The technique of transient two-dimensional infrared (T2D-IR) spectroscopy is introduced, which extends the advantage of 2D-IR spectroscopy to the investigation of a transient species with picosecond time resolution. The conformational change of a small cyclic peptide is studied in the amide-I spectral range. The conformation is controlled by means of a photoswitch integrated into the peptide backbone. Substantial changes are found in the transient 2D-IR spectra at times when the transient 1D spectra show only a minor time dependence, illustrating the information gain accessible from 2D-IR spectroscopy. In contrast to 1D spectroscopy, 2D-IR can distinguish between homogeneous and inhomogeneous broadening. The homogeneous contribution to the total width of the amide-I band changes during the course of the conformational transition, a result that is interpreted in terms of the manner in which the peptide samples its conformational space.

1. INTRODUCTION

Recent work has shown that nonlinear 2D infrared (2D-IR) spectroscopy might be a valuable experimental complement to 2D-NMR for studying molecular systems (see Ref. 1 and references therein). In the case of small peptides, 2D-IR spectroscopy allows to measure the coupling between the amide-I vibrational modes of different peptide units within the polypeptide chain. Via the couplings and the polarization dependence one can obtain structural information from a 2D-IR spectrum. This has been demonstrated recently in the case of trialanine.

The most promising potential of 2D-IR spectroscopy is the combination of this structure resolution power with its intrinsic high time resolution of about 1 ps - many orders of magnitudes faster than what 2D-NMR spectroscopy can achieve - which allows for freezing in all but the fastest sub-picosecond motions of the peptide backbone. Up to now, 2D-IR spectroscopy has only been applied to equilibrium states. This proceeding reports on our first efforts in transient 2D-IR (T2D-IR) spectroscopy of a non-equilibrium ensemble, where full advantage of the high time resolution can be taken. The nonequilibrium process investigated here is the conformational transition of a cyclic octapeptide containing a photoswitchable azobenzene based omega amino acid. Starting from the cis-azo conformation, the conformational transition was initiated by a 400 nm pulse (Figure 2). Transient 1D and 2D (T1D, T2D) spectra of the isomerization process were measured according to our published method.

2. RESULTS

Steady-State Spectroscopy. The absorption spectra of the cis and trans conformation show a broad amide-I band with only little structure (Figure 1, Top), despite in total 9 amide-I oscillators lie underneath this band. The difference spectrum between the trans and cis conformation (Figure 1, Bottom) reveals a blue shift upon isomerization. It is well established that the amide-I band is a sensitive probe of protein secondary structure. The structure sensitivity is attributed mainly to the distance and angle dependence of the coupling between individual amide-I vibrators. Applying the coupling models outlined in Ref. 9 to the cis and trans structures derived from NMR spectroscopy, the experimentally observed blue shift can be reproduced. Hence, we conclude that the blue shift directly reflects the change in the alignment of the amide groups caused by the overall conformational change of the backbone.
**Figure 1**: Absorption spectra of the *cis* (*Top*, solid line) and *trans* conformer (*Top*, dashed line), T1D spectra at selected delay times (*Middle*) and stationary cis-trans difference spectrum measured in the FTIR spectrometer (*Bottom*).

**T1D-IR Spectroscopy.** The time evolution of the peptide on its way from the start to the end conformation was investigated by T1D-IR spectroscopy⁴ (Figure 1, Middle). Two main phases can clearly be distinguished from the experimental data:

Fast Phase, 0-20 ps (*driven* and *cooling* phase): Overlapping with the fast cooling, a somewhat slower process (*τ* = 6ps) leads to a transient blue shift observed after 20 ps (Figure 1, Middle). The intensity and peak position reached after 20 ps is almost equivalent to that observed in the 1 ms spectrum. Since the blue shift of the amide-I band is directly related to the change of backbone structure, we conclude that stretching of the peptide conformation is, in a coarse sense, completed after only 20 ps.

Slow Phase, >20 ps (*biased diffusion* phase): While stretching of the peptide backbone is essentially finished after 20 ps, the further evolution of the transient spectra shows that the system is not yet in equilibrium (Figure 1). Most prominent is the formation of a shoulder at the blue side of the product band (1695 cm⁻¹) after 16 ns, the onset of which is visible already after 1.7 ns. As this shoulder develops into a well separated band in the 1 ms spectrum, we conclude that equilibration of the system is not fully completed after 16 ns. On the 1 ms time scale, however, the transient and FTIR difference spectra are identical, indicating that the system is now entirely relaxed. The whole process is summarized in Figure 2.
Figure 2: Ensemble of structures with azobenzene in the cis and trans conformation, as obtained from NMR structure analysis. After photoisomerizing the built-in azobenzene unit, the distribution of cis structures is projected onto a modified potential energy surface. During the driven phase, the ensemble relaxes very quickly on a 6-ps time scale and entirely floods the bottom area of the potential. In the subsequent biased diffusion phase, the system equilibrates on a discrete hierarchy of time scales, extending from 20 ps to 16 ns, towards the global minimum of the trans conformation.

