A (4*R*)- or a (4*S*)-Fluoroproline Residue in Position Xaa of the (Xaa-Yaa-Gly) Collagen Repeat Severely Affects Triple-Helix Formation

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*The triple-helical fold of collagen requires the presence of a glycine residue at every third position in the peptide sequence and is stabilized by proline and (4*R*)-4-hydroxyproline residues in positions Xaa and Yaa of the (Xaa-Yaa-Gly) triplets, respectively. Regular down/up puckering of these Xaa/Yaa residues is possibly responsible for the tight packing of the three peptide strands, which have a polyproline-II-like structure, into the supercoiled helix. (4*R*)-Configured electronegative substituents such as a hydroxy group or a fluorine substituent on the pyrrolidine ring of the residue in the Yaa position favor the up pucker and thus significantly stabilize the triple helix. A similar effect was expected from the corresponding (4*S*)-isomers in the Xaa positions, but the opposite effect has been observed with (4*S*)-hydroxyproline, a result that has been speculatively attributed to steric effects. In this study, (4*R*)- and (4*S*)-fluoroproline residues were introduced into the Xaa position and potential steric effects were thus avoided. Contrary to expectations, (4*S*)-fluoroproline prevents triple-helix formation, whereas (4*R*)-fluoroproline stabilizes the polyPro II conformation, but without supercoiling of the three strands. The latter observation suggests that folding of the single chains into a polyproline II helix is not directly associated with triple helix formation and that fine tuning of van der Waals contacts, electrostatic interactions, and stereoelectronic effects is required for optimal packing into a triple helix.*

Introduction

The collagen triple helix consists of three tightly packed, supercoiled polyproline-II-like chains whose sequences consist of characteristic repetitive Xaa-Yaa-Gly triplets in which the residues Xaa and Yaa are frequently proline and (2*S*,4*R*)-4-hydroxyproline (Hyp), respectively.[1] In vertebrates, enzymatic hydroxylation of the proline residues in positions Yaa occurs post-translationally in a position-dependent and stereoselective manner and leads to a significant enhancement of the thermal stability of collagen.[2] Conversely, model collagen peptides consisting of ten Hyp-Pro-Gly repeats and thus containing the natural 4-hydroxyproline isomer as the Xaa residue do not self-associate into a triple helix.[3] Similarly, incorporation of (2*S*,4*S*)-hydroxyproline ((4*S*)-Hyp) into the Xaa or Yaa positions prevents formation of the collagen structure even at low temperature.[4]

Various high-resolution crystal structures of model collagen peptides have been reported.[5] Nevertheless, the structural basis of triple-helix stabilization by Hyp residues in the Yaa positions, and of the destabilization caused by this residue in the Xaa positions is still in dispute. Since water bridges are persistently observed in crystals of $(Pro\text{-}Hyp\text{-}Gly)_n$ triple helices, a major role in the stabilization of collagen-type structures has been attributed to the hydration shell involving the Hyp residues.[2b, 5b, 6] However, the entropic cost resulting from the presence of bound water molecules argues against such a structural model.[5e, 7] In fact, fast water exchange with unbound water has been observed and the 'hopping' hydration mechanism suggested as a result means that hydration should only marginally contribute to the triple-helix stability.[8] This conclusion is further supported by the finding that substitution of Hyp residues in the Yaa positions in collagen peptides with (2*S*,4*R*)-4-fluoroproline ((4*R*)-FPro) residues greatly increases the thermal stability of the triple helix, despite the low tendency of fluorine substituents to form hydrogen bonds.[9] These findings led to an alternative structural model based on the inductive effects exerted by the 4-hydroxy group, which favor the *trans* conformation of the Pro–Hyp peptide bond, as required for the onset of the triple-helical fold.[9] However, neither the hydration model nor the favoring of the *trans* conformation can account for the adverse effects exerted on the triple-helix stability by Hyp residues in position Xaa.[3]

A close inspection of the X-ray structures of collagen peptides shows that the Pro or Hyp residues in the Yaa positions adopt an 'up' conformation, that is, a γ-*exo* pucker, and the Pro residues in the Xaa positions adopt a 'down' conformation, that is, a γ-*endo* pucker of the pyrrolidine ring (Figure 1).[5b, 5e, 5h–j] Comparative conformational studies performed on Pro, Hyp, (4*R*)-Fpro, and (4*S*)-FPro model compounds clearly revealed that the electronegative substituents energetically facilitate the *cis*/*trans* isomerization of amino acyl–proline bonds and that the 4*R* isomers

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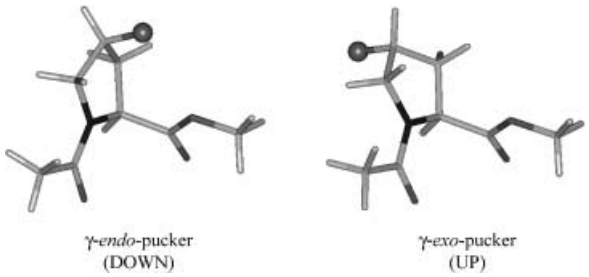

Figure 1. *Puckering of the pyrrolidine rings of Ac-(4S)-FPro-OMe (left) and Ac-(4R)-FPro-OMe (right) in the 'down' and 'up' conformations.*

favor the γ-*exo* pucker, while the 4*S* isomers favor the γ-*endo* pucker.[10] Quantum chemical calculations revealed electronic delocalization as the cause for the pucker preferences.[11] For steric reasons, the *trans* peptide bond is favored by the γ-*exo* puckering, while in the γ-*endo*-puckered state the energy difference between the *cis* and *trans* peptide bond conformations is significantly reduced, which facilitates a higher population of the *cis* conformation. Preference of the Hyp residue for the γ-*exo* pucker is also supported by conformational analysis of poly(Hyp)[12] and the X-ray structure of this amino acid.[13] Unsubstituted Pro prefers the γ-*endo* pucker.[10]

