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Amide I Two-Dimensional Infrared Spectroscopy of Proteins

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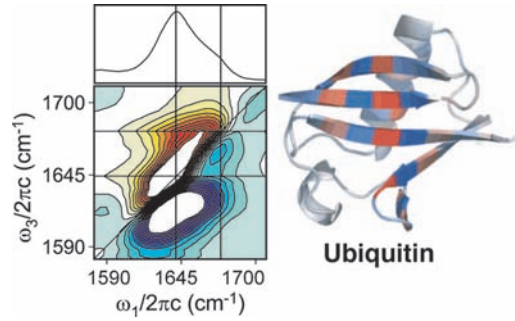
CONSPECTUS

We review two-dimensional infrared (2D IR) spectroscopy of the amide I protein backbone vibration. Amide I modes are known for secondary structural sensitivity derived from their protein-wide delocalization. However, amide I FTIR spectra often display little variation for different proteins due to the broad and featureless line shape that arises from different structural motifs. 2D IR offers increased structural resolution by spreading the spectra over a second frequency dimension to reveal two-dimensional line shapes and cross-peaks. In addition, it carries picosecond time resolution, making it an excellent choice for understanding protein dynamics.



In 2D IR spectra, cross peaks arise from anharmonic coupling between vibrations. For example, the spectra of ordered antiparallel β sheets shows a cross peak between the strong $\nu_\perp$ mode at ~1620 cm^{-1} and the weaker $\nu_\parallel$ mode at ~1680 cm^{-1}. In proteins with β-sheet content, disorder spreads the cross peaks into ridges, which gives rise to a "Z"-shaped contour profile. 2D IR spectra of α helices show a flattened "figure-8" line shape, and random coils give rise to unstructured, diagonally elongated bands.

A distinguishing quality of 2D IR is the availability of accurate structure-based models to calculate spectra from atomistic structures and MD simulations. The amide I region is relatively isolated from other protein vibrations, which allows the spectra to be described by coupled anharmonic local amide I vibrations at each peptide unit.

One of the most exciting applications of 2D IR is to study protein unfolding dynamics. While 2D IR has been used to study equilibrium structural changes, it has the time resolution to probe all changes resulting from photoinitiated dynamics. Transient 2D IR has been used to probe downhill protein unfolding and hydrogen bond dynamics in peptides. Because 2D IR spectra can be calculated from folding MD simulations, opportunities arise for making rigorous connections.

By introduction of isotope labels, amide I 2D IR spectra can probe site-specific structure with picosecond time resolution. This has been used to reveal local information about picosecond fluctuations and disorder in β hairpins and peptides. Multimode 2D IR spectroscopy has been used to correlate the structure sensitivity of amide I with amide II to report on solvent accessibility and structural stability in proteins.

Introduction

Proteins undergo structural rearrangements over a vast range of time scales, from 10^{-13} to 10^{4} s. Biophysically relevant conformational changes include short-range fluctuations of protein side chains, torsions, and hydrogen bonds (~10^{-13}–10^{-11} s); protein reorientation, chain diffusion, nucleation, and folding of secondary structure (~10^{-9}–10^{-6} s); domain folding and tertiary contact formation (~10^{-6} s); and folding, binding, or aggregation kinetics through activated barrier crossing (>10^{-3} s). Most of our insight into these events is indirectly obtained through *kinetics* measurements because the standard toolset excels at characterizing the structure and population of stable states. In con-

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trast, what are desirable for probing the mechanism of protein structural changes are *dynamics* experiments that characterize structure during its evolution along a reaction coordinate.

Two-dimensional infrared spectroscopy is a new method that can be used in such protein dynamics experiments due to its combination of time and structural resolution. 2D IR is one of a rapidly expanding class of new ultrafast coherent vibrational spectroscopies[1−4] that are finding broad use in studies of molecular structure and dynamics that probe peptides,[5] proteins,[6−10] DNA,[11] chemical exchange kinetics,[12,13] hydrogen bonding,[14,15] and rapidly initiated chemical reactions.[10,16,17] Inspired by pulsed NMR techniques, 2D IR spreads a vibrational spectrum over two frequency axes to reveal vibrational couplings through cross peaks. A 2D IR spectrum correlates the frequency of vibrational excitation ω_1 (also called ω_τ or ω_{pump}) with the frequency of detection ω_3 (or ω_t or ω_{probe}). Diagonal peaks can be assigned to chemically distinct normal vibrational modes or eigenstates. The presence and splitting of cross peaks characterizes the anharmonic couplings between vibrations. Connectivity, distance, or orientation between chemical bonds can be extracted by modeling vibrational couplings.[18] Positive and negative amplitude features in 2D IR spectra, corresponding to induced absorption or stimulated emission processes in the detection step, characterize the vibrational anharmonicity. Since the measurement is made with a picosecond or faster "shutter speed", it captures this structural information on a faster time scale than the evolution of most protein dynamics. 2D IR diagonal and antidiagonal line widths report on inhomogeneous and homogeneous broadening, respectively, and can be analyzed in more detail to describe variance in structural parameters.[19]

From its first realizations, 2D IR has been applied to the amide I backbone vibrations of proteins,[20] and the methodology for extracting structural information about peptides and small molecules has already been reviewed.[5,18] We survey 2D IR spectroscopy for the study of proteins, macromolecules with so many degrees of freedom that vibrational spectroscopy is often considered ambiguous, with emphasis on its use for dynamics and protein-folding experiments. We provide an introduction to amide I vibrations, which are widely studied, collective vibrations of the protein backbone that show spectral signatures for different secondary structural motifs. A distinctive feature of protein 2D IR spectra is that they can be accurately modeled from a set of structural coordinates. We discuss how calculating 2D IR spectra can bridge the gap between the short time scale, atomistic data from simulation and the coarse-grained experimental descriptions used to model changes over decades in time.

