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Communication: Nanosecond folding dynamics of an alpha helix: Time-dependent 2D-IR cross peaks observed using polarization-sensitive dispersed pump-probe spectroscopy

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We present a simple method to measure the dynamics of cross peaks in time-resolved two-dimensional vibrational spectroscopy. By combining suitably weighted dispersed pump-probe spectra, we eliminate the diagonal contribution to the 2D-IR response, so that the dispersed pump-probe signal contains the projection of only the cross peaks onto one of the axes of the 2D-IR spectrum. We apply the method to investigate the folding dynamics of an alpha-helical peptide in a temperature-jump experiment and find characteristic folding and unfolding time constants of 260 ± 30 and 580 ± 70 ns at 298 K. © *2015 AIP Publishing LLC.* [http://dx.doi.org/10.1063/1.4906456]

Two-dimensional infrared (2D-IR) spectroscopy is a powerful and versatile technique to investigate molecular structure and conformation in solution. The structural sensitivity of the method relies on the possibility of observing couplings between molecular vibrations (somewhat similar to the way the couplings between nuclear spins are used in multi-dimensional nuclear magnetic resonance spectroscopy). These vibrational couplings show up as cross peaks in the 2D-IR spectrum, and since the coupling strength (observed as the intensity of a cross peak) depends strongly on the distance between, and relative orientation of the coupled vibrating (groups of) chemical bonds, it provides a sensitive probe of molecular structure and conformation.[1] 2D-IR spectroscopy is actively used to study protein structure, recent examples including the identification of secondary structure elements,[2–4] the determination of domain structure,[5] the formation of amyloids,[5–7] the effects of denaturants[8] and drug or ligand binding[9,10] on protein structure, and the hydration of proteins.[11]

Since 2D-IR spectra are recorded by means of subpicosecond infrared pulses, 2D-IR spectroscopy can be used in a time-resolved manner to track structural changes at the molecular level during an externally triggered chemical or physical process. This is done by recording the change in the 2D-IR spectrum at different delay times with respect to the external trigger.[12–18] Time-resolved 2D-IR has been used to investigate peptide and protein folding kinetics (triggered by a temperature jump[14,15] or photochemical bond cleavage[13]) and photo-chemical reactions.[12,19] However, since the change in the 2D-IR spectrum in such measurements is generally small compared to the 2D-IR signal itself, time-resolved 2D-IR spectroscopy involves a much greater experimental complexity than steady-state 2D-IR spectroscopy. Here, we present a simple method to probe 2D-IR cross-peak intensities, and so the structural evolution of a molecular system, in a time-resolved manner. We achieve this by measuring a weighted difference of dispersed pump-probe (DPP) spectra recorded with parallel and perpendicular polarizations of the IR pump and probe pulses, at a series of delays after the trigger. These polarization-difference DPP spectra are the projection of the 2D-IR cross-peak spectrum onto one of the frequency axes.[1] In this time-dependent spectrum, the contribution of the diagonal 2D-IR response is eliminated[20,21] so that only the time-dependent cross peaks contribute to the observed signal.

We apply this method[22] to investigate an alanine-based α-helical peptide (see Fig. 1 for the sequence and structure), of which we have previously studied the folding dynamics using circular dichroism and conventional *T*-jump IR spectroscopy.[23] The steady-state IR spectra of the peptide at 278 K and 338 K are shown in Figs. 2(a) and 2(e), respectively. The melting temperature of the peptide is 312 K,[23] which means that at 278 K the equilibrium of the system favours the folded conformation of the peptide, whereas at 338 K, the peptide is predominantly unfolded. The structure of the unfolded peptide (338 K) is a random coil, resulting in a broad amide I' absorption band at ~1645 cm^{-1} (Fig. 2(e)). When folded, the peptide forms an α-helix, resulting in a narrower, red-shifted amide I' absorption band at ~1635 cm^{-1} (Fig. 2(a)). A perfect helical array of vibrational chromophores has only two infrared-active normal modes,[24,25] the *A* mode and the degenerate *E* mode, which have transition-dipole moments parallel and perpendicular to the helical axis, respectively. In particular, the amide I' chromophores in an ideal α-helix have *A* and *E* modes that are separated by a few cm^{-1},[25] and the *A* mode has a much larger intensity than the *E* mode, since the local amide I' transition-dipole moments are nearly parallel to the helical axis. In our 17-residue, solvated helix, *A* and *E* modes are not exactly defined,[26] but previous work has shown that in short helices, amide I' modes are still delocalized over at least 3.5 amide units,[4] and that the dominant normal modes in finite helices still have *A*-like and *E*-like characters, with a frequency splitting on the order of 10 cm^{-1}.[27] Because of the small *E*-*A* frequency difference and the low intensity of the *E* mode, an α-helix in solution effectively has a single amide I' band. The IR spectra also contain two CN-stretching modes of the arginine (Arg^+)

