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Imino Acids and Collagen Triple Helix Stability: Characterization of Collagen-like Polypeptides Containing Hyp-Hyp-Gly Sequence Repeats

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The analysis of factors contributing to the stability of proteins is a subject of intense debate. Particularly challenging is the study of structural proteins, since their function is their structure. Among these is collagen, the key structural component of bones, skin, and other connecting tissues. The collagen triple helix is characterized by the presence of hydroxyproline, whose content modulates triple helix stability. Due to the complexity and the fibrous nature of collagen, data on the stability and structure of this protein have been mainly obtained using collagen-like polypeptides.[1] Here we address the role of hydroxyproline in triple helix stability, based on a thermodynamic study of polypeptides containing repeating triplets Hyp-Hyp-Gly. In this scenario, we provide a comprehensive interpretation of the available data on collagen-like polypeptides containing proline derivatives.

Collagen triple helical structure consists of three polyproline II chains with repetitive sequence motif X-Y-Gly, (X and Y are frequently proline and hydroxyproline). In vertebrates, proline hydroxylation leading to 4*R*-hydroxy-L-proline (Hyp^R) occurs selectively at the Y position as a posttranslational modification which confers extra stability, as revealed using collagen-like polypeptides.[2] These studies also showed that the polypeptide with the repeating sequence Hyp^R-Pro-Gly does not associate in triple helix,[2b] that the stereoselectivity of the hydroxylation reaction is nonaccidental, since neither polypeptides $(Pro\text{-}Hyp^S\text{-}Gly)_{10}$ nor $(Hyp^S\text{-}Pro\text{-}Gly)_{10}$ associate in triple helix.[2c] How do we explain these stabilizing or destabilizing properties of Hyp?

The initial idea that solvation effects may stabilize the triple helix via water-mediated hydrogen bonds[3] was weakened by the observation that the polypeptide $(Pro\text{-}Flp^R\text{-}Gly)_{10}$, is hyperstable ($Flp^R$ is 4*R*-fluoro-L-proline) despite the low tendency of fluorine to form hydrogen bonds.[4] Therefore, stabilization was ascribed to inductive effects favoring the required *trans* conformation of the prolyl−peptide bond preceding Hyp^R. Since an influence of the *cis*−*trans* equilibrium on triple helix stability would have equally applied to $Pro\text{-}Hyp^R\text{-}Gly$ and $Hyp^R\text{-}Pro\text{-}Gly$ triplets, this hypothesis could not explain the triple helix destabilization induced by Hyp^R in the latter. Therefore, a new model was introduced, based on the different intrinsic conformational properties of Pro and Hyp.[5] Namely, (i) Pro adopts preferentially two distinct conformational states (down and up) which are characterized by backbone φ,ψ angles typical of the X and the Y positions, respectively,[6] (ii) Hyp^R preferentially up, with φ,ψ angles distinctive of the Y and not suitable for the X position, (iii) Hyp^S is preferentially down, requiring φ,ψ angles suitable for the X and not for the Y position. However, the insertion of Hyp^S at the X position provokes destabilizing inter-chain clashes.[5a]

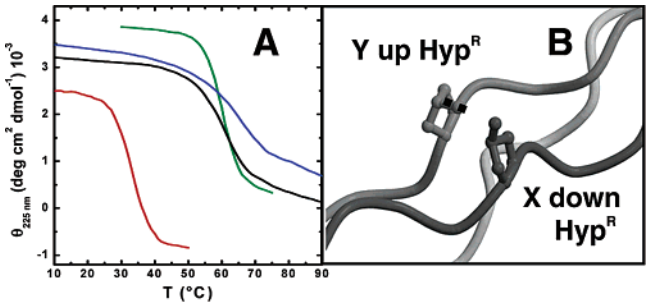

Figure 1. (A) Thermal transition curves of the collagen-like polypeptides $(PPG)_{10}$ (red), $(POG)_{10}$ (green), $(POG)_3(OOG)_4(POG)_3$ (black), and $(OOG)_{10}$ (blue). (B) Interaction between Hyp^R residues in X and Y positions of two $(OOG)_{10}$ adjacent chains, as derived by molecular modeling using O[16].

