Collagen

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Switchable Proline Derivatives: Tuning the Conformational Stability of the Collagen Triple Helix by pH Changes**

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Abstract: *(4S)-Aminoproline is introduced as a pH-sensitive probe for tuning the conformational properties of peptides and proteins. The pH-triggered flip of the ring puckering and the formation/release of a transannular H bond were used to switch the formation of collagen triple helices on and off reversibly.*

Proline residues are key for the conformational and functional properties of numerous natural peptides and proteins. They play important roles, for example, in protein folding and signal transduction.[1] Proline (Pro) and (4*R*)-hydroxyproline (Hyp) are also the predominant amino acids within the structural protein collagen, which is responsible for the high stability of skin and bones.[2] Derivatives that influence the conformational properties of proline-containing peptides and proteins are therefore important and have been used to study and tune biological processes.[3–10] Among the most valuable derivatives are proline residues that bear a substituent at C4.[3–9] The nature of this substituent influences the puckering of the pyrrolidine ring and the *trans*/*cis* conformer ratio of Xaa-Pro amide bonds (Figure 1 a) by either steric effects,[4,8] stereoelectronic gauche effects,[3,7] repulsive interactions, and/or transannular hydrogen bonding.[6,9]

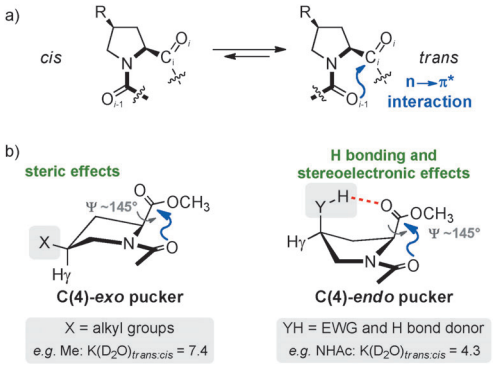

Figure 1. Conformational properties of (4*S*)-configured proline derivatives.

Within (4*S*)-configured proline derivatives, the gauche effect of electron-withdrawing groups (EWGs) favors C4-*endo* puckering.[3] If the EWG is also an H-bond donor (e.g. amide moieties), the *endo* pucker is further enhanced by a transannular H bond (Figure 1 b, right).[6] In contrast, steric effects, for example by a methyl group, favor C4-*exo* ring puckering (Figure 1 b, left).[4,8] In both cases Ψ angles of approximately 145° are realized, which are ideal for n→π* interactions[11] between the adjacent acyl groups within the *trans* conformer (Figure 1 b).[6,8,9] As a result, the *trans* conformer is favored in these derivatives over the *cis* conformer by a factor of 4–7 in aqueous environments.[6,8]

Acetamidoproline (Acp), methylproline (Mep), and several other proline derivatives with well-defined conformational properties that are insensitive to changes in the surrounding medium, have been used to tune the functional and conformational properties of peptides and proteins.[7–10] We envisioned proline derivatives that are responsive to changes in the environment as valuable for switching between different states of a peptide or protein. Herein, we present the use of (4*S*)-aminoproline (Amp) as a pH-sensitive probe that undergoes a conformational change of the ring pucker as well as the formation or release of a transannular H bond upon changes in the pH value. We show how Amp can be used to tune the conformational stability of collagen triple helices.

Previously, we showed that (4*S*)Amp adopts a C4-*endo* ring pucker in an acidic environment and has conformational properties that are similar to those of (4*S*)Acp.[6,12] The electron-withdrawing nature of the ammonium group induces an *endo* ring pucker, and a transannular H bond enforces a Ψ angle that favors the n→π* interaction and thereby the *trans* amide bond (Figure 2, right).[6] We now hypothesized

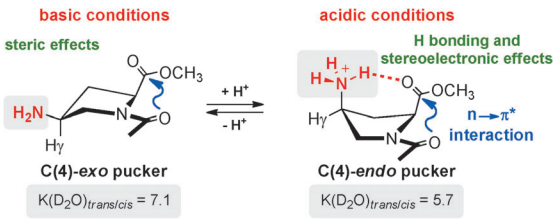

Figure 2. pH-dependent conformational switch of Ac-(4*S*)Amp-OMe.

that an uncharged amino group should exert a similar steric effect as an isosteric methyl group and favor a C4-*exo* ring pucker without a transannular H bond (Figure 2, left). Whereas the electronegativity of nitrogen ($\chi_N = 3.0$) is comparable to that of chlorine ($\chi_{Cl} = 3.0$),[13] a substituent that is known to control the conformation of 4-chloroproline deriv-

