Collagen-Based Structures Containing the Peptoid Residue *N*-Isobutylglycine (Nleu): Synthesis and Biophysical Studies of Gly-Nleu-Pro Sequences by Circular Dichroism and Optical Rotation†

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ABSTRACT: Single-chain peptide−peptoid structures, Ac-(Gly-Nleu-Pro)$_n$-NH_2 (n = 3, 6, and 10) and (Gly-Nleu-Pro)$_n$-NH_2 (n = 1 and 9), and template-assembled collagen analogs, KTA-[Gly-(Gly-Nleu-Pro)$_n$-NH_2]$_3$ (n = 3 and 6; KTA represents *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, also known as the Kemp triacid; Nleu denotes *N*-isobutylglycine), were prepared by solid-phase peptide synthesis methods. Biophysical studies using circular dichroism (CD) and optical rotation measurements show that these collagen analogs form triple-helical conformations when the chain is longer than a critical length. Unlike collagen-based structures composed of Gly-Pro-Hyp and Gly-Pro-Nleu sequences, results reveal that the presence of a positive CD peak between 220 and 225 nm is indicative of triple-helical conformations for these collagen-based structures composed of Gly-Nleu-Pro sequences. Results also indicate that the Gly-Nleu-Pro sequence possesses a higher triple-helical propensity than the Gly-Pro-Nleu sequence as demonstrated by the higher melting temperatures, the faster triple-helix folding, and the lower minimum concentration necessary to detect triple-helicity for the single-chain structures. Therefore, we conclude that the Nleu residue in the second position of the trimeric repeat is more effective in inducing triple-helix formation than Pro in the same position.

Collagen is the major protein in connective tissue. Its main structural domain is a triple helix which consists of three left-handed poly(proline-II)-like chains intertwined to form a right-handed superhelix (Ramachandran, 1963; Traub, 1974). The primary structures of these collagen sequences are characterized by repeating trimer units Gly-X-Y, where X and Y can be any amino or imino acid residues. Proline (Pro) and 4-*trans*-hydroxyproline (Hyp)[1] are frequently found in X and Y and account for about 20−25% of the residues in collagen sequences (Ramachandran & Ramakrishnan, 1976; Fietzek & Kuhn, 1975). In addition, Pro is mainly found at position X while Hyp is mostly located at position Y (Piez, 1976).

The position preference of Pro and Hyp residues found in natural collagen sequences is also confirmed in synthetic collagen models (Doyle et al., 1971; Brown et al., 1972b; Scatturin et al., 1975; Tamburro et al., 1984; Bhatnagar & Gough, 1996). Many tripeptide sequential polymers or oligomers have been prepared as collagen analogs. The most interesting results come from the comparisons of the triple-helical propensities of the Gly-Pro-X sequences and the corresponding Gly-X-Pro sequences. Brown et al. (1969, 1972a) found that poly(Gly-Pro-Ala) forms triple-helical conformations in several solvent systems, while Segal and Traub (1969) reported that poly(Gly-Ala-Pro) is mainly poly-(proline-II)-like in solution. Segal (1969) also disclosed that poly(Gly-Pro-Ala-Gly-Pro-Pro) forms more stable triple-helical conformations in solution than poly(Gly-Ala-Pro-Gly-Pro-Pro). Tamburro and co-workers (1977) reported that poly(Gly-Pro-Phe) forms triple helices in the solid state while poly(Gly-Phe-Pro) does not afford any collagen-like or poly-(proline-II)-like structures. Scatturin et al. (1975) disclosed that both poly(Gly-Pro-Leu) and poly(Gly-Leu-Pro) form triple-helical structures in the solid state. On the other hand, in solution, poly(Gly-Pro-Leu) is triple-helical in both hexafluoro-2-propanol-H_2O and ethylene glycol, while poly-(Gly-Leu-Pro) assumes the collagen-like structure only in ethylene glycol. Bertoluzza et al. (1985, 1986) reported that poly(Gly-Pro-Aze) and poly(Gly-Aze-Pro) (Aze is an azetidine-2-carboxylic acid residue) are both unordered polypeptides in most solvents except that poly(Gly-Pro-Aze) shows some signs of triple helicity in MeOH/H_2O (v/v, 97:3). Studies from several laboratories show that (Gly-Pro-Hyp)$_{10}$ easily forms triple helices in solution (Sakakibara et al., 1973; Brodsky et al., 1992), while Inouye et al. (1982) discovered that (Gly-Hyp-Pro)$_{10}$ does not exhibit a conformational transition in H_2O. These studies demonstrate that the Gly-Pro-X sequence possesses a higher triple-helical propensity than the corresponding Gly-X-Pro sequence in homopoly-tripeptide collagen mimetics.

In our approach to mimicking triple-helical structures, an unnatural peptoid residue, *N*-isobutylglycine (Nleu), has been introduced as a Pro surrogate, resulting in collagen-based peptide−peptoid oligomers composed of Gly-Pro-Nleu sequences (Goodman et al., 1996b; Feng et al., 1996a).

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[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, diisopropylcarbodiimide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; EG, ethylene glycol; HOBt, *N*-hydroxybenzotriazole; Hyp, 4-*trans*-hydroxyproline; KTA, *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid; MBHA, 4-methylbenzhydrylamine resin; Nleu, *N*-isobutylglycine; NMR, nuclear magnetic resonance; RP-HPLC, reverse-phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; TEA, triethylamine; TFA, trifluoroacetic acid.

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Biophysical studies using circular dichroism (CD), optical rotation, ultraviolet (UV) absorbance, and NMR measurements show that these peptide−peptoid structures exhibit a high triple-helical propensity (Feng et al., 1996a; Melacini et al., 1996b). Results from melting curve measurements demonstrate that the Gly-Pro-Nleu sequence is comparable to the Gly-Pro-Pro sequence in stabilizing triple-helical conformations, indicating that the peptoid residue, Nleu, possesses a higher triple-helical propensity than other amino or imino acid residues which have been studied previously (except for Pro and Hyp).

It is important to determine the triple-helical propensity for the Gly-Pro-X sequence as compared to the Gly-X-Pro sequence when X is the peptoid residue Nleu. Therefore, a series of collagen-based structures composed of Gly-Nleu-Pro sequences were synthesized and their triple-helical propensities investigated. In this paper, the synthesis of single-chain and template-assembled peptide−peptoid structures composed of Gly-Nleu-Pro sequences is reported. The biophysical studies based on circular dichroism (CD) and optical rotation measurements are also presented. The triple-helical propensity of the Gly-Nleu-Pro sequences is compared to that of the Gly-Pro-Nleu sequences which we reported previously (Goodman et al., 1996b; Feng et al., 1996a; Melacini et al., 1996b). In addition, the application of CD spectroscopy to the study of triple-helical conformations is also discussed.

