CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200601162

Photocontrol of the Collagen Triple Helix: Synthesis and Conformational Characterization of Bis-cysteinyl Collagenous Peptides with an Azobenzene Clamp

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Abstract: For the photomodulation of the collagen triple helix with an azobenzene clamp, we investigated various collagenous peptides consisting of ideal (Gly-Pro-Hyp) repeats and containing cysteine residues in various positions for a side chain-to-side chain crosslink with a suitable chromophore derivative. Comparative conformational analysis of these cysteine peptides indicated an undecarepeat peptide with two cysteine residues located in the central portion in i and i+7 positions and flanked by (Gly-Pro-Hyp) repeat sequences as the most promising for the cross-bridging experiments. In aqueous alcoholic solution the azobenzene–undecarepeat peptide formed a stable triple helix in equilibrium with the monomeric species as a *trans*-azobenzene isomer, whereas photoisomerization to the *cis* isomer leads to unfolding of at least part of the triple helix. Furthermore, the residual supercoiled structure acts like an intermolecular knot, thus making refolding upon *cis*-to-*trans* isomerization a concentration-independent fast event. Consequently, these photoswitchable collagenous systems should be well suited for time-resolved studies of folding/unfolding of the collagen triple helix under variable thermodynamic equilibria.

Keywords: azo compounds · collagen · photomodulation · transition states · triple helix

Introduction

Collagen is the most common protein in connective tissues. The defining characteristic of all types of fibril-forming and nonfibrillar collagen is the triple-helical structure, the thermal stability of which is critical to the protein's function and degradation. This native structure consists of three left-handed polyproline-II-like helices that are assembled in a parallel manner with a one-residue shift, and supercoiled into a right-handed triple helix.[1] The close packing of the three polypeptide chains requires the presence of a glycine residue at every third sequence position, leading to the characteristic $(Gly\text{-}Xaa\text{-}Yaa)_n$ motif for the triple-helical portions of collagens. A high content of imino acids in the X and Y positions is known to promote and stabilize the extended polyproline-II conformation of the individual chains, and indeed about 20% of these two positions is occupied in collagens by proline and hydroxyproline, respectively. Folding and stability of the collagen triple helix have been studied extensively over the last decades, generally by using model $(Gly\text{-}Xaa\text{-}Yaa)_n$ peptides.[2] Although the (Gly-Pro-Hyp) repeat is the most common and stabilizing tripeptide unit, the identities of the non-imino acid residues in the X and Y positions are important for functional modulation of structure and stability and, thus, for biological recognition and specificity. Indeed, the collagen triple helix is not uniform in structure and stability, but consists of multiple independent folding domains, and regions of decreased triple-helix stability have been implicated in recognition by and binding to interacting proteins in the extracellular matrix.[3]

For studying recognition and binding processes of local collagen structures, as well as for characterizing the folding/unfolding processes of triple helices, an artificial manipulation of their local stabilities by noninvasive methods could

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well represent a promising approach. In preceding studies we successfully employed the photoresponsive azobenzene chromophore inserted as ω amino acid into peptide backbones for photomodulation of conformational states of peptides.[4] Similarly, Woolley and co-workers used side chain-to-side chain crosslinking of peptides by this chromophore for light-triggered conformational transitions of α-helical conformations.[5] This latter strategy appeared to us as the most appropriate for photomodulation of triple-helix stabilities if an azobenzene clamp could be grafted intramolecularly on a collagenous peptide in a way that the *trans*-azobenzene isomer allows for folding of the peptide into a more stable triple helix than the *cis* isomer. Because 100% *trans*-azobenzene isomer can be obtained by thermal relaxation in the dark, a fully folded state would then be accessible and unfolding would be inducible by simple irradiation with light of appropriate wavelength.

In addition to the phototrigger in an optimal position, the ideal photoswitchable collagenous system should include a crosslinking of the three peptide chains to avoid all the drawbacks of the concentration-dependent kinetics of intermolecular association, which, together with the trimerization into the correct register, represents the rate-limiting step in the folding process.[2c] Such a trimeric collagenous system could be realized, in theory, by the use of an artificial cystine knot introduced C-terminally through regioselective disulfide-pairing procedures, as applied in our previous synthesis of heterotrimeric model-collagen systems.[6] Similarly, the synthetically more readily accessible native cystine knot of collagen type III[7] would lend itself for such a purpose. This procedure, however, requires a correct prefolding of collagenous peptides prior to oxidative formation of the intermolecular cystine knot.[7d,8] Because introduction of the azobenzene clamp has to be performed prior to the cystine knot, the sensitivity of the azobenzene moiety to reduction by thiols[4a] was expected to add considerable difficulties to the challenging task of synthesizing photoresponsive collagenous homotrimers. Therefore, as a first goal, our attention was addressed to the synthesis of monomeric azobenzene–collagen peptides.

Two different strategies were attempted in these efforts: 1) the use of two mercaptoproline residues suitably displayed in $(Gly\text{-}Pro\text{-}Hyp)_n$ peptides and then crosslinked at their thiol functions by the relatively rigid azobenzene derivative shown in Figure 1, and 2) incorporation of two cysteine residues into the collagenous peptide. These were expected to dampen the conformational restrictions imparted by the geometrical changes of the clamp upon *cis*/*trans* photoisomerization because of the higher flexibility of the alkane-type cysteine side chain than the pyrrolidine ring of the mercaptoprolines. Indeed, the bismercaptoproline approach led to unfolding of the collagenous peptide and, thus, to a trimer–monomer transition by *trans*-to-*cis* photoisomerization.[9] Conversely, the cysteine approach, which is the subject of the present work, induces local unfolding of a portion of the triple helix, retaining the rest of the collagenous peptide supercoiled into the homotrimeric structure.

