Transient 2D-IR Spectroscopy of Thiopeptide Isomerization

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Transient 2D-IR spectra have been recorded during the photoreaction of the thioxopeptide Boc-Ala-Gly(═S)-Ala-Aib-OMe. We demonstrate the potential of transient 2D-IR spectroscopy to resolve vibrational bands hidden in conventional pump−probe spectra. Different types of transient cross-peak signals are observed, and their information content is discussed by comparison with model spectra.

I. Introduction

Two-dimensional IR spectroscopy (2D-IR) can yield a wealth of information on a molecular system that is not accessible by linear spectroscopy.[1–5] A wide range of applications include water dynamics,[6–9] interfaces,[10] the structure of molecular devices,[11] base-pair interactions,[12] and secondary structure identification in peptides and proteins.[13–19] In peptides, 2D-IR spectroscopy can measure the couplings and relative orientation of amide I transition dipole moments, information that can be used to determine the dihedral angles between two neighboring peptide units and therefore the conformational state of the peptide backbone[20–28] The high time resolution of the experiment of 1 ps gives to 2D-IR spectroscopy the capability to reveal subpicosecond fluctuations of peptide equilibrium structures.[29,30] Ultrafast *transient* two-dimensional infrared spectroscopy (T-2D-IR) is the extension of the 2D-IR technique to observe nonequilibrium systems.[31–37] In this method, an additional pump pulse is added to the 2D-IR pulse sequence. A UV/vis pump−pulse can trigger a photoreaction, which is then probed by taking snapshot 2D-IR spectra. Alternatively, an IR-pump pulse may be used to induce a temperature jump in the solvent.[36,35] When the T-2D-IR technique was first applied to the light-triggered conformational transition of a cyclic octapeptide,[31] it was shown that, in contrast to pump−probe spectroscopy, the diagonal peaks in the transient 2D-IR spectra can be used to distinguish between homogeneous and inhomogeneous broadening in the absorption spectrum of the photoproducts. The homogeneous width of the amide I absorption band was found to diminish as the peptide adjusts to its new equilibrium structure, which was interpreted as a continuous reduction of the molecules' flexibility. However, it is the cross peaks in the equilibrium 2D-IR spectra of peptides that contain the most important information on molecular geometry,[25] because their intensity and polarization dependence can provide a quantitative measure for spacial vicinity and relative orientation of different peptide units.[38] In the same way, in T-2D-IR spectroscopy, one should be able to obtain information on conformational changes by detecting transient cross peaks and their changes in time. Transient cross peaks were first observed in a charge transfer reaction of a metal carbonyl complex, which was also studied as a model system to explore the polarization dependence of transient 2D-IR spectroscopy.[32] The observation of a transient cross peak after photocleavage of a disulfide bridge in a small model peptide has recently provided real-time structural evidence for the change of molecular geometry during the opening of a β-turn motif.[33] Still, the full information content of transient 2D-IR spectra and their relationship to peptide conformational dynamics is far from being explored in detail. For this reason, we are currently applying T-2D-IR to photoswitchable model peptides, which show well-resolved vibrational spectra and conformationally and spectrally distinct initial and final states. Here, we report our measurements on the protected tetrathioxopeptide Boc-Ala-Gly(═S)-Ala-Aib-OMe.

In a previous paper, we characterized the dynamics of photoisomerization of this thioxopeptide by UV-pump−IR-probe spectroscopy using isotope labeling to resolve individual vibrational bands.[39] The main effect of the photoisomerization of the thioamide bond is the opening of a loop conformation, stabilized by an intramolecular hydrogen bond.[40] This loop opening gives rise to a large blue-shift of the stretch vibration of the initially hydrogen-bonded carbonyl (Boc), providing spectrally distinct initial and final states. In addition, during the first 130 ps, while the thioxopeptide unit is electronically excited, the cabonyl stretch modes of its nearest neighbors Ala(1) and Ala(3) are red-shifted. An additional red shift due to excess energy in the molecule after UV excitation is also observed, which decays upon cooling on a 10 ps time scale.

Building on this information, we discuss here the transient 2D-IR spectra recorded while the molecules are in the electronically excited state and at the end of the photoreaction. With the help of simplified model spectra, we address possible origins and line shapes of transient diagonal as well as transient cross peak signals. We demonstrate that UV pump−IR probe and transient 2D-IR measurements can yield complementary information on peptide dynamics.

II. Materials and Methods

Peptide synthesis, sample handling, and the laser system are described in detail in refs 39 and 40. For the T-2D-IR measurements, the thioxopeptide was dissolved in CD_3CN at concentrations of 120 mM. The sample (1 mL) was exchanged after every 2−3 h of data acquisition to avoid accumulation of photodamaged sample.[47]

For the 2D-IR measurements, the spectrally broad, 100 fs pulses generated by a midinfrared OPA are split into three parts: an IR-pump beam with ~90% of the intensity and weak probe and reference beams. After passing the sample, the probe and reference beams are dispersed in a spectrometer and detected with a double MCT array (2 × 32 pixels) on a single-shot basis with 4 cm^{-1} resolution. The IR-pump beam passes a computer-

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controlled Fabry−Perot interferometer, generating spectrally tunable pulses of ≈10 cm^{-1} bandwidth and single-exponential time profile. By scanning the central wavelength of the pulses across the amide I region and monitoring the induced absorption changes with the broadband IR-probe pulse at 1 ps delay, 2D-IR spectra were recorded in the frequency domain.[41]

Transient 2D-IR spectroscopy can be regarded as a UV-pump−2D-IR-probe measurement. A UV laser pulse triggers the photo reaction. Subsequently, 2D-IR spectra are recorded for the UV-excited sample at a given delay. Quasi simultaneously, we also record 2D-IR spectra with the UV pulse blocked (chopper at 1/4 of the 1 kHz repetition rate of the laser). The transient 2D-IR spectrum is given by the difference between the 2D-IR spectrum recorded with the UV pulse minus the spectrum recorded without the UV pulse. Like in conventional pump−probe spectroscopy, this eliminates contributions of molecules that have not absorbed a UV photon.[31] The T-2D-IR spectra therefore show the changes in the 2D-IR spectrum induced by the photo excitation.