MATERIALS AND METHODS

Chemicals. All chiral amino acids were of the L configuration. Protected amino acids, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), diisopropylcarbodiimide (DIC), dicyclohexylcarbodiimide (DCC), and 4-methylbenzhydrylamine (MBHA) resin were purchased from Bachem. ACS-grade and HPLC-grade solvents [dichloromethane (DCM), dimethylformamide (DMF), CH_3CN, $CHCl_3$, MeOH, EtOAc, Et_2O, ethylene glycol (EG), hexane, and tetrahydrofuran (THF)] were purchased from Fisher Scientific and were used without further purification. Triethylamine (TEA) and Pd/C were purchased from Aldrich. (Benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), *N*-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA, HPLC-grade), and HCl/dioxane (4 N) were purchased from Chem-Impex International.

Chromatography. The reactions carried out in solution were monitored by thin-layer chromatography (TLC) using precoated silica gel 60F-254 plates (Merck). Compounds were visualized by UV illustration, ninhydrin or bromocresol spray reagents. Silica gel 60 (Merck, 0.040−0.063 mm, 230−400 mesh ASTM) was used to run flash column chromatography. Two types of HPLC instruments were used to analyze and purify the products: a Waters (510 pump, 484 detector) system and a MILLENNIUM 2010 system consisting of a Waters 715 Ultra WISP sample processor, a Waters TM 996 photodiode array detector, two Waters 510 pumps, and a NEC PowerMate 486/33I computer. Solvents used in HPLC included solvent A (H_2O with 0.1% TFA) and solvent B (CH_3CN with 0.1% TFA). The flow rate was 10 mL/min for the preparatory column (Vydac C-18, 25 × 2.2 cm), 4 mL/min for the semipreparatory column (Vydac C-18, 25 × 1.0 cm), and 1.0 mL/min for the analytical column (Vydac C-18, 25 × 0.46 cm).

Synthesis. The syntheses and characterization of the target collagen mimetics composed of Gly-Nleu-Pro sequences are fully described in the Supporting Information. The structures of all the single-chain and template-assembled peptide−peptoid compounds were verified by mass spectrometry [fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI) methods] and ^{1}H-NMR spectroscopy (the NMR spectra were obtained either on a Bruker AMX 500 MHz spectrometer or using an 360 MHz spectrometer assembled in-house with a Techmag pulse programmer and digitizer and an Oxford Instruments Superconducting magnet). Analytical RP-HPLC was used to determine the purity.

Sample Preparation. Except in concentration titration experiments, relatively dilute samples (0.2 mg/mL) were prepared in order to remove potential aggregations. The samples were kept in a refrigerator (4 °C) for at least 1 week so they would reach the equilibrium of triple-helix formation. The pH values of the samples were between 3 and 6. Our control experiments show that the triple-helicity (melting points and CD spectra) does not change significantly with solution pH values between 2 and 10 for collagen analogs composed of Gly-Pro-Nleu and Gly-Nleu-Pro sequences.

Circular Dichroism. Circular dichroism (CD) measurements were carried out on a modified Cary-61 spectropolarimeter equipped with a model 900 isotemp refrigerator circulator (Fisher Scientific) (Feng et al., 1996b). Spectra were obtained with either a 0.02 or a 0.05 cm path length cell by signal averaging 10 scans from 190 to 300 nm with a scan speed of 1.0 nm/s. Before the data were collected, the sample was equilibrated at the specified temperature for 2 h.

Transition Curve Measurements. The temperature dependence of the specific rotation of samples in solution was monitored with a Perkin-Elmer 241 polarimeter equipped with a model 900 isotemp refrigerator circulator (Fisher Scientific). Data were collected at 365 nm (Hg). Before an 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 time-independent. The midpoint of the melting curve was taken as the melting point.

Kinetic Experiments. Triple-helix folding was monitored by optical rotation measurements. The sample was thermally denatured at 70−75 °C for 1 h, and triple-helix folding was carried out at a temperature substantially lower than the melting temperature. In the normalization of the kinetic data, the optical rotations of both the triple-helical and the non-triple-helical structures at a specific temperature were obtained from the base lines of the thermal melting curve of each sample. The normalized triple-helix content was obtained on the basis of the following equation:

$$\text{triple-helix content} = ([\alpha]_{obs} - [\alpha]_{NTH})/([\alpha]_{TH} - [\alpha]_{NTH})$$

where $[\alpha]_{obs}$ is the observed specific rotation at different times after denaturation. The term $[\alpha]_{TH}$ is the triple-helical base line value, and the term $[\alpha]_{NTH}$ is the non-triple-helical base line value.

RESULTS

Determination of Triple-Helical Conformations in Solution. An integrated biophysical study using circular dichro-

Table 1: CD Parameters of the Synthetic Collagen-Based peptide−Peptoid Structures Composed of Gly-Nleu-Pro Sequences[a]

compounds	H_2O max (nm)	H_2O cross (nm)	H_2O min (nm)	EG/H_2O (v/v, 2:1) max (nm)	EG/H_2O (v/v, 2:1) cross (nm)	EG/H_2O (v/v, 2:1) min (nm)
Ac-Pro-NHCH$_3$[b]			201			
Gly-Nleu-Pro-NH$_2$			200 (−0.8 × 10^4)			200 (-2.1 × 10^4)
(Gly-Nleu-Pro)$_9$-NH$_2$	225 (1.1 × $10^{3)}$	220	202 (−2.5 × 10^4)	225 (2.4 × 10^3)	217	202 (-2.3 × 10^4)
Ac-Gly-Nleu-Pro-NHCH$_3$			200 (−1.2 × 10^4)			201 (-1.4 × 10^4)
Ac-(Gly-Nleu-Pro)$_3$-NH$_2$			202 (−1.4 × 10^4)			203 (−1.6 × 10^4)
Ac-(Gly-Nleu-Pro)$_6$-NH$_2$			202 (−1.4 × 10^4)	224 (3.2 × 10^3)	217	201 (−3.2 × 10^4)
Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ (1.0 mg/mL)	225 (1.0 × $10^{3)}$	220	202 (−2.6 × 10^4)			
Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$	224 (2.3 × $10^{3)}$	219	202 (−3.0 × 10^4)			
KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$			202 (−1.2 × 10^4)			202 (−1.4 × 10^4)
KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ (0 °C)				225 (1.8 × 10^3)	219	202 (−2.4 × 10^4)
KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$	225 (1.6 × $10^{3)}$	220	202 (−2.1 × 10^4)	224 (4.0 × 10^3)	218	202 (−3.1 × 10^4)