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atives by a stereoelectronic gauche effect,[7e] we reasoned that hydration of the amino group would lead to a prevailing steric effect. Thus, a change of the pH value should trigger a switch of the ring pucker as well as the formation/release of a transannular H bond (Figure 2).[14] To evaluate this hypothesis, we analyzed and compared the conformational properties of Ac-(4*S*)Amp-OMe (**1**) in aqueous environments at pH 10 and pH 3.[15]

An NMR spectroscopic analysis of Ac-(4*S*)Amp-OMe in a basic environment showed that the conformational properties do indeed differ significantly from those under acidic conditions.[16] The observed vicinal coupling constants and chemical shifts are typical for a predominant C4-*exo* ring pucker and similar to those observed for isosteric Ac-(4*S*)Mep-OMe that was prepared as a reference. In addition, a *trans*/*cis* ratio of 7.1, which is closely resembling that of Ac-(4*S*)Mep-OMe ($K_{trans/cis} = 7.4$), was observed (Figure 1 and Figure 2).

These results show that the pH-induced change between ammonium and amino groups triggers a flip between C4-*endo* (ammonium) and C4-*exo* (amine) ring puckers of the pyrrolidine ring within (4*S*)Amp. The pH value also controls an on/off switch of a transannular H bond and a switch between a charge-neutral (amine) and a cationic moiety (ammonium). Importantly, the *trans*/*cis* conformer ratio is hardly affected by the pH value, as the n→π* interaction between the carbonyl groups within Ac-(4*S*)Amp-OMe is favored, regardless of the protonation state.

Next, we investigated the value of (4*S*)Amp as a pH-sensitive probe to influence the properties of a peptide and chose the collagen triple helix as a model system. Collagen is a proline-rich fibrous protein, which serves important roles, for example, as a structural protein in bones and skin.[2] Collagen consists of single strands with the common repeat unit Xaa-Yaa-Gly, in which Pro residues are most common in the Xaa position and (4*R*)hydroxyproline (Hyp) in the Yaa position. Three single strands coil around each other to form a triple helix that is held together by interstrand hydrogen bonds between neighboring NH (Gly) and C=O (Pro) moieties (Figure 3a). Crystal structures revealed that the Pro residues in the Xaa position are in general C4-*endo* puckered, whereas the (4*R*)Hyp residues in the Yaa position adopt C4-*exo* ring puckers.[17] We and others have investigated the factors that are critical for the stability of the collagen triple helix by investigating the properties of triple helices derived from collagen model peptides (CMPs) that contain non-natural proline derivatives in place of Pro and Hyp in the Xaa and Yaa positions.[7–9] These studies showed that ring puckering, interstrand H bonds, and avoidance of steric repulsions between strands are important for the stability of the collagen triple helix. Recently, we showed that (4*S*)-configured amidoproline derivatives bearing H-bond donors, such as (4*S*)Acp, are readily tolerated in the Yaa position but weaken the collagen triple helix significantly in the Xaa position because of interference with the interstrand H bonds.[9] Sterically demanding substituents at (4*S*)-configured proline residues are tolerated in the Yaa position, but disturb the supramolecular assembly in the Xaa position where the bulky moiety points to the inside of the collagen

