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Site-Directed Spin Labeling of a Collagen Mimetic Peptide

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Collagen as the most abundant protein in mammals consists of three chains that are built up of repeating Xaa-Yaa-Gly units with all peptide bonds in *trans* conformation and most often with proline in Xaa and (4*R*)-hydroxyproline in the Yaa position. This type of sequence favors a left-handed poly-Pro-II helix conformation and the intertwining of the three chains into the unique right-handed triple helix.[1] The X-ray structure analysis of collagen mimetic peptides (CMPs) of repeating Xaa-Yaa-Gly triplets show C$^{\gamma}$-*endo* puckers of the Pro residues in Xaa and C$^{\gamma}$-*exo* puckers of the Hyp residues in Yaa position.[2] Studies on structural factors that stabilize the triple helical fold with proline derivatives led to the conclusion that besides the interchain hydrogen bond between the NH group of Gly of one strand and the CO group of Pro of the adjacent strand, stereoelectronic effects that favor the all *trans*-peptide bond conformation and the correct puckering are decisively affecting the structural stability.[1c,3] Recent studies by Erdman and Wennemers with proline derivatives in the Yaa position that exhibit preferences for the C$^{\gamma}$-*endo* puckering, but a *trans* amide conformer, contradict this conclusion showing that the ring puckering is less important for the stability of collagen if the *trans/cis* amide conformer ratio favors formation of the triple helix.[4]

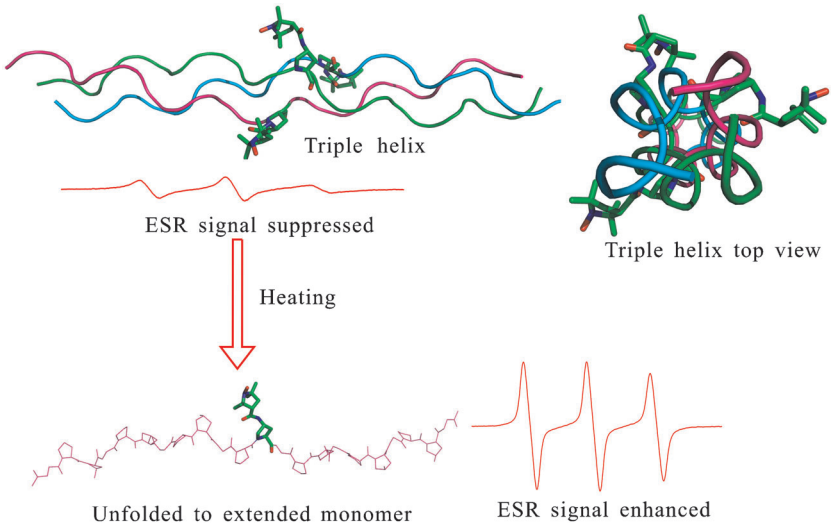

Figure 1. The relative mobility and environmental changes of spin labels with collagen folded/unfolded states.

The aim of the present study was to investigate whether electron spin resonance spectroscopy (ESR) could yield new information on the dynamics of triple helix folding/unfolding as a useful alternative to the other spectroscopic techniques applied so far. Indeed this type of spectroscopy has become popular for studying conformational changes and unfolding processes of proteins[4] as many ESR spectral parameters, such as peak-to-peak height, peak-to-peak width and rotational correlation time can accurately reflect relative mobility and environmental changes of spin labels (Figure 1).[5]

For the design and synthesis of a suitable spin-labeled CMP, the results of a recent detailed study on the equilibrium constants of acetyl-(4*R*)-Pro(X)-OMe (X = ammonium or acetylamide) by NMR spectroscopy[6] were taken into account. Although for the ammonium derivative a significantly reduced preference for the *trans* conformation was observed, a *trans* conformer preference similar to that of (4*R*)-hydroxyproline was recovered upon acetylation of the γ-amino group. Correspondingly, Ac-(Gly-Pro-Hyp)$_7$-Gly-Gly-NH$_2$[7] was selected as the CMP in which the Hyp residue of the central triplet was replaced by (2*S*,4*R*)-aminoproline

