CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.200700396

Coexistence of Hydrogen-Bonded Loop and Extended Tetrapeptide Conformations

Valentina Cervetto,[a] Rolf Pfister,[a] Christoph Kolano,[a] Harald Bregy,[a] Heinz Heimgartner,[b] and Jan Helbing*[a]

Abstract: The conformations of a protected tetrathiopeptide have been analysed by isotope labelling and two-dimensional infrared spectroscopy (2D-IR). It has been found that Boc-Ala-Gly(=S)-Ala-Aib-OMe in acetonitrile, as well as its oxopeptide analogue, can adopt a hydrogen-bonded loop conformation in coexistence with less restricted structures. The two types of conformations interconvert too quickly to be resolved on the nuclear magnetic resonance (NMR) timescale, but give rise to different cross peaks in two-dimensional infrared spectra. The hydrogen bond between the Boc terminal group and the amide proton of Aib can be broken by photoisomerisation of the thioamide bond.

Keywords: conformation analysis · hydrogen bonds · NMR spectroscopy · peptides · photoisomerization

Introduction

Nuclear Magnetic Resonance (NMR) spectroscopy has become a standard method for the determination of molecular structures in solution. For peptides, ^{1}H and relatively simple 2D-techniques such as COSY and NOESY provide scalar coupling constants and yield upper bounds for interatomic distances, which, in combination with empirical knowledge and computer simulations, are often sufficient for determining their conformation. Much of the power of NMR spectroscopy, especially in larger systems, rests on the very narrow spin resonances due to extreme motional narrowing. Solvent fluctuations and structural inhomogeneity are averaged out. However, this makes it very difficult to distinguish molecular conformations that interconvert on timescales faster than a few hundred microseconds, the minimum time required for a NMR experiment.

Infrared measurements, on the other hand, can be regarded as quasi-instantaneous on the timescale of molecular motion, and it is possible to observe spectral changes due to chemical reactions or conformational change with sub-picosecond time resolution. However, linear infrared absorption spectra often bear only limited structural information. This limitation can at least in part be overcome by two-dimensional infrared (2D-IR) spectroscopy,[1] which measures the coupling of vibrational transitions rather than the coupling between nuclear spins in 2D-NMR.[2,3] In peptides, coupling between the backbone carbonyl C=O stretch vibrations gives rise to cross-peak signals that depend on their separations and relative orientations, providing sufficient information for the determination of the backbone conformations of small peptides in solution.[4–6] Because 2D-IR spectra are recorded on a picosecond timescale, multiple conformations can, in principle, be resolved, even if they interconvert quickly.[7] If a photoswitch is incorporated into the peptide, such conformational changes can be triggered externally, which allows one to study elementary steps in protein folding with high time resolutions.[8–10] Changes in conformation cause changes in cross peak intensities, providing direct evidence for the changes in interatomic distances and molecular geometries.[11]

Thiopeptides are peptides in which one (or more) of the backbone carbonyl oxygen atoms is substituted by a sulfur atom. This one-atom substitution can serve to create a photoswitch inside the peptide backbone. This results in a red shift of the π–π^* absorption band of the substituted peptide unit, allowing for selective excitation and isomerisation of the thiopeptide bond.[12–14] The thiopeptide unit maintains the planar geometry of the oxopeptide, and the main types

[a] Dott. V. Cervetto, Chem.-Ing. R. Pfister, Dr. C. Kolano, Dipl.-Chem. H. Bregy, Dr. J. Helbing
Physikalisch-Chemisches Institut Universität Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)
Fax: (+41) 44-635-6838
E-mail: j.helbing@pci.unizh.ch

[b] Prof. Dr. H. Heimgartner
Organisch-Chemisches Institut Universität Zürich
Winterthurerstrasse 190, 8057 Zürich (Switzerland)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author.

of secondary structure are accessible to thiopeptides. Miwa and co-workers have demonstrated the possibility of incorporating thioamide linkages in β sheet and α helix secondary structures.[15,16] A β sheet structure adopted by a thiopeptide is very similar to that of the corresponding oxopeptide, while a single thio substitution even increases the thermal stability of an α helix. This is due to stronger acidity of the thioamide hydrogen, as a consequence of the longer C=S bond and the lower electronegativity of sulfur. X-ray crystal structure analyses of protected Aib-containing tripeptides have revealed similar H-bonded β-turn motifs with and without thio substitution.[17,18] Our choice of the tetrapeptide Boc-Ala-Gly-Ala-Aib-OMe (Boc: *tert*-butyloxycarbonyl; see Figure 1, top) for our present study employing two-dimen-

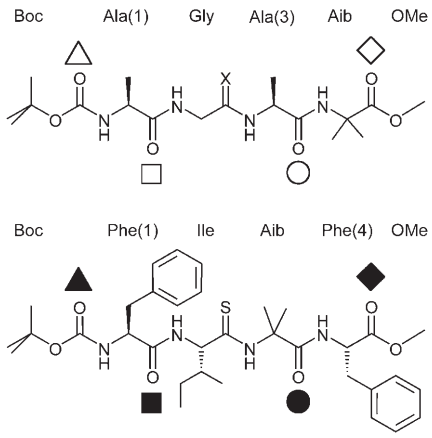

Figure 1. Structures of Boc-Ala-Gly(=X)-Ala-Aib-OMe (top) and the reference peptide Boc-Phe-Ile(=S)-Aib-Phe-OMe (bottom).

sional infrared spectroscopy in acetonitrile was in part motivated by this early work. It is well known that the achiral amino acid Aib on one hand favours turn formation and limits the number of peptide conformations in solution.[19] On the other hand, the thiopeptide analogue Boc-Ala-Gly(=S)-Ala-Aib-OMe is easily obtained by selective substitution through treatment with Lawesson's reagent.[20] The thiopeptide contains two C=O vibrators at each side of the thioamide photoswitch and is therefore ideally suited for 2D-IR investigation of the conformational change induced by the isomerisation of the thiopeptide bond. As a reference molecule we also studied the closely related tetrathiopeptide Boc-Phe-Ile(=S)-Aib-Phe-OMe (Figure 1, bottom), for which a crystal structure is available.[21] In the case of Boc-Ala-Gly(=S)-Ala-Aib-OMe we have employed isotope labelling, FTIR and 2D-IR spectroscopy as well as NMR methods to demonstrate the coexistence of a loop structure with a hydrogen bond between the carbonyl O atom of the Boc terminal group and the NH of the Aib unit, and probably several more extended conformations. Moreover, we have shown that the hydrogen bond can be broken by isomerisation of the thioamide bond upon UV irradiation. The reference molecule Boc-Phe-Ile(=S)-Aib-Phe-OMe, on the other hand, does not seem to adopt a hydrogen-bonded conformation in acetonitrile solution. These results will be the basis for a detailed future investigation of the photoisomerisation and hydrogen-bond breaking dynamics, using ultrafast IR pump-probe and transient 2D-IR spectroscopy.