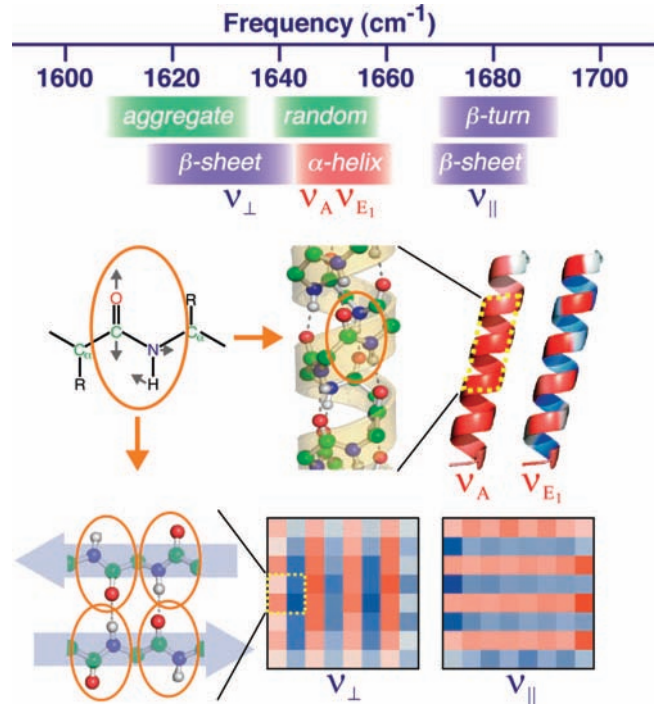

FIGURE 1. Amide I band in proteins: (top) empirical protein structure−frequency relationships in the amide I region; (bottom) a color-coded visualization of the IR active β-sheet and α-helix states where the shading intensity is proportional to the participation ratio of a unit oscillator and the color denotes its phase; blue and red are 180° out-of-phase.

Amide I 2D IR Spectroscopy

Amide I Vibrations. Amide group vibrations of the backbone receive the most attention in protein IR spectroscopy because they are native to all proteins and report on secondary conformation and solvation. These include amide I (primarily CO stretch), amide II (CN stretch and NH in-plane bend), amide III (CN stretch, NH bend, and CO in-plane bend), and amide A (NH stretch). The amide I band (1600−1700 cm^{-1}) is by far the most studied because its line shape is sensitive to the type and amount of secondary structures and is not strongly influenced by side chains.[21] Well-established empirical structure−frequency correlations, summarized in Figure 1, find that β sheets have a strong absorption band near 1630−1640 cm^{-1} and a weaker band at high frequencies (>1680 cm^{-1}). The peaks for α helices and random coils are located at 1640−1660 and 1640−1650 cm^{-1}, respectively.

The secondary structure sensitivity of amide I results from coupling between amide I oscillators that leads to vibrational states delocalized over large regions of the protein.[22−24] To illustrate, the IR-active amide I vibrational modes of ideal anti-

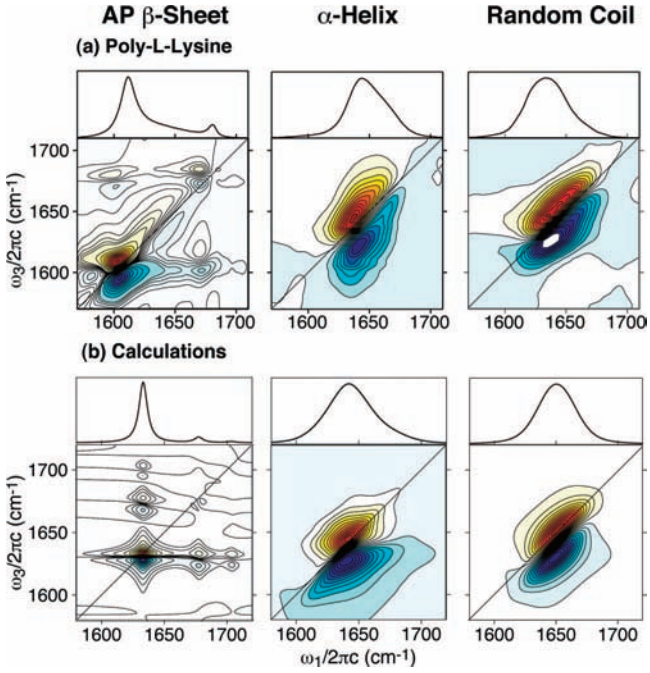

FIGURE 2. Model secondary structures and spectra: (top) FTIR and 2D IR spectra of the three forms of poly(L-lysine) compared with calculated FTIR and 2D IR spectra of idealized structures (bottom). Gaussian random site energies are sampled around a mean of 1650 cm^{-1} for the α helix and random coil (σ = 10 cm^{-1} and 12 cm^{-1}) with a homogeneous line width of γ = 12 cm^{-1} for all.

parallel (AP) β sheets and α helices are visualized in Figure 1. The color-coded diagrams indicate the vibrational amplitude and phase of the individual peptide oscillators within the normal modes of these secondary structures. Antiparallel β sheets are predicted to have two dominant IR active modes. For the intense lower frequency $\nu_\perp$ mode (also called $a-$), oscillators are in-phase perpendicular to the β strands and out-of-phase with their bonded neighbors. These relative phases are flipped for the $\nu_{||}$ mode (also called $a+$). α-Helices have two bright states corresponding to the modes shown in Figure 1. Since the carbonyls are aligned with the helix axis, most of the oscillator strength is carried by the ν_A mode with all oscillators in-phase and less by the degenerate ν_{E1} modes whose phase varies with a period of 3.6 residues.

