Collagen

DOI: 10.1002/anie.201008118

Importance of Ring Puckering versus Interstrand Hydrogen Bonds for the Conformational Stability of Collagen**

*Roman S. Erdmann and Helma Wennemers**

The fibrous protein collagen is the most abundant protein in mammals and plays a crucial role both in numerous cellular activities and as a structural protein.[1] Understanding the factors that govern the conformational stability of collagen is therefore important. In addition, there is a growing interest in collagen-based functional materials and as a result the development of synthetic collagen that bears functionalizable groups is important.[2,3]

Collagen is built up of single strands that form triple helices which then further assemble into bundles and fibres.[1] The single strands consist of repeating Xaa–Yaa–Gly units with all amide bonds in *trans* conformation. Proline (Pro) is most often found in the Xaa position and (4*R*)-hydroxyproline (Hyp) in the Yaa position. Within the triple helix the three strands are held together by hydrogen bonds between the NH group of glycine (Gly) of one strand and the C=O group of Pro of the adjacent strand (Figure 1a). Crystal structures show C(4)-*endo* ring puckers of the Pro residues in the Xaa and C(4)-*exo* ring puckers of the Hyp residues in the Yaa positions (Figure 1a).[1,4] For the dihedral angles Ψ (N_i-$C_{i\alpha}$-C_i-N_{i+1}) that are responsible for the directionality of the collagen strands average values of approximately 155° (Xaa) and approximately 150° (Yaa) are observed.[1,4]

Studies with collagen model peptides (CMPs) in which the natural Pro and Hyp residues were replaced by other proline derivatives led to the conclusion that both the ring puckering and the interstrand H bonds are crucial for the conformational stability of the collagen triple helix.[5,6] All of the studies that address the importance of the ring puckering were performed with proline derivatives in which the *trans* amide conformer is significantly favored over the *cis* conformer in the case of C(4)-*exo* ring-puckered derivatives, whereas the *trans* conformer is less favored in derivatives with C(4)-*endo* ring puckers.[3,5] In addition, the Ψ angles of the C(4)-*endo* ring puckered derivatives examined so far are typically approximately 180° and are not close to those in collagen. Thus, any C(4)-*endo* ring-puckered derivative had an unfavorable bias towards the *trans* amide bond and the Ψ angle.

We have recently introduced proline derivatives, such as (4*S*)-acetamido proline (Acp), in which intramolecular hydrogen bonding leads to a preference for the C(4)-*endo* ring pucker and the *trans* amide conformer in aqueous solutions (Figure 1b).[7] This is due to an enforced dihedral angle Ψ at approximately 140° by the intramolecular H bond, which allows for a stabilization of the *trans* isomer by a n→π* interaction.[7] This dihedral angle Ψ within (4*S*)Acp is comparable to those observed in collagen and proline derivatives such as (4*R*)Hyp with a C(4)-*exo* ring pucker.[1d] Proline derivatives such as (4*S*)Acp therefore allow for the first time to investigate, whether a C(4)-*endo* ring pucker is tolerated in the Yaa position without a concomitant unfavorable bias on the *trans* amide bond and the Ψ angle. Furthermore, the combination of the C(4)-*endo* ring pucker and the intramolecular H bond allows for probing whether the ring pucker or the interstrand H bonds are more important in the Xaa position for the stability of the collagen triple helix. Herein, we demonstrate that a mismatched ring pucker is tolerated, whereas the interstrand H bonds are crucial for the conformational stability of the collagen triple helix.

