Acetyl-Terminated and Template-Assembled Collagen-Based Polypeptides Composed of Gly-Pro-Hyp Sequences. 2. Synthesis and Conformational Analysis by Circular Dichroism, Ultraviolet Absorbance, and Optical Rotation

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Received April 17, 1996[⊗]

Abstract: Template-assembled collagen-based polypeptides KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH$_2$]$_3$ (n = 1, 3, 5, 6; KTA is *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, also known as the Kemp triacid) and acetyl-terminated single-chain collagen-based analogs Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 1, 3, 5, 6, 9) were synthesized by solid phase segment condensation methods. The triple-helical propensities of these collagen analogs were investigated using circular dichroism, ultraviolet absorbance, optical rotation, and nuclear magnetic resonance measurements. The acetyl analogs, Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 6, 9), assume a stable triple-helical conformation in H$_2$O (0.2 mg/mL) at room temperature. By contrast, Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ adopts a triple-helical conformation in H$_2$O only below 18 °C at a concentration of 0.2 mg/mL. For the template-assembled collagen analogs, results show that KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH$_2$]$_3$ (n = 5, 6) peptides form triple-helical structures which have melting temperatures above 70 °C in H$_2$O. These melting temperatures are much higher than those of the corresponding acetyl analogs, demonstrating the significant triple-helix-stabilizing effects of the KTA template. In addition, the KTA template facilitates triple-helical structures by dramatically accelerating triple-helix formation.

Introduction

Collagen is the most common protein in connective tissues. It possesses many unique properties that contribute directly to its role as a structural material. The tertiary structure of collagen is known as a triple helix. It consists of three extended left-handed polyproline-II-like peptide chains[1,2] which are arranged in a parallel direction with a one-residue shift intertwined to form a right-handed superhelix.[3,4] The primary structure of the peptide chains is composed mainly of trimer repeats. The first residue of these trimers is always a glycine followed by two variable residues: Gly-X-Y. The second (X) and third (Y) positions can be any amino acid. Proline and hydroxyproline are commonly found at these positions (X , Y), and they account for about 20% of the total amino acid composition in collagen sequences.[5,6]

Mimicry of collagen structures can help elucidate the unique triple-helical conformations and provide insights into the preparation of novel collagen-like biomaterials. Many sequential polytripeptides composed of collagen-like unique sequences Gly-X-Y have been synthesized to mimic collagen-like triple-helical structures.[7−21] Pioneering work on polytripeptides was reported in the early 1960s by Katchalski-Katzir and his colleagues.[22,23] The most interesting compounds among these collagen analogs are the monodisperse polypeptides containing Gly-Pro-Pro or Gly-Pro-Hyp sequences.[14,15,20,21,24,25] Characterization of these compounds has demonstrated the effectiveness of the Hyp residue at position Y in stabilizing triple-helical conformations. These Gly-Pro-Hyp sequence-based peptides can form stable triple-helical structures at or above room

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.

(1) Bornstein, P.; Traub, W. In *The Proteins*; Neurath, H., Hill, R. L., Eds.; Academic Press: New York, 1979; Vol. 2, pp 412−632.
(2) Ramachandran, G. N. In *Aspects of Protein Structure*; Ramachandran, G. N., Ed.; Academic Press: New York, 1963, pp 39.
(3) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75−81.
(4) Venugopal, M. G.; Ramshaw, J. A. M.; Braswell, E.; Zhu, D.; Brodsky, B. *Biochemistry* **1994**, *33*, 7948−7956.
(5) Traub, W.; Piez, K. *Adv. Protein Chem.* **1971**, *25*, 243−341.
(6) Ramachandran, G. N.; Ramakrishnan, C. In *Biochemistry of Collagen*; Ramachandran, G. N., Reddi, A. H., Eds.; Plenum Press: New York, 1976; pp 45−84.
(7) Brown, F. R., III; Carver, J. P.; Blout, E. R. *J. Mol. Biol.* **1969**, *39*, 307−313.
(8) Brown, F. R., III; Di Corato, A.; Lorenzi, G. P.; Blout, E. R. *J. Mol. Biol.* **1972**, *63*, 85−99.
(9) Brown, F. R., III; Hopfinger, A. J.; Blout, E. R. *J. Mol. Biol.* **1972**, *63*, 101−115.
(10) Ananthanarayanan, V. S.; Brahmachari, S. K. *Biopolymers* **1976**, *15*, 707−716.
(11) Bansal, M.; Ramachandran, G. N. *Int. J. Pept. Protein Res.* **1978**, *11*, 73−81.
(12) Cabrol, D.; Broch, H.; Vasilescu, D. *Biopolymers* **1981**, *20*, 269−282.
(13) Doyle, B. B.; Traub, W.; Lorenzi, G. P.; Blout, E. R. *Biochemistry* **1971**, *10*, 3052−3060.
(14) Inouye, K.; Sakakibara, S.; Prockop, D. J. *Biochim. Biophys. Acta* **1976**, *420*, 133−141.
(15) Inouye, K.; Kobayashi, Y.; Kyogoku, Y.; Kishida, Y.; Sakakibara, S.; Prockop, D. J. *Arch. Biochem. Biophys.* **1982**, *219*, 198−203.
(16) Miller, M. H.; Nemethy, G.; Scheraga, H. A. *Macromolecules* **1980**, *13*, 910−913.
(17) Segal, D. M.; Traub, W. *J. Mol. Biol.* **1969**, *43*, 487−496.
(18) Rapaka, R. S.; Bhatnagar, R. S. *Int. J. Pept. Protein Res.* **1975**, *7*, 119−128.
(19) Scatturin, A.; Tamburro, A. M.; Del Pra, A.; Bordignon, E. *Int. J. Pept. Protein Res.* **1975**, *7*, 425−435.
(20) Sakakibara, S.; Kishida, Y.; Kikuchi, Y.; Sakai, R.; Kakiuchi, K. *Bull. Chem. Soc. Jpn.* **1968**, *41*, 1273.
(21) Sakakibara, S.; Inouye, K.; Shuda, K.; Kishida, Y.; Kobayashi, Y.; Prockop, D. J. *Biochim. Biophys. Acta* **1973**, *303*, 198−202.
(22) Engel, J.; Kurtz, J.; Berger, A.; Katchalski-Katzir, E. *J. Mol. Biol.* **1966**, *17*, 255−272.
(23) Steinberg, I. Z.; Harrington, W. F.; Berger, A.; Sela, M.; Katchalski-Katzir, E. *J. Am. Chem. Soc.* **1960**, *82*, 5263−5279.
(24) Sakakibara, S.; Kishida, Y.; Okuyama, K.; Tanaka, N.; Ashida, T.; Kakudo, M. *J. Mol. Biol.* **1972**, *65*, 371−373.
(25) Kobayashi, Y.; Sakai, R.; Kakiuchi, K.; Isemura, T. *Biopolymers* **1970**, *9*, 415−425.

S0002-7863(96)01260-7 CCC: $12.00

temperature when the peptide chains contain 10 or more Gly-Pro-Hyp repeats.[14,15,21,25] All these reported compounds, (Gly-Pro-Hyp)$_n$, possess free amines on the N-termini and free acids on the C-termini.