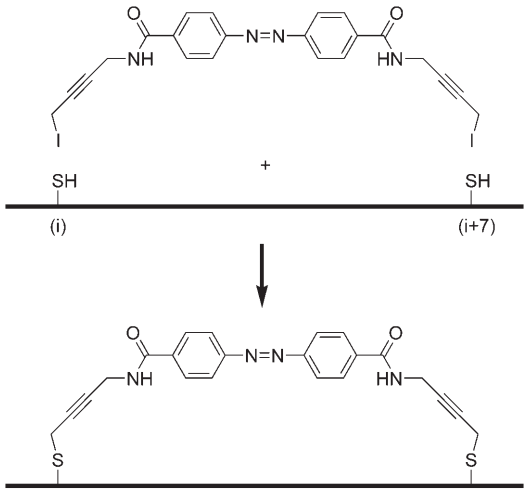

Figure 1. For optimal crosslinking of two cysteine residues in positions i and i+7 of a collagen triple helix, the azobenzene-4,4′-dicarboxylic acid was extended at both carboxylic groups with the rigid 1-amino-4-chlorobutyne as bisamide, which can be converted to the more reactive diiodo derivative.

Results and Discussion

Design and synthesis of the collagenous peptides: In our efforts to construct a reversible photoresponsive collagen triple helix, one of the strategies pursued was a crosslinking of two cysteine residues through an azobenzene-containing chromophore, similar to the approach taken by Woolley and co-workers for the photocontrol of α helices.[5,10] Molecular-modeling experiments performed on a collagen triple-helix model suggested an i, i+7 positioning of the cysteine residues to be optimal for their crosslinking by a purposely designed thiol-reactive azobenzene derivative as a sufficiently rigid clamp. This would allow formation of the triple helix in the *trans*-azobenzene isomeric state and would effect significant destabilization upon isomerization to the *cis* isomer (Figure 1). For collagenous peptides consisting of seven (Gly-Pro-Hyp) repeats (**1**) and, thus exceeding by two repeats the minimum length for a stable triple helix,[11] a midpoint of thermal transition (T_m) of 36°C has been reported.[12] Correspondingly, the N-acetylated and C-amidated heptarepeat peptide **1** of Figure 2, containing two additional C-terminal Gly residues for synthetic purposes, was selected as the parent peptide to analyze in the first instance the effects of Pro/Hyp replacements by cysteine residues in the X and Y position of the (Gly-Xaa-Yaa) repeat motif in terms of thermal stability of self-associated triple helices in aqueous solution.

From analysis of the amino-acid compositions of collagens, it is well known that Cys residues rarely occur in triple-helical sequences.[13] Host–guest studies with single Cys residues in X and Y positions clearly revealed a destabilizing effect of such Pro/Hyp replacements.[14] Side chain-to-side chain cross-bridging of the two Cys residues with the azobenzene derivative was foreseen in the central part of the collagenous peptides to achieve a maximum effect of

Figure 2. Collagenous (Gly-Pro-Hyp)$_n$ peptides containing one or two Cys residues in the X and Y position (**1**–**7**), and peptide **8**, which is side chain-to-side chain cross-bridged with the azobenzene derivative depicted in Figure 1. The Cys residues colored grey are still protected as (*S*)-*tert*-butylthio derivatives, and the glycines in italics are ^{15}N-labeled residues.

the *cis*/*trans* photoisomerization on folding/unfolding of the triple helix. Therefore, a series of peptides was planned, containing one Cys residue in the X (**2**) or Y position (**3**) and the required two Cys residues in the X and Y positions, respectively, (**4**) or both in the Y position (**5**) of the parent heptarepeat peptide **1**. Moreover, incorporation of ^{15}N-labeled glycine in some of the peptides was expected to yield detailed information on the conformational states of this portion of the collagenous peptides by NMR experiments. From the results of this first series of peptides, we expected to derive sufficient information for the design of suitable collagenous peptides containing two cysteine residues in i and i+7 positions as linear precursors for the intramolecular side-chain crosslinking with the azobenzene clamp.

In previous syntheses of collagen peptides, we generally used the tripeptide synthon Fmoc-Pro-Hyp(*t*Bu)-Gly-OH for assembly of the peptide chains to prevent deletions of single Pro/Hyp residues, as well as diketopiperazine formation as the main source of microheterogeneity.[6a–c,7d,15] However, the large excesses of synthon required in the single-coupling steps on resin makes this strategy highly labor-intensive and expensive. Comparison of crude products of collagenous peptides synthesized in a stepwise manner on batch and continuous-flow synthesizers showed that the quality of those obtained by the latter technology was significantly improved. Moreover, double couplings for all prolines largely suppressed even partial deletion of Pro residues. In some of the syntheses, nonquantitative acylation with Fmoc-Cys(S*t*Bu)-OH was observed by mass-spectrometric analysis of the crude products. However, these side products were readily separated by reversed-phase chromatography due to the hydrophobic character of the *tert*-butyl group. Correspondingly, the Cys-protected peptides **1**–**7**, listed in Figure 2, were obtained by stepwise synthesis on a continuous-flow synthesizer in satisfactory yields and at a high degree of homogeneity, as assessed by analytical HPLC and mass spectrometry.

