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Two-Dimensional Infrared Spectroscopy of Photoswitchable Peptides

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Key Words

2D-IR spectroscopy, ultrafast time-resolved spectroscopy, multidimensional spectroscopy, peptide dynamics, peptide folding

Abstract

We present a detailed discussion of the complimentary fields of the application of two-dimensional infrared (2D-IR) spectroscopy in comparison with two-dimensional nuclear magnetic resonance (2D-NMR) spectroscopy. Transient 2D-IR (T2D-IR) spectroscopy of nonequilibrium ensembles is probably one of the most promising strengths of 2D-IR spectroscopy, as the possibilities of 2D-NMR spectroscopy are limited in this regime. T2D-IR spectroscopy uniquely combines ultrafast time resolution with microscopic structural resolution. In this article we summarize our recent efforts to investigate the ultrafast structural dynamics of small peptides, such as the unfolding of peptide secondary structure motifs. The work requires two ingredients: 2D-IR spectroscopy and the possibility of triggering a structural transition of a peptide on an ultrafast timescale using embedded or intrinsic photoswitches. Several photoswitches have been tested, and we discuss our progress in merging these two pathways of research.
1. INTRODUCTION

The importance of the structural dynamics of biomolecules for their function is widely appreciated. Two-dimensional infrared (2D-IR) spectroscopy is a novel spectroscopic tool that uniquely combines ultrafast time resolution with appreciable structural resolution. The transferability of the concepts of multidimensional nuclear magnetic resonance (NMR) spectroscopy (1) to IR spectroscopy has been postulated already in the earliest publications on 2D NMR (2), however, the first 2D-IR spectrum was measured only relatively recently (3). In analogy to 2D-NMR spectroscopy, in 2D-IR spectroscopy the connectivity of various vibrational states can be related to local contacts of the corresponding molecular groups, which is the basic principle of structural determination in both cases. Equivalents of all fundamental NMR experiments [COSY (4, 5), NOESY (6), and EXSY (7–9)] have been demonstrated in the IR range in the meantime (10).

By nature of the close analogy, it might seem that 2D-IR spectroscopy’s biggest competitor will always be 2D-NMR spectroscopy, which would put the bar high! Both 2D-NMR and 2D-IR spectroscopy are capable of resolving certain functional groups in a molecule, work (mostly) in the solution phase, and aim to elucidate the structure as well as dynamics of solution-phase systems. Over the course of the past 20–30 years, NMR spectroscopy has proven to be so much more versatile that it has largely superseded conventional (1D) IR spectroscopy for standard analytic purposes for essentially two reasons: (a) NMR spectra are more structured and allow one to resolve individual resonances from much larger molecules than IR spectroscopy. (b) The interpretation and assignment of NMR spectra work on the basis of simple empirical rules, whereas the assignment of IR spectra is currently challenging to even the most powerful quantum-chemistry codes for only a midsize molecule with, for example, 30 atoms.

When we began to develop 2D-IR spectroscopy as a tool to determine molecular structure, we concentrated on a small peptide, trialanine (6, 11–13). Much to our surprise, the conformation of that molecule, or rather the distribution of conformations, was not known at the time although it has important model character: The backbone of trialanine contains only one central (\(\phi, \psi\))-pair of dihedral angles and hence reflects the intrinsic structural propensity of the peptide bond itself without the additional complication of intermolecular hydrogen bonds in larger peptides that form secondary or tertiary structure motifs. In a series of papers (6, 11–13), we determined the preferred conformation and the relative populations of conformations, as well as the flexibility of trialanine. We found that trialanine populates predominantly (approximately 80%) the so-called poly-prolin II structure (PII). Other structural elements such as \(\alpha\)-helical and \(\beta\)-sheet motifs seemed to contribute only little. This result was unexpected because the PII structure is not common in larger proteins, and standard molecular dynamics (MD) force fields predicted different structural distributions (14). Our results (among others) led to suggestions for modifications of the AMBER force field (15).

Recently, our structural prediction of trialanine has essentially been confirmed in a combined NMR and MD simulation study, going just a bit beyond the most elementary methods of the rich toolbox of NMR spectroscopy (16). This is good and
bad news: On the one hand, it validates the use of 2D-IR spectroscopy in determining the unknown structure of small peptides, even when the experimenter cannot put any biased expectation into the structural analysis. On the other hand, it suggests that if 2D-NMR spectroscopy tries hard enough, it almost always will beat 2D-IR spectroscopy in terms of pure structural-resolution capabilities.

