Unexpected Puckering of Hydroxyproline in the Guest Triplets, Hyp-Pro-Gly and Pro-alloHyp-Gly Sandwiched between Pro-Pro-Gly Sequence

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One of the major aims of studying collagen structure is to understand stabilization factors for triple-helical structures. Despite the efforts spent, the results do not provide an unambiguous answer. One obstruction is the fibrous nature of native collagen, and consequently model peptides have been used to study and define the features of the collagen triple helix. In the early stages, homopeptides with repeating sequences of a given triplet unit were studied,[1] but recently host–guest peptides,[2] in which the central region of a stable host peptide was substituted by a particular guest triplet, have been examined to investigate the physical properties of the guest sequence. In this study, $(\text{Pro-Pro-Gly})_9$, one of the best studied triple-helix peptides,[3] serves as the reference host for guest tripeptide units with Hyp and allo-Hyp, to provide new structural data on the effect of environment on the puckering of hydroxyproline.

Collagen is a major structural protein in the extracellular matrix of skin, tendons, bones, and other connective tissues. The presence of glycine as every third residue and a high content of imino acids are characteristics of collagen sequences, and its sequence may be designated as the repetition of X-Y-Gly, in which X and Y are often occupied by proline (Pro) and 4*R*-hydroxyproline (Hyp), respectively. These restrictive sequence features enable the assembly of three chains into a stable triple-helical conformation. The presence of hydroxyproline leads to significant enhancement of the thermal stability of collagen.[1a,4] The mechanism of this additional stability has

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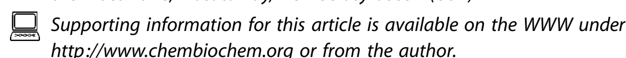
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been elucidated by calorimetry, stability measurements, and recent single-crystal analyses of synthetic model peptides. Initially, the additional stability was attributed to water-mediated hydrogen bonding involving Hyp residues.[2a,5] However, this hypothesis was weakened by the finding that the substitution of Hyp residues at the Y position by 4*R*-fluoroproline (Flp) residues increases the thermal stability of triple helices despite the low tendency of Flp substituents to participate in hydrogen bonding. The Flp studies led to an alternative hypothesis based on inductive effects[6] by the hydroxyl group, which favor the *gauche* effect and a *trans* peptide bond conformation in the *cis*–*trans* equilibrium. However, neither the hydration hypothesis nor the inductive hypothesis could explain the destabilization of Hyp at the X position shown by the fact that (Hyp-Pro-Gly)$_{10}$ could not form a triple helix, while (Pro-Pro-Gly)$_{10}$ and (Pro-Hyp-Gly)$_{10}$ did so.[7]

To explain this experimental evidence, the propensity-based hypothesis[8] was proposed. Proline residues can adopt two ring puckering conformations, up (C_γ-*exo*) or down (C_γ-*endo*) (Figure 1). Up- and down-puckering can be defined by negative and positive values of the side-chain dihedral angles χ_1 (N–C_α–C_β–C_γ), respectively. According to this hypothesis, there is an intrinsic preference of the proline ring for down-puckering at the X position and for up-puckering at the Y positions due to the geometrical features of the triple helix. On the other hand, based on the crystallographic and statistical analyses of small peptides and globular proteins, Pro residues can adopt both up- and down-puckering, while Hyp residues distinctly adopt only up-puckering. Residues of the non-natural isomer 4*S*-Hyp (alloHyp) are seen to adopt down-puckering. Therefore, the destabilization of the triple helix by Hyp at the X position is suggested to result from the conformational strain of having an “up-favored” Hyp present in an intrinsically “down-favored” X site. A similar explanation applies to the unfavorable nature of alloHyp at the Y position. However, it was found that alloHyp at the X position is also destabilizing.[1a] This was explained recently by steric clashes between the hydroxyl groups and the proline ring of the adjacent chain.[8a] On the other hand, 4*S*-fluoroproline (4*S*-Flp) present at the X position in the sequence (4*S*-Flp-Pro-Gly) formed a stable triple helix; this contradicted the above hypothesis.[9] We also recently found several cases of inconsistent puckering at the X position of (Pro-Hyp-Gly)$_n$ peptides[10] and at the Y position of (Pro-Pro-Gly)$_n$ peptides.[3] Moreover, peptides with a repeating sequence of Hyp-Thr-Gly showed higher stability than those of Pro-Thr-Gly,[11] and (Hyp-Hyp-Gly)$_{10}$[12a] and (Gly-Hyp-Hyp)$_9$[12b] peptides formed stable triple helices,[12] in spite of the inability of (Hyp-Pro-Gly)$_{10}$ to form a triple helix. These experimental inconsistencies led to a reconsideration of the propensity-based hypothesis.

