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Stereoelectronic Tuning of the Structure and Stability of the Trp Cage Miniprotein

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Proline is unique among the canonical amino acids owing to the constraints imposed by backbone cyclization and the lack of an amide proton. Proline residues in proteins are preferentially observed in specific structural roles, including turns, loops, secondary structure termination, and polyproline helices.[1,2] The central role of proline residues in proteins indicates that protein structure, function, and stability should be tunable via control of the conformation of proline residues.[3−5]

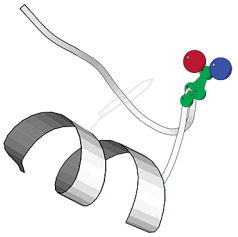
The proline side chain has two limiting conformations, the endo ring pucker, in which Cγ is puckered toward the proline carbonyl, and the exo ring pucker, in which Cγ is puckered toward H$_\alpha$. The proline ring pucker constrains the ϕ, ψ, and ω main chain torsion angles, leading to an observed correlation of proline ring pucker with main chain conformation: an exo ring pucker is more common in more compact conformations (α-helix, polyproline helix), while an endo ring pucker is favored in extended conformations.[2,3,6] Selective control of the proline ring pucker therefore provides the possibility of fixing the protein main chain conformation and thereby tuning the structure of the protein.[7]

We sought to selectively control protein structure and stability by using stereoelectronic effects to tune the protein backbone and side chain conformations, via defining the ring pucker of a critical proline residue. Incorporation of multiple 4-substituted proline derivatives has been used extensively to selectively control the stability of the repetitive proteins collagen and elastin, but these approaches have not been extensively applied to probe globular protein structure.[3−5]

Stereoelectronic tuning of protein structure was examined in the trp cage miniprotein, a designed 20-residue protein that has been the subject of considerable interest as a compact, well-folded motif (Figure 1).[8,9] Residue 12 of the trp cage is a proline residue that defines the central loop connecting the N-terminal α-helix and the C-terminal polyproline helix. In the NMR structure of the trp cage, Pro$_{12}$ exhibits an exo ring pucker. Therefore, replacement of Pro$_{12}$ with a residue stabilizing an exo ring pucker should selectively stabilize the miniprotein, while replacement of Pro$_{12}$ with a residue stabilizing an endo ring pucker should selectively destabilize the miniprotein.

Peptides containing a series of 4-substituted proline derivatives at Pro$_{12}$ were synthesized via proline editing, in which stereospecific postsynthetic modification reactions were conducted on Hyp-containing peptides to generate a series of peptides with the conformationally biased proline derivatives.[5,10,11] Peptides were synthesized with the 4*R*-(trans-substitution) or 4*S*-(cis-substitution) diastereomers of hydroxyproline and fluoroproline (Figure 2). In addition, peptides with the nitrobenzoates of hyp and Hyp, and with the trifluoromethylbenzoate of Hyp, were synthesized, on the basis of our previous[5a] observation of a strong gauche effect induced by the nitrobenzoate of hyp.[12]

The trp cage peptides were analyzed by circular dichroism (Figure 3a−c, Table 1).[11] Consistent with the trp cage NMR structure, trp cage peptides with endo-favoring residues exhibited reduced α-helicity, and peptides with exo-favoring residues exhibited enhanced α-helicity, compared to the peptide containing proline at residue 12 (trp cage-Pro). The highest α-helicities were observed in the trp cage peptides with the most exo-favoring residues, Flp, Hyp(4-NO$_2$Bz), and Hyp(4-CF$_3$Bz). Interestingly, trp cage-Pro exhibited a CD spectrum that is approximately the average of the endo (flp) and exo (Flp) CD spectra.



NLYIQWLKDGGP$_{12}$SSGRPPPS

Figure 1. Structure and sequence of the trp cage (PDB 1l2y).[8a,9h] Peptides were synthesized with proline derivatives in place of Pro$_{12}$ (green). Pro$_{12}$ 4*R* (red) and 4*S* (blue) hydrogens are indicated.