Results and Discussion

Linear IR spectroscopy: Figure 2 shows the linear absorption spectra of three different isotopomers of the protected peptide Boc-Ala-Gly-Ala-Aib-OMe in CD_3CN (solid lines). An asterisk indicates that an amino acid is ^{13}C=O-labelled. Selective substitution of S for O at Gly yielded the corresponding thiopeptides (squares). Boc-Ala-Gly(=S)-Ala-Aib-OMe contains two amide I oscillators (□ and ○ in Figure 1) separated by a thioamide bond and two C=O carbonyl groups of the urethane (Boc, △) and Aib/ester (◇) terminal units. The spectrum of the thiopeptide differs from the parental oxopeptide IR spectrum only at the low frequency side of the amide I band (see Figure 2), where we observe a loss of intensity. The thiopeptide IR spectrum has four bands centred at approximately $\tilde{\nu}$ 1673, 1688, 1713 and 1740 cm^{-1}. Figure 2b and c show that ^{13}C=O isotope labelling of either of the two alanine residues gives rise to red-shifted amide I bands of very similar frequency ($\approx$1632 cm^{-1}). This implies that the two amide I modes of the two alanine units overlap and give rise to the band centred at approximately 1673 cm^{-1} in the unlabelled molecule. The remaining three bands, which are affected only very little by isotope labelling and/or thio substitution, must therefore be due to the C=O stretch modes of the two protecting groups, both in the thiopeptide and in the oxopeptide.

Indeed, after removal of the Boc protecting group from the Ala(1)-labelled oxopeptide the bands centred at $\tilde{\nu}$ 1688 and 1713 cm^{-1} have disappeared (see Figure 3). Hence the C=O stretch of the urethane group gives rise to two bands separated by approximately 25 cm^{-1}, while the band at 1740 cm^{-1} is due to the C=O stretch of the Aib/ester group. Indeed, the presence of five absorption bands for only four C=O vibrators in the isotope-labelled samples already indicates that the molecule can adopt at least two different types of conformations. Figure 4 shows that raising the temperature of Boc-Ala-Gly(=S)-Ala*-Aib-OMe from 2 °C to 38 °C leads to a relative increase of the intensity of the urethane band at 1713 cm^{-1}, suggesting a change in equilibrium between the two spectrally distinct types of molecules.

For comparison, Figure 2d shows the FTIR absorption spectrum of the thiopeptide sequence Boc-Phe-Ile(=S)-Aib-Phe-OMe. The molecule has the same number of C=O oscillators (four), but only three bands are resolved. The redmost band contains the two amide I modes of Phe(1) and Aib, the 1740 cm^{-1} band is due to the ester C=O stretch, and only one band of the Boc protection group is seen at 1713 cm^{-1}.

Indeed, a C=O stretch absorption of the Boc protection group near 1715 cm^{-1} is typical for protected polypeptides

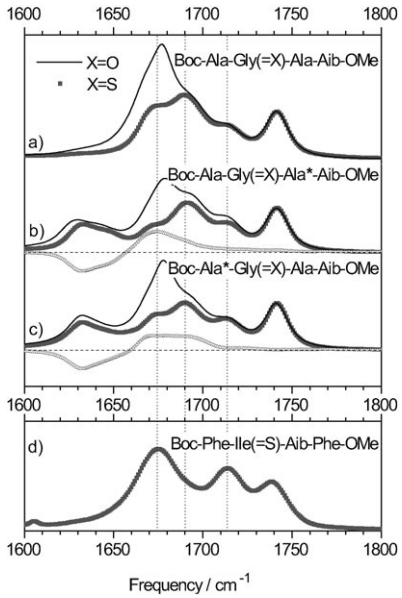

Figure 2. a)–c) FTIR absorption spectra of Boc-Ala-Gly(=X)-Ala-Aib-OMe in CD_3CN with different $^{13}C{=}O$ isotope labels (positions indicated by asterisks). The solid lines represent the oxopeptide (X = O), the squares the corresponding thiopeptide spectra (X = S). The triangles in b) and c) show the difference between the absorption of isotope-labelled and unlabelled thiopeptide. All spectra have been normalized to the ester band at 1740 cm^{-1}. d) FTIR absorption spectrum of the reference thiopeptide Boc-Phe-Ile(=S)-Aib-Phe-OMe in CD_3CN.

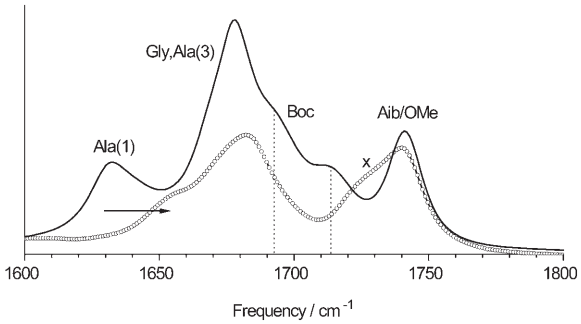

Figure 3. FTIR absorption spectra of the oxopeptides H_3N^+-Ala*-Gly-Ala-Aib-OMe (open circles) and Boc-Ala*-Gly-Ala-Aib-OMe (solid line) in CD_3CN. The vertical lines indicate the positions of the two bands assigned to Boc. The $^{13}C{=}O$ stretch vibration of the isotope-labelled alanine is blue-shifted after removal of the protecting group, due to a lowering of its effective mass, as indicated by the horizontal arrow. The shoulder labelled by a cross is due to dioxane impurities.