For side-chain cross-bridging of the bis-cysteinyl peptides with the bifunctional azobenzene-4,4′-bis-*N*-(4-chloro-2-butynenyl)carboxyamide reagent,[9] the (*S*)-*tert*-butylthio groups were removed by reduction of the asymmetric disulfide with tributylphosphine in trifluoroethanol/water, and the azobenzene-chloro derivative was converted to the bisiodo compound by the Finkelstein reaction with sodium iodide in acetone. This conversion does not occur quantitatively and minor amounts of educt as well as of monoiodo derivatives were detectable in the reaction mixture. Nonetheless the crude bisiodo derivative was used in the crosslinking experiments. Optimization of the intramolecular side-chain cross-bridging reaction was performed with the model compound Ac-Gly-Cys-Hyp-Gly-Pro-Hyp-Gly-Pro-Cys-Gly-NH$_2$ both on resin and in solution. Deprotection of the Cys(S*t*Bu) residues on resin was found to be strongly solvent- and resin-dependent, and under all examined conditions partial desulfuration was observed, a side reaction that was reported to occur even in solution.[16] Therefore, deprotection of the Cys residues was performed on the purified peptides in solution, and the subsequent intramolecular reaction between the two free thiol groups and the bifunctional azobenzene reagent (1.5 equiv) was carried out at 1 mM under argon and in the presence of an excess of diisopropylethylamine (DIPEA) (10 equiv). Despite the low concentration, in addition to the target product, various side products were formed, such as the bis-alkylated or the mono-alkylated derivatives derived from both the bisiodo or mixed iodo/chloro reagent, as well as minor amounts of unreacted peptide or of its oxidized form.

Optimized reaction conditions were then applied for the preparation of the azobenzene-cross-bridged peptide **8** (Figure 2), which could be isolated in only low yields, but in sufficient quantity for the careful analysis of its preferred structural properties in solution by CD and NMR spectroscopy.

Triple-helix stabilities of the bis-cysteinyl peptide precursors: Nucleation of the triple helix from monomeric collagenous peptides is a slow and concentration-dependent process of apparent third order.[2c,7b] Although trimerization is known to be strongly favored by the (Gly-Pro-Hyp) repeats compared to the (Gly-Pro-Pro) repeats, relatively high concentrations (1 mM) of the monomeric peptides were used to reduce the rate-limiting effect of the intermolecular association into homotrimers as the first folding step.[7b] The other rate-limiting factors in the triple-helix formation are the correct registration of the chains and the *cis*-to-*trans* isomerization of the Gly-Pro and Xaa-Hyp bonds. To assure full equilibration of the folding process, the 1-mM solutions of all peptides analyzed in the present study were preincubated for 12–24 h at 4 °C prior to the conformational characteriza-

tion. The equilibrium structures of peptides **1**–**7** were analyzed by CD spectroscopy, translational diffusion constants were determined by NMR spectroscopy, the temperature dependence of NMR chemical shifts was measured, and characteristic signal patterns and shifts of the Gly amide protons in homonuclear two-dimensional (2D) NMR spectra were recorded. The stabilities of the triple-helical fold of the peptides were compared by thermal unfolding, as monitored by changes in the dichroic intensities at 225 nm in temperature excursions at a heating rate of 0.2 °C min^{-1}. Because of the slow equilibration of the folding/unfolding of collagen molecules, this heating rate does not produce equilibrium, but rather kinetic melting curves.[17] Nonetheless, the apparent melting temperatures (T_m) obtained under standardized conditions allow for useful comparisons.

The thermal unfolding curves of peptides **1**–**7** in aqueous solution are shown in Figure 3 and the related CD parameters and NMR-derived diffusion constants and temperature-dependence of chemical shifts are summarized in Table 1. N-acetylation and C-amidation of collagenous peptides prevents electrostatic repulsions in the self-association process, thus increasing the thermal stability of the triple helix. Indeed, the parent peptide **1** is characterized by a T_m value of 43 °C, which is significantly higher than that reported for H-(Gly-Pro-Hyp)$_7$-OH (36 °C).[12] For peptide **2**, in which a Pro residue in the X position was replaced by a Cys(S*t*Bu) residue, a reduction in thermal stability by 13.1 °C was observed, compared to 11.2 °C reported by Persikov and coworkers[14a] for unprotected Cys residues. The substitution of a Hyp residue in the Y position with Cys(S*t*Bu) (**3**) leads to a similar destabilization by 13.8 °C, whereas only a decrease by 9.6 °C was measured previously for this position in host–guest studies of Ac-(Gly-Pro-Hyp)$_8$-NH$_2$.[14a] The difference most probably results from the presence of the bulky and hydrophobic *tert*-butylthio group in the cysteinyl peptides, which significantly changes in loco the hydration shell as one of the potential, but disputed, factors that stabilize the collagen triple helix.[18] The thiol protecting group, however, was retained for the conformational analysis to mimic more properly the hydrophobic character of the azobenzene moiety of the target molecules. The T_m values of peptides **4** and **5**, containing two Cys(S*t*Bu) residues in different positions, are consistent with a negative contribution of about 13 °C per Cys(S*t*Bu) residue to the melting temperature, confirming the largely additive contributions of the single Gly-Xaa-Yaa triplets to the overall triple-helix stability. Despite the slightly more favorable Y position for a Cys residue according to host–guest studies,[14a] peptide **5** is destabilized by 1.1 °C relative to **4**. In peptides **3** and **4**, the ^{15}N-labeled Gly residue is embedded into a triple-helical structure, as determined by 2D {^{1}H,^{15}N} FHSQC spectra at 4 °C, and the disappearance of its "folded" signal parallels the disappearance of the signals of the other triple-helical Gly residues (see Supporting Information). Because of the relatively low stability of the triple-helical peptides **4** and **5** (T_m = 17.9 and 16.8 °C, respectively) these appeared to be inappropriate as linear precursors.