The results of these model studies account for the lower stability of $(Pro\text{-}Pro\text{-}Gly)_n$ triple helices compared to those with the sequence $(Pro\text{-}Hyp\text{-}Gly)_n$[2] and for the strong destabilizing effects of (4*S*)-Hyp and 4*S*-FPro when placed in position Yaa of the triplets.[4, 10d, 14] These arguments suggest that 4*S*-Hyp should favor the triple helix when placed in the Xaa position; however, the opposite effect was observed experimentally.[4] This fact was explained by possible steric clashes of the 4-hydroxy group with the proline ring of the adjacent chain.[5i] To exclude such steric effects, we synthesized the model collagen peptides shown in Figure 2. These peptides contain (4*S*)-FPro (peptide **I**) and (4*R*)-FPro (peptide **II**) in position Xaa of the collagen repeats, residues with preferences for the γ-*endo* and γ-*exo* pucker, respectively. The monomeric reference peptide **III** and the trimer **IV**, each of which contains five (Pro-Hyp-Gly) repeats, were synthesized in a previous study.[15]

Ac-[(4*S*)-FPro-Hyp-Gly]$_5$-Pro-Cys(S*t*Bu)-Cys(S*t*Bu)-(Gly)$_3$-NH_2 (**I**)
Ac-[(4*R*)-FPro-Hyp-Gly]$_5$-Pro-Cys(S*t*Bu)-Cys(S*t*Bu)-(Gly)$_3$-NH_2 (**II**)
Ac-[Pro-Hyp-Gly]$_5$-Pro-Cys(S*t*Bu)-Cys(S*t*Bu)-(Gly)$_3$-NH_2 (**III**)
[Ac-[Pro-Hyp-Gly]$_5$-Pro-Cys-Cys-(Gly)$_3$-NH_2]$_3$ (**IV**)

Figure 2. *Sequence composition of the collagenous peptides with (4S)-FPro (**I**) and (4R)-FPro (**II**) residues in the Xaa position, and of the monomeric (**III**) and trimeric (**IV**) reference peptides, each with a Pro residue in position Xaa.*

Results and Discussion

Synthesis of the model collagen peptides

At a concentration of 1 mM, the monomeric reference peptide **III** was found to self-associate into a triple helix characterized by a melting temperature (T_m) of 20.3 °C in phosphate buffer (pH 7.2), and 19.1 °C in water. The propensity of this peptide for self-structuring at low temperature allows its oxidative assembly (after deprotection of the cysteine thiol groups) into the homotrimer **IV**, with a C-terminal type-III-collagen cystine knot.[15] To attempt to exploit such a cystine knot for cross-linking the FPro-containing collagen peptides to form homotrimers, peptides **I** and **II** were similarly C-terminally extended with the typical bis-cysteinyl sequence of type III collagen.

The stereochemically homogeneous intermediate derivative Z-(4*S*)-FPro-OH (**1**; Z, benzyloxycarbonyl) was readily obtained through chiral inversion at C-4 by treatment of Z-Hyp-OH with diethylaminosulfur trifluoride (DAST),[16] by analogy to other (4*S*)-FPro derivatives.[17] To synthesize Z-(4*R*)-FPro-OH (**3**), alkaline hydrolysis of Z-Hyp(Tos)-OBzl (Tos, toluene-4-sulfonyl; Bzl, benzyl)[18] was used to generate Z-(4*S*)-Hyp-OBzl (**2**), which was treated with DAST to produce the desired stereoisomer. The two fluoroproline derivatives **1** and **3** were then employed for the synthesis of the tripeptide synthons Fmoc-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH (**5**; Fmoc, 9-fluorenylmethoxycarbonyl) and Fmoc-(4*R*)-FPro-Hyp(*t*Bu)-Gly-OH (**6**), respectively, in solution by procedures established previously for the parent peptide Fmoc-Pro-Hyp(*t*Bu)-Gly-OH.[19] These tripeptides were used in the chain elongation of H-Pro-Cys(*St*Bu)-Cys(*St*Bu)-$(Gly)_3$-Rink-MBHA-resin (MBHA, 4-methylbenzhydrylamine) by essentially the same procedures as developed for the synthesis of the reference peptide **III**.[15] As a result of the greatly reduced nucleophilicity of N-terminal FPro residues in comparison to Pro residues multiple couplings with Fmoc-(4*S* or 4*R*)-FPro-Hyp(*t*Bu)-Gly-OH/TFFH/DIPEA (1.8:1.8:3.6; DIPEA, diisopropylethylamine; TFFH, fluoro-*N*,*N*,*N*′,*N*′-tetramethylformamidinium hexafluorophosphate) and prolonged reaction times (3×24 h and 1×48 h) were required for quantitative acylation steps. To avoid the use of such large excesses of these cost- and labor-intensive synthons, coupling with bis-(trichloromethyl)carbonate (BTC) was attempted. However, crude products of insufficient quality were obtained.

Upon cleavage/deprotection with trifluoroacetic acid (TFA)/$(Et)_3SiH/H_2O$ (96:2:2) and purification by HPLC, the target peptides **I** and **II** were isolated in satisfactory yields as analytically well-defined materials. Since self-association of these peptides into triple-helical structures was detectable neither in water nor in water/MeOH mixtures (see below), air oxidation of the thiol-deprotected peptides to form the disulfide-cross-linked trimers was expected to fail. Even oxidation in 1,2-propandiol containing 3% ethanolamine was unsuccessful, although these conditions were successfully applied for oxidative trimerization of H-$(Gly\text{-}Pro\text{-}Thr)_{10}$-Gly-Pro-Cys-Cys-OH despite its low propensity to form a triple helix.[20] Mixtures of products were obtained from the attempts to trimerize peptides **I** and **II**. Monomers containing an intramolecular disulfide bridge were the main components of the mixture according to an LC-MS analysis.

Conformational properties of the model collagen peptides in aqueous solution

The dichroic properties of peptides **I**, **II**, and **III** in aqueous solution at 4 °C are listed in Table 1. Although the peptides were pre-equilibrated at low temperature and at 1 mM concentration,

Table 1. *CD parameters of the collagenous peptides at 1 mM concentration and 4 °C in water (A) and in 1 mM AcOH/MeOH (2:8; B) as derived from spectra recorded after equilibration for 12 h at 4 °C.*

Collagen peptides	Solvent	λ_{min} (θ_R)	λ_{max} (θ_R)	Rpn[a]	T_m [°C]
I	A	200.7 (−21699)	–	–	–
II	A	199.2 (−20996)	224.7 (669)	0.032	–
III	A	199.7 (−19987)	226 (952)	0.048	19.1
I	B	201.5 (−20473)	–	–	–
II	B	198.4 (−30849)	225.1 (1833)	0.059	–
III	B	199.3 (−24275)	225.8 (2138)	0.088	30.4

