Hydroxylation-induced Stabilization of the Collagen Triple Helix

ACETYL-(GLYCYL-4(R)-HYDROXYPROLYL-4(R)-HYDROXYPROLYL)₁₀-NH₂ FORMS A HIGHLY STABLE TRIPLE HELIX

Received for publication, March 17, 2004, and in revised form, June 14, 2004
Published, JBC Papers in Press, July 1, 2004, DOI 10.1074/jbc.M402953200

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The collagen triple helix is one of the most abundant protein motifs in animals. The structural motif of collagen is the triple helix formed by the repeated sequence -Gly-Xaa-Yaa-. Previous reports showed that H-(Pro-4(R)Hyp-Gly)₁₀-OH (where ‘4(R)Hyp’ is (2S,4R)-4-hydroxyproline) forms a trimeric structure, whereas H-(4(R)Hyp-Pro-Gly)₁₀-OH does not form a triple helix. Compared with H-(Pro-Pro-Gly)₁₀-OH, the melting temperature of H-(Pro-4(R)Hyp-Gly)₁₀-OH is higher, suggesting that 4(R)Hyp in the Yaa position has a stabilizing effect. The inability of triple helix formation of H-(4(R)Hyp-Pro-Gly)₁₀-OH has been explained by a stereoelectronic effect, but the details are unknown. In this study, we synthesized a peptide that contains 4(R)Hyp in both the Xaa and the Yaa positions, that is, Ac-(Gly-4(R)Hyp-Pro-Gly)₁₀-NH₂ and Ac-(Gly-4(R)Hyp-Pro)₁₀-NH₂. Ac-(Gly-4(R)Hyp)-4(R)Hyp-4(R)Hyp-4(R)Hyp-NH₂ showed a polypeptide II-like circular dichroic spectrum in water. The thermal transition temperatures measured by circular dichroism and differential scanning calorimetry were slightly higher than the values measured for Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂ under the same conditions. For Ac-(Gly-4(R)Hyp-Pro-Gly)₁₀-NH₂, the calorimetric and the van’t Hoff transition enthalpies ΔH were significantly higher than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. We postulate that the denatured states of the two peptides are significantly different, with Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ forming a more polypeptide II-like structure instead of a random coil. Two-dimensional nuclear Overhauser effect spectroscopy suggests that the triple helical structure of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ is more flexible than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. This is confirmed by the kinetics of amide 1H exchange with solvent deuterium of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂, which is faster than that of Ac-(Gly-Pro-4(R)Hyp)₁₀-NH₂. The higher transition temperature of Ac-(Gly-4(R)Hyp-4(R)Hyp)₁₀-NH₂ can be explained by the higher transfer ratio of the Gly-4(R)Hyp peptide bonds than that of the Gly-Pro bonds, and this ratio compensates for the weaker interchain hydrogen bonds.

* This work was supported by a grant from the Shriners Hospital for Children. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: 4(R)Hyp, (2S,4R)-4-hydroxyproline; 4(Si)Hyp, (2S,4S)-4-hydroxyproline; 3(Si)Hyp, (2S,3S)-3-hydroxyproline; 4(R)Flp, (2S,4R)-4-fluoroproline; 4(Si)Flp, (2S,4S)-4-fluoroproline; Fmoc, N-(9-fluorenylethoxycarbonyl); NOESY, two-dimensional nuclear Overhauser effect spectroscopy; DSC, differential scanning calorimetry; CD, circular dichroism.

38072 This paper is available on line at http://www.jbc.org
Peptide Synthesis—Peptides were synthesized with an ABI 433A peptide synthesizer. Couplings were carried out on a PAL-PEG-PS resin (Perkin-Elmer Applied Biosystems, Foster City, CA. 0.16 mmol/g) using Fmoc amino acids (Fmoc-Gly-OH, Fmoc-Pro-OH, and Fmoc-4(R)Hyp-Bu-OH) and acetyl glycine (Bachem). O-(7-Azabenzotriazol-1-yl)-1.1.3.3-tetramethyluronium hexafluorophosphate (Perkin-Elmer Applied Biosystems, Foster City, CA. 4.0 eq) diisopropylethylamine was used to mediate peptide couplings. The peptides were cleaved from the resin and purified by preparative high-performance liquid chromatography on a Vydac® C18, 5 μm, 300E, 250 × 10 mm, W.R. Grace, Cincinatti, MD. All synthesized peptides were characterized by electrospray/quadrupole/time-of-flight mass spectrometry (Micromass, Q-tof micro, Manchester, UK) and amino acid analysis.

