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4-Fluoroprolines: Conformational Analysis and Effects on the Stability and Folding of Peptides and Proteins

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Abstract

Proline is unique among proteinogenic amino acids because a pyrrolidine ring links its amino group to its side chain. This heterocycle constrains the conformations of the main chain and thus templates particular secondary structures. Proline residues undergo post-translational modification at the 4-position to yield 4-hydroxyproline, which is especially prevalent in collagen. Interest in characterizing the effects of this modification led to the use of 4-fluoroprolines to enhance inductive properties relative to the hydroxyl group of 4-hydroxyproline and to eliminate contributions from hydrogen bonding. The strong inductive effect of the fluoro group has three main consequences: enforcing a particular pucker upon the pyrrolidine ring, biasing the conformation of the preceding peptide bond, and accelerating *cis/trans* prolyl peptide bond isomerization. These subtle, yet reliable modulations make 4-fluoroproline–incorporation a complement to traditional genetic approaches for exploring structure–function relationships in peptides and proteins, as well as for endowing peptides and proteins with conformational stability.

Keywords

collagen; gauche effect; $n \rightarrow \pi^*$ interaction; pyrrolidine; stereoelectronic effect

1 Introduction

Proline is unique among proteinogenic amino acids because its α -amino group is constrained within a pyrrolidine. This heterocyclic ring restricts the ϕ ($C_{i-1}'-N_i-C_i^{\alpha}-C_i'$) main-chain dihedral angle, making proline an important determinant of the conformational stability of proteins [1]. Moreover, the pyrrolidine ring promotes population of both the *cis* and *trans* conformation of the preceding peptide bond, thereby enabling structural diversity. The isomerization of this “prolyl amide” bond can limit the folding rate of a protein [2].

Consistent with the conformational restriction imposed by proline relative to other amino acids, proline is prevalent in structural proteins, especially collagen [3]. In 1901, Fischer discovered proline in gelatin hydrolysates [4]. A year later, he discovered therein the product of the earliest known post-translational modification: 4-hydroxyproline [5]. Within collagen strands, proline residues are oxidized at the 4*R* position by prolyl-4-hydroxylase (P4H [6]), an essential enzyme in animals [7–9]. Attempts to understand this modification led ultimately to the development of a wide variety of proline analogues [10]. Among these, 4-

fluoroprolines have emerged as powerful tools for studying collagen, as well as for engineering conformationally biased peptides and proteins.

4-Fluoroprolines were first employed in 1965 [11], when they were used to investigate the mechanism of collagen hydroxylation and found to be incorporated successfully into collagen proteins [12–15]. Although early studies focused mainly on collagen hydroxylation [11,16,17], 4-fluoroprolines have now been applied to study a wide variety of systems, some of which have been reviewed previously [18–21]. In this review, the utility of these important heterocycles is presented with a thorough discussion of their synthesis and conformational properties, followed by a detailed examination of their impact on the stability and folding of peptides and proteins. We conclude with an overview of the growing interest of employing 4-fluoroprolines for medicinal applications.

2 Synthesis

4-Fluoroproline syntheses have improved significantly since the original report [11]. Nearly all of the syntheses employ displacement of the hydroxyl group of 4-hydroxyproline with a fluoride source. Activation of the hydroxyl group of Cbz-4-hydroxyproline as tosylate **2** before displacement by inorganic fluoride at elevated temperatures gave 56–62% yields of (4*S*)- and (4*R*)-*N*-Cbz-4-fluoroprolines **3** to afford, after protecting group removal, (2*S*,4*S*)-fluoroproline (flp, (4*S*)-**1**) from (2*S*,4*R*)-4-hydroxyproline (Hyp) with complete stereochemical inversion, but diastereomeric mixtures of (2*S*,4*R*)-4-fluoroproline (Flp, (4*R*)-**1**) starting from (2*S*,4*S*)-4-hydroxyproline (hyp) (Scheme 1a) [11]. Subsequent reports found difficulty in generalizing this procedure for use of alternative protecting group schemes [22]. Demand existed for syntheses that could provide *both* stereoisomers starting from naturally occurring and relatively less expensive Hyp.

Preliminary work to bypass the alcohol activation step by employing organic fluorinating agents generally resulted in inseparable mixtures of the 4*R* and 4*S* diastereomers [23–26]. Successful syntheses of both 4-fluoroproline diastereomers from a single 4-hydroxyproline diastereomer were first reported in 1998 [22]. Employing diethylaminosulfur trifluoride (DAST) as the fluorine source, both Boc- and Fmoc-flp were prepared in approximately 80% yield from Boc-Hyp-OMe (Scheme 1b). Inversion of the alcohol of (4*R*)-*N*-trityl-4-hydroxyproline methyl ester by a Mitsunobu procedure using benzoic acid as the nucleophile followed by saponification and *N*-protection gave (4*S*)-**5**, which was similarly converted to Fmoc-Flp ((4*R*)-**6**), albeit in <25% yield [22]. Substitution of formic acid for benzoic acid enabled the Mitsunobu reaction in the presence of Boc-amine protection to generate (4*S*)-**4** in 78% yield (Scheme 1b) [27]. Moreover, switching from a methyl to a phenylacetyl (Pac, PhCOCH₂-) ester enabled an orthogonal cleavage with Zn in AcOH in the presence of Boc and Fmoc amine-protecting groups [27]. Fluorination of Pac ester (4*S*)-**7** with morpholinosulfur trifluoride inverted the alcohol to give 4-fluoroproline (4*R*)-**8** in 79–94% yields. The configuration of Boc-flp was assigned by X-ray diffraction, and chiral HPLC established that the synthesis produced single stereoisomers. Protecting group manipulation then provided Fmoc-fluoroproline starting materials **6** for solid-phase synthesis (Scheme 1b).

