On the Thermal Stability of β-Peptides: A Two-Dimensional Vibrational Spectroscopy Study

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A temperature-dependent 2D-IR study of the amide-I band of a β-peptide forming a *12*/*10*/*12*/*10* helix is presented. Cross-relaxation of a spectrally separated marker amide-I mode, which could be assigned with the help of the NMR structure of the molecule, can be used as measure of conformational flexibility of the molecule. We find that the conformational flexibility of the N-terminal part of the helix increases slightly upon increasing the temperature from 0° to 80°. The cross-peaks in the 2D-IR spectrum, and hence the connectivity of the corresponding peptide units, do not change, suggesting that the N-terminal part of the helix remains essentially intact at 80°. This conclusion is in agreement with previous NMR and CD measurements.

Introduction. – Protein folding is the process of transferring information from one dimension into three dimensions, where the information that determines the molecular structure of a protein is encoded in its amino acid sequence. Understanding the mechanism by which the amino acid sequence of a protein folds into its unique native conformation is one of the biggest challenges in biophysical chemistry.

The major degrees of freedom of the polypeptide chain are two σ-bonds in each amino acid, which can freely rotate. The amide group (*i.e.*, −CO−NH−) itself is close to planar since the C−N bond has partial double-bond character. The free-energy surface of the two-dimensional configuration space of each amino acid has been extensively studied. A complicated balance of different forces results in two main local free-energy minima, which are of almost equal depth. Because of the large dimensionality of the problem in polypeptides and proteins, and because of the

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uncertainty in the exact values of the free energies, protein folding is an extremely difficult problem.

A few years ago, *Seebach* and co-workers have introduced a new class of molecules with several interesting properties, the so-called β-peptides [1–6]. The essential difference compared to natural α-peptides is the introduction of an additional C-atom in the amino acid backbone. From the apparent greater flexibility of β-peptides and the additional torsional variable in the β-amino acid residue compared to the α-amino acids, one might have expected that longer β-peptidic oligomers would be required to form stable secondary structures. It was, therefore, surprising that, despite the seemingly higher configurational flexibility (three rather than two σ-bonds for each amino acid), extremely short sequences of six to seven β-amino acids are capable to form stable secondary structures, whereas, in α-peptides, 20 amino acids are necessary to form, for example, a natural α-helix in aqueous solution. Furthermore, NMR studies revealed that certain β-peptides adopt stable secondary structures that persist at high temperatures, and indicated that there is no cooperative melting of the helix. [7]. An all-atom molecular-dynamics (MD) simulation of the folding process of β-peptidic helices and of β-peptidic hairpin has been performed recently [8–10], demonstrating that their structures can be predicted from an entirely extended conformation within a simulation time of nanoseconds (without adding any NOE constraints). Natural peptides are believed to fold on a microsecond timescale, which is still considerably longer than typically achievable MD simulation times [11]. The extraordinary stability of β-peptides, therefore, suggests that their secondary structures are much better predictable. Furthermore, by choosing the side substitution pattern and configuration of the β-amino acids, a series of β-peptide secondary-structure motifs has been designed and synthesized. Apart from turns, hairpins, and various helices with 14-, 12-, and 10-membered rings, another type of helix, a *12/10* helix with alternating 12- and 10-membered H-bonded rings was identified [12][13]. The structural properties of various β-peptides with alternating β^2/β^3- or β^3/β^2-sequences have been extensively studied by NMR spectroscopy. Complementary *ab initio* calculations have verified that the (*S*)-β^2/(*S*)-β^3 and the (*S*)-β^3/(*S*)-β^2 sequences are ideal for the formation of 10- or 12-membered rings, respectively [14–16]. Since β-peptides are structurally related to α-peptides, they present an ideal model system to study and deepen our understanding of protein structure, folding, and stability.

Recently, nonlinear two-dimensional infrared (2D-IR) spectroscopy has been introduced as a novel spectroscopic tool, which allows revealing detailed structural information of small peptides [17–20]. This spectroscopy allows measurement of the coupling between certain vibrational modes of different peptide units within the polypeptide chain. The vibrational modes under study are the so-called amide-I modes (mostly the C=O stretching mode of the peptide unit) located between 1600 and 1700 cm^{-1}. Since the coupling strength is related to the relative orientation of the peptide units [21–23], one might deduce the structure from a 2D-IR spectrum, as has been demonstrated recently for the first time for a tripeptide [18][19]. The potential of this spectroscopy is the intrinsically high time resolution, which allows following structural motion on a picosecond and shorter timescale [24].

