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Cis−Trans Proline Isomerization Effects on Collagen Triple-Helix Stability Are Limited

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Abstract: We investigated the effect of restricting cis−trans proline isomerization on collagen triple-helix stability. The Pro residues at the Xaa and Yaa positions of an (Xaa-Yaa-Gly) triplet were replaced by a Pro-*trans*-Pro alkene isostere in the host−guest peptide, H-(Pro-Pro-Gly)$_{10}$-OH. The resulting alkene isostere peptide had a T_m value 53.6 °C lower than that of the control peptide. The Pro-*trans*-Pro alkene isostere peptide had a T_m value 3.9 °C higher than that of the previously reported Pro-*trans*-Gly alkene isostere peptide that did not involve cis−trans Pro isomerization (Jenkins, C. L.; Vasbinder, M. M.; Miller, S. J.; Raines, R. T. *Org. Lett.* **2005**, *7*, 2619−22). Thus, single cis−trans proline amide isomerization alone has limited contribution to the overall stability of the collagen triple helix. Since collagen has a high content of imino acid residues, the cumulative effects of cis−trans isomerization may be quite significant. The peptide containing the Pro-*trans*-Pro isostere was significantly less stable than the previously reported Gly-*trans*-Pro alkene isostere peptide that retained the backbone interchain hydrogen bond (Dai, N.; Wang, X. J.; Etzkorn, F. A. *J. Am. Chem. Soc.* **2008**, *130*, 5396−5397), which confirms that direct interchain backbone hydrogen bonding is a major force for stabilizing the collagen triple helix.

Collagen is the most important structural protein in vertebrates, comprising more than 25% of all vertebrate protein.[1] It consists of three parallel left-handed polyproline type II (PPII) chains[2] wound around a common axis.[3−6] The primary structure of collagen can be represented as tripeptide repeating units (Xaa-Yaa-Gly)$_n$, in which 10% of Xaa residues are proline (Pro) and 10−12% of Yaa residues are 4(*R*)-hydroxyproline (Hyp).[7] Pro at the Yaa position is also stabilizing,[8] and both (Pro-Hyp-Gly)$_n$ and (Pro-Pro-Gly)$_n$ triple helixes have been studied extensively.[9−11] Mutations of imino acids cause dissociation of the collagen triple helix and may cause severe damage in collagen-rich connective tissues, such as bone, tendon, cartilage, ligament, skin, and blood vessels.[2]

The collagen triple helix has a requisite Gly residue at every third position; since Gly lacks a Cα side chain, it fits into the very compact core of the collagen triple helix.[6,12] An interchain hydrogen bond between the N−H of the Gly residue of one chain and the C=O of the Xaa residue (usually Pro) in an adjacent chain is believed to be one of the major factors in stabilizing the collagen triple helix.[12] Moreover, a specific n-to-π^* stereoelectronic interaction has been found to be an additional force stabilizing the polyproline type II conformation found in the collagen triple helix.[13]

The amide bond has partial double bond character, making it planar.[14] The cis content of the amide bond preceding prolyl residues is much higher than for other amino acid residues. About 10−30% of prolyl amide bonds in peptides and unfolded proteins are in the cis conformation.[15] In the collagen triple helix, all of the amide bonds adopt the trans conformation.[3] Previous studies indicated that prolyl cis−trans isomerization is a major rate-limiting step during the collagen folding process,[16,17] but the thermodynamic effects of proline isomerization have not yet been demonstrated. The entropic barrier of bringing three all-trans chains together has been demonstrated to be significant in the kinetics of collagen triple-helix folding, indicating a zipper-like folding mechanism.[18] Raines and co-workers showed that the exo-conformation of the Hyp five-membered ring increases the trans content of the preceding amide bond, which improves the stability of the resulting triple helix,[19,20] Replacing Hyp with 4(*R*)-fluoro- or 4(*S*)-methylproline at the Yaa position