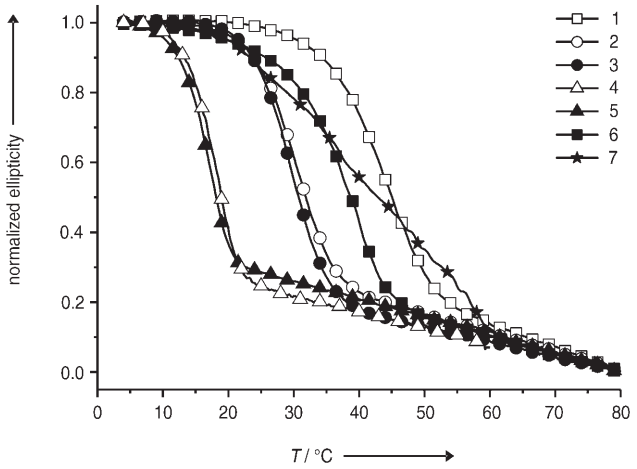

Figure 3. Thermal unfolding of the collagenous peptides **1**–**7** in water at 1 mM and at a heating rate of 0.2 °C min^{-1}, as monitored by CD at 225 nm.

Folding of the triple helix is known to occur primarily from the C to the N terminus in a zipper-like fashion.[2b,c] To facilitate nucleation of the triple helix at the C-terminal site, the Cys residues were moved to the N terminus to install five regular (Gly-Pro-Hyp) repeats at the C terminus (see compound **6**). In fact, after this study had been performed, it was confirmed experimentally that about ten triplets, that is, 3.3 on each chain, comprise the nucleation seed and that this nucleation is rate limiting in the triple-helix folding of

Table 1. CD characteristics, melting transition temperatures, NMR diffusion constants and temperature shifts of peptides **1**–**7** in aqueous solution at 1 mM.[a]

	Circular dichroism							NMR: translational diffusion		NMR: temperature shifts	
peptide	λ_{min}	θ_R^{min}	λ_{max}	θ_R^{max}	Rpn	T_m [°C]	triple helix	4 °C	27 °C	Gly	^{15}N-Gly
1	196.8	−30055	225.1	3443	0.11	43.0	yes	0.71	1.5 (2.0)	−3.6	
2	196.7	−30454	224.8	4475	0.15	29.9	yes	0.65	1.4 (1.7)	−3.4	
3	197.8	−34937	228.8	4990	0.14	29.2	yes	0.68	1.3 (1.7)	−3.6	−3.5
4	199.3	−31173	224.1	3377	0.12	17.9	yes	0.60 (0.91)	– (2.2)	−2.7	−4.2
5	199.6	−31464	224.0	2925	0.09	16.8	yes	0.64 (1.0)	– (2.1)	−2.2	
6	197.3	−35101	224.3	5044	0.14	37.8	yes	0.62 (0.84)	1.3 (1.9)	−2.7	
7	197.8	−31358	224.5	3863	0.12	42.2	yes	0.55	1.1 (1.6)	−3.7	−4.0

[a] λ is denoted in nm; θ_R in deg cm^2 dmol^{-1}; Rpn represents the ratio of positive maximum to negative minimum from CD spectra recorded at 4 °C; T_m was monitored by CD at 225 nm and at a heating rate of 0.2 °C min^{-1}; translational diffusion constants (in 10^{-10} m^2 s^{-1}) without brackets refer to the folded state, with brackets to the unfolded state; temperature shifts are given in ppb K^{-1}, the peaks were chosen in the folded region (7.8–7.6 ppm).

all-*trans* collagen peptides.[19] This finding also agrees with the observation that the pentarepeat (Gly-Pro-Hyp)$_5$ represents the shortest sequence capable of forming, even as a self-associated homotrimer, a relatively stable triple helix.[6c,11b] By moving the two Cys residues towards the N terminus, an extension of the parent heptarepeat peptide **1** to the octarepeat sequence of peptide **6** was required. Compared to **4**, the thermal unfolding of peptide **6** shows a marked increase in the transition midpoint to 37.8 °C, which indicates a weaker destabilizing effect of the two Cys(S*t*Bu) residues relative to the T_m value of 47.3 °C reported for Ac-(Gly-Pro-Hyp)$_8$-NH$_2$ under slightly different conditions.[14a] The T_m value is also significantly higher than that of Ac-(Gly-Pro-Hyp)$_5$-NH$_2$ (18 °C; ref. [11b]) and Ac-(Pro-Hyp-Gly)$_5$-Cys(*t*Bu)-Cys(*t*Bu)-Gly-Gly-NH$_2$ (20.3 °C; ref. [7d]). This increased melting temperature could derive from full involvement of the larger N-terminal extension of **6** in the triple-helical fold, or from at least partial propagation of the triple helix into the bis-cysteinyl sequence. An approximate quantification of the Gly amide protons in triple-helical and random conformation would support a triple helix spanning the entire C-terminal (Gly-Pro-Hyp)$_5$ sequence and partly protruding into the N terminus. To promote propagation of the triple helix over the whole bis-cysteinyl sequence and to stabilize it even in this peptide portion, peptide **6** was extended N-terminally by an additional three (Gly-Pro-Hyp) repeats to produce peptide **7**. As expected, for peptide **7**, the T_m value increased to 42.2 °C. For **6**, a sharp thermal transition was obtained that reflects a highly cooperative unfolding by a two-state transition mechanism (Figure 3). In contrast, the melting curve of peptide **7** is more consistent with the unfolding of multiple domains of different stabilities, possibly resulting from the bis-cysteinyl portion and the two flanking (Gly-Pro-Hyp) repeat regions of different sizes. For peptide **6**, NMR experiments confirm the presence of nonassociated monomers in equilibrium with the homotrimers even at 4 °C (Table 1), whereas for peptide **7**, at 4 °C only the trimer species was clearly identified by translational diffusion measurements. The FHSQC spectra of the ^{15}N-glycines of peptide **7**, however, indicated the presence of some locally unfolded species in addition to completely folded trimer even at 4 °C (Supporting Information). Because of the higher thermal stability of **7**, it was then used as a template to attempt a photocontrol of the triple helix by side-chain cross-bridging with the azobenzene clamp to produce the peptide **8**.

