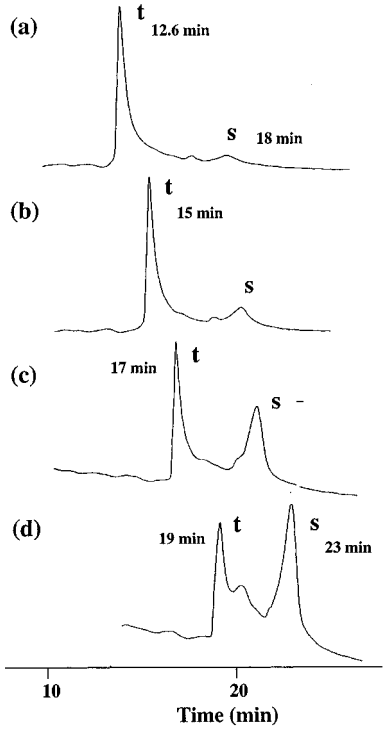

Figure 6. HPLC traces of $Ac-(Gly-Pro-Hyp)_9-NH_2$ dissolved in H_2O (0.2 mg/mL) at different gradient profiles. Conditions: Vydac column, C-18, 25 × 0.46 cm, 1.0 mL/min, monitored at 215 nm. Solvent A: H_2O. Solvent B: CH_3CN. Key (a) 10−30% B in 30 min; (b) 8−30% B in 30 min; (c) 6−30% B in 30 min; (d) 4−30% B in 30 min. In each case peak **t**, attributed to the triple helical structures, elutes when the acetonitrile content of the mobile phase reaches 18−20%.

composed of Gly−Pro−Pro or Gly−Pro−Nleu sequences[12,13,17] mainly because of the interchain and interresidue H-bonding network formed through the hydroxyl group of Hyp.[36,37] Therefore, HPLC peaks arising from the triple helical conformations of the Gly−Pro−Hyp collagen analogs are expected to be more easily observed. However, HPLC analyses show that triple helical conformations formed by these collagen analogs can be easily denatured by the reverse-phase HPLC system during the chromatography time. In addition, the HPLC pattern (the relative positions of the triple helical and non triple helical structures) is also different from those observed for collagen analogs composed of Gly−Pro−Pro and Gly−Pro−Nleu sequences.

The 27-residue peptide $Ac-(Gly-Pro-Hyp)_9-NH_2$ has been demonstrated to form very stable triple helical conformations. Its melting temperature is 67 °C in H_2O (0.2 mg/mL),[12] which is much higher than that of $Ac-(Gly-Pro-Nleu)_9-NH_2$ (39 °C in H_2O).[13] In addition, results from optical rotation measurements[12] indicate that $Ac-(Gly-Pro-Hyp)_9-NH_2$ is completely triple helical in H_2O down to a concentration of 0.02 mg/mL. Therefore, the HPLC profile of $Ac-(Gly-Pro-Hyp)_9-NH_2$ at a concentration of 0.2 mg/mL must arise from the triple helical structure. However, results presented in Figure 6 show that the HPLC profiles of this compound are significantly dependent on the chromatographic gradients (reflected by retention times). When the HPLC gradient is 10−30% B in 30 min, one major peak with a retention time of *ca.* 13 min and a small peak at *ca.* 18 min are observed (Figure 6a). The major peak at 13 min can be assigned to the triple helical conformation and is therefore denoted as peak **t**. As the retention time is gradually elongated (by changing the chromatography gradients), the intensity of peak **t** decreases while that of the second peak increases. When the retention time of peak **t** is *ca.* 19 min, the second peak is even more intense than peak **t**. In addition, a shoulder peak between the two peaks is observed (Figure 6d).