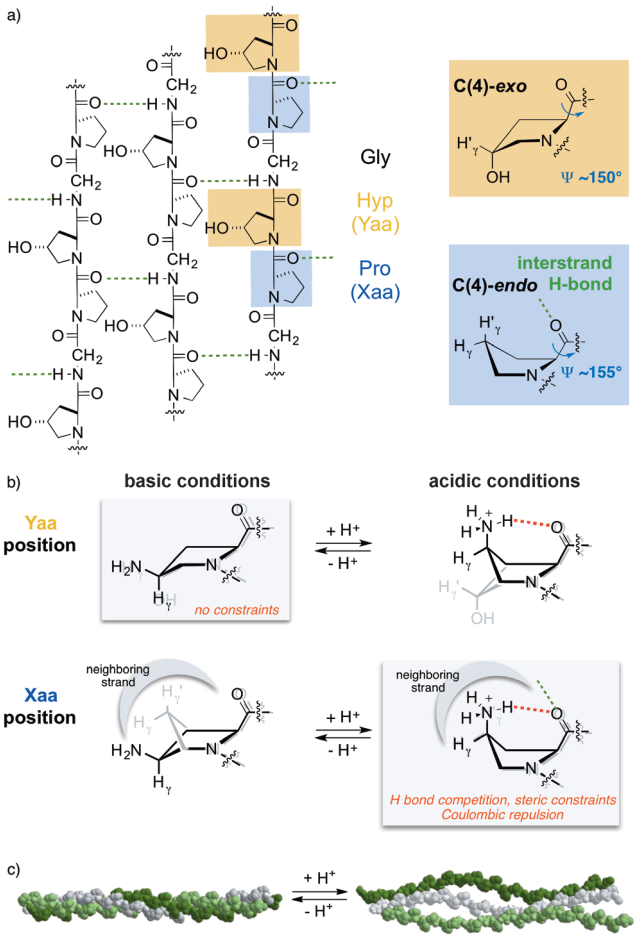

Figure 3. a) General structure of collagen. b) Schematic overlay of the preferred conformations of (4*S*)Amp under acidic and basic conditions (black) with those of the residues in natural collagen (grey). c) Cartoon of the stability of (4*S*)Amp-containing collagen triple helices under acidic and basic conditions.

triple helix. In comparison to unfavorable H bonding or steric effects, the ring puckering was found to be less important.[9]

These insights suggest that changes in the protonation state of (4*S*)Amp-containing collagen triple helices will have significant effects on the stability of the supramolecular assembly that are not only caused by Coulombic interactions[18] and should allow the switching between single and triple helical states of collagen: Under basic conditions, the triple-helix formation of CMPs that bear (4*S*)Amp in the Yaa position should proceed equally well as with CMPs that are composed of the most abundant natural residues, as neither steric nor ring puckering or H bonding constraints should hinder the supramolecular assembly (Figure 3b, top left). In contrast, under acidic conditions, repulsion between the cationic ammonium groups in neighboring strands combined with a mismatch in the ring pucker should destabilize the triple helix. (4*S*)Amp residues in the Xaa position should destabilize the collagen triple helices both under acidic and basic conditions as a result of steric constraints with the inner part of the triple helix and a mismatch in the ring pucker. Under acidic conditions, additional charge repulsion and most

importantly interference of the transannular H bond with the interstrand H bonds were expected to significantly weaken the supramolecular assembly (Figure 3b, bottom right).

To probe these hypotheses, we prepared CMPs **2Y** and **2X**, which bear (4*S*)Amp in the Yaa and Xaa position, respectively, of the middle repeat unit within a 21-mer. Such CMPs have been proven to be valuable for monitoring the effect of a single residue on the conformational stability of collagen.[19] CMP **3**, with the naturally most abundant Pro and Hyp residues in the Xaa and Yaa positions, was used as a reference. CMP **4**, with a (4*S*)Mep residue in the Yaa position, was prepared to compare the effects of amino versus methyl groups. In addition, CMP **5** with a charge-neutral but H-bonding (4*S*)Acp residue in the Xaa position was used as a reference to estimate the extent of H bonding versus Coulombic effects.

Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*S*)Amp**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**2Y**)

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

Ac-(Pro-Hyp-Gly)$_3$-(Pro-Hyp-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**3**)

Ac-(Pro-Hyp-Gly)$_3$-(Pro-(**4*S*)Mep**-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**4**)

Ac-(Pro-Hyp-Gly)$_3$-((**4*S*)Acp**-Pro-Gly)-(Pro-Hyp-Gly)$_3$-NH$_2$ (**5**)

CD spectra of solutions of CMP **2Y** and **2X** in aqueous AcOH (50 mM, pH 3) and $NaHCO_3$/NaOH buffer (pH 10.7), respectively, revealed that both CMPs form triple helices at either pH value, as indicated by a maximum at 225 nm, which is typical for the collagen triple helix (see the Supporting Information). Thermal denaturation studies, which were performed by heating the solutions and monitoring with CD spectroscopy, provided sigmoidal thermal transitions with midpoints (T_m) that gave insight into the relative stabilities of the collagen triple helices (Figure 4, Table 1). Additional denaturation experiments that monitored also the cooling process provided the free energies, ΔG, of the triple helices (Table 1).[20]