in acetonitrile.[11] The additional, strongly red-shifted Boc absorption in the thiopeptide Boc-Ala-Gly(=S)-Ala-Aib-OMe thus strongly points to the presence of a hydrogen bond. Ab initio calculations on NMA (*N*-methylacetamide) dimers[22] suggest that only hydrogen bond lengths smaller than 3.5 Å can induce a red shift of the stretching of the accepting C=O group by as much as 25 cm^{-1}. In addition, a smaller red shift is expected for the amide I mode of the peptide unit acting as hydrogen bond donor. Indeed, Figure 4 shows a shoulder of the isotope-labelled Ala(3) band, which intensifies as the temperature is raised. This

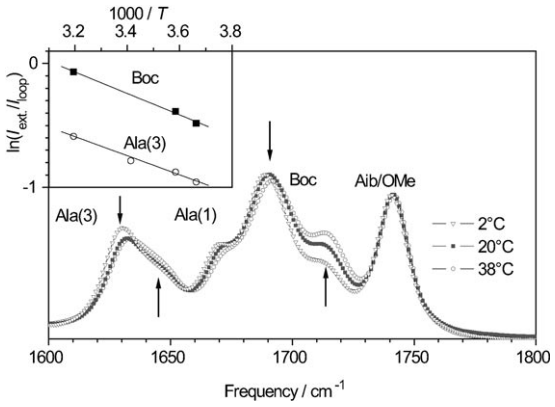

Figure 4. FTIR absorption spectra of the thiopeptide Boc-Ala-Gly(=S)-Ala*-Aib-OMe in CD_3CN at different temperatures. Arrows indicate changes with increasing temperature. The inset shows the natural logarithm of the intensity ratio of the $\tilde{\nu}$ 1688 and 1713 cm^{-1} Boc bands (solid squares) and the two Ala(3) bands (open circles). The offset between the two is attributed to a stronger gain in oscillator strength of Ala(3) upon intramolecular hydrogen bonding with respect to Boc. The data for Ala(3) are directly obtained from a fit to the Figure, while the data for Boc are extracted from similar spectra for Boc-Ala*-Gly(=S)-Ala-Aib-OMe, where intensity borrowing effects can be neglected (see also Supporting Information).

provides a first hint to identifying the amide proton of the Ala(3) residue as the H-bond donor to the C=O group of Boc.

The exact determination of the ratio of hydrogen-bonded and non-hydrogen-bonded conformations from the IR spectra is made difficult by spectral overlap, intensity borrowing effects and the possible enhancement of the C=O stretch oscillator strength in the presence of an intramolecular hydrogen bond.[23] The first two effects can be partially eliminated by combining data for the Ala(1) and Ala(3) isotope-labelled samples (see Experimental Section and Supporting Information), which reveals approximately equal Boc signals from both types of molecules at 40 °C, and a signal ratio of ≈65:35 in favour of the hydrogen-bonded molecules at 0 °C. The inset in Figure 4 shows a van't Hoff plot based on this analysis of the temperature-dependent IR spectra, indicating that the molecules giving rise to the red-shifted absorption bands are approximately 6–7 kJ mol^{-1} lower in enthalpy, while the blue-absorbing (non-hydrogen-bonded) conformations are entropically favoured by approximately 20 J mol^{-1} K^{-1}. These numbers, however, are sensitive to variations of the fit parameters, which leads us to a more conservative estimate of $\Delta H = 4$–9 kJ mol^{-1} and $\Delta S = 10$–30 J mol^{-1} K^{-1}.

2D-IR spectroscopy: In order to confirm the above band assignment, and also to obtain additional structural information, we recorded 2D-IR spectra. In the frequency-domain implementation of 2D-IR used in this study, a narrow band IR-pump pulse is scanned across the spectral region of interest for the selective excitation of individual C=O vibrations. Its central frequency defines the vertical pump-axis. Immediately afterwards (at a delay of 1 ps) a broad-band probe

pulse records the pump-induced absorption changes, which are plotted as a function of probe energy, defining the horizontal probe-axis. Bleach and stimulated emission of each selectively excited 0–1 transition give rise to a negative signal (blue in Figure 5) along the diagonal of the 2D-spectrum, and the corresponding excited state absorption (1–2 transition) is seen as a positive signal (red), which is shifted to smaller wavenumbers due to the anharmonicity of the C=O stretch mode. In addition, cross peaks can be seen if an absorption band shifts in frequency as a result of excitation of another transition to which it is coupled. Thus, each signal (diagonal and off-diagonal) consists of a pair of negative and positive peaks. To the first approximation, the intensity of a cross peak between two vibrational bands is proportional to the square of their transition dipole coupling, which varies with the inverse third power of the distance between two C=O groups. For adjacent peptide units, however, this through-space coupling is only a poor approximation, and ab initio methods are needed to include through-bond effects between nearest neighbours. Nevertheless, transition dipole coupling gives a first estimate for the distance (and orientation) dependence of cross peak intensities. In particular, two peptide units at a large distance from each other do not give rise to cross peaks.

Figure 5a and b show the 2D-IR spectra of the Ala(1)-labelled thiopeptide recorded with parallel and perpendicular polarization of pump and probe beams. Most cross peaks in these spectra are hidden because they overlap with the broad and much stronger diagonal peaks. However, because of their different polarization dependence, the diagonal peaks can be selectively suppressed by taking the weighted difference between the two sets of data.[5] These difference spectra (Figure 5c for the Ala(1)-labelled and Figure 5g for the Ala(3)-labelled thiopeptide) show almost exclusively the cross peaks.[24] For better comparison of the two isotopomers they were normalized to the Aib/ester signal (1740 cm^{-1}).