Many have argued that the large potential of 2D-IR spectroscopy, as compared with 2D-NMR spectroscopy, is its many orders of magnitude higher intrinsic time resolution. However, one must carefully specify what exactly is meant by time resolution. Figure 1a shows the time regimes that can be covered by numerous variants of NMR spectroscopy, which do in fact include the ultrafast picosecond and even subpicosecond range (17). For example, in the mid-1970s, Fung & McGaughy (18) deduced the orientational diffusion time of bulk water using NMR spectroscopy, revealing a value of 2.3 ps for D2O. Only approximately 20 years later (19), IR spectroscopy came into the position to repeat the measurement, revealing that the process is more complex. The generally accepted view now is that the anisotropy decay is biphasic with a sub-100-fs librational component prior to a 2.5–3-ps diffusive component (20, 21). Hence, although NMR spectroscopy is essentially right for the diffusive part, it completely misses the fast component and cannot differentiate the contributions in a heterogeneous process—a subtle but important difference.

The ultrafast time regime is accessed by the intrinsically very slow NMR spectroscopy indirectly through relaxation methods (T1, T2, HetNOE, T1ρ, and residual dipolar couplings; see Figure 1a). For example, in the case of the orientational diffusion of water discussed above, a simple theory exists that relates the T1 relaxation time...
of the proton spin transition, which is what is actually measured in the experiment and is quite slow (0.45 s), to the fast orientational correlation time $\tau_c$ of the molecule (18). Both quantities are inversely proportional; hence the faster the molecular structural process is, the slower the spin relaxation. The important point is that this theory relies on a model assumption—free orientational diffusion—which is not extremely well justified in the case of water (22). NMR relaxation techniques cannot validate the model assumption made to interpret the data and, hence, tend to oversimplify complex systems such as liquid water. For a truly diffusive process (e.g., the reorientation of a larger, close to spherical molecule in solution), the results from NMR spectroscopy are usually correct.

The important difference between IR and NMR spectroscopy is the time range that can be accessed in real time. That is, the experimenter modifies a time variable somewhere in the measuring instrument—e.g., the timing of one part of an NMR pulse sequence with respect to the rest, or the sledge of an optical delay line—and that time is related directly to the time a particular structural process takes in a one-to-one manner (e.g., the time for interconversion of two molecular species). (For the purpose of this review, we distinguish between structural and energy relaxation processes, the latter of which can be investigated as well by 2D-IR spectroscopy.) In the context of this definition, this is exchange spectroscopy (EXSY) and pump-probe spectroscopy (Figure 1).

In an EXSY experiment, an initial part of a pulse sequence (preparation phase) is used to label a subensemble of molecules in a certain conformation by exciting a spectrally distinct spin or vibrational state. The subensemble is then given time to change its conformation during a waiting period, after which the conformation is finally read out. By varying the waiting period, one observes the conformational transition in real time. EXSY has an upper limit of the accessible time window, which is dictated by the excitation lifetime of the spin or vibrational state used as a label. In the case of 2D-IR spectroscopy, the time window ranges, for example, from approximately 100 fs for the frequency fluctuations of the OH vibration in water (i.e., spectral diffusion) (23–26) to typically a few tens of picoseconds for hydrogen bond on and off times in solute-solvent systems (7–9).

In a pump-probe experiment, a molecular system is perturbed in some way (e.g., by triggering a photoreaction or by a temperature jump) and thereby brought into a nonequilibrium state. As a result, all molecules in the sample volume undergo a conformational transition in a concerted way, and 2D-NMR or 2D-IR spectroscopy is then used to elucidate a snapshot structure of that ensemble on the fly. The lower time limit of pump-probe spectroscopy is dictated by the spectroscopic method (approximately 1 ps for 2D-IR spectroscopy and tens of milliseconds for 2D-NMR spectroscopy), whereas no intrinsic limitation is imposed toward longer timescales.

