Enhanced Triple Helix Stability of Collagen Peptides with 4*R*-Aminoprolyl (Amp) Residues: Relative Roles of Electrostatic and Hydrogen Bonding Effects

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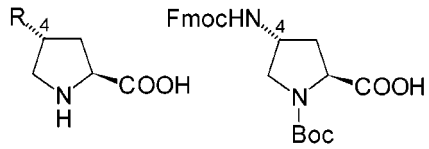
Collagen is a major structural protein found in the connective tissues of higher organisms.[1] The primary structure of collagen is composed of Gly-X-Y repeats, where X/Y are predominantly proline **1** and 4-hydroxyproline **2** (Hyp) which account for 20% of the total amino acid composition in natural collagen, the other commonly found amino acids being Ala, Lys, Arg, Leu, Val, Ser, and Thr.[2] The tertiary structure of collagen is a triple helix consisting of three extended left-handed polyproline type II chains, intertwined in a parallel fashion with one residue shift to form a right-handed superhelix.[3] The thermal stability of collagen triple helix is attributed to Hyp residues involved in the hydrogen bonded network mediated by water molecules, which connect the hydroxyl group of Hyp in one strand to the main chain amide carbonyl of another chain.[4] Substituting Pro for Hyp in the collagen chain or acetylation of the 4-OH group of Hyp significantly decreased the T_m in collagen model peptides.[4] Interestingly, collagen peptides with 4-fluoroproline **3** exhibited remarkably higher stability than peptides with Hyp residues.[5] It was explained that the stabilizing effect of the hydroxyl group of Hyp might not arise from hydrogen bonding but is a consequence of its electron withdrawing inductive effect on the proline ring conformation. The recent crystal structures reported for the triple helical peptides $(Pro.Pro.Gly)_{10}$ and $(Pro.Hyp.Gly)_{10}$ indicated that the 4-OH group of Hyp had no effect on the hydration pattern and the resulting molecular structure.[6]

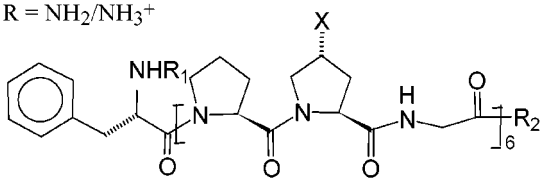
In view of these surprising findings it was thought worthwhile to study the effect of replacing the 4-OH group by another electronegative group NH_2, which unlike fluorine is also a potent hydrogen bonder. The NH_2 group is similar in size to OH and its higher basicity causes its protonation at physiological pH. Herein we report the synthesis and circular dichroic (CD) studies on a collagen model peptide analogue H-Phe.$(Pro.Amp.Gly)_6$-OH **6** containing 4*R*-NH_2-2*S*-Proline (Amp) **4** in place of Hyp **2**. It is demonstrated that the triplex formed from this analogue is more stable compared to that of unmodified collagen peptides Hyp **7** and Pro **8** and the 4-NH_2 group plays a direct role in enhancing the stability.

The naturally occurring *trans*-4-hydroxy-L-proline **2** was transformed to compound **5** via its benzylester in six steps.[7] The collagen model peptides H-Phe.$(Pro.Amp.Gly)_6$-OH **6**, H-Phe.$(Pro.Hyp.Gly)_6$-OH **7**, and H-Phe.$(Pro.Pro.Gly)_6$-OH **8** and the end-capped peptides **9** and **10** were synthesized by solid-phase synthesis and the HPLC purified peptides were characterized by MALDI-TOF mass spectrometry.[8] The amino acid Phe was included at the N-terminus to enable accurate determination of peptide concentrations by UV absorbance at 259 nm ($\epsilon = 200$ M^{-1} cm^{-1}).