Circular Dichroism—Circular dichroism spectra were recorded on an Aviv 202 spectropolarimeter using a Pelletier thermostatted cell holder and a 1-mm path length rectangular cell (Starna Cells Inc., Atascadero, CA). Peptide concentrations were determined by amino acid analysis. The wavelength spectra represent at least an average of 10 scans with 0.1-nm resolution. The CD data were analyzed with SCIENTIST for Windows (MicroMath Research, St. Louis, MO) using the cubic equation for the all-or-none model described in Frank et al. (42).

NMR Spectroscopy—NMR spectra were recorded on a Bruker AMX-400 spectrometer, operating at 400.14 MHz. The 90° pulse width was 9 μs, and a low power 2-μs presaturation pulse was applied to suppress the H2O resonance. The spectra were recorded as 16,384 points for the one-dimensional spectra and as 1,024 × 512 data point sets for the two-dimensional spectra. The NOESY data were collected with time-proportional phase incrementation in the indirect dimension, at mixing times between 30 and 120 ms, and a total recording time of about 10 h. Total correlation spectroscopy data were collected with various mixing times, ranging from 30 to 90 ms. The data were processed with Swan-MR or nmrPipe to 1024 real data sets after application of a 60° phase-shifted sin2 function and Fourier transformation for the two-dimensional spectra; baselines were straightened as needed. Spectra were referenced to 0 ppm via internal 2.2-dimethylsilapentane-5-sulfonate or via the water resonance (4.71 ppm at 30 °C). Final visualization and analyses of the two-dimensional data sets were performed using NMRView (43) or Swan-MR.

The I/D exchange experiment was performed by recording a series of one-dimensional spectra after dissolving the peptides into D2O, buffered to pH 4.7 (uncorrected) with 100 mM acetate/acetic acid-d4. The peak intensities were evaluated by measuring the heights on the same absolute scale. This was found to give essentially the same results as when using the peak integrals.

Differential Scanning Calorimetry—The temperature dependence of the partial heat capacity was measured in an N-DSC II differential scanning calorimeter (Calorimetry Science Corp.). The heating rate was 7.5 °C/h, and the data were collected using the software provided by the manufacturer. The data were analyzed using the cubic equation for the all-or-none model described in Frank et al. (42).

RESULTS

The CD spectra of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 in water at 4 °C are shown in Fig. 1A together with the spectra of Ac-(Gly-Pro-4(R)Hyp)10-NH2, Ac-(Gly-4(R)Hyp-Pro)10-NH2, and Ac-(Gly-Pro-Pro)10-NH2 measured under the same conditions. The spectrum of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 shows a positive maximum at 225 nm and a negative minimum at 197 nm. In this regard, it most closely resembles the spectrum of Ac-(Gly-Pro-4(R)Hyp)10-NH2, but both extrema are smaller than those of Ac-(Gly-Pro-4(R)Hyp)10-NH2. The spectrum of Ac-(Gly-Pro-Pro)10-NH2 is slightly red-shifted when compared with the first two spectra. The spectrum of Ac-(Gly-4(R)Hyp-Pro)10-NH2 is significantly different with a positive maximum at 222 nm and a negative minimum at 200 nm. At 95 °C the positive maximum disappears in all peptides except for Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2, which still shows a positive maximum at 225 nm. The negative minimum is at 203 nm for all peptides (Fig. 1B). The persistence of the positive maximum at 225 nm with increasing temperature is shown in Fig. 1C, which also indicates the shift of the negative minimum from...
197 to 203 nm. The change in ellipticity at 225 nm as a function of temperature shows that all peptides with the exception of Ac-(Gly-4(R)Hyp-Pro)10-NH2 show a cooperative transition (Fig. 2). The midpoint of the transition ($T_m$) of the heating scan of Ac-(Gly-Pro-4(R)Hyp)10-NH2 is at 74°C, the $T_m$ of the much broader transition of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 is near 80°C, and the $T_m$ of Ac-(Gly-Pro-Pro)10-NH2 is at 47°C. The folding and unfolding transitions of collagens and collagen-like peptides are slow, and a significant hysteresis is observed with fast scanning rates and low peptide concentrations (44, 45). For the evaluation of thermodynamic data, equilibrium transition curves were required. Fig. 3A shows the temperature dependence of the heat capacity for Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 and Ac-(Gly-Pro-Pro)10-NH2 measured at a 2 mM peptide concentration and with a heating/cooling rate of 7.5°C/h. When a high concentration and a slow heating/cooling rate were used, the unfolding/refolding curves were practically identical. The hysteresis observed at lower concentrations, even present in peptides with cross-links (46), disappeared under these conditions. Fig. 3B shows the CD transition curves measured at 235 nm under the same conditions. This wavelength was chosen because at 225 nm the CD signal fell outside the dynamic range of the instrument at this high concentration. The thermodynamic values of these transitions for the concentration-dependent all-or-none model (47) are given in Table I together with the values determined by differential scanning calorimetry. The van't Hoff enthalpy and entropy changes as well as the calorimetric enthalpy change were significantly smaller for Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 ($\Delta H_{\text{cal}} = -6.2$ kJ/mole of tripeptide unit) than the calorimetric enthalpy change for Ac-(Gly-Pro-Pro)10-NH2 ($\Delta H_{\text{cal}} = -12.0$ kJ/mole of tripeptide unit). The enthalpy change of -12.0 kJ/mole for Ac-(Gly-Pro-Pro)10-NH2 is similar to the values of -13.4 and -13.9 kJ/mole obtained for H-(Pro-4(R)Hyp-Gly)10-OH (47, 48).