A more scalable and cost effective synthesis of 4-fluoroproline was later developed, which avoided potentially explosive aminosulfur trifluoride reagents [28]. To generate flp (4*S*)-**1**, Boc-Hyp-OMe (4*R*)-**4** was treated with trifluoromethane sulfonic anhydride to provide a triflate that was treated with tetrabutylammonium fluoride (TBAF). Heating of the resulting protected (4*S*)-**5** with 2 N HCl at reflux removed the Boc group and methyl ester to provide flp (4*S*)-**1** in 16% overall yield from Hyp (Scheme 1c). Albeit lower-yielding than previous approaches, largely due to inefficiency in the safer fluorination step, this approach was scaled to kilogram quantities. To generate Flp (4*R*)-**1**, rather than employ the Mitsunobu approach, lactone **11** was synthesized by conversion of Boc-Hyp (4*R*)-**9** to methanesulfonate **10** and intramolecular displacement by the carboxylate on treatment with potassium *t*-butoxide. Opening of lactone **11** with lithium hydroxide provided Boc-hyp (4*S*)-**9** in 78% yield from Boc-Hyp (4*R*)-**9**. Conversion of Boc-hyp (4*S*)-**9** to Flp (4*R*)-**1** was performed using a similar protocol by way of triflate to yield (4*S*)-**5**. Alternatively, Hyp was transformed to its 2*R* diastereomer by treatment of with acetic anhydride at 90° C to form an *N*-acetyl lactone which was hydrolyzed *in situ* with 2N HCl at reflux.

The solid-phase synthesis of peptides possessing 4-fluoroproline residues has been achieved by conversion of Hyp residues on resin, a technique called “proline editing” [29,30]. After coupling Fmoc-Hyp to the peptide chain, the hydroxyl group was protected as trityl ether and the protected peptide chain was completed by standard Fmoc-based solid-phase peptide synthesis. The trityl group was removed selectively using 2% TFA to reveal the free hydroxyl group, which was transformed to its (4*S*)-4-hydroxyproline counterpart by Mitsunobu reaction with 4-nitrobenzoic acid and ester hydrolysis with K₂CO₃/MeOH. Transformation of the hydroxyproline containing peptides to their respective 4-fluoroproline peptides was accomplished with inversion of configuration using DAST, prior to resin cleavage and deprotection. Multiple modified proline residues bearing different 4-position substituents in the same peptide may be produced employing alternative and orthogonal protecting group strategies using *tert*-butyldimethylsilyl (TBS) ether and allyloxycarbonyl (Alloc) carbonate groups. In sum, this strategy circumvents the labor required for solution-phase purification of 4-fluoroproline building blocks.

3 Conformational Analysis

Eclipsing interactions deter population of the planar conformer of saturated five-membered rings. Instead, these rings adopt one of two predominant conformations: the envelope conformation, wherein four atoms lie in plane with the fifth distorted away; or the half-chair conformation, wherein two adjacent atoms distort on opposite sides of the plane of the other three (Fig. 1a). Early crystallographic studies [31–33], as well as data from NMR spectroscopy [34], indicated that proline generally adopts the envelope conformation. Although many envelope conformations are possible, crystallographic analysis demonstrates that two predominate: one termed *C^γ-endo*, in which C4 (otherwise, C^γ) distorts out of plane to the same side of the ring as the C1 carboxylate; and another, *C^γ-exo*, in which C4 distorts out of plane to the opposite side of the ring as the C1 carboxylate (Fig. 1b) [35]. These designations are however approximate, and many molecules labeled as “*C^γ-exo*” are better described as *C^β-endo*. Moreover, many proline crystal structures display some half-chair character. The *C^γ-endo/exo* terminology has become customary and is useful for

discussing stereoelectronic effects on proline conformations (*vide infra*), because C γ often experiences the largest change in orientation and because C γ is typically the site of substituent modification.

The energy difference between the *endo* and *exo* puckers is small for proline (~0.5 kcal/mol), and the two forms interconvert rapidly at room temperature [36]. Using NMR spectroscopy, an approximately 2:1 *endo:exo* equilibrium population has been determined for Ac-Pro-OMe [36]. Fluorination of C4 serves to bias this equilibrium. Specifically, the C–F bonds in 4-fluoroproline will orient antiperiplanar to adjacent C–H single bonds due to a gauche effect. The polarity of the C–F bond makes the substituted carbon electron-deficient and therefore a potent electron acceptor. The C–F σ^* orbital is, in particular, highly electrophilic and will accept electron density from antiperiplanar C–H bonding orbitals [37]. The C–F bond in Flp is satisfied by C–H donor orbitals in an orientation that positions the fluoro group towards the opposite side of the ring as the carbonyl carbon, thereby enforcing the C γ -*exo* pucker (Fig. 2a). Conversely, flp exists largely in the C γ -*endo* conformation due to similar hyperconjugation (Fig. 2b). These preferences were first established using ^1H -NMR spectroscopy [38]. The presence of the fluoro substituent disperses the condensed proline proton signals, thereby enabling accurate calculations using the Karplus equation [39]. Refined analysis quantified the preference of flp for the *endo* conformation to be approximately 20:1; analogously, Flp prefers the *exo* conformation at a 6:1 ratio [36].

The reorientation of the pyrrolidine ring between the two puckers influences the structure and stability of proline-containing peptides (*vide infra*). The *exo* pucker reduces the absolute value of the proline ϕ and ψ main-chain torsion angles (Table 1) [37]. Constrained by the pyrrolidine ring, the ϕ dihedral angle varies from approximately -60° in the *exo* pucker to approximately -75° in the *endo* pucker [36]. Although these changes appear modest, they can have a profound effect on the structure and stability of proline-rich peptides (*vide infra*). Two general orientations exist for the ψ ($\text{N}_i\text{--C}_i^\alpha\text{--C}_i'\text{--N}_{i+1}$) main-chain dihedral angle: one near 150° , corresponding to the polypeptide type II (PPII) helix; and another near -30° , which is consistent with α -helix geometry. In isolated proline molecules and derivatives, the former ψ value is more common, and calculated to be of lower energy [40]. Within the PPII conformer, 4-position fluorination of the pyrrolidine ring has been shown to affect the ψ dihedral angle by as much as 30° , from $\sim 140^\circ$ in the *exo* pucker of Flp to $\sim 170^\circ$ in the *endo* pucker of flp [36].

