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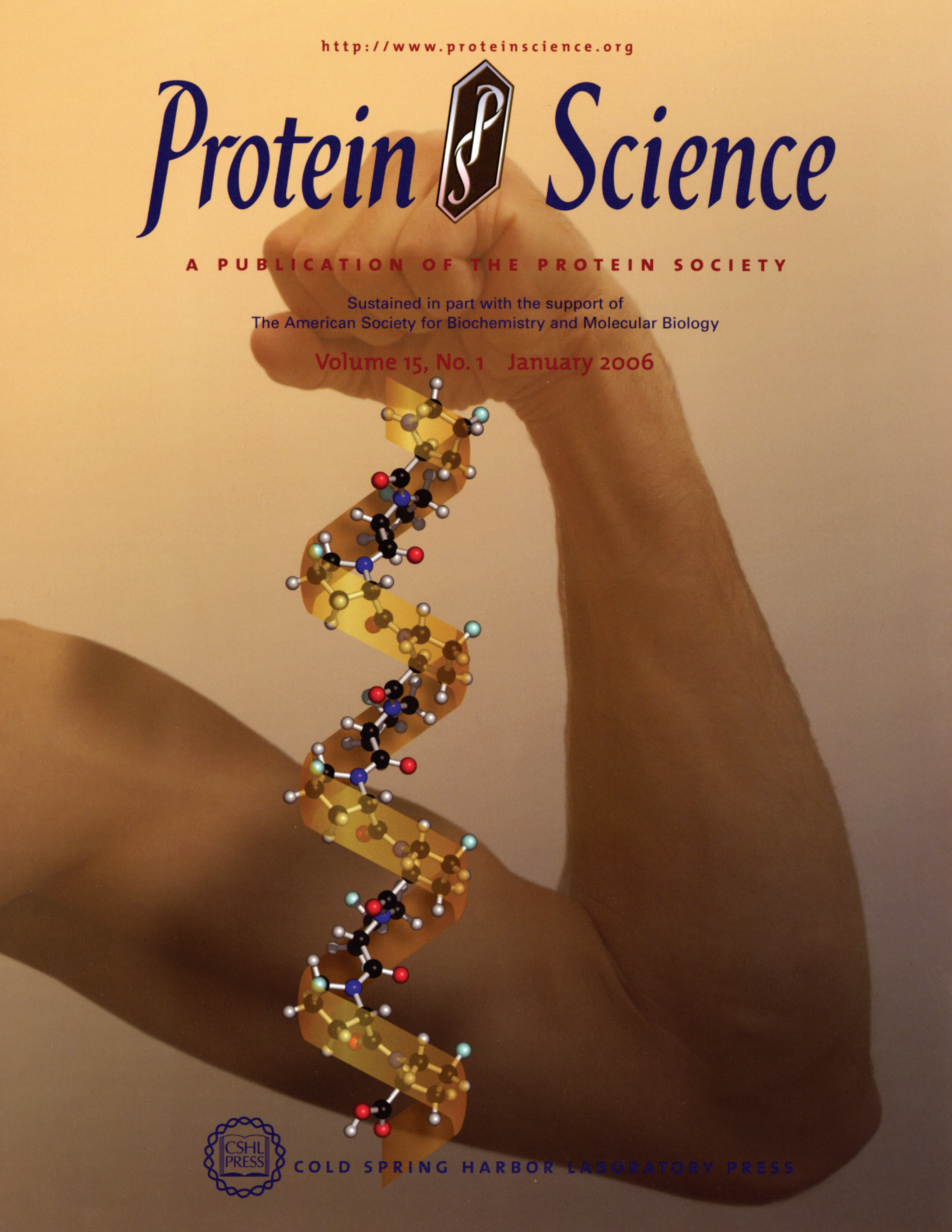

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# Stereoelectronic effects on polyproline conformation

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**Abstract**

The polyproline type II (PPII) helix is a prevalent conformation in both folded and unfolded proteins, and is known to play important roles in a wide variety of biological processes. Polyproline itself can also form a type I (PPI) helix, which has a disparate conformation. Here, we use derivatives of polyproline, $(Pro)_{10}$, $(Hyp)_{10}$, $(Flp)_{10}$, and $(flp)_{10}$, where Hyp is (2*S*,4*R*)-4-hydroxyproline, Flp is (2*S*,4*R*)-4-fluoroproline, and flp is (2*S*,4*S*)-4-fluoroproline, to probe for a stereoelectronic effect on the conformation of polyproline. Circular dichroism spectral analyses show that 4*R* electron-withdrawing substituents stabilize a PPII helix relative to a PPI helix, even in a solvent that favors the PPI conformation, such as *n*-propanol. The stereochemistry at C4 ordains the relative stability of PPI and PPII helices, as $(flp)_{10}$ forms a mixture of PPI and PPII helices in water and a PPI helix in *n*-propanol. The conformational preferences of $(Pro)_{10}$ are intermediate between those of $(Hyp)_{10}$/$(Flp)_{10}$ and $(flp)_{10}$. Interestingly, PPI helices of $(flp)_{10}$ exhibit cold denaturation in *n*-propanol with a value of $T_s$ near 70°C. Together, these data show that stereoelectronic effects can have a substantial impact on polyproline conformation and provide a rational means to stabilize a PPI or PPII helix.

**Keywords:** cold denaturation; collagen; fluoroproline; helix; hydroxyproline; polyproline; stereoelectronic effect

Polyproline adopts two stable secondary structures: the type I helix (PPI) and the type II helix (PPII) (Steinberg et al. 1960). PPI is a right-handed helix containing all *cis* peptide bonds and backbone dihedral angles of (ϕ, ψ, ω) = (–75°, 160°, 0°). PPII is a left-handed helix with all *trans* peptide bonds and backbone dihedral angles of (ϕ, ψ, ω) = (–75°, 145°, 180°). The PPI helix is compact, having a helical pitch of 5.6 Å/turn and 3.3 residues/turn. In comparison, the PPII helix is extended, having a helical pitch of 9.3 Å/turn and 3.0 residues/turn (Fig. 1). The PPI helix is favored in organic solvents, such as *n*-propanol, whereas the PPII helix dominates in aqueous solution (Knof and Engel 1974; Mutter et al. 1999; Kakinoki et al. 2005). The high conformational stability and rigidity of the PPII helix has led to the repeated use of $(Pro)_n$ sequences as a molecular "spacer" (Ungar-Waron et al. 1973) and "ruler" (Schuler et al. 2005). PPI helices are rare in biological contexts, whereas PPII helices are common in both folded proteins (Adzhubei and Sternberg 1993; Siligardi and Drake 1995; Kleywegt and Jones 1996) and unfolded polypeptides (Shi et al. 2002b), and are known to play important roles in biological signal transduction, transcription, cell motility, and immune responses (Kelly et al. 2001; Rath et al. 2005).