To ensure that we were analyzing trimeric peptides, analytical ultracentrifugation measurements were performed. Table II shows the molecular mass determinations by sedimentation equilibrium runs. Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2, Ac-(Gly-Pro-Pro)10-NH2, and Ac-(Gly-Pro-Pro)10-NH2 are trimeric triple helical molecules, whereas Ac-(Gly-4(R)Hyp-Pro)10-NH2 is monomeric.

Two-dimensional $^1$H NMR spectra were obtained for Ac-(Gly-
**Table I**

Thermodynamic values for the thermal transitions of Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2} and Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2}.

The thermodynamic values were calculated from the CD transition curves and from differential scanning calorimetry (DSC) experiments. For CD and DSC the peptide concentration was 2 mM, and the rate of heating/cooling was 7.5 °C/h. The values are given per mole of tripeptide unit.

| Peptide                     | CD                  | DSC                  |
|-----------------------------|---------------------|----------------------|
|                             | $T_m$ (°C)         | Δ$H_{vH}$ (kJ/mol)   | Δ$S_{vH}$ (J/K·mol) | $T_m$ (°C) | Δ$H_{cal}$ (kJ/mol) | Δ$S_{cal}$ (J/K·mol) |
| Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2} | 80.5\textsuperscript{a} | −7.9                | −17.8               | 81.8       | −6.2                | −13.7                |
|                             | 80.0\textsuperscript{a} | −8.2                | −19.5               | 79.8       | −6.0                | −13.3                |
| Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2} | 76.1\textsuperscript{a} | −15.5               | −40.5               | 75.7       | −12.0               | −30.6                |
|                             | 76.8\textsuperscript{a} | −14.9               | −39.0               | 74.3       | −11.5               | −29.4                |

\textsuperscript{a} Values were calculated for the heating scan.

\textsuperscript{b} Values were calculated for the cooling scan.

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**Fig. 3.** Differential scanning calorimetry of Ac-(Gly-Pro-4(R)-Hyp)\textsubscript{10}-NH\textsubscript{2} and Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2}. A, the heat capacity was measured as a function of temperature with a peptide concentration of 2 mM and a heating/cooling rate of 7.5 °C/h. For Ac-(Gly-Pro-4(R)-Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2}, the data is shown as a solid line and for Ac-(Gly-Pro-4(R)-Hyp)\textsubscript{10}-NH\textsubscript{2} as a dotted line. Positive Δ$C_p$ values are for the heating scan; negative Δ$C_p$ values are for the cooling scan. B, the transition curves monitored by CD at 235 nm measured under the same conditions for Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2} (circles) and Ac-(Gly-Pro-4(R)-Hyp)\textsubscript{10}-NH\textsubscript{2} (upward triangles). Solid symbols are for the heating curve; open symbols are for the cooling curve.

Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}-NH\textsubscript{2} Forms a Stable Collagen Triple Helix
Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} Forms a Stable Collagen Triple Helix

The molecular masses of Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}}, Ac-(Gly-4(R)Hyp-Pro)\textsubscript{10}\textsubscript{-NH\textsubscript{2}}, Ac-(Gly-Pro-Pro)\textsubscript{10}\textsubscript{-NH\textsubscript{2}}, and Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} were measured in phosphate-buffered saline at 4–6 °C. The partial specific volume used was 0.71 ml/g.

| Peptide | Experimental mass (Da) | Theoretical monomer mass (Da) | Theoretical trimer mass (Da) |
|---------|------------------------|-------------------------------|-----------------------------|
| Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} | 8300 ± 500 | 2731.9 | 8195.7 |
| Ac-(Gly-4(R)Hyp-Pro)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} | 8979 ± 500 | 2891.9 | 8675.7 |
| Ac-(Gly-Pro-Pro)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} | 2932 ± 700 | 2731.9 | 8195.7 |
| Ac-(Gly-Pro-Pro)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} | 7256 ± 500 | 2571.9 | 7715.7 |

4(R)Hyp-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} and Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} in D\textsubscript{2}O solution (Fig. 4). Both data sets showed cross-peaks between nearly all proton resonance positions, clearly demonstrating that the peptides are triple helical at 30 °C (49–51). Thus the CD, calorimetric, and analytical ultracentrifugation data about these peptides are confirmed. A comparison between the two data sets suggested that there might be additional broadening in the NOESY spectrum of Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}}. This stronger broadening might arise from less mobility of Ac-(Gly-Pro-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}} when compared with Ac-(Gly-4(R)Hyp-4(R)Hyp)\textsubscript{10}\textsubscript{-NH\textsubscript{2}}.

We therefore performed an H/D exchange experiment to test for potential unfolding of the helix by examining the rate of the Gly NH exchange (Fig. 5). The results of this H/D exchange
experiment show that at least two different Gly NH populations exist: a minor one with a faster decay, and a slower, dominant one. Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 retains a smaller fraction of the minor population at the initial time measured (1 min after mixing), and faster exchange of both populations (Table III). Thus, the H/D exchange data indicate that Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 has less overall structural integrity, insofar as H/D exchange would indicate.

**DISCUSSION**

The results show that the peptide Ac-(Gly-4(R)Hyp-4(R)Hyp-4(R)Hyp)10-NH2 forms a more stable triple helix than the peptide Ac-(Gly-Pro-4(R)Hyp-4(R)Hyp)10-NH2, when evaluated by the Tm value. The steric restrictions imposed by the proline ring play a dominant role in the stability of the triple helix. The interchain hydrogen bonds between GlyNH ... OC(Xaa) of an adjacent chain are also recognized as important for the stability. The additional stabilization observed by the hydroxylation of proline residues in the Yaa position to 4(R)-hydroxyproline was explained in two different models. In the first model the hydroxyl group of 4(R)-Hyp is thought to form water bridges between backbone groups, and in the second model the inductive effect of the hydroxyl group influences the ring puckering of the pyrrolidine ring and changes the cis/trans ratio of the peptide bond. The triple helix of (Pro-Pro-Gly)10 shows a preference of down puckering of the proline ring in the Xaa position and an up puckering of the proline ring in the Yaa position. Previous studies have shown that the -Gly-4(R)Hyp-Pro- tripeptide units destabilize the triple helix (19). This is explained by the preference of up puckering of the proline ring of 4(R)Hyp in the Xaa position, and, conversely, it also explains the stabilizing effect of 4(R)Hyp in the Yaa position. Therefore, we hypothesized that Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 should have an intermediate stability between Ac-(Gly-4(R)Hyp-Pro)10-NH2 and Ac-(Gly-Pro-4(R)Hyp)10-NH2. However, the experimental data (Figs. 2 and 3) indicate that Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 is a little more stable than Ac-(Gly-Pro-4(R)Hyp)10-NH2.