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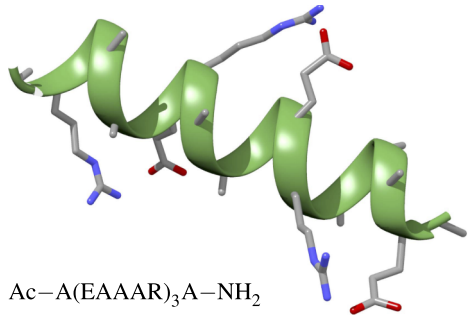

FIG. 1. Sequence and structure of the investigated α-helical peptide.

side-chain groups at 1582 cm^{-1} and 1611 cm^{-1}. The change in the IR spectrum upon thermal unfolding is predominantly a frequency shift and a slight decrease in intensity of the amide I' band, whereas the Arg^+ bands remain unchanged.

We measure 2D-IR spectra in a pump-probe double-resonance setup,[1] in which a narrow-band (10 cm^{-1} FWHM) pump pulse excites the sample and the resulting absorption change ΔA is measured over a range of frequencies ν_{probe} using a broad (>100 cm^{-1} FWHM), spectrally dispersed probing pulse. By recording a series of ΔA spectra with different pump frequencies, we obtain a two-dimensional spectrum $\Delta A(\nu_{probe},\nu_{pump})$. The 2D-IR spectra recorded at 278 and 338 K (with perpendicularly polarized pump and probe pulses) are shown in Figs. 2(b) and 2(f), respectively. The ΔA signals on the diagonal (three negative-positive doublets) arise from resonant excitation of the amide I' and Arg^+ modes of the peptide. The negative ΔA signals arise from the ground-state depletion and $\nu = 1 \rightarrow 0$ stimulated emission, whereas the positive signals are caused by the $\nu = 1 \rightarrow 2$ transition of the excited vibrational mode. Upon increasing the temperature, the maximum of the amide I' bleach shifts from 1635 cm^{-1} (in Fig. 2(b)) to 1650 cm^{-1} (in Fig. 2(f)). However, the most prominent change upon unfolding is a decrease in intensity of the amide I' signal, which is caused by a loss of excitonic delocalization as a result of α-helix unfolding.[4]

The coupling between the *A* and *E* amide I' modes and between the two Arg^+ CN-stretch modes gives rise to cross-peaks in the 2D-IR spectrum, but in the raw 2D-IR spectra, these are difficult to distinguish from the much more intense signals on the diagonal. This diagonal contribution to the 2D-IR spectrum can be eliminated efficiently by constructing the