Unlike α-helices and β-sheets, there are no characteristic backbone hydrogen bonds in PPI and PPII helices. Hence, detecting these secondary structures in folded proteins directly by X-ray crystallography or NMR spectroscopy can be problematic, often leading to their mistaken assignment as random coils (Bochicchio and Tamburro 2002; Lam and Hsu 2003). In recent work, the PPII helix has been identified as the dominant element of

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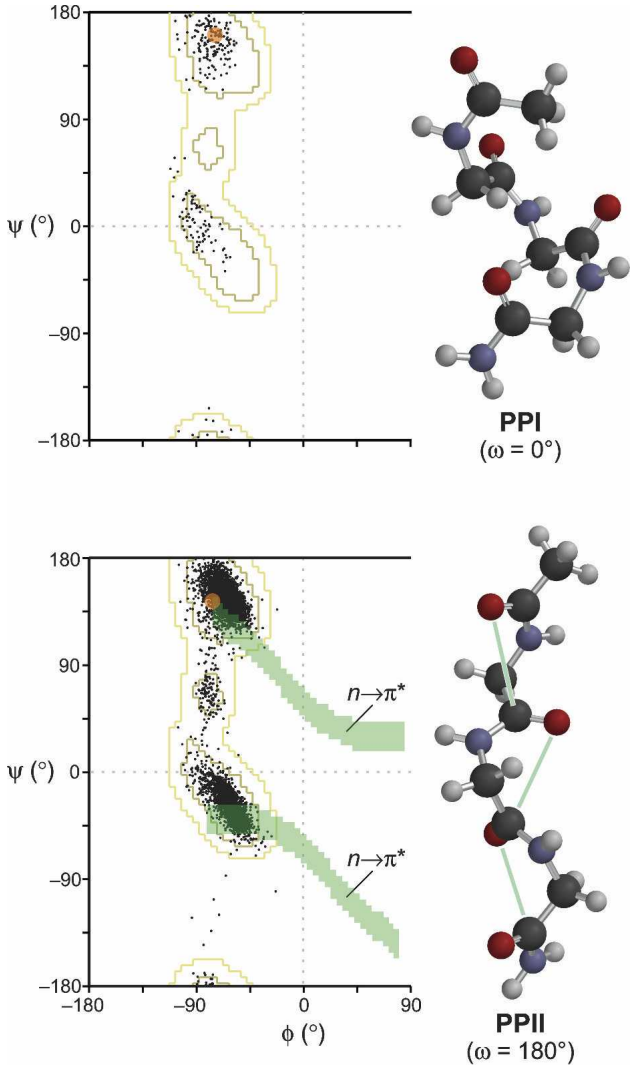



**Figure 1.** Ramachandran plots for a *cis* (*top*) and *trans* (*bottom*) proline residue and structures of $AcGly_3NH_2$ in a PPI (*top*) and PPII (*bottom*) helix. Ramachandran-plot distributions are from 500 PDB entries of crystalline protein structures known to a resolution of ≤1.8 Å. The orange dots indicate the ϕ and ψ angles for an ideal PPI (*top*) and PPII (*bottom*) helix. The green swathes (*bottom left*) indicate the ϕ and ψ angles that yield a favorable $n \rightarrow \pi^*$ interaction, and the green lines (*bottom right*) depict this interaction in a PPII helix (Hinderaker and Raines 2003).

secondary structure in unfolded proteins, and must therefore make a significant contribution to the energetics of protein folding (Hamburger et al. 2004; Whittington et al. 2005).

The basis for the intrinsic stability of the PPII helix is unclear. Most amino acid residues can adopt a PPII conformation, although each has a different propensity to do so (Woody 1992; Creamer and Campbell 2002; Rucker and Creamer 2002; Shi et al. 2002a; Ding et al. 2003; Eker et al. 2003; Chellgren and Creamer 2004; Cubellis et al. 2005; Jha et al. 2005). Aqueous solvation endows a PPII helix with conformational stability (Krimm and Tiffany 1974; Tanaka and Scheraga 1975; Creamer and Campbell 2002; Weise and Weisshaar 2003; Drozdov et al. 2004; Kentsis et al. 2004; Mezei et al. 2004), as can be inferred simply from its relative instability in nonaqueous solvents. Finally, the affinity of $O_{i-1}$ for $C'_i$ within the backbone appears to stabilize a PPII helix, which has an $O_{i-1}\cdots C'_i$ distance of 3.2 Å and an $O_{i-1}\cdots C'_i{=}O_i$ angle of 103° (Fig. 1). Enhancing this $n \rightarrow \pi^*$ interaction with stereoelectronic effects favors *trans* peptide bonds (Table 1) (Hinderaker and Raines 2003; Jenkins et al. 2004) and increases the conformational stability of collagen (Bretscher et al. 2001; Hodges and Raines 2003), which is a triple helix of PPII strands. Accordingly, we reasoned that stereoelectronic effects could mediate the propensity of polyproline to adopt a PPI or PPII helix.

Here, we report on the chemical synthesis of four peptides, $(Pro)_{10}$, $(Hyp)_{10}$, $(Flp)_{10}$, and $(flp)_{10}$ (Table 2), where Hyp is (2*S*,4*R*)-4-hydroxyproline, Flp is (2*S*,4*R*)-4-fluoroproline, and flp is (2*S*,4*S*)-4-fluoroproline, to probe for stereoelectronic effects on polyproline conformation. We use a fluoro group as an electron-withdrawing substituent because organic fluorine does not form hydrogen bonds and thus has little effect on aqueous solvation (Howard et al. 1996; Dunitz and Taylor 1997). We examine the conformation of each peptide in both aqueous and organic solvents by using temperature-dependent circular dichroism (CD) spectroscopy. We find that an electron-withdrawing substituent at the 4*R* position of proline can stabilize the PPII conformation, and that an electron-withdrawing substituent at the 4*S* position of proline can stabilize the PPI conformation. These results reveal an important role for stereoelectronic effects in the conformation of polyproline.