From all the methods indicated in Figure 1, pump-probe spectroscopy is the only one that allows access to the nonequilibrium regime. Comparing Figures 1a,b, one sees clearly that the biggest potential of IR spectroscopy, as compared with NMR spectroscopy, lies in the almost unlimited time window accessible to such nonequilibrium experiments. This is why we made the strategic decision to explore and develop this capability of 2D-IR spectroscopy: measuring snapshot structures of fast evolving
molecular systems by means of transient 2D-IR (T2D-IR) spectroscopy. This is where the dynamical aspect of NMR spectroscopy almost completely breaks down.

T2D-IR spectroscopy is a versatile technique that can be applied to all sorts of problems in chemistry and biophysics. For example, we have studied in detail the polarization dependence of the 2D-IR spectra of a metal-to-ligand charge transfer complex in its electronically excited state (27), established a technique that allows one to correlate vibrations in the electronic ground state to those in a photoproduct state (i.e., labeling vibrations by light) (28), applied that technique to elucidate the connectivity of binding sites after the photodissociation of CO in myoglobin (29), and studied solvation phenomena beyond the linear response regime (30). Recently, T2D-IR experiments have also been reported by Tokmakoff and coworkers (31, 32), as well as Kubarych and coworkers (33). In this review we focus on the folding dynamics of photoswitchable peptides (34–36). Our approach requires two ingredients that we currently explore as parallel research paths in our laboratory: (a) 2D-IR and T2D-IR spectroscopy (Section 2.1) and (b) concepts of the ultrafast photoswitching of peptides (Section 2.2). In Section 3, we report on our current progress in merging these two pathways of research.

2. METHODOLOGY

2.1. Two-Dimensional Infrared and Transient Two-Dimensional Infrared Spectroscopy

Although the first 2D-IR experiment was a pulsed-frequency domain experiment (3), a technique that has been adapted and refined by various groups (4, 6, 7, 11–13, 37–41), time domain–pulsed Fourier transform techniques have been devised as well (8, 9, 23–26, 42–57). Variants of 2D vibrational spectroscopy are 2D-Raman spectroscopy (58–60) and DOVE spectroscopy (i.e., doubly vibrationally enhanced four-wave mixing, a hybrid frequency–time domain spectroscopy) (61, 62). 2D spectroscopy has also been applied to electronic transitions (63–68), addressed extensively by theoretical groups (10, 69–82), and reviewed from various perspectives (5, 83–91).

The information content of the time domain and frequency domain implementations of 2D-IR spectroscopy is largely identical (55, 92). The pulsed-frequency domain experiment preselects the pump frequency with the help of a tunable Fabry-Perot filter, whereas the time domain–Fourier transform techniques achieve the pump process by two phase-locked, spectrally broad pulses that interfere in the sample. In the latter case, the spectral information is regained by Fourier transforming the data after the measurement. With respect to the probe process, both techniques are identical. The time domain–Fourier transform technique has conceptual advantages (highest possible spectral resolution, higher sensitivity, more control over pulse parameters of all three field interactions), which, however, come at the expense of a more difficult experiment (interferometric stability, accurate determination of the absolute phase) (92). Zanni and coworkers (54, 55) have recently proposed a hybrid method based on an acousto-optical modulator that seems to combine the advantages of both approaches.
It is more intuitive to discuss the outcome of a 2D-IR experiment in a frequency domain context. First let us consider two vibrators that are uncoupled, in which case a characteristic double-peak structure is obtained for each vibrator on the diagonal of the 2D-IR spectrum (Figure 2a). Whenever the pump frequency is resonant with one

![Figure 2a](image1)

![Figure 2a](image2)

**Figure 2**

Two-dimensional infrared spectrum of two vibrators that are (a) not coupled and (b) coupled. Negative, bleach and stimulated emission is presented in blue, and positive, excited-state absorption is presented in red.
of the vibrators, it is promoted into the first excited state ($|1\rangle$ or $|1'\rangle$), and the probe beam then probes the fundamental 0–1 transition (bleach and stimulated emission), as well as the first 1–2 overtone transition (excited-state absorption). Because chemical bonds are always weakly anharmonic with slightly different frequencies of the 0–1 and the 1–2 transitions, the two peaks separate along the probe frequency axis. Both peaks have opposite signs, and we usually color code them blue (negative, bleach and stimulated emission) and red (positive, excited-state absorption).