[a] All CD spectra were measured at 0.2 mg/mL and 20 °C (except where otherwise indicated). [b] From Madison and Schellman (1970).

ism (CD) and optical rotation measurements was carried out to determine the triple-helical propensities of these synthetic peptide−peptoid structures composed of Gly-Nleu-Pro sequences. The CD spectra (band positions and shapes) of these collagen analogs were compared to those of collagen and Ac-(Gly-Pro-Hyp)$_9$-NH$_2$ in order to provide insight into the presence of triple-helical conformations in solution. Collagen exhibits a unique CD spectrum with a small positive peak at 220 nm and a large negative peak at 197 nm (Brown et al., 1969, 1972a; Li et al., 1993). The peptide Ac-(Gly-Pro-Hyp)$_9$-NH$_2$, which has been demonstrated in our laboratory to form stable triple-helices at room temperature in H_2O by CD and optical rotation measurements, exhibits a CD spectrum in H_2O at 20 °C with a positive peak at 224 nm and a negative peak at 199 nm (Feng et al., 1996b). The CD spectral parameters obtained for the synthesized collagen-based structures composed of Gly-Nleu-Pro sequences in both H_2O and EG/H_2O (v/v, 2:1) are listed in Table 1. It is readily seen from Table 1 that the CD spectra of the following compounds are consistent with a triple-helical structure: Ac-(Gly-Nleu-Pro)$_n$-NH$_2$ ($n = 6$ and 10), (Gly-Nleu-Pro)$_9$-NH$_2$, and KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ at 20 °C and KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in EG/H_2O (v/v, 2:1) at 0 °C.

Melting curves monitored by optical rotation measurements were also used to confirm the presence of triple-helical conformations in solution. In addition, the melting points (temperature, salt concentration) obtained from these melting curves were used to evaluate the triple-helix stability, and the transition magnitudes (the difference in specific rotations immediately before and immediately after the transition range) were utilized to correlate with triple-helix percentages. The results of melting curve measurements in both H_2O and EG/H_2O (v/v, 2:1) are presented in Table 2. It can be seen from Table 2 that results from denaturation experiments are consistent with those obtained from CD spectroscopy; cooperative transition curves are observed for those samples which exhibit CD spectra similar to that of collagen.

Single-Chain Structures. For Ac-Gly-Nleu-Pro-NHCH$_3$, Gly-Nleu-Pro-NH$_2$, and Ac-(Gly-Nleu-Pro)$_3$-NH$_2$, the chains are too short to form collagen-like triple-helical structures. As shown in Table 1, their CD spectra in both H_2O and EG/H_2O (v/v, 2:1) exhibit only a negative peak. These CD spectra are very similar to that of Ac-Pro-NHCH$_3$ (Table 1), the conformation of which has been independently demonstrated to be similar to that of the proline residue in poly(proline-II)-like chains by CD and NMR studies (Madison & Schellman, 1970). The proline derivative, Ac-Pro-NHCH$_3$, is used as a model compound to study the non-triple-helical Gly-Nleu-Pro chains, since only the Pro residue in Gly-Nleu-Pro possesses a chiral center and the C terminus of the Pro residue is monosubstituted in both Ac-Pro-NHCH$_3$ and the Gly-Nleu-Pro chains (compare CH$_3$CO-Pro-NHCH$_3$ with CH$_2$CO-Pro-NHCH$_2$, the repeat in Gly-Nleu-Pro oligomers).

Table 2: Thermal Melting Results Obtained for Collagen-Based peptide−Peptoid Structures Composed of Gly-Nleu-Pro Sequences from Optical Rotation Measurements[a]

compounds	H_2O	EG/H_2O (v/v, 2:1)
Gly-Nleu-Pro-NH$_2$	no transition	no transition
(Gly-Nleu-Pro)$_9$-NH$_2$	*b*	*b*
Ac-Gly-Nleu-Pro-NHCH$_3$	no transition	no transition
Ac-(Gly-Nleu-Pro)$_3$-NH$_2$	no transition	no transition
Ac-(Gly-Nleu-Pro)$_6$-NH$_2$	26 °C	43 °C
Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$	*b*	
KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$	no transition	22 °C
KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$	36 °C	57 °C
Ac-(Gly-Pro-Nleu)$_6$-NH$_2$	18 °C[c]	35 °C[d]
KTA-[Gly-(Gly-Pro-Nleu)$_3$-NH$_2$]$_3$	no transition[d]	12 °C[d]
KTA-[Gly-(Gly-Pro-Nleu)$_6$-NH$_2$]$_3$	33 °C[d]	52 °C[d]

[a] Sample concentrations were 0.2 mg/mL. [b] The melting temperatures for these compounds could not be obtained since their solutions became cloudy with increasing temperature (>35 °C) because of triple-helix aggregation. [c] This result was obtained at a concentration of 2.0 mg/mL. [d] Feng et al. (1996a).

Ethylene glycol (EG) stabilizes triple-helical structures. Therefore, EG/H_2O (v/v, 2:1) has been widely used to detect triple-helical conformations of synthetic collagen mimetics (Brown et al., 1969, 1972a,b; Scatturin et al., 1975; Feng et al., 1996a,b). Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ forms stable triple-helical structures in EG/H_2O (v/v, 2:1) at 0.2 mg/mL. In addition to a large negative peak at 201 nm, the CD spectrum of Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ in EG/H_2O (v/v, 2:1) also possesses a small positive peak at 222 nm (Table 1), which is absent in the CD spectra of the shorter chain structures. This CD spectrum is similar to those of collagen and Ac-(Gly-Pro-Hyp)$_9$-NH$_2$ (spectral shapes and band positions) and therefore is consistent with a triple-helical conformation. The presence of triple-helical conformations is also supported by melting curve measurements. As shown in Figure 1A, the thermal melting studies using optical rotation measurements show that Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ in EG/H_2O (v/v, 2:1) exhibits a cooperative melting curve with a melting temperature of 43 °C.