A simulation study of the 2D spectroscopic response of a small β-peptide as a function of temperature has been published recently [25]. To model inhomogeneous

broadening, which is directly related to structural heterogeneity, an all-atom MD simulation was used to generate a collection of instantaneous structures of the polypeptide. For each structure, an amide-I model Hamiltonian was deduced by means of simple empirical models for the coupling between the amide-I modes of different peptide units (the off-diagonal matrix elements of the Hamiltonian) as well as for the H-bond-induced frequency shift of amide-I modes (the diagonal matrix elements of the Hamiltonian). The spectroscopic response was calculated as average over all structures. Despite of the simple model for the molecular Hamiltonian, this approach fully takes into account the complicated correlation of the different elements of the Hamiltonian and shows qualitatively that spectroscopic signatures might be expected upon melting of the helix.

In the present paper, we report on the 2D-IR spectrum of a short β-peptide consisting of β^2- and β^3-amino acid residues (*Fig. 1*) and its temperature dependence.

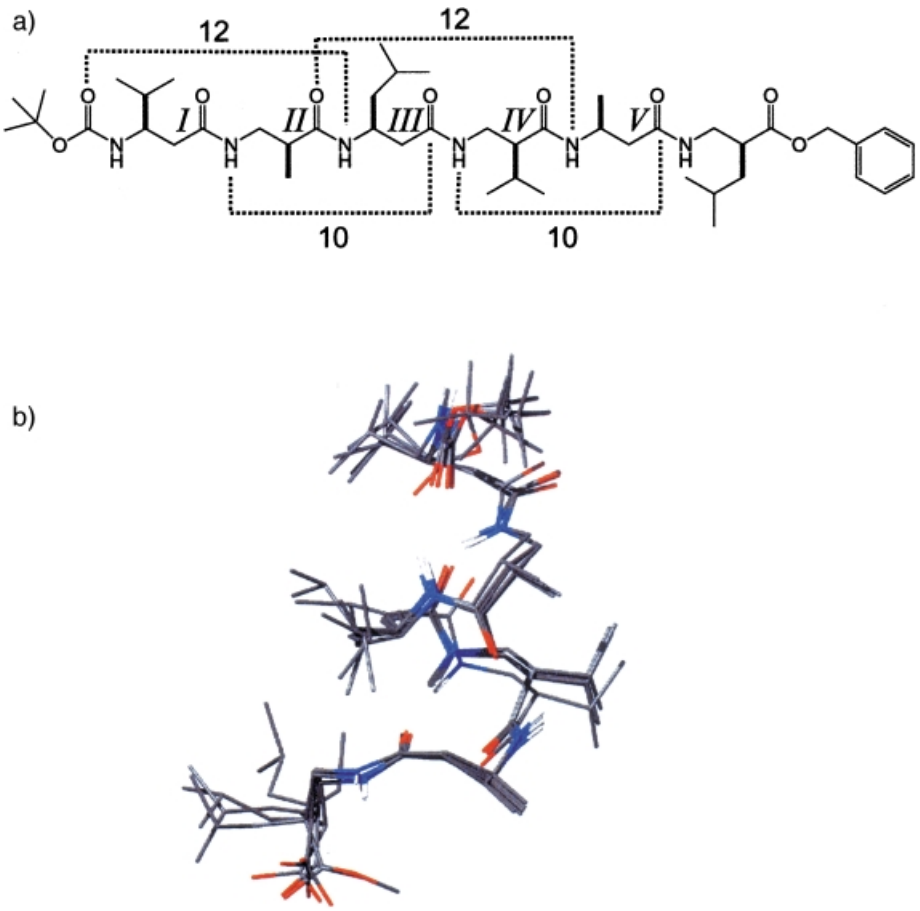

Fig. 1. a) *The β-peptide used in this investigation*. The H-bonded rings and the numbering of the peptide units are indicated. b) *NMR Solution structure of the β-peptide in MeOH obtained by simulated annealing calculations.* A bundle of 5 conformers that adopt a *12/10/12/10*-helix with the C-terminal protecting group omitted [26].

NMR Investigations revealed that this β-peptide adopts a *12*/*10*/*12*/*10*-helical structure with alternating wide 12-membered H-bonded rings and narrow 10-membered H-bonded rings, where the first 12-ring involves the N-terminal protecting group [26]. The C-terminal benzyl protecting group, on the other hand, cannot participate in the H-bond network, leading to a larger flexibility at the C-terminus of the helix.

Experimental Results. – *Linear Absorption Spectrum*. *Fig. 2* shows the (linear) IR absorption spectra of the β-peptide in the spectral range of the C=O bands at 0° and 80°, respectively. Four absorption bands are clearly resolved. The main band around 1640 cm^{-1} exhibits additional substructure, showing up as shoulders at the low- and the high-frequency wing of the band. A multi-line Lorentzian fit of the 0° spectrum is used to decompose the band[3]), yielding the peak positions at 1618, 1634, 1647, 1681, 1717, and 1737 cm^{-1} (see *Fig. 2*, dotted lines).