Amide I 2D IR of Secondary Structure. Two-dimensional infrared spectroscopy provides an extra level of discrimination by spreading congested FTIR spectra over an additional dimension to correlate different spectral features. To demonstrate, FTIR and 2D IR spectra for the three most common secondary structure motifs are shown in Figure 2. The AP β-sheet, α-helix, and random coil states of poly(L-lysine) have served as basis spectra for circular dichroism and exhibit FTIR peaks with the canonical secondary structure assignments.

For AP β sheets, the amide I FTIR spectrum shows the strong $\nu_\perp$ mode at ~1620 cm^{-1} and a weaker intensity $\nu_{||}$ mode at ~1680 cm^{-1}. The corresponding 2D IR spectrum has $\nu_\perp$ and $\nu_{||}$ peaks along the diagonal and two off-diagonal cross peaks. The frequency and intensity of $\nu_\perp$ is sensitive to the size of the β sheet,[6] which causes the $\nu_\perp/\nu_{||}$ splitting to increase as the sheet grows. Due to vibrational anharmonicity, induced absorption (positive peaks) and stimulated emission (negative peaks) appear displaced along ω_1, which causes 2D IR spectra to be asymmetric about the diagonal.

In FTIR spectra, both α helices and random coil regions appear as a single peak. For α helices, this peak is at ~1650 cm^{-1}. The corresponding 2D IR spectra show a flattened "figure-8" line shape due to their composition of a ν_A mode at ~1639 cm^{-1} and doubly degenerate ν_{E1} modes at ~1652 cm^{-1} that are typically irresolvable with natural line widths of ~15 cm^{-1}. Extended 3_{10} helices are predicted to have similar ν_A and ν_{E1} modes; however, this assignment is not straightforward in realistic 3_{10} helices that typically appear as short segments capping the ends of α helices.[25]

Unstructured proteins and random coils display a symmetric peak in the FTIR spectrum at ~1640 cm^{-1} that overlaps with the α-helical region. The 2D IR signature of this disorder is diagonal elongation caused by the stochastic variation in structure and hydrogen-bonding environments. The diagonal line width reports on inhomogeneous broadening, whereas the antidiagonal gives the homogeneous line width. Disordered regions in the experimental AP β sheet yield a random coil feature in between the $\nu_\perp$ and $\nu_{||}$ bands.

Protein 2D IR Spectra. Features from the amide I 2D IR spectra of idealized β sheets, α helices, and random coils presented in the previous section can be seen in the variety of protein spectra in Figure 3. The two extended β sheets in concanavalin A (con A) give rise to a large ~40 cm^{-1} splitting between $\nu_\perp$ and $\nu_{||}$ and a well-defined cross peak in the top left quadrant that is similar to those of the β-sheet form of poly(L-lysine) (Figure 2a). An overall "Z"-shaped contour profile appears for proteins with AP β sheets due to diagonal elongation of $\nu_\perp$ with disorder, diagonal peaks at ~1650 cm^{-1}, and constructive interference with cross peaks in the ω_1 dimension.

With decreasing β-sheet content, the splitting between $\nu_{||}$ and $\nu_\perp$ decreases and the $\nu_\perp/\nu_{||}$ cross-peak becomes a ridge extending from the $\nu_{||}$ band. This trend in splitting continues with β-lactoglobulin and RNase A, which retain AP β-sheet features despite twisted sheets. β-lactoglobulin, which contains an eight-stranded β barrel, shows distinct bifurcation of the $\nu_\perp$ band. The spectrum for RNase A shows an α-helix peak along

