A hyperstable collagen mimic

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Background: Collagen is the most abundant protein in animals. Each polypeptide chain of collagen is composed of repeats of the sequence: Gly–X–Y, where X and Y are often L-proline (Pro) and 4(*R*)-hydroxy-L-proline (Hyp) residues, respectively. These chains are wound into tight triple helices of great stability. The hydroxyl group of Hyp residues contributes much to this conformational stability. The existing paradigm is that this stability arises from interstrand hydrogen bonds mediated by bridging water molecules. This model was tested using chemical synthesis to replace Hyp residues with 4(*R*)-fluoro-L-proline (Flp) residues. The fluorine atom in Flp residues does not form hydrogen bonds but does elicit strong inductive effects.

Results: Replacing the Hyp residues in collagen with Flp residues greatly increases triple-helical stability. The free energy contributed by the fluorine atom in Flp residues is twice that of the hydroxyl group in Hyp residues. The stability of the Flp-containing triple helix far exceeds that of any untemplated collagen mimic of similar size.

Conclusions: Bridging water molecules contribute little to collagen stability. Rather, collagen stability relies on previously unappreciated inductive effects. Collagen mimics containing fluorine or other appropriate electron-withdrawing substituents could be the basis of new biomaterials for restorative therapies.

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Introduction

Collagen is an abundant fibrous protein [1]. Vertebrates produce at least 19 different types of collagen [2]. In each of these types, the polypeptide chains are composed of approximately 300 repeats of the sequence: Gly–X–Y, where X is often an L-proline (Pro) residue and Y is often a 4(*R*)-hydroxy-L-proline (Hyp) residue (Figure 1). For example, in the common type I form of collagen, Gly–Pro–Hyp triplets are the most common triplets, accounting for 12% of the sequence [3]. The remainder of type I collagen includes 22% Gly–Pro–Y, 22% Gly–X–Hyp and 44% Gly–X–Y triplets, with no imino acids. In connective tissue such as bone, tendon, cartilage, ligament, skin, blood vessels and teeth, collagen chains are wound in tight triple helices [4]. These helices are organized into fibrils of great tensile strength [5]. Varying the arrangement and cross-linking of collagen fibrils enables vertebrates to support stress in one dimension (tendons), two dimensions (skin) and three dimensions (cartilage). Abnormalities in collagen structure are associated with a wide variety of human diseases [6–8].

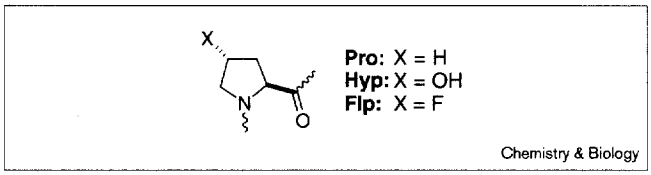
The hydroxyl groups of collagen Hyp residues have an important role. Hyp residues are not incorporated into collagen by ribosomes. Rather, the hydroxylation of proline residues occurs after collagen biosynthesis but before the chains form a triple helix. Hydroxylation could therefore be important for both collagen folding and collagen stability. In 1973, seminal work by Prockop and coworkers [9] demonstrated that the hydroxyl group of the Hyp residue dramatically increases the thermal stability of triple-helical collagen. For example, the melting temperature of a triple helix of $(Pro–Hyp–Gly)_{10}$ chains is 58°C, but that of a triple helix of $(Pro–Pro–Gly)_{10}$ chains is only 24°C [10]. In addition, the rate at which $(Pro–Hyp–Gly)_{10}$ chains fold into a triple helix is substantially greater than the corresponding rate for $(Pro–Pro–Gly)_{10}$ chains [11].

What is the basis for the stability conferred by the hydroxyl group of Hyp residues? Models based on the structure of triple-helical collagen and conformational energy calculations suggest that hydrogen bonds cannot form between the hydroxyl group of Hyp residues and the mainchain of the same triple helix [12]. Several other models have been proposed, however, in which one or more water molecules form a bridge between the hydroxyl group and a mainchain oxygen (for reviews, see [13,14]).

In 1994, the first high-resolution three-dimensional structure of triple-helical collagen was determined by X-ray diffraction analysis [15]. In this structure (Figure 2), the Hyp residues do indeed have water molecules bound to their hydroxyl groups. Individual Hyp residues bond most often to two water molecules, forming an interchain link to the amide oxygen of another Hyp residue [16].

Are water bridges responsible for collagen stability? Some experimental data are inconsistent with this hypothesis. For

Figure 1



Structure of L-proline, 4(*R*)-hydroxy-L-proline (Hyp) and 4(*R*)-fluoro-L-proline (Flp) residues.

example, triple helices of $(Pro–Pro–Gly)_{10}$ and $(Pro–Hyp–Gly)_{10}$ are stable in either methanol or propane-1,2-diol, and the Hyp residues confer additional stability in these anhydrous conditions [17]. Also, heat capacity measurements are inconsistent with collagen having more than one tightly bound water per six Gly–X–Y units [18].