As a result of the influence of ring pucker on the main-chain dihedral angles, fluorination of C4 can modulate interactions between the N-terminal amide carbonyl and the proline residue carboxamide in peptides. Carbonyl groups of adjacent peptide residues have been shown to engage in so-called “ $n \rightarrow \pi^*$ interactions” [41]. In an $n \rightarrow \pi^*$ interaction, lone-pair (n) electron density delocalizes from the oxygen of one carbonyl group into the π^* anti-bonding orbital of a proximal carbonyl group (Fig. 2c) [42]. Donation typically occurs from the $i-1$ residue to the i residue within a polypeptide [43]. Effective orbital mixing requires a distance closer than a van der Waals contact between the donor oxygen and the acceptor carbon along the Bürgi–Dunitz trajectory for nucleophilic addition [44]. The $n \rightarrow \pi^*$ interactions have energies generally greater than 0.27 kcal/mol per interaction [45]. They have been shown to be ubiquitous in proteins [43,46,47], particularly about proline residues, in which the

pyrrolidine ring pre-organizes the neighboring carbonyl groups to favor the $n \rightarrow \pi^*$ interaction [43]. Fluorination of C4 influences the pyrrolidine ring pucker and the main-chain dihedral angles in ways that have important consequences for the $n \rightarrow \pi^*$ interaction [48]. Specifically, the distance between the oxygen–carbon donor–acceptor pair of the neighboring carbonyls is generally longer in the *endo* pucker, which leads to a weaker $n \rightarrow \pi^*$ interaction [49]. Conversely, the *exo* pucker is associated with shorter donor–acceptor distances and stronger $n \rightarrow \pi^*$ interactions. In turn, the $n \rightarrow \pi^*$ interaction biases the conformation of the preceding peptide bond. An attractive $n \rightarrow \pi^*$ interaction is only possible in the *trans* conformation of the peptide bond (ω : $C_{i-1}^{\alpha}-C_{i-1}'-N_i-C_i^{\alpha}$, Fig. 1c). Accordingly, a higher *trans* peptide bond population correlates with the increased preference for *exo* pucker of the pyrrolidine ring substituted with a 4*R* fluoro group (Table 1, Figure 4). Notably, the *cis* and *trans* isomers of 4-fluoroproline have distinct dipole moments, and the *cis:trans* ratio of their peptide bonds is sensitive to solvent polarity [50]. *In toto*, modification of the proline ring with a 4-fluoro substituent has structural consequences on all three backbone dihedral angles in a protein. This principle has been exploited to modulate the conformational stability of peptides and proteins (*vide infra*).

The inductive effect of 4-fluoro substituents has additional consequences on the peptide bond N-terminal to proline residues. Electron withdrawal by the fluoro group reduces the capacity for the proline nitrogen lone pair to contribute double-bond character to the amide N-terminal to proline [51], and thereby diminishes the rotational barrier between the *cis* and *trans* isomers in the peptide [52]. 4-Fluoroproline has been used to study protein folding kinetics, because isomerization of peptide bonds N-terminal to proline can limit the rate of protein folding [2]. To enhance the influence of a single fluoro substituent, (2*S*)-4,4-difluoroproline (Dfp) has been developed to study protein folding (*vide infra*). Although detailed conformational analysis of Dfp has yet to be performed, its conformational preferences may likely be similar to those of proline.

4 Effects on Collagen Stability

4-Fluoroproline has been particularly useful in the study of collagen stability [3, 53]. Collagen is the major structural protein in animals, forming a large portion of the dry weight of the skin. Collagen is also prevalent in the extracellular matrix, in which it performs an important structural role. Collagen consists of a distinct triple-helical structure comprised of three polypeptide strands that each have a characteristic Xaa-Yaa-Gly amino acid repeat, in which prolyl residues often occupy the Xaa and Yaa positions. This sequence enables each of the three strands to adopt PPII helices that wrap around one another with an offset of a single amino acid residue [54]. A slice through a collagen triple helix will therefore contain one Xaa, one Yaa, and one Gly residue from different strands. In the PPII secondary structure, the individual peptide chain lacks typically *intra*-strand hydrogen bonding. In a slice of the triple helix, a single *inter*-strand hydrogen bond usually exists between the glycine NH and the carbonyl of the Xaa residue on different peptide chains. The relatively low prevalence of hydrogen bonding raised the question as to the source of the high thermal and mechanical stability of collagen.

One clue as to the source of collagen stability is apparent from its amino acid content. Collagen has a high prevalence of Pro and Hyp residues, which appear at approximately 28% and 38% frequencies in the Xaa and Yaa positions, respectively [55]. The importance of the Hyp hydroxyl group was shown early-on by thermal denaturation studies with both synthetic peptides [56] and protocollagen [57], which is the non-hydroxylated precursor to mature collagen. A crystal structure of a collagen mimetic peptide showed bridging water molecules between the hydroxyl substituent and main-chain carbonyl groups, and these water bridges were proposed to confer stability [58,59]. The importance of the inductive effect of the Hyp hydroxyl group was examined by the use of 4-fluoroprolines, which cannot serve as proton donors in hydrogen bonds. In the first synthetic incorporation of 4-fluoroproline into a protein mimetic, the Hyp residues at the Yaa position of a collagen mimetic were replaced with Flp, and circular dichroism (CD) spectroscopy was used to compare the thermostability of the two triple helices (Table 2) [60,61]. Relative to the Hyp triple helix mimetic, the Flp analog exhibited increased thermostability. Considering that fluoro groups do not form strong hydrogen bonds [62], the data from this comparison refuted the water-bridge proposal. Subsequent molecular dynamics simulations demonstrated the transient nature of bridging water molecules [63]. Moreover, experiments with (2*S*,4*R*)-4-methoxyproline showed that the water bridges actually diminished the stability of the collagen triple helix [64].