[a] Rpn is defined as the absolute value of the ratio of the dichroic intensity of the positive band to that of the negative band and is considered an index of triple helicity.[23]

which generally suffices for self-association of collagenous peptides into homotrimers,[21] the CD spectrum of peptide **I** ((4*S*)-FPro residue in the Xaa position) lacks a maximum at 223–226 nm and thus clearly does not have a triple-helical fold. The blue-shifted positive maximum of the CD spectrum of peptide **II**, ((4*R*)-FPro in the Xaa position) suggests a polyproline II conformation rather than a triple helix, as determined by comparison with the maximum of the reference peptide **III**. In contrast to the cooperative thermal unfolding of the reference peptide **III**, peptide **II** shows a monotonous intensity decay at 225 nm as is typically observed for peptides folded into the polyproline II conformation.[22]

The conclusions derived from the CD spectral properties are fully supported by the NMR spectra of peptides **I** and **II**, which confirm the absence of hydrogen-bonded Gly amides and thus of a triple-helical structure (Figure 3). Characteristic NOE cross peaks (*trans*: H_α(Gly)-H_δ(FPro); *cis*: H_α(Gly)-H_δ(FPro)) also revealed the presence of *trans* and *cis* Gly-(4*S*)-FPro bonds in peptide **I**, which excludes a polyproline II conformation for this model peptide and points instead to a random coil conformation. Conversely, the NMR spectra of peptide **II** show an all-*trans* conformation of the peptide bonds, which is consistent with a polyproline II structure.

Further evidence for the existence of a polyproline-II helical conformation of peptide **II** was derived from NMR diffusion experiments. NMR-derived translational diffusion coefficients depend on molecular size and structure[24, 25] and have proven useful for the determination of oligomerization states of peptides,[26] as well as in folding/unfolding studies of proteins.[24] Recently, the trimeric association and folding of a synthetic collagen peptide into a triple helix was monitored in real time by NMR diffusion measurements.[26] The homotrimeric collagen

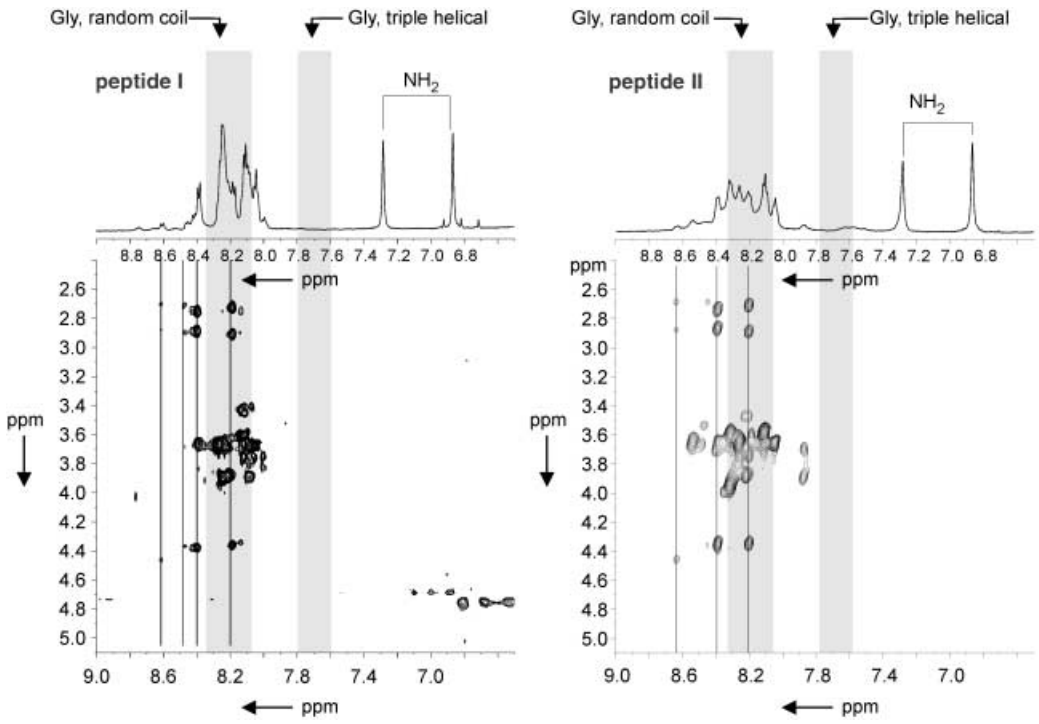

Figure 3. *Sections of the 1D NMR and 2D TOCSY spectra of the model collagen peptides **I** and **II**. The typical resonances of the hydrogen-bonded Gly NH proton observed for peptides folded into a triple helix are not detectable.*

peptide **IV** was used as a reference collagen triple helix of known size. This peptide is identical in size to the self-associated trimer of peptide **III**, except for the cystine residues that form a cystine knot in **IV** but are protected as *S-tert*-butyl derivatives in **III**. We have shown previously that the cystine knot dramatically increases the thermal stability of the triple helix in collagen peptide **IV**.[15] Diffusion experiments were performed at 4 and 27 °C (Table 2). The triple helix of **IV** is stable at these temperatures ($T_m = 68$ °C[15]). The diffusion constants of residual H_2O in the samples served as an internal control and were the same for all samples at a given temperature within experimental error. Hydrodynamic modeling suggests that peptides **I** and **II** are less compact in the polyproline II conformation (hydrodynamic radius 14 Å) than in the random coil state (hydrodynamic radius 12 ± 1 Å, see the Materials and Methods section). The random coil radius of 12 Å agrees well with the value of 12.2 Å obtained with empirical formulae for unstructured peptides.[24, 25] However, for both the polyproline II and the triple-helical conformation, a larger hydrodynamic radius is obtained by hydrodynamic modeling than expected for globular folds as a result of the elongated shape of these helices. Experimentally, a larger diffusion constant, and thus a smaller hydrodynamic radius, is observed for peptide **I** than for peptide **II** at 4 and at 27 °C, which is consistent with a polyproline II conformation of the (4*R*)-Fpro-containing peptide **II**. Diffusion experiments were performed at two concentrations (0.4 and 2.0 mM for peptides **I** and **II** and 0.2 and 0.7 mM for peptide **IV**) to exclude the possibility of aggregation. The experiment at 27 °C was repeated after cooling to 4 °C to allow detection of any possible changes in sample aggregation state (such as slow aggregation, precipitation, fibril formation). No concentration dependence or aging effects could be detected. Theoretical diffusion constants were predicted for all three peptides based on the following structural models: For peptide **IV**, which is known to form a triple helix, an ideal triple-helical conformation was used. For peptide **I**, an ensemble of single-chain random-coil conformations was generated by molecular modeling and the average of the whole ensemble is reported in Table 2. Peptide **II** was modeled in an ideal polyproline II conformation for all five triplets. As is clear from the data in Table 2, the experimental and predicted diffusion constants agree very well. This result confirms the assumption that peptide **II** has the polyproline II conformation. Furthermore, the ratio of the diffusion constant of the triple-helical peptide **IV** to that of the unordered peptide **I** is 0.6 at both temperatures, which is in perfect agreement with a previous study on the unfolding of a collagen peptide.[26]