When the coupling between the two vibrators is switched on (Figure 2b) by bringing them into close spatial proximity, additional cross-peaks appear in the off-diagonal region of the 2D-IR spectrum. This is because the $|00'\rangle \rightarrow |10'\rangle$ transition, for example—exciting the first vibrator while the second is in its ground state—is different in frequency from the $|01'\rangle \rightarrow |11'\rangle$ transition, in which the second vibrator is already in its first excited state. This so-called intermode anharmonicity is a direct manifestation of the coupling between the two vibrators (86). The coupling can be of static nature (in the sense of a level scheme as shown in Figure 2b) or of dynamic nature by the energy flow between the two vibrators (6). In any case, the intensity of the cross-peak carries information on the distance, whereas the anisotropy of the cross-peak reveals the relative orientation of the corresponding molecular groups. Both are important structural indicators that can be used to determine the unknown structure of small peptides from 2D-IR spectroscopy (4, 11, 12, 43, 49).

For reasons discussed in Section 1, we concentrate on an extension of 2D-IR spectroscopy, T2D-IR spectroscopy (27–30, 34–36). For that purpose, we introduce an additional optical or ultraviolet (UV) pulse preceding the 2D-IR pulse sequence, which triggers a photochemical reaction, or a conformational transition, of the molecular system under study. The evolving nonequilibrium ensemble is then probed by the 2D-IR part of the experiment, and we may call the outcome a UV-pump 2D-IR probe experiment.

Because the UV pump pulse in general does not convert 100% of the reactant species into the photoproduct species, the 2D-IR spectrum in the presence of the UV-switch pulse always contains contributions from both. To eliminate contributions of molecules that have not absorbed any UV photons, one records two sets of 2D-IR spectra simultaneously—one with the UV-switch pulse on and one with the UV-switch pulse off—and subtracts one from the other (34). Hence, just as it is common practice in conventional pump-probe spectroscopy, our T2D-IR spectra are in fact T2D-IR difference spectra.

To facilitate the interpretation of T2D-IR spectroscopy, we introduce the schematic response of an idealized molecule with one single homogeneously broadened oscillator, the frequency of which shifts on triggering the photoreaction. In analogy to the data presented in Figure 2, the vibrator gives rise to a 2D-IR spectrum with two peaks in each of the initial and product states: a negative (blue) bleach and stimulated emission signal at the vibrator's original frequency and a positive (red) excited-state absorption signal, which is red-shifted owing to the intrinsic anharmonicity of the oscillator. The T2D-IR spectrum, however, is the difference of the 2D-IR spectra of the initial and the transient species (Figure 3). In a transient 1D difference spectrum, negative contributions arise from the depleted initial population, whereas
Figure 3
Schematic illustration of transient one-dimensional (1D) (top panels) and two-dimensional infrared (2D-IR) (bottom panels) spectroscopy. The absorption bands shift from the steady state (a to b), each yielding a characteristic 2D-IR spectrum. Panel c shows the difference 1D and 2D-IR spectra. Negative response is depicted in blue and positive response in red. Figure adapted from Reference 34.

positive contributions stem from the transient photoproduct (Figure 3c, top panel). Accordingly, the signs of the various 2D-IR peaks of the initial and the product state are interchanged also in the T2D-IR difference spectrum (Figure 3c, bottom panel).

If the frequency shift on the photoreaction, as well as the anharmonicities of reactant and product states, is large compared to the line width of a band, four peaks are expected in total along the diagonal for each individual resonance. Principally speaking, the same is true for each cross-peak. However, we demonstrate below that this is not the case in general, and the various contributions overlap and tend to strongly cancel each other owing to their opposite signs. In particular for transient cross-peaks, in which we anticipate a change in coupling and hence a change in intermode anharmonicity, only the two peaks related to the conformation with the stronger coupling tend to survive this cancellation effect (35). However, T2D-IR lineshapes have not been thoroughly studied from the theoretical side.