(1) Ramachandran, G. N.; Reddi, A. H. *Biochemistry of Collagen*; Plenum Press: New York and London, 1976.
(2) Brodsky, B.; Shah, N. K. *FASEB J.* **1995**, *9*, 1537–1546.
(3) Ramachandran, G. N.; Kartha, G. *Nature* **1954**, *174*, 269–270.
(4) Ramachandran, G. N.; Kartha, G. *Nature* **1955**, *176*, 593–595.
(5) Rich, A.; Crick, F. H. C. *Nature* **1955**, *176*, 915–916.
(6) Rich, A.; Crick, F. H. C. *J. Mol. Biol.* **1961**, *3*, 483–506.
(7) Ramachandran, G. N.; Bansal, M.; Bhatnagar, R. S. *Biochim. Biophys. Acta* **1973**, *322*, 166–171.
(8) Persikov, A. V.; Ramshaw, J. A. M.; Kirkpatrick, A.; Brodsky, B. *Biochemistry* **2000**, *39*, 14960–14967.
(9) Kobayashi, Y.; Sakai, R.; Kakiuchi, K.; Isemura, T. *Biopolymers* **1970**, *9*, 415–425.
(10) Shaw, B. R.; Schurr, J. M. *Biopolymers* **1975**, *14*, 1951–1985.
(11) Feng, Y.; Melacini, G.; Taulane, J. P.; Goodman, M. *J. Am. Chem. Soc.* **1996**, *118*, 10351–10358.
(12) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75–81.
(13) Hinderaker, M. P.; Raines, R. T. *Protein Sci.* **2003**, *12*, 1188–1194.
(14) Pauling, L.; Corey, R. B.; Branson, H. R. *Proc. Natl. Acad. Sci. U.S.A.* **1951**, *37*, 205–211.
(15) Brandts, J. F.; Halvorson, H. R.; Brennan, M. *Biochemistry* **1975**, *14*, 4953–4963.
(16) Bächinger, H. P.; Bruckner, P.; Timpl, R.; Engel, J. *Eur. J. Biochem.* **1978**, *90*, 605–613.
(17) Bächinger, H. P. *J. Biol. Chem.* **1987**, *262*, 17144–17148.
(18) Bachmann, A.; Kiefhaber, T.; Boudko, S.; Engel, J.; Bächinger, H. P. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 13897–13902.

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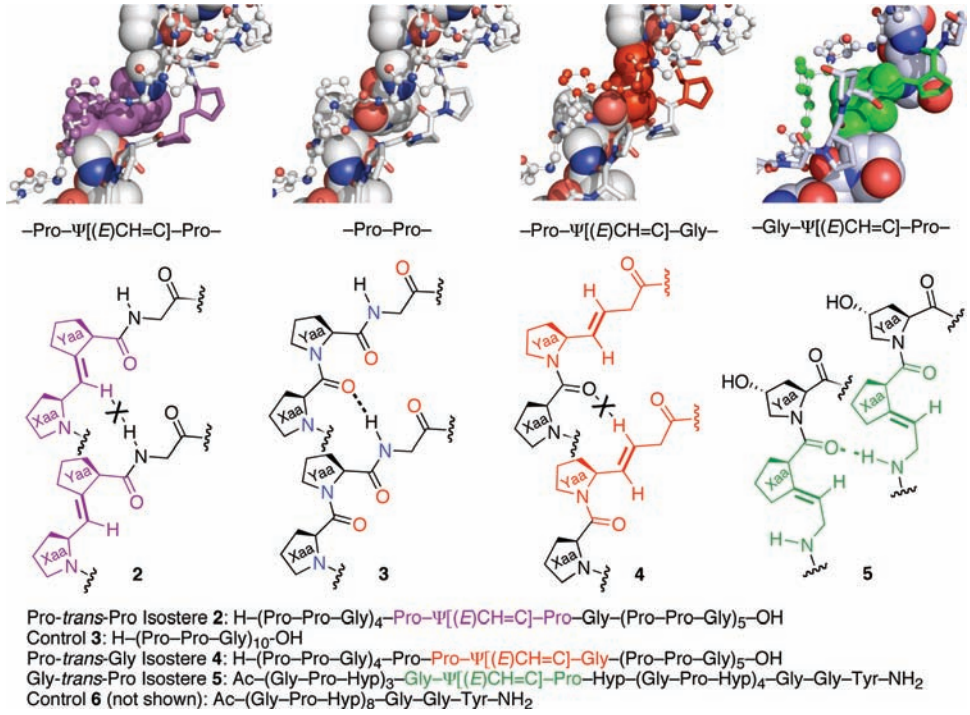
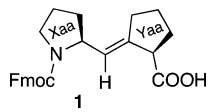