Unless stated otherwise, in the transient 2D-IR measurements, the UV pump pulse polarization was set at magic angle with respect to the IR pulses, both of which had the same polarization. Setting the UV pump polarization at magic angle with respect to the two IR pulses, the diagonal signals in the transient 2D-IR spectra are not affected by rotational diffusion.[32] The UV pulses (266 nm, generated by frequency tripling) had an energy of ≃4 μ J/pulse with a duration of 700 fs after passing a 10 cm fused silica rod. The UV spot size on the sample was ≈120 μ m (fwhm).

III. Transient 2D-IR Model Spectra

Because the transient 2D-IR spectra are double-difference spectra with positive and negative contributions and vibrational transitions of peptides in solution are subject to both homogeneous and inhomogeneous line-broadening, the shapes of transient 2D-IR signals can often be very complicated. Therefore, to facilitate the discussion of the experimental data, we first introduce idealized model spectra considering only two spectrally resolved vibrational transitions; for example, due to two carbonyl bonds. The spectra contain a diagonal peak for each oscillator and cross peaks, which are due to frequency changes of one oscillator as a result of the excitation of the other oscillator to which it is coupled. In peptides, coupling between C═O oscillators is strongest when they are close in space and point into a similar direction (transition-dipole coupling) with additional through-bond contributions for nearest neighbor peptide units. Using the localized vibrations of individual C═O groups as a basis, the spectroscopy of the system up to third order can be described by the exciton Hamiltonian:[42,41]

$$\begin{pmatrix} 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & \epsilon_1 & \beta & 0 & 0 & 0 \\ 0 & \beta & \epsilon_2 & 0 & 0 & 0 \\ 0 & 0 & 0 & 2\epsilon_1 - \Delta & 0 & \sqrt{\beta} \\ 0 & 0 & 0 & 0 & 2\epsilon_2 - \Delta & \sqrt{\beta} \\ 0 & 0 & 0 & \sqrt{\beta} & \sqrt{\beta} & \epsilon_1 + \epsilon_2 \end{pmatrix} \quad (1)$$

Here, ϵ_1 and ϵ_2 are the intrinsic transition energies of the individual oscillators, β is the coupling between the oscillators, and Δ is the diagonal anharmonicity, that is, the difference between the $0 \rightarrow 1$ and $1 \rightarrow 2$ transition frequencies of each vibrational mode. By changing some of these parameters, we have calculated model transient 2D-IR spectra for four different cases using a Bloch approximation for the correlation function[41] with 10 cm^{-1} inhomogeneous width and 20 cm^{-1} homogeneous width. We fixed $\Delta = 16$ cm^{-1} and assume that the two transition dipole moments are of equal strength (only in case 4 does the oscillator strength of one transition increase after photoexcitation). Because we do not want band intensities in the linear absorption spectrum to be affected by changes in coupling and spectral separation, which would unnecessarily complicate our model spectra, we assume perpendicular dipole moments. Orientation effects must, however, be kept in mind when analyzing experimental spectra in detail. The other parameters are varied as follows:

TABLE 1: Parameters for the Model Spectra Shown in Figure 1

initial state			final state			
			case 1	case 2	case 3	case 4[a]
ϵ_1 (cm^{-1})	1672	$\Delta\epsilon_1$	0	+1.5	−5	0
ϵ_2 (cm^{-1})	1741	$\Delta\epsilon_2$	0	−1.5	−5	0
β (cm^{-1})	10	$\Delta\beta$	−1	0	0	0

[a] Larger transition dipole moment for higher energy transition.

Case 1: Change of Coupling. In the ideal case 1 (see Figure 1, top) the two anharmonic oscillators are coupled in equilibrium, and photoexcitation switches off the coupling in the final state. Such a change may, for example, be caused by the separation of initially close peptide units, which may have been singled out by isotope labeling. Naturally, the off-diagonal peaks in the transient 2D-IR spectrum have the same shape as the cross peaks in the initial state but inverted sign. In addition, the absence of coupling in the final state results in a slight blue shift of the band at lower energy and a red shift of the band at higher energy. The red shift yields a transient 2D-IR diagonal peak having positive−negative−positive shape (red−blue−red), whereas the blue shift yields a T-2D-IR diagonal peak with a negative−positive−negative shape (blue−red−blue). The intensity of diagonal and off-diagonal signals in the T-2D-IR spectra is very similar.

Case 2: Blue and Red Shift of Two Coupled Bands. In case 2, the two anharmonic oscillators remain coupled in the final state but undergo the same small frequency shifts as in case 1. This time, however, the frequency shift originates directly from a small change in the intrinsic transition energies ϵ_1 and ϵ_2. Such changes of the diagonal energies can easily arise from small conformational changes, charge redistribution, or solvation effects, which need not affect the coupling. As a consequence of the red and blue shifts of the absorption bands, the pump−probe spectrum and the diagonal peaks in the T-2D-IR spectrum are very similar to the previous case, whereas the cross peaks are clearly different. Their shape is now similar to the shape of the T-2D-IR diagonal signals (a stronger central peak between two weaker peaks of opposite sign), and their intensity is very small compared to them.