Template-assembled synthetic protein (TASP) methods have been used in the design and synthesis of many protein mimics.[26−32] In this method, template molecules are used to fix peptide loops and to induce α-helical (or helical bundles) and β-turn structures of polypeptides. For example, Schneider and Kelly have reported the use of templates to form β-structures.[32] Muller *et al*. disclosed anthrancene-type tricyclic structures that can bridge two antiparallel peptide β-strands or induce β-turns.[28] Muller *et al*. also discovered that Kemp triacid condensed with glycine or alanine can act as a template for inducing α-helicity of an attached polypeptide.[28] Ghadiri *et al*. have prepared a 3-α-helix bundle by means of a ruthenium metal bipyridyl complex.[30]

Similar template-assembling methods can also be used to mimic collagen-like triple-helical structures. In the collagen triple helix, three parallel polypeptide chains are supercoiled around a common axis and each of them is shifted by one residue along the axis as compared to the adjacent chains. A template with three functional groups that can be connected to the three peptide chains, if designed properly, will help the three chains to adopt this special array and register. Heidemann *et al*. have used lysine dimer and 1,2,3-propanetricarboxylic acid to prepare covalently bridged synthetic polypeptides which were found to assemble into a triple helix.[33−37] Fields *et al*.[38−40] and Tanaka *et al*.[41] employed two consecutively connected lysine residues with three functional groups to link the three peptide chains at the C-termini or the N-termini. These templates are able to stabilize triple-helical structures by changing the entropy effects.

In our approach, a more conformationally constrained organic molecule, *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (the Kemp triacid, KTA, Figure 1),[42] was selected to induce the collagen-like triple-helical structure for very short peptide chains.[43] The KTA contains three axial carboxylic acid functionalities, each of which can be coupled to a strand of the triple helix. In addition, its three axial functional groups are parallel to each other, which can facilitate the interactions of the three peptide chains to form the desired triple-helical conformation.

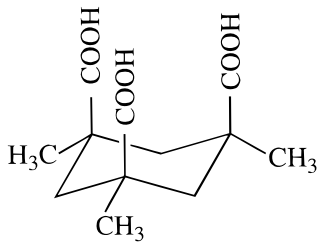
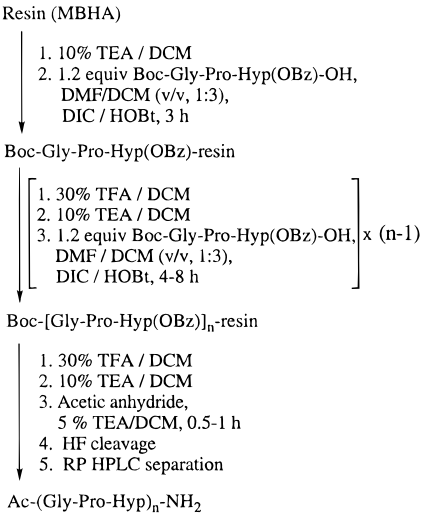


Figure 1. Structure of the Kemp triacid. According to NMR studies,[42] the most stable conformation for this tricarboxylic acid compound is a chair conformer with the carboxyl groups in the axial positions.

Scheme 1



Rather than coupling KTA directly to the N-termini of the peptides, a glycine spacer was attached to each of the carboxyl groups. The spacer reduces the steric hindrance of the three peptide chains reacting with the KTA functional groups and provides the flexibility to allow the three peptide chains to adopt the proper one-residue register shift necessary for a collagen-like triple-helical conformation. The template-assembled collagen-based polypeptides KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH$_2$]$_3$ (n = 1, 3, 5, 6), were synthesized using KTA as the template and glycine as the spacer. Acetyl compounds, Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 1, 3, 5, 6, 9), were also prepared for comparison studies to demonstrate the effects of the KTA template.

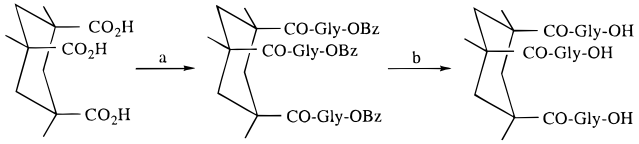
In this paper, the synthesis of these template-assembled collagen-based polypeptides is reported along with the synthesis of the N-terminal-acetylated single-chain sequential polypeptides. The biophysical characterization of both the acetyl-terminated and template-assembled collagen-based analogs is presented and is based on circular dichroism (CD), ultraviolet (UV) absorbance, optical rotation, and nuclear magnetic resonance (NMR) measurements. The stabilizing effect of the KTA template with glycine residue spacers on triple-helical conformation is shown. Solvent and concentration effects on triple helicity are also discussed.

Synthesis

Synthesis of the N-Terminal Acetyl Compounds. The peptide chains were assembled by the solid phase segment condensation method that was first introduced by Sakakibara *et al*.[20] The resin utilized was 4-methylbenzhydrylamine (MBHA), and Boc(*tert*-butyloxycarbonyl) chemistry was employed in the solid phase synthesis (Scheme 1). At each segment condensation, 1.2−1.5 equiv of building block Boc-Gly-Pro-Hyp(OBz)-OH was used with the coupling reagents diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole

(26) Tuchscherer, G.; Domer, B.; Sila, U.; Kamber, B.; Mutter, M. *Tetrahedron* **1993**, *49*, 3559−3575.
(27) Vuilleumier, S.; Mutter, M. *Biopolymers* **1993**, *33*, 389−400.
(28) Muller, K.; Obrecht, D.; Knierzinger, A.; Stankovic, C.; Spiegler, C.; Bannwarth, W.; Trzeciak, A.; Englert, G.; Labhardt, A. M.; Schonholzer, P. In *Perspective in Medicinal Chemistry*; Testa, B., *et al*., Eds.; Verlag: Basel, 1993; pp 513−531.
(29) Ikeura, Y.; Honda, Y.; Kurihara, K.; Kunitake, T. *Chem. Lett*. **1990**, 169−172.
(30) Ghadiri, M. R.; Case, M. A. *Angew. Chem., Int. Ed. Engl*. **1993**, *32*, 1594−1597.
(31) Whitesell, J. K.; Chang, H. K. *Science* **1993**, *261*, 73−76.
(32) Schneider, J. P.; Kelly, J. W. *J. Am. Chem. Soc*. **1995**, *117*, 2533−2546.
(33) Greiche, Y.; Heidemann, E. *Biopolymers* **1979**, *18*, 2359−2361.
(34) Roth, W.; Heidemann, E. *Biopolymers* **1980**, *19*, 1909−1917.
(35) Thakur, S.; Vadolas, D.; Germann, H. P.; Heidemann, E. *Biopolymers* **1986**, *25*, 1081−1086.
(36) Dolz, R.; Heidemann, E. *Biopolymers* **1986**, *25*, 1069−1080.
(37) Germann, H. P.; Heidemann, E. *Biopolymers* **1988**, *27*, 157−163.
(38) Fields, G. B. *J. Theor. Biol*. **1991**, *153*, 585−602.
(39) Fields, C. G.; Lovdahl, C. M.; Miles, A. J.; Matthias Hagen, V. L.; Fields, G. B. *Biopolymers* **1993**, *33*, 1695−1707.
(40) Fields, C. G.; Mickelson, D. J.; Drake, S. L.; McCarthy, J. B.; Fields, G. B. *J. Biol. Chem*. **1993**, *268*, 14153−14160.
(41) Tanaka, T.; Wada, Y.; Nakamura, H.; Doi, T.; Imanishi, T.; Kodama, T. *FEBS Lett*. **1993**, *334*, 272−276.
(42) Kemp, D. S.; Petrakis, K. S. *J. Org. Chem*. **1981**, *46*, 5140−5143.
(43) Goodman, M.; Feng, Y.; Melacini, G.; Taulane, J. P. *J. Am. Chem. Soc*. **1996**, *118*, 5156−5157. This is paper 1 of our series on collagen-based structures.

Scheme 2[a]



[a] (a) Gly-OBz, EDC/HOBt, DMF, room temperature, 4 h, 98%; (b) H_2/Pd, MeOH, 97%.