2.2. Photoswitchable Peptides
The second important ingredient for studying nonequilibrium structural dynamics is the possibility of initiating a conformational transition of a peptide or a protein on an ultrafast picosecond timescale. The best established approach to achieve this is a laser-induced temperature jump to initiate, for example, the folding or unfolding of a peptide (93–101). Temperature jumps, in principle, can be very fast and are only limited by the thermal equilibration time of the bulk solvent that is in the range of a few tens of picoseconds, depending on the concentration of the molecule used to absorb light and to deposit energy into the solvent (102). Nevertheless, most
temperature-jump studies have been limited to the 10-ns regime for technical reasons: Q-switched Raman-shifted Nd-YAG lasers were used to generate the intense IR pulse with an energy of typically 1 mJ needed to heat the solvent water directly by exciting an overtone of either its OH or OD stretch vibration.

However, the intrinsic time resolution of 2D-IR spectroscopy is significantly faster (1 ps) than the pulse duration of suitable Q-switched lasers (10 ns). We felt that by limiting the experiment’s overall time resolution to 10 ns, we would potentially miss important advantages of 2D-IR spectroscopy (although this time resolution might be sufficient for most of the peptide dynamics problems studied in temperature-jump experiments).

Temperature jumps introduce another problem in combination with a 2D-IR experiment as a probe process: A significant amount of energy is deposited in a relatively small volume, which causes a sudden jump of pressure in a small sample volume. Researchers have devised strategies to reduce artifacts from the resulting shock waves and the resulting index of refraction gradients for conventional pump-probe experiments (103), but 2D-IR spectroscopy, consisting of a sequence of IR pulses, is more critical in this regard. That the pulsed-frequency domain experiment is not a phase-sensitive technique has proven to be a crucial advantage. Only recently, Tokmakoff and coworkers (31, 32) figured out how to cope with the problem of phase distortions using the time domain–Fourier transform technique.

We have chosen another approach to initiate conformational transitions in small peptides. By incorporating a photoswitch into a peptide that undergoes a photochemical reaction after electronic excitation, one may alter the conformation of the peptide in a controlled way. The switching time is ultrafast in most cases (≈1 ps). Furthermore, because the photoswitch is directly incorporated into the molecular system, its action is more local than that of a temperature jump. As a consequence, the overall energy pumped into the sample volume is much smaller (at least a factor of 10); hence problems with shock waves are considerably reduced (albeit still present in some cases). We distinguish between photoswitches, which are reversible, and phototriggers, which are one-time switches. Various photoswitches have been proposed in the literature, of which we tested a few in combination with T2D-IR spectroscopy: azobenzene (Figure 4a,b) as well as disulfide bridges and thiopptides as intrinsic photoswitches (Figure 4c,d). The use of photoswitches for various applications is reviewed in References 104–106.

3. TRANSIENT TWO-DIMENSIONAL INFRARED SPECTROSCOPY OF PHOTOSWITCHABLE PEPTIDES

3.1. Azobenzene

The cis-trans isomerization of azobenzene is widely used for photoswitching (106). It is characterized by a high quantum yield (50%), high photostability, ultrafast switching speed (approximately 1 ps), and the fact that cis and trans isomers have absorption bands that are well separated, therefore allowing a selective switching in both directions (107). Various groups (108–121) have directly incorporated azobenzene
Figure 4
Photoswitchable peptides and intrinsic photoswitches studied in our lab: azobenzene (a) embedded into a peptide backbone (b) or cross-linking amino acid side chains, and (c) a photocleavable disulfide bridge and (d) thiopeptides.

into the backbone of peptides, switching the peptide conformation by photo-exciting the azo-moiety. In a first step, we concentrated on a small cyclized peptide designed by Moroder and coworkers (112): The peptide resembles the active site of thioredoxin reductase (Figure 4a) and was cyclized by an azobenzene-based ω-amino acid. Researchers have applied UV spectroscopy, reporting the response of the
Amide I band: vibrational band mainly located on the carbonyl groups of the peptide backbone, which exhibits characteristic spectral features sensitive to secondary structure motifs of peptides and proteins.
Figure 5
Time-resolved pump-probe (top of each panel) and transient two-dimensional infrared spectra (bottom of each panel) at $T = 20$ ps (top panels), 200 ps (middle panels), and 1.7 ns (bottom panels). The left column shows experimental results, whereas the right column shows simulations. Figure adapted from Reference 34.
revealed by T2D-IR may reflect the width of the conformation space accessible on a fast timescale, which becomes more confined as the backbone approaches its global energy minimum.

