Published in final edited form as:

J Phys Chem B. 2013 December 12; 117(49): . doi:10.1021/jp402942s.

2D IR Cross Peaks Reveal Hydrogen-Deuterium Exchange with Single Residue Specificity

Emily B. Dunkelberger¹, Ann Marie Woys^{1,2}, and Martin T. Zanni^{1,*}

¹Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53706-1396

Abstract

A form of chemical exchange, hydrogen-deuterium exchange (HDX), has long been used as a method for studying the secondary and tertiary structure of peptides and proteins using mass spectrometry and NMR spectroscopy. Using 2D IR (two dimensional infrared) spectroscopy, we resolve cross peaks between the amide II band and a $^{13}\text{C}^{18}\text{O}$ isotope labeled amide I band, which we show measures HDX with site-specific resolution. By rapidly scanning 2D IR spectra using mid-IR pulse shaping, we monitor the kinetics of HDX exchange on-the-fly. For the antimicrobial peptide, ovispirin, bound to membrane bilayers, we find that the amide II peak decays with a biexponential with rate constants of 0.54 ± 0.02 and $0.12 \pm 0.01 \text{ min}^{-1}$, which is a measure of the overall HDX in the peptide. The cross peaks between Ile-10 labeled ovispirin and the amide II mode, which specifically monitor HDX kinetics at Ile-10, decay with a single rate constant of $0.36 \pm 0.1 \text{ min}^{-1}$. Comparing this exchange rate to theoretically determined exchange rates of Ile-10 for ovispirin in a solution random coil configuration, the exchange rate at Ile-10 is at least 100 times slower, consistent with the known α -helix structure of ovispirin in bilayers. Because backbone isotope labels produce only a very small shift of the amide II band, site-specific HDX cannot be measured with FTIR spectroscopy, which is why 2D IR spectroscopy is needed for these measurements.

Keywords

Hydrogen-deuterium exchange; two dimensional IR; 2D IR; membrane peptide; vibrational spectroscopy; infrared spectroscopy; ovispirin

Introduction

Two-dimensional infrared (2D IR) spectroscopy correlates vibrational modes, which provides information about molecular structure and dynamics. One recent development in 2D IR spectroscopy is the study of chemical exchange processes that occur on the femto/picosecond timescales.¹⁻¹¹ If two different molecular configurations absorb at two different frequencies, then the dynamics of the cross peaks between the two reveal the timescale at which one configuration exchanges into the other. A beautiful example is from the Fayer group, who applied this principle to study the phenol hydroxyl stretch in a benzene solvent mixture.⁷ By varying the waiting time in the vibrational echo pulse sequence, Fayer showed that the time dependence of the intensity of the cross peaks reports on the dissociation time of the solute-solvent complex, which occurred in only 8 ps. Such information cannot be obtained from linear spectroscopy, like FTIR spectroscopy, because FTIR spectroscopy solely reports on the equilibrium concentrations. To obtain kinetics, one needs to know the

*Corresponding author: zanni@chem.wisc.edu, 608-262-4783.

²Current Address: Department of Chemistry, Northwestern University, Evanston, IL 60208-3113

exchange rate, which is contained in the cross peaks of 2D IR spectra. In this article, we utilize a variant of chemical exchange to study hydrogen-deuterium exchange (HDX) in a polypeptide. We show that by using isotope labeling and monitoring the cross peaks in rapidly scanned 2D IR spectra, we obtain single-residue exchange information. Such information cannot be obtained from FTIR spectroscopy.

The principle behind HDX is that the amide proton on each amino acid is in rapid exchange with hydrogen atoms in the solvent. Shown in Figure 1a is a protonated peptide bond. After adding deuterium atoms to the sample using D₂O, amide protons exchange with solvent deuterium atoms, if they are solvent accessible and in a conformation that allows proton exchange. This isotopic exchange does not perturb the structure of the protein, but does provide information on which amide bonds are solvent exposed or protected by internal hydrogen bonds, thereby providing insights into the secondary and tertiary structure of peptides. The rate of exchange also depends on other factors. In general, HDX is strongly pH-dependent; base-catalyzed HDX is more effective than acid-catalyzed exchange, as evidenced by measuring HDX rates in poly-alanine as a function of pH.^{12–15} At physiological pH, HDX occurs in random coil and solvent exposed residues on the timescale of milliseconds-seconds.¹³ Exchange times are longer for proteins with regular secondary structures. A good example is human serum albumin, a globular peptide with α -helix, β -turn, and extended chain secondary structures. The residues in the β -turns are relatively unstructured and were shown to be most exposed to deuterated solvent, thus they exchanged rapidly, on the order of less than two minutes. Random coil residues exchanged on the order of 30 minutes, and α -helix residues, the most structured portion of the protein, exchanged over a few hours. Nearly 25% of the peptide never exchanged in the four hour experimental window due to the high stability its hydrophobic core.¹⁶ HDX can take even longer or cease to happen in transmembrane proteins.^{17–18} Previous studies have shown that residues in a transmembrane protein that lie in the center of the membrane bilayer do not undergo HDX in 3–4 days following sample exposure to deuterium, but residues on the surface can exchange rapidly, and thus provide information about membrane protein structure.