**Transient 2D-IR Spectroscopy.**

Based on the T1D-IR results, we have chosen four characteristic delays $t_{uv}$ between the UV-switch pulse and the 2D-IR probe: 3 ps, 20 ps, 200 ps and 1.7 ns. The red-shift of the amide-I band at 3 ps has been attributed to (thermal or non-thermal) excitation of low frequency modes. Hence, the peptide backbone is still hot after 3 ps and contains a significant fraction of the large excess energy absorbed by the photo-switch. Despite the strong red-shifted absorption band in the T1D-IR spectrum, we do not observe any distinct contribution from the transient photo-product in the T2D-IR spectrum recorded at 3 ps (expected at arrow 1 in Figure 3).

After 20 ps, the stretching of the backbone conformation is almost finished, but the system has not fully equilibrated yet. The T1D-IR spectrum at $t_{uv} = 20$ ps shows a blue-shifted contribution from the transient photo-product at $\omega_{pe} = 1685$ cm$^{-1}$ (Figure 3, arrow 2). Again, hardly any signal is observed in the T2D-IR spectrum from the transient photo-product (Figure 3, arrow 3).

The dynamics at delays larger than 20 ps has been attributed to the peptide backbone adjusting to the stretched conformation. After 200 ps the T1D-IR spectrum has changed very little compared to that at 20 ps. In the T2D-IR spectrum, however, we now observe a negative band resulting from the transient photo-product at $\omega_{pe} = 1685$ cm$^{-1}$. (Figure 3, arrow 4).

**Modelling of the T2D-IR Data.** The most striking result of our T2D-IR experiment is the absence of an observable signal from the transient photo-product at 3 ps and 20 ps delay time, although such a signal is clearly present in the T1D-IR spectra. To gain an understanding for this observation, we have to recall that a 2D-IR measurement can be viewed as a dynamic hole burning experiment. As such, 2D-IR spectroscopy – in contrast to 1D spectroscopy - can distinguish between homogeneous and inhomogeneous broadening.
By modelling the T2D-IR spectra we found that variation of the homogeneous broadening $\gamma_t$ of the transient photo-product is - besides the band shift – the main effect responsible for the time dependence of the signal (Figure 3, Bottom Row). Good agreement with the experimental data was obtained for a homogeneous width of the photo-product decreasing from $\gamma_t = 16 \text{ cm}^{-1}$ at 3 ps, 13.5 cm$^{-1}$ at 20 ps to 12.1 cm$^{-1}$ at 1.7 ns. The simulated T2D-IR spectra can almost quantitatively explain all the experimentally observed features by a change of $\gamma_t$. In particular, the lack of an observable transient product band in the 3 ps and 20 ps T2D-IR spectra (Figure 3, arrow 1 and 3) as well as its appearance at $\omega_{pr} = 1685 \text{ cm}^{-1}$ at later times (Figure 3, arrow 4 and 5) are perfectly reproduced. Also the more subtle changes, i.e. tilts and shifts of the various T2D-IR bands, are well described.

3. DISCUSSION AND CONCLUSION
Spectral dephasing of a vibrational transition in the condensed phase is commonly related to stochastic fluctuations of its transition frequency$^{10,12}$. In this approach spectroscopy is described in terms of the transition frequency fluctuation autocorrelation function $\langle \delta \omega(t) \delta \omega(0) \rangle$, where $\delta \omega(t)$ is the instantaneous deviation of the vibrator's frequency from its average value$^{11,12}$. The vibrator is coupled to a fluctuating bath, which may include inter- or intramolecular degrees of freedom. As the transition frequency of the amide-I band is related to the backbone structure$^7$, fluctuations of the transition frequency reflect dynamics of the backbone as well as of surrounding solvent molecules. The homogeneous linewidth $\gamma_t$ in our experiment is a measure of the fast (<1 ps) part of the total fluctuations of the system$^5$. The experimental data reveal that this fast contribution is more pronounced when the system is far from equilibrium. This enhanced fluctuations of the peptide backbone reflect properties of the part of the potential energy surface the molecules sample at a given time. The time scale relevant for homogeneous broadening (<1 ps) is too fast for the system to cross any significant barrier on the order of $k_BT$$^{13,14}$. Figure 4 schematically depicts the way of the system towards its global minimum: Although the potential energy surface is rough, paths exist far from equilibrium which do not have to overcome any barrier, but may pass by barriers in a high dimensional space, so that sampling of the surface along those directions can proceed extremely quickly on time scales faster than < 1 ps. Nevertheless, owing to the large dimensionality of the problem, it may take a much
longer time until the system actually finds the global minimum. Once it is trapped, however, the conformational space is confined and the fast fluctuation amplitude is diminished accordingly as reflected by the reduced homogeneous width at longer times.

In the present paper, the discussion of the T2D-IR spectrum is limited to the effects of homogeneous and inhomogeneous broadening, since the amide-I spectrum of the peptide investigated is not resolved into individual sub-bands. For the case of spectrally resolved amide-I vibrators, it has been shown that additional structural information is contained in the cross peaks of 2D-IR spectra. There are indications for a transient cross peak between the carboxyl group of the aspartic acid side chain at 1720 cm$^{-1}$ and the amide-I band (Figure 3, arrow 6), which would allow a more site-specific discussion of the conformational transition of that side chain, and which will be addressed in a future work. The possibility of observing transient cross peaks has already been demonstrated for the excited electronic state of a transition metal complex$^{15}$. This gives rise to the hope, that combined with site specific isotope labelling the present technique may provide detailed pictures of the three-dimensional structure, or structural distribution, of the peptide as it approaches its new equilibrium state and help to construct a molecular movie.

4. REFERENCES