Table 2. *Translational diffusion constants [10^{-10} $m^2 s^{-1}$], as determined by NMR diffusion experiments at 0.4 mM concentration in D_2O solution.*[a]

Temperature	Peptide **I**	Peptide **II**	Peptide **IV**	H_2O
4 °C	0.85	0.8	0.5	8.3
27 °C	2.1	1.8	1.2	18
27 °C, calcd	2.1 ± 0.2[a]	1.7	1.3	–
structural model	random coil	polyproline II	triple helix	

[a] Experimental errors were approximately 5%. Theoretical diffusion constants are derived from hydrodynamic calculations based on structural models (see the text for details). [b] 2.1 is the average over 100 structures and 0.2 the corresponding standard deviation.

Conformational properties of the collagen model peptides in alcoholic solution

Alcohols are known to stabilize the triple-helical conformation of collagen peptides.[27] A synthetic collagenous trimer cross-linked C-terminally by a dilysine template and consisting of residues 606 – 618 of type III collagen and the N-terminal extension (Hyp-Pro-Gly)$_8$ (Hyp residue in the Xaa position) has been reported to fold into a triple helix of high thermal stability ($T_m = 58.5$ °C) under acidic conditions and at high MeOH concentration (85%).[28] Under these conditions, even the triple helix of reference peptide **III** exhibits a significantly enhanced thermal stability (Table 1), which confirms the beneficial effects of alcohols. Conversely, a cooperative transition was not observed for peptide **II** (Figure 4), although its Rpn value under these conditions is similar to that of peptide **III** in water. For peptide **I**, even a polyproline II conformation is excluded by the absence of a positive CD maximum at 223 – 226 nm (Table 1). The results obtained for peptide **II** contradict those reported above for the trimeric construct, unless the high alcohol content stabilizes a triple-helical structure in the portion of the construct consisting of the native collagen type III 606 – 618 sequence, but the polyproline II conformation is retained in the N-terminal (Hyp-Pro-Gly)$_8$ extension.

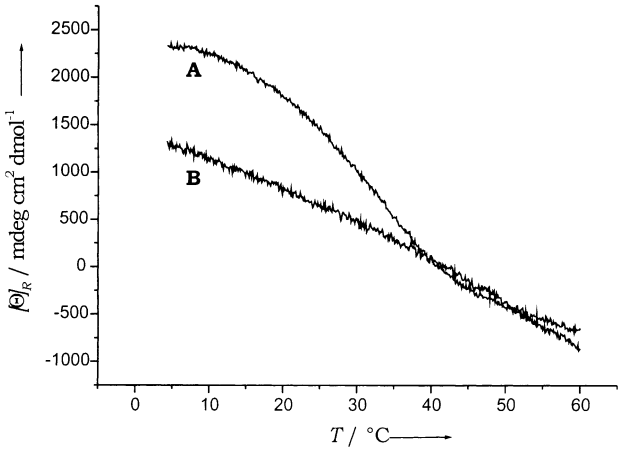

Figure 4. *Thermal denaturation of peptides **II** (A) and **III** (B) in 80% MeOH, monitored by CD at 225 nm.*

The inability of peptide **II** to fold into a triple helix even under optimized experimental conditions fully agrees with the similar behavior reported for (Hyp-Pro-Gly)$_{10}$[3] and confirms the strong effect of electronegative C-4 substituents on the Pro residue in the Xaa position. Since the (4*R*)-FPro stereoisomer favors the up conformation of the pyrrolidine ring, the experimental results suggest a rather decisive contribution of the ring puckering to the peptide folding. Although quantum mechanical calculations

for down/up and up/up conformations in the Xaa/Yaa positions of the triple helix revealed almost identical energies, optimal packing of the three chains is obtained with the down/up arrangement,[11] a fact fully consistent with the X-ray structures.[5h] However, peptide **I** has (4*S*)-FPro in the Xaa and Hyp in the Yaa position, which in combination should favor the ideal down/up arrangement of the pyrrolidine rings, but the peptide is unable to self-associate into a triple helix or even to assume a polyproline II conformation as a single chain. The expected and experimentally observed higher population of *cis* Gly-(4*S*)-FPro bonds is unlikely to be the decisive factor in the lack of triple helix, but electrostatic effects could well play an important role.

In contrast to vertebrate collagens, which are stabilized by Hyp residues in the Yaa position of the triplet repeat unit,[2] the cuticle collagen of the earthworm[29] and (as confirmed in more detail by sequence analysis) the cuticle collagen of the hydrothermal vent vestimentiferan *Riftia pachyptila*[20] have most of their Hyp residues in the Xaa position, while the Yaa position is occupied by glycosylated Thr residues. Replacement of the Pro residue in the Xaa position of (Gly-Pro-Thr(β-D-Gal))$_{10}$ model peptides by a Hyp residue led to increased thermal stability,[30] which suggests that the stability of proline-poor triple helices is governed by different rules from those that apply to proline-rich peptides.

Conclusion

The emerging picture of the stabilization of a collagen triple helix involves a carefully balanced combination of several effects. Favorable van der Waals contacts between proline rings in adjacent chains and electrostatic interactions add to the well-known hydrogen bonding network to create an optimally packed triple helix. The corresponding backbone dihedral angles are most compatible with the down/up pattern for the pucker in the Xaa and Yaa positions. While the down pucker is the natural preference for proline, up puckering can be stabilized by electronegative (4*R*)-configured substituents through electronic effects. In vivo, this stabilization is achieved by post-translational enzymatic hydroxylation. In summary, the collagen triple helix is a structural motif that is highly optimized by nature. Therefore, rational modifications, for example, for creating new biomaterials, require a thorough understanding of all the effects involved.