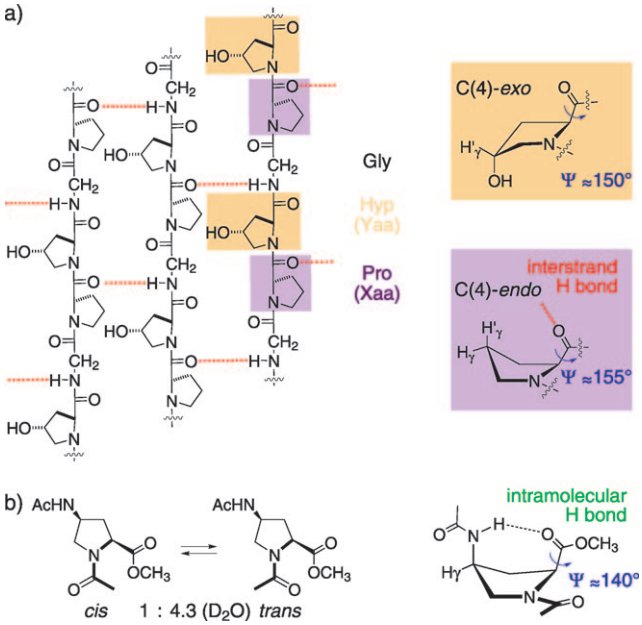

Figure 1. a) General structure of collagen. b) Ac–(4*S*)Acp–OCH_3 with C(4)-*endo* conformation by intramolecular H bonding.

We started our investigations by analyzing the possible effects of the incorporation of (4*S*)Acp in the Yaa position on the properties of the collagen triple helix (Figure 2): The ring pucker of (4*S*)Acp is C(4)-*endo* and a mismatch to that of the C(4)-*exo* pucker of the natural (4*R*)Hyp residue. Thus, if the

[*] R. S. Erdmann, Prof. Dr. H. Wennemers
Department of Chemistry, University of Basel
St. Johanns-Ring 19, 4056 Basel (Switzerland)
Fax: (+41) 61-267-0976
E-mail: helma.wennemers@unibas.ch

[**] This work was supported by the NCCR NANO and Bachem. H.W. is grateful to Bachem for an endowed professorship.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201008118.

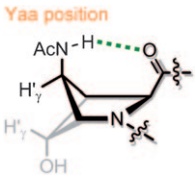

Figure 2. Structural differences between a (4*S*)Acp residue (black) and the natural (4*R*)Hyp residue (gray) in the Yaa position of collagen.

C(4)-*exo* ring puckering in the Yaa position is crucial for the stability of the collagen triple helix, incorporation of (4*S*)Acp in the Yaa position should lead to a significant destabilization.

To probe the effect experimentally we prepared CMP **1Y** bearing (4*S*)Acp in the Yaa position of the middle repeat unit within a 21mer.[8] In addition, CMP **2Y** that bears the conformationally analogous but sterically different (4*S*)-formamido proline (Fmp) in the Yaa position was prepared.[7,9] Such host–guest CMPs with one varied residue have proven valuable for the investigation of the effect of single residues on the conformational stability of collagen.[10] CMPs **3** and **4** with (4*R*)Hyp and Pro residues, respectively, in the Yaa position were prepared for comparison.

Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*S*)Acp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**1Y**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*S*)Fmp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**2Y**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-**(4*R*)Hyp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3**)
Ac-(Pro-Hyp-Gly)$_3$-(Pro-**Pro**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4**)

Thermal denaturation studies using circular dichroism (CD) spectroscopy as a monitoring tool were used to investigate the relative stabilities of the collagen triple helices derived from CMPs **1Y**, **2Y**, **3**, and **4**. All CMPs formed triple helices as indicated by the observed maxima at 225 nm, which is typical for the collagen triple helix.[1,5] Upon heating solutions of **1Y**, **2Y**, **3**, and **4**, comparable midpoints of thermal transition (T_m) were observed (Figure 3, spheres and triangles).