## Results and Discussion

### *Stabilization of PPII helices by 4*R *electron-withdrawing substituents*

Peptides $(Pro)_{10}$, $(Hyp)_{10}$, and $(Flp)_{10}$ were prepared by chemical synthesis. All three peptides have CD spectra

**Table 1.** *Effect of 4R and 4S substituents on* $K_{trans/cis}$ *of proline residues*

| Yaa | $K_{trans/cis}$ in AcYaaOMe[a] |
|---|---|
| Flp | 6.7 |
| Hyp | 6.1 |
| Pro | 4.6 |
| flp | 2.5 |

[a] Values were obtained in $D_2O$ at 25°C (Bretscher et al. 2001).

**Table 2.** *Peptides used in this study*

| Peptide | Sequence[a] |
|---|---|
| $(Pro)_{10}$ | $(Pro)_{10}$GlyTyrOH |
| $(Hyp)_{10}$ | $(Hyp)_{10}$GlyTyrOH |
| $(Flp)_{10}$ | $(Flp)_{10}$GlyTyrOH |
| $(flp)_{10}$ | $(flp)_{10}$GlyTyrOH |

[a] Each peptide has free N and C termini. Hyp is (2*S*, 4*R*)-4-hydroxyproline, Flp is (2*S*, 4*R*)-4-fluoroproline, and flp is (2*S*, 4*S*)-4-fluoroproline.

with a positive band between 220 and 230 nm and a negative band between 200 and 210 nm (Fig. 2), which are hallmarks of the PPII conformation (Woody 1992). The more intense maximum of $(Hyp)_{10}$ at 224 nm indicates that $(Hyp)_{10}$ has higher PPII content than do $(Pro)_{10}$ and $(Flp)_{10}$.

Temperature-dependent CD spectra were acquired on aqueous solutions of $(Pro)_{10}$, $(Hyp)_{10}$, and (Flp). The reversibility of PPII helix formation was found to be >90% for all three peptides. There is an apparent decrease in PPII content with increasing temperature for the peptides but without a clear unfolding transition (Fig. 3A), consistent with previous work (Kelly et al. 2001; Eker et al. 2003; Rucker et al. 2003; Chen et al. 2004). Accordingly, first-derivative curves were used to compare the conformational stability of each PPII helix (Fig. 3B). The estimated $T_m$ values from these curves are 27°C for $(Pro)_{10}$ and 53°C for $(Flp)_{10}$. The higher $T_m$ value of $(Flp)_{10}$ indicates that $(Flp)_{10}$ forms a more stable PPII helix than does $(Pro)_{10}$. Although no good estimate could be made for $(Hyp)_{10}$ because of its highly noncooperative transition, the PPII helix of $(Hyp)_{10}$ clearly has a higher $T_m$ value than does that of $(Pro)_{10}$. Thus, both $(Hyp)_{10}$ and $(Flp)_{10}$ form more stable PPII helices than does $(Pro)_{10}$.

CD spectra of all three peptides were also acquired in *n*-propanol (95% vol/vol), which is a favorable solvent for forming PPI helices (Mutter et al. 1999). Because the PPII → PPI conversion is slow, samples were incubated in *n*-propanol (95% vol/vol) for 6 d before measurements. $(Pro)_{10}$ has a CD spectrum in *n*-propanol (95% vol/vol) that differs significantly from that in aqueous solution (Fig. 4A). The maximum at 216 nm and minimum at 200 nm are diagnostic of a PPI helix (Rababal et al. 1993; Mutter et al. 1999; Crespo et al. 2002). In marked contrast, the CD spectra of $(Hyp)_{10}$ and $(Flp)_{10}$ in *n*-propanol (95% vol/vol) have similar positive and negative bands to those in aqueous solution (Fig. 4B,C), indicating that only PPII helices are present. The CD spectra of $(Hyp)_{10}$ and $(Flp)_{10}$ in *n*-propanol (95% vol/vol) did not change after a 30-d incubation (data not shown), indicating further that these two peptides form quite stable PPII helices.

High temperature disfavors the *trans* isomer of prolyl peptide bonds (Eberhardt et al. 1993, 1996). Likewise, temperature affects the thermodynamics of the PPI–PPII interconversion. CD spectra were acquired for solutions of $(Pro)_{10}$, $(Hyp)_{10}$, and $(Flp)_{10}$ in *n*-propanol (95% vol/vol) after a 4-d incubation at various temperatures. After this incubation, $(Pro)_{10}$ was observed to form a mixture of PPI and PPII helices, and to convert from PPII to PPI at higher temperatures (Fig. 5A). In comparison, no such conversion was observed for $(Hyp)_{10}$ and $(Flp)_{10}$, as only a PPII conformation was detected for those two peptides (Fig. 5B,C).

Glutamine has a higher PPII helix-forming propensity than any other nonproline residue. Hydrogen bonds between side chain and backbone atoms in glutamine residues appear to make a major contribution to this propensity (Stapley and Creamer 1999). Solvent-mediated hydrogen bonds have been indicted in the strong tendency of $(HypHypGly)_{10}NH_2$ to form a PPII helix (Schumacher et al. 2005), and could likewise contribute to the ability of $(Hyp)_{10}$ to form a PPII helix (Fig. 2). Nonetheless, because the side chain of Flp residues cannot form hydrogen bonds, another force must be responsible for its great propensity to form a PPII helix.

A hydroxyl or fluoro group in the 4*R* position increases the free energy difference between the PPI and PPII conformations (Figs. 2–5). Installing an electron-withdrawing substituent at the 4*R* position of a proline residue is known to induce a pyrrolidine ring pucker that favors the *trans* isomer of a prolyl peptide bond (because of a favorable $n \rightarrow \pi^*$ interaction) (Bretscher et al. 2001; DeRider et al. 2002; Taylor et al.

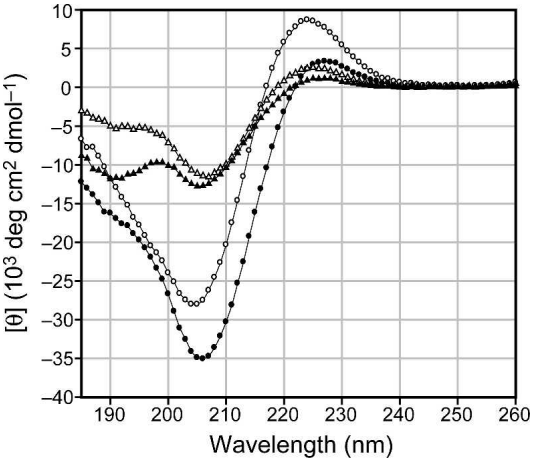



**Figure 2.** CD spectra of $(Pro)_{10}$ (●), $(Hyp)_{10}$ (○), $(Flp)_{10}$ (△), and $(flp)_{10}$ (▲). All measurements were conducted at 4°C on solutions of peptide (100 μM) in 20 mM sodium phosphate buffer at pH 7.0.

