Collagen

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# Photocontrolled Folding and Unfolding of a Collagen Triple Helix**

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Collagens consist of three identical or different polypeptide chains which are arranged in a triple-helical supercoil of variable stability. Although the overall shape is that of a soft rod, collagens also contain stretches of low triple-helix stability and even disruptions of this structure. The local stability and recognition sites for interaction with other proteins of the extracellular matrix are defined and highly fine-tuned by the amino acids in the X and Y positions of the repeating Gly-Xaa-Yaa triplets with a Gly residue mandatory at every third position for the compact intertwining of three left-handed poly-Pro-II helices into the right-handed triple helix.[1] Extensive studies on synthetic model peptides have clearly identified the tripeptide unit Gly-Pro-Hyp as the most favorable triplet for stabilizing the triple helix,[1b,2] and crystal structures of collagen peptides allowed detailed insights into the hydrogen-bonding networks of this very repetitive and regular tertiary structure.[3]

From folding studies of natural collagens and related fragments a zipper-like folding mechanism was derived with the *cis*-to-*trans* isomerization of proline as the rate-limiting step.[1d,4] In absence of *cis*-proline isomers the folding is very fast and completed within time scales comparable to the folding rates of other repetitive structures, such as the α-helix.[5] However, to increase our understanding of the folding and stability of the collagen triple helix, a system should be available that is equipped with an ultrafast conformational trigger which allows the induced folding/unfolding events to be monitored by time-resolved spectroscopy. This challenging task was realized in the present study by using a purposely designed azobenzene derivative as a photoswitchable conformational clamp which, when incorporated into the single collagen chains as side-chain-to-side-chain crosslink, provides the required changes in the conformational space to trigger folding/unfolding of the collagen triple helix.

In preceding studies from our and other laboratories the concept of using azobenzene for ultrafast photomodulation of conformational states and thus of related biophysical properties has been validated.[6] The successful application of this principle for the photocontrol of α-helices[7] and recently of β-hairpins[8] as the most simple tertiary structure motifs, led us to exploit the favorable optical properties of azobenzene, together with the regular supramolecular structure of the collagen triple helix, for the design of a model system suited for time-resolved spectroscopic studies on collagen folding and unfolding. The strategy applied was to crosslink two side chains of a collagen $(Pro\text{-}Hyp\text{-}Gly)_n$ peptide by the azobenzene chromophore (Scheme 1). As the collagen peptide an N-acetylated and C-amidated $(Gly\text{-}Pro\text{-}Hyp)_7\text{-}Gly\text{-}Gly$ was selected because of the relatively high thermal stability of its triple helix.[9] Molecular modeling served to identify suitable sequence positions in this peptide for grafting the azobenzene clamp intramolecularly so that the *trans*-azobenzene isomer should give a more stable triple helix than the *cis* isomer. Since 100% *trans*-azobenzene isomer can be obtained by thermal relaxation in the dark, a fully folded state would be accessible and unfolding can be induced by irradiation with light at an appropriate wavelength.

Moreover, to maximize the effect of chromophore isomerization on the triple-helix stability, a rather rigid acetylene-type linker between the peptide side chains and the azobenzene moiety was devised. Correspondingly, the bifunctional diiodo derivative **1** was used for reaction with the two thiols (Scheme 1). Taking into account the stabilizing role of imino acids in both the X and Y positions of collagen triplets, 4-mercaptoproline (Mpc) residues were selected to provide the required thiol functions for intramolecular crosslinking. While Cys residues in X and Y positions remarkably affect triple-helix stabilities,[10] a 4-mercapto or 4-thioether substituent in the pyrrolidine ring, as in compounds **2** and **3** (Scheme 1), respectively, was expected to affect, independent of its configuration, the triple-helix stability to a lesser extent than (4*R*)- and (4*S*)-hydroxyproline. Through stereoelectronic effects, the latter influence the *trans–cis* isomerization