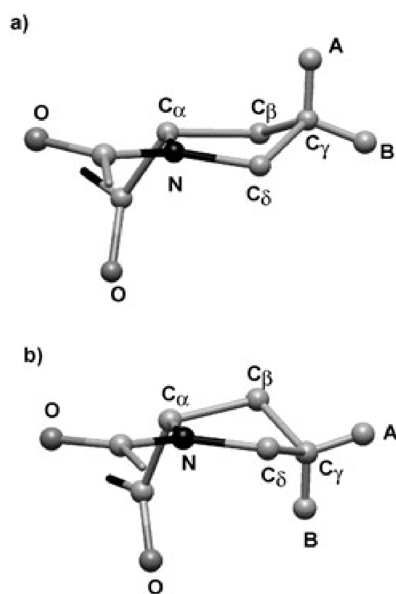

Figure 1. Illustration of proline ring conformation. a) Up-puckering and b) down-puckering were defined by negative and positive values of χ_1 (N–C_α–C_β–C_γ), respectively. Pro: A = B = H, Hyp: A = OH, B = H, and alloHyp: A = H, B = OH.

Since Hyp-Pro-Gly and Pro-alloHyp-Gly will not form stable triple helices in a repeating sequence such as (Hyp-Pro-Gly)$_{10}$[7] and (Pro-alloHyp-Gly)$_{10}$,[1a] we inserted the Hyp-Pro-Gly and Pro-alloHyp-Gly sequences as guests into a stable, host (Pro-Pro-Gly)$_9$ sequence. These host–guest peptides did form stable triple helices, and the crystal structures of (Pro-Pro-Gly)$_4$-(Hyp-Pro-Gly)-(Pro-Pro-Gly)$_4$ (denoted OPG), (Pro-Pro-Gly)$_4$-(Pro-alloHyp-Gly)-(Pro-Pro-Gly)$_4$ (denoted PaOG), and (Pro-Pro-Gly)$_4$-(Pro-Hyp-Gly)-(Pro-Pro-Gly)$_4$ (denoted POG) as well as the reference crystal structure of (Pro-Pro-Gly)$_9$ (denoted PPG9) were analyzed.

The stabilizing nature of POG and the destabilizing natures of the OPG and PaOG tripeptide units were seen in the CD thermal transition curves of the host–guest peptides. The obtained T_m values of OPG and PaOG are 2–2.2 °C lower than that of PPG9, while that of POG is 3.5 °C higher than that of PPG9 (Table 1). This result confirmed the destabilization of Hyp at the X position and alloHyp at the Y position. Although the effect of one guest triplet in a host–guest system is expected to be much less than that of 10 units in a homopeptide environment, the relative order is the same in host–guest peptides (POG > PPG9 > PaOG = OPG). Given that one OPG or one PaOG unit decreases stability by 2–2.2 °C relative to PPG9, one might predict that 10 units would be at least 20–22 °C less stable than (Pro-Pro-Gly)$_{10}$ (T_m = 32.6 °C). Therefore, this estimation of T_m is consistent with the lack of formation of a stable triple

Table 1. Helix–coil transition temperatures [T_m] of host–guest peptides (Pro-Pro-Gly)$_4$-X-Y-Gly-(Pro-Pro-Gly)$_4$ and homopeptides (X-Y-Gly)$_{10}$.

X-Y-Gly	T_m [°C] Host–guest peptide	(X-Y-Gly)$_{10}$
PPG	17.7	32.6[13]
POG	21.2	60.0[13]
OPG	15.5	no helix formation[7]
PaOG	15.7	no helix formation[1a]

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helix by $(OPG)_{10}$ and $(PaOG)_{10}$. Although the propensity-based hypothesis stated that the lack of helix formation of $(OPG)_{10}$[7] and $(PaOG)_{10}$[1a] is a result of unfavorable Hyp and alloHyp conformations (Hyp does not prefer up-puckering at the X position, and alloHyp does not prefer down-puckering at the Y position), it is not known whether Hyp and alloHyp conformations adopt down- or up-puckering in the above-cited host–guest peptides and homopeptides. Here, we have performed the first visualization of these unique triplet conformations. Although, the lateral packings of these three host–guest peptides were very similar to that of PPG9, structure analyses provided interesting results about proline-ring puckering in the guest triplet. Namely, 1) Hyp in OPG peptide showed down-puckering at the X position, which had an unfavorable conformation for Hyp, 2) alloHyp in PaOG peptide showed up-puckering at the Y position; this conflicted with the normal conformation for alloHyp, 3) Hyp in POG peptide showed up-puckering at the Y position as usual. These entire proline-ring conformations were confirmed by omit maps, as shown in Figure 2.