The unfolding of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 measured by CD and DSC shows that the thermal transition is broader than that of Ac-(Gly-Pro-4(R)Hyp)10-NH2. This is consistent with the smaller enthalpy change observed by CD and DSC for the transition of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2. However, this smaller enthalpy change is compensated by a smaller entropy change, which results in a higher Tm for Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2. The presence of 4(R)Hyp in the Xaa position, with a "wrong" pyrrolidine ring puckering for that position, results in a less tight triple helix, in which the amide hydrogens of glycine exchange with solvent faster than in the triple helix formed by Ac-(Gly-Pro-4(R)Hyp)10-NH2.

Additional stabilization of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 might come from the higher trans/cis ratio of peptide bonds in the denatured state. The magnitude of the difference in the enthalpy and entropy changes observed for the two peptides could also indicate that the two denatured states are significantly different. For Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 the CD signal at 225 nm remains positive for the denatured state. Poly(4(R)-hydroxyproline) was shown to form a hydrogen-bonded left-handed helix in aqueous solutions whose CD spectra had a negative peak at 205 nm and a positive peak at 225 nm (52). The negative peak of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 shifts from 197 to 203 nm with increasing temperature, while the positive peak remains at 225 nm, indicating that this peptide does not change the conformation drastically when transitioning to a monomeric, non-triple helical state. This is a similar finding as for the unfolded state of Ac-(Gly-3(S)Hyp-4(R)Hyp-4(R)Hyp)10-NH2, a peptide that does not form a triple helix (49). For the structure of this peptide, the possibility of a polyproline II-like conformation has been suggested. This is

![FIG. 5. Hydrogen exchange kinetics for Ac-(Gly-Pro-4(R)Hyp)10-NH2 and Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2.](image)

The data points for the hydrogen to deuterium exchange of Ac-(Gly-Pro-4(R)Hyp)10-NH2 (squares) and Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 (upward triangles) were fitted to a bi-exponential decay with the parameters in Table III.

**TABLE III**

| Peptide                        | Fractional amplitude | k (s⁻¹)  |
|--------------------------------|----------------------|----------|
| Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 | 0.80                 | 3.7 × 10⁻⁴ |
| Ac-(Gly-Pro-4(R)Hyp)10-NH2    | 0.85                 | 7.1 × 10⁻⁴ |
| Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 | 0.15                 | 3.5 × 10⁻² |

The kinetics of hydrogen exchange was measured at 30 °C with a 10 mM peptide concentration in D₂O buffered with 100 mM acetate/acetic acid at a pH of 4.7 (uncorrected).
consistent with the higher stability of poly(4(R)Hyp) helices compared with polyproline helices (52). The conformation of the thermally denatured state of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 is experimentally not very accessible, due to the high Tm.

The stability of Ac-(Gly-4(R)Hyp-4(R)Hyp)10-NH2 is also consistent with the stabilizing influence of 4(R)Hyp in the Xaa position, as long as the Yaa position is not proline (22, 23). Recently, two reports were published showing that H-(4(S)Flp-Pro-Gly)OH (17) and H-(4(S)Flp-Pro-Gly)10-OH (18) form a triple helix in aqueous solution. If the pyrrolidine ring structure and inducing dihedral angles contribute significantly to the triple helix stability, -Gly(4(S)Hyp-4(R)Hyp)- peptide should form a very stable helix, unless there is steric hindrance. Vitagliano et al. (27) predicted steric hindrance by modeling, if the proline in the Xaa position of -(Pro-Pro-Gly)10- triple helix is replaced by 4(S)Hyp. Quantum mechanical studies also come to this conclusion (25). However, this steric hindrance might be minimized by peptides with 4(S)Flp, which is smaller than 4(R)Hyp. Replacing the hydroxyl group with fluorine results in little steric hindrance.

The occurrence of 4(R)Hyp in the Xaa position is mostly found in collagens of invertebrates (9, 53). No collagen sequences with Gly-4(R)Hyp-4(R)Hyp tripeptide units have been identified. However, it was shown recently that 4(R)Hyp can be incorporated in both the Xaa and Yaa positions of a collagen-like molecule in Escherichia coli when the NaCl concentration is high (54). Our results show that comparable stabilities should be obtained for collagen-like peptides expressed in this system. Collagen has been used extensively for tissue engineering purposes (55–57). For such purposes, the regulation of the triple helical stability is important, and collagenous peptides or recombinant collagen molecules with Gly-4(R)Hyp-4(R)Hyp tripeptide units might be useful.

Acknowledgments—We thank Eric Steel for expert technical assistance and Dr. Kerry Maddox for amino acid analyses.

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