This propensity-based mechanism, supported by quantum mechanical calculations[7] and the recent finding that triple helix stabilization induced by Hyp^R is due to entropic effects,[8] afforded an explanation of the literature data. More recently, two independent studies showed that the presence of Flp^R in the X position of the Flp^R-Pro-Gly triplets does not allow triple helix folding, whereas the presence of Flp^S at the same position confers enhanced thermal stability.[9] In contrast, the polypeptide with repeating sequence Flp^S-Hyp^R-Gly, which was expected to be more stable than Flp^S-Pro-Gly, does not form the triple helix,[10] Table S1. In addition, polypeptides containing the repeating sequence Hyp^R-Thr-Gly are more stable than those containing Pro-Thr-Gly.[11] All these studies have renewed the challenge to rationalize the known experimental data and to gather further information to clarify the mechanism of triple helix stabilization.

With this aim we characterized the host−guest $(Pro\text{-}Hyp^R\text{-}Gly)_3$-$(Hyp^R\text{-}Hyp^R\text{-}Gly)_4(Pro\text{-}Hyp^R\text{-}Gly)_3$ ($(POG)_3(OOG)_4(POG)_3$) and the full-length $(Hyp^R\text{-}Hyp^R\text{-}Gly)_{10}$ ($(OOG)_{10}$) polypeptides, both containing Hyp^R simultaneously at the X and the Y positions. Despite the inability of $(Hyp^R\text{-}Pro\text{-}Gly)_{10}$ to fold in triple helix,[2b] thermal unfolding curves of the two polypeptides are typical of a triple helix transition (Figures 1A and S1, and S2). Their capability to form a triple helix is also supported by kinetic studies and crystallization experiments (Figures S3 and S4). Furthermore, thermal transition curves show that $(POG)_3(OOG)_4(POG)_3$ and $(OOG)_{10}$ consistently exhibit a moderate stabilization induced by proline hydroxylation at the X position of the Hyp^R-Hyp^R-Gly triplets, since their melting temperatures are respectively 1° and 5° higher than that of $(POG)_{10}$ (Figures 1A, S1, and S2 and Table S1). The lower slope of these curves, compared to those of $(POG)_{10}$ and $(PPG)_{10}$, indicates a lower degree of cooperative unfolding (Figure 1A). Our findings are consistent with the slight stabilization induced by the replacement of a single $Pro\text{-}Hyp^R\text{-}Gly$ triplet with $Hyp^R\text{-}Hyp^R\text{-}Gly$ in host−guest polypeptides.[1a,12] This consistency between results on host−

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guest and full-length polypeptides, which has not been observed in the case of the Pro-Flp^R-Gly triplet,[12] shows that stabilization induced by Hyp^R-Hyp^R-Gly triplets is compatible with the triple helical structure and is not to be attributed to local distortions of the triple helix.

Together with the data reported for (Hyp^R-Pro-Gly)$_{10}$ and (Hyp^R-Thr-Gly)$_{10}$,[2b,11] our results on (POG)$_3$(OOG)$_4$(POG)$_3$ and (OOG)$_{10}$ indicate that the effect on triple helix stability of Hyp^R in the X position strongly depends on the residue type allocated in the Y position (Table S1). This suggests that a contribution to the triple helix stability may derive from an interaction between residues allocated in the X and the Y positions of two adjacent chains.

On the basis of the propensity-based model,[5] the allocation of Hyp^R in the X position of the polypeptide (Hyp^R-Hyp^R-Gly)$_{10}$ should generate destabilizing effects. Indeed, for the correct building of the triple helix, the imino acid located in the X position should assume the backbone dihedral angles associated to a down puckering, whereas Hyp^R preferentially adopts the up state.[5a] However, quantum mechanical calculations have shown that the energies involved in the up−down transition of Hyp^R fall in the range 0.5−1.5 kcal/mol.[7a] Consistently, Hyp^R can occur in its less favored down state when this is concomitant with an extra-stabilizing interaction.[13] By modeling a down Hyp^R at the X position and an up Hyp^R at the Y position of the (Pro-Pro-Gly)$_{10}$ crystal structure,[6a] we observed that a direct hydrogen bond can be easily formed between Hyp^R residues belonging to two adjacent chains (Figure 1B and Supporting Information). This interaction may allow overcoming the energy gap between the up−down conformers of Hyp^R and has the advantage of keeping the correct φ,ψ angles to form the triple helix (since it keeps Hyp^R in X as a down conformer). The occurrence of a down puckering for Hyp^R in the presence of extra-stabilizing interactions is supported by the observation that the replacement of Pro with Hyp^R in the sequence Ac(Gly-Pro-Thr)$_{10}$NH$_2$ increases triple helix stability.[11] Indeed, a stabilizing either direct or water-mediated hydrogen bond between Hyp^R and Thr side chains at the X and Y positions of two adjacent helices is possible only if Hyp^R adopts a down conformation.