The spectra shown in Figure 5c and 5g reveal very different cross peak patterns for the two absorption bands assigned to the Boc protection group. The cross peaks marked

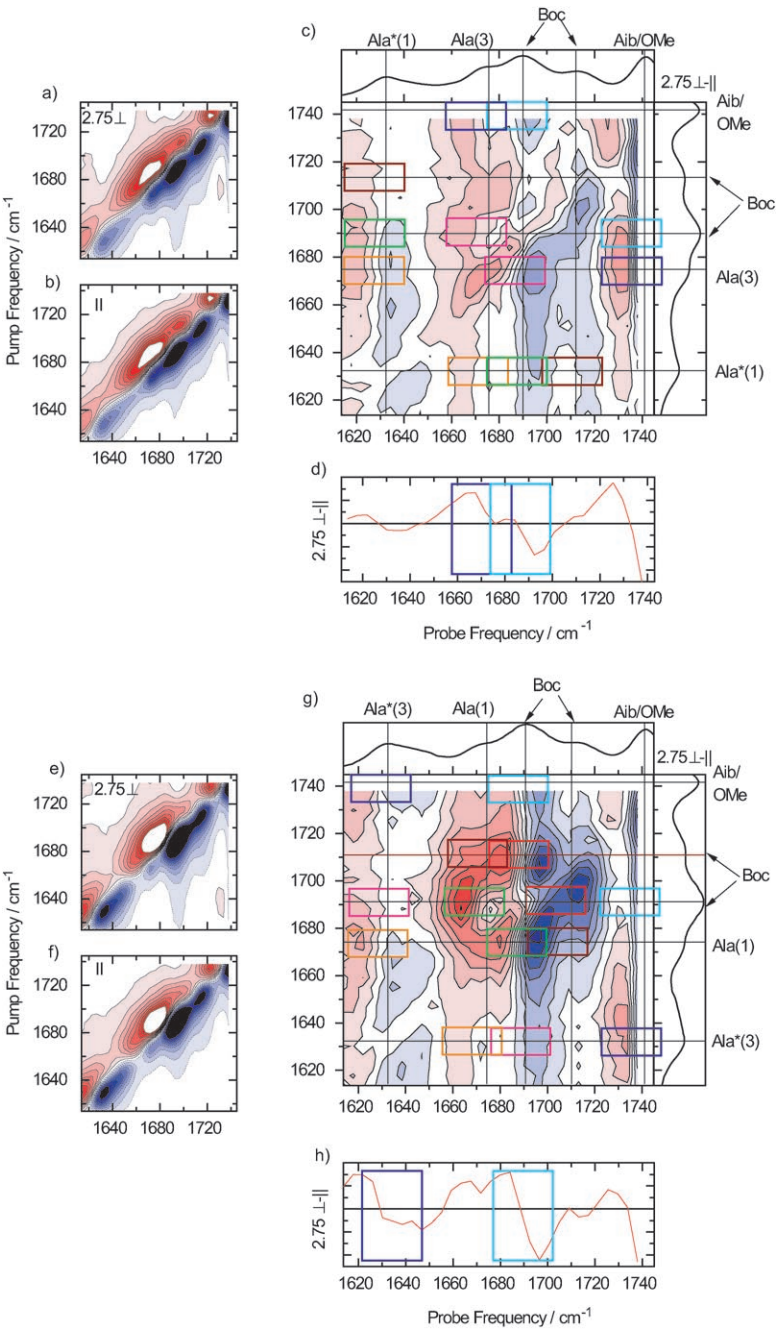

Figure 5. a)–c) 2D-IR spectra of Boc-Ala*-Gly(=S)-Ala-Aib-OMe. a) Perpendicular polarization of pump and probe beams. b) Parallel polarization. c) Cross peaks in the thiopeptide 2D-IR spectrum (weighted difference between the 2D-IR spectra in (a) and in (b)). d) Cut through the cross peak spectrum (c) at a pump frequency of 1738 cm^{-1}. e–h) The same spectra for Boc-Ala-Gly(=S)-Ala*-Aib-OMe. Spectra have been normalized to the ester signal at 1740 cm^{-1}. Coloured rectangles indicate the cross peaks discussed in the text, each consisting of a positive (red) and negative (blue) contribution.

by a cyan rectangle show that the band at 1688 cm^{-1} is coupled to the Aib/ester group at 1740 cm^{-1}, while those marked with pink rectangles are due to coupling to Ala(3). On the other hand, the Boc group absorbing at 1713 cm^{-1} does not couple—or does so only very weakly—with either of these two groups. These differences are illustrated by the cuts through the cross-peak spectra of Figure 5c and 5g at an IR-pump frequency of 1738 cm^{-1}, which are shown in Figure 5d and h, respectively. They clearly show a strong response of the red-shifted Boc absorption band to excitation of the Aib/ester vibration (cyan rectangle), which is similar to that of Ala(3) caused by nearest neighbour coupling (blue rectangle). This indicates that the molecules with the Boc absorption band at lower frequency have their Boc terminal group spatially close to the Aib/ester and to the Ala(3), while the molecules with the Boc group absorbing at higher frequency are in conformations in which the Aib/ester and the Ala(3) groups are too far away from the Boc unit for significant coupling. The 2D-IR spectra also show that the amide I modes of the Ala residues are coupled (orange rectangle), although they are separated by the thiopeptide unit. Moreover, coupling is also present between vicinal groups, as is to be expected. The Aib/ester group is coupled to Ala(3) (blue rectangles). The C=O stretches of Boc both at $\tilde{\nu}$ 1713 and at 1688 cm^{-1} couple with Ala(1) (brown and green rectangles, respectively).

The 2D-IR spectrum of Boc-Ala-Gly(=S)-Ala*-Aib-OMe shows intense cross peaks, which seem to indicate significant coupling of the bands at $\tilde{\nu}$ 1688 and at 1713 cm^{-1} (Figure 5g, red rectangles). This coupling would not be expected if the two bands were indeed due to Boc protection groups of distinct molecules in different conformations. However, this set of cross peaks is not present when the amide I band of Ala(1), which partially overlaps the 1688 cm^{-1} band, is red-shifted by isotope labelling (Figure 5c). It must therefore be possible to explain the observed cross peak pattern in terms of coupling to Ala(1). Indeed, because the Ala(1) amide I band is almost in resonance with the 1688 cm^{-1} band of Boc, coupling between the two should shift the former band to lower wavenumbers. This spectral distortion due to Ala(1)-Boc coupling is evident from the changes in the FTIR spectra induced by isotope labelling (open triangles in Figure 2c), which also indicate substantial intensity borrowing between the H-bonded urethane transition and Ala(1). In contrast, the cross peak between Ala(1) and the 1713 cm^{-1} band is probably enhanced at the high energy tail of the Ala(1) absorption, because cross-peak intensity is inversely proportional to the square of the energy difference between two coupled transitions. Together, these two effects can lead to the observed misalignment of the two pairs of off-diagonal signals due to Ala(1)-Boc nearest-neighbour coupling for two different molecular structures in Figure 5g. We do not observe population transfer between the two Boc absorption bands in the first 10 ps after IR excitation. These measurements are limited by the short vibrational lifetime of the C=O stretch excitation.