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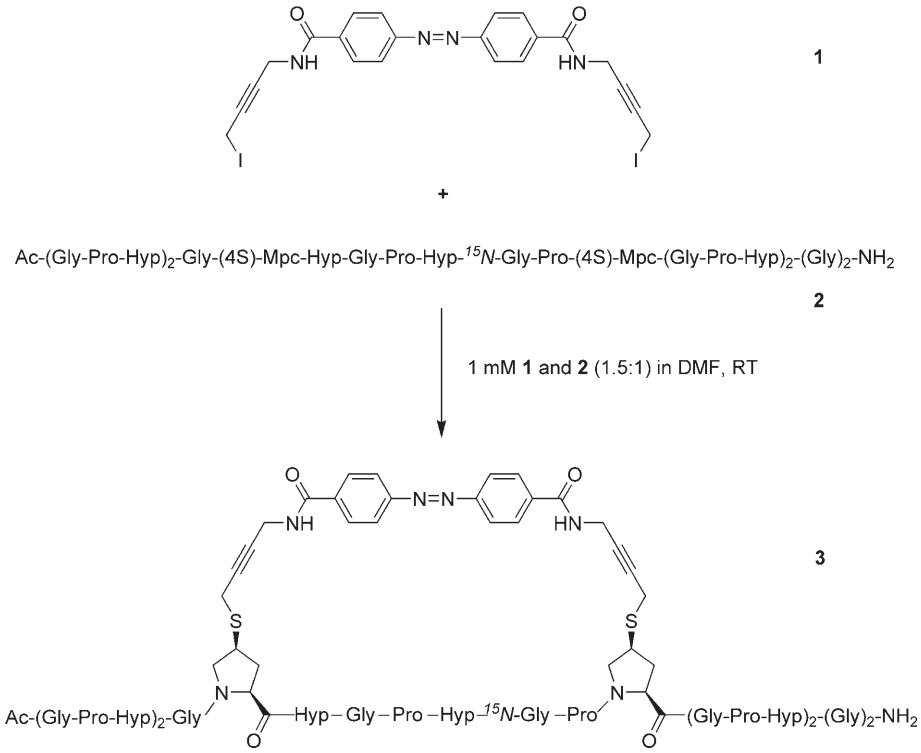


***Scheme 1.*** Synthesis of a collagen peptide with an intramolecular side-chain-to-side-chain bridge containing the photoresponsive azobenzene moiety. For synthetic purposes, the collagen peptide was extended at the C-terminal with two Gly residues to avoid diketopiperazine formation;[10a] Mpc = (2*S*,4*S*)-mercaptopyrrolidine-2-carboxylic acid (4-mercaptoproline).

as well as the $C^{\gamma}$-*exo* and $C^{\gamma}$-*endo* pucker of the pyrrolidine rings to different extents.[2b,9a,11]

According to the peptide design a Pro residue in the X and a Hyp residue in the Y position should be replaced by Mpc. Despite the known triple-helix stabilizing effect of a $C^{\gamma}$-*endo* in X and $C^{\gamma}$-*exo* in Y position,[2c,3i,9a,11] the (2*S*,4*S*)-Mpc was used to replace these imino acids in preparing peptide **2** (Scheme 1). Correspondingly, the thermal stability of the triple-helix of **2** in aqueous solution ($T_m = 34.5\,^{\circ}C$) was found to be 8.5 °C lower than that of the reference peptide Ac-(Pro-Hyp-Gly)$_7$-Gly-Gly-$NH_2$ ($T_m = 43\,^{\circ}C$; this value fully agrees with those reported for similar peptides[9a,b]).