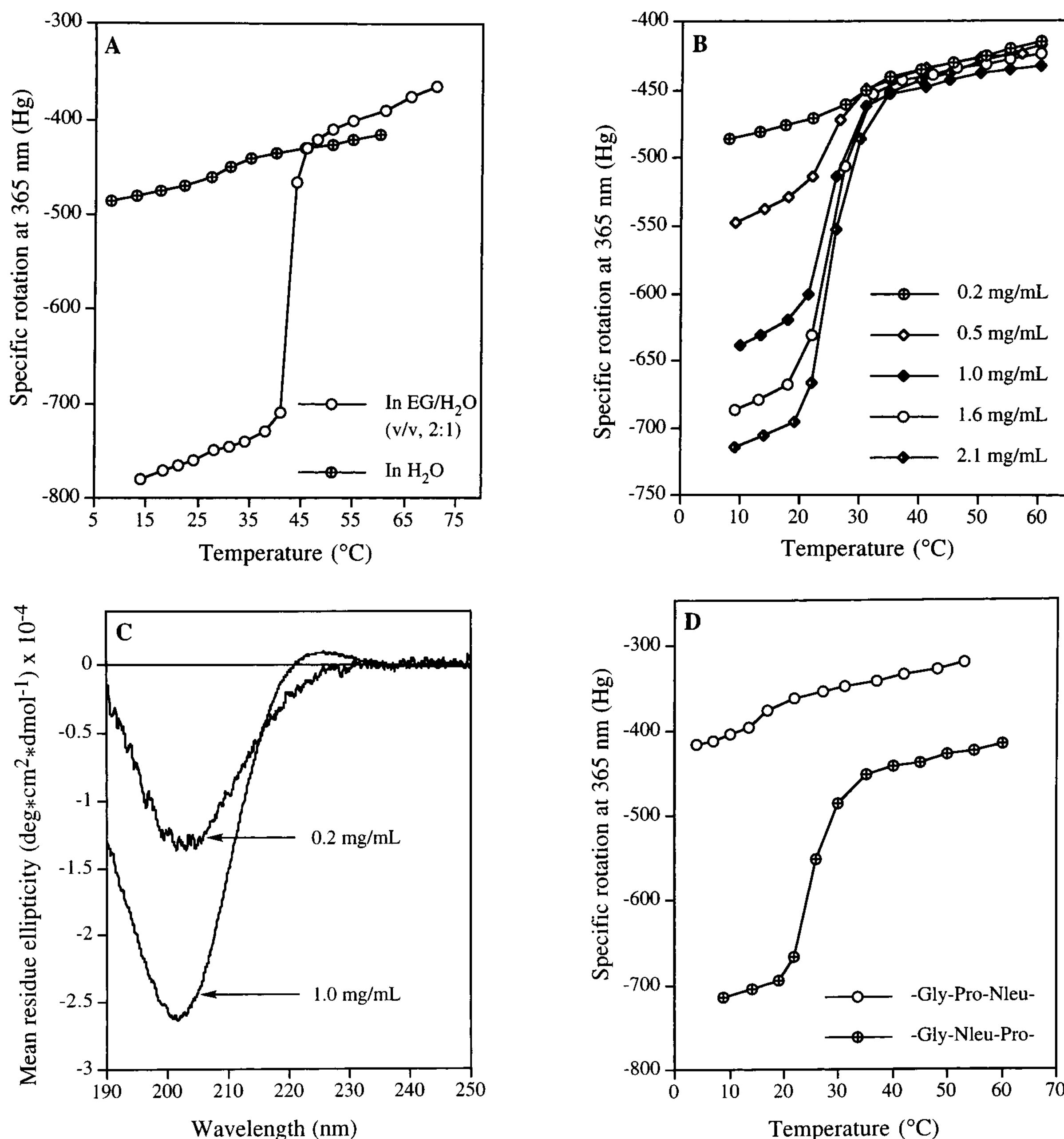

FIGURE 1: (A) Thermal melting curves obtained by optical rotation measurements for Ac-(Gly-Nleu-Pro)$_6$-NH_2 in EG/H_2O (v/v, 2:1) and in H_2O (0.2 mg/mL). (B) Thermal melting curves obtained by optical rotation measurements for Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O at different concentrations. (C) CD spectra of Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O at 0.2 and 1.0 mg/mL and 20 °C. (D) Comparison of thermal melting curves of Ac-(Gly-Pro-Nleu)$_6$-NH_2 and Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O at *ca.* 2.0 mg/mL.

The triple-helical propensity of Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O is lower than that in EG/H_2O (v/v, 2:1). Melting curve measurements demonstrate that Ac-(Gly-Nleu-Pro)$_6$-NH_2 is barely triple-helical in H_2O at a concentration of 0.2 mg/mL, since no clear transition is observed (Figure 1A). However, when the concentration is increased to 0.53 mg/mL, a transition curve with a melting temperature of 26 °C is obtained (Figure 1B). Further increases in concentration lead to larger transition magnitudes but no significant change in the melting temperature (*ca.* 26 °C), as demonstrated in Figure 1B. These results demonstrate that the triple-helicity of Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O increases with increasing concentration from 0.2 to 2.1 mg/mL. In addition, the melting experiments are consistent with those results obtained from CD spectroscopy. At a concentration of 0.2 mg/mL, Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O exhibits a CD spectrum with only a negative peak at 202 nm (Figure 1C, Table 1), which is consistent with a poly(proline-II)-like structure. However, at 1.0 mg/mL, a CD spectrum with both a negative peak and a positive peak is observed (Figure 1C, Table 1), which is characteristic of a triple-helical conformation.

Previous studies show that the corresponding Gly-Pro-Nleu sequence compound, Ac-(Gly-Pro-Nleu)$_6$-NH_2, is not triple-helical in H_2O at a concentration of 0.2 mg/mL (Feng et al., 1996a). In order to compare the triple-helical propensities between Gly-Nleu-Pro and Gly-Pro-Nleu sequences, the melting curve of Ac-(Gly-Pro-Nleu)$_6$-NH_2 in H_2O at a concentration of 2.0 mg/mL was determined. As shown in Figure 1D, only a small transition is observed. This result indicates that the triple-helix percentage of Ac-(Gly-Pro-Nleu)$_6$-NH_2 in H_2O is very low even at a concentration of 2 mg/mL. In addition, the melting temperature of Ac-(Gly-Pro-Nleu)$_6$-NH_2 in H_2O (Figure 1D) is only *ca.* 18 °C, lower than that of Ac-(Gly-Nleu-Pro)$_6$-NH_2 in H_2O (26 °C).