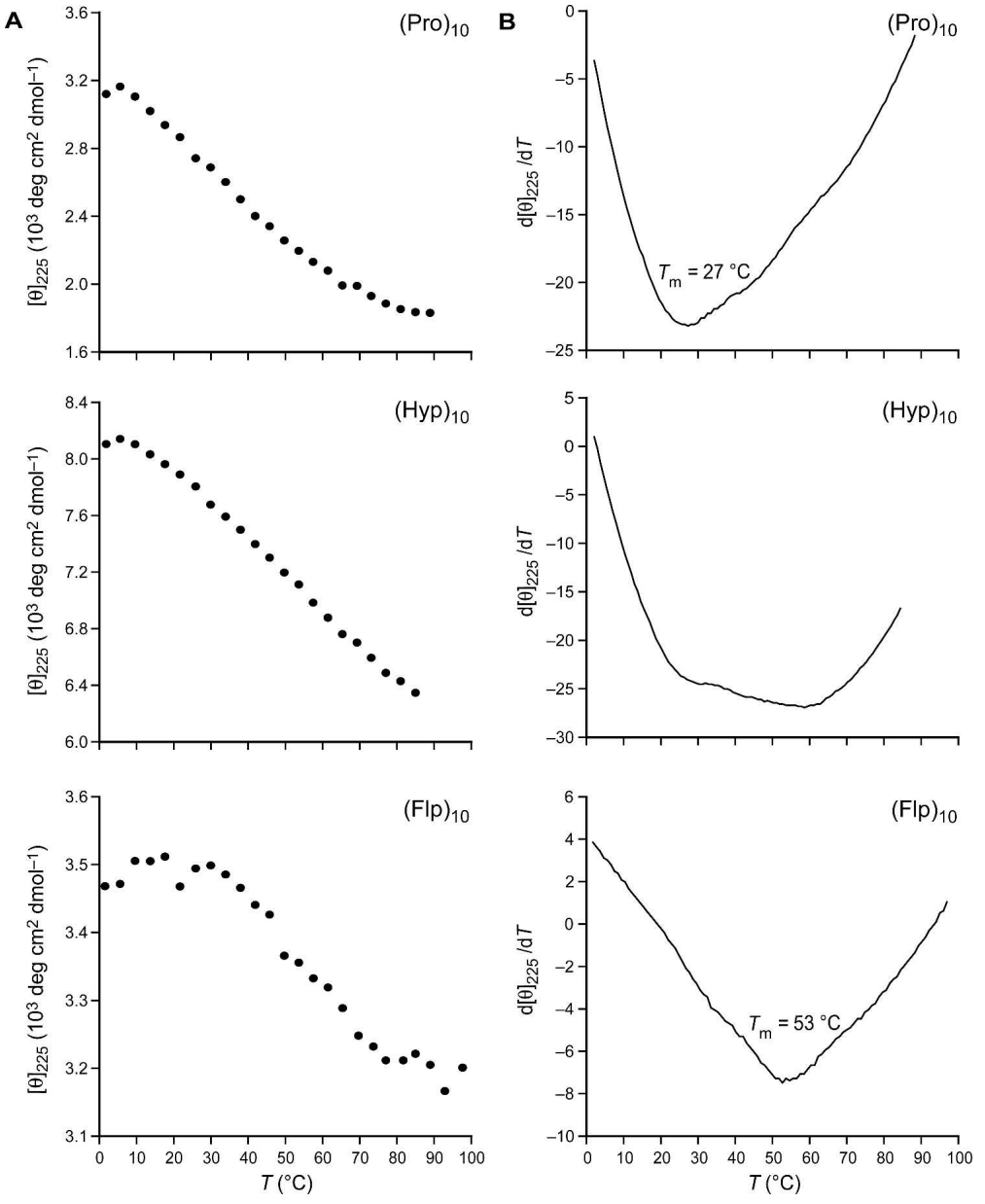



**Figure 3.** Thermal unfolding transition curves for $(Pro)_{10}$, $(Hyp)_{10}$, and $(Flp)_{10}$. All measurements were conducted on peptides (100 μM) in 20 mM sodium phosphate buffer at pH 7.0. (*A*) Effect of temperature on the molar ellipticity at 225 nm. (*B*) First-derivative curves of the data in *A* and the resultant values of $T_m$.

2005) as well as the ϕ and ψ dihedral angles found in a PPII helix (Hinderaker and Raines 2003). Thus, inductive effects from electron-withdrawing substituents in the 4*R* position stabilize the PPII conformation of polyproline.

It is noteworthy that the contribution of an $n \rightarrow \pi^*$ interaction to the conformational stability of a PPII helix could be cooperative (Hinderaker and Raines 2003). Both the negative charge on $O_i$ and the $C'_i = O_i$ bond length increase as a result of an $n \rightarrow \pi^*$ interaction between $O_{i-1}$ and $C'_i$ (Bretscher et al. 2001; DeRider et al. 2002). These effects would, in turn, serve to increase the stabilization provided by an $n \rightarrow \pi^*$ interaction between $O_i$ and $C'_{i+1}$. Such cooperativity could explain the recent finding that the aggregation of polyglutamine is suppressed by a C-terminal $(Pro)_{10}$ domain, which could impose a soluble PPII conformation on the polypeptide (Bhattacharyya et al. 2006).