The key to obtaining detailed structural information from HDX is to measure exchange rates on individual residues. To this end, solid-state and solution NMR spectroscopies are very good methods. They have been used extensively to study the structure of a variety of peptides.^{14, 17, 19–21} Electrospray ionization mass spectrometry (ESI-MS) is another good tool to measure HDX to determine the structure of membrane bound peptides and amyloid peptides.^{18, 22–25} NMR spectroscopy and mass spectrometry techniques often require conditions which may be perturbative, such as low pH to slow down HDX or quenching to stop it. Nonetheless, the information that one obtains from residue-by-residue HDX exchange kinetics has provided important insights into many problems in structural biology.

Fourier transform infrared (FTIR) spectroscopy is also often used to measure HDX,^{16, 26–31} but unlike NMR spectroscopy and mass spectrometry, it does not provide residue-level structural information even when using isotope labels. As such, it is often difficult to obtain precise structural information from the kinetics. The problem is as follows. The amide I mode is composed primarily of C=O stretch motions with a little CN stretch and typically absorbs between 1620 and 1680 cm⁻¹, depending on the secondary structure(s) of the protein.³² If a single residue is ¹³C¹⁸O isotope labeled, then it is largely decoupled from the other amide I modes and residues near 1590 cm⁻¹, which is why ¹³C¹⁸O labeling is often used in FTIR and 2D IR spectroscopy to obtain residue-level structural resolution. However, the amide I mode frequency depends very little on whether the peptide group is protonated or deuterated. At most, there is an 8 cm⁻¹ difference between the frequency of the amide I mode when there is a hydrogen atom in the amide position and when there is a deuterium atom in the amide position; thus it is not a good mode for monitoring HDX.^{16, 32} In contrast,

the amide II mode is mostly CN stretch and NH bend motions. Because of the bend, its frequency is highly dependent on whether the nitrogen is protonated or deuterated. If protonated, the amide II mode absorbs at $\sim 1550\text{ cm}^{-1}$.³² If deuterated, it absorbs at $\sim 1450\text{ cm}^{-1}$ (Figure 1c and d), which is often called the amide II' band. Thus, the amide II mode is very sensitive to HDX. However, it alone does not provide residue-level structural information because isotope labeling with ^{13}C or ^{18}O has no effect on the amide II frequency and ^{15}N only produces a red shift of $13\text{--}15\text{ cm}^{-1}$, which is much smaller than the amide II linewidth and so cannot typically be resolved for polypeptides larger than a few residues.^{33–34} Thus, FTIR spectroscopy cannot obtain site-specific HDX on proteins, because neither the amide I nor the amide II modes alone contain the necessary site-specificity and HDX sensitivity.

In this paper, we illustrate how one can take advantage of the coupling between the amide I and II vibrational modes in order to obtain residue-specific HDX using 2D IR spectroscopy. Our study builds on previous work using 2D IR spectroscopy and cross peaks between the amide I and amide II/II' modes to resolve HDX of different secondary structures in proteins.^{35–36} In combination with isotope labeling, one can also obtain site-specific HDX rates. Because the amide I and II modes both contain CN stretching motions, they are strongly coupled, and so the typical 2D IR spectrum exhibits large cross peaks between the two bands.^{33, 37} We take advantage of this fact to determine whether or not an isotope labeled residue is coupled to the amide II. Our scheme is illustrated in Figure 1. Figure 1b has a schematic 2D IR spectrum for a protonated peptide with a single $^{13}\text{C}^{18}\text{O}$ isotope label. As such, there are large diagonal peaks for all the unlabeled residues that would appear somewhere between 1620 and 1680 cm^{-1} depending on the secondary structure, which we chose to be 1650 cm^{-1} in this example. The single isotope labeled residue would appear at 1590 cm^{-1} , regardless of the secondary structure because it is largely decoupled from the other modes. A large amide II band would appear at $\sim 1550\text{ cm}^{-1}$, since all the residues are protonated. Cross peaks between the unlabeled amide I and amide II are shown, which are typical of all proteins that are not fully deuterated. These cross peaks have been measured previously using 2D IR spectroscopy.^{33, 35–38} The cross peaks that we are interested in appear between the $^{13}\text{C}^{18}\text{O}$ label and the amide II. These cross peaks appear because, before the addition of D_2O , the labeled residue is protonated. Shown in Figure 1d is a schematic for how the spectrum might look a few minutes after the addition of D_2O . The peaks along the diagonal would not change frequency, but the amide II mode would decrease in intensity as would its cross peaks to the amide I mode as amide hydrogen atoms exchange for deuterium atoms. If the isotope labeled residue has undergone HDX (which is the scenario we illustrate in Figure 1d), then the isotope labeled amide I mode is no longer coupled with the amide II mode, so that the cross peaks between these modes disappear. Thus, by monitoring the decay of the cross peaks between the labeled amide I and the amide II modes, one can measure the kinetics of HDX for a particular residue, because those particular cross peaks only report on the protonation state of the single isotope labeled residue, not the average of all the residues. Ideally, one would also monitor the cross peaks between the isotope labeled and the amide II' mode, which would increase with HDX, but that band currently lies outside our experimentally accessible frequency range.