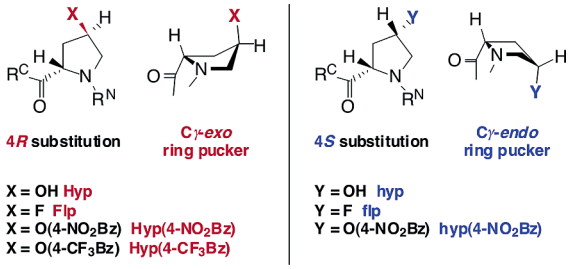


Figure 2. 4-Substituted proline derivatives incorporated at trp cage residue 12. The indicated side chain conformational preferences depend on the stereochemistry and electronics of the substitution, with a stronger conformational bias when X or Y is more electron-withdrawing.

Thermal denaturation experiments (Figure 3d, Table 1) revealed that all trp cage peptides with endo-favoring residues (4*S* substitution) at residue 12 were considerably destabilized compared to trp cage-Pro.[13] In contrast, peptides containing the strongly exo-favoring residues Flp, Hyp(4-NO$_2$Bz), or Hyp(4-CF$_3$Bz) at residue 12 exhibited increased thermal stability compared to trp cage-Pro. Overall, the thermal stability of the trp cage miniprotein was tunable by over 50 °C via the identity of the proline derivative at residue 12.

The trp cage peptides were analyzed by NMR spectroscopy at 285 K.[14] NMR revealed that all peptides, except the unfolded peptide trp cage-hyp, exhibited broadly comparable NMR spectra, similar to the described trp cage, suggesting that the peptides adopted similar structures.[11] Evidence of folding is provided by a series of chemical shift deviations described by Neidigh et al., including the diagnostic upfield shifting of the Trp$_6$ indole H$_N$.[8,11] Gly$_{11}$ H$_{\alpha 2}$ was shifted substantially upfield in all peptides except

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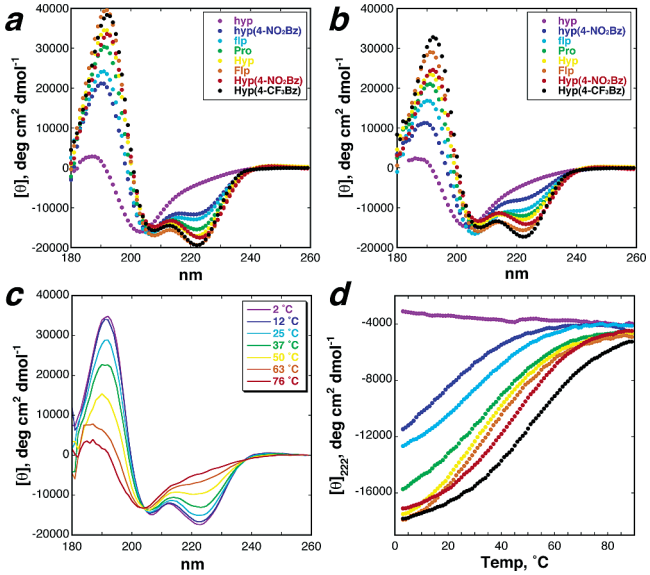

Figure 3. CD spectra of the trp cage peptides at (a) 2 °C and (b) 25 °C; (c) temperature-dependent CD spectra of trp cage-Hyp(4-NO_2Bz); (d) thermal denaturation curves for the trp cage peptides.