Case 3: Red Shift of Two Coupled Bands. In case 3, the photoexcitation red-shifts both absorption bands, again without changing the coupling. As in case 2, the cross peaks in the T-2D-IR signal are therefore only due to a frequency shift, they have the positive−negative−positive shape of the T-2D-IR diagonal signals, and they are relatively weak.

Case 4: Change of Band Intensity. In case 4, the photoexcitation is assumed to increase the intensity of the absorption band at higher energy while the coupling between the two

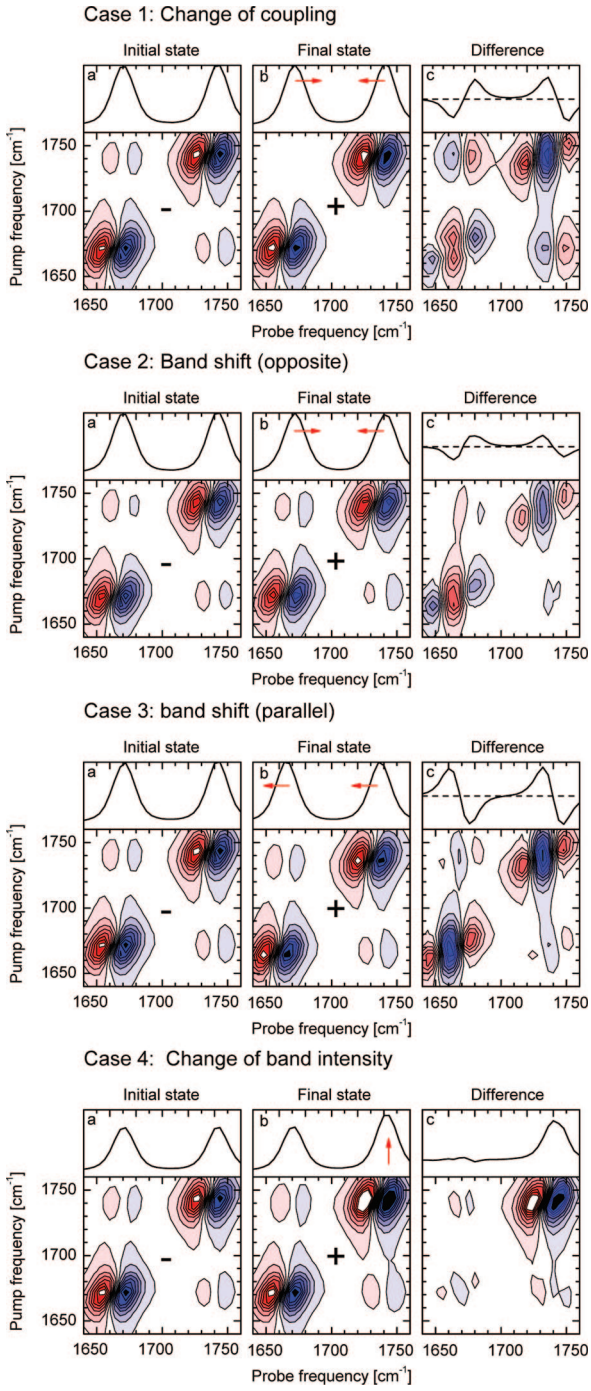

Figure 1. Model 2D-IR and transient 2D-IR spectra for four different sets of parameters (see Table 1 and text for details). (a) Absorption and 2D-IR spectrum of the initial state (two coupled oscillators). (b) Absorption and 2D-IR spectrum of the final state. Red arrows indicate spectral changes with respect to the initial state. (c) Pump−probe and transient 2D-IR spectrum given by the difference of the spectra shown in a and b.

oscillators is kept constant. As a result, diagonal and cross peaks in the photoproduct 2D-IR spectrum are stronger, yielding T-2D-IR cross peak signals with positive−negative shape.

IV. Experimental Results and Discussion

A. Equilibrium Spectra. The FTIR absorption spectrum of the protected thioxopeptide Boc-Ala-Gly(═S)-Ala-Aib-OMe contains four resolved bands (see Figure 2). As shown with the help of isotope labeling in previous work,[40] the two alanine

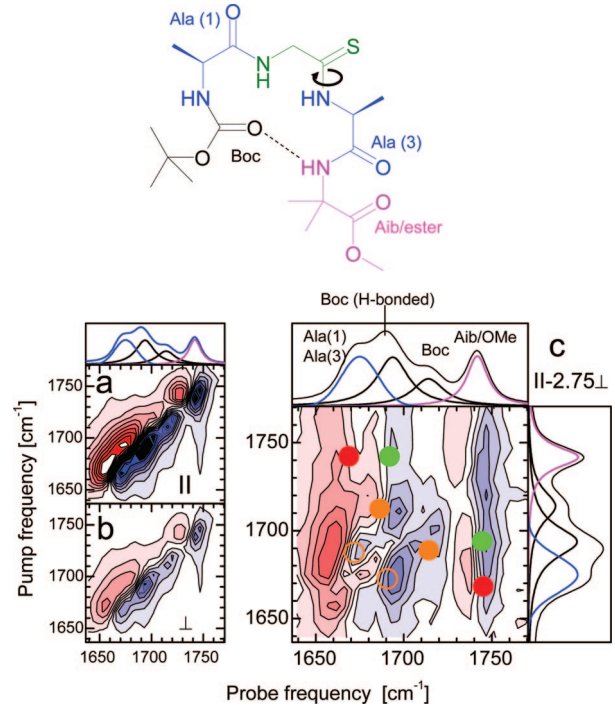

Figure 2. Top: Hydrogen-bonded loop structure of Boc-Ala-Gly(═S)-Ala-Aib-OMe, adopted by approximately half of the molecules in acetonitrile at room temperature. Bottom: FTIR absorption spectrum with bands of individual C═O groups indicated by idealized line shapes, and 2D-IR spectra for parallel (a) and perpendicular (b) polarization of IR-pump and IR-probe pulses. Diagonal signals are almost completely suppressed in the weighted [(a)-2.75(b)] difference spectrum (c). Colored dots mark different sets of cross peaks, which are discussed in the text.