We suspected that bridging water molecules do not contribute significantly to collagen stability. In addition to heeding the contrary experimental data [17,18], we reasoned that immobilizing two water molecules for each Hyp residue would evoke an enormous entropic cost. Hyp comprises approximately 10% of the residues in most forms of collagen. Immobilizing two water molecules per Hyp residue requires that > 500 water molecules be immobilized to stabilize one molecule of triple-helical collagen. Because water molecules contribute approximately half of the weight to typical protein crystals, we suspected that the water bridges observed in crystalline collagen could be artifactual rather than meaningful. We therefore sought an alternative explanation for the origin of collagen stability. A preliminary report of our findings has appeared elsewhere [19].

Results and discussion

A new hypothesis: inductive effects stabilize collagen

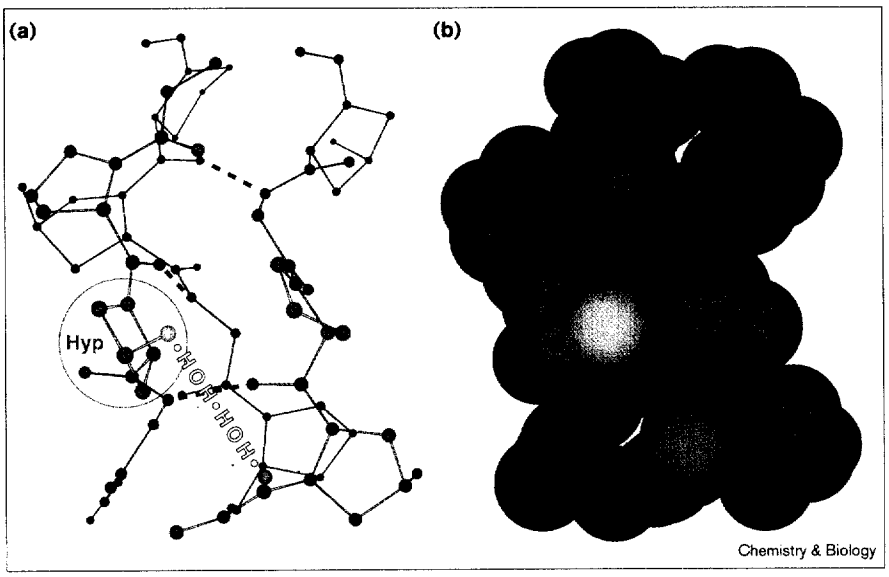
Electron-withdrawing groups can alter the properties of molecules [20]. The electronegative oxygen in a hydroxyl group is effective at withdrawing electron density by through-bond and through-space interactions [21]. This inductive effect is apparent in our previous data on the structure [22] and properties [23] of small-molecule amides related to collagen. Specifically, these data are consistent with the manifestation of an inductive effect that draws electron density towards the hydroxyl group in Hyp residues.

Testing the new hypothesis

To distinguish between the contributions of hydrogen bonding and inductive effects to collagen stability, we chose to replace the hydroxyl groups in Hyp residues with fluorine atoms [24,25]. We chose fluorine because it is the most electronegative atom and so elicits a large inductive effect, and because organic fluorine does not form hydrogen bonds. This latter attribute of fluorine warrants elaboration.

Anionic fluoride forms strong hydrogen bonds. Indeed, the hydrogen bond in gas-phase $[F\cdots HF]^{1-}$ is the strongest known [26,27]. In contrast to anionic fluoride, organic fluorine is a poor hydrogen-bond acceptor. X-ray diffraction analyses by Glusker and coworkers [28,29] as well as extensive structure database surveys [30–32] have revealed only a few crystalline organofluorine compounds that display short CF···HX distances, where X = C, N or O. In addition, a presumably intimate CF···HN interaction does not stabilize DNA double helices [33]. The weakness of the CF···HX interaction is probably due to the high charge of the fluorine nucleus, which compacts the

Figure 2



Structure of a segment (3 × 4 residues) of triple-helical collagen [15]. **(a)** Ball-and-stick model highlighting one Hyp residue and its associated interstrand water bridge. The three HypC=O···HNGly interstrand hydrogen bonds are indicated by dashed lines. **(b)** Space-filling model.

surrounding electrons. As a familiar comparison of hydrogen bonding to C–OH versus C–F, consider the properties of polyvinyl alcohol (PVA) and polytetrafluoroethylene (i.e., Teflon™ [34]). These two polymers are similar in composition, but only PVA is soluble in water. (Interestingly, no known solvent will dissolve Teflon™.) A comparison of the aqueous solubilities of CH_3OH (which is miscible with water) with CH_3F (which is virtually insoluble in water) is likewise enlightening.