NMR spectroscopy: The ^{1}H NMR spectra of the oxo- and the thiopeptide in acetonitrile at room temperature each consist of only one set of signals, which can be unambiguously assigned on the basis of a TOCSY spectrum (see Supporting Information for details). The different conformations of the peptide, which can be distinguished by IR spectroscopy in combination with isotope labelling, must therefore interconvert on a sub-millisecond timescale, which is too fast for detection in the NMR experiment. Nevertheless, NMR can provide important structural information, which is fully complementary to our infrared data. The top panel in Figure 6 shows the chemical shifts of the four amide protons

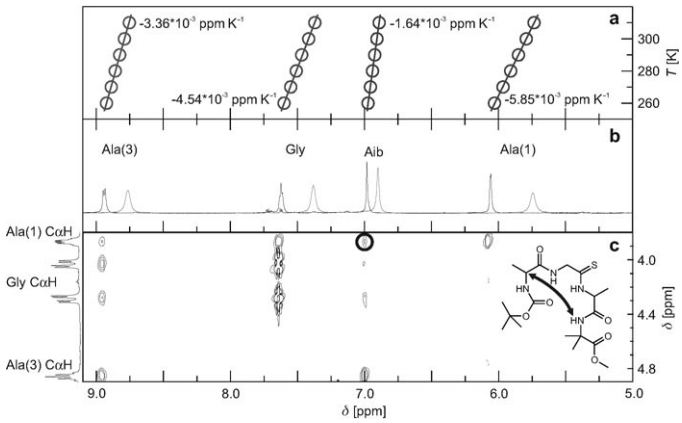

Figure 6. a) Temperature dependence of the amide proton chemical shifts of Boc-Ala-Gly(=S)-Ala-Aib-OMe, including the slopes obtained from a least-squares linear fit. b) ^{1}H NMR spectrum of Boc-Ala-Gly(=S)-Ala-Aib-OMe at 250 K (black line) and 300 K (grey line). c) Amide Cα proton coupling region of the NOESY spectrum recorded at 250 K. The NOESY peak connecting the amide proton of Aib and the Cα proton of Ala(1) is marked by a circle. All spectra were recorded on a 500 MHz spectrometer in CD_3CN.

of the thiopeptide as a function of temperature. Clearly, the chemical shift of the amide proton belonging to the Aib unit changes significantly less with temperature than the remaining NH signals. We determine a temperature coefficient of $-1.6{\cdot}10^{-3}$ ppm K^{-1}, which is evidence for strong solvent shielding and strongly points to its involvement in an intramolecular hydrogen bond.[25] All other amide protons, on the other hand, appear to be solvent-exposed. Note that for the completely solvent-exposed amide and thioamide protons of NMA and NMTAA (*N*-methylthioacetamide; the thio-substituted analogue of NMA) we measured values of -6.9×10^{-3} and $-3.8{\cdot}10^{-3}$ ppm K^{-1}, respectively, in CD_3CN. The doublet and triplet structures of the Ala and Gly NH signals due to $^3J_{C\alpha H,NH}$ coupling can only be resolved below 270 K (J = Ala(1) 2.8, Gly ≈5, Ala(3) 6.9 Hz). Moreover, the temperature-dependent FTIR spectra in Figure 4 indicate that the proportion of thiopeptides in non-hydrogen-bonded conformations is becoming small at low temperature. Indeed, the NOESY spectrum at 250 K clearly shows a cross peak between the amide proton of the Aib residue and the Cα proton of Ala(1) (lower part of Figure 6). In ad-

dition, there is a cross peak between the Aib amide proton and the Ala(1) CH_3 protons (data not shown). The presence of these NOESY cross peaks confirms the spatial proximity of the two ends of the peptide sequence, as indicated by the strong cross peak between the C=O vibrations of Boc at 1688 cm^{-1} and Aib/OMe in the 2D-IR spectra.

Photoisomerisation: The thiopeptide bond can be selectively isomerised from the *trans* to the *cis* conformation upon S_2 excitation (π–π^* transition).[12–14] On irradiation at 248 nm we can thus enhance the concentration of peptides in the *cis* state until a photoequilibrium is established by reverse photoisomerisation and thermal relaxation. The difference between the FTIR spectra recorded before and during UV irradiation (thick solid lines in Figure 7) show the absorption

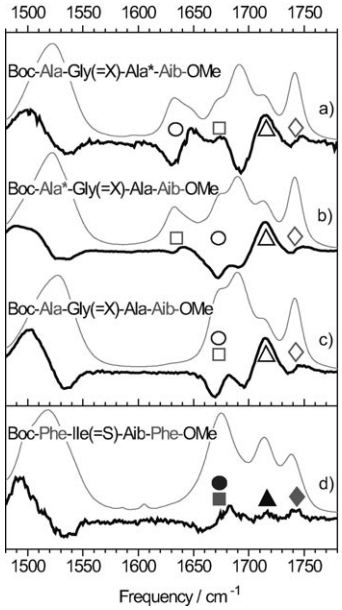

Figure 7. Thick lines: Differences between the FTIR spectra before and during 248 nm irradiation of the three isotopomers of Boc-Ala-Gly(=S)-Ala-Aib-OMe (a–c) and the reference peptide Boc-Phe-Ile(=S)-Aib-Phe-OMe (d). Adsorption bands are labelled by the symbols defined in Figure 1. The absorption spectra of the molecules before irradiation (*trans* conformation of the thioamide bond) are plotted as thin lines. The difference spectra have been normalized to the ester signal at 1740 cm^{-1} and signal intensities cannot be compared to the absorption spectra.