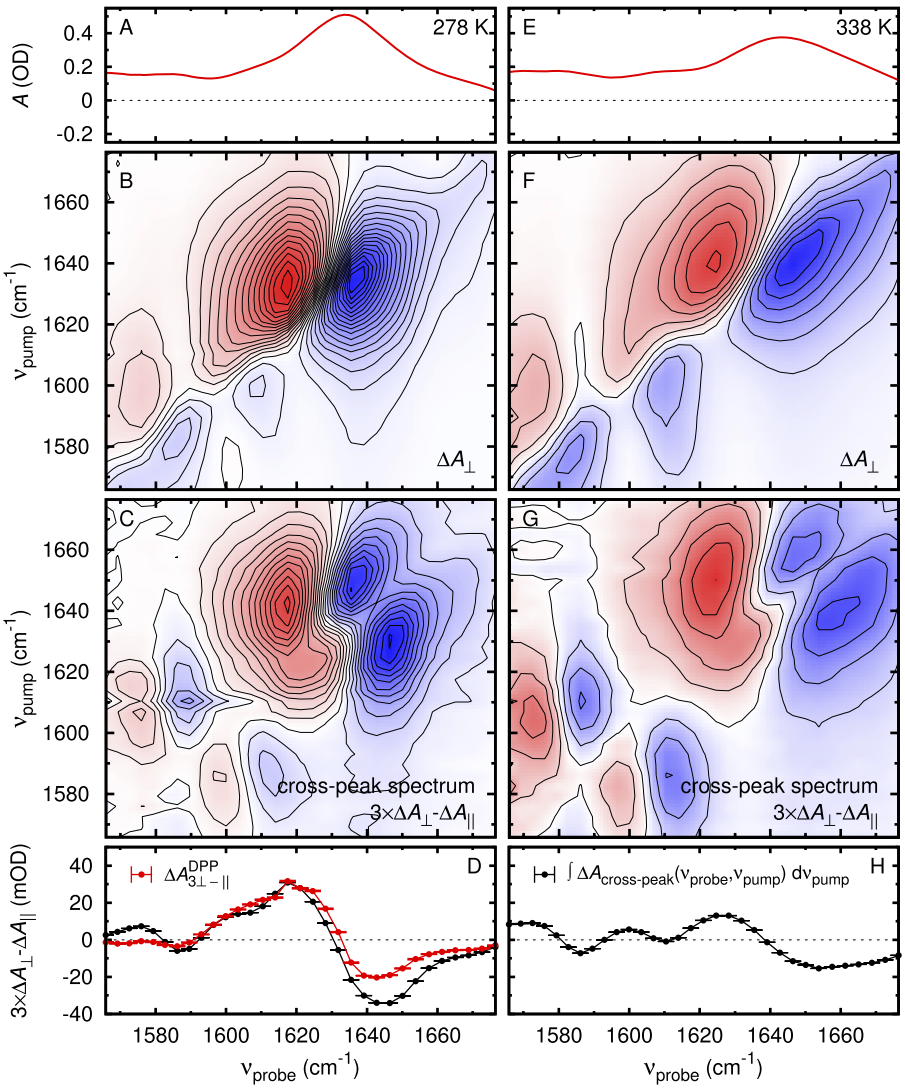

FIG. 2. (a) Solvent-corrected IR spectrum of the peptide at 278 K. (b) 2D-IR spectrum at 278 K with perpendicular polarizations of the pump and probe pulses. (c) Cross-peak 2D-IR spectrum ($3\times\Delta A_{\perp}-\Delta A_{\parallel}$) at 278 K. (d) Projection of the spectrum in panel (c) onto the probe axis (black points) and DPP spectrum at 288 K (red points). (e) Solvent-corrected IR spectrum of the peptide at 338 K. (f) 2D-IR spectrum at 338 K with perpendicular polarizations of the pump and probe pulses. (g) Cross-peak 2D-IR spectrum at 338 K. (h) Projection of the spectrum in panel (g) onto the probe axis. For the 2D-IR spectra: negative signals are indicated in blue and positive signals are indicated in red; the interval between contour lines is 0.25 mOD; the delay between pump and probe pulses is 1 ps. The delay between pump and probe pulses for the DPP and transient DPP spectra is 200 fs.

difference

$$\Delta A_{\text{cross-peak}}(\omega_{\text{probe}},\omega_{\text{pump}}) = 3\Delta A_{\perp}(\omega_{\text{probe}},\omega_{\text{pump}}) - \Delta A_{\parallel}(\omega_{\text{probe}},\omega_{\text{pump}}) \quad (1)$$

of the 2D-IR spectra recorded with parallel and perpendicular polarizations of the pump and probe pulses.[1] This 2D-IR "cross-peak spectrum" contains no diagonal signal because the diagonal peaks always have an anisotropy of $\frac{2}{5}$ (since the pumped and probed transition-dipole moments are identical), so that for these peaks, $\Delta A_{\parallel} = 3\Delta A_{\perp}$, whereas the cross peaks (for which the pumped and probed transition-dipoles are not identical) generally have an anisotropy $\neq \frac{2}{5}$.[1] The $\Delta A_{\text{cross-peak}}$ spectrum at 278 K (Fig. 2(c)) contains two sets of complementary cross peaks. Like the diagonal peaks, the cross peaks are positive-negative doublets (along the ν_{probe} axis). This is because the cross peaks arise from a small decrease in frequency of the probed mode upon excitation of the pumped mode. Since the diagonal anharmonicity (the difference between the $v = 0 \rightarrow 1$ and $v = 1 \rightarrow 2$ frequencies of a mode) and the cross anharmonicities (the lowering of $v = 0 \rightarrow 1$ frequency of a mode upon excitation of another mode that is coupled to it) are both comparable to, or smaller than the width of the absorption bands, the absorption-difference spectrum is very similar to the derivative of the absorption band with respect to the probe frequency, for both the diagonal peaks and the cross peaks. The negative extrema of the cross peaks between the *A* and *E* amide I' modes are at $(\nu_{\text{probe}},\nu_{\text{pump}}) = (1647,1630)$ and $(1637,1650)$ cm^{-1}. Note that the intensities of the two cross peaks are the same (even though the intensities of the *A* and *E* IR bands are very different), since they are both proportional to $|\mu_A|^2|\mu_E|^2$ (whereas the intensities of the diagonal peaks are proportional to $|\mu_A|^4$ and $|\mu_E|^4$).[1] The *A*-*E* cross-peak signature is typical for an α-helix,[28] and can therefore be used as a measure of folded peptide population. This can be seen from the $\Delta A_{\text{cross-peak}}$ spectrum at 338 K, in which the intensity of these α-helix cross peaks has decreased dramatically. The cross peaks between the two Arg^+ modes at $(\nu_{\text{probe}},\nu_{\text{pump}}) = (1612,1588)$ and $(1590,1610)$ cm^{-1} remain unchanged upon thermal unfolding.