(HOBt). Trifluoroacetic acid (TFA) was used to remove the Boc protecting group after each coupling step, and the resin was subsequently neutralized with triethylamine (TEA). The Kaiser ninhydrin test was used to monitor the coupling reaction. After a specific chain length was reached, the N-terminal amines were acetylated by treatment with acetic anhydride. The products were removed from the resin using HF cleavage methods. Since MBHA resin was used, the final products were all amides on the C-termini. Reverse phase HPLC was utilized to separate the products and determine the purity. The structures were all verified using mass spectrometry and ^{1}H-NMR spectroscopy.

Generally, in solid phase peptide synthesis more than 2 equiv of the free acid component is used to drive each coupling reaction to completion. Here only 1.2−1.5 equiv of the building block tripeptide Boc-Gly-Pro-Hyp(OBz)-OH was used at each coupling step because of the synthetic effort required for the preparation of this tripeptide. Nevertheless, this coupling strategy was found to be successful up to the ninth coupling stage.

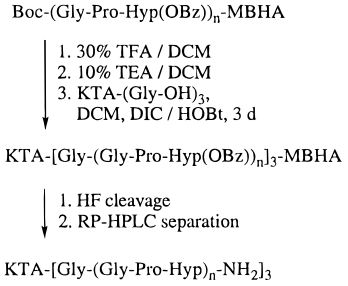
Synthesis of the Template KTA-(Gly-OH)$_3$. Peptide-coupling methods in solution were used to prepare the template structure KTA-(Gly-OH)$_3$, and the synthesis is described in Scheme 2. The KTA-(Gly-OBz)$_3$ was prepared by coupling the amino acid ester Gly-OBz to the carboxyl groups of KTA using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) and HOBt as the coupling reagents. The benzyl groups were then removed by hydrogenation to yield the template with three attached glycine residues bearing free carboxyl groups, KTA-(Gly-OH)$_3$.

Synthesis of the Template-Assembled Compounds. To prepare template-assembled collagen-like polypeptides, the peptide chains were built up on the resin and the template was directly coupled to the N-termini of these chains before removal from the resin. The synthetic route is described in Scheme 3, and the coupling reagents were DIC and HOBt. In order to obtain a high yield of the desired three-chain product and to minimize side products, KTA-(Gly-OH)$_3$ was used as the limiting reagent. The couplings were monitored by the Kaiser ninhydrin test to completion, and the HF cleavage method was used to remove the products from the resin. Reverse phase HPLC was utilized to separate the template-assembled compounds and determine their purity. Mass spectrometry and ^{1}H-NMR spectroscopy were used to characterize the peptide structures.

Biophysical Studies

CD Spectra. Collagen-like triple-helical and polyproline-II-like structures exhibit CD spectra in solutions which are characterized by a large negative peak around 200 nm and a small positive peak around 215−227 nm.[4,7,8,44−49] These

Scheme 3



features have been used as a basis to provide information about the presence of a polyproline-II-like or collagen-like triple-helical structure in solution for natural and synthetic peptides.[4,7,15,49] In order to investigate the triple-helical profiles of our synthetic collagen-based peptides, their CD spectra in both H_2O and ethylene glycol/H_2O (EG/H_2O, v/v, 2:1) solvents were determined. Ethylene glycol is known to stabilize helical structures and therefore can be very useful to amplify and detect very weak triple-helical propensities.[7,8,27,50] Typical CD spectra are shown in Figure 2. The spectroscopic parameters for the polypeptides are listed in Table 1. The CD parameters listed in Table 1 for collagen[7,8] and (Gly-Pro-Hyp)$_{10}$-OH[4] are based on previously reported data.

The 30-residue peptide (Gly-Pro-Hyp)$_{10}$-OH has been reported to be triple helical in H_2O,[3,4,15,21,51,52] while Ac-Gly-Pro-Hyp-NH_2 and KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$ lack the structural requirements to form a triple-helical structure.[53] However, these three compounds along with all the other compounds listed in Table 1 show similar CD spectral shapes and peak positions for the minimum, crossover, and maximum, which are similar to those of a typical polyproline-II-like conformation.[7,45,46,48,54] These CD spectral shapes and peak positions do not show whether the polyproline-II-like peptide chains are associated to triple-helical structures. Therefore these features, although necessary, can not be used conclusively to establish the presence of a collagen-like triple-helical conformation.

A parameter related to the CD peak intensities was found to be useful in establishing triple-helical conformations in solution. This parameter, Rpn, denotes the ratio of positive peak intensity over negative peak intensity. The Rpn values for the synthetic collagen-based peptides are listed in Table 1. The Rpn values for natural collagen and (Gly-Pro-Hyp)$_{10}$-OH are also included in Table 1 and are estimated from previously reported CD spectra.[4,7,8] From Table 1, it can be seen that a sharp increase in Rpn values occurs at specific chain lengths. For the acetyl compounds, Ac-(Gly-Pro-Hyp)$_n$-NH_2, the sharp increase is seen when $n = 6$ in H_2O and $n = 5$ in EG/H_2O (v/v, 2:1). For the template-assembled analogs, KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$, there is a sharp increase in Rpn values at $n = 3$ in both H_2O and EG/H_2O (v/v, 2:1). The Rpn value at the point of sharp increase is solvent dependent and found to be 0.12 in H_2O and 0.15 in EG/H_2O (v/v, 2:1). From Table 1, it can also be seen that the Rpn values for our collagen-like polypeptides are essentially the same as the Rpn values exhibited by collagen

(44) Brodsky-Doyle, B.; Leonard, K. R.; Reid, K. B. M. *Biochem. J.* **1976**, *159*, 279−286.
(45) Rabanal, F.; Ludevid, M. D.; Pons, M.; Giralt, E. *Biopolymers* **1993**, *33*, 1019−1028.
(46) Madison, V.; Schellman, J. *Biopolymers* **1970**, *9*, 511−567.
(47) Siligardi, G.; Drake, A. F. *Biopolymers (Pept. Sci.)* **1995**, *37*, 1−12.
(48) Sreerama, N.; Woody, R. W. *Biochemistry* **1994**, *33*, 10022−10025.
(49) Toumadje, A.; Johnson, W. C., Jr. *J. Am. Chem. Soc.* **1995**, *117*, 7023−7024.
(50) Choma, C. T.; Lear, J. D.; Nelson, M. J.; Dutton, P. L.; Roberson, D. E.; DeGrado, W. F. *J. Am. Chem. Soc.* **1994**, *116*, 856−865.
(51) Brodsky, B.; Li, M.; Long, C. G.; Apigo, J.; Baum, J. *Biopolymers* **1992**, *32*, 447−451.
(52) Li, M.; Fan, P.; Brodsky, B.; Baum, J. *Biochemistry* **1993**, *32*, 7377−7387.
(53) Melacini, G.; Feng, Y.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10359−10364.
(54) Ramachandran, G. N. In *Treatise of Collagen*, 1st ed.; Ramachandran, G. N., Ed.; Academic Press: London, 1967; Vol. 1, pp 103−184.

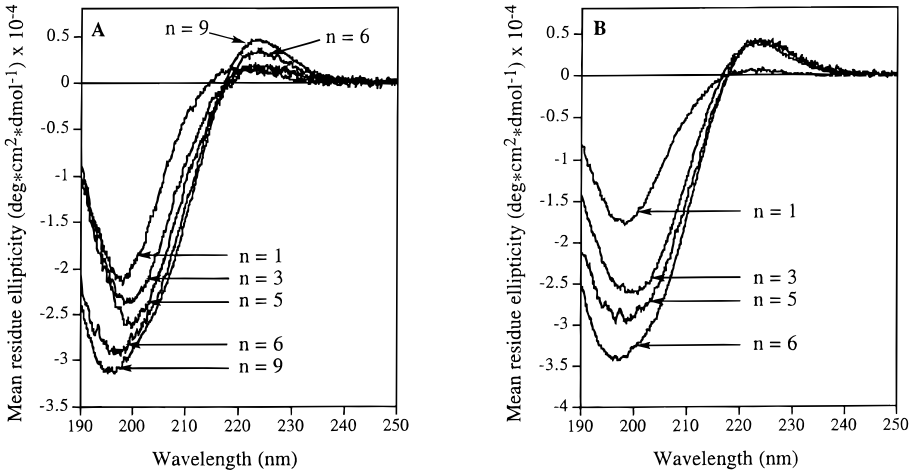


Figure 2. CD spectra of Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 1, 3, 5, 6, 9) (A) and KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ (n = 1, 3, 5, 6) (B) in H_2O (0.2 mg/mL) at 20 °C.