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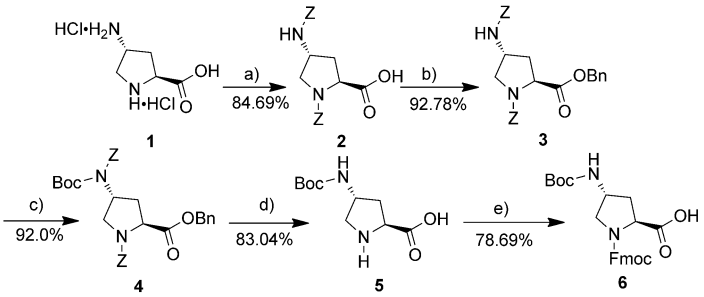
(Amp) to allow for insertion of an nitroxide radical by side-chain acylation of the related Amp containing host–guest CMP with a suitable spin-label derivative.

Among the nitroxide spin probes frequently utilized for site-specific labeling of peptides and proteins, such as 2,2,5,5-tetramethyl-1-oxyl-3-methylmethane thiosulfonate (MSTL),[8] 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-carboxylic acid (TOAC),[9] 2,2,6,6-tetra-methylpiperidine-*N*-oxyl (TEMPO),[10] and 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-yloxy free radical (3-carboxy-PROXYL)[11] we have selected the latter reagent as it is readily prepared from the commercially available 2,2,6,6-tetramethyl-4-piperidine by known procedures.[12]

The synthesis of the Amp-CMP (2*S*,4*R*)-Fmoc-Amp-(Boc)-OH (**6**) was prepared from the readily available Amp hydrochloride in five steps (Scheme 1). After Z protection



Scheme 1. Synthesis of (2*S*,4*R*)-Fmoc-Amp(Boc)-OH: a) i) 2 N NaOH (4.0 equiv), 0 °C; ii) ZCl (2.5 equiv), RT, 4 h; iii) 6 N HCl, pH 1, 84.7 %; b) BnBr (1.5 equiv), TEA (10 equiv), RT, 22 h, 92.8 %; c) Boc_2O (2.0 equiv), DMAP (0.1 equiv), THF, RT, 17 h, 92.0 %; d) 10 % Pd/C, 9:1 EtOH/H_2O (v/v), H_2, RT, overnight, 83.0 %; e) Fmoc-OSu (1.5 equiv), 2:1 THF/H_2O (v/v), $NaHCO_3$ (2.5 equiv), RT, overnight, 78.7 %.

of the γ-amino and α-amino groups of Amp to yield **2**, the carboxy group was protected as benzyl ester **3**. Subsequent double acylation of the γ-amino group of **3** with $(Boc)_2O$ by DMAP catalysis led to compound **4**. Hydrogenation of **4** over Pd/C catalyst for removal of the Z and benzyl protecting groups produced intermediate **5** for reaction with Fmoc-OSu to afford the target derivative **6**. It was obtained in an overall yield of 47 %, which is about 30 % higher than that of the previously reported synthesis.[13]

The suitably protected Amp derivative was then applied for the synthesis of Ac-(Gly-Pro-Hyp)$_3$-Gly-Pro-Amp-(Gly-Pro-Hyp)$_3$-Gly-Gly-NH_2 by standard Fmoc/*t*Bu synthesis on solid support. As shown in Scheme S1 in the Supporting Information, the resulting Amp-containing host–guest CMP (**8**) was acylated in solution with an excess of 3-carboxy-PROXYL (**7**) in the presence of HBTU/HOBt/DIEA (1:1:2). Both the Amp-CMP (**8**) and the spin-labeled collagen peptide (SLP) **9** were obtained as highly homogeneous and well-characterized compounds.

Based on previous detailed studies on kinetics of folding/unfolding of CMPs into the triple helical structure[14] aqueous solutions of peptides **8** and **9** at 1 mM concentration were incubated at 4 °C for 24 h. The CD spectra with a positive band near 225 nm and a negative maximum around 198–200 nm showed the typical pattern of poly-Pro-II helices (Figure 2 A). The CD spectra shows that CMP and SLP actually both fold into the typical collagen triple helix, but the dichroic intensity of the negative maximum was found to be lower for the SLP than for the Amp-peptide. Thus, the CD signature of SLP would indicate a lower content of triple helix than for the Amp-CMP. To test the influence of