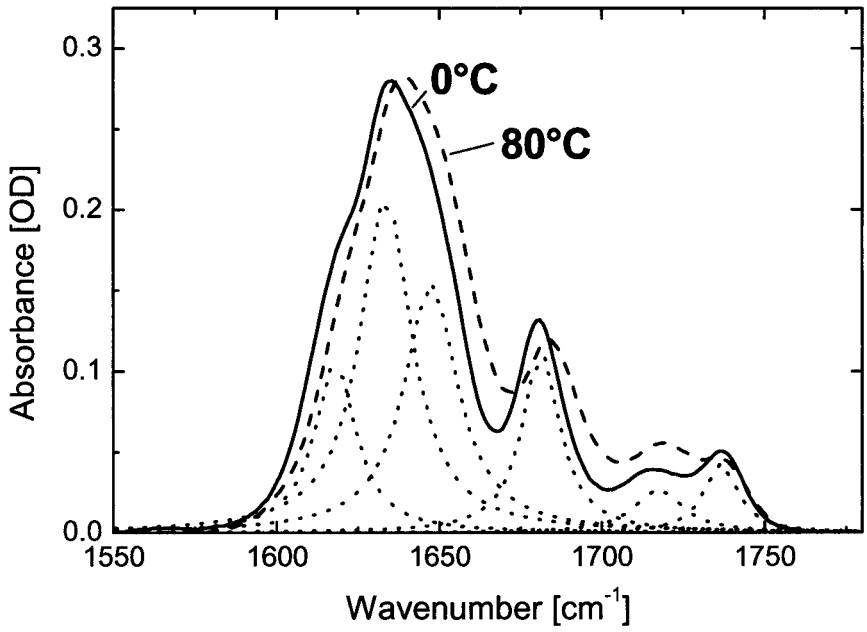

Fig. 2. *The IR absorption spectrum of the investigated β-peptide in the spectral range of the* C=O *bands.* Solid line: 0° and dashed line: 80°. A weak contribution of the solvent, CD_3OD, was measured independently and subtracted from the data. The dotted lines depict the decomposition of the 0°-spectrum by a multi-line Lorentzian fit.

As the temperature is increased to 80° (dashed line), a general blue-shift (*ca.* + 5 cm^{-1}) together with a broadening of all spectral features is observed. The first effect is a consequence of an overall weakening of inter- and intramolecular H-bonds, while the

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[3]) A Lorentzian line shape is as arbitrary as a Gaussian line shape would be. The spectra are neither purely homogeneously nor purely inhomogeneously broadened, but dynamic processes have to be taken into account [27]. Therefore, the result from this multi-line decomposition should be understood more as a labeling of the states underneath the absorption spectrum rather than as a physical interpretation.

second is due to increased fluctuations and faster dephasing. In addition, the 1717-cm^{-1} absorption band gains some oscillator strength from the 1681-cm^{-1} amide-I state. This anti-correlation indicates coupling between the two states. Since the intensities of the $\nu = 0 \rightarrow 1$ transitions of two coupled vibrational modes depend on the degree of mixing and the relative orientation of the corresponding transition dipoles, this result suggests that the average peptide conformation changes somewhat upon heating.

Nonlinear 2D-IR Spectrum. *Fig. 3* shows the 2D-IR spectrum at 0° and a pump/probe delay of 750 fs[4]). Pump and the probe beams were polarized parallel in *Fig. 3*,*a* and perpendicular in *Fig. 3*,*b*. *Fig. 3*,*c* shows a weighted difference of both, with the weighting factor chosen in such a way that the otherwise dominating diagonal contribution (whose anisotropy ratio ideally is 1/3) is suppressed, and only the off-diagonal contribution remains [18][28]. Along the diagonal, where the strongest signal is observed in *Fig. 3*,*a* and *b*, each transition responds with a negative signal (bleach and stimulated emission; blue colors) and excited-state absorption, which is red-shifted by (diagonal) anharmonicity (red colors). The diagonal contribution essentially reflects the linear absorption spectrum. In the off-diagonal region, on the other hand, cross-peaks emerge, each of which also consists of a negative bleach and positive excited-state absorption. These cross-peaks represent the anharmonic shift of one transition, when the other is excited, and manifest the couplings between the corresponding states. A series of cross-peaks can be identified (see arrows in *Fig. 3*,*b* and *c*): cross-peaks between the high-frequency amide-I state (1681 cm^{-1}) and all states underneath the main band emerge as a broad ridge elongated along the pump-frequency axis. These cross-peaks are well-separated from the diagonal ridge, and hence are directly evident