Table 1. Circular Dichroism Data for the Synthesized Collagen-Based Polypeptides Composed of Gly-Pro-Hyp Sequences[a]

	H_2O				EG/H_2O (v/v, 2:1)			
compounds	min (nm)	cross (nm)	max (nm)	Rpn[b]	min (nm)	cross (nm)	max (nm)	Rpn[b]
Ac-Gly-Pro-Hyp-NH_2	198 (-2.1×10^4)	214	220 (1.4×10^3)	0.07	198 (-1.9×10^4)	214	221 (1.9×10^3)	0.10
Ac-(Gly-Pro-Hyp)$_3$-NH_2	200 (-2.3×10^4)	218	223 (1.8×10^3)	0.08	199 (-2.4×10^4)	217	223 (2.2×10^3)	0.09
Ac-(Gly-Pro-Hyp)$_5$-NH_2	200 (-2.6×10^4)	218	224 (1.7×10^3)	0.07	199 (-2.9×10^4)	217	223 (4.4×10^3)	0.15
Ac-(Gly-Pro-Hyp)$_6$-NH_2	198 (-2.9×10^4)	218	224 (3.4×10^3)	0.12	198 (-3.4×10^4)	217	223 (5.3×10^3)	0.16
Ac-(Gly-Pro-Hyp)$_9$-NH_2	198 (-3.1×10^4)	218	224 (4.4×10^3)	0.14	197 (-4.0×10^4)	217	223 (6.7×10^3)	0.17
KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$	199 (-1.7×10^4)	217	222 (0.8×10^3)	0.05	199 (-1.7×10^4)	217	223 (1.0×10^3)	0.06
KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH_2]$_3$	200 (-2.6×10^4)	217	223 (3.7×10^3)	0.14	200 (-3.1×10^4)	217	224 (5.0×10^3)	0.16
KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH_2]$_3$	198 (-2.9×10^4)	218	224 (4.0×10^3)	0.14	200 (-2.7×10^4)	218	224 (4.4×10^3)	0.16
KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$	198 (-3.4×10^4)	218	224 (4.1×10^3)	0.12	199 (-2.2×10^4)	218	224 (3.7×10^3)	0.17
collagen[c]	198 (-5.4×10^4)	213	220 (7.1×10^3)	0.13	197 (-5.7×10^4)	213	220 (9.5×10^3)	0.17
(Gly-Pro-Hyp)$_{10}$-OH[c]	198 (-3.4×10^4)	218	225 (4.3×10^3)	0.13				

[a] CD spectra were obtained at 20 °C using a peptide concentration of 0.2 mg/mL. The peak intensities are included in parentheses. [b] Rpn represents the ratio of positive peak intensity over negative peak intensity (absolute values). [c] Estimated from published CD spectra: refs 8 and 9 for collagen in H_2O, ref 7 for collagen in EG/H_2O (v/v, 2:1), and ref 4 for (Gly-Pro-Hyp)$_{10}$-OH in H_2O.

and (Gly-Pro-Hyp)$_{10}$-OH. On the basis of these Rpn values, the presence of triple-helical conformations is established for the following synthetic polypeptides, at 20 °C, at a concentration of 0.2 mg/mL: Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 6, 9) and KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ (n = 3, 5, 6) in both H_2O and EG/H_2O (v/v, 2:1) and Ac-(Gly-Pro-Hyp)$_5$-NH_2 in EG/H_2O (v/v, 2:1).

Melting Studies. In addition to CD spectroscopy, melting curve measurements were also used to determine the triple helicity of the synthetic collagen-based polypeptides. Measurements of the optical rotation (α)[15,20,21] and the CD molar ellipticity (θ)[4,39,51,52] as a function of temperature have been used to monitor the thermal denaturation of collagen-like triple-helical conformations. Similarly, UV absorbance has also been used for this purpose.[55] For natural collagen, denaturation is always accompanied by a hyperchromic effect in UV absorbance.[56] A similar effect is observed for our synthetic acetyl-terminated and template-assembled peptides.

Both optical rotation and UV absorbance measurements were used to construct the melting curves for our synthetic collagen-based peptides. The melting temperatures obtained in both H_2O and EG/H_2O (v/v, 2:1) are listed in Table 2. The melting curves obtained by optical rotation measurements in H_2O are presented in Figure 3. The results obtained from both UV and optical rotation methods are consistent with each other. For example, for Ac-(Gly-Pro-Hyp)$_6$-NH_2 in H_2O (0.2 mg/mL), melting temperatures of 37 and 36 °C (Table 2) were obtained from UV absorbance and optical rotation measurements, respectively.

Based on the results of melting studies (Table 2), it can be concluded that KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ (n = 3, 5, 6) and Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 6, 9) in both H_2O and EG/H_2O (v/v, 2:1) as well as Ac-(Gly-Pro-Hyp)$_5$-NH_2 in EG/H_2O (v/v, 2:1) form triple-helical conformations at 20 °C, at a concentration of 0.2 mg/mL. However, Ac-(Gly-Pro-Hyp)$_5$-NH_2 is triple helical in H_2O only below 18 °C, at a concentration of 0.2 mg/mL. It is important to point out that the results obtained from these melting studies are consistent with those obtained from Rpn values with regard to the presence of triple-helical conformations in solution.

Acetyl-Terminated Collagen Analogs. Tables 1 and 2 show that Ac-Gly-Pro-Hyp-NH_2 and Ac-(Gly-Pro-Hyp)$_3$-NH_2 cannot assume triple-helical structures in either H_2O or EG/H_2O (v/v, 2:1) solvents. This is indicated by their low Rpn values (Table 1) and the lack of observable melting transitions (Table 2). On the other hand, Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 6, 9) peptides form very stable triple-helical structures with melting temperatures of 36 °C (n = 6 in H_2O), 52 °C (n = 6 in EG/H_2O, v/v, 2:1), and 67 °C (n = 9 in H_2O). At a concentration of 0.2 mg/mL, Ac-(Gly-Pro-Hyp)$_5$-NH_2 forms a stable triple-helical conformation in EG/H_2O (v/v, 2:1) with a melting temperature of 32 °C. However in H_2O, a triple-helical structure is observed only below a temperature of 18 °C (Table 2). These results suggest that this compound represents a transition between the triple-helical and the non-triple-helical conformations, in H_2O. Additions of alcohol solvents such as EtOH and ethylene glycol

(55) von Hippel, P. H. In *Treatise on Collagen*, 1st ed.; Ramachandran, G. N., Ed.; Academic Press: London, 1967; Vol. 1, pp 253−338.
(56) Wood, G. C. *Biochem. Biophys. Res. Commun.* **1963**, *13*, 95.