Abbreviations

Standard abbreviations are used as recommended by the IUPAC-IUB commission on biochemical nomenclature and the ACS Style Guide. By convention, the abbreviation Hyp corresponds to the most common naturally occurring hydroxyproline, that is, (2*S*,4*R*)-4-hydroxyproline. Since all C-4-substituted proline analogues used and discussed herein are of the L-configuration, only the chirality at C-4 is given.

Addendum

During review of this article two reports appeared on the effect of (4*R*)-FPro and (4*S*)-FPro in the Xaa positions of (Xaa-Pro-Gly)$_7$[31] and (Xaa-Pro-Gly)$_{10}$[32] model collagen peptides. In both cases, an (4*S*)-FPro residue in the Xaa position was found to significantly enhance the stability of the triple helix in a manner consistent with structural arguments (see the Introduction). Conversely, in our peptide, in which the (4*S*)-Fpro residue at position Xaa is combined with a Hyp residue in the Yaa position, a triple helix is not formed. These results clearly show that contributions of substituents at the pyrrolidine ring in the Xaa and Yaa positions to the conformation of the peptide are not additive and confirm our conclusion that a concerted interplay of various factors is necessary for optimal packing of the collagen structure.

Experimental Section

Materials and methods: Reagents and solvents were of the highest quality commercially available and were used without further purification, except dimethylformamide (DMF), which was freshly distilled over ninhydrin. Amino acid derivatives were purchased from Fluka (Taufkirchen, Germany), TFFH and BTC from Aldrich (Taufkirchen, Germany), and the Fmoc-Gly-Rink-MBHA resin (linker: 4-[(*R*,*S*)-α-amino-2′,4′-dimethoxybenzyl]phenoxyacetyl-norleucine-amidobenzhydryl) from Calbiochem – NovaBiochem (Läufelfingen, Switzerland). Peptide synthesis was performed manually in a polypropylene syringe fitted with a polyethylene disk. Precoated silica gel 60 TLC plates were purchased from Merck AG (Darmstadt, Germany) and compounds were visualized with chlorine/tolidine. Analytical reversed-phase (RP)-HPLC was performed with Waters equipment (Eschborn, Germany), reversed-phase Nucleosil C18 columns (0.4 × 25 cm, 10 μm) from Macherey & Nagel (Düren, Germany), and linear gradients of CH_3CN/2% H_3PO_4 (from 5:95 to 90:10 in 15 min) as eluents at a flow rate of 1.5 mL min^{-1}. UV absorbance was monitored at 210 nm. Preparative RP-HPLC was carried out with Abimed equipment (Langenfeld, Germany) on reversed-phase Nucleosil C18 columns (2.1 × 25 cm, 5 μm endcapped) by elution with a linear gradient of 0.08% TFA in CH_3CN and 0.1% TFA from 2:8 to 8:2 in 50 min. Elution profiles were monitored by UV absorbance at 210 nm. ESI MS was carried out on a PE Sciex API 165 instrument.

Peptide synthesis:

Z-(4*S*)-FPro-OH (**1**): DAST (9.3 mL; 71.2 mmol) was added to Z-Hyp-OH (4.7 g; 17.8 mmol) in CH_2Cl_2 at 0 °C and the reaction mixture was stirred for 5 h at RT and poured onto ice. The solvents were evaporated and the dark red residue was distributed between EtOAc and 5% $NaHCO_3$. The organic layer was extracted with 5% $NaHCO_3$ and the combined aqueous extracts were acidified with 1 M HCl and again extracted with EtOAc. These organic extracts were dried ($MgSO_4$) and evaporated to a solid residue. Yield: 3.4 g (71.2%); HPLC: $t_R = 9.5$ min (> 95%); m.p.: 118 °C; $[\alpha]_D^{24} = -49$ ($c = 0.1$, MeOH) [ref. [33]: -44 ($c = 1$, MeOH) for the cyclohexylamine salt]; ^{1}H NMR (400 MHz, d_4-MeOH, 27 °C): $\delta = 7.40 - 7.25$ (m, 5H, arom.), 5.32 – 5.15 (m, 1H, FPro$^{\gamma}$, $^2J_{HF} = 51$ Hz), 5.17 – 5.10 (m, 2H, benzyl CH_2), 4.56 – 4.49 (m, 1H, FPro$^{\alpha}$), 3.80 – 3.66 (m, 2H, FPro$^{\delta}$), 2.58 – 2.39 (m, 2H, FPro$^{\beta}$) ppm; HRMS (ESI): calcd for $C_{13}H_{15}NO_4F$: $m/z = 268.0980$ $[M+H]^+$; found: 268.0979.

Z-(4*S*)-Hyp-OBzl (**2**): Z-(4*R*)-Hyp(Tos)-OBzl[18] (27.5 g; 54 mmol) was stirred in dioxane (162 mL) and 1 M NaOH (162 mL) at 80 °C for 4 h. The bulk of the solvent was evaporated and the residue distributed

between EtOAc and 5% $NaHCO_3$. The aqueous layer was extracted with EtOAc and then acidified with 1 M HCl to pH 2.5. The product was extracted with EtOAc and the combined extracts were dried ($MgSO_4$) and evaporated. To remove contaminating TosOH, the crude product was treated with triethylamine (7.5 mL; 53.5 mmol) and benzyl bromide (6.43 mL; 53.5 mmol) in tetrahydrofuran (75 mL) at 0 °C. After stirring overnight at RT, the bulk of the solvent was removed and the residue dissolved in EtOAc. The solution was washed with H_2O, 5% $KHSO_4$, 5% $NaHCO_3$, and brine, dried ($MgSO_4$), and evaporated to an oily residue. Yield: 15.45 g (80.4%); HPLC: t_R = 11.60 min (> 99%); $[\alpha]_D^{24} = -54$ ($c = 0.1$, MeOH); ^{1}H NMR (400 MHz, d_4-MeOH, 27 °C): δ = 7.38 – 7.22 (m, 10H, arom.), 5.18 – 5.00 (m, 4H, benzyl), 4.47 (dd, 1H Hyp$^{\alpha}$, $^3J_{\alpha\beta}$ = 3.2, 9.2 Hz), 4.36 (m, 1H, Hyp$^{\gamma}$), 3.66, 3.42 (m, 2H, Hyp$^{\delta}$), 2.39, 2.11 (m, 2H Hyp$^{\beta}$) ppm; HRMS (ESI): calcd for $C_{20}H_{22}NO_5$: m/z = 356.1492 $[M+H]^+$; found: 356.1495.