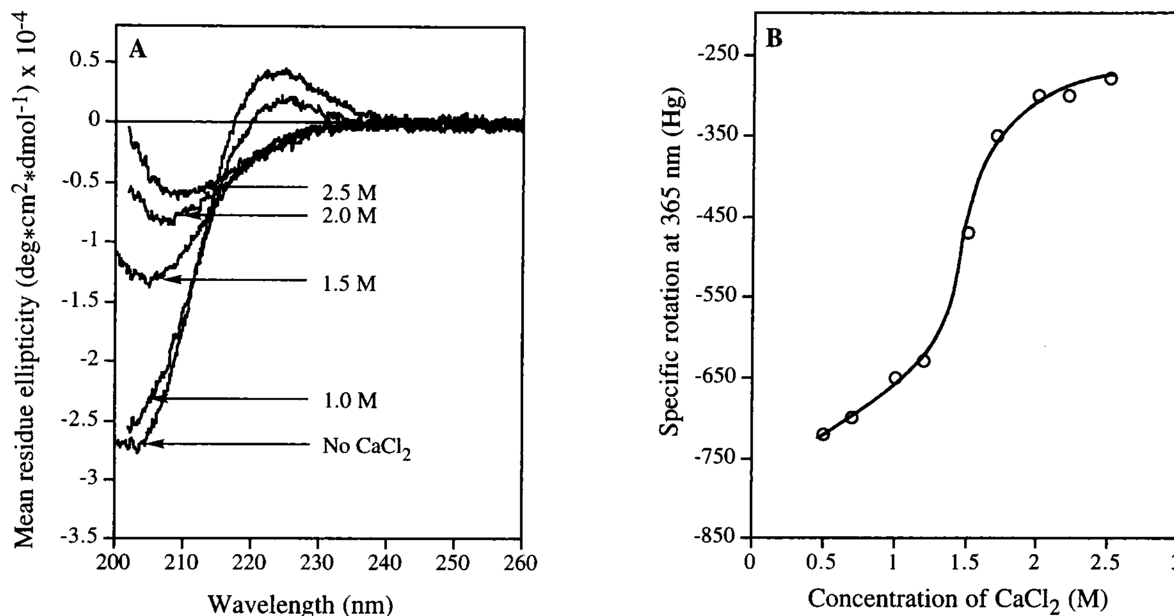

FIGURE 2: (A) CD spectra of Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$ in H_2O (0.1 mg/mL) at 20 °C at different concentrations of $CaCl_2$. (B) Dependence of specific rotation (365 nm, Hg) on the concentration of $CaCl_2$ for Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$ in H_2O (0.1 mg/mL) at 20 °C.

The triple-helical conformations of Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$ and (Gly-Nleu-Pro)$_9$-NH$_2$ in H_2O at 20 °C were established by CD spectroscopy. Results given in Table 1 show that both analogs exhibit a CD spectrum with a small positive peak at 224−225 nm in addition to a large negative peak at 202 nm, as expected for a collagen-like triple-helical conformation. The solutions of these two collagen analogs in H_2O become cloudy with increasing temperature. Therefore, neither the CD spectra after thermal denaturation nor the thermal melting curves could be obtained. To determine the triple-helical propensities below the cloud point, melting experiments were carried out by salt ($CaCl_2$) denaturation at 20 °C. As demonstrated in Figure 2A, CD spectra show that Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$ is triple-helical in H_2O (0.1 mg/mL) at 20 °C until 1.0 M $CaCl_2$. At a $CaCl_2$ concentration of 1.5 M or higher, the triple-helical conformations are denatured to poly(proline-II)-like and/or unordered structures since the positive CD peak disappears. Optical rotations were measured for Ac-(Gly-Nleu-Pro)$_{10}$-NH$_2$ in H_2O (0.1 mg/mL) at different $CaCl_2$ concentrations and at 20 °C. A melting transition curve is obtained by plotting the specific rotation against the $CaCl_2$ concentration (Figure 2B), which yields a melting point of *ca.* 1.5 M $CaCl_2$. These melting experiments present information similar to that of thermal denaturation and support the presence of triple-helical structures in solution as independently determined by CD spectroscopy. The denaturation of triple-helical structures by $CaCl_2$ is believed to occur through the binding of Ca^{2+} to the peptide backbone carbonyl, which alters the conformational flexibility around the C^{α}−C(=O) bond (Tiffany & Krimm, 1968; Bhatnagar & Gough, 1996).

Template-Assembled Structures. Unlike the acetyl-terminated analog, Ac-(Gly-Nleu-Pro)$_3$-NH$_2$, for which no evidence of triple helicity has been found in either H_2O or EG/H_2O (v/v, 2:1), the template-assembled collagen mimetic with only three Gly-Nleu-Pro repeats, KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$, forms a triple-helical conformation in EG/H_2O (v/v, 2:1) with a melting temperature of 22 °C. According to CD spectroscopy, this molecule is not triple-helical in H_2O at 20 °C, since only a negative CD peak is detected (Figure 3A, curve a). However, at 0 °C, a typical triple-helical CD spectrum with both a large negative peak at 202 nm and a small positive peak at 225 nm (Figure 3A, curve b) is observed in EG/H_2O (v/v, 2:1). Results from melting curve measurements are consistent with those obtained from CD spectra. As shown in Figure 3B, a cooperative melting curve with a midpoint at *ca.* 22 °C was obtained in EG/H_2O (v/v, 2:1). It is important to point out that these results support our previous finding that three Gly-X-Y trimer repeats attached to an appropriate template are sufficient for formation of an incipient triple-helical conformation. This is observed when X and Y are residues with high triple-helical propensity, such as Pro, Hyp, and Nleu (Goodman et al., 1996a; Feng et al., 1996a).

KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ exhibits a higher triple-helical propensity than KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ because of the longer chains. As demonstrated in Figure 4A, KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ shows typical triple-helical CD spectra in both H_2O and EG/H_2O (v/v, 2:1) at 20 °C. In addition, optical rotation measurements (Figure 4B) indicate that the melting temperatures of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ are 36 °C in H_2O and 57 °C in EG/H_2O (v/v, 2:1), both higher than that obtained for KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in EG/H_2O (v/v, 2:1) (22 °C).

