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# Two prolines with a difference: contrasting stereoelectronic effects of 4*R*/*S*-aminoproline on triplex stability in collagen peptides [Pro(X)-Pro(Y)-Gly]$_n$†

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***Received (in Cambridge, UK) 24th July 2003, Accepted 28th August 2003***
***First published as an Advance Article on the web 17th September 2003***

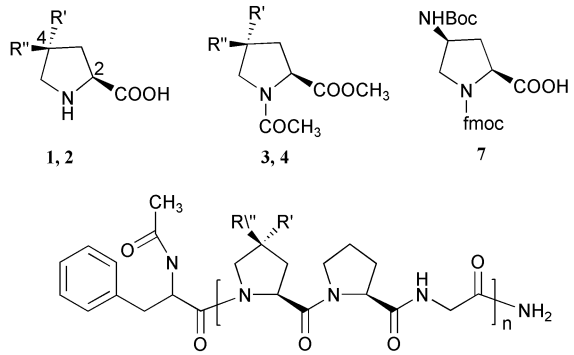
**4*R*/*S*-Aminoprolines when present in the X-position of collagen peptide [pro(X)-pro(Y)-Gly]$_n$ exhibit pH- and stereochemistry-dependent effects on triplex stability.**

Collagen is the most abundant structural protein in mammals and its mechanical properties are related to the high thermal stability of its triple helical structure.[1] The primary structure of collagen is characterized by a repeating X–Y–Gly triplet motif. Proline is the most abundant residue in the triple helix, with the Y-position mostly occupied by *trans*-4*R*-hydroxyproline. While this amino acid causes remarkable collagen stabilization when present in the Y position, polypeptides with this moiety at the X-position , Pro(OH)-Pro-Glycine, do not form a triplex in aqueous solution.[2] Differing conformational preferences for the two prolines at the X and Y positions are thought to play a key role in imparting unusual stability to collagen triple helices[3] and understanding the conformation–stability interplay is presently an area of active research. It is now emerging that the conformational preferences of the two prolines are strongly dictated by the nature of the 4-substituent.[4] Others[5] and we[6] have respectively demonstrated that 4*R*-F and 4*R*-$NH_2$/$NH_3^+$ (**1**) substituted prolines in the Y postion show improved triplex properties of the derived collagen peptides, but the true molecular origin of the stability is yet to be fully deciphered. Fluorine at the 4-position has been shown to contribute to stability by its strong electronegativity and the concomitant influence on pyrrolidene ring conformation.[5b] The situation is not that clear for the stability offered by 4-$NH_2$/$NH_3^+$ functions, where the conformational control may be exercised by a combination of hydrogen bonding, electronegativity and long range interstrand electrostatic contributions from the protonated $NH_3^+$ groups.

To decipher the influence of these factors, we analyzed the dependence of pyrrolidene ring conformation on the stereochemistry of 4-$NH_2$/$NH_3^+$ by vicinal $^1$H–$^1$H-coupling constants in $^1$H-NMR of 4*R*-*trans* (**1**) and 4*S*-*cis* (**2**) aminoprolines. Analysis, done according to previously established methods,[7] indicated that 4*R*-*trans*-aminoproline **1** prefers a C4-*exo* pucker (Fig. 1) for the pyrrolidene ring as in 4*R*-*trans*-hydroxyproline, whereas the preferred conformation for 4*S*-*cis*-aminoproline **2** is C4-*endo*. Although for proline with no C4 substituent, these two puckers are isoenergetic, theoretical calculations indicate that C4-*exo* is not favourably accommodated in the X-position.[3,4] The preferred C4-*endo* conformation for 4*S*-*cis*-aminoproline **4** prompted us to examine its compatibility at the X-position in collagen peptide **6** and compare the stability of the derived triplex with that of the analogous triplex from 4*R*-aminoproline collagen peptide **5**.