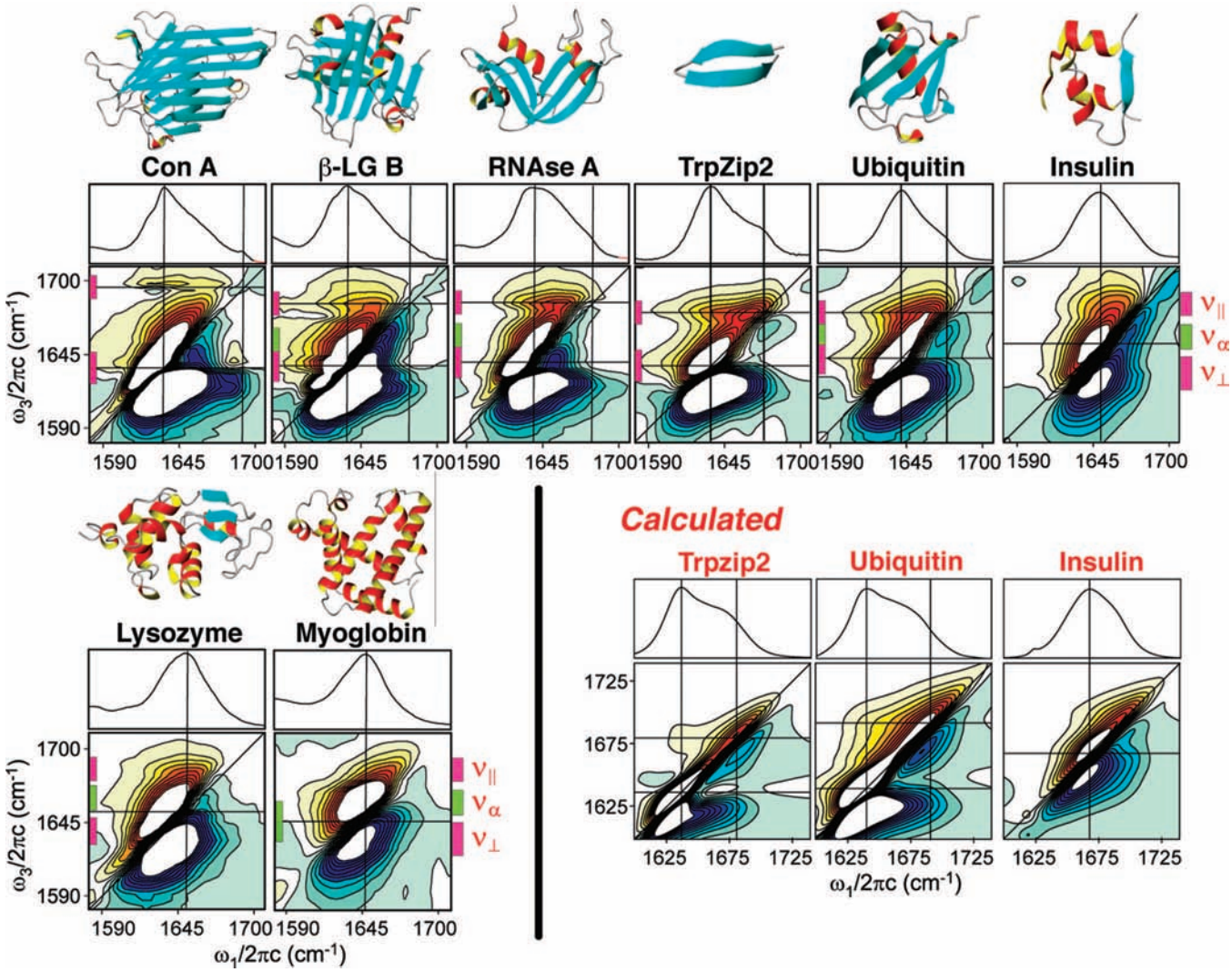

FIGURE 3. Amide I protein 2D IR spectra: experimental FTIR and 2D IR spectra for various proteins compared with the calculated spectra for trpzip2, ubiquitin, and insulin.

the diagonal at 1650 cm^{-1}. The β hairpin trpzip2 has a clear $\nu_{||}$ peak and a strong ridge indicative of its stability.[26] Ubiquitin shows the same "Z"-shape, although with smaller splitting, even though its five-stranded sheet contains a mixture of AP and parallel contacts. Lysozyme, insulin, and myoglobin display nearly identical FTIR spectra, but a comparison of their 2D IR spectra shows subtle plateaus for lysozyme both above and below the diagonal that originate in its small sheet.

Modeling of Amide I Spectroscopy

The feature that sets 2D IR apart from other fast protein probes is that the data can readily be calculated from an atomistic structure or molecular dynamics (MD) simulation. A parametrized model uses a structure from simulations, X-ray crystallography, NMR, or a trial construction to assign a so-called local amide Hamiltonian (LAH), which contains local amide I frequencies (site energies) and vibrational couplings between sites. The LAH is diagonalized to calculate FTIR spectra or scaled to include two-quantum states and diagonalized to calculate 2D IR spectra. This provides an avenue for comparing experiment and simulation in which the simulation predicts how atomistic structural changes appear in spectroscopic data and the experiment benchmarks the dynamical validity of computer models.

Spectral calculations are only feasible because amide I vibrations are largely isolated from the other protein vibrations and can be described as linear combinations of local amide I vibrations at each peptide unit. The underlying physics of an amide I subspace have been known for over 30 years through the pioneering work of Miyazawa[27] and Krimm[28] and were first applied to calculate FTIR spectra of proteins by Torii and Tasumi.[29] Hamm and Hochstrasser[20] adapted this force field into a model useful for 2D IR by

describing amide I states as vibrational excitons. The general features of 2D IR spectra of β sheets and α helices can be understood by analytical models for the spectra of idealized secondary structures (Figure 2b),[6,30,31] which have produced intuitive descriptions of the effects of amide group symmetry layout and disorder.

For the purpose of comparing with experimental 2D IR spectra of proteins, several groups have contributed to the development of numerical methods required to convert transient structures into a LAH.[32–40] Although harmonic molecular mechanics (MM) force fields are not accurate enough to calculate 2D IR spectra, MM structures and electrostatics can be used to calculate LAH matrix elements by high-level quantum chemistry parametrizations. The amide I site energies, or diagonal LAH elements, are defined by the local hydrogen-bonding environment. Stronger hydrogen bonds to the amide carbonyl lead to lower site energies and are quantified using a linear correlation between the amide I frequency and the electrostatic potential[34–36,39] or electric field[36,38,40] at the site. The off-diagonal LAH elements or couplings between sites include both through-bond and through-space interactions. Through-space interactions can be treated with transition dipole coupling[28] or interactions between transition charges at each atomic site of the peptide unit.[33,40] Through-bond couplings are parametrized from calculations on small peptides based on backbone dihedral angles.[32,34,37,40] The strongest couplings are through-space between hydrogen-bonded contacts across β strands or in α helices. A summary of the different approaches and a comparison of different models was presented in ref 41.