A 4(*R*) fluorine atom has substantial effects on the properties of proline residues. For example, we showed previously that the nitrogen pK_a of the conjugate acid of 4(*R*)-fluoro-L-proline (FlpOH; 9.23) is lower than that of HypOH (9.68) and ProOH (10.8) [23]. To reveal any such effects in a system related to collagen, we synthesized compounds of the form AcYOMe, where Y is a Pro, Hyp or Flp residue. In these compounds, the inductive effects of the hydroxyl group and fluorine atom are propagated to the nitrogen. For example, the nitrogen of AcFlpOMe is more pyramidal than that of AcHypOMe or AcProOMe [22]. This result indicates that the nitrogen of AcFlpOMe has greater sp^3 character and hence higher electron density. In addition, amide I vibrational modes, which result primarily from the C=O stretching vibration, decrease in the order: AcProOMe > AcHypOMe > AcFlpOMe. Moreover, the rate constants for amide-bond isomerization are greater for AcFlpOMe than for AcProOMe [23]. Each of these results is consistent with the traditional picture of amide resonance [35,36] coupled with an inductive effect that results in a higher bond order in the amide C=O bond and a lower bond order in the amide C–N bond [23].

A hyperstable collagen mimic

Here, we have compared directly the stability conferred to a collagen triple helix by a 4(*R*) hydroxyl group with that conferred by a 4(*R*) fluorine atom. To do so, we synthesized a collagen-like peptide containing Pro–Flp–Gly units. Recently, Goodman and coworkers [37] have incorporated non-natural amino acid residues into small templated mimics of collagen, fostering elegant analyses. Nonetheless, to enable comparisons with the majority of extant data, we chose to prepare untemplated mimics. Protected Flp was produced from protected 4(*S*)-hydroxy-L-proline by using morpholinosulfur trifluoride (morph-DAST). Fmoc–Pro–Flp–Gly-OH units were assembled by standard solution-phase procedures from Flp and commercial reagents. $(Pro–Flp–Gly)_{10}$ was synthesized by segment condensation of Fmoc–Pro–Flp–Gly-OH units on a solid support.

Flp residues allow triple-helix formation. Sedimentation velocity experiments with an analytical ultracentrifuge indicate that $(Pro–Flp–Gly)_{10}$ chains form a complex of molecular mass (8.0 ± 0.1) kDa. The expected molecular mass of a $(Pro–Flp–Gly)_{10}$ trimer ($C_{360}H_{480}N_{90}O_{90}F_{30}$) is 8078 Da. The fluorescence of 1-anilinonaphthalene-8-sulfonate (ANS) [38], which has affinity for molten globules [39], is unchanged by the presence of an excess of $(Pro–Flp–Gly)_{10}$ trimer. This result suggests that the tertiary structure of the trimer is packed tightly. At low temperature, the circular dichroism (CD) spectrum of the complex formed by $(Pro–Flp–Gly)_{10}$ chains is indistinguishable from CD spectra of complexes composed of $(Pro–Hyp–Gly)_{10}$ or $(Pro–Pro–Gly)_{10}$ chains (Figure 3). All three polymers have a CD spectrum with a positive peak at 225 nm and a stronger negative peak at 200–210 nm, which are defining characteristics of a collagen triple helix [40]. The ellipticity at 225 nm of each triple helix decreases with increasing temperature, which is characteristic of denaturation of the triple helix. This temperature-dependent change in conformational stability was observed in two solvents: 50 mM acetic acid, which stabilizes triple helices by protonating the carboxy-terminal carboxylates and thereby eliminating unfavorable Coulombic interactions, and phosphate-buffered saline (PBS), which mimics the relevant physiological environment.

Flp residues enhance triple-helix stability. In both 50 mM acetic acid and PBS, the values of T_m and $\Delta\Delta G_m$ for the three triple helices differ dramatically, increasing in the order: $(Pro–Pro–Gly)_{10} < (Pro–Hyp–Gly)_{10} < (Pro–Flp–Gly)_{10}$ (Figure 3; Table 1). This order is inconsistent with collagen stability arising largely from bridging water molecules, but it is consistent with the manifestation of an inductive effect from the electronegative substituent.

The $(Pro–Flp–Gly)_{10}$ triple helix is hyperstable. On average, each of the 30 hydroxyl groups in a $(Pro–Hyp–Gly)_{10}$ triple helix contributes $\Delta\Delta G_m = 0.2$ kcal/mol to stability (Table 1). In marked contrast, each fluorine atom in a $(Pro–Flp–Gly)_{10}$ triple helix contributes $\Delta\Delta G_m = 0.4$ kcal/mol. The stability of the $(Pro–Flp–Gly)_{10}$ triple helix far exceeds that of any untemplated collagen mimic of similar size.