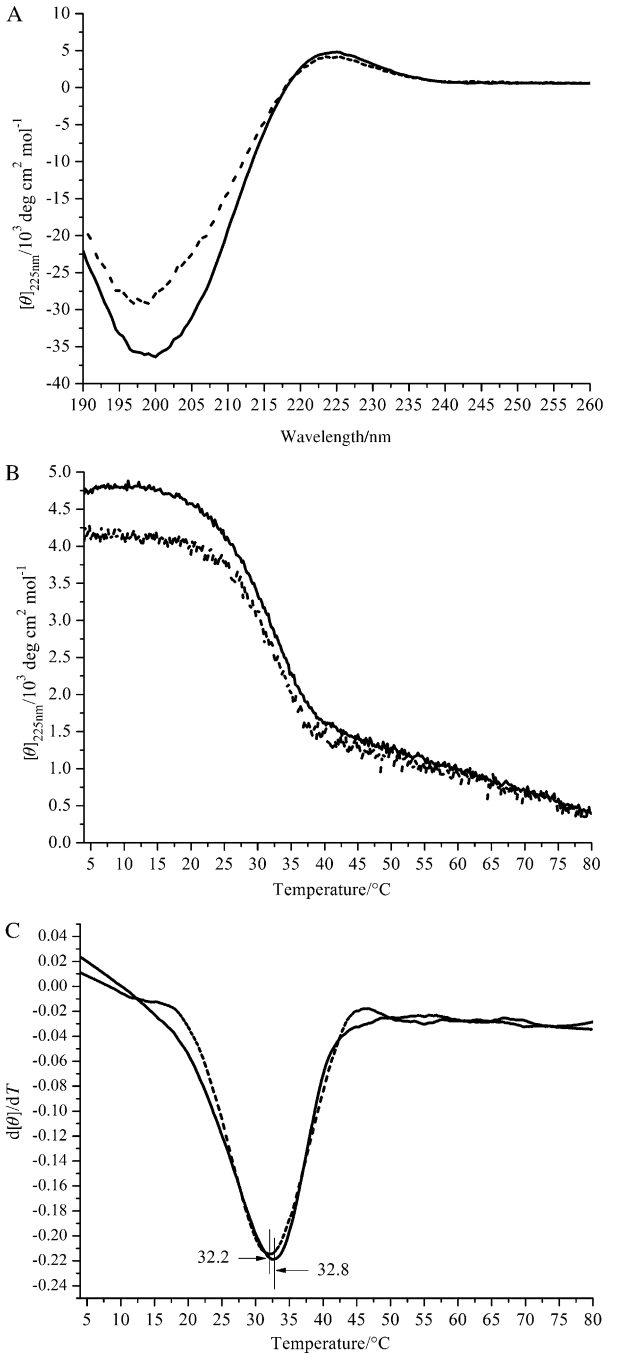


Figure 2. CD spectra of CMP (**8**) and SLP (**9**). A) CD spectra of **8** (solid line) and **9** (dashed line) at 4 °C; B) CD melting curves of **8** (solid line) and **9** (dashed line) triple helices; C) the first derivative of melting curves of **8** and **9** triple helices versus temperature.

Amp and its spin-labeled derivative on the stability of the triple helix, the thermal denaturation was monitored by changes in dichroic intensities at 225 nm in the temperature range from 5 to 70 °C. As shown in Figure 2B both peptides display a cooperative transition with very similar midpoints T_m of 32.8 and 32.2 °C for **8** and **9**, respectively, as determined by the derivative of the melting curves (Figure 2C). Compared to the parent CMP, that is, Ac-(Gly-Pro-Hyp)$_7$-Gly-Gly-NH$_2$, for which a T_m of 43 °C was reported under identical conditions,[7] the stability of the two collagen peptides **8** and **9** is reduced by 10 °C. For the Amp-peptide as trifluoroacetate salt, such weaker stability was predictable on the basis of the lower *trans* conformer preference as induced by the γ-ammonium group,[6] whereas the unprotonated Amp, under basic conditions, was reported to significantly stabilize the triple helix.[15] The reduced triple helix stability of the spin-labeled peptide was unexpected as acylation of the γ-amino group was predicted to restore, to a large extent, the preference for the *trans*-conformation.[6] Such benefit may well be annulled by sterical clashes of the nitroxide label that lead to a stronger breathing of the triple helix in its central part. More information on this aspect can possibly be expected from ESR measurements.