The site energies and couplings derived from one structure cannot reproduce 2D IR line shapes without including dynamics.[41] Structural fluctuations much slower than the amide I dephasing time of ~1 ps appear in the spectrum as static disorder. The most computationally efficient models work in this static limit and treat LAH matrix elements as random variables sampled from an appropriate distribution[20,31] or by summing spectra from a distribution of protein structures.[41] More accurate methods include "motional narrowing" effects into line shapes by calculating Fourier transforms of transition dipole time-correlation functions,[42–44] but one cannot treat full proteins (N > 30 residues) without simplifying approximations.[45] Presently, computation of 2D IR spectra is limited by the $O(N^4)$ scaling of the number of two-quantum matrix elements in protein residues.

A demonstration of 2D IR spectra calculated from molecular dynamics simulations for trpzip2, insulin, and ubiquitin appears in Figure 3. The crystal structures are solvated and equilibrated in explicit water, and then spectra are calculated and summed over an ensemble of structures from short (~2 ns) MD simulations. Frequency splittings, cross-peak ridges, and line shapes, as well as many subtle features, are reproduced with accuracy sufficient to make experimental predictions and assign frequency regions.

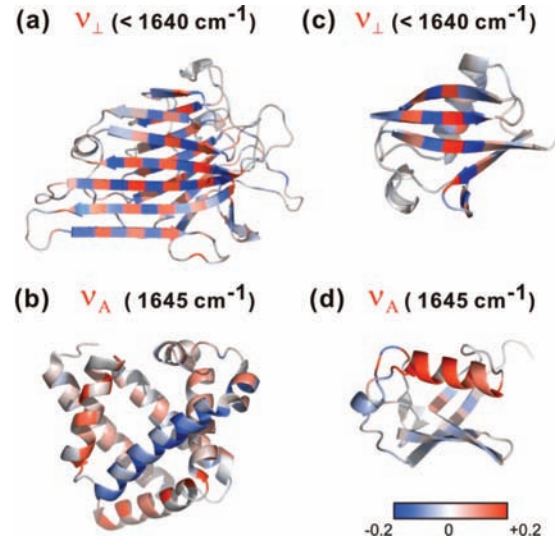

FIGURE 4. Doorway modes: Bright states are pictured for the $\nu_\perp$ vibration in concanavalin A (a) and ubiquitin (c) and the ν_A mode in myoglobin (b) and ubiquitin (d) to demonstrate the delocalization over secondary structures and separability of β-sheet and α-helical modes. Adapted from ref 24.

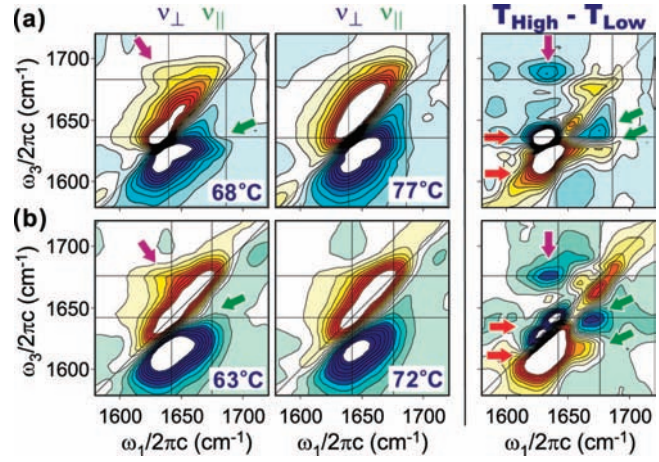

FIGURE 5. Thermal unfolding 2D IR: Thermal changes in the 2D IR of RNase A (a) and ubiquitin (b), below and above the melting temperature and difference spectra (left, middle, and right, respectively). Red arrows highlight the loss of diagonal features. Purple and green arrows highlight the loss of cross peak features above and below the diagonal.

One can use spectra in agreement with experiment to investigate the structural composition of amide I modes using doorway mode visualization, a method we have developed[24] by extending the work of Torii and Tasumi.[22] The eigenstates within a narrow frequency region are decomposed into bright