These studies revealed significant differences in the thermal stabilities of the (4*S*)Amp-containing triple helices. The most stable triple helix is formed by CMP **2Y** under basic conditions (Table 1, entry 1). T_m and ΔG values of 44 °C and −12.1 kcal mol^{-1}, respectively, were observed, which are comparable to those of the triple helix derived from the reference CMP with the natural Pro-Hyp-Gly motive (T_m = 45 °C, ΔG = −11.9 kcal mol^{-1}, Table 1, entry 3) and that derived from the CMP **4** bearing a methyl in place of the amino group (T_m = 42 °C, ΔG = −10.8 kcal mol^{-1}, Table 1, entry 4). This result demonstrates that a (4*S*)Amp residue in the Yaa position does not destabilize the supramolecular assembly in a basic environment and shows that amino and methyl groups behave as isosteric groups also in the supramolecular assembly. As expected, in acidic media, the triple helix derived from CMP **2Y** is destabilized by the non-ideal ring pucker and the repulsion of positive charges in three symmetry-related positions, which is reflected in lower T_m and ΔG values (T_m = 37 °C, ΔG = −9.2 kcal mol^{-1}, Table 1, entry 5).

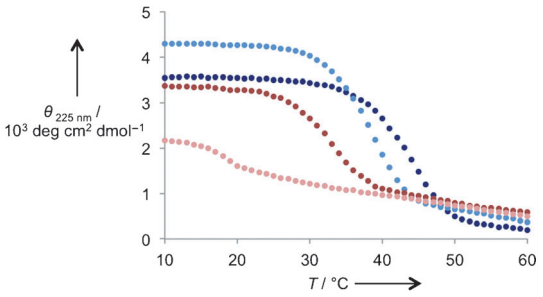

Figure 4. Thermal denaturation curves of triple helices formed by solutions of CMPs **2Y** (0.20 mM) at pH 10.7 (dark blue), **2Y** at pH 3 (light blue), **2X** at pH 10.7 (dark red), and **2X** at pH 3 (light red).

Table 1: Thermodynamic parameters of triple helices derived from the functionalized CMPs **2X** and **2Y** under basic and acidic conditions and comparison with reference compounds **3**–**5**.

Entry	CMP	Xaa [middle triplet]	Yaa [middle triplet]	T_m[a] [°C]	ΔG[b,c] [kcal mol^{-1}]
pH 10.7					
1	**2Y**	Pro	(4*S*)Amp	44	−12.1
2	**2X**	(4*S*)Amp	Pro	33	−8.6
3	**3**	Pro	Hyp	45	−11.9
4	**4**	Pro	(4*S*)Mep	42	−10.8
pH 3					
5	**2Y**	Pro	(4*S*)Amp	37	−9.2
6[d]	**2X**	(4*S*)Amp	Pro	13	−6.4
7[e]	**3**	Pro	Hyp	43	−12.4
8[e]	**5**	(4*S*)Acp	Pro	32	−8.9

[a] T_m at a heating rate of 1 °C/100 s (±2 °C). [b] Data at a heating rate of 0.1 °C/72 s. [c] ΔG at 25 °C (±0.4 kcal mol^{-1}). [d] T_m values below 20 °C have a higher error of ±5 °C. [e] Data taken from Ref. [9b].

Steric constraints were expected to destabilize the collagen triple helices that bear (4*S*)Amp in the Xaa position (CMP **2X**) and they have indeed at either pH value lower thermal stabilities compared to those derived from CMP **2Y** (Table 1, entries 2 and 6). In particular at pH 3, the T_m and ΔG values of 13 °C and −6.4 kcal mol^{-1}, respectively, demonstrate a strong destabilization, which is to the best of our knowledge the highest yet observed for a single residue in the Xaa or Yaa position upon changes in the environment. Thus, the combination of a transannular H bond that competes with the interstrand H bond, steric constraints and charge repulsion affects the supramolecular assembly strongly. Comparison with the stability of the triple helix derived from CMP **5**, which bears a charge-neutral H-bonding acetyl group and is considerably more stable (T_m = 32 °C, ΔG = −8.9 kcal mol^{-1}, Table 1, entry 8), suggests that the Coulombic repulsion contributes to the destabilization. Denaturation experiments in acidic aqueous solution with 0.1M NaCl showed that the triple helices of CMPs **2X** and **2Y** are more stable (T_m = 15 °C and 40 °C, respectively), but not to a large extent. This result suggests that the main contributors to the observed destabilization of the triple helix of CMP **2X** are the interference with the interstrand H bond and steric effects.[21]

The significant differences in the stability of the triple helices formed by CMPs **2Y** and **2X** in basic and acidic

environments demonstrate that changes in the pH value allow tuning the conformational and thereby supramolecular properties of (4*S*)Amp-containing collagen triple helices.