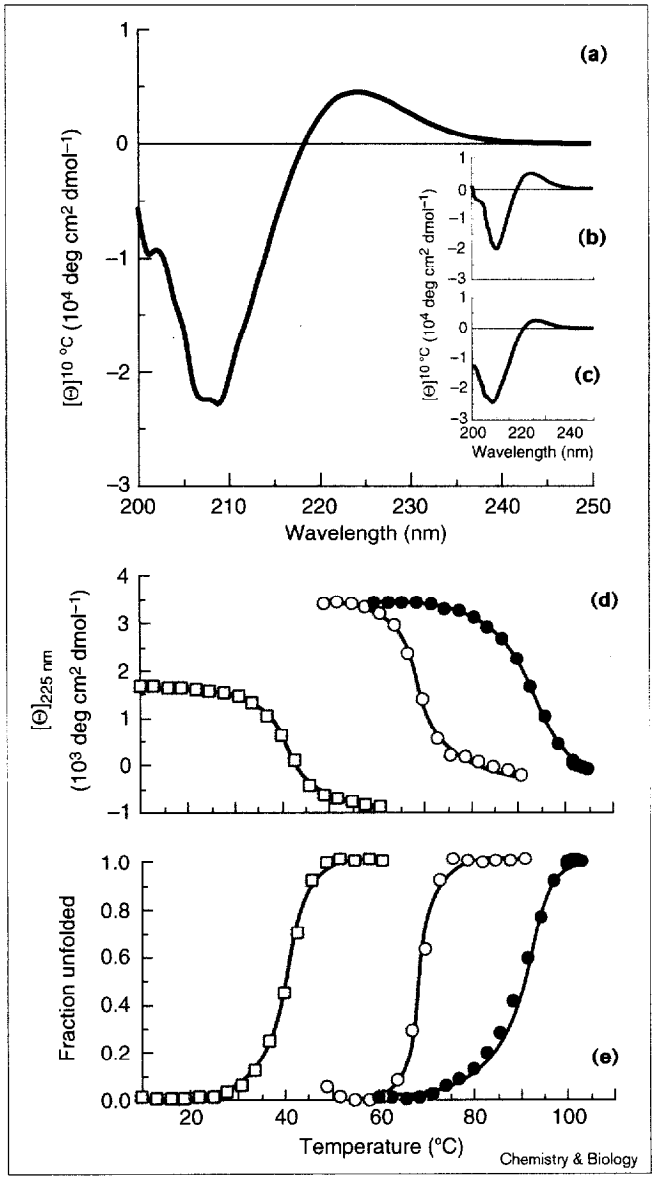
Basis for hyperstability

Our data provide strong evidence that inductive effects of the 4(*R*) hydroxyl group contribute more than do water-mediated hydrogen bonds to the stability of triple-helical collagen. In addition, we have described the most stable known mimic of triple helical collagen. What is the precise basis for the additional stability conferred by the 4(*R*) fluorine atom? We have identified three factors that could be responsible, alone or in concert, for this hyperstability.

Preorganization of prolyl peptide bond conformation

The Hyp residues in crystalline collagen do not have unusual van der Waals contacts, or ϕ or ψ bond angles. But, ω angles (which are the dihedral angles of the peptide bond) merit consideration. The *trans* isomer (i.e., the isomer with $\omega = 180°$) of a prolyl peptide bond is only

Figure 3



Structure and stability of collagen mimics in 50 mM acetic acid. Data in phosphate-buffered saline were similar. **(a)** Circular dichroism (CD) spectrum of a $(Pro–Flp–Gly)_{10}$ triple helix at 10°C. **(b)** CD spectrum of a $(Pro–Hyp–Gly)_{10}$ triple helix at 10°C. **(c)** CD spectrum of a $(Pro–Pro–Gly)_{10}$ triple helix at 10°C. **(d)** Effect of temperature on the ellipticity at 225 nm of $(Pro–Flp–Gly)_{10}$ (red circles), $(Pro–Hyp–Gly)_{10}$ (blue open circles), and $(Pro–Pro–Gly)_{10}$ (green open squares) triple helices. **(e)** Effect of temperature on the conformational stability of $(Pro–Flp–Gly)_{10}$ (red circles), $(Pro–Hyp–Gly)_{10}$ (blue open circles), and $(Pro–Pro–Gly)_{10}$ (green open squares) triple helices.

slightly favored over the *cis* isomer (i.e., the isomer with $\omega = 0°$). Yet, all of the peptide bonds in triple-helical collagen are in the *trans* conformation [15]. This requirement leads to the hypothesis that Hyp residues could enhance collagen stability by favoring the *trans* conformation, thereby preorganizing the strands to more closely resemble

Table 1

Thermal stabilities of triple-helical collagen mimics.