1. R = H
2. R = OH
3. R = F
4. R = NH_2/NH_3^+

5



6. X = NH_2, R_1=H, R_2=OH; **7**. X = OH, R_1=H, R_2=OH;
8. X = R_1=H, R_2=OH; **9**.X = NH_2, R_1=$COCH_3$; R_2= NH_2
10. X = OH, R_1=$COCH_3$; R_2= NH_2

CD spectroscopic studies: Collagen-like structures in solution show a fingerprint CD profile consisting of a weak positive band around 215−227 nm and a large negative band around 200 nm.[9] Figure 1A,B shows CD spectra of 0.2 mM solutions of peptides **6**−**8** measured in acidic (pH 3.0, 20 mM acetate buffer) and alkaline (pH 12.0, 20 mM borate) conditions in the presence of 0.1 M NaCl. The ratio of the intensity of positive to negative band (R_{pn})[10] for **6** measured over a different concentration range indicated a critical triple-helical concentration of 0.15 mM and hence peptide **6** is fully aggregated at the concentration of 0.2 mM used in all experiments. At both pH values, the peptides Amp **6** and Hyp **7** show collagen-type spectra, unlike that of Pro peptide **8**. Figure 1C,D shows temperature-dependent CD data (ellipticity at 225 nm) of the three peptides at pH values of 3 and 12 and the derived helical thermal stability (T_m) values are given in Table 1. At all pH values, both peptides Amp **6** and Hyp **7** show sigmoidal transitions indicative of cooperative melting, unlike Pro **8** that shows a linear decrease in ellipticity. The T_m of peptides **6** and **7** decreases with an increase in pH over the range 3.0−9.0, followed by increase again at pH 12.0, as seen in collagen peptides.[9,11]

Significantly, the triplex stability of Amp peptide **6** is always more than that of Hyp peptide **7** in the entire pH range ($\Delta T_m \sim$ 19 °C, pH 3.0; 10 °C, pH 7.0; 3 °C, pH 9.0, and 17 °C, pH 12.0). This suggests that the 4-NH_2 group (pK_a 10.5) in both protonated and unprotonated forms has a positive role in stabilizing the structure of collagen peptide **6**. The higher triplex stabilities at either extremes of pH (3.0 and 12.0) compared to that at intermediate pH (7.0 and 9.0) arise from the N- and C-terminal effects.[9] At extremes of pH, only one of the terminal groups is completely ionized (NH_3^+/COOH at pH 3.0 and COO^-/-NH_2 at

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(8) Phe.$(Pro.Amp.Gly)_6$ **6**: M_{obsd} = 1758.1, M_{calc} = 1761.9; Phe$(Pro.Hyp.Gly)_6$ **7**: M_{obsd} = 1768.1, M_{calcd} = 1768.9; Phe.$(Pro.Pro.Gly)_6$ **8**: M_{obsd} = 1672.8, M_{calcd} = 1672.9. For all experimental details see the Supporting Information.
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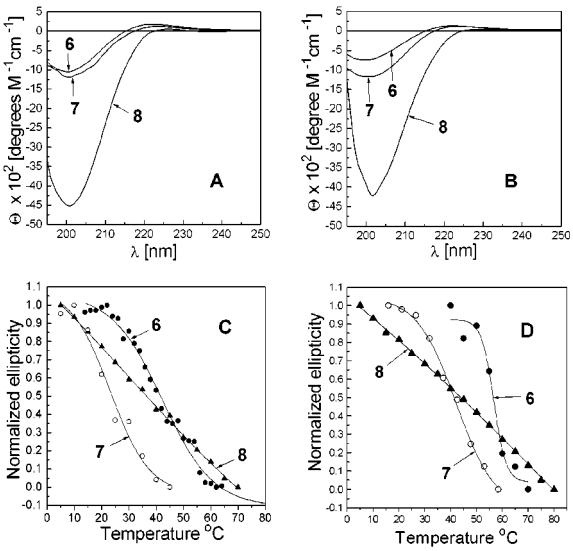

Figure 1. (A, B) CD spectra and (C, D) thermal denaturation profiles with normalized ellipticity $[(\theta - \theta_{min})/(\theta_{max} - \theta_{min})$, where θ_{max} and θ_{min} are maximum and minimum values in each data set] for peptides **6**−**8** (0.2 mM), monitored at 225 nm. Conditions: (A, C) pH 3.0 and (B, D) pH 12.0, both with 0.1 M NaCl.

