Time-Resolved Infrared Spectroscopy of Thiopeptide Isomerization and Hydrogen-Bond Breaking

Valentina Cervetto, Rolf Pfister, and Jan Helbing*

Physikalisch-Chemisches Institut, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

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The photoisomerization of the protected tetrathioxopeptide Boc-Ala-Gly(═S)-Ala-Aib-OMe was followed using time-resolved infrared spectroscopy in the amide I region in combination with isotope labeling. In acetonitrile at room temperature, approximately half of the molecules are found in a loop conformation, restrained by an intramolecular hydrogen bond, while the other half adopts more extended conformations. UV-excitation of the thioxopeptide unit immediately weakens the intramolecular hydrogen bond. After the molecules have relaxed to the electronic ground state with a 130 ps time-constant, a delayed re-formation of the intramolecular hydrogen bond is observed for molecules returning to the initial *trans* conformation of the thioamide bond, while the loop structure is permanently broken when the molecules isomerize to the *cis* conformation.

I. Introduction

The investigation of the dynamic aspects of peptide folding can help us understand how functional secondary and tertiary structures of proteins arise from primary sequences. In this respect, experimental results on fast conformational changes in peptides can, for example, provide test cases for computer models, which are in some cases already capable of predicting static protein structure.[1,2]

Most of the dynamic information on fast peptide folding so far has been inferred from thermal unfolding experiments, in which the reaction is initiated by a laser-induced temperature jump (T-jump).[3−5] In principle, the time resolution of T-jump experiments is only limited by the equilibration of thermal energy in the solvent (∼10 ps),[6] however, for technical reasons, T-jumps are usually achieved by nanosecond-lasers, limiting the time resolution to the 10 ns time scale.[6−8] Picosecond time resolution can be reached using ultrashort light pulses in combination with a photoswitchable element in the peptide. Conformational changes may then be followed by UV/visible or infrared spectroscopy. To this end, different approaches have been taken. Using azobenzene as a cross-linker, Woolley and co-workers have connected two cysteine side chains of a polypeptide.[9] This method proved particularly well suited for studying the dynamics of α-helix folding and unfolding.[10,11] Photomodulation of the structure of cyclic peptides has been achieved by incorporating an azobenzene-based ω-amino acid directly into the peptide backbone. [12−15] A similar approach[16] has very recently been used to study phototriggered hairpin folding and unfolding.[17] UV light can also cleave an intramolecular disulfide bridge that links together the ends of a polypeptide chain,[18−20] avoiding the incorporation of an artificial chromophore.

In order to trigger conformational dynamics in native peptides, it would, in principle, be possible to photoisomerize the CONH units of the peptide backbone itself by exciting the $\pi-\pi^*$ transition near 200 nm.[21] Trans−cis isomerizations about the peptide bond at a proline (in the electronic ground state) is well-known to occur in proteins and can, for example, trigger the opening of the pore of an ion channel.[22] Apart from creating photodamage, far UV-excitation of a polypeptide would not, however, be unit selective and yield a random distribution of isomers at different sites. Replacing one oxygen atom of a backbone carbonyl group with sulfur, on the other hand, can single out one peptide unit by creating a thioamide bond, which can be selectively excited and isomerized by light near 260 nm.[23−26]

Our group has previously investigated[27,28] the photo isomerization mechanism of the thioamide bond by means of time-resolved infrared spectroscopy of *N*-methylthioacetamide (NMTAA), with support from *ab initio* calculations by de Vico and Olivucci.[27] At the same time, Satzger at al. studied a series of peptides, where one peptide bond has been replaced by its thioxo congener, using ultrafast spectroscopy in the visible and in the near UV.[26] It could be shown that the excited molecules remain trapped in a low-lying electronically excited state, without any significant barrier nor driving force along the isomerization coordinate (torsion of the thioamide bond). Only the decay of the excited-state after a few hundred picoseconds leads to the formation of the *cis* form of the thioamide bond or the return to the initial *trans* conformation.