bands in the *trans* state as negative signals and the absorption bands in the *cis* state as positive signals. In both samples the predominant signal is due to bleaching of the band at 1688 cm^{-1} and an absorption increase at 1713 cm^{-1} (△). Moreover, we observe that only Ala(3) (○) shows a significant response (blue shift) to thiopeptide isomerisation, while Ala(1) (□) shows only a small signal similar to that of the Aib/ester group (◇), although both alanines are nearest neighbours of the thioamino acid. The fact that the largest response to photoisomerisation comes from the Boc and the Ala(3) groups is in agreement with the presence of a hydrogen bond between the C=O part of Boc and the NH of Aib (this group forms the amide bond with the C=O of Ala(3)), which is broken upon photoisomerisation of the thioamide unit. Further evidence is provided by comparison of the unlabelled molecule (Figure 7c) with our reference peptide Boc-Phe-Ile(=S)-Aib-Phe-OMe (Figure 7d). Despite the presence of a similar signal in the amide II region at 1520 cm^{-1}, which is evidence of successful isomerisation, 248 nm irradiation of this peptide, without a strongly hydrogen-bonded Boc group, leads only to very weak absorption changes in the C=O stretch region. Note that for all samples the original absorption spectra were fully recovered within minutes after irradiation was stopped, due to thermal back-relaxation of the isomerised molecules.

Discussion

Our characterization of the protected tetrapeptide Boc-Ala-Gly(=X)-Ala-Aib-OMe is based on a number of complementary experiments: linear IR spectroscopy in combination with isotope labelling shows that the peptide can adopt two types of conformations in acetonitrile at room temperature, as reflected by two different C=O stretch transitions of the urethane group. In the conformation that dominates at low temperatures the C=O stretch of Boc is red-shifted by 25 cm^{-1} with respect to the dominant conformations at high temperature, which is compatible with a strongly hydrogen-bonded urethane carbonyl in the former case. Because of the similarity of the IR spectra of the thiopeptides and their oxopeptide analogues, the same kind of conformational heterogeneity seems to be present in the oxopeptides. The 2D-IR spectra clearly distinguish the two types of structures associated with different Boc absorption bands and show that the hydrogen-bonded molecules are characterized by spatial proximity of the two terminal groups, which is confirmed by the observation of NOESY peaks in low-temperature 2D-NMR measurements. Finally, the spectral changes observed upon photoisomerisation of the thiopeptide bond, together with ^1H NMR peak shift data, unambiguously localize the intermolecular hydrogen bond between the amide proton of Aib and the carbonyl group of Boc. Thus, in acetonitrile at room temperature, the peptide under study can adopt a loop structure with an *i*–*i*+4 hydrogen bond, as illustrated in Figure 8. This conformation coexists with an entropically favoured second class of molecules without an intramolecular hydrogen bond, probably comprising several different conformations, which are not resolved.

The ^1H and 2D-NMR spectra do not on their own reveal the coexistence of these different types of structures. This initially even misled us to a wrong interpretation of the infrared spectra of the unlabelled thiopeptide, in which the four C=O groups seemed to be nicely matched by the four resolved bands in the amide I region (Figure 2a). The much higher time resolution that is needed to resolve the looped conformation is indeed the main advantage of the infrared method over NMR, especially when direct structural information about the vicinity of molecular groups is gained

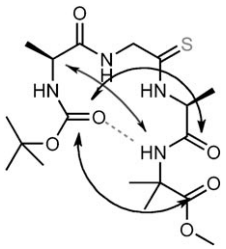

Figure 8. Identification of the hydrogen-bonded conformation of Boc-Ala-Gly(=S)-Ala-Aib-OMe in acetonitrile. The circular arrows indicate the through-space couplings between C=O stretch modes that give rise to 2D-IR cross peaks, while the long, less tightly curved arrow connects the two hydrogen atoms for which a 2D-NMR NOESY peak is observed at low temperatures. The hydrogen bond (dotted line) is identified with the help of temperature-dependent IR and ^{1}H NMR spectra.

from 2D-IR spectra. At the same time the spectral congestion already encountered for a peptide with only four C=O vibrators also illustrates the main difficulty of this new approach, which is at present best overcome by systematic isotope labelling.

Our finding of an *i*–*i*+4 hydrogen bond in Boc-Ala-Gly(=X)-Ala-Aib-OMe is somewhat surprising, and we rather expected a β-turn structure, stabilized by an *i*–*i*+3 hydrogen bond due to the presence of the achiral Aib residue. A hydrogen bond between C=O of Boc and Ala(3) would gain additional strength in the thiopeptide because of the greater acidity of the thioamide proton. Indeed, the crystal structures of the protected tripeptides Boc-Gly-Ala-Aib-OMe and Boc-Gly-Ala(=S)-Aib-OMe, with only one alanine residue less than the molecule investigated here, each exhibit such a hydrogen bond, while Boc-Gly(=S)-Ala-Aib-OMe does not.[17,18] Two *i*–*i*+3 hydrogen bonds between C=O of Boc and NH of Aib (the thioamide proton) and C=O of Phe(1) and NH of Phe(4) have also been identified in the crystal structure of our reference peptide Boc-Phe-Ile(=S)-Aib-Phe-OMe.[21] In CD_3CN solution, however, we find no evidence for this 3_{10}-helical structure nor of any other hydrogen bond for the latter molecule, given that the amide I IR spectrum of the molecule is only weakly perturbed by the isomerisation of the thiopeptide bond. Thus, even in the presence of Aib and of a more strongly hydrogen-bond-donating thioamide unit, the solution structures of these small peptides seem to be mainly governed by their different side-chain interactions.[26] Furthermore, these results confirm that thioacylation of one peptide bond does not significantly perturb the solution structure, thereby creating a photoswitch for the investigation of conformational dynamics in an essentially native peptide.