Reaction of peptide **2** with 4,4′-(diazene-1,2-diyl)bis(*N*-(4-iodobut-2-ynyl)benzamide (**1**) led to the azobenzene-peptide (**3**) as an analytically well characterized compound. This peptide derivative is not sufficiently soluble in aqueous media for spectroscopic characterization. Therefore [$D_3$]MeOH/0.1 M AcOH (4:1) was used as a solvent, since it is well-known that the triple-helical structure of collagen peptides is stabilized in aqueous/alcoholic mixtures.[2a,12] For CD, NMR, and IR spectroscopic measurements solutions of **3** at 1 mM concentration were prepared as required for fast self-association of the monomers into triple-helical homotrimers.[1d,13] Upon incubation at 4 °C, the CD spectrum of peptide **3** (Figure 1) as *trans*-azobenzene isomer is consistent with a triple-helical fold with an Rpn value of 0.190,[14] a value which is identical to that of peptide **2** (Rpn = 0.190) but higher than that of the reference peptide Ac-(Gly-Pro-Hyp)$_7$-Gly-Gly-$NH_2$ (Rpn = 0.161). However, CD spectral shapes, peak positions, and intensities are not fully conclusive, since the monomeric forms of these proline-rich peptides adopt a poly-Pro-II conformation that generates CD spectra very similar to those of triple helices. Compared to poly-Pro-II helices which unfold thermally without cooperativity, the thermal transitions of collagen triple helices are very cooperative.[1d] Because of the high stability of the collagen superhelix in aqueous MeOH and the limits imposed on heating by this alcoholic solution, a direct comparison of the thermal transitions of the reference peptide Ac-(Gly-Pro-Hyp)$_7$-Gly-Gly-$NH_2$ and the parent peptide **2** with the azobenzene-peptide **3** was not possible. However, by comparing the initial decrease of dichroic intensities at 225 nm in the temperature range 4 °C to 60 °C, a qualitative rank order of triple-helix stability can be derived: **3** > reference peptide > **2**. The conformation of the triple-stranded collagen structure of **3** was derived from NMR spectroscopic measurements. The chemical shifts of the $^{15}$N-Gly amide protons around $\delta = 7.7$ ppm are indicative of the folded structure (unfolded and poly-Pro-II species exhibit shift values higher than $\delta = 8.0$ ppm) and the temperature shifts of the same amide protons are less negative than $-4.5\ \mathrm{ppb\,K^{-1}}$ indicating that hydrogen bonding is present in the triple helix, and diffusion measurements demonstrate the trimeric nature of the peptide under these conditions.[15] 2D $^1$H–$^{15}$N correlation spectra (FHSQC) allowed the selective detection of the glycine residue preced-

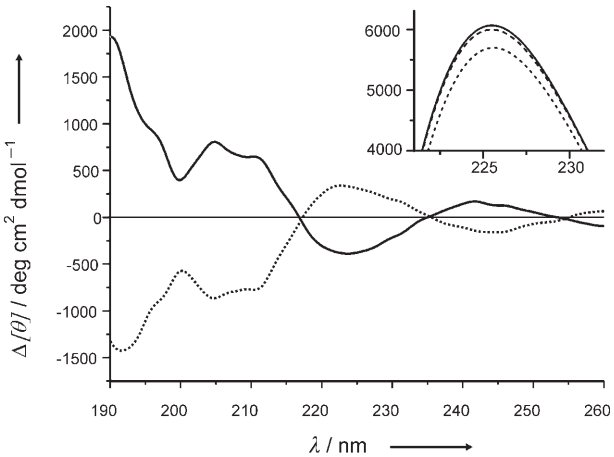


***Figure 1.*** CD difference spectra of peptide **3** at 1 mM concentration in [$D_3$]MeOH/0.1 M AcOH (4:1) obtained by subtraction of the spectra upon irradiation at 330 nm for *trans*-to-*cis* isomerization (——) and at 420 nm from *cis*-to-*trans* isomerization (•••••). Inset: CD spectra of the thermally relaxed 100% *trans*-azobenzene peptide **3** (——), after irradiation at 330 nm to generate the maximum amount of the *cis* isomer (•••••), and again at 420 nm for conversion of the *cis* isomer back into the *trans* isomer (-----).