In this Article, we apply this approach to the antibiotic peptide ovispirin bound to lipid bilayers. Ovispirin is a surface bound α -helical peptide whose structure is known from solid-state NMR, 2D IR, and molecular dynamics and thus is a good test system for our approach.^{39–40} By initially measuring 2D IR spectra in H_2O , we observe cross peaks between the amide I and amide II modes as well as between the isotope label and amide II modes. Upon deuterating the sample via addition of D_2O , the cross peaks between the amide I and amide II decrease in intensity as amide hydrogen atoms exchange for deuterium atoms, like illustrated in Figure 1. At the same time, the cross peak between the isotope label and

amide II also decreases in intensity, because the amide hydrogen located on the same amino acid as the isotope label has exchanged for deuterium. In addition, Arg residues in ovispirin also become deuterated, which create new diagonal peaks. For all discussions in this paper, the $^{13}\text{C}^{18}\text{O}$ labeled residue will be described as 'isotope label(ed)', and while exchanging hydrogen atoms for deuterium atoms is also a form of isotope labeling, this will be described as HDX.

Experimental

Peptide synthesis and isotope labeling Ile-10

Ovispirin, with the sequence $\text{H}_2\text{N-KNLRRIIRKIIHIIKKYG-COOH}$, was synthesized using standard Fmoc peptide synthesis techniques. One $^{13}\text{C}^{18}\text{O}$ carbonyl group was incorporated into Ile-10. To synthesize the isotope labeled residue, Fmoc-protected Ile, labeled with ^{13}C in the carbonyl position, underwent acid-catalyzed exchange with $^{18}\text{OH}_2$, to produce the fully-labeled carbonyl group. Further details of the isotope labeling procedure can be found elsewhere.^{41–43} Following peptide synthesis, ovispirin was purified by HPLC using a 0.1% TFA water/acetonitrile solvent system. Masses were confirmed using MALDI-MS.³⁹

Preparation of membranes

For membrane studies, a mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) (Avanti Polar Lipids) was produced. The lipids were purchased as chloroform solutions, and mixed at a molar of 3:1 POPC:POPG. The mixture was dried under a stream of nitrogen for four hours and lyophilized overnight. The dry film was rehydrated in 0.1 mM phosphate buffer that contained isotope labeled ovispirin, and the final peptide-to-lipid ratio was larger than 1:21.³⁹ A complete description of ovispirin synthesis, purification, and incorporation into membranes can be found elsewhere.³⁹

Two-dimensional infrared spectroscopy

A home-built Ti:Sapphire oscillator was used to seed a Nd:YLF-pumped regenerative amplifier, producing ultra-fast (~ 60 fs) 800 nm pulses. The 800 nm light was sent to a Type II BBO crystal-based optical parametric amplifier, producing signal and idler beams. The signal and idler were difference frequency mixed in a AgGaS₂ crystal and produced 6 μm limited bandwidth mid-IR light. The mid-IR light was specifically tuned to the frequency of the isotope labeled amide I peak ($\sim 1595\text{ cm}^{-1}$). Due to the bandwidth of the mid-IR light, the amide II' mode was not observed. The mid-IR light was split into pump and probe pulses; the pump passed through a Ge-based mid-IR pulse shaper before overlapping with the probe and focused to a spot of $\sim 120\text{ }\mu\text{m}$ at the sample cell. The probe was then directed to a monochromator and the signal was detected with a HgCdTe detector. All spectra were collected with parallel pump probe polarization. Further details of the experimental setup and pulse shaper can be found elsewhere.^{44–45}