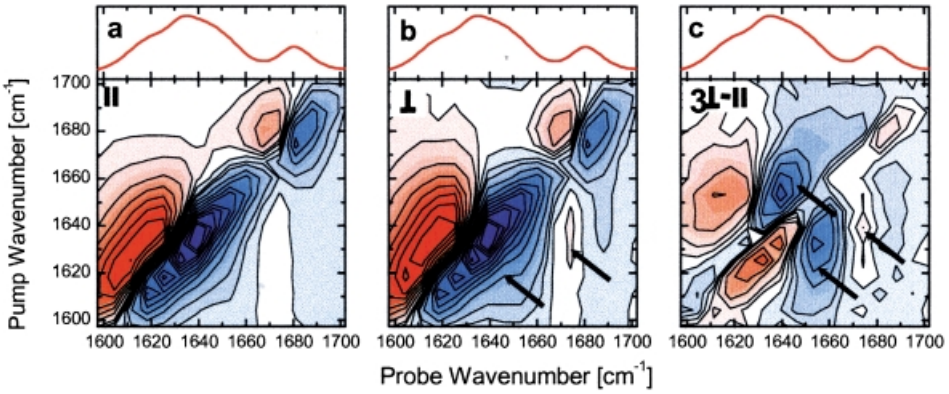

Fig. 3. *2D-IR Spectrum of the* 12/10/12-*helix measured for a pump/probe delay of* 750 *fs and at a temperature of* 0°. *a*) and *b*) show the response with parallel and perpendicular polarization of pump and the probe beam, respectively, *c*) shows a weighted difference of both, with a weighting factor chosen to suppress the dominating diagonal contribution. The arrows mark prominent cross-peaks.

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[4]) The separation between the fast-rising onset of the pump pulse and the probe pulse was chosen $T = 750$ fs. At this delay-time position, the signal strength approaches its maximum. On the other hand, the temporal overlap between the slowly falling wing of the pump pulse (see *Exper. Part*) and the probe pulse is small at this delay time so that contribution to the signal due to non-time-ordered pump and probe-field interactions (the so-called coherence peak) can be neglected in the analysis of the data.

in the parallel and perpendicular 2D spectrum. Another strong cross-peak between the 1634 and 1647-cm^{-1} bands, which overlaps with the diagonal contribution, is more clearly visible in the diagonal-suppressed representation of *Fig. 3*, *c*.

Fig. 4 shows the nonlinear response of the 1681-cm^{-1} band upon exciting the 1717-cm^{-1} hand. An anharmonic shift of the 1681-cm^{-1} band is clearly resolved, indicating that the corresponding molecular groups are strongly coupled, and hence in close contact. One can estimate the coupling strength using the perturbative expression [17][29]:

$$\Delta\varepsilon_{12} = -4\Delta\frac{\beta^2}{(\varepsilon_1-\varepsilon_2)^2} \tag{1}$$

which is valid in the localization limit where $|\beta| \ll |\varepsilon_1-\varepsilon_2|$. Here, $\Delta\varepsilon_{12}$ is the anharmonic shift of the 1681-cm^{-1} band upon excitation of 1717-cm^{-1} band, $\Delta \approx 16\ \text{cm}^{-1}$ the intrinsic anhamonicity of a C=O group, which gives rise to the response of the excited state itself, and $(\varepsilon_1-\varepsilon_2) = 36\ \text{cm}^{-1}$ the frequency separation of both bands. From the ratio of the absorption band intensities and the ratio of the nonlinear pump/probe signals, one can estimate for the coupling constant $|\beta| = 4 \pm 2\ \text{cm}^{-1}$.

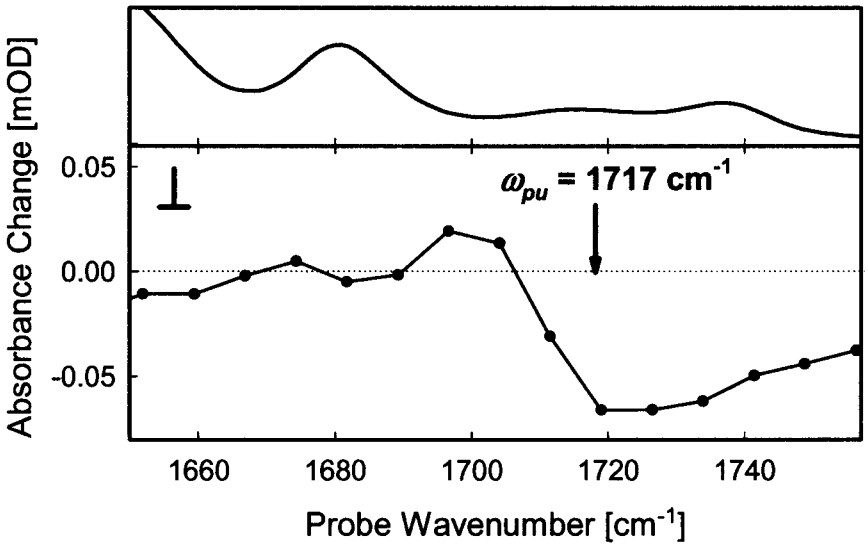

Fig. 4. *Nonlinear response of the 1681-cm^{-1} band after pumping the C=O band of the N-terminal protecting groups at 1717 cm^{-1}.* Pump/probe delay was set to 750 fs. The arrow marks the pump frequency. Pump and probe pulse polarization are perpendicular.