	50 mM Acetic acid		Phosphate-buffered saline	
Chain	T_m (°C)	$\Delta\Delta G_m$ (kcal/mol)	T_m (°C)	$\Delta\Delta G_m$ (kcal/mol)
$(Pro–Pro–Gly)_{10}$	41 ± 1	0	36 ± 2	0
$(Pro–Hyp–Gly)_{10}$	69 ± 1	6.4 ± 0.4	67 ± 1	6.5 ± 0.5
$(Pro–Flp–Gly)_{10}$	91 ± 1	12 ± 1	87 ± 2	11 ± 1

Values of $\Delta\Delta G_m$ are calculated at the T_m of $(Pro–Pro–Gly)_{10}$.

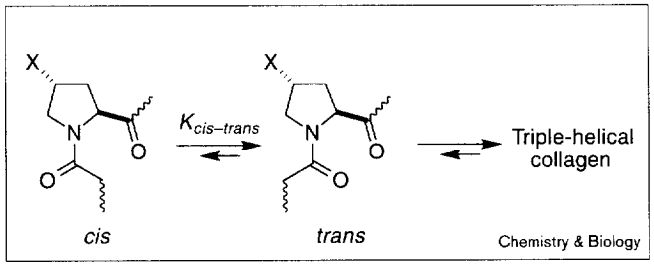
a collagen triple helix (Figure 4). Existing data support this hypothesis. At 37°C in water or dioxane, $K_{cis–trans}$ increases in the order: AcProOMe < AcHypOMe < AcFlpOMe [23]. This ordering has also been observed for Pro and Hyp residues in other contexts [41,42]. Moreover, the amide bond of crystalline AcProOMe is in the *cis* conformation, which is the minor isomer in solution. In crystalline AcHypOMe and AcFlpOMe, however, the amide bonds are in the *trans* conformation.

How does the inductive effect increase $K_{cis–trans}$? The lengths of the bonds between sp^3-hybridized carbon atoms (particularly $C^\gamma–C^\delta$) in the pyrrolidine ring are significantly shorter in AcHypOMe and AcFlpOMe than in AcProOMe [22]. A decrease in the length of the $C^\gamma–C^\delta$ bond could diminish steric clashes in the *trans* isomer, and could thereby favor the *trans* isomer found in the collagen triple helix.

Dipole–dipole interactions mediated by a gauche effect

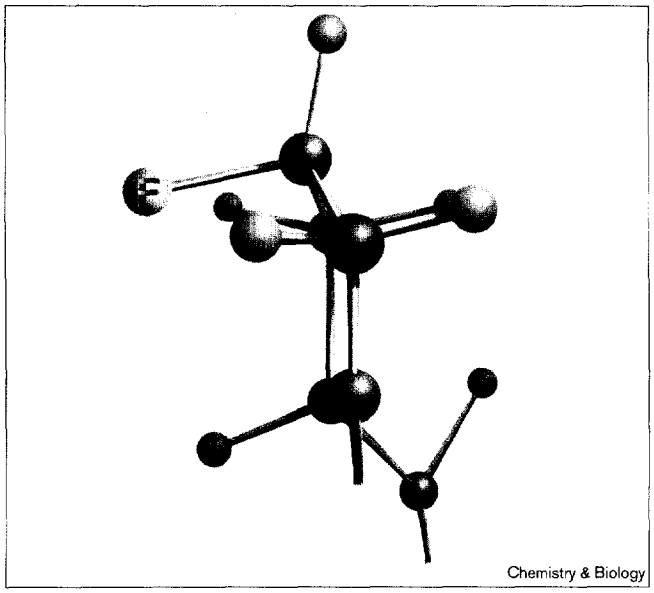
Electron-withdrawing groups can alter the preferred conformation of molecules. For example, the tendency of molecules to adopt the conformation that has the maximum number of gauche interactions between adjacent polar bonds has been termed the 'gauche effect' (for leading references, see [43–45]). The gauche effect is

Figure 4



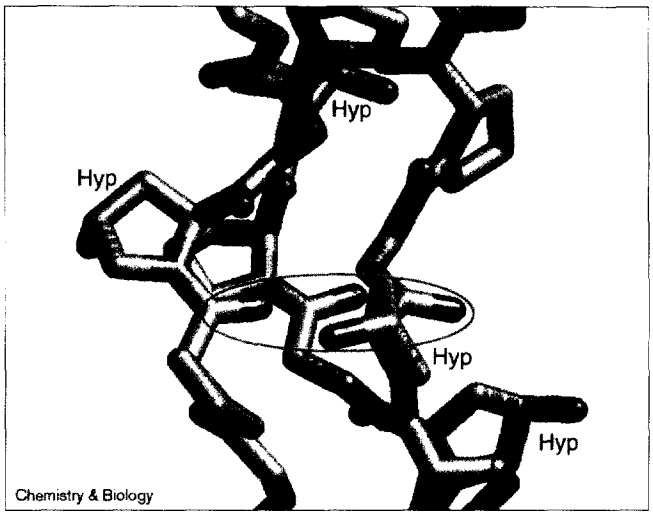
Relationship between *cis–trans* prolyl peptide bond isomerization and the formation of collagen triple helix, which contains only *trans* peptide bonds. X = H (Pro), OH (Hyp) or F (Flp).