C═O stretch bands overlap and give rise to a band centered at approximately 1673 cm^{-1}. The bands at both ∼1688 and 1713 cm^{-1} belong to the Boc protection group and allow us to distinguish between molecules in a looped conformation with an intramolecular hydrogen bond (peak at 1688 cm^{-1}, see sketch) and peptides in more extended conformations without this hydrogen bond (peak at 1713 cm^{-1}). The band at ≈1740 cm^{-1} is due to the Aib/OMe terminal group.

The equilibrium 2D-IR spectra of Boc-Ala-Gly(═S)-Ala-Aib-OMe, recorded for parallel and perpendicular polarization of IR-pump- and -probe pulses, are shown in Figure 2a and b. Since diagonal and cross-peak intensities depend differently on polarization, the weighted difference between these two spectra in Figure 2c suppresses the diagonal peaks and, ideally, only shows the cross peaks.[20] Three pairs of cross peaks, which will also be of importance in the transient 2D-IR experiments, are highlighted. Their assignment is supported by the 2D-IR data for the isotope-labeled molecules, which have been reported in ref 40. The ester C═O stretch band at 1740 cm^{-1} is sensitive to excitation of its nearest neighbor Ala(3), giving rise to the cross peak marked by a red dot at the bottom right corner of Figure 2c. Nearest-neighbor coupling between Ala(1) and non-hydrogen-bonded Boc is at the origin of the cross peaks marked in orange. The corresponding cross peaks with the hydrogen-bonded Boc band are indicated by an open orange circle. Both sets of cross peaks disappear when Ala(1) is shifted out of the spectral window by ^{13}C-isotope labeling.[40] Their pattern reflects the fact that Ala(1) in the hydrogen-bonded conformation absorbs at lower frequencies than in the more extended conformations due to excitonic coupling with Boc.[40] Finally, through space coupling produces a cross peak between the

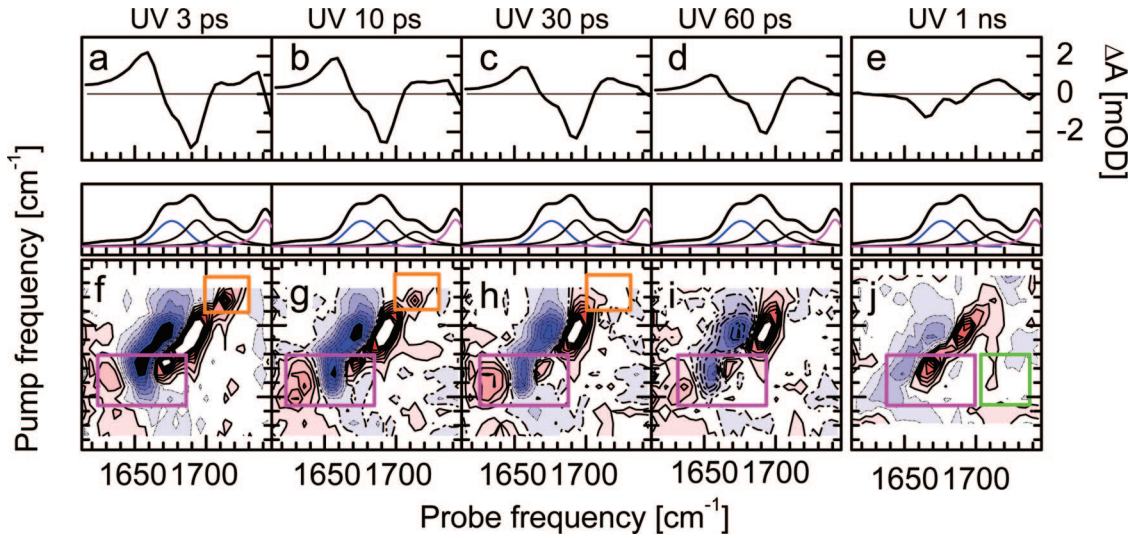

Figure 3. (a−e) UV-pump−IR-probe spectra of Boc-Ala-Gly(═S)-Ala-Aib-OMe at different delay times between the UV pump and the IR probe. (g−j) Transient 2D-IR spectra (2D-IR spectra in the presence of the UV pulse minus equilibrium 2D-IR spectrum) at different delay times between UV pump and 2D-IR probe. The orange and magenta rectangles highlight diagonal peaks discussed in the text, and the green rectangle indicates the spectral region where transient cross peaks can be observed. For each UV delay, the FTIR spectrum is shown for comparison.

hydrogen-bonded C═O group of Boc and that of Aib/ester (green dots).