Next we explored whether the pH-triggered folding and unfolding of (4*S*)Amp-containing collagen triple helices is reversible. Toward this goal, base (2 M NaOH) and acid (2 M HCl) were added in turn to a solution of CMP **2X** and the CD spectroscopic signal at 225 nm, which is most indicative of the collagen triple helix, was monitored over time (Figure 5a).

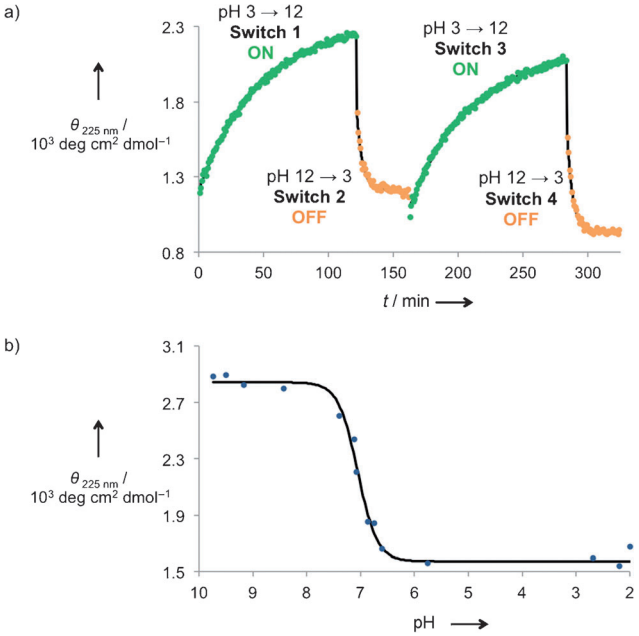

Figure 5. a) Cycles of pH-triggered repeated folding and unfolding of the triple helix derived from CMP **2X** ($c = 0.20$ mM) at 7 °C. b) Dependence of the unfolding of the triple helix derived from CMP **2X** on the pH value.

These experiments also shed light on the speed with which the switch occurs. While the addition of acid to a basic solution of CMP **2X** led to an immediate decrease of the signal intensity, the signal increased significantly slower upon addition of base to an acidic solution of CMP **2X**. Thus, acid triggers a rapid unfolding, but the base-induced folding of the triple helix is slow. Rate constants and half-lives of $k = 0.015\ s^{-1}$ and $t_{1/2} =$ 45 min, respectively, for the folding, and $k = 0.12\ s^{-1}$ and $t_{1/2} =$ 6 min, respectively, for the unfolding, were determined using an exponential fit that takes a "zipper mechanism" into account.[22] These kinetic parameters are comparable to those known for thermally induced unfolding and folding of collagen triple helices, which suggests that even in the case of pH-induced folding, the folding rate is limited by the *cis–trans* isomerization of the prolyl peptide bonds and the propagation step.[23] Repeated alternating additions of base and acid showed that the switch is reversible and can be triggered several times (Figure 5a).[24]

Finally we examined at which pH value the switch occurs by adding small portions of HCl (1 M) to a basic solution of CMP **2X**, upon which we monitored both the pH value and the CD signal at 225 nm (for details see the Supporting Information). This titration revealed that the switch takes place at a pH value of approximately 7. As the amino group of Amp at C4 has a pK_a value of around 8, this experiment indicates that the environment within the supramolecular assembly affects the pK_a value.

In conclusion, we have introduced (4*S*)-aminoproline as a versatile probe for inducing pH-triggered conformational changes within peptides and proteins. Changing the protonation state leads to a switch of the ring pucker of (4*S*)Amp and the formation or release of a transannular H bond. We have highlighted the value of (4*S*)Amp for tuning the conformational properties and thereby the stability of collagen triple helices. These findings open intriguing possibilities for the design of pH-sensitive collagen-based materials that might be useful for the encapsulation and specific release of drugs in an acidic environment that is present, for example, around certain cancer cells.[25] Studies toward this goal are currently ongoing in our laboratory and will be reported in due course.

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[21] The T_m value of collagen triple helices derived from an analogue of CMP **5** bearing a pivaloyl in place of an acetyl group was found to be 26 °C (Ref. [9b]), supporting the importance of steric effects on the stability of the supramolecular assembly. The steric demand of the ammonium ion is likely enhanced by solvation.

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[24] The differences in the signal intensities are a result of the dilution effect caused by the addition of the NaOH and HCl solutions.

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