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Fluoroproline Flip-Flop: Regiochemical Reversal of a Stereoelectronic Effect on Peptide and Protein Structures**

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The de novo design of protein structure relies on the ability to uniquely define a polypeptide chain conformation through introduction of specific stabilizing interactions into the amino acid sequence, which act cooperatively to overcome the loss of conformational entropy associated with the protein folding process. Within this concept, the stereoelectronic effect has recently emerged as a means to restrict the local chain conformation of polypeptide sequences and to modulate the thermodynamic stability of secondary and supersecondary structural elements.[1–5]

Substituted proline residues are particularly susceptible to the influence of stereoelectronic effects that alter the conformational energetics of the pyrrolidine ring. Introduction of an electronegative substituent (X = N, O, F) at the C3 or C4 position of the pyrrolidine ring establishes a vicinal N-C-C-X arrangement between the prolyl amide group and the electronegative atom.[6,7] A preference for a *gauche* stereochemical relationship is observed between the two substituents as a result of hyperconjugative delocalization, in which the magnitude of the effect depends on the electronegativity of the substituent. These stereoelectronic interactions strongly influence the equilibrium conformational population of the pyrrolidine ring-pucker isomers, such that epimeric pairs of substituted proline derivatives have opposite ring-pucker preferences and often display antagonistic effects on protein stability that depend on structural context.[1,8,9] Most comparative analyses have employed the (4*S*)- and (4*R*)-fluoroproline epimeric pair, in which strong conformational preferences are observed for the C^{γ}-*endo* and C^{γ}-*exo* puckers, respectively. Herein, we report a structural comparison between the (3*S*)- and (3*R*)-fluoroproline epimers, in which the altered regiochemistry results in a reversal of conformational preferences compared to the corresponding 4-fluoroproline epimers, which has structural implications for protein design and engineering.

To evaluate the effect of 3-fluoro substitution on pyrrolidine ring conformation, the epimeric proline derivatives **1** and **2** were synthesized and their structures were determined by single-crystal X-ray diffraction analysis (Figure 1). Prior structural investigations of substituted *N*-acetylproline methyl ester derivatives[6,10] have established that these compounds provide reliable structural models to assess the conformational preferences of the pyrrolidine ring. The structural data indicated that both **1** and **2** crystallized with a *trans* configuration of the prolyl–peptide bond; however, significant differences were observed between **1** and **2** with respect to the conformation of the substituted pyrrolidine ring. The values of the N-C^{α}-C^{β}-F torsion angles for **1** and **2** were 90.7 and −87.4°, respectively, which indicated a *gauche* arrangement of the vicinal fluorine and amide substituents, as expected for the strong fluorine–amide stereoelectronic

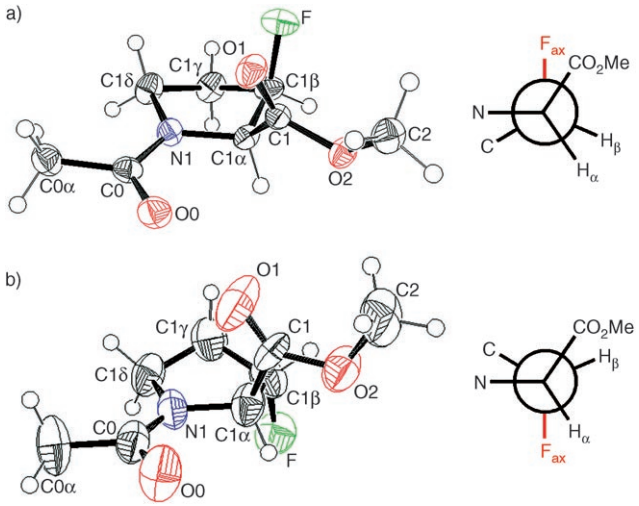

Figure 1. Crystallographically determined structures of a) *N*-acetyl-(2*R*,3*R*)-3-fluoroproline methyl ester (**1**) and b) *N*-acetyl-(2*R*,3*S*)-3-fluoroproline methyl ester (**2**) in conjunction with the Newman projections along the C^{α}–C^{β} bond vector, which depict the *gauche* relation between the amide and fluorine substituents.