In the case of Hyp^R-Pro-Gly triplets,[2b] no interactions would be available to overcome the down−up energy gap, with the result of requiring φ,ψ angles not suitable for the X position. Along this line, the strong destabilizing effect induced by Flp^R-Hyp^R-Gly and Flp^R-Pro-Gly triplets may be ascribed to (i) the absence of stabilizing interactions between the side chains of the residues in X and Y position and to (ii) the higher energetic barrier between the up and the down state of Flp^R. Therefore, these peptides strictly follow the rules imposed by the propensity-based model.

Conformational preferences are also able to explicate the higher stability conferred to the triple helix by replacement of Pro-Pro-Gly triplets with Flp^S-Pro-Gly[9] or with Amp+^{S}-Pro-Gly[14] (Amp+^{S} = protonated 4*S*-amino-L-proline), independent results which cannot be explained invoking the *cis*−*trans* equilibrium.[4] Indeed, Flp^S and plausibly the charged Amp+^{S} have a higher tendency toward the *cis* conformer than Hyp^S and, therefore, should be destabilizing.[4,14b] Alternatively, the stabilization arising from the intrinsic higher propensity of Flp^S and likely of the charged Amp+^{S} (compared to Hyp^S)[15] to adopt a down conformation, with φ, ψ angles adequate for the X position, may compensate for the steric collisions generated by the pyrrolidine substituents, which appear to render Hyp^S destabilizing in X.[5a] Consistently, the protonated status of NH$_2$ in Amp^S is a prerequisite for triple helix formation.[14b] In addition, we have observed by molecular modeling that severe steric clashes exist in the structure of (Flp^S-Hyp^R-Gly)$_{10}$, due to the presence of the Hyp^R hydroxyl group at the Y position. This finding justifies the apparent contrasting result that Flp^S stabilizes the triple helix when in the X position of Flp^S-Pro-Gly triplets,[9] whereas it is strongly destabilizing in Flp^S-Hyp^R-Gly triplets (Table S1).[10]

Collectively, these considerations show that several factors modulate triple helix stabilization. A combination of imino-acid propensities and either attractive or repulsive side-chain interactions can rationalize all the apparently intricate literature data available. Finally, it should be noted that Hyp^R stabilizes in the Y position independent of the residue type in X. Conversely, stabilization in X occurs only when the residue located in Y is able to provide extra-stabilizing interactions. It is tempting to believe that this is the reason prolylhydroxylases almost exclusively act upon proline residues at the Y position of vertebrate collagens, with no need to control which residue is located in X. Namely, that is the reason Nature chose Y.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Persikov, A. V.; Ramshaw, J. A. M.; Brodsky, B. *Biopolymers* **2001**, *55*, 436−450. (b) Jenkins, C. L.; Raines, R. T. *Nat. Prod. Rep.* **2002**, *19*, 49−59. (c) Berisio, R.; Vitagliano, L.; Mazzarella, L.; Zagari, A. *Protein Pept. Lett.* **2002**, *9*, 107−116. (d) Okuyama, K.; Nagarajan, V.; Kamitori, S. *Proc. Indian Acad. Sci., Chem. Sci.* **1999**, *111*, 19−34.

(2) (a) Berg, R. A.; Prockop, D. J. *Biochem. Biophys. Res. Commun.* **1973**, *52*, 115−120. (b) Inouye, K.; Kobayashi, Y.; Kyogoku, Y.; Kishida, Y.; Sakakibara, S.; Prockop, D. J. *Arch. Biochem. Biophys.* **1982**, *219*, 198−203. (c) Inouye, K.; Sakakibara, S.; Prockop, D. J. *Biochim. Biophys. Acta* **1976**, *420*, 133−141.

