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Origin of the stability conferred upon collagen by fluorination

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ABSTRACT

According to a prevailing theory, (2*S*,4*R*)-4-hydroxyproline (Hyp) residues stabilize the collagen triple helix via a stereoelectronic effect that preorganizes appropriate backbone torsion angles for triple-helix formation. This theory is consistent with the marked stability that results from replacing the hydroxyl group with the more electron-withdrawing fluoro group, as in (2*S*,4*R*)-4-fluoroproline (Flp). Nonetheless, the hyperstability of triple helices containing Flp has been attributed by others to the hydrophobic effect rather than a stereoelectronic effect. We tested this hypothesis by replacing Hyp with 4,4-difluoroproline (Dfp) in collagen-related peptides. Dfp retains the hydrophobicity of Flp, but lacks the ability of Flp to preorganize backbone torsion angles. Unlike Flp, Dfp does not endow triple helices with elevated stability, indicating that the hyperstability conferred by Flp is not due to the hydrophobic effect.

Collagen is the principal structural protein in animals. Collagen is composed of a right-handed triple helix formed from three parallel polyproline type-II helices.[1] The individual strands contain repeats of the sequence XaaYaaGly, where Xaa and Yaa are often (2*S*)-proline (Pro) and (2*S*,4*R*)-4-hydroxyproline (Hyp), respectively. The repetitive sequence, large size, and insolubility of native collagen has motivated the study of triple-helix structure and stability with short (⩽30-residue) collagen-related polypeptides (CRPs). Much of our current understanding of the triple helix derives from such studies.[1]

Hyp is introduced in protocollagen strands by the post-translational hydroxylation of prolines in the Yaa position. This modification, which provides dramatic conformational stability to triple-helical CRPs,[2,3] is essential for animal life.[4,5] We have proposed that Hyp stabilizes collagen via a stereoelectronic effect—the gauche effect—which imposes a C^{γ}-exo ring pucker[6] upon the pyrrolidine ring in Hyp and Flp residues (Fig. 1).[7–10] Because the proline backbone torsion angles that accompany a C^{γ}-exo ring pucker are required in the Yaa position of collagen triple helices, Hyp and Flp could stabilize the collagen triple helix by preorganization of the appropriate backbone dihedral angles for triple-helix formation.[1,7,11] Flp is more effective than Hyp at this preorganization because fluorine is more electronegative than oxygen. Later findings

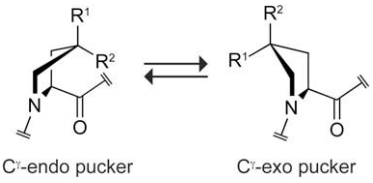

Figure 1. Ring conformations of Pro and its derivatives. The C^{γ}-endo:C^{γ}-exo ratio is ~2 when $R^1 = R^2 = H$ (Pro).[9] The C^{γ}-exo conformation is favored strongly by stereoelectronic effects when $R^1 = OH$ (Hyp) or F (Flp), $R^2 = H$.[6]

are in accord with these conclusions. For example, CRPs containing a diverse manifold of Pro derivatives with C^{γ}-exo pucker in the Yaa position are also hyperstable.[12–15]

A stereoelectronic origin for collagen stability has, however, been questioned by others.[16,17,34–36] In particular, Hyp and Flp have been proposed to impart stability by distinct means. Differential scanning calorimetry experiments show that the hyperstability of triple helices formed from $(ProFlpGly)_{10}$ is dominated by entropic effects, whereas that of $(ProHypGly)_{10}$ is dominated by enthalpic effects.[16,17] Because the hydrophobic effect is manifested in entropy, Flp has been proposed to stabilize the triple helix by the energetically favorable segregation of hydrophobic fluorine atoms[19] from water upon triple-helix folding. (It is noteworthy, however, that triple-helix stabilization by preorganization would likewise arise from an entropic effect.)