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interaction.[11] As observed for the 4-fluoroproline epimeric pair, the *gauche* interaction enforced opposing pyrrolidine ring puckers between **1** and **2**. The conformation of **1** corresponded to a C^{β}-*endo*/C^{γ}-*exo* arrangement (displacements from C^{δ}-N-C^{α} mean plane: C^{β}, 0.112 Å; C^{γ}, 0.460 Å), whereas that of **2** corresponded to a C^{β}-*exo*/C^{γ}-*endo* arrangement (displacements from C^{δ}-N-C^{α} mean plane: C^{β}, 0.308 Å; C^{γ}, 0.230 Å). The relatively small displacement of C^{β} for **1** may result from steric hindrance as a result of close contacts between the fluorine atom and the carbon (C1) and oxygen (O1) atoms of the carbonyl group of the methyl ester, in which the nonbonding distances ($d_{F\cdots C} = 2.67$ and $d_{F\cdots O} = 2.94$ Å) were less than the sum of the corresponding van der Waals radii (3.17 and 2.99 Å, respectively).

Hodges et al. have reported computational analyses of the conformational energetics of **1** and **2**,[7] which indicate that the preferred ring pucker in each case corresponded to that observed in the respective crystal structure. However, the thermodynamic preference of **2** for the C^{γ}-*endo* isomer (estimated 97% population) was significantly greater than that of **1** for the C^{γ}-*exo* isomer (estimated 69% population). The steric congestion surrounding the C^{β} atom observed in the crystal structure of **1** may account for the weaker conformational preference in the latter case. The persistence of the C^{γ}-*endo* conformation of **2** in solution may be inferred from the very small value of $^{3}J_{H\alpha H\beta}$ (≤ 1 Hz) in the ^{1}H NMR spectrum in D_2O, which is consistent with the corresponding value of 0.79 Hz that was calculated from the Karplus equation for the observed H^{α}-C^{α}-C^{β}-H^{β} dihedral angle of −92.6° in the crystal structure of **2**.

The availability of crystallographic and computational data permits a comparison of the structural and thermodynamic parameters of the 3-fluoroproline (3-FPro) derivatives with the corresponding 4-FPro regioisomers (Table 1). Notably, the ring-pucker conformational preferences were reversed between *N*-Ac-FPro-OMe species that had similar *syn* (**1** and **3**) or *anti* (**2** and **4**) orientations of the fluoro substituent with respect to the fixed L-configuration of C^{α}. Thus, an *anti* orientation of the fluoro group resulted in a predominant C^{γ}-*endo* pucker for the 3-fluoroproline derivative **2** and a predominant C^{γ}-*exo* pucker for the 4-fluoroproline derivative **4**, whereas the opposite was true for the respective *syn*-oriented fluoroproline epimers **1** and **3** (Table 1).

Table 1: Thermodynamic data and main-chain torsion angles for the *N*-acetylfluoroproline methyl ester derivatives.

Ac-FPro-OMe	$K_{trans/cis}$[a]	$\Delta E_{exo/endo}$ [kcal mol^{-1}][b]	ϕ [°][c]	ψ [°][c]
(3*R*)-**1** (*syn*,*exo*)	8.9	−0.48	−56.4	151.5
(3*S*)-**2** (*anti*,*endo*)	4.3	2.13	−71.2	158.4
(4*S*)-**3** (*syn*,*endo*)	2.5	0.61	−76.4	172.0
(4*R*)-**4** (*anti*,*exo*)	6.7	−0.85	−55.1	140.5

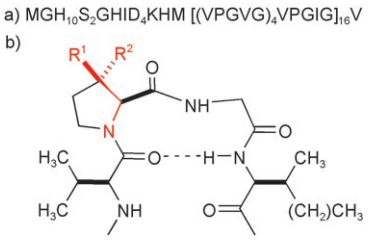
[a] The values of the equilibrium constants $K_{trans/cis}$ were calculated from integration of the well-resolved H^{α} peaks in the ^{1}H NMR spectra of **1** and **2** in D_2O solution at 25 °C. [b] Energy values were obtained from DFT calculations with B3YLP at 6-311 + G(2d,p).[6,7] [c] Dihedral angles were determined from the crystal structures of **1**, **2**, and **4** and from DFT calculations for **3**.[6,7]