Table 1. CD−T_m Data for Collagen Peptides[a]

	6	**7**	**9**	**10**
pH 3.0	42.5	23.0	60	27
pH 7.0	31.3	21.6	56.5	28
pH 9.0	18.5	15.5	26	27
pH 12.0	56.6	39.6	49	27

[a] pH 3.0, 20 mM acetate; pH 7.0, 20 mM phosphate; pH 9.0 and 12.0, 20 mM borate buffers, all with 0.1 M NaCl. T_m values are ±0.5 °C.

pH 12.0), while at intermediate pH values both groups are ionized to varying degrees. The presence of similar charged residues at termini in a parallel triplex leads to its destabilization, which is maximal when both termini are ionized as at pH 9.0. The terminal effects may be nulled by end capping, which also stabilizes the triplexes significantly.[10]

The contributions from terminal effects to triplex stability may relatively differ in Amp **6** and Hyp **7** peptides due to the presence of additional ionizable 4-NH_2 groups (pK_a 10.5) in **6**. To delineate this factor, the triplex stabilities of the corresponding end-capped peptides **9** and **10** were measured at different pH values (Table 1). In the pH range 3.0−9.0, the end capping led to a remarkable enhancement in T_m of Amp peptide **9** over that of uncapped **6** ($\Delta T_m \sim 18°$, pH 3.0; 25°, pH 7.0; 8 °C, pH 9.0). In contrast, the effects were much lower for the capped Hyp peptide **7** ((ΔT_m, 4−12°). The T_m values of both capped peptides **9** and **10** at pH 12.0 are lower than those of the corresponding uncapped peptides **6** and **7**. The T_m of Hyp **10** was invariant with pH unlike that of the capped Amp **9**. This fact along with a very large difference in T_m values of capped Amp **9** and Hyp **10** peptides (**9** > **10**, $\Delta T_m \sim 33°$) implicates the ionizable 4-NH_2 groups in Amp **6** and **9** to play a direct role in stabiliztion via electrostatic interactions, hydrogen bonding, or a combination of both.

The T_m values measured at pH 7.0 for Amp peptide **6** in the presence of increasing concentrations of NaCl (0.1 M, 31.3 °C; 0.6 M, 35 °C; 1 M, 44 °C; 2 M, 37.3 °C) indicate a steady increase up to 1 M, and a slight decrease beyond. As compared to that of Hyp peptide **7** under similar conditions (0.1 M, 21.6 °C; 0.6 M, 15.3 °C; 1 M, 33.6 °C; 2 M, 27.4 °C), the T_m of **6** was uniformly higher. This stabilization by NaCl arises from the screening of interstrand electrostatic repulsion caused by terminal charged amino or carboxyl groups by counterions[9,12] and should be similar in both **6** and **7**. However**,** the observed larger T_m effects in **6** suggest an additional electrostatic screening effect on 4-NH_3^+ groups by the salt anion. Beyond 2 M NaCl, breaking of the hydrogen bond network and water structure may lead to a lowering of T_m. Ethylene glycol stabilizes collagen triple helices and is useful for detecting weak triple helical propensities.[13] In EG:H_2O (3:1), the CD T_m for Amp peptide **6** (T_m = 23 °C) is 8° lower compared to that of Hyp peptide **7** (T_m = 31 °C). This reversal of stability in EG:H_2O arises from interstrand electrostatic repulsions of 4-NH_3^+ groups, which remain unscreened in the absence of salt.

In conclusion, it is shown that the replacement of 4-OH prolyl residues in collagen peptides by 4-NH_2 proline leads to a significant stabilization of the derived triple helices. The observed pH and salt effects in capped and uncapped peptides suggest different mechanisms to be responsible for enhancing the triplex stability at different conditions. The higher stability at lower pH could arise from increased electronegativity and the hydrogen bonding potential of the protonated amine moiety. At higher pH, the stabilizing effect may be a consequence of hydrogen bonding and the absence of electrostatic repulsion in nonprotonated 4-NH_2 groups. The results have a direct bearing on the current interest in collagen structure[5] and mimetics.[14] Since the 4-OH group in natural collagen can be potentially transformed chemically to 4-NH_2, the properties of this analogue may have significance in the design of new collagen-based biomaterials,[15] including chimeric collagens. Future studies are directed toward synthesis of such collagen hybrids and structural characterization of triplexes using NMR.

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Supporting Information Available: ^1H, ^{13}C NMR spectra and FAB-MS of **5**; synthesis of **6**−**10**; MALDI-TOF MS of **6**−**8**; HPLC of **6** and **9**; CD spectra and melting profiles of (A) **6**−**8** at pH values of 7.0 and 9.0 and in EG:H_2O and (B) **9** and **10** at pH values of 3.0, 9.0, and 12.0; pH titration of 4*R*-NH_2-N^1-Boc-proline; and R_{pn} vs concentrated plot of **6** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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