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The hydrophobic effect is a dominant force in the folding of globular proteins.[20] Moreover, substantial precedence exists for enhancing protein stability by incorporating hydrophobic, fluorinated amino-acid residues within protein cores.[21–25] The role for the hydrophobic effect in collagen stability is, however, minimized by collagen being a fibrous protein that lacks a substantive core. Indeed, earlier studies suggested that the hydrophobic effect is not important for triple-helix stability.[26,27] Still, we sought to ascertain whether triple-helix stabilization by Flp is due to a hydrophobic effect or a stereoelectronic effect. We reasoned that (2*S*)-4,4-difluoroproline (Dfp; Fig. 1 with $R^1 = R^2 = F$) would be useful in this regard, because Dfp would exhibit a similar hydrophobic effect but ambiguous stereoelectronic effects.

First, we determined whether Dfp and Pro do indeed have similar preorganizational capacity. Substantial experimental evidence supports this treatise. Moroder and co-workers used NMR spectroscopy to conclude that Ac-Dfp-OMe prefers the C^γ-endo ring pucker in water.[28] Our own conformational analyses using ^1H and ^{19}F NMR spectroscopy reveal a significant population of both the C^γ-exo and C^γ-endo conformers of Ac-Dfp-OMe in both water and chloroform (see Supplementary data), suggesting that the C^γ-exo and C^γ-endo ring puckers of Dfp are of similar energy. This finding indicates that the preorganizational capacity of Dfp is much closer to that of Pro (which has only a slight preference for the C^γ-endo ring pucker[9]) than to that of Flp (which prefers the C^γ-exo ring pucker strongly due to the gauche effect[9]).

Additional experimental evidence that Pro and Dfp have similar triple-helix preorganizational capacity can be obtained by observing the equilibrium constant ($K_{trans/cis}$) for the isomerization of their peptide bonds. Typically, the $K_{trans/cis}$ value of a Pro derivative is correlated to its ring pucker.[9,28,29] Thus, $K_{trans/cis}$ values for Ac-Xaa-OMe model systems can provide a valuable measure of the relative conformational preferences of Pro derivatives. We used ^1H NMR spectroscopy to show that Ac-Dfp-OMe has $K_{trans/cis} = 3.6$ in water (see Supplementary data). This value is similar to that of Ac-Pro-OMe, which has $K_{trans/cis} = 4.6$, but divergent from that of Ac-Flp-OMe, which has $K_{trans/cis} = 6.7$.[7] Finally, replacement of Pro with Dfp in a barstar variant does not alter its conformational stability, whereas the monofluorinated derivatives Flp and its diastereomer, (2*S*,4*S*)-4-fluoroproline, have marked effects.[28]

Next, we resorted to hybrid density functional theory to explore further the conformational preferences of Dfp. Geometry optimizations and frequency calculations were performed at the B3LYP/6-311+G(2d,p) level of theory on four preferred conformations of Ac-Dfp-OMe in the gas phase. Briefly, we found that the C^γ-endo ring pucker is favored by 0.3 kcal/mol for Ac-Dfp-OMe in the cis conformation, whereas the C^γ-exo ring pucker is favored by 0.5 kcal/mol over a slightly distorted C^γ-endo ring pucker in the trans conformation. These calculated conformational preferences are closer to those of Ac-Pro-OMe than to those of Ac-Flp-OMe.[9] Hence, Pro and Dfp should have roughly similar triple-helix preorganization capacity. Importantly, the C^γ-exo conformations adopted by Ac-Dfp-OMe have appropriate dihedral angles for the Yaa position of the collagen triple helix, indicating that Dfp should be acceptable in the Yaa position of a triple helix.