Similar to collagen-like structures composed of Gly-Pro-Nleu sequences (Feng, 1996), the triple-helices of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ are denatured by the addition of 25 mM sodium dodecyl sulfate (SDS) in H_2O (Figure 4A, curve c). This denaturation is most likely due to the hydrophobic interactions between the interior of micelles and the side chain of the Nleu residue. One major triple-helix-stabilizing force in Gly-Pro-Nleu and Gly-Nleu-Pro sequences is the interchain, interresidue interactions involving the hydrophobic side chains of Pro and Nleu residues (Melacini et al., 1996b, 1997). The interactions with the surfactant compete with the inter-peptide chain hydrophobic interactions and therefore destroy the triple-helical structures. Control experiments show that the corresponding collagen mimetics composed of Gly-Pro-Hyp sequences cannot be denatured by the same amount of SDS. This result is consistent with

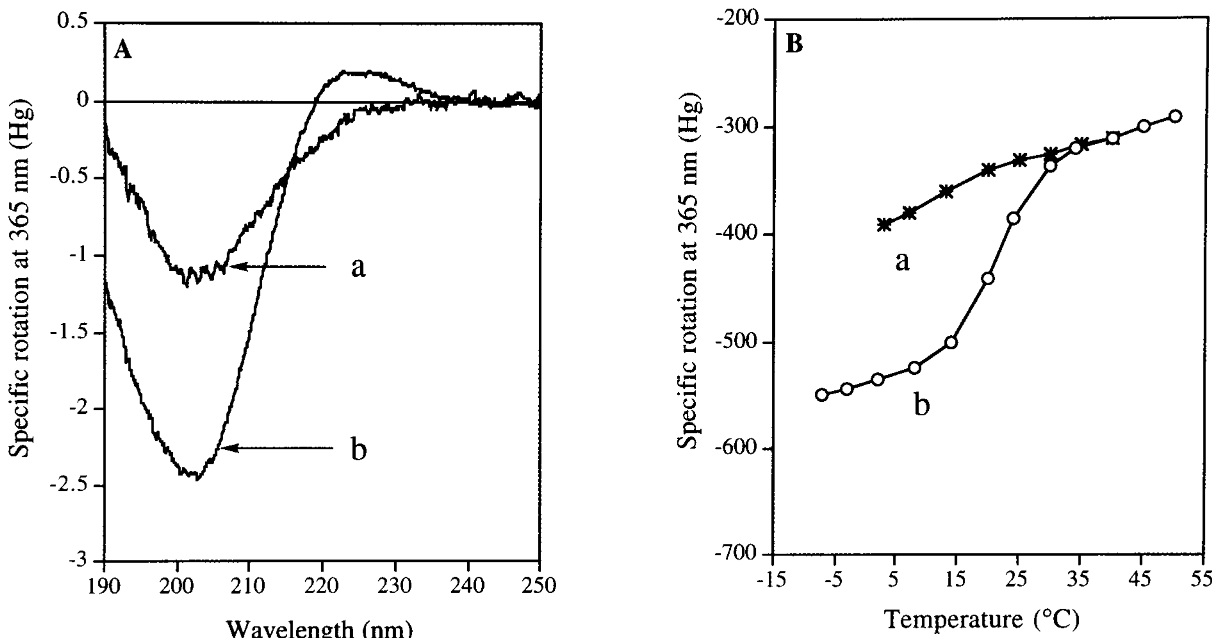

FIGURE 3: (A) CD spectra of KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in H_2O (0.2 mg/mL) at 20 °C (a) and in EG/H_2O (v/v, 2:1, 0.2 mg/mL) at 0 °C (b). (B) Thermal melting curves of KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in H_2O (0.2 mg/mL) (a) and in EG/H_2O (v/v, 2:1, 0.2 mg/mL) (b).

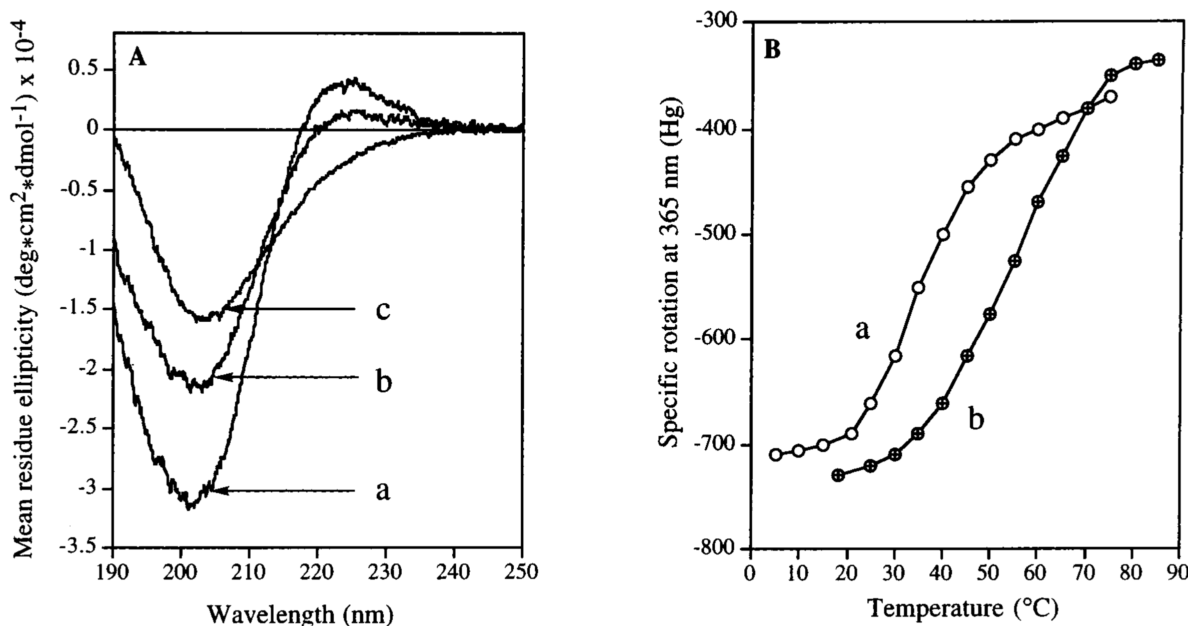

FIGURE 4: (A) CD spectra of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in EG/H_2O (v/v, 2:1, 0.2 mg/mL) at 20 °C (a), in H_2O (0.2 mg/mL) at 20 °C (b), and in H_2O (0.2 mg/mL) with 25 mM SDS at 20 °C (c). (B) Thermal melting curves of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in H_2O (0.2 mg/mL) (a) and in EG/H_2O (v/v, 2:1. 0.2 mg/mL) (b).

the view that Gly-Pro-Hyp and natural collagen triple-helices are stabilized by a solvent (H_2O)-bridged H-bonding network through the hydroxyl group of Hyp (Bella et al., 1994, 1995). The hydrophobic interactions play almost no role in affecting the triple-helix stability (Privalov, 1982; Jones & Miller, 1991; Brodsky & Shah, 1995; Shah et al., 1996). Therefore, the triple-helical structures in Gly-Pro-Hyp sequences are not affected by SDS.