Figure 1. Pro-*trans*-Pro (*E*)-alkene isostere containing collagen host−guest peptide **2**, control **3**, Pro-*trans*-Gly isostere peptide **4**,[22] Gly-*trans*-Pro isostere peptide **5**, and its control **6** (not shown).[23] The (*E*)-alkene isosteres and interchain hydrogen-bonding patterns (---), or lack thereof (X), are shown in the structures.

increased the trans content of the resulting peptides and also formed very stable triple helixes.[20,21] Thus, we hypothesized that the entropy loss of locking cis−trans isomerization would thermodynamically stabilize the collagen triple helix.



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We now report a dipeptide isostere, Fmoc-Pro-Ψ[(*E*)CH═C]-Pro-OH (**1**), designed to tease out how much cis-trans isomerization affects the stability of a Pro-*trans*-Pro alkene isostere containing peptide **2** compared with its host collagen triple-helix peptide **3** (Figure 1). In this isostere, the (*E*)-alkene bond was locked in a *trans*-Pro conformation, and one key hydrogen bond between Gly N−H and Xaa C═O was removed, combining the effects of two previously reported isosteres, **4** and **5** (Figure 1).[22,23] Those two studies showed that substituting (*E*)-alkenes for amide bonds destabilized the collagen triple helix, but for different reasons.[22,23]

Experimental Section

Solid-Phase Peptide Synthesis. H-(Pro-Pro-Gly)$_4$-Pro-Ψ[(*E*)-CH═C]-Pro-Gly-(Pro-Pro-Gly)$_5$-OH (**2**) and H-(Pro-Pro-Gly)$_{10}$-OH (**3**) were synthesized by manual solid-phase peptide synthesis. 2-Chlorotrityl chloride resin (400 mg, 1.2 mmol/g) was placed in a 5 mL polypropylene tube, swelled in CH_2Cl_2 (2 mL) for 1.5 h, capped with MeOH (2 mL), and dried in vacuo for 14 h. A sample of the resin (10 mg) was dried in vacuo for 16 h, stirred in 30% piperidine in DMF (0.5 mL) for 30 min, and then diluted with 19.5 mL of ethanol. UV analysis showed that the loading was 0.67 mmol/g (56%). The capped 2-chlorotrityl chloride resin was separated into two polypropylene tubes (110 mg, 0.074 mmol). To couple single amino acids (Gly and Pro), HATU (85 mg, 0.22 mmol), HOAt (30 mg, 0.22 mmol), Fmoc-amino acid (0.22 mmol), and DIEA (0.075 mL, 0.43 mmol) were dissolved in DMF (2 mL), added to the resin, and shaken at 30 °C for 30 min, and the coupling was repeated. Fmoc-Gly-Pro-Pro-OH (74 mg, 0.15 mmol), HATU (57 mg, 0.15 mmol), HOAt (20 mg, 0.15 mmol), and DIEA (0.050 mL, 0.29 mmol) were dissolved in DMF (2 mL), and the mixture was shaken at 30 °C for 2 h. Fmoc-Pro-Ψ[(*E*)CH═C]-Pro-OH (33 mg, 0.079 mmol), HATU (57 mg, 0.15 mmol), and HOAt (20 mg, 0.15 mmol) were dissolved in DMF (2 mL), and the mixture was shaken at 30 °C for 2.5 h. The coupling was monitored by reverse-phase HPLC. After each coupling, the peptide was capped with 10% Ac_2O and 10% DIEA in CH_2Cl_2 (2.5 mL) for 15 min. The Fmoc group was removed with 30% piperidine in DMF for 20 and 15 min. The peptide was cleaved from the resin by treatment with 2% H_2O and 3% triisopropyl silane in TFA (5 mL) for 3.5 h. After removal of the resin by filtration and concentration of the solution in vacuo, Et_2O (10 mL) was added, and the white solid that precipitated was collected. Preparative HPLC: 10 μm protein C4 22 × 250 mm column, (A) 0.1% TFA/H_2O, (B) 0.1% TFA/CH_3CN, 10% to 95% B gradient over 20 min, flow rate 10 mL/min, λ = 210 nm. The pure peptides were obtained as white solids and stored at −20 °C under N_2. Analytical HPLC (Supporting Information): 5 μm protein C4, 4.6 × 250 mm column, 10% to 95% B gradient over 20 min, flow rate 1.0 mL/min, λ = 210 nm. Peptide **2** retention time 8.3 min. Peptide **3** retention time 7.9 min. LC-MS was performed with a C18, 2.1 × 100 mm column, 5% to 95% CH_3CN/H_2O over 35 min, positive electrospray, and a triple-quadrupole mass separator. The molecular ion peaks were not available because of the1500 Da cutoff of the detector. Isostere peptide **2**: retention time 8.3 min; MS *m*/*z* $[M + 2H]^{2+}/2$ calcd for $C_{121}H_{175}N_{29}O_{30}/2$