Figure 5



Structure of the pyrrolidine ring in crystalline AcFlpOMe. The ring pucker is C^{γ}-exo, with an N–C^{δ}–C^{γ}–F dihedral angle of (−85 ± 1)° for the two molecules in the unit cell.

Figure 6



Structure of a segment (3 × 4 residues) of triple helical collagen [15]. The C–O bond of each Hyp residue is oriented antiparallel to three proximal C = O bonds, as indicated by the oval. The view is different from that in Figure 2.

caused by the donation of electron density from molecular orbitals that are electron-rich to those that are electron-poor [46]. In X–CH_2–CH_2–X systems, the σ* anti-bonding orbital of the carbon atom in a C–X bond is electron-poor if X is an electronegative atom. This deficiency can be overcome by the donation of electron density from the relatively electron-rich σ bonding orbital of a C–H bond. Both C–X σ* anti-bonding orbitals can receive electron density from C–H bonding orbitals only if the two X atoms are gauche, that is, have a dihedral angle of +60° or −60°. Gauche effects within ribose and deoxyribose sugars have a strong influence on the conformational preferences of nucleic acids [47,45].

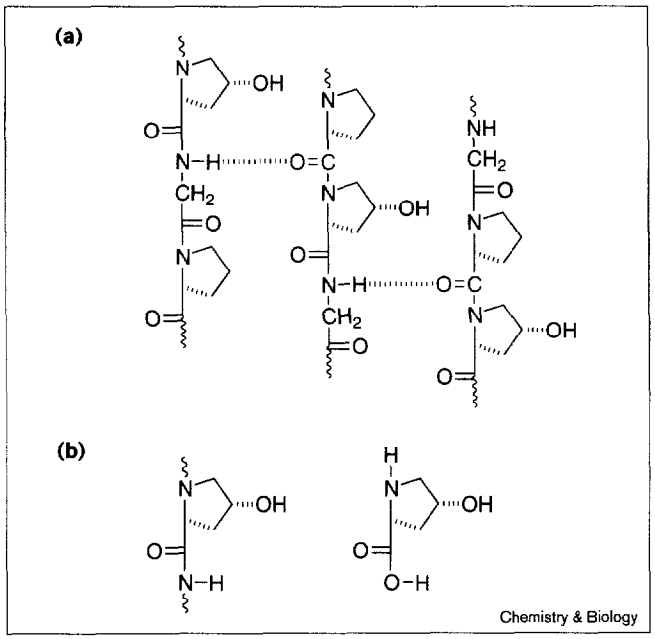
Manifestation of a gauche effect in the N–C^{δ}–C^{γ}–O^{δ} dihedral angle of an Hyp residue would impose a C^{γ}-exo pucker upon the pyrrolidine ring. The rings in crystalline AcHypOMe [14], as well as in crystalline AcFlpOMe [22] and in FlpOH [48], do indeed have a C^{γ}-exo pucker (Figure 5). In contrast, the ring in crystalline AcProOMe has a C^{γ}-endo pucker [22]. Moreover, the rings in all of the Hyp residues in crystalline collagen have a C^{γ}-exo pucker, whereas the rings in half of the Pro residues have C^{γ}-exo puckers and the remainder have C^{γ}-endo puckers [15]. In solution, Hyp and Flp residues are much more rigid than are Pro residues [49–51], and strongly prefer a C^{γ}-exo pucker [48,52–55]. These data are consistent with the manifestation of a gauche effect in Hyp and Flp residues. In a collagen triple helix, the gauche effect organizes the dipole of the C^{γ}–O^{δ} bond of Hyp residues to be antiparallel to proximal amide-bond dipoles (Figure 6). The analogous dipole in the C^{γ}–F bond of Flp residues is stronger, and could thus partake in stronger dipole–dipole interactions. To our knowledge, the gauche effect has not been invoked previously to explain any aspect of protein structure.