The results from ESR spectroscopy experiments are presented in Figure 3A and Figure S9 in the Supporting Information as the first-derivative lines of original ESR absorption spectra. They all show three-line ESR spectra referred to as center-, low- and high-field lines, for the hyperfine interaction of unpaired electron spins in water. Indeed, the aqueous solution of nitroxide shows a typically three-line ESR spectrum of approximately equal heights reflecting its fast motion.[5] Varying degrees of broadening of the ESR spectra are observed in Figures S9 and S10 in the Supporting Information, which indicate the influence of the peptide backbone on spin-probe mobility, consistent with experiences with other nitroxide-labeled peptides.[9,16] It is well known that ESR spectroscopy is highly sensitive to the local structure and environmental changes around the spin label in proteins. Upon heating the solution of peptide **9** from 5 to 70 °C, the ESR spectrum shows strong changes with a broad signal, corresponding to the transition from folded to unfolded state of the triple helix as shown by CD spectroscopy. The unfolding process of the triple helix results in an increased rotational tumbling of the nitroxide probe, which is consistent with a reduced immobilization as expected from an increased fluctuation of the spin label in unfolded single chains. Similar effects of reduced constraints upon unfolding were reported for other spin-labeled peptides and structured proteins.[4b] In addition, the spin–spin interaction in the triple helix, which depends on distance between the labels, may also contribute to the broader lines of ESR spectrum.[17] Therefore, interspin distance measurements using ESR technique could well add further information on triradical interactions in the triple helix, and thus allow the dynamics of local unfolding and folding to be monitored.

The ESR spectra show a combined spectroscopy of three spin labels for they share the same interaction environment and mobility. According to the ESR spectra in Figure 3A, we could confirm the dynamics of the spin-labeled collagen mimetic peptide as a fast motional model. Thus, the motion of peptide can be reflected by the rotational correlation time, τ_c, which is the time necessary for the spin label to rotate through an angle of one radian given by Equation (1).[18] It accurately reflects relative motilities and changes in the spin label motion.