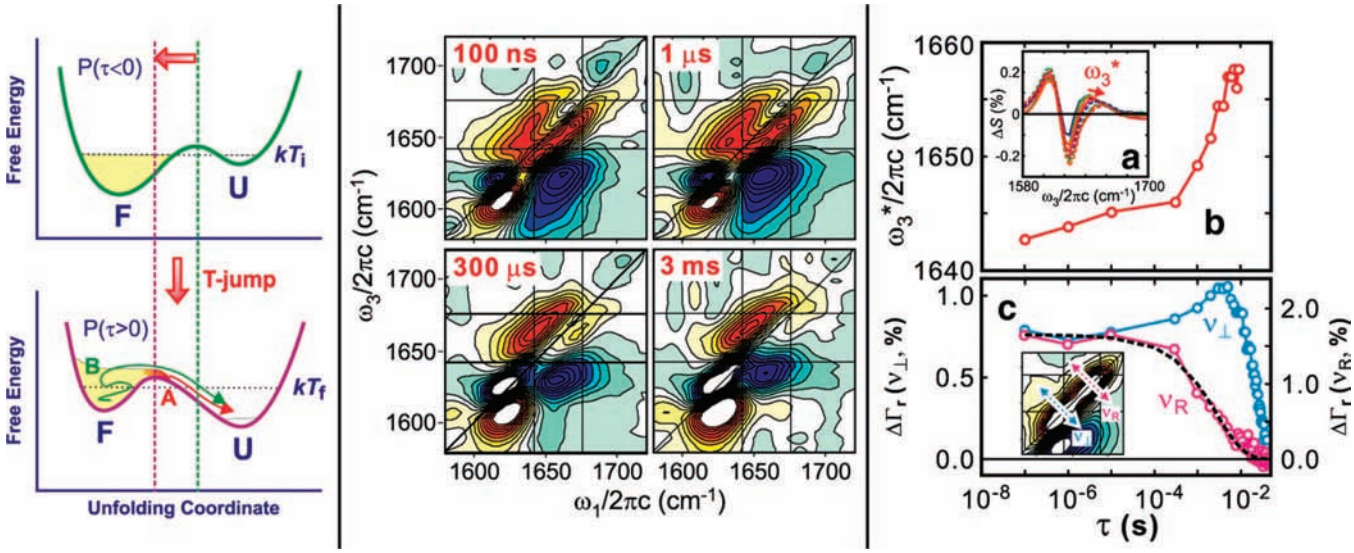

FIGURE 6. Ubiquitin unfolding dynamics: (left) a schematic view of the temperature-jump unfolding experiment preparing burst phase, downhill unfolding subensembles (A) and ensembles undergoing activated barrier crossing (B); (middle) transient 2D IR spectra at indicated time delays after *T*-jump; (right) slices at $\omega_1 = 1640$ cm^{-1} of the transient spectra (a) showing the blue shift in cross peak (b) and antidiagonal line widths (c) reporting on fluctuations in the random coil and β-sheet regions after *T*-jump. Adapted from ref 10.

states that carry the IR oscillator strength, using singular value decomposition. The contribution of each oscillator to these modes can be visualized in a manner similar to the idealized modes (Figure 1). Figure 4 shows bright states for the $\nu_\perp$ mode in con A (panel a) demonstrating that the mode is delocalized over the entire β sheet and it retains the symmetry properties of the idealized case. In myoglobin (Figure 4b), asymmetrical α helix arrangements mix ν_A modes on different helices. Figure 4c,d demonstrate that β-sheet and α-helix modes in ubiquitin can roughly be separated by frequency.

Applications of Protein 2D IR Spectroscopy

Protein Unfolding. The ability of 2D IR to reveal the degree of secondary structure contacts makes it a useful tool to study equilibrium thermal unfolding,[8,9,26] which is demonstrated with RNase A[8] (Figure 5a) and ubiquitin[9] (Figure 5b). The changes to the contour profile across the melting curve show the loss of β-sheet modes (red arrows) and a blue shift of the band center caused by an increase in the random coil region. Diagonal elongation can also be seen by inspecting the (negative) overtone transition. Cross-peak ridges extending along ω_1, both above and below the diagonal (purple and green arrows, respectively) are mostly lost upon heating, but residual β-sheet content is seen by elongation and asymmetry in the line shapes compared with a random coil spectrum (Figure 2c).

While 2D IR provides a unique way to study equilibrium structural changes, its picosecond time resolution makes it a natural transient probe of photoinitiated dynamics.[16,41] In the case of folding, the ability of laser temperature jumps to create nonequilibrium states in unstable regions of the free energy surface can be used to characterize conformational dynamics and folding transition states. In our view, when a ~7 ns *T*-jump laser pulse heats the solvent faster than the protein can reconfigure, it shifts the free-energy bias to favor the unfolded state and moves the barrier toward the folded state (Hammond−Leffler principle) as illustrated in Figure 6 (left).[9,46] A subensemble that was formerly in the folded well suddenly experiences a gradient, which drives quasi-barrierless unfolding on the nanosecond to microsecond time scale and is observed as the "burst phase" in kinetics experiments. The remainder of the ensemble equilibrates on the millisecond or longer time scale through activated barrier crossing. The validity of this "downhill unfolding" scenario, a contested prediction of energy landscape theory,[47,48] would provide an opportunity to characterize elusive transition states. We believe this methodology is general insomuch as the folded state encompasses some structural flexibility, which provides an entropic handle for thermal changes in free energy.

We have studied the *T*-jump unfolding of ubiquitin with a combined experimental and simulation approach.[9,10] The transient 2D IR data on nanosecond to millisecond time scales, presented as transient difference spectra relative to the equilibrium spectrum, are shown in Figure 6 (middle). Decreased couplings between ubiquitin's β-sheet modes are observed on the fastest time scales by several signatures. The two diagonal $\nu_\perp$ and $\nu_{||}$ modes of the β sheet lose intensity, and there is a concerted rise in the random coil region. The cross-peak ridge between these two modes also gradually decreases

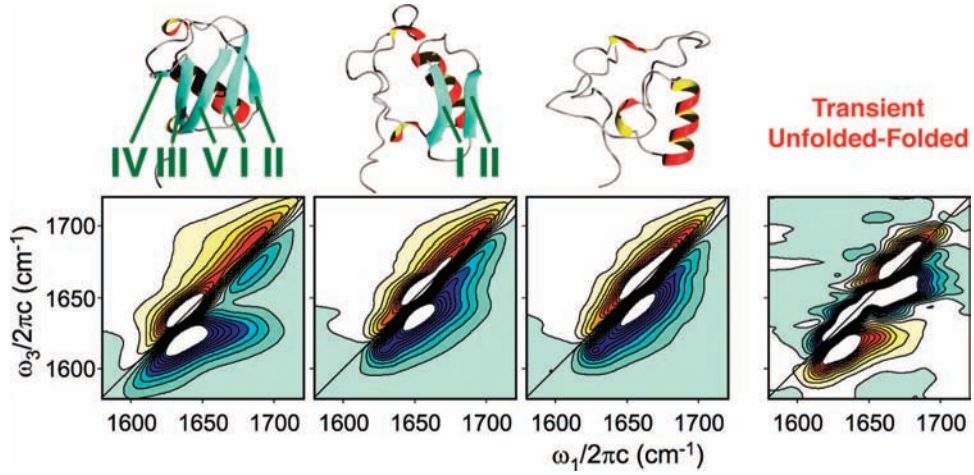

FIGURE 7. Ubiquitin unfolding dynamics: calculated 2D IR spectra for equilibrium ubiquitin, solvated snapshots corresponding to the structures shown, and difference spectrum. Adapted from ref 10.