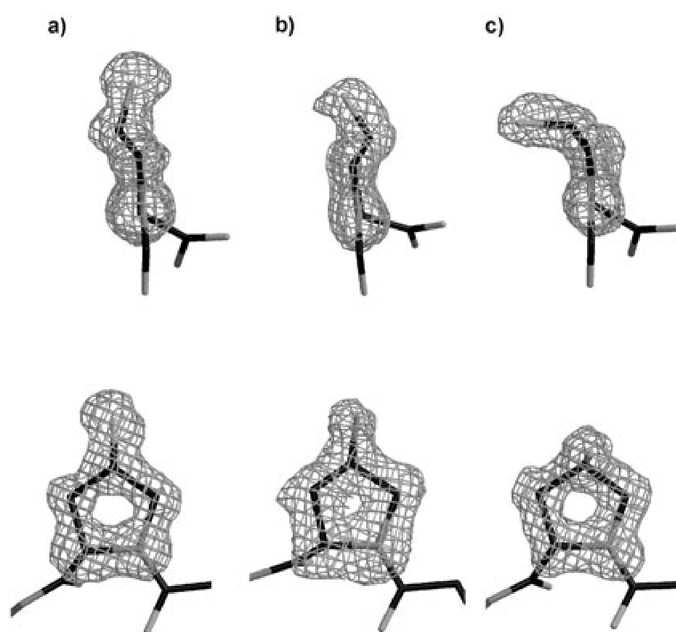

Figure 2. Omit map (F_o-F_c) contoured at 3σ of Hyp in three sequences. a) AlloHyp at the Y position in PaOG showed up-puckering. b) Hyp at the X position in OPG showed down-puckering. c) Hyp at the Y position in POG showed up-puckering.

Hyp puckering at the X position was demonstrated to be in the down conformation in the Hyp-Pro-Gly sequence, in accordance with the propensity-based hypothesis. Likewise, alloHyp at the Y position in the Pro-alloHyp-Gly sequence was proven to adopt the up-puckering, thus also supporting the propensity-based hypothesis. Therefore, main reason for the destabilization of OPG and PaOG could be explained by the less favorable down-puckering of Hyp and the less favorable up-puckering of alloHyp. These unfavorable ring puckerings are consistent with the decreased T_m values of OPG and PaOG compared with PPG9.

Here, the structures of triplets that tend to destabilize the triple-helical conformation were visualized by using crystallography and thermal-stability studies on designed host–guest peptides. Hyp and alloHyp, which typically adopted only up- and down-puckering, respectively, unexpectedly adopted opposing puckering in the above host–guest peptides: Hyp adopted down-puckering at the X position and alloHyp adopted up-puckering at the Y position. The results showed that the pucker of hydroxyproline depends on positional preference, the diastereoisomer form, and the environment. The puckering preference is seen to effect triple-helix stability.

Experimental Section

Peptide synthesis: The peptides PPG9, POG, OPG, and PaOG were synthesized by a solid-phase method. The details of syntheses will be reported elsewhere.[3a] The crude product was purified by Sephadex G-50 (40% acetic acid), and corresponding fractions were collected, concentrated, and lyophilized. The purity and identity of the peptides for CD measurement were confirmed by using reversed-phase analytical high-pressure liquid chromatography (C-18 column) and by MALDI-TOF mass spectrometry.

Crystallography: The peptides were crystallized by the hanging drop vapor diffusion method.[3a] X-ray data collection of these crystals was performed at the BL40B2 beamline of the SPring-8 synchrotron at 100 K. The diffraction patterns showed almost similar strong reflections on the layer lines corresponding to a helical repeat of 20 Å, together with weak reflections on the layer lines corresponding to 80 Å. The diffraction images were indexed and integrated by using CrystalClear and HKL2000.[14] Details of data collection statistics are reported in the Supporting Information. In the molecular-replacement structure analyses, positional refinement was performed by X-PLOR,[15] and the structure refinement was carried by using SHELX-L[16] with the previously determined triple-helical structures as a starting model. An anisotropic treatment of the atomic-displacement parameters was used for non-hydrogen atoms in the peptides. Five percent of the reflections were used for R_{free} monitoring. Peaks in the F_o-F_c maps were identified as potential water sites by using distance cut-off criteria and hydrogen-bonding geometry. Only water molecules with improving R and R_{free} were retained. Data-collection parameters and refinement statistics are reported in the Supporting Information.

Circular dichroism measurements: CD spectra were recorded on an Aviv model 202–01 spectrometer with a Peltier thermoelectric temperature controller. Samples were prepared at a concentration of 1.0 mg mL^{-1}, with the peptides dried in vacuo over P_2O_5 for 48 h prior to weighing. Final concentrations were determined by the absorption at 214 nm (assuming $\in = 2.2\times10^3$). Peptides in NaCl (0.15 M), sodium phosphate (0.01 M), pH 7.0, were equilibrated at 0 °C for more than 48 h prior to analysis. Wavelength scans were collected from 210 to 260 nm at 0 °C. For melting transitions, the ellipticity at 225 nm was monitored at an average rate of 0.1 °C min^{-1}. The T_m value was taken as the temperature at which the fraction folded is equal to 0.5.

PaOG ($(Pro\text{-}Pro\text{-}Gly)_4$-Pro-alloHyp-Gly-$(Pro\text{-}Pro\text{-}Gly)_4$) has been registered at the Protein Data Bank with reference 1X1K.

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