For the known reference compounds **3** and **4**, T_m values of 43 °C and 40 °C, respectively, were observed, which are consistent with previous studies.[3,11] The T_m value of CMPs **1Y** and **2Y** are 40 °C and 39 °C, respectively, and identical or close to those of CMPs **4** and **3**. These results demonstrate that the C(4)-*endo* ring puckering of (4*S*)Amp and (4*S*)Acp does not destabilize the collagen triple helix to a significant extent. It shows that a C(4)-*exo* ring pucker is not strictly required in the Yaa position of collagen. An overlay of (4*S*)Acp with a (4*R*)Hyp residue in the crystal structure[4b] of a collagen triple helix shows the mismatched puckering. It also illustrates that the Ψ angles of (4*S*)Acp and the natural collagen strand are in good agreement and that no steric constraints arise (Figure 4). In addition, the overlay shows

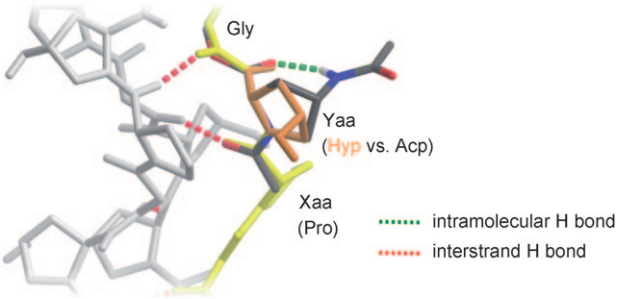

Figure 4. Overlay of the lowest-energy structure of Ac-(4*S*)Acp-OMe (gray)[7] with a (4*R*)Hyp residue (orange) in the Yaa position of collagen (PDB 1 V7H).[4b]

that the intramolecular H bond within the (4*S*)Acp residue does not interfere with the interstrand H bonds that hold the three strands together.[12]

To further analyze the importance of the ring puckering for the stability of the collagen triple helix, we also incorporated (4*S*)Acp and (4*S*)Fmp in the Xaa position and prepared CMPs **1X** and **2X**.[13]

Ac-(Pro-Hyp-Gly)$_3$-(**(4*S*)Acp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**1X**)
Ac-(Pro-Hyp-Gly)$_3$-(**(4*S*)Fmp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**2X**)

In the Xaa position, the C(4)-*endo* ring puckering of (4*S*)Fmp and (4*S*)Acp matches that of the natural Pro residue (Figure 5a). However, since the carbonyl group of Pro is involved in the interstrand H bonds of collagen, the intramolecular H bond within (4*S*)Acp and (4*S*)Fmp can be

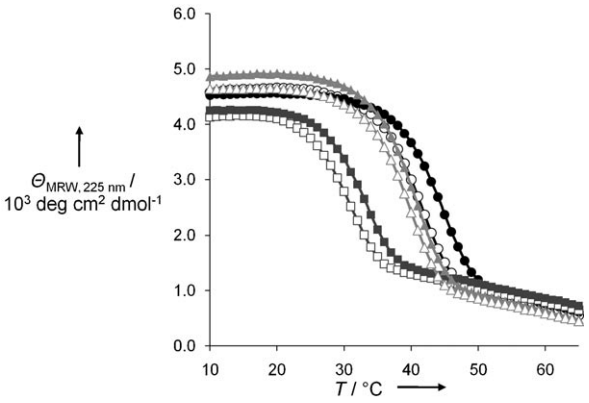

Figure 3. CD thermal denaturation curves for CMPs **1Y** (▲), **2Y** (△), **3** (○), **4** (●), **1X** (■), and **2X** (□), in aqueous 50 mM AcOH.

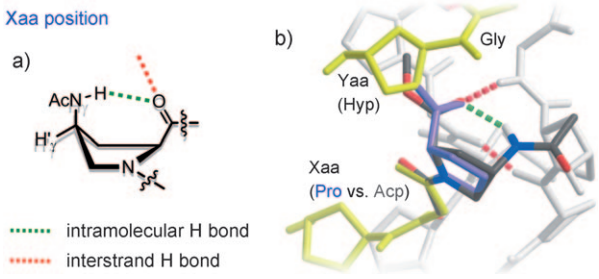

Figure 5. a) Structural differences between a (4*S*)Acp residue (black) and the natural Pro residue (gray) in the Xaa position of collagen. b) Overlay of the lowest-energy structure of Ac-(4*S*)Acp-OMe (gray)[7] with a Pro residue (pink) in the Xaa position of collagen (PDB 1V7H).[4b]

expected to compete with these interstrand H bonds that hold the three collagen strands together. Incorporation of a (4*S*)Acp residue in the Xaa position will therefore allow for comparing the importance of the ring pucker relative to that of the interstrand H bonds.