The results listed in Table 2 demonstrate the stabilizing effect of the KTA-based template. KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ possesses melting temperatures of 36 °C in H_2O and 57 °C in EG/H_2O (v/v, 2:1). On the other hand, Ac-(Gly-Nleu-Pro)$_6$-NH$_2$ melts at temperatures of 26 °C in H_2O and 43 °C in EG/H_2O (v/v, 2:1). The triple-helix-stabilizing effect of the KTA template results from a reduction of the entropy loss involved in triple-helix formation (Feng et al., 1996b; Roth & Heidemann, 1980), which is an intramolecular process in the KTA conjugate and an intermolecular process in the single-chain compound.

In addition to stabilizing triple-helical structures, template assembling also accelerates the folding of triple-helical conformations (Feng et al., 1996b). For the non-template-assembled acetyl analogs composed of Gly-Nleu-Pro sequences, control experiments (Feng, 1996) showed that high concentrations (>10 mg/mL) are required to incubate the triple helix (equilibrium is reached within 3 days). In order to determine the time required for triple-helix formation in the corresponding template-assembled analogs, triple-helix

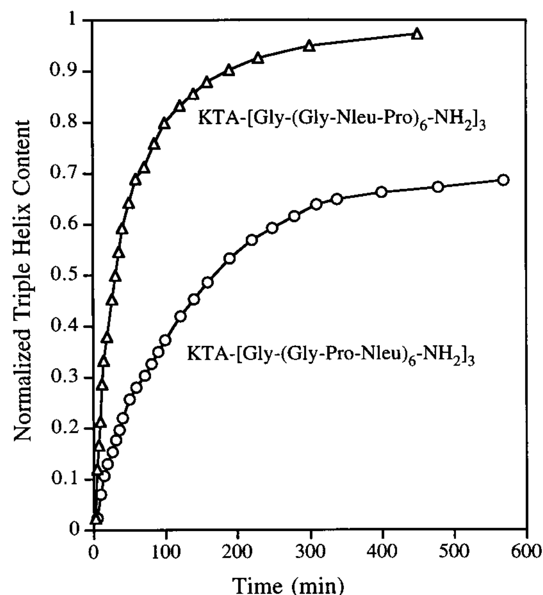

FIGURE 5: Comparison of the normalized triple-helix folding profiles monitored by optical rotation experiments (see descriptions in Materials and Methods) between KTA-[Gly-(Gly-Pro-Nleu)$_6$-NH$_2$]$_3$ and KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ at 16 °C in H_2O at 0.2 mg/mL.

folding experiments were carried out for KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in EG/H_2O (v/v, 2:1, 0.2 mg/mL) at 5 °C and KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in H_2O (0.2 mg/mL) at 16 °C. The two temperatures (5 and 16 °C) were selected since they are below the temperatures necessary to cause the triple helices of these two analogs to denature (Figure 3B, curve b; Figure 4B, curve a). The samples were thermally denatured, and the triple-helix refolding was monitored by optical rotation measurements at the specific temperatures. Results show that the half-time of triple-helix formation for KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$ in EG/H_2O (v/v, 2:1) at 5 °C is *ca.* 25 min, and that for KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in H_2O at 16 °C is *ca.* 30 min (Figure 5). These results clearly demonstrate that the KTA template dramatically accelerates triple-helix folding.

DISCUSSION AND CONCLUSIONS

CD Spectra of Triple-Helical Conformations. CD spectroscopy is widely used in the characterization of natural collagens and synthetic collagen analogs, since all collagen-like triple-helical structures exhibit a CD spectrum with a small positive peak at 215−230 nm and a large negative peak at 195−205 nm. However, there has been confusion in the application of CD spectroscopy. The most vexing problem arises from the fact that the triple-helical structure and the individual poly(proline-II)-like chain possess similar CD spectral shapes and band positions for some synthetic polypeptide collagen models (Woody, 1992), such as those composed of Gly-Pro-Hyp, Gly-Pro-Pro, and Gly-Pro-Nleu sequences (Feng et al., 1996a,b). Therefore, the CD spectral shapes and band positions alone cannot be used as an unambiguous criterion to establish triple-helical conformations. To differentiate a triple-helical conformation from a poly(proline-II)-like structure, thermal melting curves have been followed using the temperature dependence of the (positive) CD band intensity (Fields & Fields, 1992; Li et al., 1993), the specific rotation (Sakakibara et al., 1968, 1973; Feng et al., 1996a,b), the UV absorbance at 223 nm (Feng et al., 1996a,b), and the NMR resonance intensity (Melacini et al., 1996a,b). The observation of a cooperative transition curve together with a proper CD spectrum is indicative of triple-helical conformations. In addition, the ratio of the two CD bands is also found to be sensitive to the peptide conformation in solution (Jenness et al., 1976; Feng et al., 1996b).

Unlike the cases discussed above, for Gly-Nleu-Pro sequences, the CD spectral shapes and band positions were found to be highly indicative of triple-helical conformations. As demonstrated by the results given in Table 1 and Figures 1−4, the CD spectra for the non-triple-helical Gly-Nleu-Pro compounds exhibit only a negative peak at 200−203 nm. On the other hand, the CD spectra for the triple-helical analogs show both a large negative peak at 201−202 nm and a small positive peak at 224−225 nm. For those samples possessing CD spectra with both a large negative peak and a small positive peak, cooperative transition curves are observed (compare results in Tables 1 and 2). These results demonstrate that the presence of a positive peak at 224−225 nm in the CD spectra can be used as conclusive evidence of collagen-like triple-helical conformations for Gly-Nleu-Pro sequences.