Replacing the Hyp residues in collagen with 4(*S*)-hydroxy-L-proline residues prevents triple-helix formation [56]. One explanation for this result is based on the formation of a hydrogen bond between the hydroxyl group and the amide oxygen, which are situated endo on the pyrrolidine ring of 4(*S*)-hydroxy-L-proline residues. Such a hydrogen bond would perturb the orientation of the amide from its conformation in a collagen triple helix. An alternative explanation invokes the gauche effect. The effect of dipole–dipole interactions depends on the orientation of one dipole relative to another. The manifestation of a gauche effect would enforce a C^{ψ}-endo pucker on the pyrrolidine rings of 4(*S*)-hydroxy-L-proline residues. In that pucker, the N–C^{δ}–C^{γ}–O^{δ} dihedral angle differs by nearly 120° from that in Hyp residues. In this orientation, the dipole of the C^{γ}–O^{δ} bond of 4(*S*)-hydroxy-L-proline residues is nearly orthogonal to proximal amide bond dipoles.

ProC=O···HNGly hydrogen bond strength

A ladder of interstrand ProC=O···HNGly hydrogen bonds runs through the middle of the collagen triple helix (Figures 2 and 7a). These solvent-inaccessible hydrogen bonds are likely to contribute greatly to collagen stability. The strength of a hydrogen bond increases with the acidity of the hydrogen-bond donor [27]. The HNGly proton is likely to be made more acidic by an inductive effect from the neighboring Hyp residue. The NH proton in the

Figure 7



Hydrogen bonding in triple-helical collagen. **(a)** Ladder of interstrand ProC=O···HNGly hydrogen bonds [15], which is also apparent in Figure 2. **(b)** Isologous relationship between the NH proton in the ProC=O···HNGly hydrogen bond and the C(O)OH proton of HypOH.

ProC=O···HNGly hydrogen bond of a HypC(O)NHGly segment of collagen is isologous to the C(O)OH proton of HypOH (Figure 7b). The pK_a of the C(O)OH proton of HypOH is 1.82, and that of ProOH is 1.95 [57]. Thus, an inductive effect from the 4(*R*) hydroxyl group of the adjacent Hyp residue is likely to increase the strength of the ProC=O···HNGly hydrogen bond.

The strength of a hydrogen bond also increases with the basicity of the hydrogen-bond acceptor [27]. The inductive effect from the 4(*R*) hydroxyl group of the adjacent Hyp residue should decrease the basicity of the ProC=O oxygen. Direct evidence for this effect is apparent from amide I vibrational modes, which indicate the amide C=O bond order increases with electron-withdrawal by the 4(*R*) substituent. This trend suggests that the ProC=O oxygen has lower charge and is less basic in ProC(O)NHFlp and ProC(O)NHHyp segments than in ProC(O)NHPro segments. This inductive effect should decrease the stability of triple-helical collagen. The net contribution of the inductive effect to the ProC=O···HNGly hydrogen bond will be the sum of these stabilizing and destabilizing effects, mediated by the effects on the H_2O···HNGly and ProC=O···H_2O hydrogen bonds in unfolded strands [58,59].

Significance

A long-standing paradigm has held that the stability of triple-helical collagen is mediated by hydrogen bonds from the hydroxyl groups of the prevalent 4(*R*)-hydroxy-L-proline (Hyp) residues. We have refuted this model. Collagen chains in which fluorine atoms replace the hydroxyl groups form triple helices with extraordinary stability. This finding provides the foundation for the development of new collagen-based biomaterials [60,61] useful for therapies such as tissue welding [62] and skin grafting [63] as well as for other restorative procedures.

Materials and methods

Synthesis of (Pro–Flp–Gly)$_{10}$

(Pro–Fly–Gly)$_{10}$ was synthesized by a convergent route based on FmocProFlpGlyOH tripeptide units, where Fmoc is *N*-9-fluorenylmethoxycarbonyl. We chose to synthesize a peptide comprised of ten tripeptide units because (Pro–Pro–Gly)$_n$ triple helices are stable at ambient temperature only if n = 10 [10]. By placing a glycine residue at the carboxyl terminus of these units, we avoided problems caused by racemization (via azlactone formation) during the solid-phase coupling of activated peptide fragments [64]. The Flp residues were synthesized by treatment of protected 4(*S*)-hydroxy-L-proline with morph-DAST, as we described previously [22,23]. This reagent converts a hydroxyl group into a fluorine atom with inversion of stereochemistry.

FmocProFlpGlyOH was synthesized by using standard solution-phase techniques [65]. The unit was assembled with N^a-*tert*-butyloxycarbonyl (Boc) rather than Fmoc protecting groups because Fmoc cannot withstand the Pd/C-catalyzed hydrogenolysis that is necessary to deprotect the glycine residue [64]. Briefly, reaction of BocFlpOSu with GlyOBn gave a protected dipeptide, BocFlpGlyOBn. Removal of the Boc group in acidic dioxane followed by coupling with BocProOH gave a protected tripeptide, BocProFlpGlyOBn. Removal of the benzoyl group by hydrogenolysis gave BocProFlpGlyOH, which was converted to FmocProFlpGlyOH by removal of the Boc group and reaction with FmocOSu.