B. Pump−Probe Spectra. The transient IR data for Boc-Ala-Gly(═S)-Ala-Aib-OMe upon $\pi-\pi^*$ excitation of the thioamide bond has been reported and analyzed in detail in reference 39. To summarize the results obtained in that study, we show in the first row of Figure 3 the UV-pump−IR-probe spectra at selected delay times after 266 nm excitation. The UV excitation leads to an instantaneous red shift of the alanine bands (signal near 1660 cm^{-1} and of the Aib/OMe ester band (signal near 1740 cm^{-1}) due to strong heating of the molecule by excess laser energy. Although this signal almost fully decays with a 10 ps time constant for the terminal Aib/OMe unit, a red shift persists for ∼100 ps for the C═O stretch bands of the alanine residues. Indeed, these nearest neighbors of the thio-substituted peptide unit are sensitive to the changes in charge distribution and dipole moment while the molecules remain trapped in the lowest-lying electronic excited-state with a lifetime of 130 ps. The excited state is characterized by a thioamide single bond without any significant barrier to isomerization.[43]

The band due to the hydrogen-bonded C═O group of Boc at 1688 cm^{-1} bleaches immediately after photoexcitation. In ref 39, we have attributed this instantaneous signal to only a small increase in hydrogen bond distance, which nevertheless strongly reduces the oscillator strength of this band and leads to a blue shift. The opening of the loop structure, expected on a 100 ps time scale, then leads to only comparatively small spectral changes in the Boc region. However, at the end of the photoreaction, the H-bonded Boc signal at 1688 cm^{-1} partially recovers with a 230 ps time constant when the intramolecular hydrogen bond is reforming in molecules that have returned to the initial trans conformation of the thioamide bond in the electronic ground state (∼70% of all excited peptides).[39] The formation of cis species permanently increases the number of molecules without this hydrogen bond and leads to an enhanced Boc absorption around 1715 cm^{-1}. Spectra recorded with ^{13}C-labeled samples further reveal a slight blue shift of all C═O stretch bands in the cis conformation of the thioamide bond.[39,40]

C. Transient 2D-IR Spectra. On the basis of these results, we have chosen five characteristic delay times between the UV-pump pulse and the 2D-IR probe for the recording of transient 2D-IR data: 3, 10, 30, and 60 ps and 1 ns (Figure 3). At the first four delays, the peptide molecules are predominantly in the electronically excited state, characterized by a thioamide single bond.[43] After 1 ns, the photoreaction is essentially finished, and all molecules are again in the electronic ground-state and have either adopted the original trans or the new cis conformation of the thioamide bond.

1. Diagonal Signals. The transient 2D-IR spectra shown in Figure 3f−j are highly structured along the diagonal and undergo important changes in the course of the photo reaction. In particular, we have marked by orange and pink rectangles two diagonal signals which contain interesting new information on the photoreaction.

The orange squares highlight an initially well-resolved diagonal peak, which decreases significantly from 3 to 10 ps and completely disappears after 30 ps. The origin of this signal becomes clear when we look at the horizontal cuts trough the T-2D-IR spectra at $\omega_{pump} = 1717$ cm^{-1} (Figure 3a).

At the first two delay times, the cuts in Figure 3a show a positive peak at the frequency of the urethane group of molecules in non-hydrogen-bonded conformations. It decays on a ≈10 ps time scale. Very similar kinetics were found in the UV-pump−IR-probe spectra for the other terminal group, the Aib/OMe unit, absorbing near 1740 cm^{-1} (see Figure 3b). The C═O group of Aib, which is at a similar distance from the photoexcited moiety in the peptide sequence, is nearly unaffected by the change of the electronic state or isomerization, and red-shifts only at early times due to anharmonic coupling to low-frequency vibrations, which are excited before the excess laser energy is dissipated to the solvent.[39] The short-lived T-2D-IR peak for the non-hydrogen-bonded Boc group points to a similar heat-induced shift of the 1717 cm^{-1} band. Note that in the pump−probe spectra, the signal due to non-hydrogen-bonded Boc is largely hidden by the much larger signal of the hydrogen-bonded Boc group of the molecules initially in the loop conformation. The capability of transient 2D-IR spectroscopy to resolve the dynamics of bands hidden in the pump−probe spectra can be explained by the additional IR excitation, which can individually address single IR bands. The UV-induced absorption changes are thereby enhanced and disentangled in the two-dimensional representation of the data.

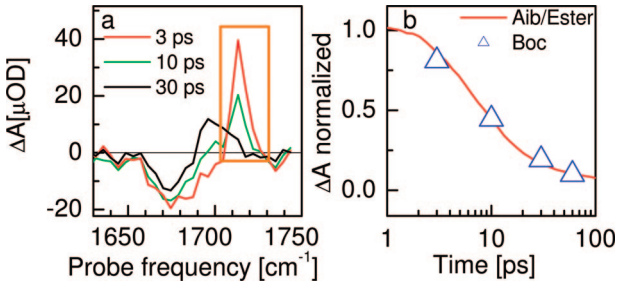

Figure 4. (a) Cuts through the transient 2D-IR spectra at ω_{pump} = 1717 cm^{-1}. The orange square highlights the diagonal peak of the Boc group of the molecule in non-hydrogen-bonded open conformations. (b) Normalized intensity of this transient diagonal peak as a function of delay between UV pump and 2D-IR probe (blue triangles). For comparison, the normalized UV-pump−IR-probe signal of the ester protection group at 1740 cm^{-1} is shown by a red solid line.