In *Fig. 5*, *a* and *b*, we compare the 2D-IR spectrum at 0° with that at 80° (perpendicular polarizations, pump/probe delay of 750 fs). The 2D-IR spectrum remains essentially the same upon heating, except for a slight broadening of all spectral features. In particular, the cross-peak structure remains unchanged. *Fig. 5*, *c* and *d* shows the 2D-IR spectrum with the delay time between pump and probe pulses increased to 4.5 ps. The overall decay of the signal due to vibrational T_1 relaxation has been normalized out in *Fig. 5*. The most pronounced effect of the increasing delay is an

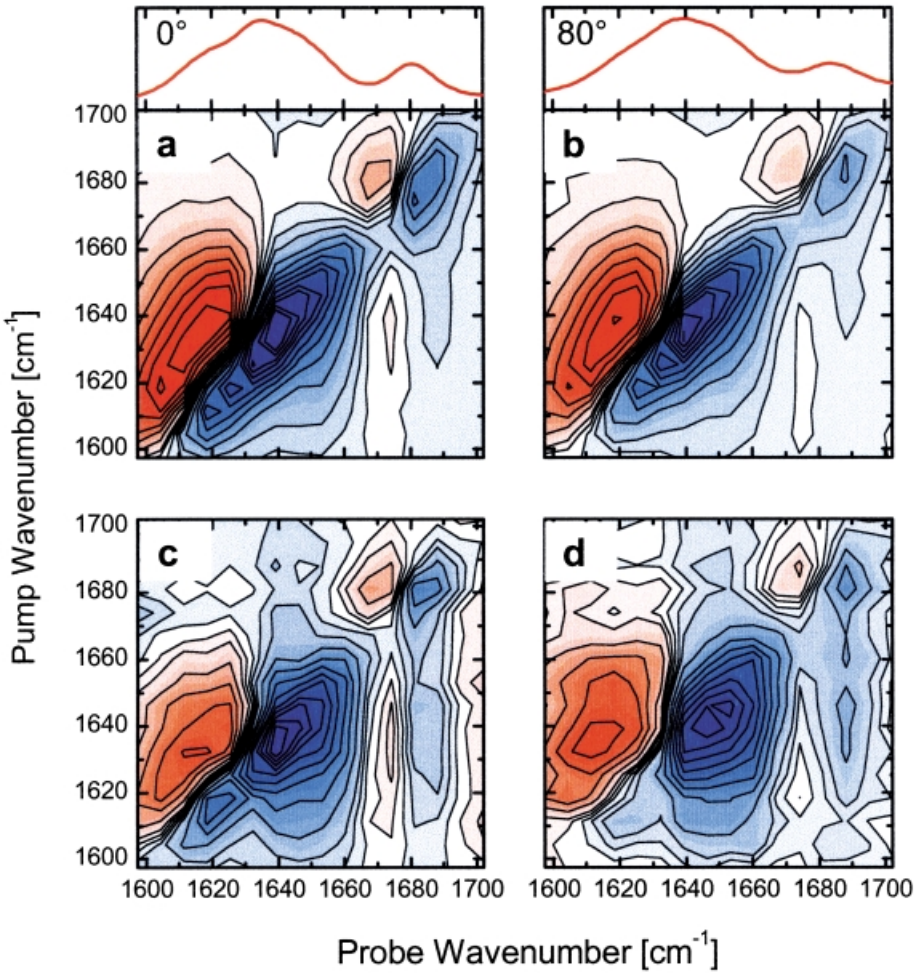

Fig. 5. a) *2D-IR Spectrum of the* 12/10/12/10-*helix measured for a temperature of* 0° *and a pump/probe delay time of 750 fs* (same data as in *Fig. 3, b*). b) *The 2D-IR spectrum measured at* 80° *under otherwise identical conditions.* c) *and* d) *The same conditions as in* a *and* b, *except for the delay time between pump and probe pulse increased to 4.5 ps* (*b* and *d*). Pump and probe pulse were polarized perpendicular in all experiments.

increase of the cross-peak intensity (measured relative to the diagonal-peak intensity), reflecting an irreversible population cross-relaxation between the corresponding states. This effect has been investigated in detail for a small tripeptide [24], and it has been suggested that population transfer is related to ultrafast fluctuations of the peptide backbone. As a measure of the population ratio, the ratio of the bleach signal intensity of the 1634-cm^{-1} band after selective excitation, and that of the cross-coupled band at 1681-cm^{-1} is shown in *Fig. 6*. Population transfer is *ca.* 50% more efficient at 80° as compared to that at 0°.

