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Stabilization of the Collagen Triple Helix by O-Methylation of Hydroxyproline Residues

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The hydroxylation of proline residues in collagen is the most common posttranslational modification in humans. The hydroxylation is stereoselective, affording (2*S*,4*R*)-4-hydroxyproline (Hyp) in the Yaa position of the canonical Xaa–Yaa–Gly triad and thereby bestowing marked stabilization upon the collagen triple helix.¹ The means by which Hyp stabilizes collagen has engendered dispute. One hypothesis suggests that a network of water molecules links the Hyp hydroxyl groups and main-chain carbonyl groups.^{2,3} An alternative hypothesis invokes a stereoelectronic effect by which the electronegative oxygen preorganizes the main chain in the proper conformation for triple-helix formation.⁴

The latter explanation originates from the observation that replacing Hyp with (2*S*,4*R*)-4-fluoroproline (Flp) increases triple-helix stability; the fluoro group is strongly electron-withdrawing but cannot participate effectively in a putative hydrogen-bonded network. Similar results have been obtained with (2*S*,4*R*)-4-chloroproline.⁵ This explanation has been challenged by a host–guest study in which a single Hyp→Flp substitution was shown to destabilize a triple helix.⁶ A similar study has, however, reported a stabilization.⁷ So the question remains: does Hyp stabilize collagen by serving as a template for a water network or through stereoelectronic effects?

To differentiate between these hypotheses, we have made perhaps the simplest of covalent modifications to Hyp: *O*-methylation. Similar alkylations are known to decrease the hydration of alcohols,^{8,9} nucleobases,¹⁰ and phospholipids.¹¹ Yet, *O*-methylation conserves the stereoelectronic effects of a hydroxyl group, as the electron-withdrawing¹² and hyperconjugative ability¹³ of OH and OCH₃ are similar. Moreover, the *O*-methylation of Hyp introduces less steric encumbrance than does *O*-acetylation, which is known to destabilize the collagen triple helix.¹⁴

We used commercial (ProHypGly)₁₀ (**1**) as a basis for comparison. Then, we synthesized (2*S*,4*R*)-4-methoxyproline (Mop)¹⁵ and incorporated it into a collagen-related peptide: (ProMopGly)₁₀ (**2**). We then used circular dichroism (CD) spectroscopy to discern the effect of *O*-methylation. Peptides **1** and **2** were observed to form a triple helix at 4 °C, as evidenced by a weak positive CD signal near 225 nm and a strong negative signal near 200 nm (Fig. 1A). In addition, both were found to undergo cooperative transitions upon heating (Fig. 1B), indicative of an unfolding triple helix. Most interestingly, triple helices of **2** were discovered to have substantially more conformational stability than those of **1** (Table 1). As in water, **2**₃

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Supporting Information. Procedures and additional data for syntheses and analyses reported herein. This material is available free of charge via the Internet at <http://pubs.acs.org>.

was found to be more stable than **1**₃ in aqueous ethylene glycol (EG; Fig. 1B; Table 1), which is known to stabilize the collagen triple helix.^{4c,16}

Next, we used differential scanning calorimetry (DSC) to reveal the thermodynamic basis for the greater conformational stability of triple-helical **2**. The stability of **1**₃ relies more on enthalpy and less on entropy than does that of triple-helical (ProFlpGly)₁₀ (**3**), indicative of a lesser reliance on a water network.¹⁷ The thermodynamic parameters for **2**₃ lie between those for **1**₃ and **3**₃ (Fig. 1C; Table 1), suggesting that **2**₃ is hydrated to an intermediate extent. The decrease in hydration and increase in conformational stability in the series **1**₃→**2**₃→**3**₃ is consistent with hydration being *deleterious*, rather than advantageous, to the collagen triple helix.