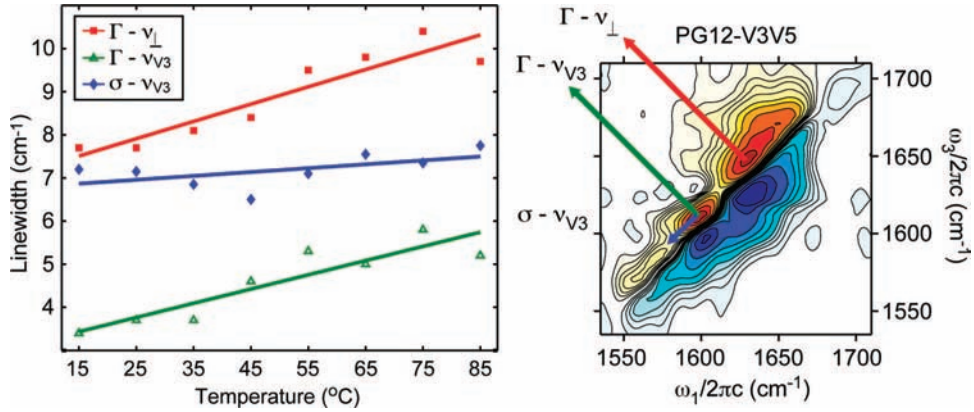

FIGURE 8. Thermal change in homogeneous broadening: Measurements of 2D line widths for several peaks in the 2D IR spectra of PG12-V3V5 from 15 to 85 °C. Antidiagonal (Γ) and diagonal (σ) half-width at half-maximum of the peaks are shown with linear fits with the following slopes: $\Gamma-\nu_{\perp} = 0.040$ cm^{-1}/°C, $\Gamma-\nu_{V3} = 0.033$ cm^{-1}/°C, and $\sigma-\nu_{V3} = 0.009$ cm^{-1}/°C. Adapted from ref 53.

while blue-shifting in frequency, indicating a decrease of the amide I delocalization as a result of β-sheet unfolding. As the sheet unfolds, increased fluctuations and solvent exposure of the β-sheet amide groups are also characterized by increases in homogeneous line width (Figure 6, right). The nanosecond to microsecond nonexponential relaxation observed is consistent with the expectations of a downhill unfolding process.[9,10]

These transient spectral changes are in qualitative agreement with structural changes observed in a *T*-jump ubiquitin unfolding simulation study.[49] The simulation shows a sequential loss of strands V-III-IV from the β sheet, leaving a stable core consisting of the α helix and the N-terminal β hairpin (I-II). In the latter stages of unfolding, the hairpin disassociates. To provide a quantitative basis for comparison, the experimental frequency and intensity shifts were directly modeled using the simulation.[49] 2D IR spectra were calculated from several persistent structures along the strand-by-strand unfolding pathway (Figure 7) and averaged over solvation environments. Calculated transient difference spectra were in agreement with the experimentally observed intensity changes for the β-sheet and random coil spectral features and the frequency shift of $\nu_{\perp}$ on unfolding. Although this comparison is made only to a single unfolding trajectory, this work demonstrates the general principles of using a structural model to interpret 2D IR data from MD simulation and identify unfolding pathways.

Transient 2D IR techniques have also been used to watch hydrogen-bond dynamics of a disulfide-bridged β turn.[17] Structural changes following UV photolysis ascribed to β-turn opening and radical recombination were observed on time scales of 160 ps and 2.6 ns, respectively, and corroborated with nonequilibrium MD simulations. This finding is significant because it places an unusually fast limit on the inherent dynamical time scale for opening of a β turn. Further applications of transient 2D IR spectroscopy to phototriggered peptides have recently been reviewed.[50]

Site-Specific Amide I Spectroscopy. Although local structural information is obscured by the delocalized vibrations in