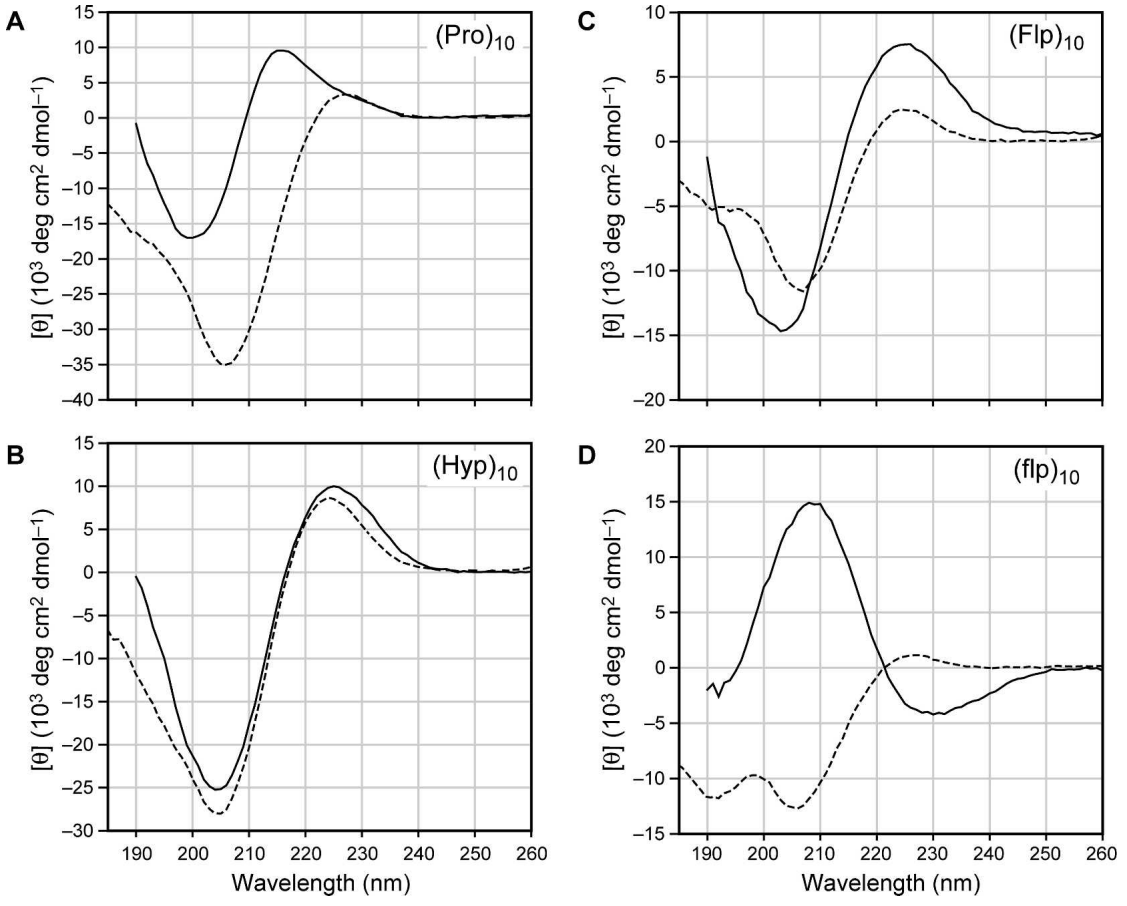



**Figure 4.** CD spectra in aqueous solution (dashed line) and *n*-propanol (95% vol/vol) (solid line) of (*A*) $(Pro)_{10}$, (*B*) $(Hyp)_{10}$, (*C*) $(Flp)_{10}$, (*D*) $(flp)_{10}$. All measurements were conducted at 4°C on either peptides (100 μM) in 20 mM sodium phosphate buffer at pH 7.0 or peptides (60–100 μM) in *n*-propanol (95% vol/vol).

*Stereoelectronic effects on polyproline conformation*

The peptide $(flp)_{10}$ was also prepared by chemical synthesis. In aqueous solution, $(flp)_{10}$ has a CD spectrum with a weak positive maximum at 225 nm and two moderate negative minima at 205 and 190 nm (Fig. 2), which differs from the typical CD spectrum of a PPII helix. The difference likely arises from $(flp)_{10}$ forming a mixture of PPI and PPII helices. Although the spectral characteristics of a PPII helix are present in aqueous solution of $(flp)_{10}$, the PPI content diminishes the positive band intensity in the region of 220–230 nm and generates local negative minima at 205 and 190 nm. Apparently, $(flp)_{10}$ has a significant propensity to form PPI helices, even in aqueous solution.

In *n*-propanol (95% vol/vol), $(flp)_{10}$ has a CD spectrum significantly different from that of a PPII helix. The positive maximum is blue-shifted away from the 220–230-nm region, and the CD spectrum has strong positive ellipticity at 208 nm and weak negative ellipticity at 230 nm (Fig. 4D). Although the spectrum differs slightly from that of $(Pro)_{10}$ in its PPI conformation, its characteristics are similar to those of PPI helices reported in the literature (Wu et al. 2003). In comparison to $(Pro)_{10}$ (Fig. 4A), $(flp)_{10}$ has enhanced positive and negative ellipticity, indicating that $(flp)_{10}$ has a greater propensity than does $(Pro)_{10}$ to form a PPI helix in *n*-propanol (95% vol/vol).

A fluoro group in the 4*S* position decreases the free energy difference between the PPI and PPII conformations (Fig. 4). Installing an electron-withdrawing substituent at the 4*S* position of a proline residue is known to induce a pyrrolidine ring pucker that obviates the $n \rightarrow \pi^*$ interaction and thereby disfavors the *trans* isomer of a prolyl peptide bond (Bretscher et al. 2001; DeRider et al. 2002; Taylor et al. 2005). Thus, inductive effects from electron-withdrawing substituents in the 4*S* position stabilize the PPI conformation of polyproline.

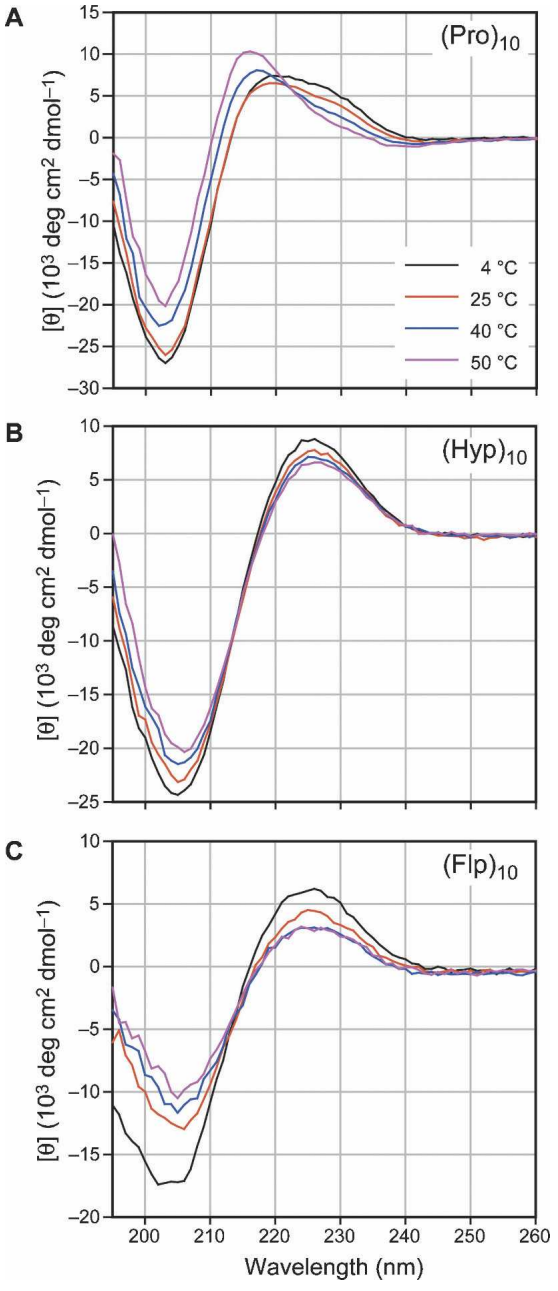



**Figure 5.** Effect of temperature on the CD spectrum in *n*-propanol (95% vol/vol) of (*A*) $(Pro)_{10}$, (*B*) $(Hyp)_{10}$, and (*C*) $(Flp)_{10}$. Solutions of peptide (50–100 μM) were incubated at 4°C for 4 d before measurements.