CD-spectroscopic studies demonstrate that also CMPs **1X** and **2X** form collagen triple helices. However, thermal denaturation studies demonstrate that the stability of the triple helices derived from **1X** and **2X** (Figure 3, squares) are significantly lower ($T_m \approx 30\,°C$) compared to those of CMPs **1Y**, **2Y**, **3**, and **4**. Thus, despite the matching ring puckering of (4*S*)Acp and (4*S*)Fmp in the Xaa position the collagen triple helices derived from **1X** and **2X** are significantly destabilized. An overlay of the (4*S*)Acp residue with a Pro residue in the Xaa position of collagen illustrates that this destabilization can be easily explained by the interference of the intramolecular H bond within (4*S*)Acp or (4*S*)Fmp with the interstrand H bond (Figure 5b).[14] The results show that the formation of interstrand H bonds is significantly more important for the stability of the collagen triple helix than the ring puckering.

The observed T_m values provide insight into the relative stabilities of the collagen triple helices. To gain a deeper understanding of the conformational stabilities of the collagen triple helices derived from CMPs **1Y**, **1X**, **2Y**, and **2X**, we performed additional thermal denaturation experiments at a lower heating rate and also monitored the refolding process. From the resulting data, the thermodynamic parameters were derived using a model introduced by Bächinger, Engel, and co-workers (see the Supporting Information).[15]

The free energies (ΔG) of the triple helices derived from the CMPs reflect the order observed in the melting temperatures (Table 1). Whereas the free energies of the triple helices of CMPs **1Y** and **2Y** (entries 1 and 2) are comparable to those of the triple helices formed by the parent CMP **4** (entry 6), those of **1X** and **2X** (entries 3 and 4) are approximately 2–3 kcal mol^{-1} lower in energy.

Table 1: Thermodynamic parameters for the thermal denaturation of the CMPs.[a]

Entry	CMP	T_m [°C][b]	ΔH [kcal mol^{-1}]	$-T\Delta S$ [kcal mol^{-1}]	ΔG [kcal mol^{-1}][c]
1	**1Y**	40	−69.3	58.3	−11.0
2	**2Y**	39	−71.1	60.7	−10.4
3	**1X**	32	−68.4	59.5	−8.9
4	**2X**	29	−67.5	59.1	−8.4
5	**3**	43	−74.5	62.1	−12.4
6	**4**	40	−71.6	60.4	−11.1

[a] Data accumulated at a heating rate of 5 °C h^{-1} unless noted otherwise. [b] Observed T_m at a heating rate of 1 °C/100 s. [c] ΔG at 25 °C.

A closer analysis of the thermodynamic data demonstrates that the destabilization of the collagen triple helices derived from **1X** and **2X** is due to an approximately 4 kcal mol^{-1} lower enthalpy compared to the energies for the triple helices derived from **3** and **4**. This enthalpic cost reflects at least in part the hindered formation of the three interstrand H bonds because of the engagement of the carbonyl groups in the intramolecular H bonds within the (4*S*)Acp and (4*S*)Fmp residues. The thermodynamic parameters of the collagen triple helix derived from CMP **1Y** and **2Y** are comparable to those of the parent CMP **4**. This result demonstrates that the enthalpic and entropic contributions of residues with C(4)-*endo* puckering do not differ significantly from those of residues with C(4)-*exo* puckering. The slight differences in the enthalpy and entropy for the triple helix formation observed for CMPs **1Y** and **2Y** are likely caused by either differences in the solvation properties of formamido versus acetamido groups[16] or steric effects, which will be further explored in future studies.