The N1-Fmoc-N$^4$-Boc protected 4*S*-aminoproline **7** was synthesized from 4*R*-hydroxyproline in eight steps by sequential protection/deprotection strategies (ESI).† This was then incorporated by solid phase synthesis on Rink resin into the collagen model peptide Phe(X-Pro-Gly)$_6$ at the X-position, followed by capping of the N-terminal residue. TFA cleavage of the product directly yielded the N-acetylated C-terminal amidated peptide **6**. Similarly, the collagen peptide **5**, having 4*R*-aminoproline at the X position, was synthesized from 4*R*-aminoproline. The peptides **5** and **6** were purified on a semi-preparative RP-C8 HPLC column and characterized by MALDI-TOF mass spectrometry.[8] The amino acid Phe included at the N-terminus enabled accurate determination of the peptide concentrations by UV absorbance at 259 nm ($\varepsilon$ = 200 $M^{-1}$ $cm^{-1}$). The N,C-end capped peptides **5** and **6** were used for biophysical studies in order to eliminate the effects arising from charge–charge repulsion in terminally ionized forms, a phenomenon well established in collagen peptides.

Collagen-like triple helical structures in solution exhibit fingerprint CD spectra with a characteristically large negative band around 200 nm, a crossover at around 213 nm, and a small positive band around 215–227 nm.[9] The ratio of the intensiy of the positive to the negative band ($R_{pn}$), 0.07–0.18, is an established criterion for triplex formation.[10] All CD spectra were recorded at 0.2 mM concentration which is higher than the critical triple helical concentration seen in these systems. Fig. 2 shows the CD spectra of peptides **5** and **6** measured at different pHs. Based on the $R_{pn}$ criterion, 4*S*-Amp-peptide **6** forms a triplex at pHs 3, 7 and 9, while 4*R*-Amp-peptide **5** forms a triplex only at pHs 3 and 7 and at pH 12.0 neither form triplexes. The formation or non-formation of triplexes was further




For **1**, **3** and **5**, R' = $NH_2$, R" = H (4*R*, Amp)
For **2**, **4** and **6**, R' = H, R" = $NH_2$ (4*S*, amp)

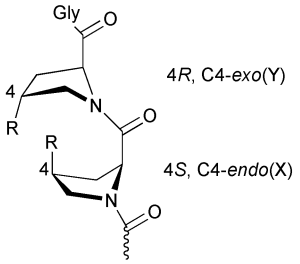



**Fig. 1** Position dependent preferred proline puckers in collagen.

† Electronic supplementary information (ESI) available: $^1$H NMR spectra and coupling constant analysis of **3** and **4**; synthetic schemes for **3**, **4**, **5** and **6**; FABMS of **3** and **4**; MALDI-TOF MS of **5** and **6**; HPLC of peptides **5** and **6**. See http://www.rsc.org/suppdata/cc/b3/b308581c/

DOI: 10.1039/b308581c

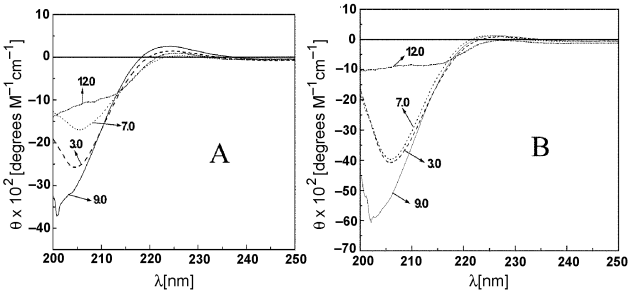



**Fig. 2** CD spectra of X-peptides at different pHs. A, **5**; B, **6**.

confirmed by CD spectra recorded as a function of temperature at different pHs as shown in Fig. 3. The observed sigmoidal transitions (Fig. 3 A and B) confirmed the presence of triplexes while the non-sigmoidal transitions indicated non-formation of triplexes (Fig. 3C for **5** and D). The CD-triplex melting temperatures obtained from the corresponding derivative curves are shown in Table 1.

The thermal stability of 4*S*-Amp X-peptide **6** at pH 3.0 and 7.0 was higher compared to that of 4*R*-Amp X-peptide **5** ($\Delta T_m$, 5–8 °C). At pH 9.0, only the 4*S*-amp X-peptide formed a triplex and at pH 12.0 both X-peptides failed to show any triplex. This is in contrast to the 4*R*-Amp Y-peptide **8** that formed a triplex at all pH ranges. The triplex from X-peptides **5** and **6** were however of lower stability than that of the 4*R*-Amp-Y-peptide **8** at pH 3 and 7, while 4*S*-Amp X-peptide **3** was better than **8** at pH 9.0. The p$K_a$ of the 4-$NH_2$ group is 10.5 and these results suggest that protonation of the 4-amino group seems to be essential for triplex formation of X-peptides. In ethylene glycol, which stabilizes triple helices through hydrogen bonding interactions,[11] both X-peptides exhibited significantly higher stability ($\Delta T_m$ 12–16 °C) than the Y-peptide **8**.