Thorough conformational analysis of 4-fluoroproline monomers provided compelling evidence that collagen is stabilized by the stereoelectronic effects of the Hyp residue that are augmented by replacement with the Flp residue [42]. For example, collagen mimetics bearing Flp at the Yaa position exhibited higher thermostability than their Pro counterparts. On the other hand, incorporation of flp at the Yaa position caused a relative decrease in thermostability (Table 2), demonstrating the importance of 4-position stereochemistry. Inspection of available crystal structures indicated a prevalence of *exo* ring puckers in the Yaa position of collagen [58]. The fluoro group in Flp enforces the *exo* ring pucker, which stabilizes collagen by organizing proper dihedral angle orientations. Moreover, Flp favors the prolyl amide *trans* isomer, organizing the proper ω dihedral angle featured in collagen (Table 1) [54]. These effects are evident with even a single Pro→Flp substitution within a collagen strand [65]. Increased and decreased stability relative to the parent peptide were respectively observed by CD and NMR spectroscopies upon substitution of Hyp by Flp and flp in a triple-helical model of the $\alpha 1(\text{IV})1263\text{--}1277$ sequence of type IV (basement membrane) collagen, which is known to promote melanoma cell adhesion, spreading, and signaling [66]. Melanoma cell adhesion and spreading on the triple-helical models correlated with stability demonstrating the dramatic influences of fluoro group changes at the Hyp residue [66].

Although Flp-incorporation at the Yaa position increased thermostability in collagen relative to proline in Pro-Yaa-Gly repeats, decreased thermostability was observed with Flp relative to proline in Xaa-Hyp-Gly repeats [67]. Conversely, flp demonstrated respectively stabilizing and destabilizing effects at the Xaa and Yaa positions [68]. Inspection of available crystal structures demonstrated the Xaa residue prefers *endo* ring pucker, which is enforced by flp. The slight preference of Pro for the *endo* pucker is consistent with the

stronger effect of Flp at the Yaa position relative to the milder influence of flp at the Xaa position (Table 2).

Although respective incorporation of flp and Flp at Xaa and Yaa residues in the same peptide was predicted to template the appropriate main-chain dihedral angles and confer greater stability than either substitution alone, the resulting collagen triple helix exhibited decreased thermostability relative to the peptide composed of repeats of Pro-Pro-Gly (Table 2) [69,70]. Molecular modeling revealed a potential inter-strand steric clash between the fluoro groups of 4-fluoroproline residues on adjacent strands that prevented self-association of the peptide. Stable triple helices could be formed by mixing different ratios of (flp-Flp-Gly)₇ and (Pro-Pro-Gly)₇, [70]. The (flp-Flp-Gly)_n sequence binds preferably other collagen strands but not itself. In biological collagen samples, such as in wounds, disrupted helices have provided annealing sites for (flp-Flp-Gly)_n sequences coupled to fluorescent dyes to visualize damaged collagen [71]. Moreover, such annealing strands have been conjugated with moieties, such as growth factors, that modulate the physiology of the surrounding environment to aid in wound healing [72,73].

Differential scanning calorimetry was employed to provide more insight into the thermodynamics of the influences of fluorination on collagen stability [74]. In comparisons with (Pro-Pro-Gly)₁₀ triple helices, the (Pro-Hyp-Gly)₁₀ and (Pro-Flp-Gly)₁₀ counterparts were shown to retard thermal denaturation by enthalpic and entropic contributions, respectively. Analysis of molecular volumes indicated that the enthalpic contribution to the stability of (Pro-Hyp-Gly)₁₀ was mediated significantly by enhanced water solvation. Water desolvation due to the hydrophobic effect of fluoro substitution may account for the entropic stabilization of (Pro-Flp-Gly)₁₀ because the fluoro group of Flp is a much weaker hydrogen bond acceptor than the hydroxyl group of Hyp [62], and cannot donate a hydrogen bond. This hypothesis was tested in a follow-up study employing Dfp, which retains the hydrophobicity of Flp, but lacks the strong preference for the *exo* ring pucker [53]. Accordingly, if the hydrophobic effect was dominant to triple-helical stability, Dfp and Flp should have similar effects; however, Dfp decreased stability relative to Flp at the Yaa position, arguing against a strong contribution from the hydrophobic effect in driving collagen-mimetic peptide assembly.

Albeit application has been limited to date [75], 4-fluoroprolines have served as NMR probes due to the utility of the ¹⁹F nucleus. For example, the mechanism of strand association and dissociation was studied in a collagen triple helix model possessing a single Flp residue at the central Yaa position using ¹H-¹⁹F and ¹⁹F-¹⁹F exchange experiments to measure populations of native and nonnative structures in solution [76]. As the temperature increased, denaturation of the native collagen strands was observed to give rise to multiple intermediates, contradicting the two-state model often assumed for the denaturation of collagen-mimetic peptides. Isolated monomer strands were found to possess both *cis* and *trans* peptide bonds, but collagen assembly was concluded to take place only from monomers with all *trans* peptide bonds. Off-pathway intermediates were characterized having misaligned triple helices with more than one residue offset. Misaligned helices required dissociation to all-*trans* monomer strands to convert to the native state.

Besides imparting thermostability and binding specificity to collagen strands, 4-fluoroproline has also been used to investigate the mechanism by which collagen strands are hydroxylated by proline 4-hydroxylase (P4H). Proline derivatives such as flp, which prefer relatively the *endo* pucker and the *cis* peptide bond conformation proved better P4H substrates [77]. Enzymatic discrimination during substrate binding and not during turnover was evident from the hydroxylation of peptides bearing flp and the absence of binding to peptide counterparts containing Flp residues. Oxidation of flp to 4-ketoproline (Kep) by P4H was used to develop a probe of enzymatic activity by measuring fluoride-ion release in a continuous assay [78]. Shedding light on the mechanism of collagen hydroxylation, these studies have provided information for designing inhibitors of P4H to treat fibrotic diseases associated with an overabundance of collagen.