  

ing the second Mpc residue in **3**. The left panel of Figure 2 shows that the $^{15}$N-Gly residues of the three strands that form the triple helix are not equivalent, in line with the known stagger of one amino acid between the individual chains.[1]

Upon irradiation at 330 nm at 4 °C, photoisomerization of the azobenzene moiety was detected giving 27 % *cis* isomer,[16] but the changes in the 2D $^{1}$H–$^{15}$N FHSQC spectra were not indicative of triple-helix unfolding. On increasing the temperature to 27 °C, photoisomerization leads to 45 % *cis* isomer and appearance of several new peaks in the NMR spectrum (Figure 2, right panel) corresponding to unfolded peptide and to "distorted" triple helical forms. By irradiation at 420 nm the triple helix is almost quantitatively restored and the content of *cis*-azobenzene isomer is $<8\,\%$. As the thermal relaxation of the *cis*-azobenzene to the *trans* isomer is slow even at 37 °C ($t_{1/2}=8.9$ h for **3**, $t_{1/2}=25.4$ h for **1** as dichloro derivative) the photostationary state of **3**, with a maximum *cis*-azobenzene content of approximately 45 % as obtained by irradiation at 330 nm can be investigated at 27 °C by NMR spectroscopy.

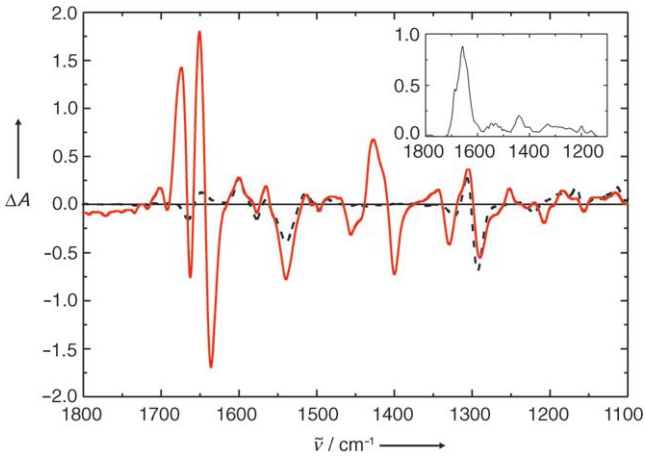


***Figure 3.*** FTIR difference spectra ("*cis* minus *trans*") of peptide **3** (red) in comparison with chromophore **1** as the dichloro derivative (black dashed), both in [D$_6$]DMSO upon irradiation. For the peptide, additional bands can be identified in the regions 1650 cm$^{-1}$ (amide I) and 1450 cm$^{-1}$. Inset: IR spectrum of peptide **3** in [D$_6$]DMSO.

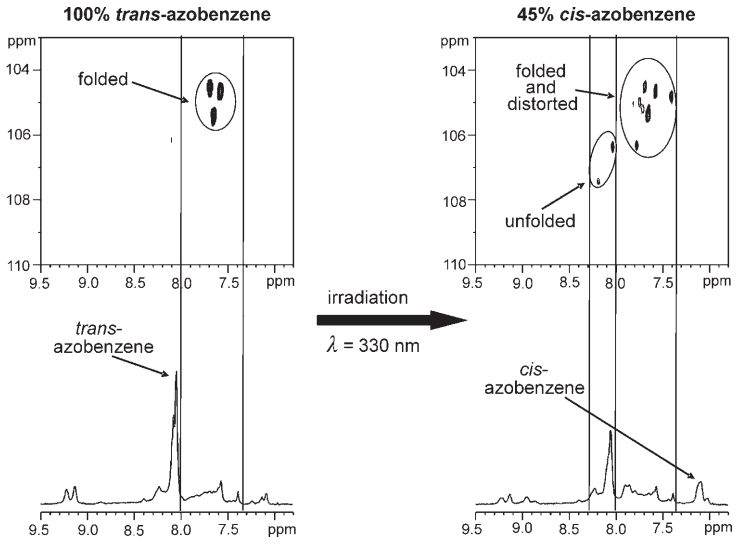


***Figure 2.*** $^{1}$H–$^{15}$N FHSQC (top) and $^{1}$H NMR spectra (bottom) of 1 mM peptide **3** in [D$_3$]MeOH/0.1 M AcOH (4:1) recorded at 27 °C and 500 MHz before (left) and after (right) irradiation at 330 nm. After irradiation new signals are present which are assigned to "distorted" triple helices and unfolded monomers; the two signals seen for the unfolded monomer correspond to the *trans* and *cis* conformations of the ($^{15}$N-Gly)–Pro peptide bond.

