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Triple-Helix Propensity of Hydroxyproline and Fluoroproline: Comparison of Host−Guest and Repeating Tripeptide Collagen Models

Anton V. Persikov,[‡] John A. M. Ramshaw,[§] Alan Kirkpatrick,[§] and Barbara Brodsky*,[‡]

Department of Biochemistry, UMDNJ - Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, New Jersey 08854, and CSIRO, 343 Royal Parade, Parkville, Australia

Received June 13, 2003; E-mail: brodsky@umdnj.edu

Imino acids are critical for the conformational stability of the collagen triple-helix. The close packing of three supercoiled chains in the triple-helix generates the requirement for a $(Xaa\text{-}Yaa\text{-}Gly)_n$ repeating sequence, while a high content of imino acids is necessary to stabilize the extended polyproline II-like structure of the individual chains.[1] Pro is the most common occupant of the X-position, while the Y-position is frequently occupied by 4R-hydroxyproline (Hyp), which arises from posttranslational modification of Pro. Peptide models have proved important in defining the structural features of the collagen triple-helix. The earliest models are based on multiple repeats of a given tripeptide unit,[2] while more recent host−guest models include an individual tripeptide unit substituted within a constant repeating framework.[3] In the present study, we report unexpected differences between the effect of imino acids in these two types of model systems.

The sequence Pro-Hyp-Gly is the most common tripeptide unit in collagens, and peptides with repeating Pro-Hyp-Gly units, e.g. $(Pro\text{-}Hyp\text{-}Gly)_{10}$, have served as models for the triple-helix conformation by X-ray diffraction,[4] NMR,[5] and thermodynamic studies.[2a,6] Early evidence for an additional stabilizing effect of Hyp in the Y position came from the greater stability of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ compared with $(Pro\text{-}Pro\text{-}Gly)_{10}$.[2a,6] The mechanism of Hyp stabilization has proved controversial. No direct interaction of the hydroxyl group of Hyp is possible within the triple-helix molecule. It was suggested a water-mediated network involving Hyp contributes to its enthalpic stabilization,[7] and more recently, evidence of an electron-inductive effect has been reported.[2c,8] High-resolution X-ray crystallography indicates imino acids in the X-position adopt the *endo* ring pucker, while the Y-position favors the *exo* pucker.[4d] Hyp alone or in proteins favors the *exo* pucker as a consequence of its electron withdrawing effect,[8] providing additional stability when it is found in the Y-positions in collagen.[4d] This helps explain the position dependence for Hyp stabilization as shown by the inability of the peptide $(Hyp\text{-}Pro\text{-}Gly)_{10}$ to form stable triple-helices.[9] Raines et al. showed that the electron-withdrawing effect of F in fluoroproline promotes the gauche (*exo*) ring pucker,[8] consistent with the very high stability of peptide $(Pro\text{-}Flp\text{-}Gly)_{10}$ (where Flp is 4(*R*)-fluoroproline), which is even more stable than $(Pro\text{-}Hyp\text{-}Gly)_{10}$.

While repeating tripeptides are uniquely useful as models for collagen, the effects of individual Xaa-Yaa-Gly sequences have also been successfully investigated using a host−guest system of the form $Ac\text{-}Gly\text{-}(Pro\text{-}Hyp\text{-}Gly)_3\text{-}Xaa\text{-}Yaa\text{-}Gly\text{-}(Pro\text{-}Hyp\text{-}Gly)_4\text{-}Gly\text{-}NH_2$.[3] The propensities of the 20 common amino acid residues in the X- and Y-positions were determined, and all formed stable triple-helices, with a 25 °C range of stabilities. The stabilizing effect and mobility of a given residue in the X-position was not equivalent to the Y-position, consistent with their different environments within the triple-helix.[10] In the present study, Pro, Hyp, and Flp residues were incorporated in X- or Y-positions of a guest triplet in the host−guest peptide design, to compare with repeating tripeptides.[11] These host−guest peptides formed stable triple-helices at low temperatures, as shown by their characteristic circular dichroism (CD) spectrum, with a maximum near 225 nm and a minimum near 198 nm[12] (Figure 1). Measurement of CD thermal transitions at pH 7.0 in phosphate-buffered saline (PBS) indicated that Pro-Hyp-Gly,[13] as well as Hyp-Hyp-Gly showed the highest T_m values, followed by Pro-Pro-Gly, Pro-Flp-Gly, and Hyp-Pro-Gly (Figure 2). Even though a triple-helix cannot be formed by $(Hyp\text{-}Pro\text{-}Gly)_{10}$,[9] a single Hyp-Pro-Gly unit can be incorporated into a collagen conformation with only a small amount of destabilization. The lack of triple-helix formation by $(Hyp\text{-}Pro\text{-}Gly)_{10}$ has been attributed to the unfavorable placement of the *exo* puckered Hyp in the X-position, although it is not known whether the Hyp ring remains *exo* puckered in the host−guest environment.