(19) Bretscher, L. E.; Jenkins, C. L.; Taylor, K. M.; DeRider, M. L.; Raines, R. T. *J. Am. Chem. Soc.* **2001**, *123*, 777–778.
(20) Shoulders, M. D.; Hodges, J. A.; Raines, R. T. *J. Am. Chem. Soc.* **2006**, *128*, 8112–8113.
(21) Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, *392*, 666–667.
(22) Jenkins, C. L.; Vasbinder, M. M.; Miller, S. J.; Raines, R. T. *Org. Lett.* **2005**, *7*, 2619–2622.
(23) Dai, N.; Wang, X. J.; Etzkorn, F. A. *J. Am. Chem. Soc.* **2008**, *130*, 5396–5397.

1257.9, found 1257.8; $[M + 3H]^{3+}/3$ calcd for $C_{121}H_{176}N_{29}O_{30}/3$ 838.9, found 838.8. Control peptide **3**: retention time 7.9 min; MS *m/z* $[M + 2H]^{2+}/2$ calcd for $C_{120}H_{174}N_{30}O_{31}/2$ 1266.4, found 1266.3; $[M + 3H]^{3+}/3$ calcd for $C_{120}H_{175}N_{30}O_{31}/3$ 844.6, found 844.6.

CD Analysis. The concentrations of peptides in PBS (0.27 mM KCl, 13.7 mM NaCl, 0.15 mM KH_2PO_4, 0.81 mM Na_2HPO_4 in H_2O, pH 7.4) were determined by weight; peptide **2** was 1.32 mg/mL, and control peptide **3** was 1.26 mg/mL. The peptide solutions were incubated at 5 °C for more than 48 h. The CD spectra were obtained with a spectropolarimeter in 0.5 nm increments, 1 nm bandwidth, and 0.2 cm path length at a scan speed of 100 nm/min. The spectra were averaged over four consecutive scans. The solutions were heated from 2 to 70 °C in 3 °C increments with a 10 min equilibration time at each temperature before measurement. The temperature was measured in the cell. The ellipticity at 227 nm was monitored at each temperature and averaged over four consecutive measurements. The unfolding process of each peptide was measured twice, and the data obtained were processed separately and averaged to obtain the T_m value (Supporting Information, Figures S1 and S2). The data were fit to the following equations using SigmaPlot v.10.0:[24,25]

$$\Theta = \frac{(a_n + b_n T) + (a_d + b_d T)\exp\left(\frac{-\Delta G_U^0(T)}{RT}\right)}{1 + \exp\left(\frac{-\Delta G_U^0(T)}{RT}\right)}$$