Flp, Pro, and flp are isologous residues that lack the ability to form hydrogen bonds. Accordingly, these three residues are likely to interact in a similar manner with aqueous and other solvents. Yet, $(Flp)_{10}$, $(Pro)_{10}$, and $(flp)_{10}$ have a markedly different propensity to form a PPII helix (Figs. 2–5). Although aqueous solvation provides empirical stabilization to the PPII conformation (Creamer and Campbell 2002), the relative stabilities of the PPII helices formed by $(Flp)_{10}$, $(Pro)_{10}$, and $(flp)_{10}$ derive from intrinsic stereoelectronic effects and not differential solvation.

### *Cold denaturation of the PPI helix*

Temperature-dependent CD spectra of $(Pro)_{10}$ and $(flp)_{10}$ were recorded in *n*-propanol (95% vol/vol). Although both peptides form PPI helices in *n*-propanol (95% vol/vol), these helices have distinct thermal properties. The PPI content of $(Pro)_{10}$ decreases with increasing temperature (Fig. 6A). In contrast, the PPI content of $(flp)_{10}$ is higher at 70°C than at temperatures less than or greater than 70°C (Fig. 6B). To characterize the behavior of $(flp)_{10}$ in more detail, the molar ellipticity at 209 nm was determined in *n*-propanol (95% vol/vol) as a function of temperature. The resulting data indicate that $(flp)_{10}$ has maximal PPI content at $T_s \approx 70$°C (Fig. 7). Such cold denaturation has been observed for molten

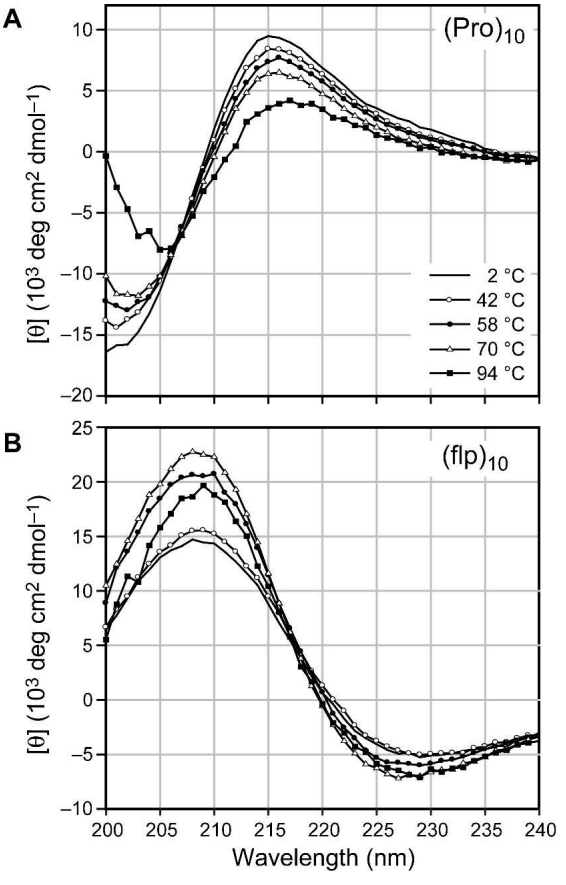



**Figure 6.** Effect of temperature on the CD spectrum in *n*-propanol (95% vol/vol) of (*A*) $(Pro)_{10}$ and (*B*) $(flp)_{10}$. Solutions of peptide (70–100 μM) were incubated at 4°C for > 6 d before measurements.

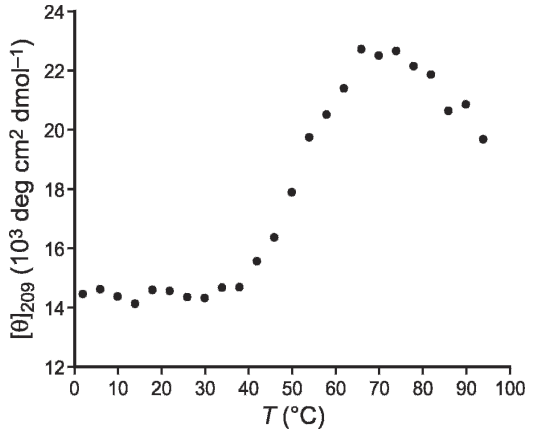



**Figure 7.** Effect of temperature on the molar ellipticity at 209 nm of $(flp)_{10}$ in *n*-propanol (95% vol/vol). The solution of $(flp)_{10}$ (70 μM) was incubated at 4°C for 6 d before measurements.

globule states of proteins, small helical peptide oligomers, and β-hairpin peptides (Kitakuni et al. 1994; Andersen et al. 1996, 1999; Bhattacharjya and Balaram 1997; Maynard et al. 1998; Searle et al. 1999; Griffiths-Jones and Searle 2000), although not with such a large value of $T_s$.