Z-(4*R*)-FPro-OH (**3**): DAST (4.1 mL; 30.8 mmol) was added to an ice-cold solution of **2** (5.48 g; 15.4 mmol) in CH_2Cl_2 and the mixture was stirred at RT for 5 h then poured onto ice and diluted with dioxane (100 mL) and 1 M NaOH (30.8 mL; 30.8 mmol). After 5 h stirring, the mixture was neutralized with 1 M HCl and concentrated to a small volume. The residue was distributed between EtOAc and 5% $NaHCO_3$. The aqueous layer was washed with EtOAc, acidified with 1 M HCl, and extracted with EtOAc. The combined organic extracts were dried ($MgSO_4$) and evaporated to an oil. Yield: 4.1 g (96%); HPLC: t_R = 8.7 min (> 95%); $[\delta]_D^{24} = -62$ ($c = 0.1$, MeOH) [ref. [33]: −40 ($c = 1$, MeOH) for the cyclohexylamine salt; ^{1}H NMR (400 MHz, d_4-MeOH, 27 °C): δ = 7.40 –7.25 (m, 5H, arom.), 5.25 (m, 1H, FPro$^{\gamma}$, $^2J_{HF}$ = 51 Hz), 5.18 – 5.8 (m, 2H, benzyl CH_2), 4.43 (dd, 1H, FPro$^{\alpha}$, $^3J_{\alpha\beta}$ = 9 Hz), 3.88, 3.63 (m, 2H, FPro$^{\delta}$), 2.64, 2.18 (m, 2H, FPro$^{\beta}$) ppm; HRMS (ESI): calcd for $C_{13}H_{15}NO_4F$: m/z = 268.0980 $[M+H]^+$; found: 268.0978.

Z-(4*S*)-FPro-Hyp-Gly-OBzl (**4**): Z-(4*S*)-FPro-OH (**1**; 3.4 g 12.67 mmol) was converted into the *N*-hydroxysuccinimide ester by the standard reaction with *N*-hydroxysuccinimide (HOSu; 2.19 g; 19 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (3.92 g; 19 mmol) in EtOAc/dioxane (1:2; 45 mL). Yield: 4.6 g (100%) oil.

H-Hyp-OH (2.49 g; 19 mmol) was dissolved in 40% Triton B in MeOH (8.5 mL) and evaporated. The residue was taken up in DMF (50 mL) and Z-(4*S*)-FPro-OSu (4.6 g; 12.67 mmol) was added. After 12 h at RT, the solvent was evaporated and the residue distributed between EtOAc and 5% $NaHCO_3$. The aqueous layer was washed with EtOAc, acidified to pH 1.5 with 1 M HCl and extracted with EtOAc. The combined organic layers were dried ($MgSO_4$) and evaporated to an oily residue. Yield: 1.8 g (37%); HPLC: t_R = 7.5 min (> 85%); ESI MS: calcd for $C_{18}H_{21}N_2O_6F$: M_r = 380.38; found: m/z = 381.0 $[M+H]^+$.

The dipeptide derivative Z-(4*S*)-FPro-Hyp-OH (1.8 g; 4.73 mmol) was coupled with H-Gly-OBzl · TosOH (1.92 g, 5.58 mmol) in DMF (50 mL) by treatment with *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide · HCl (1 g; 5.2 mmol), 1-hydroxybenzotriazole (HOBt; 639 mg; 4.73 mmol), and DIPEA (9.46 mmol; 1.9 mL). After 12 h, the solvent was evaporated and the residue dissolved in EtOAc and washed with 5% $KHSO_4$, 5% $NaHCO_3$, and brine. The solution was dried ($MgSO_4$) and evaporated to an oil. Yield: 1.3 g (52%); HPLC: t_R = 10.9 min (> 90%); ESI MS: calcd for $C_{27}H_{30}N_3O_7F$: M_r = 527.55; found: m/z = 528.2 $[M+H]^+$.

Fmoc-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH (**5**): The tripeptide **4** (1.3 g; 2.46 mmol) was treated with isobutene (25 mL) in CH_2Cl_2 (25 mL) in the presence of H_2SO_4 (48 µl) for 7 days at RT. The solution was cooled and neutralized with 5% $NaHCO_3$ (100 mL) and the excess isobutene was evaporated. The organic layer was diluted with CH_2Cl_2 and washed with 5% $NaHCO_3$. The solvent was removed and the residue dissolved in EtOAc and washed with H_2O. The solution was dried ($MgSO_4$) and evaporated to an oil. The crude product (1.45 g) was hydrogenated in MeOH/H_2O (9:1; 120 mL) over Pd/C in the presence of TosOH · H_2O (0.49 g; 2.48 mmol). After 14 h, the catalyst was filtered off and the solution evaporated to dryness. Fmoc-OSu (0.88 g; 2.62 mmol) in dioxane (20 mL) was then added to a solution of the resulting H-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH · TosOH (1.2 g; 2.18 mmol) in H_2O/dioxane (2:1; 60 mL) containing $NaHCO_3$ (0.46 g; 5.45 mmol). After 12 h at RT, the reaction mixture was neutralized with 1 M HCl and evaporated to a small volume. The residue was distributed between 5% $NaHCO_3$ and EtOAc. The aqueous layer was acidified with 5% $KHSO_4$ and the product extracted with EtOAc. The combined extracts were dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica gel (5 × 17 cm) by elution with CH_2Cl_2/MeOH/HOAc (95:5:0.1) followed by CH_2Cl_2/MeOH/HOAc (85:15:0.1). Fractions containing homogeneous product were pooled and evaporated to a solid. Yield: 0.6 g (42.3% over three steps); HPLC: t_R = 12.2 min (> 98%); R_f = 0.3 (CH_2Cl_2/MeOH/0.05% HOAc, 95:5:0.1); m.p.: 168 °C; $[\delta]_D^{24} = -40$ ($c = 0.1$, MeOH); ^{1}H NMR (400 MHz, d_4-MeOH, 27 °C): δ = 7.83 – 7.30 (m, 9H, Fmoc), 5.23 (m, 1H, FPro$_c^{\gamma}$), 5.21 (m, 1H, FPro$_t^{\gamma}$), 4.65 (m, 1H, FPro$_c^{\alpha}$), 4.59 (m, 2H, Hyp′$^{\alpha}$ + Hyp′$^{\gamma}$), 4.49 (m, 2H, Hyp′$^{\alpha}$ + Hyp′$^{\gamma}$), 4.45 (m, 1H, FPro$_t^{\alpha}$), 4.45 – 4.38 (m, 2H, Gly$^{\gamma}$), 3.79, 3.48 (m, 2H, Hyp′$^{\delta}$), 3.74 (m, 2H, FPro$^{\delta}$), 3.71, 3.18 (m, 2H, Hyp$^{\delta}$), 2.57, 2.44 (m, 2H, FPro$^{\beta}$), 2.23, 2.09 (m, 2H, Hyp$^{\beta}$), 2.23, 2.09 (m, 2H, Hyp′$^{\beta}$), 1.22 – 1.16 (s, 9H, *t*Bu) ppm; the *cis* and *trans* urethane bond species are denoted by subscripts to the proton positions; HRMS (ESI): calcd for $C_{31}H_{37}N_3O_7F$: m/z = 582.2610 $[M+H]^+$; found: 582.2617.