In this paper we study the isomerization dynamics of a small thioxopeptide by means of UV-pump−IR-probe spectroscopy, which is directly sensitive to backbone conformational changes,[14] using isotope labeling in order to resolve dynamics of individual vibrational bands. The thioxopeptide we have chosen, Boc-Ala-Gly(═S)-Ala-Aib-OMe, in acetonitrile solution at room temperature, can adopt two types of conformations (both with a *trans*-thioamide bond), which give rise to distinct IR spectra:[29] a loop structure, in which the amide proton of Aib forms a $i-i+4$ hydrogen bond with the C═O carbonyl of the urethane group (see Figure 1) in coexistence with more extended conformations. In equilibrium, the interchange between these two types of structures takes place on a time scale that is slower than the picosecond vibrational lifetime of the backbone C═O stretch mode. The presence of only one set of bands in the NMR

* Corresponding author. E-mail: j.helbing@pci.unizh.ch.

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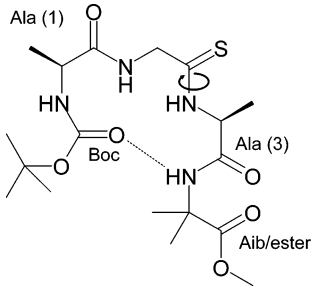

Figure 1. Structure of Boc-Ala-Gly(═S)-Ala-Aib-OMe in acetonitrile. At room temperature, approximately half of the molecules adopt a loop conformation stabilized by an intramolecular hydrogen bond (dashed line). The circular arrow indicates the thioamide bond, which can photoisomerize from *trans* to *cis* after absorption of a UV photon.

spectra, on the other hand, indicated that loop opening in the electronic ground state is faster than hundreds of microseconds.[29] Here we investigate the isomerization of the thioamide bond after excitation to the $\pi-\pi^*$ (S_2) electronic excited state, which phototriggers the breaking of the hydrogen bond. The molecule is used as a model to investigate how the isomerization of a thiopeptide unit can induce structural changes in peptides in the presence of a conformational constraint similar to those found in secondary structure motifs.

II. Materials and Methods

Sample. The oxopeptides Boc-Ala-Gly-Ala-Aib-OMe, Boc-Ala*-Gly-Ala-Aib-OMe, Boc-Ala-Gly-Ala*-Aib-OMe (the asterisk stands for $^{13}C{=}O$ labeled amino acids) were synthesized by stepwise elongation and fragment condensation. Subsequent site-selective thioxylation using Lawesson's reagent yielded the thioxopeptide Boc-Ala-Gly(═S)-Ala-Aib-OMe, as described in detail in ref.[29] The thioxopeptide was dissolved in CD_3CN at a concentrations 40−60 mM. The sample was circulated in a flow cell consisting of 2-mm thick CaF_2 windows separated by a 50 μm spacer at a rate sufficient to ensure the complete exchange of excited sample volume between subsequent excitation pulses.[30]

Time-Resolved Spectroscopy. Femtosecond pulses at 800 nm (1 kHz, 600−700 μJ/pulse, 80−100 fs) were obtained from an amplified titanium−sapphire laser system (Spectra Physics). These were used to generate mid-infrared pulses (100−150 fs, 1.8 μJ/pulse, 200−250 cm^{-1} full width at half-maximum (fwhm)) in a home-built double-stage optical parametric amplifier (OPA) followed by frequency mixing in a $AgGaS_2$ crystal.[31] The OPA output was split into two beams. One part (the probe pulses), was focused in spacial overlap with the UV pump beam in the sample cell. The second part was used as a reference beam to correct for intensity fluctuations and crossed the flow cell approximately 500 μm further upstream. The IR probe and the reference beams were dispersed in a spectrometer and detected with a double mercury cadmium telluride (MCT) array (2 × 32 pixels) on a single shot basis with 4 cm^{-1} resolution. The UV pump light at 266 nm was generated by frequency tripling of the 800 nm IR light in two β-barium borate (BBO) crystals. The third harmonic beam was isolated by dielectric mirrors, and the UV pulses were stretched to 700 fs duration by guiding them through 10 cm of fused silica. The UV pulse energy measured at the sample position was $\simeq$ 1 μJ/pulse, with a focal spot size of ∼120 μm (fwhm).