Table 2. Melting Results of Collagen-Based Polypeptides Composed of Gly-Pro-Hyp Sequences from UV and Optical Rotation Measurements

compounds	H_2O	EG/H_2O (v/v, 2:1)
Ac-Gly-Pro-Hyp-NH_2	no transition observed	no transition observed
Ac-(Gly-Pro-Hyp)$_3$-NH_2	no transition observed	no transition observed
Ac-(Gly-Pro-Hyp)$_5$-NH_2	18 °C[b]	32 °C[b]
Ac-(Gly-Pro-Hyp)$_6$-NH_2	37 °C,[a] 36 °C[b]	52 °C[a]
Ac-(Gly-Pro-Hyp)$_9$-NH_2	67 °C[b]	
KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$	no transition observed	no transition observed
KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH_2]$_3$	30 °C[b]	50 °C[b]
KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH_2]$_3$	72 °C,[a] 70 °C[b]	*ca*. 95 °C[a,c]
KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$	82 °C,[a] 81 °C[b]	>95 °C[a,c]
Ac-(Gly-Pro-Hyp)$_5$-NH_2	23 °C,[b] in 50% aqueous EtOH	
(Gly-Pro-Hyp)$_5$-OH[d]	5 °C, in 50% aqueous EtOH	
(Gly-Pro-Hyp)$_{10}$-OH[d]	58 °C, in 50% aqueous EtOH	

[a] Results obtained from UV measurements. The concentrations are 0.04 mg/mL. [b] Results obtained from optical rotation measurements. The wavelength was set at 365 nmHg. Peptide concentrations are 0.2 mg/mL. [c] The denaturation commences at about 90 °C. Complete melting curves could not be obtained because the measurements could not be carried out above 100 °C. [d] Reference 21.

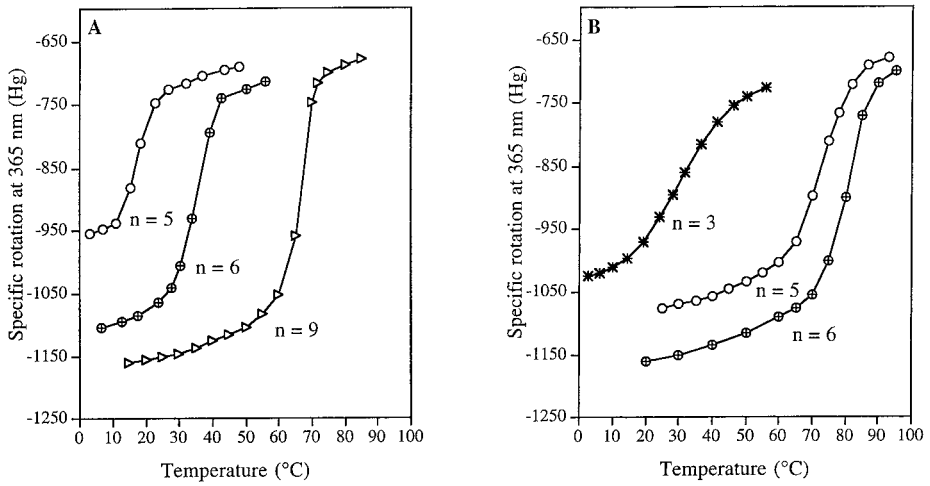


Figure 3. Temperature dependence of specific rotations at 365 nmHg for Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 5, 6, 9) (A) and KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ (n = 3, 5, 6) (B) in H_2O (0.2 mg/mL).

increase the melting temperatures, indicating an enhancement of triple helicity (Table 2). In addition, in more concentrated H_2O solutions, higher melting temperatures were obtained presumably because of concentration effects (see discussion below). However, Ac-(Gly-Pro-Hyp)$_3$-NH_2 does not show any evidence of triple helicity even at a concentration of *ca*. 2 mg/mL, at 5 °C.[53] These results indicate that at least five Gly-Pro-Hyp repeats are required to form a collagen-like triple-helical structure in H_2O, in the absence of a template.

Template-Assembled Collagen Analogs. The effect of the KTA template on triple-helical structures can be demonstrated by comparing the properties of KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ with those of the corresponding acetyl compounds, Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 3, 5, 6). For the n = 5 analogs, only the template-assembled compound shows a typical triple-helical CD spectrum in H_2O, at 20 °C (Table 1). The melting temperatures of KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH_2]$_3$ are 70 °C in H_2O and *ca*. 95 °C in EG/H_2O (v/v, 2:1). In contrast, much lower melting temperatures are seen for Ac-(Gly-Pro-Hyp)$_5$-NH_2: 18 °C in H_2O and 32 °C in EG/H_2O (v/v, 2:1). Similar results were also obtained for the n = 6 analogs. As shown in Table 2, a melting temperature of 81 °C was observed for KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH_2]$_3$ in H_2O, and a melting temperature of 36 °C was obtained for Ac-(Gly-Pro-Hyp)$_6$-NH_2 in H_2O (0.2 mg/mL). The increase in melting temperatures for the template-assembled analogs shows the significant stabilizing effects of the KTA-based template on triple-helical conformations. Sakakibara *et al*. observed a melting temperature of 58 °C for (Gly-Pro-Hyp)$_{10}$-OH in 50% EtOH/H_2O,[21] which is 53 °C higher than that obtained for (Gly-Pro-Hyp)$_5$-OH (5 °C in 50% EtOH/H_2O.[21] Therefore, the effect of the KTA template, with glycine residue spacers, on the thermal stability of the triple helices formed with five Gly-Pro-Hyp repeat analogs is similar to the addition of five more trimer repeats to the peptide chain. The triple-helix-inducing effect of the KTA template is even more dramatic in the n = 3 compounds. KTA-[Gly-(Gly-Pro-Hyp)$_3$-NH_2]$_3$ is triple helical in H_2O at room temperature. In contrast, no signs of triple helicity were observed for Ac-(Gly-Pro-Hyp)$_3$-NH_2 at lower temperatures in H_2O and EG/H_2O (v/v, 2:1) solvents (Table 2) as well as at higher concentrations (*ca*. 2 mg/mL).[53]

In addition to the triple-helix-stabilizing effects discussed above, the KTA template also facilitates triple-helical structures by accelerating triple-helix formation. Triple-helix denaturation−reformation experiments were carried out in H_2O, at concentrations of 0.2 mg/mL. The samples were thermally denatured, and the triple-helix reformation was monitored by optical rotation measurements at a temperature lower than the melting temperature of each sample. It was found that several hours were necessary for the triple helices of Ac-(Gly-Pro-Hyp)$_n$-NH_2 (n = 5, 6) to reform. For the longer chain acetyl analog Ac-(Gly-Pro-Hyp)$_9$-NH_2, the triple helix reformed in 1 h. In contrast the template-assembled analogs, KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH_2]$_3$ (n = 3, 5, 6), reformed their triple helices instantaneously (<1 min). These results substantiate the dramatic kinetic effects of the KTA-based template on triple-helix formation.