Prior analyses of proteins within the structural database,[12] as well as small-molecule proline analogues,[6–8] suggested that the pyrrolidine ring pucker strongly influenced the main-chain torsion angles ϕ and ψ that define the peptide backbone conformation. Moreover, the main-chain dihedral angles appeared to be correlated with the pyrrolidine ring pucker. Thus, the C^{γ}-*exo* pucker of **4** displayed a less negative value of the angle ϕ and a smaller value of the angle ψ than did the C^{γ}-*endo* pucker of **3** (Table 1). A similar, although less pronounced, difference was observed between the corresponding torsional angles for **1** and **2**. The observed values of ϕ and ψ for the C^{γ}-*exo* ring pucker were rationalized on the basis of an energetic stabilization associated with a nonbonded n→π* interaction between the p-type lone pair of the amide oxygen atom and the antibonding orbital of the ester carbonyl group.[6a] Raines et al. have postulated that the close contact between the O0 and C1 atoms ($\delta_{BD} = 2.76$ Å) and the relatively large value of the O0···C1=O1 angle (98°) that were observed in the crystal structure of **4** resembled the Bürgi–Dunitz (BD) trajectory[13] for approach of a nucleophile to a carbonyl group.[1a,b,6a] Similarly, a close contact (2.81 Å) was observed between O0 and C1 in the crystal structure of **1**, albeit with a more acute value of the O0···C1=O1 angle (91°) than that observed in the crystal structure of **4**. These data imply the presence of an n→π* interaction in the C^{γ}-*exo* conformation of **1** and **4**, although the apparently stronger interaction in the latter case reduces the corresponding values of the ϕ and ψ dihedral angles to a greater extent than for **1**. Notably, the nonbonded contacts observed between O0 and C1 for the energetically preferred C^{γ}-*endo* conformations of **2** and **3** ($\delta_{BD} = 3.08$ and 3.23 Å, respectively) were beyond the range in which a significant energetic stabilization could be reasonably expected.

The origin of the observed differences in ϕ and ψ between **1** and **4** could conceivably arise from the anticipated steric repulsion between the *syn*-oriented fluorine atom and the carbonyl group of the methyl ester. This repulsive interaction presumably distorts the C^{γ}-*exo* conformation of **1** from that observed in the crystal structure of **4**. A similar repulsive interaction was invoked to rationalize the variance of the ϕ and ψ torsions that was observed in a computational analysis of the conformational energetics of **3**.[6a] In the latter case, a 1,3-diaxial steric interaction between the *syn*-oriented fluoro and carbomethoxy substituents significantly distorts values of the ϕ and ψ torsions of the C^{γ}-*endo* ring pucker in comparison to the corresponding conformations for the unsubstituted *N*-acetylproline methyl ester or the *anti*-substituted derivatives **2** and **4**. These results suggest that lone pair/lone pair repulsion between the *syn*-oriented fluorine atom and the carbonyl oxygen atom (O0) of the ester can significantly alter the peptide torsions of the *syn*-substituted fluoroproline derivatives **1** and **3**. Thus, two potentially conflicting structural considerations can modulate the conformational energetics of fluoroproline derivatives: the stereoelectronic *gauche* effect and the steric repulsion between *syn*-oriented substituents. The interplay between these two factors may determine the

energetic effect of fluoroproline substitution into polypeptide sequences.

The subtle differences in conformational properties between the C$^{\gamma}$-*exo* and C$^{\gamma}$-*endo* ring puckers suggested that fluoroproline substitution may be employed as a mechanism to interrogate local structural effects that arise from the presence of proline residues in polypeptide sequences.[1–5,7,14] We recently demonstrated that stereoelectronic effects that result from 4-fluoroproline substitution altered the thermodynamics of self-assembly of an elastin-mimetic polypeptide.[9] The differences in macromolecular properties between the elastin derivatives were interpreted on the basis of differential stabilization of the β-turn structures[15–20] that develop in the VPGVG structural repeats above the phase transition (Scheme 1). The greater stability of the (4*R*)-



Scheme 1. a) Amino acid sequence of the elastin-mimetic model polypeptide. b) Structural representation of the β-turn unit of the elastin pentapeptide repeat. The bond vectors that geometrically define the stereoelectronic fluorine–amide *gauche* interactions within the substituted proline residues are highlighted in red for elastin-1 ($R^1 = F$; $R^2 = H$) and elastin-2 ($R^1 = H$; $R^2 = F$).

fluoroproline-substituted elastin (elastin-4) was attributed to the close correspondence between the observed values of the angles ϕ and ψ for the preferred C$^{\gamma}$-*exo* pucker of **4** and the range of ϕ and ψ angles associated with proline residues in the $(i+1)$ position of a type II β-turn conformation (ideal β_{II} ϕ, ψ: −60°, 120°).[21] In contrast, the deviation of the angles ϕ and ψ for the preferred C$^{\gamma}$-*endo* conformation of **3** from those of the type II β-turn conformation caused a destabilization of the corresponding elastin derivative (elastin-3). DFT calculations on model turn segments provided evidence for a relative destabilization of the type II β-turn structure in relation to the type I β-turn structure in the latter situation, which was supported by the conformational analysis of the corresponding elastin-mimetic polypeptides.[9]

To assess the effect of 3-fluoroproline substitution in a well-defined polypeptide model system, the elastin-mimetic proteins elastin-1 and elastin-2 were synthesized for comparison to the 4-fluoroproline derivatives elastin-3 and elastin-4 (Scheme 1). On the basis of the observed regiochemical reversal of ring-pucker preferences for **1** and **2** in relation to **3** and **4**, we expected that elastin-1 and elastin-2 would display opposite tendencies in conformational and thermodynamic behavior compared to the corresponding 4-fluoroproline derivatives.