UV-pump−IR-probe spectra were recorded with the UV-pump light polarized parallel and perpendicular to the IR-probe pulses. From these two signals, recorded quasi-simultaneously by rotating a $\lambda/2$ plate every 300 laser shots, magic angle signals were calculated. The signal anisotropy decays with an ∼47 ps time constant (data not shown), similar to other peptides of comparable size and shape in acetonitrile.[20]

III. Results and Discussion

The amide I (C═O stretch) region of the Fourier transform infrared (FTIR) spectra of Boc-Ala-Gly(═S)-Ala-Aib-OMe, and the two isotope labeled molecules Boc-Ala*-Gly(═S)-Ala-Aib-OMe and Boc-Ala-Gly(═S)-Ala*-Aib-OMe (the asterisk stands for $^{13}C{=}O$ labeled amino acids) are displayed in the first line of Figure 2. As discussed in ref 29, at room temperature approximately half of the molecules are in a looped conformation with a strong hydrogen bond between the C═O of the urethane group (Boc) and the NH of the Aib residue. The remaining molecules adopt more extended conformations without this intramolecular hydrogen bond. For the molecule in the loop conformation, the urethane C═O stretch band is strongly red-shifted and is centered near 1688 cm^{-1}, while the non-hydrogen-bonded Boc absorption is found at 1713 cm^{-1}. This significant red-shift of the hydrogen-bonded C═O band is a signature of a very tight H-bond, which also influences the C═O stretch frequency of the donating peptide unit.[32] Indeed, the isotope-labeled Ala(3) residue gives rise to two bands at 1634 cm^{-1} and ∼1650 cm^{-1} (hydrogen-bonded and non). The amide I band of the isotope-labeled Ala(1) residue also has its maximum at 1634 cm^{-1}. This band, too, is strongly asymmetric, most likely as a result of different degrees of hydrogen bonding to the solvent. In the unlabeled molecule, the Ala(3) bands therefore overlap with the Ala(1) band and, partially, with the H-bonded Boc band. The band at 1740 cm^{-1} is due to the C═O stretch vibration of the Aib/ester unit.

The dynamics of the labeled and unlabeled thioxopeptide induced by 266 nm excitation was monitored by transient IR spectroscopy in the spectral range of 1600−1760 cm^{-1}. Figure 2a−c shows the UV-pump−IR-probe spectra for short delay times; spectra at longer delays are enlarged by a factor of 1.5 in Figure 2d−f. Immediately after UV excitation, we observe a red shift of the two alanine amide I bands as well as of the Aib/OMe ester band. This shift can be attributed to excess laser energy, which leads to the excitation of low-frequency vibrations that anharmonically couple to the C═O stretch modes.[33] The Aib/OMe ($\cong$ 1740 cm^{-1}) red shift subsequently diminishes with a time constant of approximately 10 ps, reflecting the dissipation of energy to the solvent (magenta diamonds in Figure 3). However, the red shift of the Ala(1) band persists also after the first 10 ps (Figure 2b), thus it must have a different origin. Previous work on the photo isomerization of molecules containing the thioamide bond[26,27] have shown that, after $\pi-\pi^*$ excitation, the thioxopeptides first relax to the lowest-lying electronically excited-state where they remain trapped for a few hundred picoseconds. *Ab initio* calculations indicate that the molecule is free to rotate about the thioamide bond in this excited state, and only relaxation to the electric ground state stabilizes the molecule either in the *trans* or in the *cis* conformation.[27] The persisting red-shift of the C═O stretch vibration of Ala(1), which is a nearest neighbor of the thioamide bond, is thus a response to the change of the electronic state and dipole moment, and this signal only decays with a 130 ps time-constant (red squares in Figure 3). This time-constant is assigned to the recovery of the electronic ground state. On the other hand, the Aib/ester group, which is two peptide units away from the thio-switch is hardly affected by the change in electronic configuration, and the ester C═O band is primarily sensitive to changes in temperature. Both heat-induced anhar-