Finally, it should be noted that T_1 relaxation of the amide-I states is essentially the same for all peptide units and nearly independent on temperature. Within the

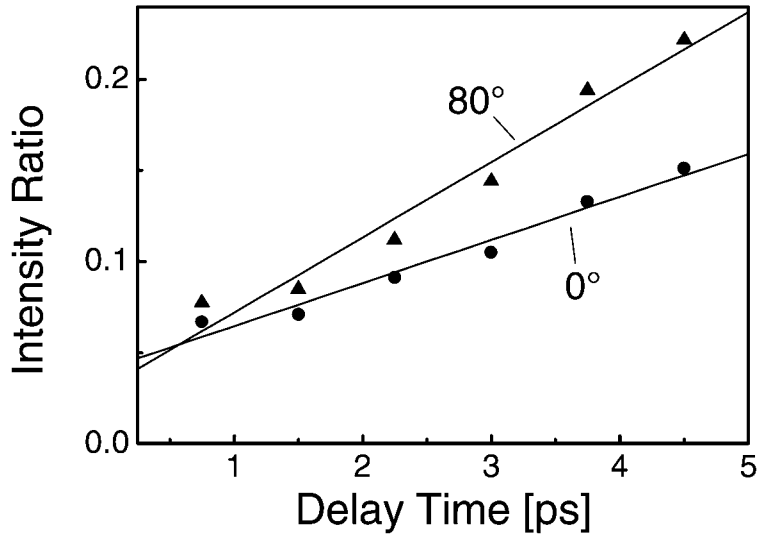

Fig. 6. *Ratio of the bleach signal of the 1634-cm^{-1} band after selective excitation and that of the cross-coupled band at 1681 cm^{-1}.* These data are compared for temperatures of 0° (*circles*) and 80° (*triangles*).

accessible time window, the decay proceeds single-exponentially with a time constant of *ca.* $T_1 = 1.5 \pm 0.2$ ps at 0° and 1.3 ± 0.2 ps at 80°, respectively (data not shown). Hence, as has been noted before [24][29][30], T_1 relaxation of amide-I modes appears to be an intrinsic property of the peptide unit, and is not significantly affected by the local chemical structure (*e.g.*, the amino acid residue or the difference between α- and β-peptides), the secondary structure, the solvent, the temperature [31], or the extent of delocalization.

Assignments. – Two observables can, in principle, be deduced for each pair of vibrational bands from the nonlinear 2D spectra: the coupling strength, given by the cross-peak amplitude, and the angle between corresponding dipole moments, given by the cross-peak anisotropy [18][19]. With the help of quantum-chemical models, which describe these quantities as a function of the conformation of the molecule [22][23], one might invert the problem and deduce the structure from the 2D experiment. This has been demonstrated recently for the smallest possible system, tri-alanine, which has only two peptide units [18][19]. However, in total seven C=O bands contribute to spectra of the *12/10/12/10*-helix, some of which strongly overlap. Due to the crudeness of the coupling models currently available, a structure determination based on 2D-IR spectroscopy is not possible at this point and remains to be developed. We, therefore, assign only some of the C=O bands, using the known NMR structure of the molecule [26].

Owing to their frequency, which is typical for ester groups, the two highest frequency bands (1717 and 1738 cm^{-1}) can with certainty be assigned to both terminal protecting groups (see *Fig. 1*). The remaining four bands (*i.e.*, 1618, 1634, 1647, and 1681 cm^{-1}) are assigned to the residual C=O groups, namely the amide-I vibrations of

the five peptide units of the molecule. In a system of coupled states, individual states can gain significant oscillator strength from others, depending on the degree of mixing and the orientation of the corresponding transition dipoles. Hence, the fact that only four (instead of the expected five) transitions are observed experimentally can readily be understood.

One of the amide-I states (1681 cm^{-1}) is unusually high in frequency and well-separated from all others. We will use this amide-I band as marker mode in the following. Two mechanisms potentially can spread out amide-I frequencies over a large spectral range:

Chemical shift: Amide-I vibrations respond extremely sensitively to electron-drawing and pushing effects in their vicinity. In particular, inter- or intramolecular H-bonding sensitively affects the amide-I frequency. *Ab initio* calculations [32] on *N*-methylacetamide (NMA) have suggested that the amide-I frequency downshifts by *ca.* 20 cm^{-1}, when the C=O group is H-bonded. An even larger shift of *ca.* 40 cm^{-1} is observed experimentally for the amide-I frequency of NMA in H_2O compared with that in DMSO [30].