The elastin-mimetic polypeptides elastin-1 and elastin-2 were prepared through the biosynthetic approach that we reported earlier for elastin-3 and elastin-4.[22] The purified proteins were obtained in comparable yield to the 4-fluoroproline elastin derivatives. MALDI-TOF mass spectrometry of the elastin-1 and elastin-2 indicated virtually complete substitution of proline with the respective analogue. The ^{1}H–^{1}H NOESY NMR spectra of elastin-1 and elastin-2 revealed that the *trans* configuration of the prolyl–peptide bond was the dominant one in aqueous solution. In addition, the values of the coupling constants, $^3J_{H\alpha H\beta}$ and $^3J_{H\alpha F\beta}$, for the substituted proline residues in the elastin-mimetic polypeptides were similar to the corresponding values for **1** and **2**, which indicated that the ring-pucker preferences of the analogues were conserved within the respective polypeptides.

Circular dichroism (CD) studies of elastin-mimetic polypeptides have established that a conformational rearrangement occurs as the temperature increases through the transition point, which corresponds to a conversion of the local secondary structure of the pentapeptide repeats from a random coil conformation to a more ordered type II β-turn conformation. This conformational transition can be detected in the CD spectra of elastin-1 as a function of temperature, in that the random coil signature (negative ellipticity near 196 nm) is gradually replaced with the type II β-turn signature (positive ellipticity near 208 nm) as the temperature is increased through the transition point (Figure 2).[15,18,23] In contrast, the CD temperature manifold for elastin-2 displays a different spectroscopic profile in which a strong negative ellipticity (minimum at 223 nm) develops above the transition temperature. This spectroscopic feature is not consistent with the formation of a type II β-turn conformation, but more closely resembles the CD spectrum of a type I β-turn structure.[24] Thus, the CD spectroscopic data indicate a formal reversal of turn preferences for the 3-fluoroproline-substituted elastin sequences with respect to those previously observed for the 4-fluoroproline derivatives. The C$^{\gamma}$-*exo* pucker of (4*R*)-fluoroproline significantly stabilized the type II β-turn conformation for elastin-4, whereas the C$^{\gamma}$-*endo* pucker of (4*S*)-fluoroproline in elastin-3 appreciably destabilized the type II β-turn conformation relative to alternative turn structures.[9] The CD spectroscopic data for both sets of fluoroproline-substituted elastin derivatives suggest that the C$^{\gamma}$-*exo* pucker is more compatible with a type II β-turn structure, and, conversely, the C$^{\gamma}$-*endo* pucker is less compatible with a type II β-turn structure to the point that the alternative type I β-turn conformation becomes energetically competitive, if not dominant, for the ordered state of the polypeptide.

In addition, the *syn* versus *anti* stereochemistry of the proline analogue may strongly influence the thermodynamic parameters associated with the elastin assembly. The CD thermal transition curves indicated that the disappearance of the low-temperature random coil conformation adheres to a quasi-two-state transition for both elastin-1 and elastin-2 (Figure 2c).[23] The mathematical fits of these thermal transitions provided estimates for the respective transition temperatures (T_t) of 30.1 and 10.9 °C. Differential scanning calorimetry (DSC) measurements provided T_t values of 35.9 and 13.6 °C for elastin-1 and elastin-2, respectively, which correlate well with those determined from the CD temperature profiles. These T_t values[25] provide an indication of the