Triple-helix stability of the azobenzene-crosslinked collagenous peptide: Peptide **8** was found to be poorly soluble in water at the concentration of 1 mM required for efficient self-association into triple-helical homotrimers and for NMR experiments. Conformational studies on collagenous peptides have often been performed in aqueous alcohols such as EtOH, MeOH, or ethylene glycol to increase their solubility and to increase the thermal stability of the triple-helical fold.[1a,20] Such increased stability is well evidenced by the T_m values, for example, of peptides **3** (46.9 °C), **4** (35.2 °C), and **5** (25.6 °C) in MeOH/0.1 M AcOH (4:1), that are higher than those determined in water (see Table 1). For peptide **6**, the T_m value in aqueous solution is 37.8 °C, but in MeOH/0.1 M AcOH (4:1) it cannot be determined because of the limits imposed on the temperature excursion by MeOH evaporation. We used this solvent mixture to prepare a 1-mM and 0.5-mM solution of the linear precursor peptide **7** and the azobenzene peptide **8**, respectively. After storage in the cold, the CD parameters of both peptides were fully consistent with a triple-helical structure (Table 2). As expected, the thermal stability of **7** in this aqueous alcoholic medium is significantly higher than in aqueous solution, but cannot be determined. A comparison of the initial decrease of dichroic intensities at 225 nm of peptide **7** and of the azobenzene peptide **8** within the temperature range of 4–60 °C would suggest only a minor destabilizing effect of the cross-bridging with the azobenzene chromophore on the stability of the triple helix. An approximate quantification of the triple-helical glycines of **7** by the NMR signal in the 7.8–7.6 ppm region versus nonfolded glycines would indicate a large part of these residues to be hydrogen bonded at 4 °C. Moreover, from the FHSQC spectrum of **7** at 4 °C in aqueous methanol it can be concluded that the labeled glycines are completely folded (Supporting Information); therefore, the triple helix has to span almost the entire peptide sequence of **7**. Conversely, by comparing the one-dimensional (1D) ^{1}H NMR spectra of the linear precursor **7** (Figure 4D) and the azobenzene peptide **8** (Figure 4E) at 4 °C, fewer triple-helical glycines are present in the bridged peptide. Translational diffusion experiments of **8** indicate the presence of monomers already at this low temperature (Table 2). By monitoring the characteristic signal of ^{15}N-Gly at 27 °C (Figure 4G), the presence of the triple-helical species was confirmed, but also the presence of unfolded species that can derive from the monomeric (Table 2) or locally unfolded species.

Table 2. CD characteristics, melting transition temperatures, NMR diffusion constants and temperature shifts of **7** and **8** at 1 mM in MeOH/0.1 M AcOH (4:1).[a]

	Circular dichroism							NMR: translational diffusion		NMR: temperature shifts	
peptide	λ_{min}	θ_R^{min}	λ_{max}	θ_R^{max}	Rpn	T_m [°C]	triple helix	4 °C	27 °C	Gly	^{15}N-Gly
7	198.4	−38269	224.6	6460	0.17	n.d.	yes	0.73	1.0	−2.2	−2.8
8	197.7	−32945	224.8	5275	0.16	n.d.	yes	0.61 (1.0)	1.1 (1.4)	−1.5	−3.8

[a] λ is denoted in nm; θ_R in deg cm^2 dmol^{-1}; Rpn represents the ratio of positive maximum to negative minimum from CD spectra recorded at 4 °C; T_m was monitored by CD at 225 nm and at a heating rate of 0.2 °C min^{-1}; nd: not determined; translational diffusion constants (in 10^{-10} m^2 s^{-1}) without brackets refer to the folded state, with brackets to the unfolded state; temperature shifts are given in ppb K^{-1}, the peaks were chosen in the folded region (7.8–7.6 ppm).