Table 1. CD Data (T_m and Data at 25 °C) for Trp Cage Peptides[a]

residue 12 of trp cage	T_m °C	$[\theta]_{222}$[b]	$[\theta]_{208}$[b]	$[\theta]_{190}$[b]	λ_{max} nm	$[\theta]_{222}/[\theta]_{208}$
hyp	<0	−4 370	−12 780	1 490	186	0.34
hyp(4-NO_2Bz)	11	−7 870	−13 870	11 250	189	0.57
flp	25	−10 570	−15 670	16 870	190	0.67
Pro	37	−12 030	−14 110	20 820	191	0.85
Hyp	40	−13 150	−14 160	22 840	191	0.93
Flp	42	−15 630	−15 800	28 320	192	0.99
Hyp(4-NO_2Bz)	50	−14 020	−13 250	23 110	192	1.06
Hyp(4-CF_3Bz)	59	−17 250	−14 900	30 340	192	1.16

[a] CD spectral data were collected at 25 °C in 15 mM phosphate buffer pH 7.0. [b] $[\theta]$ = mean residue ellipticity (deg cm^2 dmol^{-1}) at the indicated wavelength (nm). α-Helicity and folding are indicated by the magnitude of the bands at 222 or 190 nm, by the λ_{max} of the positive band near 190 nm, or by the ratios $[\theta]_{222}/[\theta]_{208}$ or $-[\theta]_{190}/[\theta]_{208}$, with a larger ratio indicating greater α-helicity and a more folded peptide.[11]

trp cage-hyp, consistent with a Trp_6-Gly_{11} interaction that is central to trp cage stability. Notably, the largest Gly_{11} $H_{\alpha 2}$ upfield shifts were observed in the peptides with the most exo-favoring residues (Hyp(4-CF_3Bz), 0.40 ppm; Hyp(4-NO_2Bz), 0.49 ppm; Flp, 0.70 ppm; Pro, 0.96 ppm), consistent with the CD data, providing additional evidence that enhanced protein stability may be achieved by optimizing the proline ring pucker via stereoelectronic effects.

We have demonstrated that the structure and stability of the trp cage miniprotein may be exquisitely tuned via the identity of the side chain of a single proline residue. In peptide and miniprotein motifs, the hydrophobic effect alone is often insufficient to define three-dimensional structure. Additional structural constraints imparted by conformational optimization can provide key stabilization of the defined epitopes required for biological function.[3−5,15] In view of the prominent roles of proline residues in proteins, the use of stereoelectronic effects should be a general mechanism to tune protein structure, stability, and function.

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Supporting Information Available: Experimental procedures, characterization data, and CD and TOCSY spectra for all peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) All eight peptides were synthesized without solution phase synthesis, although only Pro, Hyp, and Flp are commercially available as Fmoc amino acids. Peptides were acetylated on the N-terminus and contained C-terminal amides. The reported trp cage peptides (Tc5b)[8a] have free termini.
(11) See the Supporting Information for details.
(12) The ring pucker bias for 4-substituted prolines is manifested in the X-Pro amide cis−trans equilibrium ($K_{trans/cis}$).[3,4a,6] $K_{trans/cis}$ for all proline derivatives herein, except Hyp(4-NO_2Bz) and Hyp(4-CF_3Bz), was reported previously in the context TYPN.[5a] TYHyp(4-NO_2Bz)N and TYHyp(4-CF_3Bz)N, synthesized via on-resin acylation of TYHypN with DCC/DMAP and 4-NO_2-benzoic acid or 4-CF_3-benzoic acid, respectively, were more trans-favoring ($K_{trans/cis}$ = 8.2 and $K_{trans/cis}$ = 8.2, respectively) than TYFlpN ($K_{trans/cis}$ = 7.0) or TYHypN ($K_{trans/cis}$ = 5.6), compared to TYPN ($K_{trans/cis}$ = 2.7). These data indicate that benzoates enhance the gauche effect of hydroxyproline residues. Nitrobenzoate was also an effective hydroxyl protecting group that could be selectively removed: Gomez-Vidal, J. A.; Forrester, M. T.; Silverman, R. B. *Org. Lett.* **2001**, *3*, 2477−2479.
(13) Trp cage-hyp was poorly folded even in 30% TFE at 2 °C, suggesting that the trp cage may adopt a different structure when an endo ring pucker is enforced than is observed with an exo ring pucker.
(14) NMR spectroscopy revealed a single species for all peptides, indicating that the reduced stabilities of trp cage peptides containing endo-favoring residues were not due to cis−trans isomerism.
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