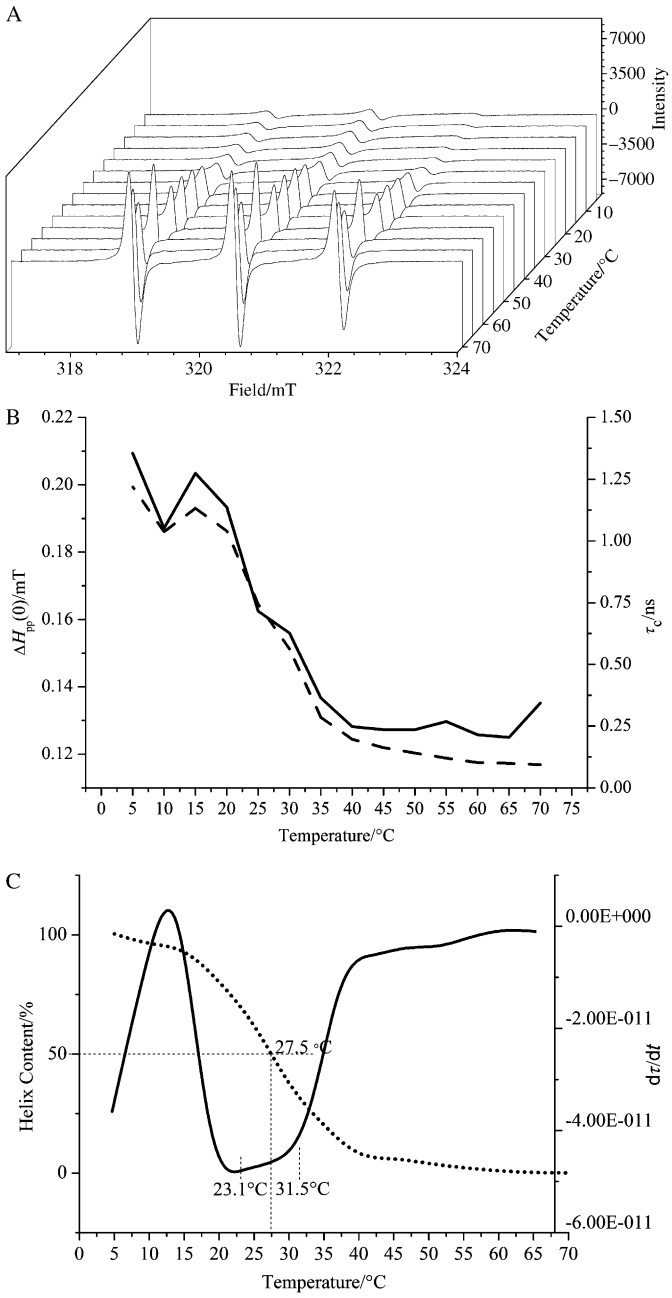


Figure 3. A) ESR spectra of SLP (**9**) triple helix at different temperatures in three-dimensional view. B) The relationship between $\Delta H_{pp}(0)/\tau_c$ and temperatures of SLP (**9**). Dotted line: rotational correlation times (τ_c) at different temperatures; solid line: the peak-to-peak width ($\Delta H_{pp}(0)$) of the center line at different temperatures. **C**) The first derivative of rotational correlation time versus temperatures (solid line); normalized τ_c values versus temperatures (dotted line).

$$\tau_c = 1.19 \times 10^{-9} \times \frac{\sqrt{3}}{4}\left\{\sqrt{\frac{A(0)}{A(+1)}} + \sqrt{\frac{A(0)}{A(-1)}} - 2\right\} \times \Delta H_{pp}(0) \quad (1)$$

Calculated values of the peak-to-peak width of the center line ($\Delta H_{pp}(0)$) and the correlation time (τ_c) by varying the temperatures are shown in Table S1 in the Supporting Information. The sensitivity of the ESR spectrum to the spin-label mobility is influenced by both the rate and amplitude of the motion. Consequently, a clear correlation between ESR spectral shape and conformational status of the triple helix could be established. In Figure 3, $\Delta H_{pp}(0)$ and τ_c are shown with increasing temperatures. The changes of these values reflect the thermal transition from triple helix to the unfolded state, which are consistent with the CD spectra. From 5 to 40 °C, the τ_c values decrease almost linearly from 1.21865 to 0.19669 ns and $\Delta H_{pp}(0)$ values also show a linear decrease from 0.20935 to 0.12818 mT, proving the unfolding process of the triple helix and the related weakening of the restricted local structure and spin–spin interactions. The relationship between τ_c and temperature can be viewed as ESR melting transition of the triple helix of peptide **9**. For comparative analysis of thermal transitions monitored by CD and ESR very slow heating rates (12 °C h^{-1}) were applied as required for almost equilibrium transitions of CMPs even at high peptide concentrations.[14b,19]

In the first derivative of τ_c versus T curve, a transition is observed from 23.1–31.5 °C (Figure 3C). Deconvolution of this temperature excursion curve allows the identification of two transition temperatures. The first T_m of 23.1 °C is significantly lower than the T_m of 32.2 °C derived from CD spectroscopy. It could be interpreted as a pretransition of misaligned trimeric species present at equilibrium with correctly registered chains in self-assembled CMPs triple helices. Such pretransitions of CMP triple helices have been identified by NMR spectroscopy studies on the dynamics of thermal unfolding of CMP triple helices.[20] The unfolded monomers realign into correctly registered triple helices in a continuous-equilibrium process. The second transition midpoint T_m of 31.5 °C is practically identical to the T_m monitored by CD, which should reflect the transition midpoint of the correctly aligned triple helices. The normalized thermal unfolding curve suggests that the exact T_m is at 27.5 °C. Little fluctuation of the τ_c values was observed confirming complete unfolding of triple helix at higher temperatures as also observed in the CD melting curve.

The results obtained with the spin-labeled CMP fully confirm the usefulness of ESR spectroscopy for measuring the thermodynamic stability of collagen triple helices. Compared to CD spectroscopy, it could allow the monitoring of the dynamics of CMP triple helix unfolding similar to very detailed NMR spectroscopy experiments[20] and thus provide further insights into collagen structure, stability and folding dynamics.

Experimental Section

All the experimental procedures and are reported in the Supporting Information.

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Keywords: (2*S*,4*R*)-aminoproline · collagen · ESR spectroscopy · proteins · site-directed spin labeling

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