Concentration Effects on Triple Helicity. For single-chain acetyl-terminated collagen analogs, triple-helix formation involves the interaction of three molecules. Therefore, the

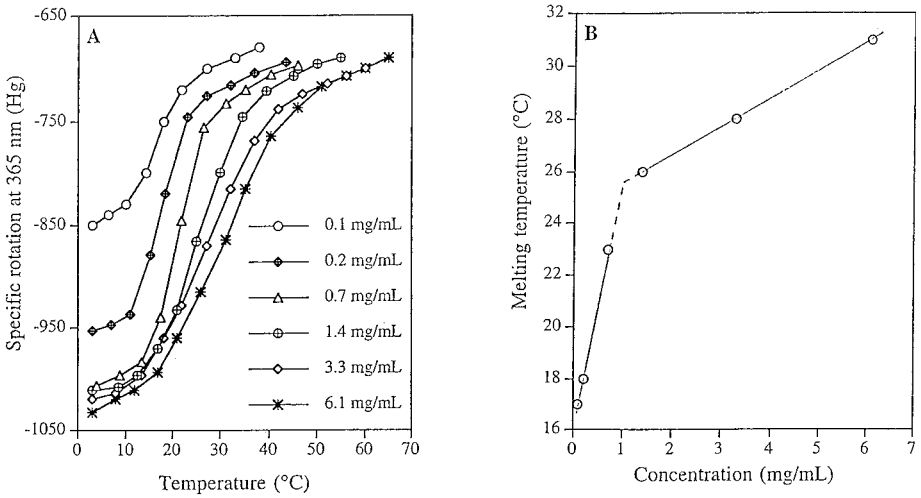


Figure 4. (A) Temperature dependence of specific rotations at 365 nmHg for Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ in H$_2$O at different concentrations and (B) relationship between the melting temperature and the concentration for Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ in H$_2$O. The critical triple-helical concentration (CTHC) is *ca.* 1 mg/mL.

concentration is expected to have a significant effect on triple-helical propensity, especially when the peptide chain is short. Ac-(Gly-Pro-Hyp)$_5$-NH$_2$, in H$_2$O, is a good candidate to study concentration effects since it has a low melting temperature (18 °C at 0.2 mg/mL) and is the non-template-assembled collagen analog with the shortest chains capable of forming a triple-helical conformation near room temperature.

As shown in Figure 4A, the melting temperature of Ac-(Gly-Pro-Hyp)$_5$-NH$_2$, in H$_2$O, increased from 17 to 31 °C when the concentration was varied from 0.1 to 6.1 mg/mL. The relationship between melting temperature and concentration is provided in Figure 4B. Two linear relationships are observed. In the lower concentration range, the melting temperature increases significantly with increasing concentration, with a slope of 8.2 °C/mg. However, when the concentration is higher than *ca.* 1 mg/mL, the melting temperature increases with concentrations with a slope of only *ca.* 1 °C/mg. The intersection of these two slopes defines a critical triple-helical concentration (CTHC). For Ac-(Gly-Pro-Hyp)$_5$-NH$_2$, in H$_2$O, the CTHC is *ca.* 1 mg/mL. On the other hand, our control experiments show that the concentration dependence of the melting temperature of KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ is small at high dilution; the melting temperature increases *ca.* 1 °C from 0.2 to 1.5 mg/mL. This dependence is similar to that of Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ above its CTHC.

In addition to stabilizing triple helices, higher concentrations for the single-chain peptides also increase the percentage of triple-helical conformations in solution. This is evident for Ac-(Gly-Pro-Hyp)$_5$-NH$_2$, in H$_2$O, where the magnitude of melting transition range increases with increasing concentration (Figure 4A). The magnitude of melting transition is defined as the difference in specific rotations immediately before and immediately after the transition range. The larger the transition magnitude, the greater the percentage of triple-helical conformations. The percentage of triple-helical structures in H$_2$O for Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ is close to a maximum when the concentration is greater than its CTHC. This is demonstrated by the similar transition magnitudes between Ac-(Gly-Pro-Hyp)$_5$-NH$_2$, at concentrations higher than its CTHC (Figure 4A), and KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$, in H$_2$O (Figure 3B). Our NMR results show that the average number of Pro residues in each chain in a triple-helical environment for both KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ and Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ in H$_2$O (*ca.* 2 mg/mL) is 4 (out of 5 total Pro residues on each chain), which is consistent with a maximal triple helicity considering the end effect and the one-residue register shift in a collagen-like triple helix.[53]

The CTHC values for Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 6, 9) occur at a concentration of 0.02 mg/mL in H$_2$O or less. As demonstrated in Figure 3, the transition magnitude for Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ is almost the same as that obtained for KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH$_2$]$_3$. On the basis of the above discussion for Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ and KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$, at a concentration of 0.2 mg/mL, the triple helicity of Ac-(Gly-Pro-Hyp)$_6$-NH$_2$ is similar to that of KTA-[Gly-(Gly-Pro-Hyp)$_6$-NH$_2$]$_3$, which is maximal according to our NMR studies.[53] The specific rotations for Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 6, 9) do not vary from 0.2 to 0.02 mg/mL in H$_2$O (20 °C), which indicates that the polypeptides Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 6, 9) are completely triple helical even at a concentration of 0.02 mg/mL in H$_2$O.

NMR Spectra. The presence of a triple-helical conformation in solution can also be established by NMR spectroscopy. The formation of a triple-helical structure results in unique interchain NOEs and the appearance of a new set of NMR resonances which can not be observed for the fully unfolded analogs.[51,52] The 1-D spectra of KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ is presented here to demonstrate the use of NMR to study triple-helical conformations. A comprehensive conformational analysis by NMR for our synthetic collagen analogs is presented in the following paper.[53] Among the resonances of the triple-helical set, the high field δ-H of the proline residue at 3.2 ppm is well resolved and not overlapped with any resonances of the denatured conformations. The resonance at 3.2 ppm (Figure 5) can therefore be used unambiguously to identify the triple-helical structure.[53] For KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ (D$_2$O, 19 °C, 2 mg/mL), the peak at 3.2 ppm is strong, but after heating to 75 °C it almost disappears, indicating denaturation of the triple-helical structure which is fully consistent with the melting transition measurements (Table 2).

Conclusions

The chemistry presented in this paper describes a unique synthetic approach to collagen-based polypeptides. The assembling of peptide chains and the coupling of the template to these chains are both carried out on a resin. It is necessary to insert glycine residues as spacers between the template and the peptide chains considering both the steric effects involved in the synthesis and the conformational requirements for triple-helical conformations.

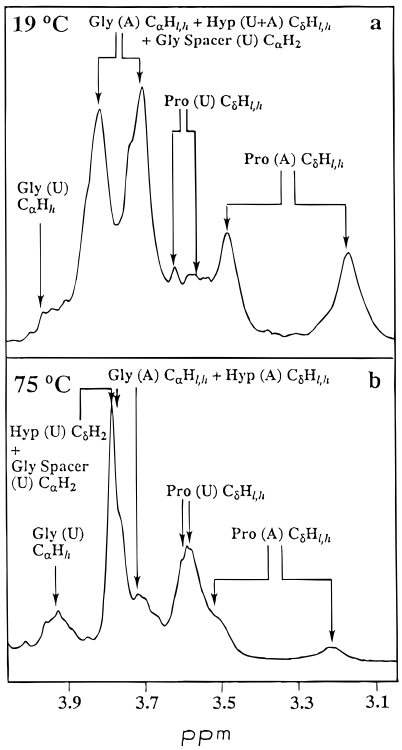


Figure 5. NMR spectra of KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ in D_2O at 19 and 75 °C. The sample concentration was 2 mg/mL. Gly (U) and Pro (U) represent the signals of Gly and Pro which are not in the triple-helical environment (unassembled); Gly (A) and Pro (A) denote the signals of Gly and Pro which are in the triple-helical array (assembled). The intensities of Gly (A) and Pro (A) are proportional to the percentage of triple-helical conformations in solution.

A CD parameter, the Rpn, was introduced as an empirical criterion to establish the presence of triple-helical conformations in solution. It was used to detect the formation of a triple-helical conformation on increasing the length of the peptide chain. For collagen analogs composed of Gly-Pro-Hyp sequences, triple-helix formation is always accompanied by a sharp increase in Rpn values. A critical triple-helical concentration (CTHC) of *ca.* 1 mg/mL in H_2O was observed for the single-chain acetyl-terminated analog Ac-(Gly-Pro-Hyp)$_5$-NH$_2$. Above the CTHC, its triple-helical percentage in solution is close to that of the corresponding template-assembled analog KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$, which is completely triple helical in H_2O according to NMR spectroscopy. The single-chain structures Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ ($n = 6, 9$) are completely triple helical even at a concentration of 0.02 mg/mL, in H_2O.