Fmoc-(4*R*)-FPro-Hyp(*t*Bu)-Gly-OH (**6**): The tripeptide derivative was prepared from Z-(4*R*)-FPro-OH (**3**) by procedures identical to those described for **4** and **5**. Yield: 0.93 g (11% over 6 steps); HPLC: t_R = 12.8 min (> 90%); TLC: R_f = 0.3 (CH_2Cl_2/MeOH/0.05% HOAc, 95:5:0.1); m.p.: 104 °C; $[\delta]_D^{24} = -65$ ($c = 0.1$, MeOH); ^{1}H NMR (400 MHz, d_4-MeOH, 27 °C): δ = 7.83 – 7.30 (m, 9H, Fmoc), 5.27 (m, 1H, FPro$^{\gamma}$), 4.67 (m, 1H, FPro$^{\alpha}$), 4.60 (m, 1H, Hyp$^{\alpha}$), 4.52 (m, 1H, Hyp$^{\gamma}$), 4.45 (m, 2H, Hyp′$^{\gamma}$ + Hyp′$^{\alpha}$), 4.40 – 4.49 (m, 2H, Gly$^{\alpha}$), 3.75, 3.35 (m, 2H, Hyp′$^{\delta}$), 3.84, 3.60 (m, 2H, Hyp$^{\delta}$), 3.63 (m, 2H, FPro$^{\delta}$), 2.68, 2.18 (m, 2H, FPro$^{\beta}$), 2.18 – 2.06 (m, 1H, Hyp′$^{\beta}$), 2.12 – 2.22 (m, 2H, Hyp$^{\beta}$), 1.22 – 1.06 (s, 9H, *t*Bu) ppm; HRMS (ESI): calcd for $C_{31}H_{37}N_3O_7F$: m/z = 582.2610 $[M+H]^+$; found: 582.2620.

Ac-[(4*S*)-FPro-Hyp-Gly]$_5$-Pro-Cys(*St*Bu)-Cys(*St*Bu)-Gly-Gly-Gly-NH_2 (**I**): The syntheses were carried out on Fmoc-Gly-Rink-MBHA-resin (106 mg; 0.4 mmol g^{-1}) according to standard Fmoc chemistry procedures. For chain elongations with Fmoc amino acids, double couplings with Fmoc-Xaa-OH/HBTU/HOBt/DIPEA (1:1:1:2, 4 equiv; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide) in DMF (2 × 1 h) were applied, followed by washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), and MeOH (3 × 1 min). The first chain elongation with Fmoc-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH (**5**) was carried out by a single coupling (2 h) with Fmoc-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH/TFFH/DIPEA (1:1:2, 1.8 equiv) in DMF followed by washings with DMF (3 × 1 min), acylation with $(Boc)_2O$ (10 equiv) in DMF (20 min), and additional washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), and MeOH (3 × 1 min). The subsequent couplings of the tripeptide **5** with N-terminal FPro-peptides were performed by treatment with Fmoc-(4*S*)-FPro-Hyp(*t*Bu)-Gly-OH/TFFH/DIPEA (1:1:2, 1.8/0.8/0.8 equiv) for 3 h in DMF and in *N*-methylpyrrolidone/CH_2Cl_2 for the last two steps, followed by washings with DMF (3 × 1 min), acetylation with $(Boc)_2O$ (10 equiv) in DMF (20 min), and washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), and MeOH (3 × 1 min). Coupling efficiency was monitored by using the Kaiser test,[34] except for N-terminal proline residues, for which the chloranil test[35] was used. Fmoc cleavage from Fmoc-Pro- or Fmoc-FPro-peptidyl-resin was carried out by treatment with 2% piperidine

and 2% 1,8-diazabicyclo[5.4.0]undec-7-ene in DMF (0 °C, 1 × 60 sec, 1 × 30 sec) and washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), and MeOH (3 × 1 min). N-terminal acetylation of the peptides on the resin was performed by treatment with Ac_2O/DIPEA (1:2, 4 equiv) in DMF (2 × 30 min) followed by washings with DMF (3 × 1 min), MeOH (3 × 1 min), CH_2Cl_2 (3 × 1 min), and MeOH (3 × 1 min). For deprotection/cleavage, the peptidyl resin (462 mg) was treated with TFA/Et_3SiH/H_2O (96:2:2; 1 × 15 min, 2 × 40 min, and 1 × 60 min), filtered off, and washed with CH_2Cl_2. The combined filtrates were concentrated and the crude product was precipitated from trifluoroethanol with Et_2O, collected by filtration, and lyophilized from *t*BuOH/H_2O (5:1). The crude product was purified by preparative HPLC. Yield: 75 mg (83%); HPLC: $t_R = 9.2$ min (>98%); ^{1}H NMR (500 MHz, D_2O, 4 °C): $\delta = 5.33$ (d, 5H, 5 × $Fpro_t^{\gamma}$), 5.25 (d, 5H, 5 × $Fpro_c^{\gamma}$), 5.03 (m, 5H, 5 × $Fpro_c^{\alpha}$), 4.86 (m, 5H, 5 × $Fpro_t^{\alpha}$), 4.60 (m, 2H, 2 × Cys^{α}), 4.53 (m, 10H, 5 × $Hyp^{\alpha,\gamma}$), 4.33 (m, 1H, Pro^{α}), 4.12, 3.92 (m, 10H, 5 × Gly_t^{α}), 3.97,3.66 (m, 10H, 5 × Gly_c^{α}), 4.16–3.86 (m, 6H, 3 × Gly^{α}), 3.76 (m, 10H, 5 × $Fpro_t^{\delta}$), 3.61 (m, 10H, 5 × $Fpro_c^{\delta}$), 3.62 (m, 10H, 5 × Hyp^{δ}), 3.55 (m, 2H, Pro^{δ}), 3.11, 2.96 (m, 4H, 2 × Cys^{β}), 2.56–2.31 (m, 10H, $FPro_t^{\beta}$), 2.63–2.48 (m, 10H, $FPro_c^{\beta}$), 2.29, 1.98 (m, 10H, 5 × Hyp^{β}), 2.20, 1.91 (m, 4H, $Pro^{\beta,\gamma}$), 1.24 (s, 18H, 2 × *St*Bu) ppm; the *cis* and *trans* urethane bond species are denoted by subscripts to the proton positions; HRMS (ESI): calcd for $C_{87}H_{129}N_{22}O_{27}S_4F_5$: $m/z =$ 1068.4095 $[M+2H]^{2+}$; found: 1068.4092.