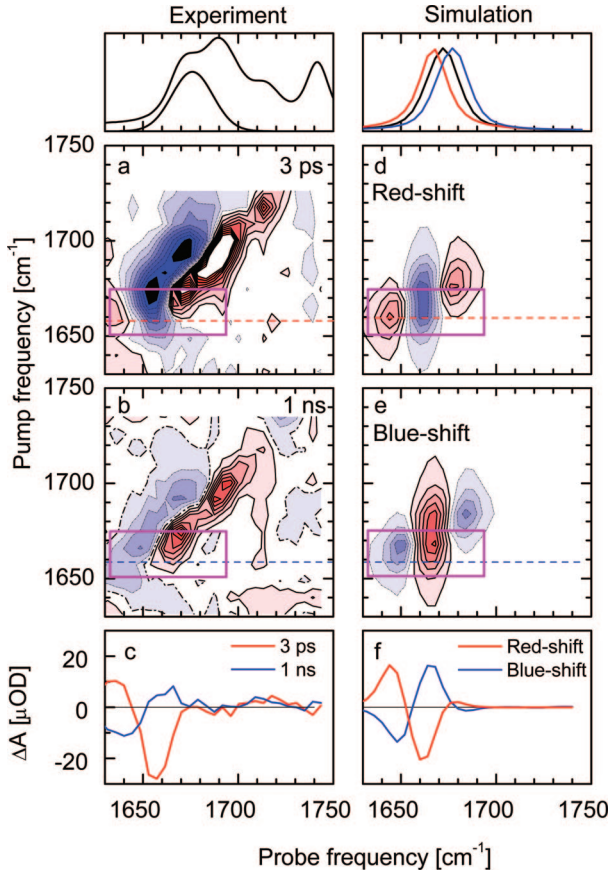

Figure 5. (a−b) Transient 2D-IR spectra (2D-IR spectra in the presence of the UV pulse minus equilibrium 2D-IR spectrum) at different delay times between UV pump and 2D-IR probe. (c) Cuts through the T-2D-IR spectra with the IR pump pulse centered at 1657 cm^{-1}. (d−e) Calculated T-2D-IR spectra for a single oscillator, the frequency of which red-shifts (d) or blue-shifts (e) upon photoexcitation. (f) Cuts through d and e. Linear absorption spectra are shown in the top panels. The absorption band of the two alanines is indicated by an idealized line shape. Black, blue and red lines in the simulated linear spectra show the equilibrium, blue-shifted and red-shifted absorption bands.

The signal marked by magenta squares in Figure 3 is due to the C═O groups of the two alanines. During the first four delay times, the transient 2D-IR signal is characterized by a positive−negative−positive shape, whereas at 1 ns, the signs are inverted (negative−positive−negative). The spectra at short and late delay times are compared in Figure 4 and 5 to model spectra based on two simple simulations. We have considered a molecule with only one vibrational band, which red-shifts (d) or blue-shifts (e) upon triggering a photoreaction. The red shift yields a positive−negative−positive signal; the blue shift gives a similar signal as the red shift, but with inverted signs, in agrement with the experimental spectra. Horizontal cuts at ω_{pump} = 1657 cm^{-1}, where overlap with the strong signal due to hydrogen-bonded Boc is small (Figure 5 bottom row), confirm this similarity. The red shift of the alanine bands at early delay times is also seen in the pump−probe spectra in Figure 3a and b, whereas the blue shift at long delay times is not directly evident in Figure 3e because the positive induced absorption of the blue-shifted alanine bands overlaps with the bleach of the Boc band near 1690 cm^{-1}. However, the spectral evolution indicated by the transient T-2D-IR data is clearly confirmed by pump−probe measurements in combination with isotope labeling.[39] Again, the additional IR excitation in the T-2D-IR experiment can be regarded as a "light-label",[44] yielding similar information as ^{13}C isotope-labeling in pump−probe spectroscopy.

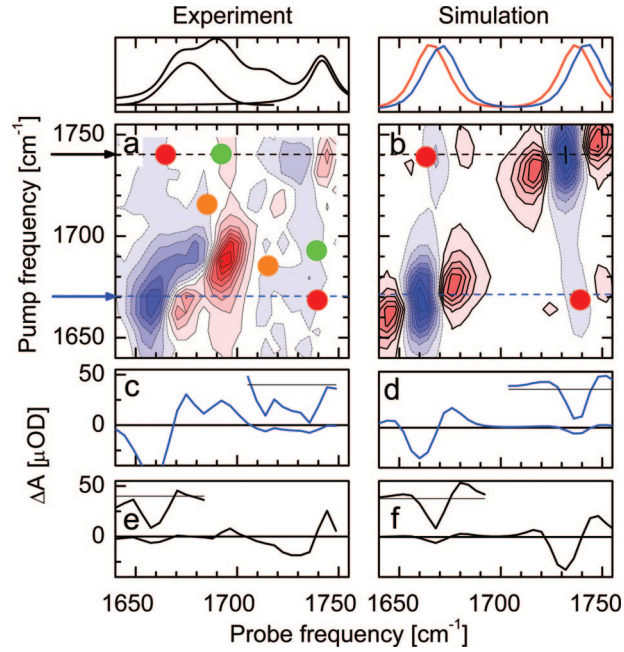

Figure 6. (a) Transient 2D-IR spectrum of Boc-Ala-Gly(═S)-Ala-Aib-OMe at 3 ps delay between UV pump and 2D-IR probe. Unlike in the other measurements, the UV-pump polarization was set parallel to the IR probe and perpendicular to the IR pump polarizations to enhance the cross peaks. The blue and black arrows indicate the pump frequencies (1670 and 1740 cm^{-1}) of the cuts shown in c and e, respectively. Colored dots mark different sets of transient cross peaks. In the FTIR spectrum shown on top, the two C═O stretch bands of the alanines and Aib are indicated by idealized line shapes. (b) Model T-2D-IR spectrum for case 3. Blue and red lines on top show the absorption spectra of initial and final species. (c, d) Horizontal cuts through the T-2D-IR spectra at 1670 cm^{-1}. (e, f) Horizontal cuts through the T-2D-IR spectra at 1740 cm^{-1}. The cross peak signals are enlarged by a factor of 5 in the insets.