The amide I' cross peak is a convenient and sensitive structural probe to follow the thermal re-equilibration of the α-helical peptide in real time, in particular, in T-jump experiments in which the folded ⇌ unfolded equilibrium is rapidly disturbed and the subsequent re-equilibration is probed in real time.[29–32] However, instead of measuring the complete 2D-IR cross-peak spectrum at different delays with respect to the *T*-jump, the cross-peak intensity can be measured efficiently by using a DPP measurement, in which the sample is excited using a spectrally broad pump pulse, and the absorption change is measured using a spectrally dispersed probe pulse.[1] According to the projection-slice theorem, the signal obtained in this way is the projection of the 2D-IR spectrum onto the probe axis.[1] The same holds for the 2D-IR cross-peak spectrum

$$\Delta A^{\text{DPP}}_{3\perp-\parallel}(\omega_{\text{probe}}) \equiv 3\Delta A^{\text{DPP}}_{\perp}(\omega_{\text{probe}}) - \Delta A^{\text{DPP}}_{\parallel}(\omega_{\text{probe}}) = \int \Delta A_{\text{cross-peak}}(\omega_{\text{probe}},\omega_{\text{pump}})\, d\omega_{\text{pump}}. \quad (2)$$

Hence, in the $\Delta A^{\text{DPP}}_{3\perp-\parallel}(\omega_{\text{probe}})$ spectrum, the diagonal response is eliminated in the same fashion as in the 2D-IR cross-peak spectrum (Eq. (1)). The integral over ω_{pump} of the $\Delta A_{\text{cross-peak}}(\omega_{\text{probe}},\omega_{\text{pump}})$ signal is indeed very similar to the $\Delta A^{\text{DPP}}_{3\perp-\parallel}(\omega_{\text{probe}})$ spectrum, see Fig. 2(d). In the $\Delta A^{\text{DPP}}_{3\perp-\parallel}(\omega_{\text{probe}})$ spectrum, the Arg^+ cross-peak feature at ~1575 cm^{-1} is weaker than in the integrated 2D-IR spectrum. This discrepancy in intensity probably arises from the difference in IR pump-probe delay in the two experiments (1 ps and 200 fs for the 2D-IR and DPP spectra, respectively). The Arg^+ cross-peak intensity increases with delay due to energy transfer between the two CN-stretch modes, which occurs on a ps time scale.[33] At 1 ps, more energy transfer has occurred than at 200 fs, resulting in more intense cross peaks in the integrated 2D-IR spectrum than in the $\Delta A^{\text{DPP}}_{3\perp-\parallel}$ spectrum.

We use the $\Delta A^{\text{DPP}}_{3\perp-\parallel}$ spectrum to probe the evolution of the 2D-IR cross peaks of the α-helical peptide in a nanosecond *T*-jump experiment.[22] Figure 3(a) shows the $\Delta A^{\text{DPP}}_{3\perp-\parallel}$ spectrum at different delays with respect to the *T*-jump. The intensity of the integrated cross peak decreases as a function of delay. As the system equilibrates to its new temperature, the fraction of helical content decreases, and the concomitant loss of secondary structure causes a decrease in the $A \leftrightarrow E$ cross-peak intensity. The sharp feature at 1618 cm^{-1} is caused by the high-frequency Arg^+ cross-peak doublet, which is spectrally much narrower than the *A*-*E* cross-peak of the α-helix. Note that the magnitude of the change in integrated cross-peak intensity (Fig. 3A) is much smaller than the difference between Figs. 2(c) and 2(g), because the *T*-jump is only a few K, whereas the temperature difference between the two 2D-IR spectra is 60 K. The time-dependence of the integrated cross-peak intensity (the difference between the $\Delta A^{\text{DPP}}_{3\perp-\parallel}$ signals at