Ac-[(4*R*)-FPro-Hyp-Gly]$_5$-Pro-Cys(*St*Bu)-Cys(*St*Bu)-Gly-Gly-Gly-NH_2 (**II**): Peptide **II** was synthesized and isolated as described for **I**, with Fmoc-(4*R*)-FPro-Hyp(*t*Bu)-Gly-OH (**6**) as a synthon. Yield: 54 mg (60.2%); HPLC: $t_R = 9.8$ min (>90); ^{1}H NMR (500 MHz, D_2O, 4 °C): $\delta = 5.45$–5.26 (m, 5H, 5 × $FPro^{\gamma}$), 4.85 (m, 5H, 5 × $FPro^{\alpha}$), 4.60 (m, 2H, 2 × Cys^{α}), 4.54 (m, 10H, 5 × $Hyp^{\alpha,\gamma}$), 4.34 (m, 1H, Pro^{α}), 4.27–3.81 (m, 16H, 8 × Gly^{α}), 3.94, 3.73 (m, 10H, 5 × $FPro^{\delta}$), 3.85, 3.75 (m, 10H, 5 × Hyp^{δ}), 3.55 (m, 2H, Pro^{δ}), 3.13, 2.98 (m, 4H, 2 × Cys^{β}), 2.64, 1.94 (m, 10H, 5 × $FPro^{\beta}$), 2.29, 2.01 (m, 10H, 5 × Hyp^{β}), 2.24–1.83 (m, 4H, $Pro^{\beta,\gamma}$), 1.24 (s, 18H, 2 × *St*Bu) ppm; HRMS (ESI): calcd for $C_{87}H_{129}N_{22}O_{27}S_4F_5$: $m/z =$ 1068.4095 $[M+2H]^{2+}$; found: 1068.4097.

CD measurements: The CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a thermostated cell holder and connected to a PC for signal averaging and processing. All spectra were recorded in the 190–250-nm range in quartz cuvettes of 0.01-cm optical path length. The average of 10 scans is reported and expressed in terms of ellipticity units per mole peptide residues ($[\theta]_R$). The measurements were performed on peptide solutions pre-equilibrated at 4 °C for at least 12 h, at a concentration of 1 mM. The concentrations were determined by weight and an estimated peptide content of about 80%, in analogy to previously synthesized collagenous peptides of similar sequence composition.[14] The thermal denaturation curves were recorded by following the change in intensity of the circular dichroic signal at 225 nm with temperature from 4 to 60 °C with a heating rate of 0.2 °C min^{-1} in quartz cuvettes of 0.1-cm path length. T_m values were derived from the original transition curves by using the manufacture's software.

NMR measurements: NMR spectra were recorded in 90% H_2O/10% D_2O or 100% D_2O at 4 °C on a Bruker DRX 500 spectrometer equipped with pulsed-field-gradient accessories, at a proton frequency of 500.13 MHz. 2D TOCSY experiments with spin-lock periods of 70 ms and the MLEV-17 sequence for isotropic mixing,[36] and 2D NOESY spectra[37] with mixing times between 75 and 300 ms were used for partial assignment according to the method of Wüthrich.[38] For peptide **I**, which contains the (4*S*)-FPro residue, two sets of resonances were observed. The major set of signals could be identified as the result of a *trans* configuration of the peptide bond preceding the (4*S*)-FPro residue by observation of the characteristic H_α(Gly)-H_δ(FPro) NOE. The minor signals correspond to the *cis* peptide bond configuration, as evidenced by the characteristic H_α(Gly)-H_α(FPro) NOE.

For ^{1}H diffusion measurements, stimulated echo experiments with bipolar gradients and diffusion times between 15 and 250 ms were performed in a pseudo-2D fashion by changing the gradient strength from 1 G cm^{-1} to 40 G cm^{-1} in 10 increments. The gradient strength was calibrated to a diffusion constant of 18×10^{-10} m^2 s^{-1} for H_2O in D_2O at 27 °C. Only well-resolved signals were used for extracting diffusion constants from the monoexponential signal decay. NMR diffusion experiments were performed with D_2O solutions at 4 and 27 °C at peptide concentrations of 0.4 mM for peptides **I** and **II** and 0.2 mM for peptide **IV**. The absence of significant aggregation was confirmed at 27 °C by diffusion experiments at a higher concentration (2 mM for peptides **I** and **II** and 0.7 mM for peptide **IV**). The diffusion experiment at 27 °C was repeated after measurements had been made at 4 °C to exclude any change in sample condition due to heating or cooling (such as slow aggregation, precipitation, fibril formation).

Hydrodynamic calculations: Calculation of hydrodynamic radii and the corresponding translational diffusion constants was performed with the HYDROPRO 5a program[39] by using a solvent viscosity of 0.0094 Poise and an effective atomic radius of 3.1 Å. The choice of the effective atomic radius was found to have little influence on the results. Diffusion constants obtained in this way are directly proportional to the solvent viscosity used. For calculation of the hydrodynamic parameter for the unfolded random coil state of (Pro-Hyp-Gly)$_n$ peptides, 100 random conformations were generated by an unrestrained distance geometry protocol followed by a short molecular dynamics refinement, as described in ref. [40]. The average of all individual diffusion constants is reported with the standard deviation for the whole ensemble.

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