$$\Delta G_U^0(T) = \Delta H^0(T_m)\left(1 - \frac{T}{T_m}\right) - \Delta C_p^0\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right]$$

$$\Delta C_p^0 = 0$$

Results

Synthesis. The Pro-*trans*-Pro isostere **1** was designed with the (*S,R*)-stereochemistry to mimic the all-L natural amino acids found in collagen. Due to the challenges of the synthetic chemistry, and in order to compare peptide **2** directly with Pro-*trans*-Gly isostere peptide **4**, we chose the same host peptide, H-(Pro-Pro-Gly)$_{10}$-OH (**3**) (Figure 1).[22] The Pro-*trans*-Pro alkene isostere **2** eliminated cis−trans isomerization by locking one Pro amide bond in the trans conformation, and it also lacked an essential Gly N−H to Xaa Pro C═O hydrogen bond.

Our method for the stereoselective synthesis of the Ser-Ψ[(*E*)-CH═C]-Pro and Gly-Ψ[(*E*)CH═C]-Pro isosteres has been reported.[23,26] Dipeptide isostere **1** has a structure similar to that of these compounds, so we used our well-developed method for the synthesis. The synthesis of a Pro-*trans*-Pro (*E*)-alkene isostere with slight variations from our method has been reported, but the key Ireland−Claisen rearrangement was the same.[27] The α-hydroxycarboxylic acid intermediate was reduced to the alcohol and crystallized to prove the stereochemistry,[27] while we used NMR to establish the stereochemistry. The details of the synthesis and characterization of **1** are included in the Supporting Information.

Peptide **2**, which contains the Pro-*trans*-Pro alkene isostere, and control peptide **3** were synthesized by coupling Fmoc-Pro-Pro-Gly-OH tripeptide units in solid-phase peptide synthesis.[23] The Gly residue of the isostere triplet was coupled during solid-phase synthesis to reduce the number of steps in the solution-phase organic synthesis of the isostere. The dipeptide alkene isostere **1** was coupled with HATU−HOAt, with no external base added, to prevent isomerization to the α,β-unsaturated activated ester,[23] and the coupling was monitored by reverse-phase HPLC. No double bond migration was observed. There was only one peak with the desired mass in the LC-MS chromatogram, and the presence of the alkene proton was confirmed by NMR (Supporting Information). Peptides **2** and **3** were obtained as white solids after purification by RP HPLC on a protein C4 column.

CD Analysis. The isostere peptide **2** lacked an interchain backbone hydrogen bond that was present in the Pro-Pro-Gly host system, so we expected that peptide **2** would have a lower T_m value than **3**. The melting point would not be measurable in ordinary phosphate-buffered saline (PBS) if T_m was too low. Trimethylamino *N*-oxide (TMAO) has been used to study the unfolding stability of peptides and proteins.[22,28,29] TMAO is a natural osmolyte, which can thermodynamically stabilize folded proteins against the denaturation process by reducing the degree of backbone solvation.[29−32] Beck et al. found that the T_m value of unstable collagen peptides was linearly dependent on the concentration of TMAO.[28] Jenkins et al. used the same method to measure the melting temperatures of an ester isostere and Pro-*trans*-Gly isostere **4** collagen peptides by extrapolating the data to 0 M TMAO.[22]

For optimal comparison, we measured T_m under exactly the same conditions that were reported for the Pro-*trans*-Gly isostere peptide **4**.[22] Both the Pro-*trans*-Pro isostere peptide **2** and control peptide **3** were dissolved in 9.6 mM PBS buffer pH 7.4 with different concentrations of TMAO. For the Pro-*trans*-Pro isostere peptide **2**, the full-range CD spectrum showed a maximum near 225 nm when the TMAO concentration was higher than 2.5 M, which indicated the presence of the PPII helix, and the ellipticity of the peptide increased with increasing TMAO concentration (Figure 2). When the TMAO concentration was greater than 2.5 M, peptide **2** showed cooperative denaturation with increasing temperature, indicating the presence of a collagen triple helix (Figure 3). Peptide **3** showed cooperative denaturation with increasing temperature at all TMAO concentrations (Figure 3).