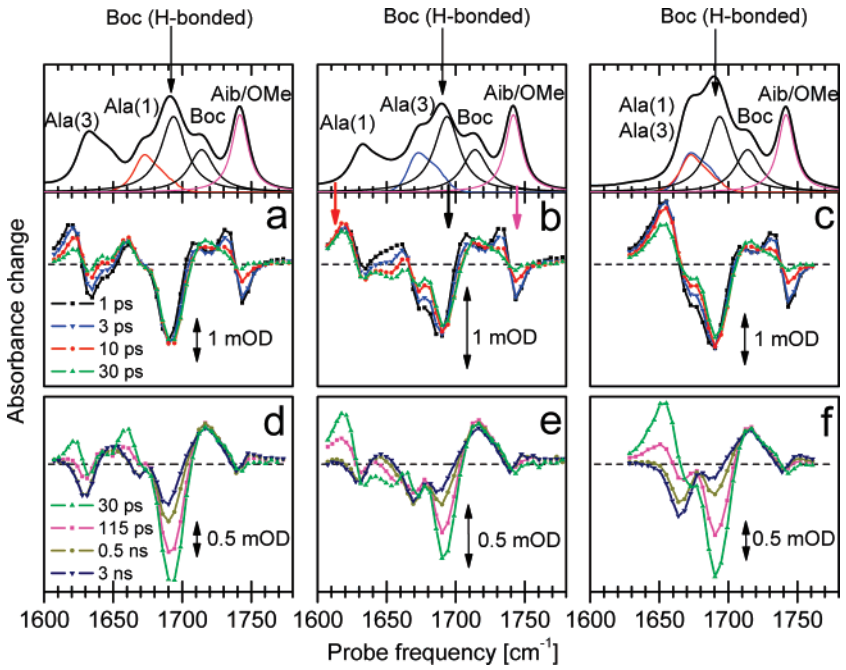

Figure 2. UV-pump−IR-probe spectra at different delay times after 266 nm excitation of Boc-Ala-Gly(═S)-Ala*-Aib-OMe (left), Boc-Ala-Gly-(═S)-Ala*-Aib-OMe (center) and Boc-Ala-Gly(═S)-Ala-Aib-OMe (right). FTIR absorption spectra are shown on top. All spectra are scaled to yield 1 ps Aib/OMe signals (near 1740 cm^{-1}) of equal magnitude. The lines underneath the FTIR spectra in the top panels indicate the approximate spectral shape of the individual C═O bands. The Ala bands are taken from the ^{13}C═O spectra, corrected for the isotope shift of 41 cm^{-1}. The Lorentzian lines for the two Boc bands are the result of a fit after subtraction of the Ala bands from the full FTIR spectra.

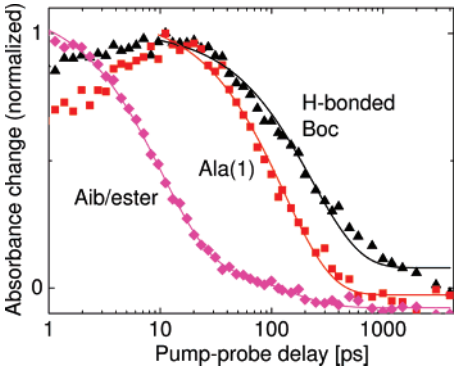

Figure 3. Normalized transient absorption signals at the spectral positions indicated by the down arrows in Figure 2b. Magenta diamonds: Aib/ester; red squares: Ala(1); black solid triangles: hydrogen-bonded Boc. Solid lines represent monoexponential fits with time constants of 130 ps (Ala(1)), 230 ps (H-bonded Boc), and a biexponential fit with a 10 ps plus a fixed 130 ps time constant (Aib/ester).

monic shifts and the change of electronic state affect the C═O stretch vibration of Ala(3), and this signal changes both on the 10 ps and on the 130 ps time scale.