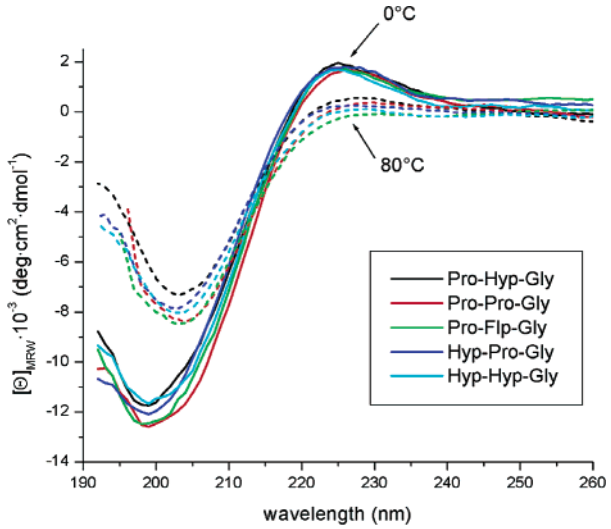

Figure 1. CD spectra of host−guest peptides at 0 °C (solid line) and 80 °C (dashed line).

The changes in thermal stability are very small (<5 °C) among the five host−guest peptides compared to the large changes (>90 °C) seen among $(Pro\text{-}Hyp\text{-}Gly)_{10}$, $(Pro\text{-}Pro\text{-}Gly)_{10}$, $(Hyp\text{-}Pro\text{-}Gly)_{10}$, and $(Pro\text{-}Flp\text{-}Gly)_{10}$ (Figure 2, Table 1). The effect of one Xaa-Yaa-Gly tripeptide in the host−guest system is expected to be much less than in a $(Xaa\text{-}Yaa\text{-}Gly)_{10}$ context, consistent with the small changes observed. If stabilization or destabilization of the triple-helix occurs by the same mechanism, the host−guest T_m values are anticipated to follow the relative order of repeating peptides, but with a decreased magnitude. The simplest model predicts the stability of $(Xaa\text{-}Yaa\text{-}Gly)_{10}$ would differ from $(Pro\text{-}Hyp\text{-}Gly)_{10}$ by 10 times the difference between Xaa-Yaa-Gly and Pro-Hyp-Gly in host−guest peptides (Table 1, T_m^{pred} repeating). The relative order

[‡] UMDNJ - Robert Wood Johnson Medical School.
[§] CSIRO.

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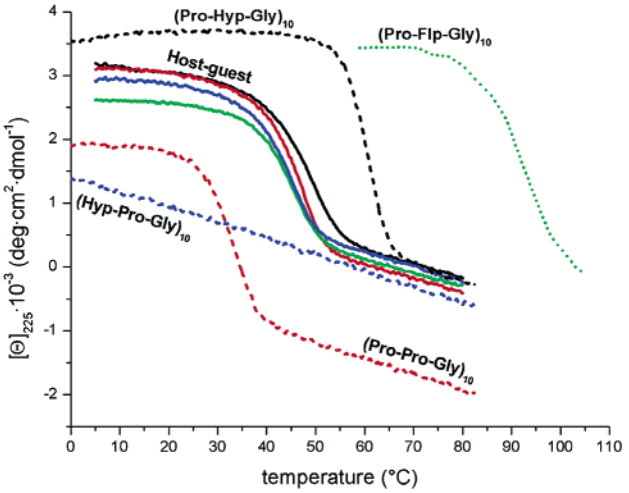

Figure 2. CD thermal transition curves for host−guest peptides (solid lines: Pro-Hyp-Gly, black; Pro-Pro-Gly, red; Pro-Flp-Gly, green; Hyp-Pro-Gly, blue) and repeating tripeptides (dashed lines). The curve for (Pro-Flp-Gly)$_{10}$ (dotted line) was taken from Holmgren et al.[8a]