Previous studies show that CD band positions of triple-helical conformations for collagen and synthetic collagen analogs depend upon the percentage of imino acid residues in the peptide sequences. The higher the percentage of imino acid residues, the higher the band positions (Rippon & Walton, 1971; Walton & Blackwell, 1973). This relation is also confirmed in the CD spectra of collagen analogs composed of Gly-Nleu-Pro sequences (Table 1). The percentage of imino acid residues in Gly-Nleu-Pro sequences is the same (67%) as that in Gly-Pro-Hyp sequences. Their CD band positions are therefore expected to be similar. Results given in Table 1 show that the CD spectra of collagen-like structures composed of Gly-Nleu-Pro sequences possess a positive peak at 224−225 nm and a negative peak at 202 nm, close to those obtained for collagen-like polypeptides composed of Gly-Pro-Hyp sequences (224−225 nm for the positive peak and 200 nm for the negative peak) (Venugopal et al., 1994; Feng et al., 1996b).

Comparisons of Triple-Helical Propensities between Gly-Nleu-Pro and Gly-Pro-Nleu Sequences. Previous studies show that the triple-helical propensity of the Gly-Pro-X sequences is higher than that of the corresponding Gly-X-Pro sequences in homopolytripeptide collagen mimetics (Brown et al., 1972b; Scatturin et al., 1975; Doyle et al., 1971) with the only exception being when X is Ile (Tamburro et al., 1984). These results on synthetic collagen mimetics agree with the distribution of Pro residues in natural collagen sequences where the Gly-Pro-X trimer units are much more abundant than the Gly-X-Pro trimer units (Piez, 1976). However, our results lead to the opposite conclusion for collagen analogs composed of Gly-Nleu-Pro and Gly-Pro-Nleu sequences. The Gly-Nleu-Pro sequence possesses a higher triple-helical propensity than the Gly-Pro-Nleu sequence, as supported by a number of experimental observations.

(1) The Gly-Nleu-Pro sequences form more stable triple-helices than the Gly-Pro-Nleu sequences as demonstrated by the melting temperatures (Table 2). Ac-(Gly-Nleu-Pro)$_6$-NH$_2$, KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH$_2$]$_3$, and KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH$_2$]$_3$ in both H_2O and EG/H_2O (v/v, 2:1)

exhibit melting temperatures higher than those of the corresponding analogs composed of Gly-Pro-Nleu sequences (Feng et al., 1996a).

(2) The minimum concentration necessary to detect triple-helicity is much lower in the single-chain Gly-Nleu-Pro sequences than in the corresponding Gly-Pro-Nleu sequences. For example, results in Figure 2B show that Ac-(Gly-Nleu-Pro)$_6$-NH_2 presents a very clear melting transition curve in H_2O at *ca.* 0.5 mg/mL. On the other hand, the transition curve exhibited by the corresponding Gly-Pro-Nleu sequence-based analog, Ac-(Gly-Pro-Nleu)$_6$-NH_2, is barely noticeable in H_2O even at a concentration of 2.0 mg/mL (Figure 2D).

(3) Results of triple-helix folding studies show that the triple-helix formation of collagen analogs composed of Gly-Nleu-Pro sequences is faster than that of the corresponding collagen analogs composed of Gly-Pro-Nleu sequences. The half-time of triple-helix folding for KTA-[Gly-(Gly-Nleu-Pro)$_3$-NH_2]$_3$ in EG/H_2O (v/v, 2:1) at 5 °C is *ca.* 25 min, while that of KTA-[Gly-(Gly-Pro-Nleu)$_3$-NH_2]$_3$ in EG/H_2O (v/v, 2:1) at 0 °C is *ca.* 100 min. In addition, the half-time of KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH_2]$_3$ in H_2O at 16 °C is *ca.* 30 min (Figure 5), while that of KTA-[Gly-(Gly-Pro-Nleu)$_6$-NH_2]$_3$ in H_2O at 16 °C is *ca.* 170 min (Feng, 1996; also see Figure 5).

In an attempt to explain the fact that the triple-helical propensity is higher in Gly-Pro-Ala sequences than in Gly-Ala-Pro sequences, Doyle et al. (1971) suggested that this difference in triple-helical propensities is caused by the solvent−polypeptide interactions. In a triple-helical packing, the NH of Ala in Gly-Ala-Pro sequences points toward the inside core of the triple-helix and is not exposed to the solvent. Therefore, the solvent (H_2O) disrupts the triple-helical packing of the Gly-Ala-Pro sequences by forming H bonds with the NH of Ala. On the other hand, the NH of Ala in the triple-helical structure of Gly-Pro-Ala sequences points toward the outside of the triple-helix core and is more exposed to the solvent. As a result, the solvent−polypeptide interactions (H bonding) do not disrupt the triple-helical structures. The same rule can also be applied to the differences in triple-helical propensities between Gly-Pro-X and Gly-X-Pro sequences when X represents other amino acid residues such as Leu, Phe, and Ser (Scatturin et al., 1975). However, this hypothesis cannot explain the differences in triple-helical propensities between Gly-Pro-Nleu and Gly-Nleu-Pro sequences or with any other imino acid residues in place of Nleu, since these residues are not hydrogen bond donors.

On the basis of the biophysical studies on (Gly-Pro-Pro)$_{10}$, Bhatnagar and co-workers suggested that the major stabilizing force in the Gly-Pro-Pro triple-helix is the interchain and/or interresidue nonbonded interactions, including close van der Waals contacts and hydrophobic interactions (Bhatnagar et al., 1988; Bhatnagar & Gough, 1996). Molecular modeling and NMR studies on collagen mimetics containing Nleu in our laboratory (Melacini et al., 1996b. 1997) also verified the significance of interactions between hydrophobic side chains in stabilizing triple-helical structures. The importance of these interactions is further supported by the fact that the Gly-Pro-Nleu and the Gly-Nleu-Pro triple-helices are easily denatured by SDS (Feng, 1996; also see Figure 4A). In a triple-helical structure, the side chains of the second and third residues in the trimer units are packed toward the outside of the triple-helix core and are exposed to the solvent, with the second residue more exposed than the third residue (Jones & Miller, 1991). As a result, triple-helix packing and interchain hydrophobic interactions are more favored when the bulkier and more hydrophobic Nleu is at the second rather than at the third position. Therefore, it is concluded that the hydrophobic interactions contribute to the fact that the triple-helical propensity is greater in Gly-Nleu-Pro sequences than in Gly-Pro-Nleu sequences.

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SUPPORTING INFORMATION AVAILABLE

Description of the syntheses and the detailed synthetic procedures for all collagen mimetics and some intermediates (9 pages). Ordering information is given on any current masthead page.

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