Several independent experimental observations demonstrate that the two major peaks and the shoulder in Figure 6d are interconvertable. From the mass spectrometric analysis it was seen that they possess the same parent molecular weight (2465). Collection and reinjection of peak **t** in Figure 6d yields an HPLC profile similar to that shown in Figure 6d under the same HPLC conditions. The Gly−Pro−Hyp sequences possess side chains with strongly hydrophilic groups. These hydrophilic side chain groups are exposed to the solvent in the triple helical packing as demonstrated by molecular modeling and NMR studies.[25,31] Therefore, the triple helical conformation is more hydrophilic than the non triple helical structures. As a result, unlike collagen analogs composed of Gly−Pro−Nleu and Gly−Pro−Pro sequences, the retention time of the triple helical peak is shorter than those of the non triple helical peaks for the Gly−Pro−Hyp sequences. On the basis of the retention times, it can be concluded that, in Figure 6, the second peak arises from the non triple helical structure [denoted as peak **s**] while the shoulder peak might arise from a partially dissociated triple helical packing.

The observation of several peaks instead of one single peak in Figure 6 indicates that denaturation of the triple helical conformation of $Ac-(Gly-Pro-Hyp)_9-NH_2$ by the HPLC system is significant during the time of the chromatography (<30 min). Results in Figure 6 demonstrate that the denaturation depends on the time the triple helical structure is retained on the column. In each case the peptide elutes when the percentage of

CH_3CN reaches 18−20%. It is the longer retention time rather than the composition of the elution solvents that leads to more significant triple helix denaturation under the HPLC conditions. A very small amount of peak **s** is observed when the retention time for the triple helix peak **t** is 12.6 min. As the elution time of peak **t** increases, the amount of peak **s** increases substantially. The triple helix denaturation is caused by the combined effects of the elution solvents (CH_3CN with 0.1% TFA), the peptide dilution, the length of time the material is exposed to the mobile phase, and the interactions between the peptide sequences and the stationary phase.[32,33] A decrease in the retention time can lessen these effects and therefore reduce the denaturation. If the triple helix denaturation caused by the HPLC system is negligible during the time course of chromatography as in the case of Ac−(Gly−Pro−Nleu)$_9$−NH_2, the peak intensities will be retention time independent. It must be pointed out that triple helix denaturation by the HPLC system is expected for any collagen-like peptide sequence. It is not observed in the Gly−Pro−Nleu sequence, because the triple helix denaturation (and folding) of this sequence is slow in a dilute solution and the time of detectable denaturation by the HPLC system is longer than the chromatography time (<30 min). These results show that caution must be taken in the purification of synthetic collagen analogs by RP-HPLC, especially for the Gly−Pro−Hyp sequence based analogs.

Conclusions

The analyses of RP-HPLC profiles of synthetic collagen analogs composed of different tripeptide sequences clearly demonstrate that the reverse-phase HPLC technique can be used to characterize triple helical conformations. For synthetic homogeneous collagen analogs, the physical and chemical properties of the triple helical conformation and the non triple helical structures are distinct so different retention times are observed for different structures in RP-HPLC profiles. The most important property that determines the retention times is the hydrophobicity of different conformations. For collagen analogs composed of sequences which do not have hydrophilic groups on the side chains, the triple helical conformations possess longer retention times than the non triple helical structures. On the other hand, for collagen analogs composed of Gly−Pro−Hyp sequences, the triple helical conformations have shorter retention times than the non triple helical structures in RP-HPLC profiles.

Several control experiments have been employed for HPLC peak assignments. Mass spectra are used to confirm that different peaks have the same parent molecular weight. Reinjection experiments are utilized to verify the interconversion of different peaks. Variation of chromatographic conditions is employed to determine the denaturation of triple helices by the reverse-phase HPLC system. UV spectra and thermal denaturation are used to confirm peaks arising from the triple helical conformations.

The denaturation of triple helices by the HPLC system for collagen analogs composed of Gly−Pro−Nleu sequences is negligible during the time of chromatography (<30 min). On the other hand, the denaturation of triple helices formed by collagen analogs composed of Gly−Pro−Hyp sequences by the HPLC conditions during the chromatography time (<30 min) can be easily detected. This denaturation is significantly affected by the retention times (chromatography conditions): the longer the retention time, the more significant the denaturation. The RP-HPLC technique provides a novel method to characterize triple helical conformations of synthetic collagen analogs.

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