The light-induced folding and unfolding of **3** can also be monitored with CD spectroscopy (Figure 1). The changes in CD intensity, however, are weak because of the small differences between the CD spectra of triple-helical and poly-Pro-II structured collagen peptides. Irradiation of **3** leads to significant changes in the IR spectrum (Figure 3). Comparison with the light-induced difference spectrum of **1** (as the stable dichloro derivative) allows the bands related to the *trans*-to-*cis* isomerization of the azobenzene moiety to be identified and also reveals strong additional peptide bands in the regions around 1650 (amide I) and 1450 cm$^{-1}$, indicative of conformational changes upon triple-helix unfolding.[20a] Photoswitching between the *trans*- and *cis*-azobenzene is fully reversible as shown by the CD spectra in the inset to Figure 1, and no signs of photobleaching or decomposition were observed even after extensive periods of irradiation. As, according to the NMR spectra, at lower temperatures (< 20 °C) even the *cis*-azobenzene isomer is folded into the triple helix, photomodulation of the folded fraction is possible only at temperatures where the difference in stability between the triple-helical and the monomeric form is comparatively small, with *trans*-azobenzene molecules more biased to the triple helix and *cis* isomers favoring the unfolded form.

Combined with the ultrafast isomerization of the chromophore,[17] the collagen peptide **3** should allow folding experiments to be performed with unprecedented time resolution, down to the picosecond time regime, by the use of ultrafast CD[18] and particularly, IR spectroscopy.[19] The IR spectra of triple-helical collagen peptides are characterized by well defined amide bands[20] and when recorded in time-resolved manner, could yield valuable information on the rate constants of formation for the periodic hydrogen-bonding network. Thus, comparison with molecular dynamics calculations that are limited to the nanosecond time scale would become feasible. Indeed, recently it was observed that the fastest folding/unfolding processes of the triple-helical structure are beyond the microsecond resolution of the stopped-flow techniques used.[5]

In summary, we have succeeded in constructing a collagen model peptide where the stability of the triple-helical structure is modulated by the isomeric state of an azobenzene-derived chromophore. At ambient temperatures folding or unfolding can be induced by photo-isomerization of the "light switch". The model peptide should allow ultrafast folding/unfolding experiments to be performed, thus provid-

  

ing a link between experimental and theoretical studies on the triple-helical collagen structure.

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[14] Rpn denotes the ratio of the positive maximum intensity over the negative maximum intensity and is used as index of triple-helical fold and stability (see also ref. [12]).

[15] The chemical shifts of the folded labeled glycine residues are $\delta =$ 7.691, 7.653, and 7.574 ppm at 27 °C and the temperature shifts relative to these values are −2.5, −3.8, and −4.1 ppb $K^{-1}$, respectively. The translational diffusion constants (given in $10^{-10}$ $m^2 s^{-1}$) at 4 °C for peptide **2** (1.09) and peptide **3** (0.90 before and 0.82, after irradiation) are indicative of trimeric species; at 27 °C the values for peptide **2** (1.34) and for peptide **3** before irradiation (1.15) correspond again to trimeric species, while after irradiation of **3** in addition to the trimeric species (0.61 and 1.07) a second species is formed, which shows a diffusion constant of 1.55 supporting the presence of an unfolded monomeric form.

[16] The relative amounts of *trans* and *cis* isomers were determined by integrating the signals of the amidic protons of the azobenzene moiety in the 1D $^1$H NMR spectra at $\delta = 9.34$ and 9.08 ppm (4 °C), 9.21 and 8.94 ppm (22 °C), and 9.18 and 8.86 ppm (27 °C), respectively.

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