2. Off-Diagonal Signals. The green square in Figure 3j marks the region of the transient 2D-IR spectra, where interesting off-diagonal signals can be observed. A better signal-to-noise ratio in the 1 ns spectrum due to additional averaging has allowed us to resolve them best at the end of the photoreaction. In addition, for time delays shorter than the rotational diffusion time of the molecule (anisotropy decay in 47 ps), the relative intensity of the off-diagonal peaks can be enhanced by changing the polarization of the UV and IR pump pulses with respect to the IR probe pulse.[32] In Figure 6a, we therefore show the T-2D-IR spectrum recorded 3 ps after UV excitation in a slightly

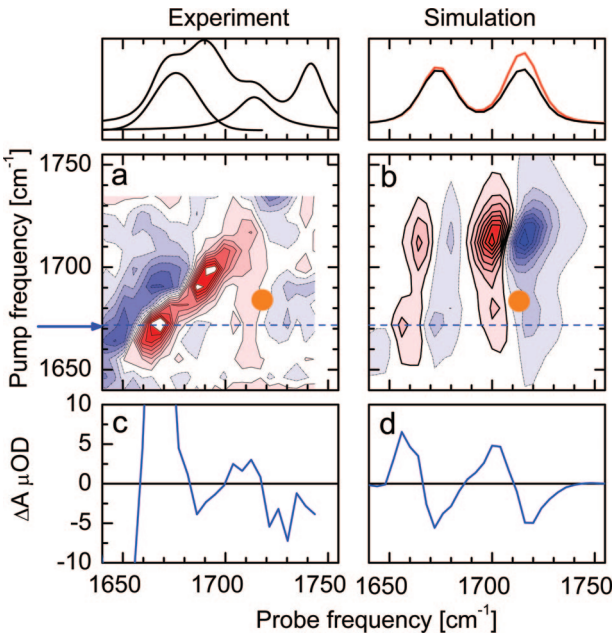

Figure 7. (a) Transient 2D-IR spectrum of Boc-Ala-Gly(=S)-Ala-Aib-OMe at 1 ns delay time between the UV pump and 2D-IR probe. The arrow indicates the pump frequency of the cuts shown in c and d. In the FTIR spectrum shown on top, the two C=O stretch bands of the alanines and non-hydrogen-bonded Boc are indicated by idealized line shapes. (b) Model T-2D-IR spectrum for case 4 with transition frequencies adapted to the experiment ($\epsilon_1 = 1673$ cm^{-1}, $\epsilon_2 = 1713$ cm^{-1}). Black and red lines on top show the absorption spectra of initial and final species. (c, d) Horizontal cuts through the T-2D-IR spectra at $\omega_{pump} = 1670$ cm^{-1}.

different spectral window for perpendicular polarization of the UV and IR pump pulses.

In Figure 6, it is possible to identify the corresponding transient off-diagonal signals for all three pairs of cross peaks we have already highlighted in the equilibrium 2D-IR spectrum in Figure 2. Red dots mark transient cross peaks between the Aib/ester band and the alanines; green dots, those between Aib/ester and hydrogen-bondend Boc; and orange dots indicate cross peak changes between the alanines and non-hydrogen-bonded Boc. Knowing that the absorption bands of the two alanines and of the ester group are initially red-shifted after photoexcitation and that the ester group is coupled to its nearest neighbor Ala(3), we compare this T-2D-IR spectrum at 3 ps with the model spectrum for case 3 (Figure 6b). In this simulation, two coupled bands are red-shifted after photoexcitation but do not change their coupling. In both spectra (experimental and calculated), the transient cross peaks are dominated by the negative (blue) central part. Horizontal cuts at $\omega_{pump} = 1670$ cm^{-1} in Figure 5c show this negative off-diagonal peak near 1740 cm^{-1}. In the experimental spectrum, there is also a second cross peak near 1715 cm^{-1} due to Boc, which partially covers the positive part of the ester−alanine crosspeak. Spectral overlap is less important in the cut at $\omega_{pump} = 1740$ cm^{-1} in Figure 6e, which agrees very well with the model spectrum 6f. The similarity of the experimental and the simulated spectra suggests that these off-diagonal peaks can be attributed mainly to the red shift of the alanines and ester bands upon photoexcitation. The cross peaks in the 2D-IR spectrum are thereby also shifted to lower frequencies, and no change of coupling is required to explain the data. This appears reasonable, because no significant dihedral angle changes between Ala(3) and Aib are expected only 3 ps after excitation.

The T-2D-IR spectrum recorded at the end of the photoreaction, 1 ns after UV excitation, shows very different cross peak signals (Figure 7a). Indeed, the red shift of both the alanine and the ester band have long decayed, and both bands are now slightly blue-shifted. The cut through the T-2D-IR spectrum at $\omega_{pump} = 1670$ cm^{-1} in Figure 7c now shows a dominant off-diagonal signal near 1715 cm^{-1}, the spectral position of the Boc protection group of molecules in non-hydrogen-bonded conformations. From the UV-pump−IR-probe data, we know that the photoisomerization leads to an increase in the number of molecules in non-hydrogen-bonded conformations, which is reflected by the enhancement of the corresponding Boc band at 1715 cm^{-1}. We compare, thus, the T-2D-IR spectrum at 1 ns with the simulation of case 4, with the transition frequencies adjusted to match the experimental data. In this simulation (Figure 7b), the photoexcitation is assumed to increase the intensity of the higher-frequency band, but the coupling does not change. The horizontal cuts at 1670 cm^{-1}, which are shown in Figure 7c and d, reveal the similarity of the experimental and calculated transient cross peaks. This suggests that this off-diagonal signal can be attributed primarily to the increase of the number of the molecule in the open conformation. However, there are also important differences between the experimental and the model spectrum, and other contributions also need to be considered. The diagonal alanine signal is certainly enhanced by the blue shift of the corresponding absorption band, as discussed above, whereas the Boc absorption band is clearly too strong in the model simulation, leading to a similar enhancement of the T-2D-IR diagonal signal. On the other hand, the cross peak between Ala(1) and non-hydrogen-bonded Boc (nearest neighbor coupling) may also be enhanced at the end of the photoreaction because the two bands are spectrally closer in the cis conformation of the thiopeptide bond. This situation is partially described by case 2 in Figure 1.