Finally, we determined the effect of the methoxy group on the conformation of a Mop residue. To do so, we synthesized the model compound Ac-Mop-OMe and determined its crystal structure (Fig. 2A). The pyrrolidine ring of Mop adopts a C^γ-exo ring pucker, which likely derives from a *gauche* effect between N_i and O^{δ1}_i.^{4c,18,19} In addition, the conformation of Ac-Mop-OMe appears to rely on another stereoelectronic effect—the O_{i-1}...C'_i=O_i distance of 2.84 Å and O_{i-1}...C'_i=O_i angle of 94.6° are indicative of a favorable *n*→*π** interaction (Fig. 2B).^{4c,20} This stereoelectronic effect would stabilize the *trans* (*Z*) isomer of the amide bond in Ac-Mop-OMe. Indeed, Ac-Mop-OMe has a *trans*:*cis* ratio of *K*_{*t/c*} = 6.7 (Table 2), which is among the largest reported in a derivative of Ac-Pro-OMe.¹ Thus, these two stereoelectronic effects appear to preorganize the main-chain dihedral angles of Ac-Mop-OMe (as well as Ac-Hyp-OMe, and Ac-Flp-OMe) close to those in **1**₃ (Table 2).

The conformational stability conferred upon the collagen triple helix by *O*-methylation provides strong evidence that the hydroxyl group of Hyp acts primarily through stereoelectronic effects and that its hydration provides little (if any) benefit. This finding could have practical consequences. Replacing a hydroxyl group in a protein with a fluoro group while retaining stereochemical configuration (as in Hyp→Flp) is not possible with extant reagents. In contrast, *O*-methylation is a readily achievable transformation. Moreover, Hyp is much more abundant in human collagens than are the other two amino acids containing a hydroxyl group, Ser and Thr,²¹ and host–guest studies indicate that Ser and Thr are not especially beneficial to collagen stability.²² Thus, we believe that *O*-methylation could be a simple means to stabilize natural collagen, and thereby enhance its utility as a biomaterial.²³

Supplementary Material

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Acknowledgments

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Figure 1.

CD spectroscopy and DSC data for peptides **1** and **2**. (A) CD spectra of **1** (E) and **2** (J) (100 μM) at 4 $^{\circ}\text{C}$ in 50 mM HOAc (pH 3.0). (B) Thermal denaturation of **1** and **2** (200 μM) in 50 mM HOAc(aq) (E,J) and 2:1 EG/50 mM HOAc (pH 3.0) (A,F). (C) DSC scans of **1** (231 μM) and **2** (129 μM) in 50 mM HOAc (pH 3.0); scan rate = 15 $^{\circ}\text{C}/\text{h}$.

Figure 2. (A) Molecular drawing of crystalline Ac-Mop-OMe (50% probability ellipsoids). (B) Conformation of crystalline Ac-Mop-OMe and Ac-Hyp-OMe showing the putative $n \rightarrow \pi^*$ interaction.

Table 1

Thermodynamic data for the unfolding of collagen triple helices

Peptide	Sequence	circular dichroism			DSC	
		T_m , water ^a (°C)	T_m , EG(aq) ^b (°C)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
1	(ProHypGly) ₁₀	62.0	77.1	-35.2 ^c	-33.2 ^c	-2.0 ^c
2	(ProMopGly) ₁₀	70.1	89.1	-27.9	-25.2	-2.7
3	(ProFlpGly) ₁₀	91 ^d	ND	-20.5 ^c	-17.2 ^c	-3.3 ^c

ND = Not determined.

^a 50 mM HOAc (pH 3.0).

^b 2:1 EG/50 mM HOAc (pH 3.0).

^c Values from ref 17.

^d Value from ref 4a.

Table 2Values of φ , ψ , ω , and $K_{t/c}$ for Ac-Mop-OMe and analogs

parameter	Ac-Mop-OMe	Ac-Hyp-OMe ^a	Ac-Flp-OMe ^a	13 ^b
φ (°)	-58.1 ± 0.1	-57.0	-55.0	-59.6
ψ (°)	147.7 ± 0.1	150.8	140.5	149.8
ω (°)	-179.7 ± 0.1	-178.8	-178.9	178.5
$K_{t/c}$	6.7 ± 0.3^c	6.1	6.7	∞

^aMean values of φ , ψ , and ω from two molecules in ref 18; values of $K_{t/c}$ from ref 19.^bMean values for Hyp in **13**.^{2a}^cDetermined in 94:6 D₂O/CD₃OD by ¹³C NMR spectroscopy using [¹³CH₃]Ac-Mop-OMe.