The most prominent signal in Figure 2 is that of the Boc carbonyl of the molecules which are initially in the hydrogen-bonded loop conformation. Immediately after UV excitation this band looses intensity (bleach at 1688 cm^{-1}) while a positive, but much weaker absorption near 1713 cm^{-1} is induced.[37] This could be interpreted as an instantaneous breaking of the hydrogen bond between the Boc group and the amide proton of Aib due to the opening of the initial loop structure. For example, it has been shown that *cis* → *trans* isomerization of an azobenzene-based photoswitch within a cyclic peptide can trigger ultrafast (picoseconds) conformational changes due to a very strong driving force.[14] However, contrary to azobenene, which undergoes an ∼5 Å change in end-to-end distance upon photoisomerization within a few hundred femtoseconds, there is only little directed structural rearrangement of the thio-substituted peptide unit upon photoexcitation, according to the *ab initio* calculations for NMTAA. Only the C−S and C−N bonds elongate, and the carbon and nitrogen centers pyramidalize during relaxation from S_2 to the lower-lying S_1 or triplet states, while the distance between the two α carbons remains almost unchanged.[27] Theory thus suggests that loop opening in the photoexcited state must take place diffusively, aided by the single-bond character of the thioamide bond in the lowest-lying electronically excited state.

It is nevertheless possible to reconcile this prediction with our observation of the immediate bleaching of the hydrogen bond signal if we consider that the transition energy and oscillator strength of an amide I vibration is a highly nonlinear function of intramolecular hydrogen bond distance, especially in the present case of a strong hydrogen bond. Models used in molecular dynamics (MD) simulations[34] as well as *ab initio* calculations on *N*-methylacetamide dimers[32] show that H-bond elongation by less than 0.5 *Å* can already reduce the initial 25 cm^{-1} red-shift of the stretch vibration of the accepting Boc C═O group by more than 10 cm^{-1}. Even without the opening of the peptide's loop structure, such a small effective elongation of the hydrogen bond distance is conceivable as a result of backbone fluctuations induced by the excess laser energy, as well as small conformational changes in response to the altered thioamide bond.

While the induced absorption at 1713 cm^{-1} slightly grows up to 30 ps after UV-excitation, the bleach signal at 1688 cm^{-1} stays almost constant during the time the molecules are in an electronically excited state. This indicates that the intramolecular hydrogen bond remains weakened even after the dissipation of the initial excess energy in the molecule to the solvent. On a longer time scale, rotation about the thioamide bond[27] may indeed lead to the diffusive opening of the loop structure and completely disrupt the hydrogen bond. This would be consistent with recent findings of transient 2D-IR spectroscopy in com-

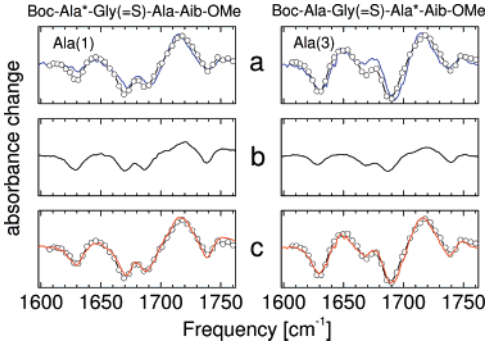

Figure 4. Contribution of the rise in solvent temperature to the pump−probe data: (a) UV-pump−IR-probe spectrum at 3 ns delay (open circles) and the difference between FTIR spectra during and after irradiation at 248 nm (solid blue line). (b) Difference between the FTIR spectra taken at 25 °C and 24 °C. (c) UV-pump−IR-probe spectrum at 3 ps delay (open circles) and sum of the FTIR difference spectra shown in a and b (solid red line). The contribution of solvent heating to the pump−probe signal can be seen from the relative size of the FTIR spectra (solid lines) in a and b. Left column: Boc-Ala-Gly(═S)-Ala*-Aib-OMe; right column: Boc-Ala-Gly(═S)-Ala*-Aib-OMe.

bination with MD simulations that a hydrogen-bonded β-turn motif in a peptide of comparable size opens diffusively on a 100 ps time scale in the same solvent.[35]