### *Conclusions*

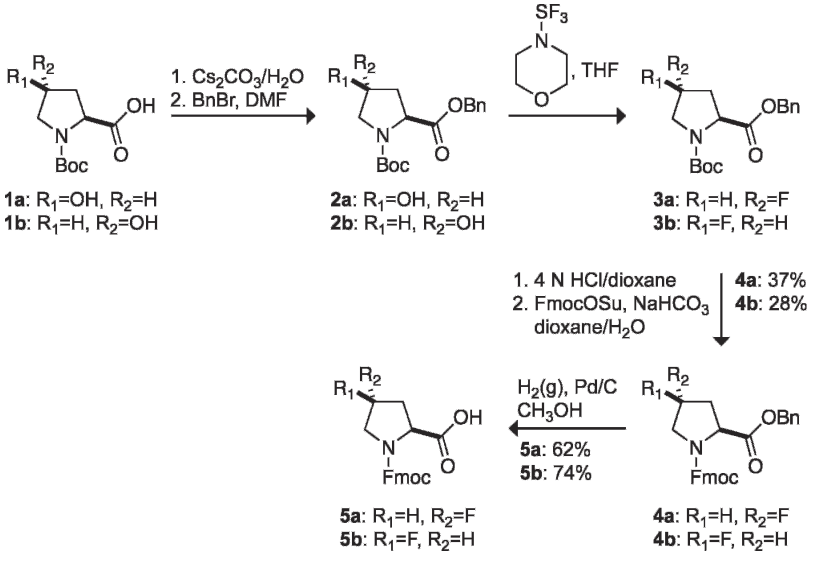
Electron-withdrawing substituents on C4 of proline residues have a profound influence on polyproline conformation. The outcome relies on the stereochemical configuration of the electron-withdrawing substituent, and is thus a manifestation of a stereoelectronic effect. An electron-withdrawing substituent in the 4*R* position constrains the ϕ and ψ dihedral angles to be close to those in a PPII helix and favors its requisite *trans* peptide bond by enhancing the $n \rightarrow \pi^*$ interaction between $O_{i-1}$ and $C'_i$ (Bretscher et al. 2001; DeRider et al. 2002; Hinderaker and Raines 2003; Taylor et al. 2005). An electron-withdrawing substituent in the 4*S* position obviates the $n \rightarrow \pi^*$ interaction and thereby alters the relative free energy in favor of the PPI helix. The absence of an electron-withdrawing substituent in proline itself leads to less determinate preferences for the ϕ, ψ, and ω dihedral angles and, consequently, an intermediate preference for a PPI or PPII helix. Accordingly, stereoelectronic effects play an important role in the relative free energy of PPI and PPII helices, and provide a rational means to modulate polyproline conformation.

## Materials and methods

### *General*

Chemical reagents were obtained from Aldrich Chemical or Fisher Scientific and used without further purification. Amino acids and their derivatives were obtained from OmegaChem, Novabiochem, or Chem-Impex International. DMF and THF were drawn from a Baker CYCLE-TAINER.

Boc-(2*S*,4*S*)-4-hydroxyproline benzyl ester (**2a**), Boc-(2*S*,4*R*)-4-hydroxyproline benzyl ester (**2b**), Boc-(2*S*,4*R*)-4-fluoroproline benzyl ester (**3a**), and Boc-(2*S*,4*S*)-4-fluoroproline benzyl ester (**3b**) were synthesized as described previously (Williams and Rapoport 1994; Hodges and Raines 2003). Fmoc-(2*S*,4*R*)-4-fluoroproline (**5a**) was either obtained from Bachem Bioscience or synthesized by the route in Scheme 1. Fmoc-(2*S*,4*S*)-4-fluoroproline (**5b**) was synthesized by the route in Scheme 1.




**Scheme 1.**

NMR spectra were recorded with a Bruker DMX-400 spectrometer in the National Magnetic Resonance Facility at Madison (NMRFAM). CD experiments were performed with an Aviv Model 202SF circular dichroism spectrometer at the University of Wisconsin-Madison Biophysics Instrumentation Facility.

### *Synthesis of Fmoc-(2S,4R)-4-fluoroproline benzyl ester (**4a**)*

To a solution of **3a** (1.94 g, 6.0 mmol) in anhydrous dioxane (30 mL) was added 4 N HCl/dioxane (23.3 mL, 93.2 mmol). The mixture was stirred at room temperature for 1 h and then concentrated by rotary evaporation to an oil-like residue. Dioxane (30 mL), $H_2O$ (25 mL), FmocOSu (2.02 g, 6.0 mmol), and $NaHCO_3$ (1.56 g, 18.6 mmol) were added to the residue. The reaction mixture was stirred at room temperature overnight and then concentrated by rotary evaporation. The residue was extracted with ethyl acetate (50 mL) and washed with $H_2O$ ($3 \times 50$ mL). The organic layer was dried over anhydrous $MgSO_4$(s) and concentrated by rotary evaporation. The crude product was purified by silica-gel column chromatography, eluting with $CH_3OH$ (1% vol/vol) in $CH_2Cl_2$ to yield **4a** as a colorless oil (1.0 g, 37%). $^1$H NMR (400 MHz, $CDCl_3$, two rotamers): δ 7.82–7.19 (m, 13H), 5.34–5.06 (m, 3H), 4.66–4.28 (m, 3H), 4.16–3.91 (m, 2H), 3.81–3.64 (m, 1H), 2.77–2.59 (m, 1H), 2.25–2.04 (m, 1H).

### *Synthesis of Fmoc-(2S,4R)-4-fluoroproline (**5a**)*

A small amount of $CH_3OH$ was added to **4a** (1.0 g, 2.24 mmol), and the mixture was flashed with Ar(g) before the addition of Pd/C (0.1 g) and more $CH_3OH$ (50 mL). The mixture was stirred under $H_2$(g) at room temperature for 5 h, filtered through Celite, and concentrated by rotary evaporation. The crude product was purified by silica-gel column chromatography, eluting with ethyl acetate/$CH_3OH$/HCOOH (99:1:0.1) to yield **5a** as a white powder (0.50 g, 62%). $^1$H NMR (400 MHz, $CDCl_3$, two rotamers): δ 7.74–7.26 (m, 8H), 5.22, 5.17 (two d, $J$ = 50.8, 52.0 Hz, 1H), 4.58–4.34 (m, 3H), 4.25, 4.13 (two t, $J$ = 7.2, 6.0 Hz, 1H), 4.05–3.87 (m, 1H), 3.68–3.56 (m, 1H), 2.72–2.57 (m, 1H), 2.31–2.07 (m, 1H). $^{13}$C NMR (100 MHz, $CDCl_3$, two rotamers): δ 177.0 (175.6), 155.7 (154.6), 143.7, 141.4, 127.9 (127.8), 127.3 (127.2), 125.1 (125.0), 120.2 (120.1), 91.5 ($J_{C-F}$ = 177 Hz), 90.9 ($J_{C-F}$ = 180 Hz), 68.2 (68.0), 57.9 (57.2), 53.3 ($J_{C-F}$ = 22.3 Hz), 53.7 ($J_{C-F}$ = 23.1 Hz), 47.2, 36.3 ($J_{C-F}$ = 22.9 Hz), 37.7 ($J_{C-F}$ = 23.1 Hz). ESI-MS: $m/z$ = 378.1133 ([M + $Na^+$]); 378.1118 ([M + $Na^+$], calcd.).