The T_m values at each TMAO concentration were determined by fitting the data to a two-state model. Extrapolating the data to 0 M TMAO gave a melting point of −22.0 ± 1.9 °C for isostere peptide **2** (Figure 4 and Table 1). The melting point of control peptide **3**, obtained by extrapolating the T_m value to 0 M TMAO, was determined to be 31.6 °C (Figure 4 and Table 1). This T_m value was very close to the measured T_m value of 31.5 °C in 9.6 mM PBS buffer with no TMAO present. It was also close to the literature T_m value of 32.8 °C determined in the same PBS buffer.[33]

Discussion

The Gly-Pro isostere peptide **5** showed a ΔT_m of −21.2 °C, the Pro-Pro isostere peptide **2** showed a ΔT_m of −53.6 °C, and the Pro-Gly isostere peptide **4** showed a ΔT_m of −57.5 °C compared to each of their control peptides, respectively (Table

(24) Becktel, W. J.; Schellman, J. A. *Biopolymers* **1987**, *26*, 1859–1877.
(25) Horng, J. C.; Hawk, A. J.; Zhao, Q.; Benedict, E. S.; Burke, S. D.; Raines, R. T. *Org. Lett.* **2006**, *8*, 4735–4738.
(26) Wang, X. J.; Xu, B.; Mullins, A. B.; Neiler, F. K.; Etzkorn, F. A. *J. Am. Chem. Soc.* **2004**, *126*, 15533–15542.
(27) Bandur, N. G.; Harms, K.; Koert, U. *Synlett* **2005**, *2005*, 773–776.
(28) Beck, K.; Chan, V. C.; Shenoy, N.; Kirkpatrick, A.; Ramshaw, J. A.; Brodsky, B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4273–4278.
(29) Baskakov, I.; Bolen, D. W. *J. Biol. Chem.* **1998**, *273*, 4831–4834.
(30) Liu, Y.; Bolen, D. W. *Biochemistry* **1995**, *34*, 12884–12891.
(31) Wang, A.; Bolen, D. W. *Biochemistry* **1997**, *36*, 9101–9108.
(32) Bolen, D. W.; Baskakov, I. V. *J. Mol. Biol.* **2001**, *310*, 955–963.

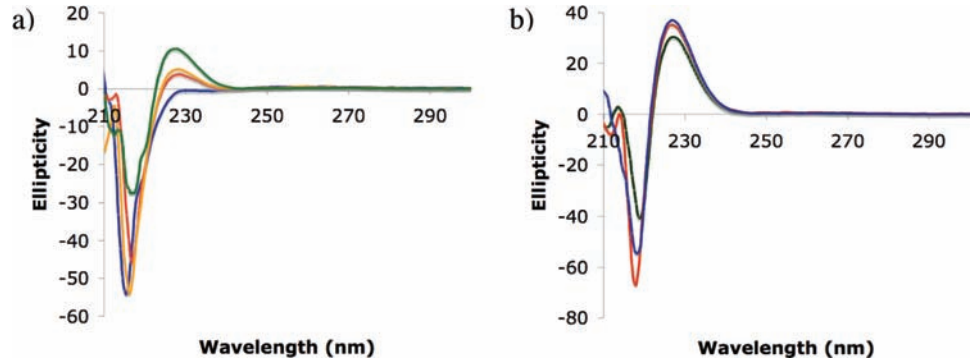

Figure 2. Full-range CD spectra. (a) Isostere peptide **2** (0.99 mg/mL in 3.25 M TMAO, 1.3 mg/mL for the rest) in PBS buffer (9.6 mM, pH 7.4) at 3 °C. Blue, 2.5 M TMAO; red, 3.0 M TMAO; yellow, 3.25 M TMAO; green, 3.5 M TMAO. (b) Control peptide **3** (1.3 mg/mL) in PBS buffer (9.6 mM, pH 7.4) at 3 °C. Green, 0 M TMAO; red, 0.5 M TMAO; blue, 1.0 M TMAO.