With the decay of the electronic excited-state, the original hydrogen bond signal partially recovers. However, while changes in the Ala(1) amide I signal are only very small after 500 ps, the Boc signal seems to follow a somewhat slower kinetics and continues to change even after this time. Between 0.5 and 3 ns the bleach of the hydrogen-bonded band clearly diminishes, and the positive signal attributed to non-hydrogen-bonded Boc slightly decreases. A single-exponential fit to the Boc signal at 1694 cm^{-1} in Figure 3 (solid triangles) yields a time-constant of 230 ps, compared to only 130 ps for the isotope-labeled Ala(1) band. In addition, the single-exponential fit only poorly reproduces the Boc data for delays longer than 500 ps, which seems to reflect even slower kinetics. This could hint at a delayed re-formation of the original intramolecular hydrogen bond in the electronic ground state, most likely in molecules that have returned to the *trans* conformation of the thioamide bond. Note, however, that no corresponding intensity redistribution is seen between the two Ala(3) bands. Rather, both isotope-labeled samples show an increase in absorption near 1650 cm^{-1} and a decrease at 1630 cm^{-1} and 1670 cm^{-1} on the very long time scale. This signal may, however, be dominated by molecules sensing a rise in solvent temperature (see below).

After 3 ns, the photoreaction is completely finished, and the transient spectra, characterized by a blue shift of all bands in the amide I region, no longer change. The strongest stationary signals are due to Boc and Ala(3), with a loss in intensity of the bands corresponding to the hydrogen-bonded conformation and an increase in intensity of the bands belonging to the more extended structures. This indicates that the hydrogen bond giving rise to the lower frequency bands in the looped *trans* species does not exist in the *cis* species. Yet, as shown in Figure 4a, the pump−probe data does not fully coincide with the FTIR difference spectra recorded under steady-state irradiation. Indeed, we found that the thioxopeptide absorption spectra are very sensitive to changes in temperature (Figure 4d). In the time-resolved experiments, the temperature is estimated to increase by approximately 0.5 K in the probed sample volume after dissipation of the pump−pulse energy, while the temperature change is negligible during irradiation in the FTIR spectrometer. The pump−probe spectra at long delays can be well reproduced by combining the FTIR difference spectra due to irradiation and temperature change (Figure 4a and b), revealing that the long-time pump−probe signal of Ala(1) arises primarily from the rise in solvent temperature, while the Boc and Ala(3) signals are mainly due to the conformational change induced by photoisomerization. In total, approximately 20% of the long delay transient signal is caused thermally and may include contributions from molecules that were not directly excited.

Quantum Yield. For the photoisomerization of the thioamide bond of NMTAA, we could, in previous work, directly determine the isomerization quantum yield (∼40% in the *trans* → *cis* direction) from the transient IR data, because photoexcitation lead to a complete bleach of the CNC stretch vibrations of the thioamide bond, and *cis* and *trans* bands are well separated.[27,28] In the larger thioxopeptide studied here, the stretch vibrations of the thioamide bond are not resolved, and the amide I bands only act as spectator modes, undergoing relatively small spectral shifts as a function of temperature, electronic excitation, and isomerization. A large spectral shift exists, however, for the Boc absorption band between the thioxopeptides with and without an intramolecular hydrogen bond. The absorption increase at 1713 cm^{-1} at the end of the photoreaction in Figure 2 amounts to approximately 0.5% ($\Delta A \approx 0.5$ mOD for an absorption of non-hydrogen-bonded Boc carbonyls before excitation of ∼100 mOD). Estimating from laser power and spot size that only approximately 1.5−3% of all molecules in the probed sample volume have absorbed a UV photon, we arrive at a quantum efficiency for the breaking of the intramolecular hydrogen bond of 15−30%. Similar quantum efficiencies have been reported for the *trans* → *cis* isomerization of other small secondary thioxopeptides by monitoring the formation of the photostationary state under continuous irradiation.[25] (Thermally activated back-relaxation to the *trans*-state within seconds makes such measurements very unreliable for the peptide studied here.) On a short time scale, the equilibrium between more extended and hydrogen-bonded conformations may also have shifted in favor of the former for molecules returning to the initial *trans* conformation of the thioamide bond. We have, however, extended pump−probe scans up to microsecond delays and found no signal changes beyond the times shown in Figure 2.