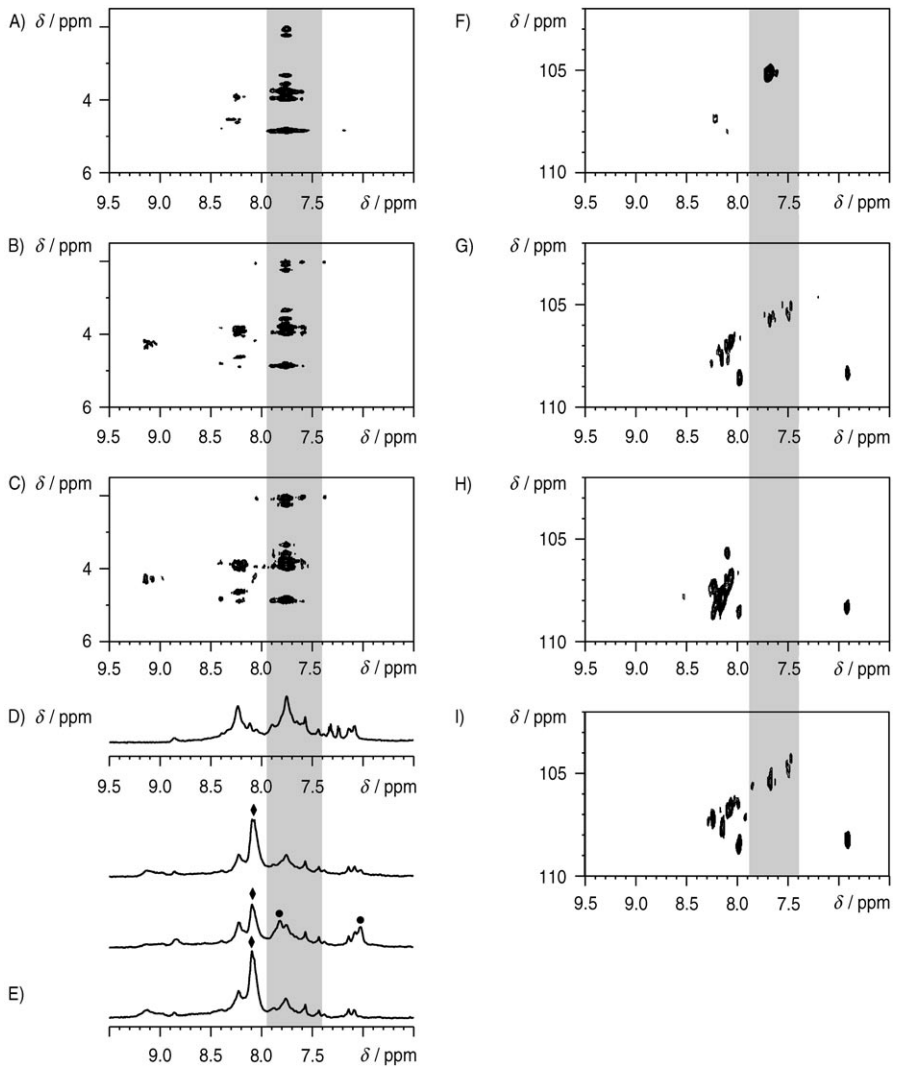

Figure 4. NMR spectra of peptide **8** in MeOH/0.1 M AcOH (4:1) at 27 °C, with the ppm region of the triple helix highlighted. A)–C) NOESY spectra of **7**, **8** (as all-*trans*-azo isomer), and **8** (after irradiation at 330 nm and thus, partial *trans*-to-*cis* photoisomerization), respectively. D) 1D ^{1}H NMR of peptide **7**. E) 1D ^{1}H NMR of peptide **8** as all *trans*-azo isomer (top), after irradiation at 330 nm with partial *trans*-to-*cis* photoisomerization (middle), and after irradiation at 420 nm with *cis*-to-*trans* photoisomerization (bottom); protons of the azobenzene moiety as *trans* isomer are marked with ◆ and as *cis* isomer with ●. F)–I) FHSQC spectra of **7**, **8** as all-*trans*-azo isomer, **8** after irradiation at 330 nm (*trans*-to-*cis* photoisomerization), and **8** after irradiation at 420 nm (*cis*-to-*trans* photoisomerization), respectively.

In the triple-helical structure the glycines are not equivalent because of the one-residue shift in the stagger of the three chains. Indeed, the 2D {^{1}H,^{15}N} correlation spectrum shows the related peaks within the typical ppm range (Figure 4G). Additional peaks related to an unfolded form are detectable, however, a quantitative evaluation of the folded and unfolded form cannot be derived as the peak intensities in these NMR experiments are strongly affected by the local flexibility of the peptide backbone. However, a significant increase to a mostly unfolded form is observed in this region of the molecule upon *trans*-to-*cis* photoisomerization by irradiation at 330 nm and 27 °C (Figure 4H). This leads to 35 % *cis*-azobenzene isomer, as derived from quantification of the azobenzene-related NMR signals (Figure 4E, middle). Although the main differences in the 1D ^{1}H NMR spectra are due to isomerization of the azobenzene moiety and only in part to an increase in unfolded peptide backbone, an inspection of the NOESY spectra (Figure 4B and C) confirms the presence of triple-helical structure even after irradiation. Conversely, the FHSQC spectrum (Figure 4H) indicates unfolding of the labeled glycines. Therefore, a residual triple helix has to span most probably the C terminus with its five consecutive (Gly-Pro-Hyp) repeats. As a logical consequence, it has to be assumed that only part of the triple-helical structure of **8** becomes unfolded by photoisomerization of the azobenzene moiety, and that the folded portion acts like a knot capable of retaining the homotrimeric state for a large population of molecules.

The original FHSQC spectrum is fully recovered upon *cis*-to-*trans* photoisomerization by irradiation at 420 nm (Figure 4I). This fact confirms the reversibility of the effects promoted by the *cis*/*trans* isomerization of the azobenzene clamp. From these NMR experiments it can be concluded that compound **8** consists of a photoswitchable triple helix in the N-terminal portion, whereas the C terminus remains tightly packed in the homotrimeric structure.

Conclusion

By cross-bridging two mercaptoproline residues with the *trans*-azobenzene derivative of Figure 1 in the i and i+7 positions of the collagenous peptide Ac-(Gly-Pro-Hyp)$_7$-Gly-Gly-NH$_2$, we succeeded recently in producing a triple-helical model collagen system that unfolds completely upon *trans*-to-*cis* photoisomerization of the azobenzene moiety.[9] Such a system is expected to allow time-resolved studies of the dynamics of triple-helix unfolding by ultrafast spectroscopy. The photoswitchable collagenous peptide **8** described in the present manuscript exhibits largely a triple-helical fold in

the *trans*-azobenzene isomeric state, but unfolds at least in the region spanned by the azobenzene clamp upon *trans*-to-*cis* photoisomerization, retaining the homotrimeric state like a knot most probably in the C-terminal portion. As expected from intermolecular crosslinked collagenous peptides in which the folding kinetics are concentration independent and *cis*/*trans* isomerization of the Gly−Pro and Pro−Hyp bonds becomes the rate-limiting factor,[2c] refolding of the collagenous peptide **8** upon *cis*-to-*trans* photoisomerization is expected to occur at high rates. Thus, the fully reversible folding/unfolding processes should allow the use of this system for time-resolved studies of the relaxation of the conformational states into thermodynamic equilibria, that is, of folding.

Experimental Section

Materials: All solvents and reagents used were of the highest quality commercially available. Fmoc-amino acids and coupling reagents were purchased from Iris Biotech (Marktredwitz, Germany) and ^{15}N-labeled glycine was from Cambridge Isotope Laboratories (Andover, USA). Resins were from Rapp Polymere (Tübingen, Germany) or Novabiochem (Nottingham, UK), and the chemicals were from Roth (Karlsruhe, Germany), Fluka (Buchs, Switzerland), Sigma–Aldrich (Taufkirchen, Germany), Bachem (Bubendorf, Switzerland), and Merck (Darmstadt, Germany).