In ongoing work, we concentrate on an alternative approach designed by Woolley and coworkers (110, 113–115), who used an azobenzene moiety to cross-link two amino acid side chains of a polypeptide (Figure 4b). Depending on its end-to-end distance, the photoswitch either increases or decreases the helix propensity of the peptide upon cis-trans isomerization (110, 113–115). However, in contrast to the previous example, the photoswitch couples much less directly to the conformational transition of the peptide backbone. In particular, the photoswitch does not force the peptide into an α-helical structure upon cis-trans isomerization; it just opens the folded state as a new region of conformational space. Helix folding occurs in a diffusive manner and hence on orders of magnitude slower timescales of 100 ns to 1 μs than in the example discussed above (128–130). Helix folding is thermally activated, and the folding rate varies strongly with temperature. Furthermore, at lower temperatures, the folding kinetics deviates strongly from mono-exponential, providing clear evidence that it is not a trivial two-state process. T2D-IR experiments are currently underway.

3.2. Intrinsic Photoswitches

The disulfide bridge between two cysteines, which occurs in natural peptides and proteins, may be used as a predetermined breaking point that photodissociates upon electronic excitation at ≈260 nm and hence as a phototrigger. Hochstrasser and coworkers (131, 132) first reported this concept in their pioneering work. A 20-residue peptide was designed to fold into an α-helix after photocleavage, while no distinct secondary structure was possible in the initial bridged state. Unfortunately, geminate recombination of the liberated thiyl radicals occurred before helix formation could be detected. We have recently used this approach for a much smaller peptide (Figure 4c) (133) that forms a β-turn structure in the disulfide-bridged configuration. Upon photocleavage of the disulfide bridge, the hydrogen bond in the β-turn is destabilized and the ring structure opens. In contrast to the molecule studied by Hochstrasser and coworkers (131, 132), this molecule is much more strained owing to the shortness of the backbone and stays open on long timescales. In fact, only approximately half the radicals are quenched after 1 ms either intra- or intermolecularly (134).

The peptide features a completely resolved and easy to assign IR spectrum of the five C==O groups without having to invoke isotope labeling. This fortunate situation made it the ideal test case for T2D-IR spectroscopy and allowed us to directly extract microscopic structural information on an ultrafast timescale for the conformational transition of the molecule (35). Figure 6b shows an equilibrium 2D-IR spectrum of the molecule that is rather congested because essentially all C==O groups are coupled to each other. Figure 6d, in contrast, shows a T2D-IR spectrum 100 ps after photocleavage of the disulfide bridge. The diagonal is dominated by signals similar to those in Figure 5. More important with regard to the structural resolution, only one dominant peak remains in the off-diagonal region, related to the coupling...
Figure 6

(Left column) Equilibrium and (right column) transient nonequilibrium spectra 100 ps after photocleavage of cyclo-(Boc-Cys-Pro-Aib-Cys-OMe). The top panels (a,c) show one-dimensional infrared (1D-IR) spectra, and the bottom panels (b,d) show the corresponding two-dimensional infrared (2D-IR) spectra. The equilibrium 2D-IR spectrum (b) is the weighted difference between parallel and perpendicular polarization measurement (to enhance the cross-peak contribution), whereas the transient 2D-IR spectrum (d) is with all three laser beams polarized parallel. The cross-peak (circled in red) in either spectrum relates to the coupling across the hydrogen bond in the β-turn structure. The example structures were obtained from an accompanying molecular dynamics simulation. Figure adapted from Reference 35.

between Cys(1)-Aib(3) across the intramolecular hydrogen bond. The corresponding cross-peak is weak in the equilibrium case (Figure 6b) and is hidden underneath the wings of the much stronger cross-peaks of Boc-Cys(1) and Cys(1)-Pro(2), both related to nearest neighbors in the sequence. In contrast, Cys(1) and Aib(3) are far in sequence, but close in space in the folded structure. Therefore, upon opening of
the ring structure, changes in coupling are expected only for Cys(1) and Aib(3) in the T2D-IR spectrum, which is a difference 2D-IR spectrum between the photoproduct state and reactant state. A time series showed that the transient cross-peak rises on a 100-ps timescale, in good agreement with accompanying results from MD simulations (35). The transient cross-peak constitutes a direct and easy to interpret structural indicator.