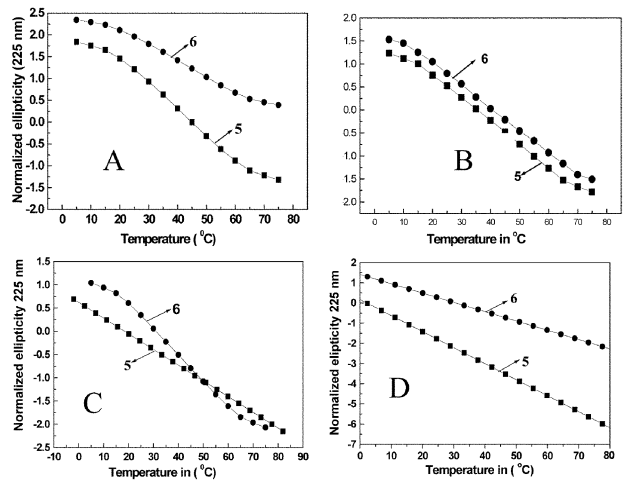



**Fig. 3** CD thermal denaturation profiles, with normalized ellipticity for peptides **5** and **6** monitored at 225 nm. A, pH 3.0; B, pH 7.0; C, pH 9.0; D, pH 12.0. For buffer conditions, see Table 1, footnote a.

**Table 1** CD–$T_m$ data for collagen peptides[a]

| | pH | 5 | 6 | 8[b] |
|---|---|---|---|---|
| 1 | 3.0 | 36 (0.08) | 44 (0.12) | 60 (0.17) |
| 2 | 7.0 | 33 (0.08) | 37 (0.08) | 56.3 (0.15) |
| 3 | 9.0 | ND | 34 (0.08) | 26 (0.09) |
| 4 | 12.0 | ND | ND | 49 (0.17) |
| 5 | EG : W (3 : 1) | 35 | 39 | 23 |

[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; [b] Data from ref. 6, values in parentheses indicate $R_{pn}$ values.

The results presented here demonstrate that the 4*R*/*S*-aminoprolines are one of the first proline derivatives that stabilize the collagen triplex when present in the X-position and the 4*S*-Amp is better than 4*R*-Amp. The cause of the stabilization is the C4-*endo* pyrrolidene conformation adopted by 4*S*-Amp that is inherently favoured at the X-position. The pH dependent stabilities in both 4*R* and 4*S* aminoprolines also suggest that protonation of $NH_2$ is a prerequisite for triplex formation in the X-position, while it is not so in the Y-position. It is possible that the conformation of pyrrolidine ring may also depend on the protonation status of the 4-amino group and influence the *cis–trans* amide rotameric equilibrium.[3a] The results reinforce the important role of stereoelectronic effects, well elucidated in 4-fluoroprolines,[12] also to be determinants of stability of 4-aminoproline collagen. Future potential of this work lies in rationally combining the stabilities offered by the two diastereomers to design collagens (*e.g.* Amp-Hyp/Amp-Gly)$_n$ or chimeric collagens of unusual stability for applications in collagen based biomaterials.[13] The results also have implications for peptidomimetic designs and the control of peptide conformations through 4-substituent effects on proline.

M. U. and I. R. B. thank CSIR, New Delhi, for Research fellowships.

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8 Ac-Phe(Amp-Pro-Gly)$_6$-$NH_2$ **5**: $C_{83}H_{122}N_{26}O_{20}$, $M_{calc}$ = 1084, $M_{obs}$ = 1085 (M + H); Ac-Phe(amp-Pro-Gly)$_6$-$NH_2$ **6**: $C_{83}H_{122}N_{26}O_{20}$, $M_{calc}$ = 1084, $M_{obs}$ = 1086 (M + 2H) and 1089 (M + 5H+).

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