5 Effects on Peptide Conformation

Beyond the triple-helical domain of collagen, the conformations of other peptides, especially polyproline helices, have been explored through incorporation of 4-fluoroproline. Two major conformations of oligoproline are known: the polyproline type I (PPI) helix, which is marked by *cis* orientation of the peptide bonds, and the aforementioned PPII helix, which features prolyl amide *trans* isomers. The PPII conformation is common in both the folded [79] and unfolded states [80] of proteins, and has found utility in molecular rulers [81] and scaffolds [82]. The PPII conformation is favored in relatively polar solvents, such as water. The PPI conformation is formed preferentially in less polar solvents, such as *n*-propanol [83]. The transition between these helices involves isomerization of the peptide bond. Modulating the conformation of the pyrrolidine ring was predicted to have a profound effect on oligoproline structure, because of the connection between pyrrolidine ring pucker and main-chain dihedral angles (*vide supra*). To evaluate this hypothesis, Pro₁₀, Flp₁₀, and flp₁₀ were synthesized by solid-phase methods and subjected to analysis by CD spectroscopy [84]. In contrast to native Pro₁₀, Flp₁₀ adopted a PPII conformation in both aqueous and organic solutions. Conversely, flp₁₀ exhibited low PPII content in water and a high population of PPI geometry in *n*-propanol. Considering the conformational preferences of the amino acid monomers, the favored *exo* ring pucker of Flp enforced $n \rightarrow \pi^*$ interactions between adjacent carbonyl groups, leading to increased populations of prolyl amide *trans* isomers and PPII helix. Subsequently, (4*R*)- and (4*S*)-fluoroproline were shown to respectively increase and decrease the transition state barrier for PPII $\rightarrow$ PPI conversion [85]. This dichotomy was consistent with Flp stabilizing the prolyl amide *trans* isomer. In a follow-up study, the effects of 4-fluoroproline were shown to be more pronounced at the C versus the N terminus of the peptide [86], consistent with poly-proline interconversion occurring by a mechanism that initiates at the C terminus [87].

Polyproline helices have attracted interest as motifs, because of their roles in protein–protein interactions [88]. For example, SH3 domains are a ubiquitous peptide module that bind often to proline-rich protein substrates, which adopt PPII conformations [89]. In an effort to design high-affinity ligands to SH3 domains for applications such as cancer chemotherapeutic agents, 4-fluoroproline was introduced into various proline-rich peptides derived from the hematopoietic progenitor kinase 1 (HPK1) [90]. In affinity assays using the SH3 domain of hematopoietic-lineage cell-specific (HS1) protein, the 4-fluoroproline

peptide analogs failed to exhibit enhanced binding, in spite of increasing the population of P_{II} conformer, suggesting that ligand recognition involved features beyond secondary structure, albeit the fluoro groups may perturb specific binding interactions.

To study the importance of *exo* ring pucker in the proline–aromatic interaction with Trp64, F_lp and f_lp were respectively substituted for Pro62 at the N terminus of the C terminal helix in the subdomain of the villin headpiece (HP36), a small mostly α -helical miniprotein [88]. Although F_lp and f_lp were respectively expected to enforce and perturb the *exo* ring pucker of Pro62 [89] with consequences of improved and depressed thermostability, thermal unfolding and urea-induced denaturation measurements indicated that the former fluoroproline significantly destabilized HP36 and the latter had little effect on its structure and protein stability, unexpected consequences suggested to be due to steric and hydrophobic effects.

To explore the importance of the *endo* pucker for Pro37 to pack into the hydrophobic core of the Pin1 WW domain, 4-fluoroprolines were incorporated into this small, three-stranded β -sheet protein of about 40 residues [91]. Consistent with the crystal structure of the parent protein [92] and the hypothesis that the native *endo* pucker is preferred for the Pin1 WW domain, F_lp decreased thermostability by favoring the *exo* pucker. Although relatively hydrophilic *endo*-favoring prolines, such as hyp and (4*S*)-methoxyproline (mop) destabilized the Pin1 WW domain, f_lp increased stability, likely due in part to its hydrophobic nature. Moreover, f_lp-incorporation increased the affinity between the protein and its phosphorylated peptide ligand. The advantages of 4-fluoroprolines to perturb selectively pyrrolidine ring conformation were thus highlighted by their capacity to probe peptide structure without complications from hydrogen bonding.

4-Fluoroprolines have been used to study the significance of the *exo* ring pucker of Pro12 in the loop that joins the C-terminal polyproline helix to the N-terminal α -helix in the Trp cage miniprotein [93], a 20-residue peptide that displays tertiary structure and cooperative folding [94], physical characteristics associated with full proteins. Employing proline editing, Trp cage peptides bearing F_lp and f_lp at position-12 were synthesized and found respectively to increase and decrease thermostability relative to the native proline peptide. The destabilizing effect of f_lp was greater than the stabilizing effect of F_lp, effects consistent with the *exo* pucker of the native proline, and the sensitivity of small proteins that lack a hydrophobic core.

The critical role of prolyl amide *cis*–*trans* isomerization in the folding of the N-terminal domain of the cysteine-rich terminal region of minicollagen-1 was confirmed by replacement of Pro24 with F_lp and f_lp, respectively [95]. During the folding of this important structural protein of lower animals, a fully oxidized intermediate accumulates in which the N-terminal domain possesses a prolyl amide *trans* isomer and three disulfide bonds. Conversion to the native folded peptide occurs by slow rate-determining *trans*-to-*cis* isomerization in the absence of enzyme catalysis [96]. Relative to the parent proline peptide, f_lp gave efficient folding with formation of the correct isomer and negligible amounts of intermediate, and F_lp slowed folding by favoring the *trans* isomer in the folding intermediate. Molecular dynamics simulations and kinetic analyses demonstrated that f_lp

decreased the population of the *trans* isomer preventing trapping of nonnative disulfide bonds. Similarly, 4-fluoroprolines were used to investigate stability, and rates of folding and the role of disulfide bond formation in model collagen triple helices [97].

4-Fluoroprolines have been used to modulate the DNA-binding properties of a mimic of the integration host factor (IHF) [98]. Crystal structures of the complex of IHF and a 35-base pair chain of DNA have demonstrated that the so-called “ α -arm” binds the DNA sequence in part by intercalating an *endo*-puckered proline residue between base pairs [99]. In the mimic, a lysine dendrimer was tethered to a cyclopeptide that adopted a β -sheet hairpin conformation, which presented the intercalating proline in homology to the IHF α -arm. Replacement of proline, by flp in the mimic increased sequence-specific DNA binding.