### *Synthesis of Fmoc-(2S,4S)-4-fluoroproline benzyl ester (**4b**)*

To a solution of **3b** (5.57 g, 17.2 mmol) in anhydrous dioxane (50 mL) was added 4 N HCl/dioxane (43 mL, 172 mmol). The mixture was stirred at room temperature for 1 h and then concentrated by rotary evaporation to an oil-like residue. Dioxane (50 mL), $H_2O$ (25 mL), FmocOSu (5.81 g, 17.2 mmol), and $NaHCO_3$ (2.89 g, 34.4 mmol) were added to the residue. The reaction mixture was stirred at room temperature overnight and then concentrated by rotary evaporation. The residue was extracted with ethyl acetate (50 mL) and washed with $H_2O$ (three times with 50 mL). The organic layer was dried over anhydrous $MgSO_4$(s) and concentrated by rotary evaporation. The crude product was purified by silica-gel column chromatography, eluting with $CH_3OH$ (1% vol/vol) in $CH_2Cl_2$ to yield **4b** as a colorless oil (2.15 g, 28%). $^1$H NMR (400 MHz, $CDCl_3$, two rotamers): δ 7.77–7.22 (m, 13H), 5.37–5.05 (m, 3H), 4.78–4.23 (m, 3H), 4.08 (t, $J$ = 9.6 Hz, 1H), 3.96–3.84 (m, 1H), 3.81–3.64 (m, 1H), 2.67–2.49 (m, 1H), 2.46–2.26 (m, 1H).

### *Synthesis of Fmoc-(2S,4S)-4-fluoroproline (**5b**)*

A small amount of $CH_3OH$ was added to **4b** (2.15 g, 4.83 mmol), and the mixture was flashed with Ar(g) before adding Pd/C (0.22 g) and more $CH_3OH$ (50 mL). The mixture was stirred under $H_2$(g) at room temperature for 6 h, filtered through Celite, and concentrated by rotary evaporation. The crude product was purified by silica-gel column chromatography, eluting with ethyl acetate/$CH_3OH$/HCOOH (99:1:0.1) to yield **5b** as a white powder (1.28 g, 74%). $^1$H NMR (400 MHz, $CDCl_3$, two rotamers): δ 7.77–7.28 (m, 8H), 5.25, 5.19 (two d, $J$ = 52.4, 52.8 Hz, 1H), 4.65–4.36 (m, 3H), 4.27, 4.17 (two t, $J$ = 6.8, 5.6 Hz, 1H), 3.90–3.79 (m, 1H), 3.75–3.59 (m, 1H), 2.66, 2.56 (two t, $J$ = 16.4, 16.8 Hz, 1H), 2.43–2.26 (m, 1H). $^{13}$C NMR (100 MHz, $CDCl_3$, two rotamers): δ 175.5 (176.5), 155.3 (154.5), 143.8 (143.7), 141.5 (141.3), 127.8, 127.3 (127.1), 125.2 (125.1), 120.1 (120.0), 92.0 ($J_{C-F}$ = 179 Hz), 91.1 ($J_{C-F}$ = 179 Hz), 68.1 (67.8), 57.8 (57.2), 53.1 ($J_{C-F}$ = 24.3 Hz), 53.7 ($J_{C-F}$ = 23.1 Hz), 47.2, 36.3 ($J_{C-F}$ = 22.9 Hz), 37.7 ($J_{C-F}$ = 23.1 Hz). ESI-MS: $m/z$ = 378.1113 ([M + $Na^+$]); 378.1118 ([M + $Na^+$], calcd.).

### *Peptide synthesis and purification*

Peptides were synthesized on a 25-μmol scale by solid-phase methods using Fmoc-protected amino acids, HBTU-mediated coupling, and standard reaction cycles on Applied Biosystems Model 432A automated peptide synthesizers with standard reaction cycles in the University of Wisconsin—Madison Biotechnology Center. All peptides contain GlyTyr at their C terminus to facilitate concentration determination (vide infra). The coupling time for $(Flp)_{10}$ and $(flp)_{10}$ was extended to 2 h for each cycle because of the difficulty of coupling reactions with 4-fluoroproline. Use of a Wang resin (which was preloaded with tyrosine) generated a free C terminus following cleavage from the resin with trifluoroacetic acid (TFA)/triisopropylsilane/$H_2O$ (38:1:1). Each peptide has a free N terminus. Peptides were purified by reverse-phase HPLC with a Varian $C_{18}$ semipreparative column. $H_2O$/acetonitrile gradients containing TFA (0.1% vol/vol) were used for the purification of $(Pro)_{10}$ and $(Hyp)_{10}$, and $H_2O$/isopropanol gradients containing TFA (0.1% vol/vol) were used to purify $(Flp)_{10}$ and $(flp)_{10}$. All peptides were >90% pure according to HPLC analysis. The identities of all peptides were confirmed by using matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. The calculated and observed molecular masses were $(Pro)_{10}$, calculated 1208.6, observed 1209.1; $(Hyp)_{10}$, calculated 1368.6, observed 1368.7; $(Flp)_{10}$, calculated 1388.5, observed 1388.6; $(flp)_{10}$, calculated 1388.5, observed 1388.7.

### *Circular dichroism (CD) spectroscopy*

Far-UV CD spectra were obtained at 4°C in 20 mM sodium phosphate buffer at pH 7.0 or *n*-propanol (95% vol/vol) using a 1-mm pathlength quartz cuvette and a spectrometer bandwidth of 1 nm. The peptide concentration was 100 μM in aqueous solution and 50–100 μM in *n*-propanol (95% vol/vol). Temperature-dependent measurements were made in a 1-cm pathlength quartz cuvette for aqueous samples (2.5-min equilibration at each temperature) and in a 1-mm pathlength quartz cuvette for nonaqueous samples (5-min equilibration). The reversibility of thermal denaturation was judged by the recovery of the signal at the conclusion of the experiment. All samples in *n*-propanol (95% vol/vol) were incubated at 4°C for 4 to >6 d before measurements. Peptide concentrations were determined by absorbance measurement in 6 M guanidine hydrochloride at pH 6.5 using $\varepsilon = 1450\ M^{-1}\ cm^{-1}$ at 276 nm (Gill and von Hippel 1989).

### *Estimation of* $T_m$ *values*

The temperature-dependent molar ellipticity at 225 nm was smoothed twice with Sigmaplot software (SPSS) to give a transition curve before calculating the first derivative of the molar ellipticity as a function of temperature. Values of $T_m$ were estimated from the minimum of the first-derivative curves.

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