V. Conclusions

The transient 2D-IR spectra recorded for our model peptide are much more structured than the UV-pump−IR-probe and the equilibrium 2D-IR spectra, and yield additional information on the photo dynamics. Indeed, on the diagonal of the transient 2D-IR spectra in Figure 6a, four signals due to different C=O oscillators can be clearly distinguished. We have demonstrated in two cases that the spectral dynamics of individual vibrational modes can thus be resolved, even when they are hidden in the UV-pump−IR-probe data.

In addition, the observation of transient 2D-IR off-diagonal signals has the potential to yield time-dependent structural information beyond what can be learned from linear absorption changes. This is, indeed, possible if we are able to detect changes in cross-peak signals that are related to changes of coupling due to distance and orientational rearrangements of two coupled chromophors. However, we have shown here that transient cross peaks can also arise from a number of different reasons other than coupling changes, such as spectral shifts, loss or gain of oscillator strength, or intensity changes of absorption bands. The transient off-diagonal signals observed for our model thioxopeptide can be reproduced at least qualitatively by simulations, which do not take into account any coupling changes. They rely only on spectral changes known from pump−probe spectroscopy and from the diagonal T-2D-IR signals. Thus, the various transient cross peaks in the T-2D-IR spectra recorded during the loop opening of Boc-Ala-Gly(=S)-Ala-Aib-OMe seem to provide no additional information on the conformational dynamics.

This is due in part to the fact that the largest change in conformation of the thioxopeptide under study is the opening of the intramolecular hydrogen bond, which is already very apparent from the UV-pump−IR-probe spectra. As far as the Boc carbonyl stretch vibration is concerned, the hydrogen-bonded and non-hydrogen-bonded molecules can also be distinguished in the equilibrium 2D-IR spectra, where both classes of conformations coexist and show different coupling patterns.[40] While this is no new information, the observation of an enhanced cross peak between Ala(1) and non-hydrogen-bonded Boc in the T-2D-IR spectra is, of course, a clear signature of the turn-opening. Although coupling changes certainly contribute to the transient cross peak signals observed here, current signal-to-noise levels are not sufficient to separate them from the dominant contributions that can be identified by our simplified model spectra. A more sophisticated theoretical treatment is, of course, possible and has been used to simulate equilibrium 2D-IR spectra of peptides.[45] However, the accurate modeling of conformational equilibria, spectral shifts, and dynamic line-broadening mechanisms and their changes, population transfer, vibrational relaxation and dipole orientations, all of which would have to be taken into account to "fit" the transient experiment, is not realistic at present. Finally, the large effect of photo excitation of the thio-substituted peptide unit on the C═O stretch frequency of the neighboring Ala residues and the long lifetime of the electronically excited state add further complexity to the analysis of the T-2D-IR data. Coupling changes involving the two alanines, which we expected to be significant, are therefore very difficult to observe. In this respect, our design of a model peptide with two coupled amide I oscillators separated by a small photoswitch did not yield the desired clear-cut results. The peptide nevertheless proved to be a good learning example, illustrating the need for a very cautious interpretation of transient cross-peak signals in the presence of strong spectral dynamics.

An experimentally much more favorable case, the appearance of a single transient cross peak in concomitance with the light-triggered β-turn opening in a peptide of similar size, has been presented in ref 33. The transient cross peak was found between two initially hydrogen-bonded peptide units. The opening of this relatively weak hydrogen bond led to much smaller spectral shifts than those observed here for the thioxopeptide. Nevertheless, a particular shift and change in oscillator strength of the two C═O vibrators involved in the hydrogen bond may have contributed to the selective enhancement of one transient cross peak during the turn opening of the initially disulfide-bridged molecule.

Despite the difficulties arising from the long-lived electronically excited state, there should, however, be continued interest in the 2D-IR spectroscopy of thiopeptide isomerization. The possibility to induce conformational changes in an active enzyme via the isomerization of a single thioamide bond has been demonstrated recently,[46] and the underlying mechanism should be further explored. Indeed, we have shown that spectral shifts due to the change of electronic state are almost negligible, already two peptide units away from the thio-substituted peptide unit. We may regard the small turn structure investigated here as a building block for larger thioxopeptides with selectively isotope-labeled carbonyls or other localized vibrational modes further away from the photoexcited moiety. Highly sensitive transient 2D-IR spectroscopy could then be very well suited for resolving structural detail during the conformational transitions triggered by the thioamide bond isomerization.

The high sensitivity of T-2D-IR spectroscopy to small variations in the vibrational spectrum of a molecule is due to the double difference technique. 2D-IR spectra recorded without photo excitation are subtracted from those recorded after photoexcitation to observe only the *changes* induced. This leads to very complex and very small signals, which, at least for the off-diagonal peaks in peptides, are close to our current noise level. In recent T-2D-IR temperature jump unfolding experiments, this referencing was not done, because it can be assumed that all probed molecules are equally affected by the rise in the solvent temperature.[36] However, as is best seen for an effective two-state problem, even a change in solvent temperature may often lead to the reaction of only a part of all molecules, leading to similar problems in identifying the spectrum of the reacted sample. Both the recording and the interpretation of transient 2D-IR data will thus remain a difficult challenge for the future.

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References and Notes

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