6 Effects on Protein Folding

Broadening the applications of 4-fluoroprolines, methods have been developed to incorporate these heterocyclic amino acids into full-length proteins. For example, selective pressure incorporation (SPI) has resulted in the global substitution of 4-fluoroproline for proline by conditioning bacterial protein synthesis through genetic engineering and the control of environmental factors such as amino acid supply and fermentation parameters [100–105]. Alternatively, the site-specific incorporation of 4-fluoroproline into proteins bearing multiple proline residues has been achieved by the chemical ligation of peptides possessing 4-fluoroproline residues to protein fragments produced by recombinant DNA technology [106].

The first complete replacement of the proline residues in a protein with 4-fluoroprolines was performed on an engineered barstar variant, the cognate inhibitor of a *Bacillus* ribonuclease (barnase) [107]. Wild-type barstar has two prolines, one in each peptide bond conformation. Genetic manipulation yielded a variant containing only the *cis* proline. Efficient recognition of 4-fluoroprolines by the endogenous proline amino-acyl-tRNA synthetase was demonstrated by production of protein with approximately equal efficiency by feeding bacteria proline or either 4-fluoroproline diastereomer. In accordance with their favoring of prolyl *cis* and *trans* isomers, barstar variants containing flp and Flp exhibited respectively higher and lower thermostability than the parent protein. The fluorinated barstar variants were employed to study the mechanism of peptide bond isomerization by peptidyl prolyl *cis*–*trans* isomerases [108].

Bacterial strains with improved efficiency for incorporating 4-fluoroproline have been used to prepare elastin derivatives [109]. In bacterial synthesis of proline from glutamate, the *proA* gene encodes 5-glutamylphosphate reductase to generate glutamate-5-semialdehyde, which spontaneously cyclizes to a ¹-pyrroline intermediate. Proline is produced by reduction of the pyrroline by pyrroline carboxylate reductase, encoded by the *proC* gene (Scheme 2). Knockout of the *proC* gene, followed by supplementation of the growth medium with 4-fluoroproline resulted in efficient incorporation of 4-fluoroproline throughout the biosynthetic elastin protein, containing eighty proline residues.

Proline residues adopt the central positions of type II β -turns within the [Val-Pro-Gly-Val-Gly] repeats of elastin as the temperature approaches the phase transition of the polypeptide [110]. Elastins substituted uniformly with either Flp or flp were prepared to explore the influence of prolyl residues on the thermodynamics of the phase transition and the physiologically relevant reversible aggregation state of native elastin [111]. Differential scanning calorimetry and CD spectroscopy confirmed that Flp stabilized the structured state and lowered the critical transition temperature, likely by enforcing the *exo* conformation commonly observed in type II β -turns. Conversely, elastins incorporating flp had less stable thermal transitions at a higher temperature, indicating weaker β -turn stability. Analysis of the flp elastin using ^{19}F -NMR spectroscopy detected significant *cis* isomer population at the valyl-prolyl peptide bond, which may exacerbate the destabilizing effect of this modification.

Ubiquitin has three native proline residues that were replaced with 4-fluoroproline by using the SPI method to study the influence of proline ring pucker on protein folding [112]. Ubiquitylation serves as a key post-translational modification with a wide variety of cellular effects, notably targeting proteins for proteasomal degradation [113]. The three prolines in native ubiquitin all reside in the *trans-exo* conformation, according to high-resolution crystal structure data [114]. Ubiquitin uniformly incorporating Flp was efficiently expressed from a bacterial auxotroph in high yield; however, attempts failed to incorporate flp, likely due to aggregation or degradation of unfolded protein. Both wild-type and Flp-ubiquitin exhibited CD spectra characteristic of native ubiquitin, and similar folding pathways involving a single intermediate detected by stopped-flow fluorescence measurements. The barrier between the unfolded state and the intermediate was lower for Flp ubiquitin, which exhibited slower unfolding from the native state to the intermediate. In thermal and guanidine-HCl induced denaturation experiments, Flp-ubiquitin exhibited greater stability than the native protein. Furthermore, Flp-ubiquitin served as substrate for ubiquitin-processing enzymes in self-ubiquitylation. In sum, (4*R*)-fluoroproline increased thermostability, accelerated protein folding, and maintained biological function.

Fluoroprolines have also been used to improve the physical properties of antibody fragments for potential uses in therapeutics, diagnostics, and biotechnology [115]. Lack of thermostability relative to their full-length parents has prevented applications of antibody fragments, such as single chain Fv (scFv) units prepared by linking the variable domains of IgG. Employing the SPI method, anti-c-Met scFv fragments were prepared possessing >90% substitution of 4-fluoroprolines at the five conserved proline positions as determined by mass spectrometric analysis. As observed with ubiquitin, incorporation of Flp and flp resulted respectively in the expression of properly folded soluble and mis-folded, insoluble protein. Moreover, Flp-scFv demonstrated higher activity in ELISA assays at elevated temperature compared to the wild-type protein.

Applying this technology to the fluorescent protein EGFP, flp and Flp were fed to a bacterial proline auxotroph to produce respectively soluble, flp-protein, and unfolded Flp-protein that was detectable only in inclusion bodies [116]. Attempts to fold the Flp-protein failed, indicating an irrecoverable effect on the structure, which was unexpected because Flp is known to promote the prolyl amide *trans* isomer found throughout GFP. Characterization of

the expressed EGFP containing flp by X-ray diffraction analysis detected no noticeable differences from native protein (Fig. 4a). Moreover, refolding experiments demonstrated that flp-incorporation accelerated the folding of EGFP relative to native protein. The only 4-fluoroproline protein reported to date that folded with flp instead of Flp residues, the flp-EGFP analog may undergo accelerated folding to the wild-type structure, due to the reduced barrier for prolyl amide isomerization of flp relative to Pro [52].

In another fluorescent protein, mRFP1, the opposite trend was observed: flp-incorporation produced only insoluble aggregates, and Flp-incorporation resulted in a soluble mRFP1, albeit without fluorescence [117]. Molecular modeling demonstrated a steric clash between the chromophore and the fluoro group of Flp63. By genetically mutating Pro63 to alanine before incorporating Flp globally, a fluorescent mRFP1 was obtained which folded faster and exhibited increased thermostability.