In conclusion, we have shown that a C(4)-*endo* ring pucker is tolerated in the Yaa position of collagen triple helices. This result shows that the ring puckering is less important for the stability of collagen, provided that the dihedral angles Ψ and the *trans*/*cis* amide conformer ratio favor the formation of a triple helix. Furthermore, we have demonstrated that the interstrand H bonds are significantly more important for the stability of the collagen triple helix than the ring puckering. The results are not only important for the basic understanding of the factors that determine the stability of collagen but also provide insight into which positions of collagen can be derivatized with functional groups without significantly disturbing the stability. Such functionalized collagen model peptides are becoming increasingly important as biocompatible functional materials.[2]

Received: December 22, 2010
Revised: May 3, 2011
Published online: June 8, 2011

Keywords: *cis*/*trans* amide bonds · collagen · PPII helix · proline · protein engineering

[1] a) G. B. Fields, D. J. Prockop, *Biopolymers* **1996**, *40*, 345–357; b) J. Engel, H. P. Bachinger, *Top. Curr. Chem.* **2005**, *247*, 7–33; c) B. Brodsky, G. Thiagarajan, B. Madhan, K. Kar, *Biopolymers* **2008**, *89*, 345–353; d) M. D. Shoulders, R. T. Raines, *Annu. Rev. Biochem.* **2009**, *78*, 929–958.

[2] a) U. Kusebauch, S. A. Cadamuro, H.-J. Musiol, M. O. Lenz, J. Wachtveitl, L. Moroder, C. Renner, *Angew. Chem.* **2006**, *118*, 7170–7173; *Angew. Chem. Int. Ed.* **2006**, *45*, 7015–7018; b) F. W. Kotch, R. T. Raines, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 3028–3033; c) M. A. Cejas, W. A. Kinney, C. Chen, G. C. Leo, B. A. Tounge, J. G. Vinter, P. P. Joshi, B. E. Maryanoff, *J. Am. Chem. Soc.* **2007**, *129*, 2202–2203; d) S. Rele, Y. Song, R. P. Apkarian, Z. Qu, V. P. Conticello, E. L. Chaikof, *J. Am. Chem. Soc.* **2007**, *129*, 14780–14787; e) C. M. Yamazaki, S. Asada, K. Kitagawa, T. Koide, *Biopolymers* **2008**, *90*, 816–823; f) J. A. Fallas, V. Gauba, J. D. Hartgerink, *J. Biol. Chem.* **2009**, *284*, 26851–26859; g) G. B. Fields, *Org. Biomol. Chem.* **2010**, *8*, 1237–1258.

[3] R. S. Erdmann, H. Wennemers, *J. Am. Chem. Soc.* **2010**, *132*, 13957–13959.

[4] a) J. Bella, M. Eaton, B. Brodsky, H. M. Berman, *Science* **1994**, *266*, 75–81; b) K. Okuyama, C. Hongo, R. Fukushima, G. G. Wu, H. Narita, K. Noguchi, Y. Tanaka, N. Nishino, *Biopolymers* **2004**, *76*, 367–377.