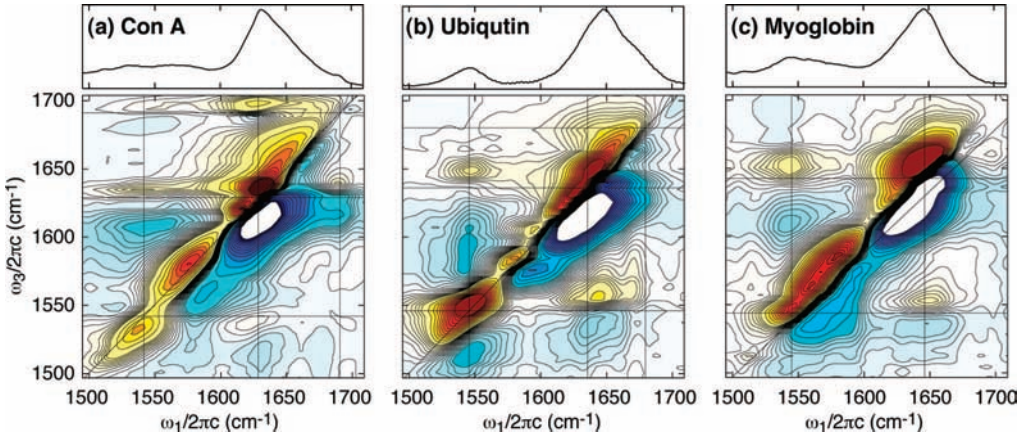

FIGURE 9. hydrogen−deuterium exchange 2D IR: partially exchanged 2D IR spectra at 5 °C in the amide I/I′/II region of concanavalin A (a), ubiquitin (b), and myoglobin (c) with arginine side chain vibrations at 1570−1580 cm^{-1}. Unexchanged structural motifs show an amide I/II cross-peak. Adapted from ref 55.

the native amide I band, site-specific resolution can be recovered with backbone site-specific ^{13}C or ^{18}O isotope labels or both, which isolate an amide I oscillator by shifting its site energy by 30 to 65 cm^{-1}. A distinctive strength of isotope-labeled 2D IR spectroscopy is the ability to independently measure homogeneous and inhomogeneous line shape parameters, which reveal local information on picosecond fluctuations and disorder in polypeptides. For a 27-residue helical fragment of the CD3ζ membrane protein, individual $^{13}C{=}^{18}O$ isotope labels placed at different sites along the peptide quantified the sequence-dependent disorder that peaks at the membrane−water interface.[51] After incorporation of $^{13}C{=}^{16}O$ isotope labels into the β-hairpin peptide trpzip2,[52] comparison of the diagonal elongation indicates that structural disorder at the N-terminus is greater than that near the β-turn. In the isotope-labeled PG12 β hairpin (Figure 8),[53] the homogeneous line width corresponding to a labeled site in the middle of the peptide was compared with the $\nu_{\perp}$ band to probe thermal denaturation. The labeled site, which primarily senses interstrand hydrogen bonding, shows considerably smaller fluctuations than the main band, which also surveys hydrogen bonding to water, and both show increased fluctuations with higher temperature. Isotope-labeled 2D IR spectroscopy has also been demonstrated as a structural biology tool in applications such as revealing vibrational delocalization though tertiary contact in a pair of transmembrane helical peptides.[54]

Multimode 2D IR Spectroscopy. While the sensitivity of the amide I vibration to secondary structure comprises an important coordinate, protein folding hinges upon understanding other variables such as side chain packing, hydrophobicity, and solvent interactions. Correlations between backbone, side chain, and solvent vibrations can be quantified by extending 2D IR to probe multiple vibrational regions. The amide II vibration is an effective solvent probe because it red shifts 100 cm^{-1} upon deuteration of the amide group (amide II′). This feature has been used to report on solvent accessibility and structural stability in proteins through the use of hydrogen/deuterium (H/D) exchange 2D IR spectroscopy.[55] Figure 9 shows the selectivity of the amide I/II cross peak to rigid secondary structures by correlating the structure sensitivity of amide I with the solvent-exposure sensitivity of amide II. The presence and position of amide I/II cross peaks for con A and myoglobin in D_2O indicate that protons in their stable secondary structures do not exchange rapidly, as random coil regions do. In the case of ubiquitin, an amide I/II cross peak to the α-helical region of the amide I spectrum indicates that the protons of the helix remain tightly bound, whereas those of the β sheet are labile.

From these types of experiments, one can imagine a number of 2D IR experiments that more critically address the nature of protein−water interactions. Through the use of isotopic labels and solvent mixtures, it is possible to perform multimode 2D IR probes of water−protein hydrogen bonds at a site-specific level or indirect observations of solvent exposure through H/D exchange on amide II and II′ modes. Implementing these model experiments[56,57] into equilibrium and folding studies on proteins would expand the types of questions that 2D IR can answer.

Outlook

Protein 2D IR spectroscopy remains a developing technique, yet it has matured enough to impact a variety of problems in protein structure, dynamics, and kinetics. The combination of fast time resolution with vibrational structure resolution makes 2D IR an excellent choice for understanding protein dynam-

ics. Amide I 2D IR spectroscopy allows for the study of real-time conformational changes in a protein ensemble for problems including folding, binding of substrates, dimerization, and aggregation. Spectroscopy on vibrations of prosthetic groups, side chains, and substrates provide an avenue for investigating local conformational changes and enzyme dynamics.

2D IR spectroscopy has the capability to provide detailed benchmarks for simulation. Transient 2D IR probes conformational dynamics on MD protein folding time scales, which can test *mechanisms* rather than thermodynamics or kinetics. Increasingly, accurate calculated 2D IR spectra have begun to pave the way for experiments to provide constructive feedback to MD force field developers.[58] We hope to see new efficient algorithms for calculating spectra and expanded parametrizations to model proline, side chains, and coupling to water modes and other amide backbone vibrations.

Continued advances are still needed to expand the scope of problems 2D IR can address. Efforts must be directed toward revealing more detailed structural data on proteins utilizing isotope labeling, labeling with vibrational chromophores, and multimode 2D IR techniques. New fast triggers for dynamical processes in proteins are required to take full advantage of the time resolution of 2D IR spectroscopy. With development of multimode probes that correlate protein−water configurations, 2D IR is uniquely suited to characterize the nature of protein−water interactions at hydrophobic and hydrophilic interfaces. Advances into the above directions promise to establish a new framework for understanding the dynamics of proteins.

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BIOGRAPHICAL INFORMATION

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