The integrated biophysical studies utilizing CD, UV, and optical rotation measurements demonstrate that the KTA template, with glycine residue spacers, can induce and facilitate triple-helix formation and also substantially stabilize triple-helical structures. Specifically, the KTA template reduces the minimal chain length necessary to form a triple-helical structure to only three Gly-Pro-Hyp repeats. In addition, the KTA template significantly increases the melting temperatures of triple-helical structures. Furthermore, triple-helix formation for the template assembled collagen analogs is instantaneous (<1 min), while some time is required for triple-helix formation of the single-chain acetyl analogs.

Experimental Section

Materials. All chiral amino acids used were of the L-configuration. Protected amino acids, EDC, and DCC were purchased from Bachem. Deuterated solvent (D_2O) was purchased from Cambridge Isotope Labs. Reagent-grade and HPLC-grade solvents (DCM, DMF, H_2O, CH_3CN, $CHCl_3$, MeOH, EtOAc, hexane, and THF) were purchased from Fisher Scientific. Triethylamine, Pd/C, benzyl chloride, and *p*-nitrophenol were purchased from Aldrich. HOBt, TFA (HPLC-grade), and HCl/dioxane (4 N) were purchased from Chem-Impex International.

General Procedures. All reactions in solution were monitored by thin-layer chromatography (TLC) carried out on precoated silica gel 60F-54 plates (Merck) using various solvent systems. Compounds were visualized by UV illustration or ninhydrin or bromocresol spray reagents. Silica gel 60 (Merck, 230−400 mesh ASTM) was used for column chromatography. Two HPLC instruments were used to analyze and purify the products. The first was a Waters (510 pump, 484 detector) system. The second was the Waters MILLENNIUM 2010 system (715 Ultra WISP sample processor, 996 photodiode array detector, two 510 pumps) with a NEC PowerMate 486/33I computer. Solvents used in HPLC included (A) H_2O with or without 0.1% TFA and (B) CH_3CN with or without 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).

To allow for proper equilibration of triple-helix formation, all solutions for biophysical studies were stored in a refrigerator (*ca.* 4 °C) for at least 24 h prior to each experiment and for another 2 h at the specified temperature before acquiring data. CD measurements were carried out on a modified Cary 61 spectropolarimeter which was modified by replacing the original Pockel cell with a 50 kHz photoelastic modulator (Hinds International FS-5/PEM-80). The original Cary linear polarizer was replaced with a MgF_2 linear polarizer supplied by AVIV, Inc. An EGG Princeton Applied Research model 128A lock in amplifier was used to integrate the phase-detected output of the original end-on PMT and preamp. System automation, multiple scan signal averaging, and base line subtraction were accomplished with an AT286 PC interfaced directly to both the Cary 61 and the 128A amplifier. The system software and custom hardware interfaces were designed by Allen MicroComputer Services Inc. and the UC San Diego Department of Chemistry & Biochemistry Computer Facility. The CD spectra were obtained using a 0.02 or 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. The UV melting curves were constructed using a Cary-1E UV spectrometer. Data were collected at 223 nm with a heating rate of 0.2 °C/min. Optical rotations were measured with a Perkin-Elmer 241 polarimeter equipped with a model 900 isotemp refrigerator circulator (Fisher Scientific). Data were collected at 365 nmHg. At each temperature point, the samples were allowed to equilibrate until the optical rotation was time independent. Triple-helix denaturation−reformation experiments were carried out by optical rotation measurements. The samples were thermally denatured in an oven at temperatures significantly higher than the melting temperatures, for a period of 2 h. The triple-helix reformation was followed by monitoring the optical rotations at a temperature lower than the melting temperature, at different time intervals. The transfer of samples from the oven to the polarimeter was done in less than 1 min.

NMR spectroscopy was used to verify the structures of intermediates and final products and to establish the conformations of KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$. For some compounds, 2-D NMR spectra were measured to determine peak assignments. These spectra were obtained on either a Bruker AMX 500 MHz spectrometer or a 360 MHz spectrometer assembled in house with a Techmag pulse programmer and digitizer and an Oxford Instruments superconducting magnet. For conformational analysis, the NMR sample was prepared by dissolving KTA-[Gly-(Gly-Pro-Hyp)$_5$-NH$_2$]$_3$ in D_2O to give a concentration of 2 mg/mL. The sample was then stored in a refrigerator (4 °C) for at least 24 h and equilibrated for another 2 h at the specified temperature before data acquisition. NMR spectra were recorded with a spectral width of 7507.507 Hz and a time domain of 16K. For each spectrum, 80 scans were acquired with a relaxation delay of 1.3 s. An exponential multiplication was used as the window function for the processing. Mass spectra were obtained at UV Riverside and the Scripps Research Institute. Fast atom bombardment (FAB), electrospray ionization (ESI), or matrix-assisted laser desorption ionization (MALDI) methods were used to verify product structures.

General Solid Phase Synthesis. A Boc chemistry approach was used in the general solid phase synthesis. An MBHA resin·HCl (Bachem) (200−400 mesh, 1% DVB, 0.45 mmol/g substitution) was

swollen in DCM for 2 h and washed with a solution of 10% TEA in DCM followed by washing with DCM. A solution of 25% DMF in DCM was used as the solvent (DMF was used to help dissolve the HOBt). DIC (2 equiv) and HOBt (2 equiv) were used as the coupling reagents along with 1.2−1.5 equiv of Boc-Gly-Pro-Hyp(OBz)-OH. After the completion of each coupling step, the solution was removed by filtration and the resin (with peptides attached) was washed with MeOH (1×) and then DCM (3×). The N-terminus was deprotected (removing Boc protection group) using a solution of 30% TFA in DCM (0.5−1.0 mL of anisol was added) for 0.5 h. After the deprotection, the solution was removed by filtration and the resin was washed with MeOH (1×) and then DCM (2×). The resin was neutralized by washing with a solution of 10% TEA in DCM (2×) and DCM (2×). The peptide chain was then built up by consecutive coupling reactions to achieve the desired length. To prepare the acetyl compound, the N-terminal amine was acetylated using acetic anhydride in a solution of 5% TEA in DCM. To obtain the template-assembled compounds, the templates were attached to the free N-termini of the peptide chains using DIC and HOBt as the coupling reagents. The resin with acetyl-terminated or template-assembled peptides was then washed several times with DCM and dried in vacuum overnight. The peptides were cleaved from the resin using standard HF cleavage methods with an apparatus manufactured by Immuno-Dynamics Inc. The HF cleavage reactions were allowed to proceed for 0.5−1 h at −5−0 °C in the presence of anisol (1−3 mL depending on the amount of the resin). The resulting mixture (resin and product) was washed (3×) with either anhydrous Et_2O or a mixture of Et_2O and hexane on a sintered glass filter. The product was then separated from the resin by extracting with H_2O.

KTA-(Gly-OBz)$_3$. A solution of the Kemp triacid (KTA) (1.0 g, 3.9 mmol), tosyl salt of Gly-OBz (6.5 g, 19 mmol), and HOBt (2.6 g, 19 mmol) in DMF (50 mL) was cooled to 0 °C. TEA (5 mL, 36 mmol) was then added slowly. After the solution was stirred for 5 min, EDC (3.7 g, 19 mmol) was added and the stirring was continued for 4 h, at room temperature. The DMF was distilled under reduced pressure, and the remaining mixture was poured into EtOAc (300 mL). The EtOAc solution was washed with H_2O (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), brine (20 mL), saturated $NaHSO_4$ (2 × 20 mL), and brine again until the brine layer was neutral. The organic layer was dried with Na_2SO_4, and the solvent was removed under reduced pressure to obtain the crude product. Column chromatography using silica gel (EtOAc/hexane, 3:2) was carried out to obtain KTA-(Gly-OBz)$_3$ as a white powder (2.65 g, 97.8%): $R_f = 0.55$ (EtOAc/hexane, 3:1).