Excitonic delocalization: Excitonic coupling further splits the amide-I states. However, since the coupling strength is typically on the order of $|\beta| < 10$ cm^{-1} [22][23], the 50-cm^{-1} separation of the 1681-cm^{-1} band from the main peak is too large to be explained by excitonic splitting only.

Hence, we conclude that the up-shift of the 1681-cm^{-1} peak is a chemical-shift effect, and reflects the absence of a H-bond on one of the C=O bands. According to the NMR structure, only the two peptide units I and IV do not form intramolecular H-bonds in the *12/10/12/10* helix (see *Fig. 1*, *a* and *b*). Both units are moreover efficiently separated from the surrounding MeOH molecules by amino acid side chains and the terminal protecting groups and, therefore, are unlikely to form intermolecular H-bonds with solvent molecules. The strongly blue-shifted 1681-cm^{-1} peak is, therefore, due to either peptide unit I or IV. Distinction between both possibilities is based on the nonlinear 2D-experiment, which yields a pronounced coupling between the 1681-cm^{-1} band and one of the terminal C=O groups at 1717 cm^{-1} (see *Fig. 4*). Hence, the corresponding peptide unit must be in the vicinity of one of the terminal protecting groups. Also the temperature-dependent anti-correlation of the 1681- and the 1717-cm^{-1} band indicates connectivity of both states. According to the NMR structure, the distance between unit I and the C=O of the N-terminal protecting group is 4.5 Å, while that between unit IV and the C-terminal ester group is considerably larger (6.5 Å). Furthermore, the relative orientation of peptide unit I and the N-terminal C=O group is favorable for a relatively strong electrostatic coupling. Using the NMR structure, one can estimate a coupling strength in the order of $\beta = -3 \pm 1$ cm^{-1}, in reasonable agreement with the experimental result $|\beta| = 4 \pm 2$ cm^{-1}. The coupling between the C-terminal ester group and unit IV, on the other hand, is estimated to be $|\beta| < 1$ cm^{-1}. Combining these arguments, we assign the 1681- and the 1717-cm^{-1} bands to states that are mostly localized on the C=O group of unit I and the N-terminal protecting group, respectively. This assignment is consistent with the frequency of the 1717-cm^{-1} band, which is indeed expected to be lower than that of the C-terminal ester group, since both *a*) the amide group and *b*) the intramolecular H-bond forming the first 12-membered ring of the helix tend to lower the frequency of the corresponding C=O vibration.

Discussion and Conclusions. – Having assigned the 1681-cm^{-1} band to peptide group I, we will now use this band as marker mode to study structure and conformational flexibility of the helix. *Figs. 5* and *6* show intermode cross-relaxation between the marker mode and the remaining vibrational states of the system. It has been shown recently that inter-mode cross-relaxation between two modes is related to conformational dynamics through the expression [24].

$$k = \frac{1}{\hbar^2} \int_{-\infty}^{\infty} \langle \delta\beta(t)\delta\beta(0)\rangle e^{i\Delta\varepsilon t/\hbar} dt \qquad (2)$$

where k is the transfer rate, $\Delta\varepsilon$ the energy gap between both states, and $\delta\beta(t) = \beta(t) - \langle\beta\rangle$ the deviation of the coupling constant from its average value. Since the instantaneous coupling $\beta(t)$ is directly related to the distance and relative orientation of the corresponding peptide units [21–23], the transfer rate is a measure of structural flexibility of the peptide backbone. MD Simulations have revealed that the decay of the correlation function $\langle\delta\beta(t)\delta\beta(0)\rangle$ contains at least two contributions [24]: an ultrafast inertial process ($\tau_c \approx 100$ fs) and a much slower, diffusive process ($\tau_c \gtrsim 3$ ps). The cross-relaxation rate is in particular sensitive to the fast contribution, for which $1/\tau_c \approx \Delta\varepsilon$ roughly matches the energy gap between both amide-I states, while it is essentially blind to the slower, diffusive part. As expected, the decay rate, and hence structural flexibility of the peptide backbone, increases with temperature. From *Fig. 6*, it is seen that upon increasing the temperature from 0° to 80°, the cross-relaxation rate increases by *ca.* 50%. Since this rate depends linearly on $\langle\delta\beta^2\rangle$ (see *Eqn. 2*), this result implies that the structural flexibility (rms of the fluctuations) increases by *ca.* 25%. The NMR-structure bundle indicates that peptide-backbone structure is relatively rigid at the N-terminus of the helix, while it is unstructured towards the carboxy end (see *Fig. 1,b*) [26]. The 1681-cm^{-1} marker mode, *i.e.*, peptide unit I, lies in the rigid part of the helix, where (according to the 2D-IR result) structural flexibility increases by only *ca.* 25% upon heating.