Table 1. Thermal Stabilities and Calorimetric Enthalpies for Host−Guest Peptide Series and (Xaa-Yaa-Gly)$_{10}$ Repeating Tripeptides

triplet	T_m host−guest, °C	ΔH^{cal} host−guest, kJ/mol	T_m^{pred} repeating, °C	T_m^{obs} repeating, °C	ΔH^{cal} repeating, kJ/mol
Pro-Hyp-Gly	47.3	215	–	60.0	390
Pro-Pro-Gly	45.5	213	42	32.6	180
Pro-Flp-Gly	43.7	204	24	87.0[8a]	unknown
Hyp-Pro-Gly	43.0	204	17	<0	–
Hyp-Hyp-Gly	47.3	217	60	unknown	unknown

(Pro-Hyp-Gly) > (Pro-Pro-Gly) > (Hyp-Pro-Gly) is the same in host−guest and repeating tripeptides. However, a lower stability is observed for repeating tripeptides than predicted by using a simple additive method based on the host−guest system. This suggests a Pro-Hyp-Gly host environment has a more stabilizing effect on Pro-Pro-Gly and Hyp-Pro-Gly than its own repetition. This greater stability could be related to a hydration network established in the Pro-Hyp-Gly host environment, which is supported by similar calorimetric enthalpy[14] values for all host−guest peptides (Table 1). Previous studies showed the stability of a repeating Pro-Arg-Gly peptide was much lower than expected on the basis of the host−guest system,[15] supporting the importance of the context, although charge repulsion may also be a factor.

In only one case, that of Pro-Flp-Gly, the relative order in repeating tripeptides and the host−guest system are reversed. The peptide (Pro-Flp-Gly)$_{10}$ is more stable than (Pro-Hyp-Gly)$_{10}$,[8a] and Fields and colleagues[16] reported that a single substitution of a Hyp by Flp in a peptide containing a type IV collagen sequence led to a slight increase in stability in water. Thus, it was not anticipated to find that the Pro-Flp-Gly host−guest peptide is somewhat less stable than Pro-Hyp-Gly (Figure 2, Table 1). This failure of Flp in the Y-position to increase stability of the host−guest peptide suggests different mechanisms are in place when one Pro-Flp-Gly unit is embedded within the Pro-Hyp-Gly context, compared with (Pro-Flp-Gly)$_{10}$. This discrepancy may be attributed to contributions in the unfolded as well as the folded states. The increased stability of (Pro-Flp-Gly)$_{10}$ is likely to be entropically driven, as a result of the favored *exo* ring in the Y-position of the unfolded form, and its polyproline II-like nature is supported by the high 225 nm ellipticity at elevated temperatures in its unfolded state (Figure 2).[8a,17] No preferential polyproline II structure is found for Pro-Flp-Gly in host−guest peptides, where all ellipticities and temperature-dependent slopes are similar (Figures 1, 2). The impact of sequence environment on residual monomer structure and on the native-state hydration network may be responsible for the differences in stability contributions of the same tripeptide unit in host−guest versus repeating tripeptide systems.

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(11) Fmoc-4(*R*)-*trans*-fluoroproline was obtained from Bachem Holding AG, Bubendorf Switzerland. Host−guest peptides were synthesized by solid-phase chemistry on a PerSeptive Pioneer Peptide Synthesis System using Fmoc chemistry with an Fmoc-PAL-PEG-PS resin and 2% DBU/2% piperidine as the Fmoc removal solution. Peptides were purified to >90% purity using a SHIMADZU reverse-phase HPLC system on a C-18 column. Laser desorption mass spectrometry (MALDI) confirmed peptide identity. (Pro-Pro-Gly)$_{10}$, (Pro-Hyp-Gly)$_{10}$, and (Hyp-Pro-Gly)$_{10}$ were obtained from Peptide International.

(12) Circular dichroism (CD) spectra measurements were made on an Aviv model 62DS spectrometer at concentration of 1.0 mg/mL in PBS, pH 7.0. The 225-nm ellipticity value was monitored as a function of temperature at a rate of 0.1 °C/min to obtain the melting curve. T_m was determined as a midpoint of the melting curve. Experimental error in T_m determination did not exceed ±0.5 °C.

(13) The host−guest peptides are designated by the three-letter amino acid sequence of its guest Gly-Xaa-Yaa triplet.

(14) Differential scanning calorimetry (DSC) experiments were performed on a Nano-DSC II (Calorimetry Sciences Corp., model 6100) instrument at scan rates 0.05−1.0 °C/min. The peptide concentration was 1.0 mg/mL in PBS, pH 7.0. Calorimetric enthalpy values obtained by temperature integration of excess heat capacity experimental data were independent of the scanning rate within the experimental error of ±10 kJ/mol.

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