Examples of protected tetrapeptides featuring *i*–*i*+4 hydrogen bonds have been reported in the literature. More recently, an α-helical turn conformation has been proposed for the sequence Boc-Ala-Ile-Ile-Gly-OMe, on the basis of a characteristic CD signature in trifluoroethanol-containing methanol solution.[27] The CD spectrum of the oxopeptide Boc-Ala-Gly-Ala-Aib-OMe in acetonitrile, on the other hand, shows only a weak negative signal at 230 nm, with a negative minimum near 198 nm. As the temperature is lowered, the 198 nm minimum becomes more pronounced, while a small positive signal near 215 nm partially fills the 240 nm dip observed at room temperature (data not shown). This CD spectrum is clearly incompatible with a right-handed conformation. A more detailed analysis of the thiopeptide 2D-IR spectra, including the cross-peak anisotropies, which could in principle yield the backbone conformation of the molecule,[4,5] is hampered by the coexistence of structures with very similar amide I spectra at room temperature. In the future, however, we will attempt to determine backbone dihedral angles of the peptide in the H-bonded looped conformation directly from low-temperature 2D-IR measurements.

Conclusion

In this paper we have analysed the conformations of a small thiopeptide by linear and two-dimensional IR spectroscopy, as well as by NMR methods. We have shown that in acetonitrile solution at room temperature, 2D-IR spectroscopy can resolve a loop structure in which the amide proton of Aib forms a hydrogen bond with the C=O carbonyl of the urethane group, in coexistence with more extended conformations, which remain unresolved. Loop-opening and -closing happens on a timescale slower than 10 ps, and cannot therefore be detected by 2D-IR exchange spectroscopy,[28] but it is also too fast to be detected by NMR measurements (hundreds of microseconds). At higher temperatures the equilibrium is shifted away from the H-bonded conformation. While the structural details for this particular example of a fully protected peptide in an organic solvent may be very different from those pertaining in naturally occurring peptides and their conformational dynamics in the biological solvent water, the technique very well illustrates the time-resolution advantage of 2D-IR spectroscopy for detecting conformational heterogeneity in biomolecules, which is applicable quite generally. Furthermore, we were able to show that photoisomerisation of the thioamide bond by UV irradiation allows us to phototrigger the breaking of an intramolecular hydrogen bond in a peptide that closely resembles its "native" oxopeptide analogue. The thiopeptide is thus an ideal model system for studying non-equilibrium dynamics. The results of this time-resolved study will be reported soon.

Experimental Section

Synthesis and spectroscopic characterization: The peptide sequences Boc-Ala-Gly-OMe and Boc-Ala-Aib-OMe were obtained by stepwise elongation by the classical method in solution. Fragment condensation yielded the oxopeptide Boc-Ala-Gly-Ala-Aib-OMe. The thiopeptide Boc-Ala-Gly(=S)-Ala-Aib-OMe was then obtained by selective substitution of the Gly C=O group by C=S by treatment with Lawesson's reagent.[17] Solid-phase peptide synthesis of smaller amounts of the isotope-labelled samples followed similar procedures. Intermediate and final

products were tested for purity by ^{1}H NMR spectroscopy, mass spectrometry and TLC (see Supporting Information for details). The synthesis of the thiopeptide Boc-Phe-Ile(=S)-Aib-Phe-OMe by the azirine/oxazolone method has been reported in Reference [21]. Samples were dissolved in deuterated acetonitrile (Armar Chemicals, 99.5 atom% D) at concentrations of 120 mM for the 2D-IR experiments. Tenfold dilution did not change the FTIR spectra. FTIR and 2D-IR measurements were performed in a home-built small-volume flow cell made of two 2 mm-thick CaF_2 windows with a 50 μm pathlength.[29] NMR spectra during synthesis were recorded on a Bruker ARX 300 spectrometer (300.13 MHz). The TOCSY spectrum at room temperature was measured on a Bruker AV 600 spectrometer (600.13 MHz) and the ^{1}H, TOCSY and NOESY spectra at low temperature on a Bruker AV2 500 spectrometer (500.13 MHz) by the NMR service of the University of Zürich. A FTIR spectrometer fitted with an MCT detector (BioRad FTS175) was used to record FTIR spectra at 2 cm^{-1} resolution before and during short irradiation by a KrF excimer laser (248 nm, Lambda Physik).

Population analysis: The relative populations of the two interconverting, spectrally distinct types of conformations were estimated from the FTIR absorption spectra. The spectrally isolated Ala(3) signal of the isotope-labelled sample Boc-Ala-Gly(=X)-Ala*-Aib-OMe was first fitted by two Lorentzian lineshapes of fixed equal width (20 cm^{-1}) with centre frequencies adjusted to the difference spectrum in Figure 7. In order to reduce overlap and intensity borrowing effects for the determination of the relative intensities of the two Boc absorption bands, these fitted lineshapes were then blue-shifted by 40 cm^{-1} (isotope shift) and subtracted from the spectrum of Boc-Ala*-Gly(=X)-Ala-Aib-OMe, recorded at the same temperature and normalized to the Aib/OMe signal at 1740 cm^{-1}. The remaining Boc signals could then be reproduced by two Lorentzian lines of equal width (fixed at 20 cm^{-1} for all temperatures). These fits are not perfect (see Supporting Information), and the use of Gaussian instead of Lorentzian lines or a 10% variation of their width led to variations of the slope in the van't Hoff plot in the 500–1100 K range, equivalent to enthalpy differences of 4–9 kJ mol^{-1}. From the *y*-intercept of these plots the entropic contribution was found to lie between 10 and 30 J mol^{-1} K^{-1}.

2D-IR spectroscopy: Femtosecond pulses for 2D-IR measurements were obtained from an amplified titanium-sapphire laser system (Spectra Physics), operating at 800 nm. Mid-infrared pulses (100–150 fs, 2 μJ per pulse, 200–250 cm^{-1} FWHM) were produced in a home-built double-stage optical parametric amplifier (OPA) followed by frequency mixing in a $AgGaS_2$ crystal.[30] Small fractions of the OPA output were used as probe and reference beams, which were focused at different spots on the sample cell. They were then imaged onto the entrance slit of a spectrometer, dispersed and detected with a double MCT array (2×32 pixels) on a single shot basis with 3 cm^{-1} resolution. The main portion of the mid-IR OPA output was passed through a computer-controlled Fabry Perot interferometer providing narrow-band tuneable IR pump pulses (bandwidth ≈10 cm^{-1}), which were focused onto the sample in spatial overlap with the probe beam. Through scanning of the central wavelength of the pump pulses across the amide I region and monitoring of the induced absorption changes with the broad-band IR-probe pulse at 1 ps delay, 2D-IR spectra were recorded in the frequency domain.[3]

Acknowledgements

We thank Dr. Simon Jurt from the NMR service team of the University of Zürich for recording temperature-dependent ^{1}H and NOESY spectra. This work was supported by the Swiss National Science Foundation (SNF grant 200020–107492) and the Alfred-Werner Legat foundation of the University of Zürich.