More important, however, is the observation that the overall characteristics of the 2D-IR spectra do not change significantly upon heating. In particular, the largely unaffected coupling pattern of the 1681-cm^{-1} marker mode to the remaining amide-I modes of the helix indicate that the helical structure remains intact in the N-terminal part of the helix. In fact, it has been shown in a recent temperature-dependent MD simulation study of a β-peptide that the 2D-IR spectrum should change remarkably upon melting of the helix [25]. Since we do not observe this, we conclude that the β-peptide does not unfold even above the (normal pressure) boiling point of the solvent, MeOH. In contrast, α-helices (with natural α-peptides) do exhibit thermal unfolding, which gives rise to considerable changes in the amide-I absorption spectrum [33]. Accordingly, a 2D-IR study of an α-helix has shown that the coupling between an isotopically labeled amino acid and the rest of the helix disappeared upon melting of the helix [34].

The standard methods to elucidate helical structures of peptides are NMR and CD spectroscopy. By these methods, *Seebach* and co-workers have concluded that β-peptides do not unfold at 80° [7]. This result was initially in contradiction with MD simulations, which predicted a melting temperature (*i.e.*, the temperature where the peptide is to 50% unfolded) of *ca.* 67° [9–11]. Only after modifying the force field, *van*

Gunsteren and co-workers were able to explain a melting temperature $>87°$ [35]. However, the information content of NMR and CD spectroscopy has been questioned recently in particular for very small peptides, which undergo extremely rapid conformational jumps. NMR Spectra contain no information about the fraction of folded molecules on this timescale [36]. Also the information content of CD spectra, which has been developed mostly as an empirical method for natural α-proteins, seems to be limited in the case of small flexible helices [37].

Clearly, new structure-sensitive spectroscopic methods are needed. 2D-IR spectroscopy is not yet developed to an extent that allows to determine the structure of a peptide as 'big' as the one investigated here. Nevertheless, the existence of cross-peaks unambiguously establishes spatial connectivity of the corresponding groups. Since the cross-peak structure does not change with temperature, we conclude that the helix remains intact even at 80°. The measurement-time scales of a NMR experiment and that of a 2D-IR experiment are completely different. Conformational substrates interchange very rapidly on the NMR timescale (1 ms – 1 s) so that NMR spectra relate to the time-averaged structure of the molecule [36]. In contrast, the 2D-IR timescale (1 ps) is much faster than conformational jumps of the molecule. 2D-IR Spectroscopy is, therefore, not blurred by the conformational jumps, and the total 2D-IR spectrum is a weighted superposition of the 2D-IR spectra of each of the conformational substates of the ensemble [38]. In that sense, our results are complementary to the work reported in [7], and they strongly support the conclusions of that work concerning the remarkable stability of β-peptides.

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Experimental Part

Material and Methods. The experimental laser setup has been described in detail in [39]. The output of a commercial Ti:Sapphire amplifier was used to pump a white-light seeded, two-stage optical parametric amplifier based on BBO. Difference-frequency generation of signal and idler in $AgGaS_2$ was used to obtain mid-infrared pulses, which, at 1650 cm^{-1}, have a duration of 100 – 150 fs, and an energy and bandwidth of 1 μJ and 200 cm^{-1}. A small fraction of the mid-infrared pulses was split off to obtain broadband probe and reference pulses. The remainder was passed through an IR *Fabry-Perot* filter to obtain narrow-band pump pulses (bandwidth *ca.* 10 cm^{-1}, pulse duration *ca.* 750 fs FWHM), the center frequency of which could be varied by adjusting the *Fabry-Perot* filter. Transient absorption spectra were measured by frequency-dispersed detection of the probe and reference pulses by means of a 2×16 HgCdTe detector array. 2D Vibrational spectra were obtained by scanning the pump frequency and recording transient spectra for each pump frequency. Scans with parallel or perpendicularly polarized pump and probe pulses are recorded by adjusting a zero-order $\lambda/2$ wave plate placed in the pump beam.

The sample was dissolved in CD_3OD and kept between two CaF_2 windows separated by a 50-μm spacer. From the absorbance of the amide-I band ($A = 0.2$), a sample concentration of *ca.* 15 mM was estimated (assuming for the extinction coefficient of one amide-I vibrator $\varepsilon = 1000$ M^{-1} cm^{-1}). Full D exchange of the amide H-atom was checked by monitoring the amide-II band. The brass CaF_2 window mount was thermalized between 0° and 80°, and it has been verified that the sample temp. was equal to the holder temp. to within 2 K.

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