(3) (a) Ramachandran, G. N.; Chandrasekharan, R. *Biopolymers* **1968**, *6*, 1771−1775. (b) Bella, J.; Eaton, M.; Brodsky, B.; Berman, H. M. *Science* **1994**, *266*, 75−81. (c) Bella, J.; Brodsky, B.; Berman, H. M. *Structure* **1995**, *3*, 893−906.

(4) (a) Holmgren, S. K.; Taylor, K. M.; Bretscher, L. E.; Raines, R. T. *Nature* **1998**, *392*, 666−667. (b) Holmgren, S. K.; Bretscher, L. E.; Taylor, K. M.; Raines, R. T. *Chem. Biol.* **1999**, *6*, 63−70.

(5) (a) Vitagliano, L.; Berisio, R.; Mazzarella, L.; Zagari, A. *Biopolymers* **2001**, *58*, 459−464. (b) Vitagliano, L.; Berisio, R.; Mastrangelo, A.; Mazzarella, L.; Zagari, A. *Protein Sci.* **2001**, *10*, 2627−2632.

(6) (a) Berisio, R.; Vitagliano, L.; Mazzarella, L.; Zagari, A. *Protein Sci.* **2002**, *11*, 262−270. (b) Kramer, R. Z.; Vitagliano, L.; Bella, J.; Berisio, R.; Mazzarella, L.; Brodsky, B.; Zagari, A.; Berman, H. M. *J. Mol. Biol.* **1998**, *280*, 623−638. (c) Hongo, C.; Nagarajan, V.; Noguchi, K.; Kamitori, S.; Okuyama, K.; Tanaka, Y.; Nishino, N. *Polym. J.* **2001**, *33*, 812−818.

(7) (a) Improta, R.; Benzi, C.; Barone, V. *J. Am. Chem. Soc.* **2001**, *123*, 12568−12577. (b) Improta, R.; Mele, F.; Crescenzi, O.; Benzi, C.; Barone, V. *J. Am. Chem. Soc.* **2002**, *124*, 7857−7865. (c) Mooney, S. D.; Kollman, P. A.; Klein, T. E. *Biopolymers* **2002**, *64*, 63−71.

(8) Berisio, R.; Granata, V.; Vitagliano, L.; Zagari, A. *Biopolymers* **2004**, *73*, 682−688.

(9) (a) Hodges, J. A.; Raines, R. T. *J. Am. Chem. Soc.* **2003**, *125*, 9262−9263. (b) Doi, M.; Nishi, Y.; Uchiyama, S.; Nishiuchi, Y.; Nakazawa, T.; Ohkubo, T.; Kobayashi, Y. *J. Am. Chem. Soc.* **2003**, *125*, 9922−9923.

(10) Barth, D.; Milbradt, A. G.; Renner, C.; Moroder, L. *ChemBioChem* **2004**, *5*, 79−86.

(11) (a) Bann, J. G.; Bachinger, H. P. *J. Biol. Chem.* **2000**, *275*, 24466−24469. (b) Mizuno, K.; Hayashi, T.; Bachinger, H. P. *J. Biol. Chem.* **2003**, *278*, 32373−32379.

(12) Persikov, A. V.; Ramshaw, J. A.; Kirkpatrick, A.; Brodsky, B. *J. Am. Chem. Soc.* **2003**, *125*, 11500−11501.

(13) Garbay, C.; Arnoux, B.; Prangè, T.; Wehri, S.; Pascard, C.; Roques, B. P. *J. Am. Chem. Soc.* **1979**, *102*, 1827−1837.

(14) (a) Babu, I. R.; Ganesh, K. N. *J. Am. Chem. Soc.* **2001**, *123*, 2079−2080. (b) Umashankara, M.; Babu, I. R.; Ganesh, K. N. *Chem. Commun.* **2003**, *20*, 2606−2607.

(15) DeRider, M. L.; Wilkens, S. J.; Waddell, M. J.; Bretscher, L. E.; Weinhold, F.; Raines, R. T.; Markley, J. L. *J. Am. Chem. Soc.* **2002**, *124*, 2497−2505.

(16) Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard *Acta Crystallogr.* **1991**, *A47*, 110−119.

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