Then, we compared the effect of Dfp and Flp on triple-helix stability. Our model of a $(ProFlpGly)_n$ triple helix shows that the fluorine atoms protrude tangentially from the triple helix and are partially buried (Fig. 2A). In a hypothetical $(ProDfpGly)_n$ triple helix, the fluorine atoms corresponding to those in a $(ProFlpGly)_n$ triple helix are buried to the same extent. The additional fluorine atoms protrude radially from the triple helix and are exposed fully to solvent (Fig. 2B). Because triple-helix folding partially buries one fluorine atom per XaaYaaGly triplet in both CRPs, any stability arising from the hydrophobic effect should be similar in the two fluorinated triple helices. Accordingly, if the effects of fluorination on

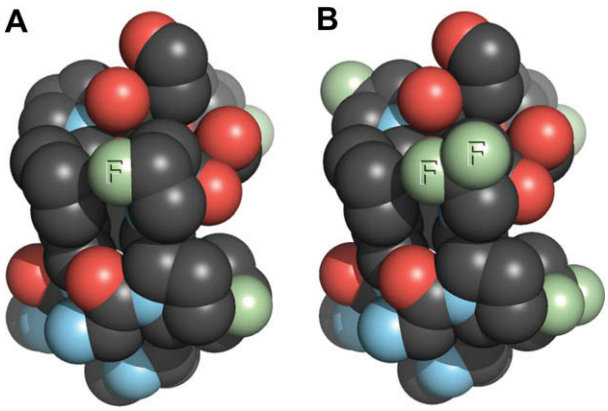

Figure 2. Space-filling models of fluorinated triple helices constructed from the three-dimensional structure of a $(ProHypGly)_n$ triple helix (PDB entry 1CAG[18]) by replacing an OH, or OH and H of Hyp with F by using the program PyMOL (Delano Scientific, Palo Alto, CA). (A) Segment of a model $(ProFlpGly)_n$ triple helix. (B) Segment of a model $(ProDfpGly)_n$ triple helix.

triple-helix stability are due primarily to the conformational preferences of Pro derivatives, then replacing Pro with Dfp in a CRP should have little effect on triple-helix stability. In contrast, replacing Pro with Flp should enhance triple-helix stability. On the other hand, if the partial burial of hydrophobic fluorine atoms is important for the hyperstability of $(ProFlpGly)_n$, then both Dfp- and Flp-containing triple helices should have markedly enhanced thermal stability relative to $(ProProGly)_n$ triple helices. Peptides appropriate for this experiment were prepared by segment condensation on a solid-phase using Fmoc-XaaYaaGly-OH amino acid trimers prepared in solution (see Supplementary data). We could not analyze the conformational properties of the CRP $(ProDfpGly)_{10}$ because of its poor solubility in water. Host–guest CRPs are generally reliable models for the effects of amino acid substitution on triple-helix structure and stability.[27,30] Hence, we synthesized the host–guest CRPs Ac-$(ProProGly)_{10}$-NH_2 (**Pro-CRP**), Ac-$(ProProGly)_4$–ProDfpGly–$(ProProGly)_5$-NH_2 (**Dfp-CRP**), and Ac-$(ProProGly)_4$–ProFlpGly–$(ProProGly)_5$-NH_2 (**Flp-CRP**). We introduced N-terminal acetyl and C-terminal amido groups to eliminate unfavorable Coulombic interactions between the CRP termini.

We analyzed the conformational stability of the triple helices formed by the three CRPs with circular dichroism (CD) spectroscopy. Values of T_m, which is the temperature at the midpoint of the thermal transition, depend on CRP concentration.[31] Accordingly, the concentrations of stock solutions were determined by amino-acid analysis and then adjusted to ensure that each CRP was analyzed at the same concentration. After incubation at $\leqslant 4$ °C for $\geqslant 24$ h to allow triple helices to form, all three CRPs displayed the signature CD spectrum for a collagen triple helix at 4 °C, with maxima near 225 nm (Fig. 3A). Upon heating, all three CRPs underwent cooperative thermal transitions (Fig. 3B). We found that the **Dfp-CRP** and the **Pro-CRP** triple helices had T_m values within experimental error. The T_m value of a **Flp-CRP** triple helix was, however, ~6 °C higher (Fig. 3B). These data suggest that the triple-helix stabilizing effects of Flp are attributable predominantly to its enhanced preference for the C^γ-exo ring pucker. Apparently, the thermodynamic advantage afforded by the partial burial of hydrophobic fluorine atoms near the center of the triple helix is minimal.[32]