IV. Summary and Conclusions

We have shown that the *trans* → *cis* photoisomerization of the thioamide bond in the protected thioxopeptide Boc-Ala-Gly-(═S)-Ala-Aib-OMe is an efficient process, and causes the opening of a loop conformation, stabilized by an intramolecular hydrogen bond. Loop opening appears to take place while the molecule is in an electronically excited-state for 130 ps, despite the fact that *ab initio* calculations suggest that there is no substantial driving force along the isomerization coordinate.

The opening of the loop structure is indicated by the permanent bleaching of the C═O stretch band of Boc that is strongly red-shifted because of the intramolecular hydrogen bond. However, the spectral shift (and gain in oscillator strength) induced by this hydrogen bond is a highly nonlinear function of CO−H distance and orientation. The transient IR spectra can thus not distinguish between an immediate H-bond weakening after photoexcitation, most likely due to thermal fluctuations and small conformational rearrangement, and larger scale conformational change on longer time scales. Still, our observation of a delayed recovery of the hydrogen-bond signal after the decay to the electronic ground state indicates that even

molecules returning to the initial *trans* conformation of the thioamide bond have undergone conformational change in the excited state. This finding also shows that conformational dynamics in thioxopeptides can be observed on a 100 ps time scale despite the relatively slow completion of the isomerization reaction by the photoswitchable element.

Analyzing the response of individual peptide units to the excitation and isomerization of the thioamide bond, we found that the C═O stretch vibrations of adjacent amino acids respond strongly (red shift) to the electronic excitation of the thioxopeptide unit. On the other hand, the ester terminal group is hardly affected, and mainly shows a short-lived spectral shift due to excess energy in the molecule after UV-excitation. Backbone carbonyl groups in larger thioxopeptides that are two or more peptide units away from the thioamide bond thus promise to be much more sensitive probes of photoinduced conformational changes.

While the particular loop-structure of the tetrapeptide we have studied is favored by our use of acetonitrile, and time constants and quantum efficiencies are expected to be slightly different in other solvents, we believe that the main mechanistic conclusions to be drawn from our results are solvent-independent and of relevance also under aqueous conditions. Most importantly, the successful photoisomerization of a thioxopeptide that is stabilized by a hydrogen bond, and even more so, as was recently shown by Wiedemann et al., the photoswitching of enzymatic activity for a site-specific modified version of ribonuclease S obtained by thioxylation at a single peptide bond,[36] suggests that the photoswitching of peptide conformations does not necessarily require a strong driving force as exerted, for example, by azobenzene-based photoswitches. Rather, essentially one new degree of freedom, here the torsional motion about the thioamide bond, seems to modify the peptide's highly balanced free energy surface sufficiently to favor the population of a new region of conformational space on the time scale of 100 ps. The peptide studied here is sufficiently small to respond fully within this short time, but conformational changes in larger molecules capable of changing enzymatic activity can only take place much more slowly. In this case, it appears likely that local changes as observed for our model peptide occur in the vicinity of the thio-substituted peptide unit within the lifetime of the photoexcited state, permitting isomerization and the formation of the *cis*-thioamide bond. The metastable *cis* conformation in the electronic ground state is then sufficiently long-lived (seconds to minutes) to allow for the relaxation of the whole peptide to a new equilibrium structure, which may take hundreds of nanoseconds or longer. This type of photoinduced conformational change seems to mimic as closely as possible the thermally activated processes taking place in natural peptides and proteins.

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(37) The Boc absorption of the molecules without the intramolecular hydrogen bond is slightly red-shifted upon photoexcitation, and essentially behaves like the ester band. However, this signal is very small, and the dynamics of the 1713 cm^{-1} band is better resolved in transient 2D-IR spectra, which will be reported elsewhere.