Optimized conditions were devised to replace the 32 native proline residues of the 60-kDa KlenTaq DNA polymerase with Flp at 92% efficiency [118]. Attempts to incorporate flp did not result in protein production. The resulting fluorinated enzyme was less thermostable than wild-type, but functioned with normal DNA replication kinetics and error rates. A refined preparation of the fluorinated protein with a substitution efficiency of 98% produced crystals of sufficient quality for diffraction to 2.4-Å resolution (Fig. 4b) [119]. The fluoro groups on the exterior of the protein were suggested to improve crystallinity by mediating contacts between individual protein molecules within the lattice. Pucker assignments were possible for 28 out of 32 proline residues in both the Flp and wild-type KlenTaq DNA polymerase crystal structures, which respectively exhibited 89% and 43% *exo* pucker, demonstrating that the strong *gauche* effect in Flp can alter the ring conformation in the context of structural bias from nonlocal interactions. Incorporation of Flp did not alter the two prolyl amide *cis* isomers in KlenTaq DNA polymerase, which adopted *endo* pucker, and accounted for half of the *endo*-puckered residues. Moreover, the electron density for 36% of the prolines in the wild-type polymerase was consistent with a superposition of puckers, only 7% of Flp residues in the fluorinated protein were consistent with multiple puckers in the electron-density map, indicating reduced conformational flexibility of the 4-fluoropyrrolidine ring.

Dissecting the impact of a modification at single residue is challenging using SPI, which inherently results in global substitution. To probe a single proline position with 4-fluoroproline, a variant of *Escherichia coli* thioredoxin was conceived in which the four *trans* prolines were replaced by alanine, and the single conserved *cis* proline was retained [120]. Both Flp- and flp-proteins were expressed, folded and shown to be stable in chemical denaturation experiments. Both modifications stabilized the reduced and destabilized the oxidized form of the protein. Both substitutions also improved the cooperativity of folding, which had been compromised by the four proline-to-alanine substitutions. Measures of catalytic activity revealed little effect from fluorination. The lack of significant differences of diastereomeric substitutions was explicable from high-resolution crystal structure analysis of all three single-proline proteins (Fig. 4c). Although Flp and flp favor usually *exo* and *endo* pyrrolidine ring puckers, respectively, both were observed in the *endo* conformation in their respective crystal structures. The Flp residue was predicted to adopt the *endo* pucker to avoid a steric clash between the 4-fluoro substituent and Cys35. The tertiary structure of

thioredoxin was suggested to nullify the usual conformational preferences of 4-fluoroproline.

The first total synthesis of a 4-fluoroproline protein was achieved using β 2-microglobulin (β 2m), the causative agent of dialysis-related amyloidosis [121]. In the native structure of β 2m, the amide of one of the five proline residues, Pro32, adopts a *cis* isomer [122], which isomerizes to the *trans* conformation in the amyloid, as shown by solid-state NMR spectroscopy [123]. Accordingly, prolyl amide isomerization was predicted to be a key step in the mechanism of amyloid formation. Traditional mutagenic experiments yielded contradictory results: substitution of Pro32 with glycine induced fibril formation, but substitution with valine or alanine did not [124]. 4-Fluoroprolines (Flp, flp, and Dfp) were employed to subtly and selectively evaluate the role of isomerization in the mechanism of β 2m amyloid formation. To incorporate site-specifically the 4-fluoroprolines, β 2m variants were produced using native chemical ligation to combine three peptide segments that were generated using solid-phase synthesis. Temporary protection of Cys25 as a thiazolidine allowed for assembly of the full-length protein, which was folded and oxidized into the native structure. Thermal denaturation of the substitution variants demonstrated that *cis*- and *trans*-favoring flp and Flp respectively increased and decreased stability of the folded state relative to wild-type. Although Dfp exhibits similar conformational preferences as Pro, the Dfp variant was the least stable of the series. The Dfp variant displayed the least cooperative unfolding, the highest association with a fluorescent probe of unstructured hydrophobic patches on proteins, and the most facile amyloidogenesis. In contrast to wild-type and the monofluoroproline variants, the Dfp variant formed fibrils spontaneously after two weeks at neutral pH. Although the relative prolyl *cis* and *trans* isomer populations alone were demonstrated to be insufficient, the rate of amide isomerization appeared key for dictating β 2m amyloid formation. Consistent with observations employing a fluorescent probe, rapid *cis*–*trans* isomerization at Pro32 may enhance flexibility of the BC loop to expose hydrophobic regions of the protein and increase the rate of amyloid formation.

7 Impact in Medicinal Chemistry

Fluorination has emerged as a powerful, general strategy for combating the oxidative metabolism of various pharmaceutical candidates [125]. In this light, 4-fluoroprolines are convenient building blocks for introducing fluorine into peptide-based pharmaceuticals [126], such as thrombin inhibitors [127]. Thrombin is an important protease for blood clotting, and targeted by inhibitors in therapeutic approaches to treat clotting diseases. Substitution of the central proline residue of a tripeptide inhibitor with flp and Flp gave respectively 200-fold reduced potency and retained potency without oxidative metabolism. In crystal structures of the thrombin-inhibitor complexes, the central proline residue preferred the *exo* ring pucker [128]. These results enabled production of compounds with improved metabolic stability and selectivity toward thrombin over related proteases like trypsin.

4-Fluoroprolines have similarly been employed in the development of peptide inhibitors of Stat3 phosphorylation [129]. Phosphorylation of Stat3 occurs after cytokines bind to their receptors and results in dimerization and translocation of Stat3 to the nucleus, where it

effects changes in gene expression. High activation of Stat3 has been observed in a number of disease states, including cancers, making Stat3 an important pharmaceutical target. Mimics of the SH2 phosphopeptide hold promise as inhibitors of dimerization, because phosphorylation occurs on the SH2 domain of Stat3. Replacement of a key proline residue within a phosphopeptide prodrug with either flp or Dfp resulted in analogs exhibiting increased potency in cellular assays, in spite of decreased affinity for full-length Stat3 *in vitro*, suggesting that the 4-fluoroproline substitution enhanced potency by retarding deleterious metabolism.