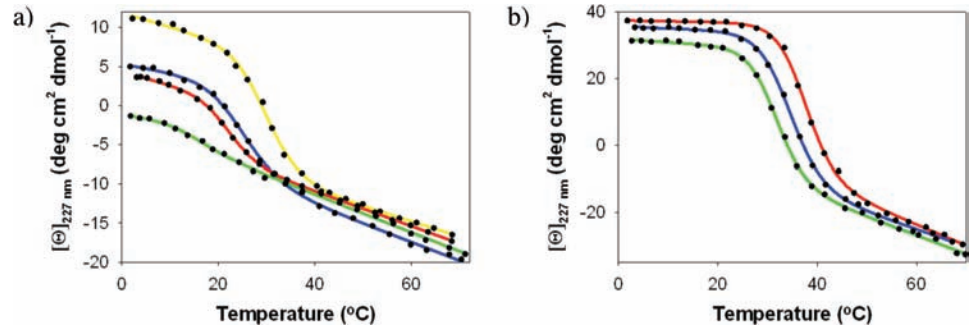

Figure 3. Measurement of T_m: CD molar ellipticity−temperature dependence in different concentrations of TMAO. (a) Isostere peptide **2**. Yellow, 3.5 M TMAO (1.3 mg/mL); blue, 3.25 M TMAO (0.99 mg/mL); red, 3.0 M TMAO (1.3 mg/mL); green, 2.5 M TMAO (1.3 mg/mL). (b) Control peptide **3** (concentration 1.3 mg/mL). Red, 1.0 M TMAO; blue, 0.5 M TMAO; green, 0 M TMAO.

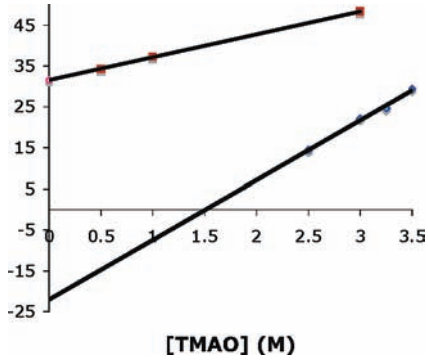

Figure 4. Dependence of T_m of peptide isostere **2** and control peptide **3** on TMAO concentration. Blue diamonds, Pro-*trans*-Pro isostere peptide **2**; red squares, control peptide **3**; red circle, measured T_m (not included in linear fit).

Table 1. Melting Point Comparison of Alkene Isostere Peptides and Their Controls

peptide	T_m (°C)	ΔT_m (°C)
Pro-*trans*-Pro peptide **2**	−22.0[a]	−53.6
Pro-Pro-Gly control **3**	+31.6[a]	
Pro-*trans*-Gly peptide **4**[22]	−24.7[a]	−57.5
Gly-*trans*-Pro peptide **5**[23]	+28.3[b]	−21.7
Pro-Hyp-Gly control **6**[23]	+50.0[b]	

[a] 9.6 mM PBS pH 7.4, varying [TMAO]. T_m value obtained by extrapolation to 0 M TMAO. [b] 10 mM PBS pH 7.0.

1). This order of melting point difference from each control (ΔT_m) is in accord with the entropic cost of folding due to cis−trans isomerization and the loss of interchain hydrogen bonds.