Another potential intrinsic photoswitch is the –CO–NH– peptide unit itself, which is thermodynamically more stable in the trans state, but which can be switched to the cis conformation by electronic ππ* excitation (135), forcing the peptide to change its secondary structure (136, 137). However, the required excitation wavelength lies deep in the UV (190 nm), which is hard to reach technically and can destroy the molecule. Excitation at 190 nm is also nonselective and can randomly switch any peptide unit in a polypeptide. One can avoid both problems by replacing one peptide unit with a thiopeptide (i.e., –CSNH–) unit (Figure 4d) (138–142). This red-shifts the wavelength of the ππ* transition of the trans isomer to ≈260 nm (143, 144), which is easily accessible by frequency tripling a Ti:S laser. Furthermore, incorporating a thioamide linkage results in only minor changes of a peptide’s secondary structure (145, 146). A combination of theoretical and time-resolved experimental studies on the photoswitch itself (N-methylthioacetamide) has shown that the isomerization quantum yield is high (30%–40% in the trans-cis and 60–70% in the cis-trans direction) although the molecule remains trapped in a relatively long-lived excited state for 200–300 ps, in which it is almost free to rotate about the thioamide bond (147, 148). Nevertheless, thiopeptides are particularly useful as model systems for T2D-IR spectroscopy because the isomerization of the thioamide bond changes the relative orientation of the adjacent peptide units, which are sufficiently close to each other for amide I mode coupling. In analogy to trialanine in 2D-IR spectroscopy, the smallest test molecule for T2D-IR is thus a tetrathiopeptide, with two amide I oscillators separated by the thio-photoswitch. The first thiopeptide we have investigated using T2D-IR spectroscopy [Boc-Ala-Gly(==S)-Ala-Aib-OMe] (36, 149) in addition contains two protecting groups (Boc and Aib/OMe, providing two more C=O oscillators), which stabilize the trans molecules in a looped conformation via an intramolecular hydrogen bond. In the T2D-IR spectra, we observed resolution enhancement along the diagonal as well as a transient cross-peak due to the breaking of that hydrogen bond (36).

4. DISCUSSION
Comparing the results of Reference 34 (Figure 5) with those of Reference 35 (Figure 6), one may suggest concrete design criteria to render T2D-IR spectroscopy an expressive method to time resolve transient structures. In the case of the azobenzene-switched cyclic octapeptide of Reference 34, the first example investigated above, the amide I’ band was not spectrally resolved; hence, we could not interpret the T2D-IR spectra in terms of structural motifs. The structural information did not go any deeper than that of the corresponding 1D spectroscopy; nevertheless, additional dynamic information could be extracted. In contrast, in the
case of the disulfide-bridged β-turn (35), the somewhat smaller peptide has a fully resolved amide I′ spectrum. As a result, we can identify specific local contacts whose time dependence we can interpret in terms of the breaking of certain loop structures.

In the examples in References 35 and 36, bands are resolved because the different C=O groups are sitting in different chemical environments. For larger peptides, this is not the case in general. However, spectral resolution can always be regained when employing isotope labeling (12, 46, 49, 149). The synthesis of site-selectively isotope-labeled peptides is not particularly demanding. For example, in our studies of the folding of a photoswitchable α-helix (Figure 4b), we employed double 13C16O and 13C18O isotope labeling to single out two specific sites from the unstructured main amide I′ band of unlabeled groups. 13C16O labeling causes a frequency downshift of the amide I′ band of approximately 30 cm$^{-1}$, whereas 13C18O shifts it by 50 cm$^{-1}$. In that way, one can identify the local contact between a specific pair of amino acids [e.g., the $i \rightarrow i + 4$ configuration of a α-helical loop (49)] and potentially measure its time dependence. These experiments promise to give unprecedented structural insights into which helix loop is forming at what time and to what extent during the folding process.