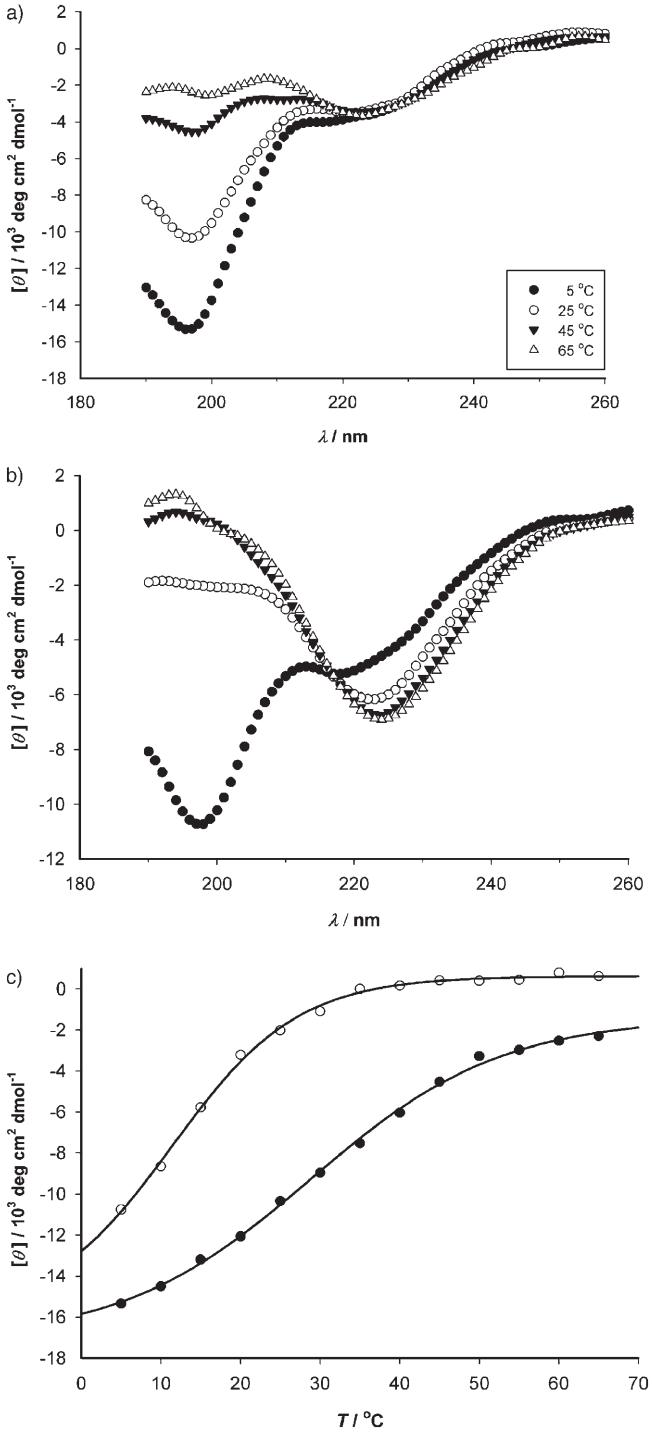


Figure 2. a, b) Temperature-dependent CD spectroscopic manifolds for elastin-1 and elastin-2, respectively. c) Thermal transition curves for the disappearance of the random coil CD signal $[\theta]_{198}$ for elastin-1 (●) and elastin-2 (○).

facility with which the elastin assembly occurs, which, as a disorder-to-order conformational transition, depends on the thermodynamic stabilization of the turn structures in relation to the random coil conformation. The DSC data indicate that the self-assembled state of elastin-2 is stabilized with respect to that of elastin-1 by approximately 22 °C. Similarly, a stabilization of 20 °C was observed for the ordered state of elastin-4 versus that of elastin-3.[9] In both cases, incorporation of the *syn*-fluoroproline derivative significantly raises the transition temperature for self-assembly of the elastin-mimetic polypeptide with respect to that of the corresponding *anti*-fluoroproline derivative. These data strongly suggest that the steric repulsion that develops within the *syn*-oriented fluoroproline derivatives can adversely influence the energetics of elastin assembly, and, more generally, of local turn-folding events compared to the *anti*-fluoroproline derivatives. Moreover, manifestation of this effect is independent of the intrinsic ring-pucker preference of the proline derivative and the observed turn type of the elastin analogue.

In conclusion, we have demonstrated that the conformational energetics of a polypeptide can be altered through a combination of stereoelectronic and steric effects that arise from the presence of substituted proline residues at structurally critical positions. In the specific case under consideration, the assembly of a series of elastin-mimetic polypeptides could be interpreted in terms of the influence of the preferred fluoroproline ring pucker on the conformational energetics of the local β-turn structures. This "proline editing"[26] strategy—based on a set of four related fluoroproline derivatives[1–5,7,9]—may represent a general approach to interrogating the structural and functional role of specific proline residues within a polypeptide sequence, through comparison of the differential effect of fluoroproline substitution on the thermodynamics of folding and assembly among the resulting group of structural variants.

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