[5] a) S. K. Holmgren, L. E. Bretscher, K. M. Taylor, R. T. Raines, *Chem. Biol.* **1999**, *6*, 63 – 70; b) L. Vitagliano, R. Berisio, L. Mazzarella, A. Zagari, *Biopolymers* **2001**, *58*, 459 – 464; c) R. Berisio, L. Vitagliano, L. Mazzarella, A. Zagari, *Protein Sci.* **2002**, *11*, 262 – 270; d) M. L. DeRider, S. J. Wilkens, M. J. Waddell, L. E. Bretscher, F. Weinhold, R. T. Raines, J. L. Markley, *J. Am. Chem. Soc.* **2002**, *124*, 2497 – 2505; e) R. Improta, F. Mele, O. Crescenzi, C. Benzi, V. Barone, *J. Am. Chem. Soc.* **2002**, *124*, 7857 – 7865; f) J. A. Hodges, R. T. Raines, *J. Am. Chem. Soc.* **2003**, *125*, 9262 – 9263; g) C. L. Jenkins, L. E. Bretscher, I. A. Guzei, R. T. Raines, *J. Am. Chem. Soc.* **2003**, *125*, 6422 – 6427; h) K. Mizuno, T. Hayashi, D. H. Peyton, H. P. Bachinger, *J. Biol. Chem.* **2004**, *279*, 38072 – 38078; i) M. D. Shoulders, J. A. Hodges, R. T. Raines, *J. Am. Chem. Soc.* **2006**, *128*, 8112 – 8113.

[6] a) C. L. Jenkins, M. M. Vasbinder, S. J. Miller, R. T. Raines, *Org. Lett.* **2005**, *7*, 2619 – 2622; b) N. Dai, F. A. Etzkorn, *J. Am. Chem. Soc.* **2009**, *131*, 13728 – 13732.

[7] M. Kuemin, Y. A. Nagel, S. Schweizer, F. W. Monnard, C. Ochsenfeld, H. Wennemers, *Angew. Chem.* **2010**, *122*, 6468 – 6471; *Angew. Chem. Int. Ed.* **2010**, *49*, 6324 – 6327.

[8] For the synthetic protocol, see: R. S. Erdmann, H. Wennemers, *Synthesis* **2009**, 143 – 147 and the Supporting Information.

[9] The model compound Ac–(4*S*)Fmp–OCH_3 like Ac-(4*S*)Acp-OCH_3 adopts a C(4)-*endo* pucker preferentially and has a *cis*/*trans* ratio of 1:4.7 around the Ac-amid bond as revealed by ^{1}H NMR spectroscopic analysis. For details, see the Supporting Information.

[10] a) A. V. Persikov, J. A. M. Ramshaw, A. Kirkpatrick, B. Brodsky, *Biochemistry* **2000**, *39*, 14960 – 14967; b) A. V. Persikov, J. A. M. Ramshaw, A. Kirkpatrick, B. Brodsky, *J. Am. Chem. Soc.* **2003**, *125*, 11500 – 11501.

[11] a) S. A. Cadamuro, R. Reichold, U. Kusebauch, H.-J. Musiol, C. Renner, P. Tavan, L. Moroder, *Angew. Chem.* **2008**, *120*, 2174 – 2177; *Angew. Chem. Int. Ed.* **2008**, *47*, 2143 – 2146.

[12] The intramolecular H bond might also enhance the acidity of the Gly NH group and thereby enhance the strength of the interstrand H bond.

[13] Pro instead of (4*R*)Hyp was used in the Yaa position of these CMPs because a steric clash is observed upon modification of both the Xaa and the Yaa positions, see: J. A. Hodges, R. T. Raines, *J. Am. Chem. Soc.* **2005**, *127*, 15923 – 15932.

[14] This observation is consistent with a study on the effect of (4*S*)Hyp in the Xaa position of CMPs, see: a) M. D. Shoulders, F. W. Kotch, A. Choudhary, I. A. Guzei, R. T. Raines, *J. Am. Chem. Soc.* **2010**, *132*, 10857 – 10865; b) K. Inouye, S. Skakibara, D. J. Prockop, *Biochim. Biophys. Acta Protein Struct.* **1976**, *420*, 133 – 141.

[15] K. Mizuno, S. P. Boudko, J. Engel, H. P. Bächinger, *Biophys. J.* **2010**, *98*, 3004 – 3014.

[16] S. Z. Wan, R. H. Stote, M. Karplus, *J. Chem. Phys.* **2004**, *121*, 9539 – 9548.