Peptide synthesis: The syntheses of peptide **1** and azobenzene-4,4′-bis-*N*-(4-chloro-2-butynenyl)carboxamide are reported elsewhere.[9] Peptides were synthesized by using a Pioneer Peptide Synthesis System (Model GEN600611, PerSeptive Biosystems, Framingham, USA) on a 0.1-mmol scale on TentaGel S Ram resin (Rapp Polymere, Tübingen, Germany) by applying the Fmoc/*t*Bu strategy and *tert*-butylthio protection of the Cys residues. Fmoc cleavage was achieved with 20% piperidine in DMF for 5 min and couplings were performed with 4 equiv of Fmoc-Xaa-OH/HOBt/HBTU/DIPEA (1:1:1:2) (Fmoc = 9-fluorenylmethyloxycarbonyl, HOBt = 1-hydroxybenzotriazole, HBTU = 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate) in DMF for 60 min, whereas Fmoc-Pro-OH required double couplings. Peptides were N-terminally acetylated with 5% acetic anhydride and 6% lutidine in DMF for 10 min. Final deprotection and cleavage from resin was carried out by treatment with TFA/TIPS/H_2O (96:2:2) (TFA = trifluoracetic acid, TIPS = triisopropylsilane) for 60 min and then twice for 25 min. Crude products were purified by preparative reversed-phase chromatography (column I: 250/21 Nucleosil 300–5 C4, column II: 250/21 Nucleosil 100–5 C8 HD from Machery & Nagel, Düren, Germany) or in a semipreparative manner (column III: 250/10 Nucleosil 100–5 C8 HD from Macherey & Nagel) by using linear gradients of 0.1% aqueous TFA (eluent A) and 0.08% TFA in acetonitrile (eluent B) at a flow rate of 10 mL min^{-1} (6 mL min^{-1} for column III).

Ac-(Gly-Pro-Hyp)$_2$-Gly-Cys(S*t*Bu)-Hyp-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH$_2$ (2): The peptide was synthesized according to the general procedures and the crude product was purified by preparative HPLC (column II) by using a linear gradient from 0–30% B in eluent A for 10 min and then to 60% B in 45 min; yield: 22.5 mg (23%); LC-HRMS: $t_R = 9.0$ min; *m*/*z* calcd for $C_{92}H_{136}N_{24}O_{31}S_2+2H$: 1069.46995 [$M+2H^+$]; found: 1069.47080.

Ac-(Gly-Pro-Hyp)$_4$-^{15N}Gly-Pro-Cys(S*t*Bu)-(Gly-Pro-Hyp)$_2$-Gly-Gly-NH$_2$ (3): The product was obtained as described for **2**; yield: 45.0 mg (40%); LC-HRMS: $t_R = 9.4$ min; *m*/*z* calcd for $C_{92}H_{136}N_{24}O_{30}S_2+2H$: 1061.97102 [$M+2H^+$]; found: 1061.97474.

Ac-(Gly-Pro-Hyp)$_2$-Gly-Cys(S*t*Bu)-Hyp-Gly-Pro-Hyp-^{15N}Gly-Pro-Cys-(S*t*Bu)-(Gly-Pro-Hyp)$_2$-Gly-Gly-NH$_2$ (4): The product was obtained as described for **2**; yield: 48.4 mg (40%); LC-HRMS: $t_R = 10.5$ min, *m*/*z* calcd for $C_{94}H_{142}N_{24}O_{30}S_4+2H$: 1108.96647 [$M+2H^+$]; found: 1108.96739.

Ac-(Gly-Pro-Hyp)$_2$-Gly-Pro-Cys(S*t*Bu)-Gly-Pro-Hyp-Gly-Pro-Cys-(S*t*Bu)-(Gly-Pro-Hyp)$_2$-Gly-Gly-NH$_2$ (5): The peptide was obtained as described for **2**; yield: 28.3 mg (30%); LC-HRMS: $t_R = 10.2$ min, *m*/*z* calcd for $C_{94}H_{142}N_{24}O_{29}S_4+2H$: 1100.47057 [$M+2H^+$]; found: 1100.473060.

Ac-Gly-Cys(S*t*Bu)-Hyp-Gly-Pro-Hyp-Gly-Pro-Cys(S*t*Bu)-(Gly-Pro-Hyp)$_5$-Gly-Gly-NH$_2$ (6): The peptide was synthesized according to the general procedures. Fmoc-Cys(S*t*Bu)-OH was introduced by double couplings with 4 equiv amino-acid derivative/HOBt/HBTU in 0.5-M collidine solution in DMF. The crude product was purified by using preparative HPLC on column I by elution with a linear gradient from 0–90% B in eluent A in 35 min; yield: 68.7 mg (43%); LC-HRMS: $t_R = 9.8$ min, *m*/*z* calcd for $C_{106}H_{159}N_{27}O_{34}S_4+2H$: 1242.02898 [$M+2H^+$]; found: 1242.03303.

Ac-(Gly-Pro-Hyp)$_3$-Gly-Cys(S*t*Bu)-Hyp-Gly-Pro-Hyp-^{15N}Gly-Pro-Cys-(S*t*Bu)-(Gly-Pro-Hyp)$_5$-Gly-Gly-NH$_2$ (7): The peptide was synthesized and purified as described for **2**; yield: 31.0 mg (23%); LC-HRMS: $t_R =$ 10.2 min, *m*/*z* calcd for $C_{142}H_{210}N_{36}O_{46}S_4+2H$: 1095.80945 [$M+2H^+$]; found: 1095.80463.