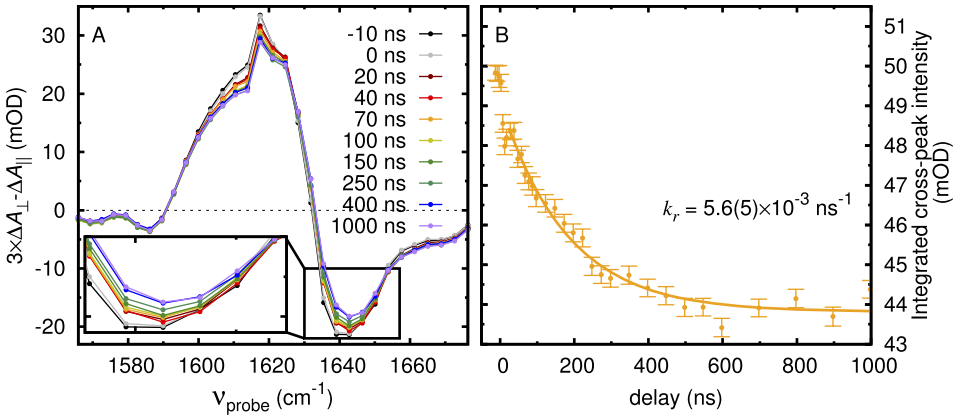

FIG. 3. (a) $\Delta A^{\text{DPP}}_{3\perp-\parallel}$ spectra at different delays after a *T*-jump from 288 to 298 K (the IR-pump/IR-probe delay is 200 fs). The inset shows a magnification of the decrease of the integrated helical ($A - E$) cross-peak intensity. (b) Time dependence of the helical cross-peak intensity (the difference between the signals at 1643 cm^{-1} and 1618 cm^{-1}). The solid line is a least-squares fit of the data to a single-exponential decay.

1643 cm^{-1} and 1618 cm^{-1}) is shown in Figure 3(b). From a single-exponential least-squares fit, we find that the structural relaxation occurs with a time constant of 180 ± 20 ns, close to the value obtained from conventional T-jump IR experiments.[23] It is interesting to note that in contrast to a conventional *T*-jump IR-probe experiment, and just like the 2D-IR spectrum, the $\Delta A^{DPP}_{3\perp-\parallel}$ signal contains no solvent contribution (which in conventional *T*-jump IR-probe experiments can lead to complications, and always must be separately measured and subtracted to isolate the protein or peptide response[34]).

We observe a small (≈1.5 mOD) instantaneous drop in the cross-peak intensity within the *T*-jump pulse duration (~ 5 ns), which is mainly caused by the intrinsic temperature dependence of the amide I' mode (a blue shift of the absorption maximum and a decrease in cross section[35]), and by the increase in the optical transparency of the solvent with increasing temperature (Jones *et al.*[36] have investigated these effects in detail for time-resolved heterodyned-echo 2D-IR experiments. Note that in contrast to such heterodyned-echo 2D-IR experiments, in our experiments, transient refractive-index changes do not influence the observed signal).

The time constant for relaxation of the cross-peak after the *T*-jump is the same as that observed for the linear amide I response[23] and for the diagonal 2D-IR response (as obtained from the $\Delta A^{DPP}_{\parallel}(\omega_{probe})$ signal[22]). Since these responses are each sensitive in a different manner to the length of an α-helix,[4] the observation of identical time constants for each suggests that the helix behaves effectively as a two-state folder, in agreement with previous studies on α-helical folding. Combining the observed relaxation constant with the known folded ⇌ unfolded equilibrium constant, we obtain folding and unfolding time constants of 260 ± 30 and 580 ± 70 ns at 298 K, in good agreement with our previous 1D-IR *T*-jump study on this peptide.[23]

In conclusion, we have demonstrated a simple method to measure time-dependent 2D-IR cross-peak intensities. The technique is less complex than transient 2D-IR spectroscopy, and provides similar information provided that the 2D-IR spectra are not too congested. This latter requirement is perhaps the most important limitation of the method. In the case of several cross peaks at the same probe frequency, one observes the time dependence of the sum of their intensities, see Eq. (2); the observed dynamics is then the sum of the dynamics of each of the contributing cross peaks. Our results on the α-helical peptide show that the polarization-resolved DPP spectra can be used as a probe for time-dependent secondary protein structure, and from the different signals, we conclude that the α-helical peptide behaves effectively as a two-state folder. Finally, it may be noted that since the polarization-elimination method to isolate 2D-IR cross peaks can also be applied in time-domain 2D-IR methods,[21] the method demonstrated here can also be used to measure non-equilibrium cross-peak dynamics in experiments using such time-domain 2D-IR methods. In particular, it can be implemented in transient dispersed vibrational-echo experiments,[37–39] which also measure a projection of the 2D-IR spectrum onto one of its axes.

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