Crystal structures of the complex between neurotensin (NT) and its G protein-coupled receptor (GPCR) NST1 have been used in structure-based drug development programs to yield new analgesics [130]. NT features a key proline residue that binds within a small pocket of NST1 as confirmed by mutagenic studies. To develop selective inhibitors of the related receptor NST2 (which also binds to neurotensin), NT8-13 analogues were produced that contain a variety of proline modifications [131]. A cellular assay was employed to determine the ability of the analogues to inhibit binding of the native ligand, and demonstrated a preference of the receptor for *exo*-puckered prolines, including Flp. Further elaboration of the lead peptide with a single peptoid moiety produced a 4-fluoroproline-based ligand with nearly 10⁴-fold selectivity for NST2 over NST1, and provided a probe for ¹⁹F MRI imaging.

The effect of a fluoro substituent on ring conformation or peptide bond isomerization is usually the dominant contribution to systems incorporating *N*-acyl-fluoroprolines. Another consequence arises in *N*-alkyl-fluoroproline derivatives. In attempts to develop γ -aminobutyric acid (GABA) reuptake inhibitors related to the *N*-alkyl (*R*)-nipecotic acid drug Tiagabine, 4-fluorination of proline and homoproline analogs decreased potency [132], likely due to their decreased amine basicity.

In addition, ¹⁸F 4-fluoroproline analogs have begun to be developed as probes for positron emission tomography (PET), due to the relatively short half-life (110 min) of the fluorine isotope, the lack of necessary chelators, and opportunity for incorporation into nascent proteins, such as collagen. In spite of challenges to radio-synthesize, administer, and allow for trafficking and biosynthetic incorporation of the analog, ¹⁸F 4-fluoroprolines have been made [133–136], and their pharmacokinetics have been studied [137,138]. Their future application in imaging of tumors [138–141] or collagen synthesis [142,143] remains to be advanced.

8 Outlook

Research to date has clearly demonstrated the utility of 4-fluoroprolines for the rational probing of peptide and protein stability, dynamics and activity. Conformational analysis of 4-fluoroproline monomers has provided predictive insight into their effects upon incorporation into biopolymers, albeit complications have been observed due to the chemical and structural constraints imposed by these complex molecules. Complementing traditional genetic approaches for exploring protein structure–function relationships, the application of 4-fluoroprolines is expected to continue to find utility for probing key proline residues in a

wide variety of proteins due to their subtle, yet reliable modulation of polypeptide structure. Incorporation of 4-fluoroprolines has increased conformational stability and accelerated folding of a variety of important proteins. Future application of 4-fluoroprolines is thus well merited to enhance physical, chemical, and biological attributes in the development of chemotherapeutic agents and protein-based technologies.

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Fig. 1. Conformational attributes of proline residues. **a** Conformations of saturated five-membered rings. **b** Pyrrolidine ring puckers. **c** Peptide bond isomers.

Fig. 2. Stereoelectronic effects in 4-fluoroprolines. $\sigma(C-H) \rightarrow \sigma^*(C-F)$ Gauche interactions stabilizing the **a** $C\gamma$ -exo or **b** $C\gamma$ -endo conformations of Ac-Flp-OMe or Ac-flp-OMe, respectively. **c** The $n \rightarrow \pi^*$ interaction between adjacent carbonyl groups stabilizing the *trans* conformation of the Ac-Flp amide bond in Ac-Flp-OMe.

Fig. 3.
a Conformational preferences of Ac-flp-OMe. **b** Conformational preferences of Ac-Flp-OMe. Relative populations (%) were measured in dioxane at 25 °C with NMR spectroscopy [36].

Fig. 4. Three-dimensional structures of representative proteins containing 4-fluoroproline residues (green sticks). **a** EGFP containing flp (PDB entry 2q6p) [116]. **b** *Taq* DNA polymerase containing Flp (4dle) [119]. **c** Thioredoxin containing Flp (4hua) [120].

Scheme 1.

Routes for the chemical synthesis of 4-fluoroprolines. Protecting group manipulations are indicated with double arrows. **a** Original synthetic route employing inorganic fluoride [11]. **b** Synthesis of 4-fluoroproline from a single 4-hydroxyproline stereoisomer using sulfur trifluoride reagents [22,27]. **c** Synthesis of 4-fluoroproline from a single 4-hydroxyproline stereoisomer using organic fluoride [28].

Scheme 2.

Route for the biosynthesis of proline from glutamate.

Table 1

Conformational preferences of proline residues

Compound	Ring Pucker ^a	ϕ (°) ^a	ψ (°) ^a	$K_{\text{trans/cis}}$ ^b
Ac-Pro-OMe	<i>endo</i>	−79	177	4.6
Ac-Hyp-OMe	<i>exo</i>	−57	151	6.1
Ac-Flp-OMe	<i>exo</i>	−55	141	6.7
Ac-flp-OMe	<i>endo</i>	−76	172	2.5

^aDetermined with X-ray crystallography [51] or density functional theory calculations (Ac-flp-OMe) [36].^bMeasured in D₂O at 25 °C with NMR spectroscopy [42].

Table 2

Thermostability of triple-helical CMPs

(Xaa-Yaa-Gly) _n	<i>T</i> _m (°C) ^a	Reference
(Pro-Pro-Gly) ₇	No Helix	[70]
(Pro-Hyp-Gly) ₇	36	[42]
(Pro-Flp-Gly) ₇	45	[42]
(Pro-flp-Gly) ₇	No Helix	[42]
(flp-Pro-Gly) ₇	33	[67]
(Flp-Pro-Gly) ₇	No Helix	[67]
(flp-Flp-Gly) ₇	No Helix	[70]
(Pro-Pro-Gly) ₁₀	31–41	[61]
(Pro-Hyp-Gly) ₁₀	61–69	[61]
(Pro-Flp-Gly) ₁₀	91	[61]
(flp-Pro-Gly) ₁₀	58	[68]
(Flp-Pro-Gly) ₁₀	No Helix	[68]
(flp-Flp-Gly) ₁₀	30	[69]

^aThe value of *T*_m refers to the temperature at the midpoint of the thermal transition between the triple-helical and single-stranded states.