An important, but not thoroughly explored issue is the lineshapes of T2D-IR spectra. If the frequency shifts induced by the photoreaction are larger than the line width of the band, we expect a total of four spectrally resolved bands for each diagonal and cross-peak in the T2D-IR spectrum (Figure 3). For example, this is the case for the metal-to-ligand charge transfer of a metal-carbonyl compound we studied extensively ([Re(CO)$_3$(dmbpy)Cl], dmbpy = 4,4′-dimethyl-2,2′bipyridine) (27). However, with regard to photoswitchable peptides, the transient frequency shifts induced by a conformational transition are typically very small—much smaller than the line width of the amide I band. In this case, the two contributions (Figure 3a,b) overlap and tend to cancel each other. If the lineshape functions of reactant and product species are identical (which is not the case in general), then we expect the T2D-IR to be symmetric (Figure 3c). Owing to the strong cancellation between reactant and product contributions, however, small changes in the lineshape functions of either contribution may cause strong effects for the resulting difference T2D-IR spectrum (see Figure 5b).

The theoretical modeling of these cancellation effects is quite demanding and has not been fully achieved. First, it requires very high accuracy to obtain still reasonable results after subtracting the two almost identical contributions from the reactant and product state; second, almost all lineshape calculations are based on an equilibrium assumption [i.e., in the sense that the frequency fluctuation correlation function, which is at the basis of any lineshape analysis, is assumed to depend on the time difference only and not on absolute time: $\langle \omega(t_1)\omega(t_2) \rangle = \langle \omega(0)\omega(t_2-t_1) \rangle$ (70)]. However, it is not clear after which time one can think about an evolving structural ensemble as being in a quasi-equilibrium. We have shown that this assumption is definitely not valid on a 10-ps timescale (30), but we do not know whether it might become reasonable on a 100-ps to 1-ns timescale (34, 35). This problem calls for significant effort from the theoretical side [preliminary results suggest that the small change in homogeneous linewidth used in our empirical fits (Figure 5b, j,l) can indeed be reproduced from a nonequilibrium all-atom MD simulation] (150).
5. CONCLUSION

NMR is a high-resolution spectroscopy, capable of spectrally isolating thousands of individual resonances. However, the narrow line width implies that direct time resolution of NMR spectroscopy is intrinsically slow. The time resolution is ultimately limited by the dephasing (T2) time of the spectroscopic transition used as molecular probe (typically 1 ms for spin transitions), rather than by the measurement instrument. In contrary, typical dephasing times of vibrational transitions in the solution phase are in the range of 1 ps; hence IR bands are broad, and the number of resolved bands in a given spectral window is restricted. Nevertheless, the fast dephasing time is responsible for the high direct time resolution of 2D-IR spectroscopy. Spectral congestion can be circumvented to a significant extent by isotope labeling. In that sense, 2D-IR and 2D-NMR spectroscopy should not be viewed as competing methods; rather 2D-IR spectroscopy should be further developed as a complementary method. In the above sections we focus on transient nonequilibrium 2D-IR spectroscopy as one of the most promising strengths of 2D-IR spectroscopy (it certainly is not the only one). Learning to use the structural-resolution capabilities of 2D-IR spectroscopy, also in equilibrium, is of course an inevitable prerequisite before extending it to the nonequilibrium regime, and work in this direction is ongoing and, of course, still required.

Because the typical length and time scales of T2D-IR spectroscopy coincide exactly with what is accessible to MD simulations, the latter can directly be tested against experiment. MD simulations currently are the only approach to understand and visualize the complicated structure and dynamics—and hence the function—of biomolecules on an atomic level. Yet the MD approach clearly is based on, in part crude, approximations, whereas experimental techniques that can provide pictures of similar clarity are scarce. 2D-IR spectroscopy combines ultrafast time resolution with microscopic structural resolution in an unprecedented manner; hence it has the potential to become as expressive as all-atom MD simulations. The big challenge of 2D-IR spectroscopy is to extend the method to larger molecular systems (i.e., proteins rather than peptides), circumventing spectral congestion with intelligent strategies of isotope labeling, the introduction of new vibrational probes, or well-designed methods of difference spectroscopy. Nevertheless, as summarized above, significant steps toward a molecular movie of ultrafast structural dynamics extracted directly from experimental data have already been made.

SUMMARY POINTS

1. One of the biggest strengths of 2D-IR spectroscopy is the investigation of nonequilibrium structural processes.
2. T2D-IR spectroscopy uniquely combines ultrafast time resolution with microscopic structural resolution.
3. 2D-IR and 2D-NMR spectroscopy should be viewed as complementary rather than competing methods.
DISCLOSURE STATEMENT
The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Errata

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