(Pro–Flp–Gly)$_{10}$ was synthesized by solid-phase coupling of FmocProFlpGlyOH units on 2-chlorotrityl resin (Advanced ChemTech; Louisville, KY), which is amenable to solid-phase synthesis with Fmoc amino acids [66]. 2-Chlorotrityl chloride resin was modified with FmocProFlpGlyOH by using standard reaction conditions [67]. Nine FmocProFlpGlyOH units were coupled to the modified resin by using an Applied Biosystems 432A peptide synthesizer. The peptide was cleaved from the resin and purified by high-pressure liquid chromatography (HPLC) on a Vydac C-18 reversed-phase column to give the desired peptide in 36% overall yield. The (Pro–Flp–Gly)$_{10}$ product was judged by HPLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry to be >90% pure.

(Pro–Pro–Gly)$_{10}$ and (Pro–Hyp–Gly)$_{10}$ were from Peptides International (Louisville, KY), and were purified by HPLC on a Vydac C-18 reversed-phase column. The resulting (Pro–Pro–Gly)$_{10}$ and (Pro–Hyp–Gly)$_{10}$ were judged by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry to be >90% pure.

Peptide concentration

Peptide concentrations in solutions of 30-mers were determined by measuring A at 214 nm ($\varepsilon = 6.4 \times 10^4$ $M^{-1}cm^{-1}$ in 50 mM acetic acid; $\varepsilon = 6.0 \times 10^4$ $M^{-1}cm^{-1}$ in PBS). These extinction coefficients were derived from the ratio of A at 214 nm and A at 274.6 nm ($\varepsilon = 1.42 \times 10^3$ $M^{-1}cm^{-1}$) of (Pro–Pro–Gly)$_{10}$Tyr, which was synthesized by the solid-phase segment condensation of ten FmocProPro-GlyOH units on resin that had been modified with FmocTyrOH.

Complexation

The complexation of (Pro–Flp–Gly)$_{10}$ was assessed by sedimentation velocity experiments using a Beckman XL-A analytical ultracentrifugation.

Briefly, three samples of peptide ($A = 0.1–1.0$ at 220 nm) in 50 mM acetic acid were spun at 30,000–60,000 rpm at 20°C. Data were analyzed with IGOR PRO (WaveMetrics; Lake Oswego, OR).

The structural integrity of $(Pro–Flp–Gly)_{10}$ complexes was probed by monitoring the binding of ANS, as described previously [68]. Briefly, a solution of peptide (20 μM) and ANS (2.5 μM; Sigma Chemical; St. Louis, MO) in 50 mM acetic acid was excited at 370 nm. Emission was recorded at 400–600 nm. Fluorescence was recorded with a Photon Technology International QM-1 fluorescence spectrometer.

Triple helix formation

Triple helices were allowed to form by incubating 30-mers (0.23 mM in 50 mM acetic acid; 0.088 mM in PBS) for 24 h at 4°C in 50 mM acetic acid or PBS (0.20 g KCl, 0.20 g KH_2PO_4, 8.0 g NaCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$ in 1.00 l). The triple-helical structure of collagen has a characteristic CD spectrum . The formation of triple helices was assessed at 10°C by CD spectroscopy on an Aviv 62A DS instrument equipped with an automated temperature controller.

Thermal stability

The conformational stabilities of the $(Pro–Pro–Gly)_{10}$, $(Pro–Hyp–Gly)_{10}$ and $(Pro–Flp–Gly)_{10}$ triple helices were evaluated by determining values of T_m, which is the temperature at the midpoint of the thermal transition. Values of T_m were determined by thermal denaturation experiments monitored by CD spectroscopy on an Aviv 62A DS instrument equipped with an automated temperature controller, as described [69]. Specifically, the decrease in ellipticity at 225 nm was monitored as the temperature was increased in 3-°C increments with a 5-min equilibration at each temperature. Data were fitted to a two-state model to determine the value of T_m, The value of $\Delta\Delta G_m = \Delta T_m\ \Delta S_m$ was estimated at the T_m of $(Pro–Pro–Gly)_{10}$ by the method of Becktel and Schellman [70]. This method is valid because $\Delta C_p \approx 0$ for the denaturation of triple-helical collagen ([71]; K.M.T and R.T.R, unpublished results). For $(Pro–Pro–Gly)_{10}$, $\Delta S_m = (0.23 \pm 0.01)$ kcal/mol in 50 mM acetic acid and $\Delta S_m = (0.21 \pm 0.01)$ kcal/mol in PBS.

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