[1] P. Hamm, M. Lim, R. M. Hochstrasser, *J. Phys. Chem. A* **1998**, *102*, 6123–6138.
[2] M. Khalil, N. Demirdöven, A. Tokmakoff, *J. Phys. Chem. A* **2003**, *107*, 5258–5279.
[3] S. Woutersen, P. Hamm, *J. Phys. Condens. Matter* **2002**, *14*, R1035.
[4] J. Bredenbeck, P. Hamm, *J. Chem. Phys.* **2003**, *119*, 1569–1578.
[5] S. Woutersen, P. Hamm, *J. Phys. Chem. A* **2000**, *104*, 11316–11320.
[6] M. T. Zanni, M. C. Asplund, R. M. Hochstrasser, *J. Chem. Phys.* **2001**, *114*, 4579–4590.
[7] S. Woutersen, R. Pfister, P. Hamm, Y. Mu, D. S. Kosov, G. Stock, *J. Chem. Phys.* **2002**, *117*, 6833–6840.
[8] J. Bredenbeck, J. Helbing, A. Sieg, T. Schrader, W. Zinth, C. Renner, R. Behrendt, L. Moroder, J. Wachtveitl, P. Hamm, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6452.
[9] M. Volk, Y. Kholodenko, H. S. M. Lu, E. A. Gooding, W. F. DeGrado, R. M. Hochstrasser, *J. Phys. Chem. B* **1997**, *101*, 8607–8616.
[10] J. Bredenbeck, J. Helbing, J. R. Kumita, G. A. Woolley, P. Hamm, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 2379–2384.
[11] C. Kolano, J. Helbing, M. Kozinski, W. Sander, P. Hamm, *Nature* **2006**, *444*, 469–472.
[12] J. Helbing, H. Bregy, J. Bredenbeck, R. Pfister, R. H. P. Hamm, J. Wachtveitl, L. D. Vico, M. Olivucci, *J. Am. Chem. Soc.* **2004**, *126*, 8823–8834.
[13] H. Satzger, C. Root, P. Gilch, W. Zinth, D. Wildemann, G. Fischer, *J. Phys. Chem. B* **2005**, *109*, 4770–4775.
[14] J. Zhao, J.-C. Micheau, C. Vargas, C. Schiene-Fischer, *Chem. Eur. J.* **2004**, *10*, 6093–6101.
[15] J. H. Miwa, A. K. Patel, N. Vivatrat, S. M. Popek, A. M. Meyer, *Org. Lett.* **2001**, *3*, 3373–3375.
[16] J. H. Miwa, L. Pallivathucal, S. Gowda, K. E. Lee, *Org. Lett.* **2002**, *4*, 4655–4657.
[17] O. E. Jensen, S. O. Lawesson, R. Bardi, A. M. Piazzesi, C. Toniolo, *Tetrahedron* **1985**, *41*, 5595–5606.
[18] R. Bosch, G. Jung, W. Winter, *Liebigs Ann. Chem.* **1982**, *1982*, 1322–1329.
[19] R. Nagaraj, P. Balaram, *Acc. Chem. Res.* **1981**, *14*, 356–362.
[20] I. Thomsen, K. Clausen, S. Scheibye, S.-O. Lawesson, *Org. Synth.* **1984**, *62*, 158–162.
[21] J. Lehmann, A. Linden, H. Heimgartner, *Tetrahedron* **1999**, *55*, 5359–5376.
[22] S. Ham, M. Cho, *J. Chem. Phys.* **2003**, *118*, 6915–6922.
[23] H. Torii, T. Tatsumi, T. Kanazawa, M. Tasumi, *J. Phys. Chem. B* **1998**, *102*, 309–314.
[24] The factor by which the perpendicular signal was scaled in order to suppress the amide I diagonal signals in the difference spectra (factor used: 2.75) is not suitable for the ester band (factor: 2.64). As a result, in the weighted difference between the 2D-IR spectra recorded with parallel and perpendicular polarization the diagonal peak of the ester band is still weakly present. This difference (and the deviation from the theoretical scaling factor of 3) is tentatively assigned to faster fluctuations in the orientation of the ester C=O mode, which leads to a partial anisotropy decay before the probe pulse arrives 1 ps after the pump pulse.
[25] H. Kessler, *Angew. Chem.* **1982**, *94*, 509–520; *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 512–523.
[26] Another reason for the differences between the two tetrapeptides, in addition to that between crystal and solution structures, may be the conformation of the peptide bond between the Boc protection group and the first amino acid, which can adopt both the *Z* and the *E* forms. Crystalline Boc-L-phenylalanine, for example, is found in the *E* form, while the *Z* form typical of a regular peptide bond seems to dominate in solution (refs. [31,32]).
[27] S. Ganesh, R. Jayakumar, *J. Pept. Res.* **2002**, *59*, 249–256.
[28] S. Woutersen, Y. Mu, G. Stock, P. Hamm, *Chem. Phys.* **2001**, *266*, 137–147.
[29] J. Bredenbeck, P. Hamm, *Rev. Sci. Instrum.* **2003**, *74*, 3188–3189.
[30] P. Hamm, R. A. Kaindl, J. Stenger, *Opt. Lett.* **2000**, *25*, 1798–1800.
[31] E. Benedetti, B. Di Blasio, V. Pavone, C. Pedone, C. Toniolo, G. M. Bonora, *Int. J. Biol. Macromol.* **1980**, *2*, 217–224.
[32] J. W. Bats, H. Fuess, H. Kessler, R. Schuck, *Chem. Ber.* **1980**, *113*, 520–530.

Received: March 9, 2007
Revised: June 22, 2007
Published online: August 15, 2007

