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COMMUNICATIONS

Published on Web 02/14/2008

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-withdrawing[12] and hyperconjugative ability[13] of OH and OCH_3 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 (Figure 1A). In addition, both were found to undergo cooperative transitions upon heating (Figure 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, $\mathbf{2}_3$ was found to be more stable than $\mathbf{1}_3$ in aqueous ethylene glycol (EG; Figure 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

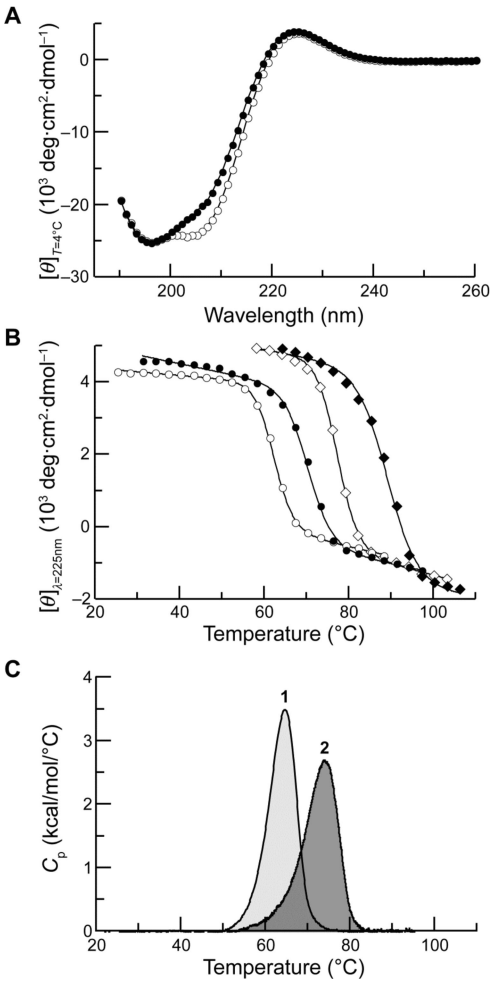

Figure 1. CD spectroscopy and DSC data for peptides **1** and **2**. (A) CD spectra of **1** (○) and **2** (●) (100 μM) at 4 °C in 50 mM HOAc (pH 3.0). (B) Thermal denaturation of **1** and **2** (200 μM) in 50 mM HOAc(aq) (○, ●) and 2:1 EG/50 mM HOAc (pH 3.0) (◇, ◆). (C) DSC scans of **1** (231 μM) and **2** (129 μM) in 50 mM HOAc (pH 3.0); scan rate = 15 °C/h.

of triple-helical **2**. The stability of $\mathbf{1}_3$ 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 $\mathbf{2}_3$ lie between those for $\mathbf{1}_3$ and $\mathbf{3}_3$ (Figure 1C; Table 1), suggesting that $\mathbf{2}_3$ is hydrated to an intermediate extent. The decrease in hydration and increase in conformational stability in the series $\mathbf{1}_3 \rightarrow \mathbf{2}_3 \rightarrow \mathbf{3}_3$ 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

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10.1021/ja800225k CCC: $40.75

Table 1. Thermodynamic Data for the Unfolding of Collagen Triple Helices

		circular dichroism		DSC		
peptide	sequence	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]

[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. ND = Not determined.

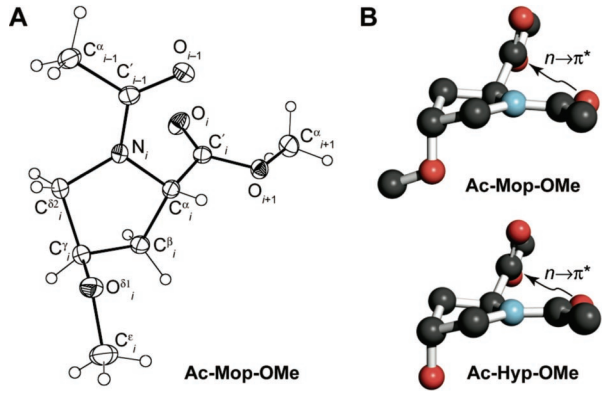

Figure 2. (A) Molecular drawing of crystalline Ac-Mop-OMe (50% probability ellipsoids). (B) Conformation of crystalline Ac-Mop-OMe and Ac-Hyp-OMe showing the putative $n \rightarrow \pi^*$ interaction.

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

parameter	Ac-Mop-OMe	Ac-Hyp-OMe[a]	Ac-Flp-OMe[a]	$\mathbf{1}_3$[b]
ϕ (deg)	−58.1 ± 0.1	−57.0	−55.0	−59.6
ψ (deg)	147.7 ± 0.1	150.8	140.5	149.8
ω (deg)	−179.7 ± 0.1	−178.8	−178.9	178.5
$K_{t/c}$	6.7 ± 0.3[c]	6.1	6.7	∞

[a] Mean values of ϕ, ψ, and ω from two molecules in ref 18; values of $K_{t/c}$ from ref 19. [b] Mean values for Hyp in $\mathbf{1}_3$.[2a] [c] Determined in 94:6 D_2O/CD_3OD by ^{13}C NMR spectroscopy using [$^{13}CH_3$]Ac-Mop-OMe.

(Figure 2A). The pyrrolidine ring of Mop adopts a C^{γ}-exo ring pucker, which likely derives from a *gauche* effect between N_i and $O^{\delta 1}{}_i$.[4c,18,19] In addition, the conformation of Ac-Mop-OMe appears to rely on another stereoelectronic effect; the $O_{i-1}\cdots C'_i{=}O_i$ distance of 2.84 Å and $O_{i-1}\cdots C'_i{=}O_i$ angle of 94.6° are indicative of a favorable $n \rightarrow \pi^*$ interaction (Figure 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 $\mathbf{1}_3$ (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 the 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]

Acknowledgment. We are grateful to M. D. Shoulders and D. R. McCaslin for contributive discussions. This work was supported by Grant AR044276 (NIH) and Instrumentation Grants BIR-9512577 (NSF) and RR13790 (NIH). F.W.K. was supported by Postdoctoral Fellowship AR050881 (NIH).

Supporting Information Available: Procedures and additional data for syntheses and analyses reported herein. Full citation for ref 15. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA800225K