The Pro-Gly secondary amide bond naturally exists almost exclusively (99.9+%) in the trans conformation, so the Pro-*trans*-Gly alkene isostere **4** did not have any effect on cis−trans isomerization (Figure 1).[22,34] This isostere does not contain the N−H of Gly, so one interchain backbone hydrogen bond was missing.[22] The Pro-*trans*-Pro isostere **2** locked one prolyl amide bond in the trans conformation and showed a 3.9 °C higher ΔT_m value than for **4** (Table 1). This difference in stability is close to the ΔT_m value of +3.5 °C for replacing a Pro residue with a Hyp in the middle of the host−guest peptide, H-(Pro-Pro-Gly)$_9$-OH, in which the trans isomer content was increased by the exo-puckering of Hyp.[35] We believe this +3.9 °C difference can be attributed to the elimination of cis−trans isomerization by locking the prolyl amide bond at the Xaa position to the trans conformation. This result indicates that locking one prolyl amide in the trans conformation has a small contribution to the overall collagen triple-helix stability. In the α1 chain of human type I collagen, there are 278 imino acid residues; thus, the cumulative effects of cis−trans isomerization may be quite significant.[36,37]

Both Gly-*trans*-Pro (**5**)[23] and Pro-*trans*-Pro (**2**) isostere peptides eliminated a cis−trans isomerization by locking one prolyl amide bond in the trans conformation with an (*E*)-alkene bond (Figure 1). Peptide isostere **5** retained all interchain

(33) Jenkins, C. L.; Bretscher, L. E.; Guzei, I. A.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 6422–6427.
(34) Scherer, G.; Kramer, M. L.; Schutkowski, M.; Reimer, U.; Fischer, G. *J. Am. Chem. Soc.* **1998**, *120*, 5568–5574.
(35) Jiravanichanun, N.; Hongo, C.; Wu, G.; Noguchi, K.; Okuyama, K.; Nishino, N.; Silva, T. *ChemBioChem* **2005**, *6*, 1184–1187.
(36) Fietzek, P. P.; Kuhn, K. *Mol. Cell. Biochem.* **1975**, *8*, 141–157.
(37) Kadler, K. *Protein Profile* **1995**, *2*, 491–619.

backbone hydrogen bonds, while peptide isostere **2** eliminated the C═O of the Xaa Pro residue that is required for a key interchain backbone hydrogen bond with the N−H of Gly in another chain. Peptide **2** was less stable than **5** by 31.9 °C, which we attribute to the missing interchain backbone hydrogen bond (Table 1).[23] Both peptides **2** and **5** were less stable than their control peptides, **3** and **6**, respectively.[23] This shows that intrinsic properties of the amide bond, including n-to-π^* interactions,[13] have a major influence on the stability of the collagen triple helix.

Jenkins et al. also studied the peptide with an ester bond replacing one Pro-Gly amide bond.[22] This ester isostere also removed one interchain backbone hydrogen bond due to the removal of a Gly N−H. The T_m value of the ester peptide was 22.1 °C lower than that of the control peptide $(Pro\text{-}Pro\text{-}Gly)_{10}$.[22] By comparison with the −57.5 °C ΔT_m value of the Pro-Gly isostere peptide **4**, the importance of the hydrogen-bonded carbonyl groups is shown to be significant. Our results confirm that the backbone interchain hydrogen bond is a major factor in stabilizing the collagen triple helix.

Conclusions

Replacing any amide within collagen triple-helix peptides with an (*E*)-alkene bond causes a significant decrease in stability.[22,23] We replaced one Pro-*trans*-Pro in a collagen peptide with an (*E*)-alkene isostere and found that it melted 53.6 °C lower than its control peptide **3**. Comparison with our previously reported Gly-*trans*-Pro isostere peptide **5** confirmed that the backbone interchain hydrogen bond between Gly N−H and Xaa C═O of the adjacent chain is one of the major forces in stabilizing the collagen triple helix.[23] Because alkene isosteres are all destabilizing, intrinsic properties of the amide bond, including n-to-π^* interactions, are also significant.[13] The Pro-*trans*-Gly isostere peptide **4** reported by Jenkins et al.[22] was 3.9 °C less stable than our Pro-*trans*-Pro isostere peptide **2**. Thus, cis−trans isomerization appears to have a small effect on triple-helix stability. Even so, the small contribution from cis−trans isomerization may be cumulatively significant, considering the large number of prolyl residues in full-length natural collagen.

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Supporting Information Available: Experimental procedures and characterization for the synthesis of **1**; NMR spectra and HPLC chromatograms for **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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