KTA-(Gly-OH)$_3$. Nitrogen gas was passed through a solution of KTA-(Gly-OBz)$_3$ (2.65 g, 3.8 mmol) and MeOH (200 mL) for 10 min in order to remove the dissolved air. Then, 10% Pd/C (0.2 g) was added, and hydrogen was led into the solution. The hydrogenation was continued until the consumption of hydrogen stopped. The catalyst was removed by filtration, and MeOH was distilled under reduced pressure to give the product as a white powder (1.55 g, 97%): R_f = 0.05 ($CHCl_3$/MeOH/acetic acid, 85:15:3); analytical HPLC profile showed a single homogeneous peak with $t_R = 14$ min (0.1% TFA in the elution solvents, 10−20% B, 30 min); FAB-MS (M + H) calcd for $C_{18}H_{27}N_3O_9$ 430, obsd 430; ^{1}H-NMR (360 MHz, DMSO-d_6, 20 °C) δ 7.93 (s, 3H, NH), 3.64 (s, 6H, Gly-α), 2.54 (d, 3H, CH_2-equatorial), 1.24−1.18 (m, 12H, 9H for CH_3, 3H for CH_2-axial).

Ac-Gly-Pro-Hyp-NH$_2$. Boc-Gly-Pro-Hyp(OBz)-MBHA (0.3 mmol based on the resin substitution) was prepared following the procedures described in the General Solid Phase Synthesis section. The Boc group was removed using a solution of 30% TFA in DCM (15 mL), and anisol (1 mL) was added as scavenger. The resin was washed with DCM (2 × 10 mL), MeOH (2 × 10 mL), and DCM (2 × 10 mL) followed by 10% TEA in DCM (2 × 15 mL) and DCM (2 × 15 mL). The N-termini were acetylated by treatment with a solution of acetic anhydride (0.5 mL) in DCM (15 mL) with 5% TEA to give Ac-Gly-Pro-Hyp(OBz)-MBHA. After carrying out the HF cleavage, the crude material (72 mg) was obtained after lyophilizing. Preparatory RP-HPLC was carried out to obtain the final product Ac-Gly-Pro-Hyp-NH_2 as a white powder (45 mg, 46%): analytical HPLC gave a homogeneous single-peak chromatogram, $t_R = 5.7$ min (3−30% B in 25 min); FAB-MS accurate mass calcd for $C_{14}H_{22}N_4O_5$ 327.1668, obsd 327.1678; ^{1}H-NMR (500 MHz, D_2O, using H_2O for obtaining NH signals, 27 °C, assignment by DQF-COSY method) δ 8.14 (s, 1H, Gly-NH), 7.75 (s, 1H, C-terminal NH_2), 7.01 (s, 1H, C-terminal NH_2), 4.95 (dd, 0.15H, Pro-α), 4.74 (m, covered in H_2O signal, Pro-α), 4.62 (m, 1H, Hyp-γ), 4.53 (t, 1H, Hyp-α), 4.15 (d, 1H, Gly-α-*l*, 4.00 (d, 1H, Gly-α-*h*), 3.88 (d, 1H, Hyp-δ-*l*), 3.81 (dd, 1H, Hyp-δ-*h*), 3.60 (m, 2H, Pro-δ), 2.34 (m, 2.1H, Hyp-β-*l*, Pro-β-*l*), 2.06 (m, 5.6 H, Hyp-β-*h*, Pro-γ, acetyl CH_3), 1.93 (m, 1.3H, Pro-β-*l*).

Ac-(Gly-Pro-Hyp)$_n$-NH$_2$ (n = 3, 5, 6, 9). The syntheses of these compounds are similar to that of Ac-Gly-Pro-Hyp-NH_2. The detailed procedures and the physicochemical results are provided in the Supporting Information.

KTA-[Gly-Gly-Pro-Hyp-NH$_2$]$_3$. Boc-Gly-Pro-Hyp(OBz)-MBHA (0.3 mmol based on resin substitution level) was prepared following the procedures described in the General Solid Phase Synthesis section. The Boc group was removed using a solution of 30% TFA in DCM (15 mL) (1.0 mL of anisol was added as scavenger). The resin was washed using DCM (2 × 10 mL), MeOH (2 × 10 mL), and DCM (2 × 10 mL) followed by a solution of 10% TEA in DCM (2 × 15 mL) and DCM (2 × 15 mL) to give Gly-Pro-Hyp(OBz)-MBHA. KTA-(Gly-OH)$_3$ (35 mg, 0.08 mmol) and HOBt (50 mg) were added to the reaction vessel, and a solution of 25% DMF in DCM (15 mL) was used as the solvent (DMF was added to help dissolve the HOBt). Then, DIC (1.0 M) in DCM (4 mL) was added. The Kaiser test showed the absence of free amines after 3 days. The resin was washed with DCM and MeOH and dried in vacuum overnight. The HF cleavage methods were carried out to remove the peptide from the resin. The resulting mixture of resin and product was washed with a mixture of hexane and Et_2O (3×). The product was separated from the resin by extracting with H_2O. Preparatory RP-HPLC was carried out to give the final product as a white powder (55 mg, 56% yield based on the template used): analytical HPLC profile showed a single homogeneous peak with $t_R = 13.2$ min (no TFA, 7−50% B, 25 min); ESI-MS (M + Na) calcd for $C_{54}H_{81}N_{15}O_{18}$ 1251, obsd 1251; ^{1}H-NMR (500 MHz, D_2O, 27 °C, using H_2O at 5 °C to obtain NH signals, DQF-COSY) δ 8.84 (m, 3H, Gly-NH), 8.57 (m, 3H, spacer Gly-NH), 7.89 (m, 3H, terminal NH_2), 7.33 (m, 3H, terminal NH_2), 4.61 (m, 3H, Hyp-γ), 4.49 (t, 3H, Hyp-α), 4.06 (q, 6H, Gly-α), 3.86−3.70 (m, 12H, spacer Gly-α, Hyp-δ), 3.67−3.54 (m, 6H, Pro-δ), 2.65 (d, 3H, methylene-equatorial), 2.37−2.26 (m, 6H, Pro-β, Hyp-β), 2.10−2.00 (m, 9H, Pro-γ, Hyp-β), 1.97−1.90 (m, 3H, Pro-β), 1.33 (d, 3H, methylene-axial), 1.26 (s, 9H, methyl).

KTA-[Gly-(Gly-Pro-Hyp)$_n$-NH$_2$]$_3$ (n = 3, 5, 6). The syntheses of these compounds are similar to that of KTA-[Gly-Gly-Pro-Hyp-NH_2]$_3$. The detailed procedures and the physicochemical results are provided in the Supporting Information.

Acknowledgment. This project is funded by grants from the National Science Foundation (NSFDMR-9201133) and Ciba-Vision Inc. We thank the Scripps Research Institute and the University of California at Riverside for carrying out MS spectrometric analyses and Ms. Yonghong Lai for synthesizing Ac-(Gly-Pro-Hyp)$_9$-NH_2. We also wish to thank Dr. Elizabeth Jefferson for her critical comments and discussion.

Supporting Information Available: Synthesis procedures, physicochemical data of all collagen-based structures, and 1-D ^{1}H-NMR spectra, mass spectra, and analytical RP-HPLC profiles for some key intermediates and the acetyl-terminated and template-assembled collagen-based polypeptides (39 pages). See any current masthead page for ordering and Internet access instructions.

JA961260C