Azobenzene-bridged peptide 8: Peptide **7** (23.3 mg, 7.1 μmol, 1 equiv) was deprotected with $P(C_4H_9)_3$ (35 μL, 0.14 mmol, 20 equiv) in trifluorethanol/water (95:5, 2 mL) and stirred under argon at RT for 5 h. After dilution with water, the deprotected peptide was lyophilized. It was dissolved in DMF (4.75 mL), and DIPEA (12.2 μL, 71.2 μmol, 10 equiv) in 0.38 mL DMF was added. In all further steps the peptide solution was kept under argon and reagents were added by using a syringe. Azobenzene-4,4′-bis-*N*-(4-chloro-2-butynenyl)carboxamide (4.7 mg, 10.7 μmol, 1.5 equiv) and NaI (48 mg, 321 μmol, 30 equiv) were refluxed for 5 h in acetone (15 mL) under argon. The solvent was removed, the crude bisiodide derivative was dissolved in DMF (2 mL) and added to the solution of the deprotected peptide in DMF to result in a final peptide concentration of 1 mM. The mixture was stirred overnight at RT and the product mixture was purified by semipreparative HPLC on column III by gradient elution (0–90% B in A, in 90 min); yield: 1.2 mg (5%); LC-HRMS: $t_R = 7.2$ min, *m*/*z* calcd for $C_{156}H_{210}N_{40}O_{48}S_2+2H$: 1739.2392 [$M+2H^+$]; found: 1739.23523.

Mass spectrometry: High-resolution mass spectra were recorded by using a micro-TOF-LC mass spectrometer from Bruker Daltonics (Bremen, Germany) equipped with an Agilent HPLC system Series 1100 (Palo Alto, USA). For the HPLC, a C18 Hypersil Gold column (100 × 2.1 mm, 5 μm from Thermo Electron Corporation, Waltham, USA) was used and elution was at a flow rate of 250 μL min^{-1} with a gradient of 95% eluent A (0.05% TFA in water) to 95% eluent B (0.05% TFA in acetonitrile) in 17 min for the peptides, and 80% eluent A to 95% eluent B in 20 min for the azobenzene peptides. All masses are monoisotopic.

Circular dichroism spectroscopy: CD spectra were recorded by using a Jasco spectropolarimeter J-715 (Groß-Umstadt, Germany) with a Peltier element PFD-350S and a thermostated cell compartment by using quartz Suprasil cuvettes 110 QS (Hellma, Müllheim, Germany) of 0.1-cm optical path length, or two quartz Suprasil lantern slides of 0.01-cm layer thickness. Alternatively, a Jasco spectropolarimeter J-810 with a cryostatic temperature regulator and a specific cell compartment for 0.01-cm quartz Suprasil cuvettes 114 QS was used. Data were processed by using the software spectra manager for Windows 95/NT, Version 1.53.00, for the J-715 and Version 1.54.03 for the J-810 polarimeter. Spectra were registered in the 190–250 nm range with a resolution of 0.1 nm, a response of 1 s, and a bandwidth of 1 nm. The average of four scans is reported and expressed in terms of ellipticity per mole of peptide residues (θ_R). The measurements were performed by using 1-mM solutions of peptides in 90% H_2O/10% D_2O and [D_3]MeOH/0.1 M AcOH (4:1), except for compound **8**, which was used at 0.5 mM in [D_3]MeOH/0.1 M AcOH (4:1). Concentrations were determined by weight and all solutions were pre-equilibrated at 4 °C for 12–24 h. Thermal denaturations were monitored in the same solutions by monitoring the change in dichroic intensities at 225 nm as a function of increasing temperature within the range of 4–80 °C in water and 4–60 °C in MeOH/0.1 M AcOH solution. The heating rate was

$0.2\,°C\,min^{-1}$, the resolution 0.1 nm, response 16 s, and the bandwidth 1 nm. At 4, 60, and 80 °C, respectively, spectra were recorded by using the parameters described above to analyze the conformational changes.

Photoisomerization: A xenon lamp 450 XBO from Osram (München, Germany) was used for irradiation at 330 nm with UG11 and BG12 filters and at 420 nm with GG435 and BG12 filters from Itos (Mainz, Germany). The photostationary states were reached after irradiation for about 10 min at the required wavelengths.

NMR spectroscopy: NMR experiments were carried out by using a Bruker DRX 500 spectrometer (Karlsruhe, Germany); the NMR spectra were recorded by using the identical peptide solutions used for CD measurements. TOCSY spectra were recorded with spin-lock periods of 60 ms by using the MLEV-17 sequence for isotropic mixing.[21] 2D NOESY[22] experiments were performed with a mixing time of 100 ms. Temperature-shift coefficients for the amide protons were obtained from 1D and from {^{1}H,^{15}N}-FHSQC[23] spectra recorded at temperatures between 277 and 300 K. Amide hydrogen bonds were assigned when temperature-shift coefficients were less negative than $-4.5\,ppb\,K^{-1}$. Water suppression was achieved by using the WATERGATE sequence.[23] For ^{1}H diffusion measurements, stimulated echo experiments with bipolar gradients and a diffusion time of 100 ms were performed in a pseudo-2D fashion by incrementing the gradient strength from 1 to $43\,G\,cm^{-1}$ in five steps. The gradient strength was calibrated to a diffusion constant of $18\times10^{-10}\,m^2\,s^{-1}$ for H_2O in ^2H_2O at 300 K. Only well-resolved signals were used for extracting diffusion constants from the monoexponential signal decay. Data processing and assignment was performed by using XWINNMR-v3.

Acknowledgements

This study was partly supported by the Deutsche Forschungsgemeinschaft (SFB 563, grant C4 and SFB533, grant A8). The excellent technical assistance of Mrs. E. Weyher-Stingl is gratefully acknowledged.

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Received: August 10, 2006
Published online: January 4, 2007