Two caveats to this conclusion must be considered. First, although fluorine is often regarded as an isosteric replacement for hydrogen, it does have a larger covalent radius ($r_H = 0.31$ Å; $r_F = 0.57$ Å).[33] Hence, we must consider whether appending a second fluoro group to C^γ engenders any steric hindrance. Molecular

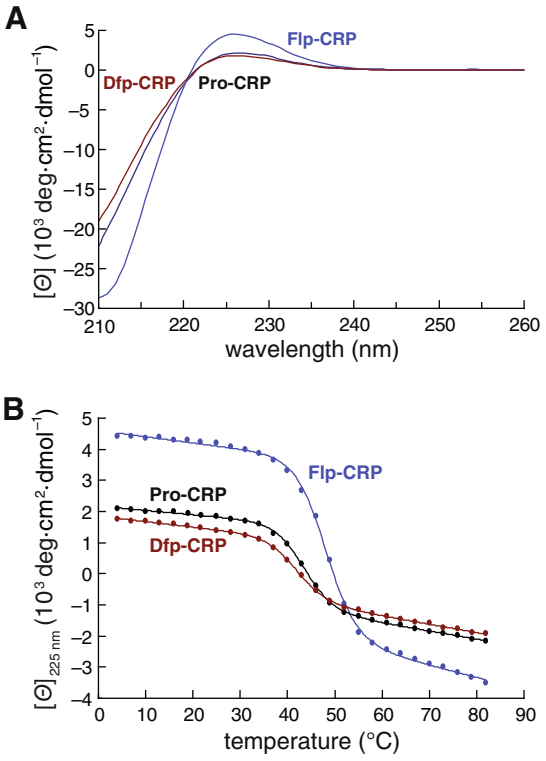

Figure 3. Conformational analysis of **Pro-CRP**, **Flp-CRP**, and **Dfp-CRP** by CD spectroscopy. (A) Spectra of peptide solutions (90 μM in 50 mM acetic acid) incubated at ⩽4 °C for ⩾24 h. (B) Effect of temperature on thermal ellipticity at 225 nm. Data were recorded at 3-°C intervals after a ⩾5-min equilibration. Values of T_m (±1 °C) from the average of at least three experiments were **Pro-CRP**, 43 °C; **Flp-CRP**, 48 °C; and **Dfp-CRP**, 42 °C.

modeling (Fig. 2B) suggests that steric hindrance is not a complicating factor. More convincingly, we demonstrated previously that a methyl group can be incorporated in the same location without deleterious effects on triple-helix stability.[13] Thus, steric hindrance should not confound our conclusions. Secondly, we must consider whether any favorable or unfavorable electrostatic interaction is introduced by the additional fluoro group. Molecular modeling does not reveal any new electrostatic interactions between proximal polarized atoms (Fig. 2B). Moreover, the energetic consequences of any such electrostatic interaction are minimized by our use of a host–guest model system.

In conclusion, we find that the hydrophobic effect is not a dominant stabilizing force for the collagen triple helix (A role for hydrophobicity in higher-order collagen assembly is, however, plausible.[26,27]) Specifically, our findings validate the theory that $(ProFlpGly)_{10}$ triple helices are stabilized by preorganization derived from a stereoelectronic effect that favors backbone torsion angles appropriate for triple-helix formation, rather than by an enhanced hydrophobic effect. Dfp, which has a preorganizational capacity similar to that of Pro and a hydrophobic effect similar to that of Flp, provides no net stabilization to the collagen triple helix. Hence, the burial of fluoro groups upon triple-helix formation provides negligible benefit. Our findings are consistent with the role of the 4*R*-hydroxyl group of Hyp being to stabilize the collagen triple helix via preorganization.

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Supplementary data

Supplementary data (detailed procedures for the syntheses and analyses of Ac-Dfp-OMe and the peptides) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.168.

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