Sample preparation

Experiments were performed first by measuring spectra of Ile-10 ovispirin hydrated in H_2O . A total of 2 μL of sample was deposited in 0.5 μL increments onto the same spot in the center of a CaF_2 window, and excess H_2O was removed under a gentle stream of nitrogen between each deposit. Bulk water was removed from the sample to reduce the signal from the water bend mode that absorbs near the amide I carbonyl mode. The sample was then covered with half of a CaF_2 window, sliced across the diameter. The sample was placed in the spectrometer such that the laser beam passed through the sample and both CaF_2 windows, within 2 mm of the edge of the half-window. Data collection was then initiated and after a few minutes we added 2 μL of D_2O to the sample cell at the edge of the half-

window. The D₂O was whisked between the two CaF₂ windows by capillary action in a fraction of a second. Using our rapid-scan pulse shaping technology, a 2D IR spectrum was collected every 0.4 s. Thus, the HDX exchange process was monitored continuously for 1 h with nearly no dead time. All diagonal slices are normalized to the unlabeled amide I band. All pump and probe slices shown in later figures are normalized to the Ile-10 amide I band, and all kinetics traces are normalized to the kinetics of the Ile-10 amide I band. The Ile-10 amide I band remains virtually unchanged during HDX, and thus by normalizing the kinetic features to this peak, any random laser fluctuations are eliminated from the data.

The HDX rates measured in this experiment exceed the permeability coefficient of water across a membrane.^{46–48} HDX light scattering experiments of vesicles measured the permeability coefficient of water to be $\sim 10^{-6}$ – 10^{-7} cm/s⁴⁷, while other experiments measured the permeability coefficient to be $\sim 10^{-1}$ – 10^{-3} cm/s.^{46, 48} The water permeability is dependent on lipid chain length, temperature, and vesicle size. However, estimating the thickness of a 3:1 mixture of POPC:POPG lipids to be 30 Å, water will permeate the membranes in this experiment in no more than ~ 2 s, and likely much faster. Thus, the exchange rates measured are not dependent on the mixing time of D₂O and the sample.

Results

Shown in Figure 2 are 2D IR spectra of Ile-10 labeled ovispirin in both H₂O and D₂O (30 min after D₂O addition), along with respective diagonal slices through the spectra. Consider the spectrum in H₂O. The pair of diagonal peaks at 1654 cm⁻¹ is due to the unlabeled (¹²C¹⁶O) amide I modes, the C-terminus carboxyl group, and various side chains. The pair of diagonal peaks at 1587 cm⁻¹ is the ¹³C¹⁸O isotope labeled amide I mode of Ile-10. The diagonal peaks at 1547 cm⁻¹ are due to the amide II mode.³² Other important features to point out are the cross peaks at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1654, 1547)$ cm⁻¹ and its conjugate (small-dash boxes). This set of cross peaks has been measured previously using 2D IR spectroscopy and contain many overlapping peaks since they result from coupling between the unlabeled amide I and the amide II mode.^{35–36, 49} The peaks that we are interested in are those connecting the isotope labeled amide I mode to the amide II mode, which appear at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1587, 1547)$ and $(1547, 1587)$ cm⁻¹ (solid boxes). These cross peaks are created by coupling from a single residue in the peptide.

Shown in Figure 2 is a spectrum (d) and diagonal slice (c) of Ile-10 ovispirin taken 30 min after addition of D₂O. The linewidth of the unlabeled amide I peak changes upon deuteration, due to the small shift in frequency of the amide I mode upon HDX (< 8 cm⁻¹); however, this frequency shift is not significant enough to use as a measure of HDX. Thus, HDX cannot be measured by the unlabeled amide I mode. The amide II mode has decreased by a factor of 3 in intensity. Additional features appear along the diagonal at 1607 cm⁻¹ due to deuteration of the (CH₃H₅)⁺ Arg sidechain (large-dash box). Ovispirin contains three Arg residues, and so the deuterated sidechain features prominently along the diagonal of the 2D IR spectrum. Original experiments performed on ovispirin were performed strictly in H₂O in order to avoid overlap of the Arg sidechain with the ¹³C¹⁸O isotope label.³⁹ But overlap is not a problem here since the cross peaks are still well-resolved, as we show below.

In addition to changing the intensities of the amide II and Arg sidechain peaks, HDX causes the intensities of the cross peaks to change as well. With the decrease in intensity of the amide II peak, the cross peaks between the unlabeled amide I and amide II $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1653, 1547)$ and $(1547, 1653)$ cm⁻¹ drop in intensity (small-dash boxes), but these features are difficult to interpret as mentioned above. More interestingly, the cross peaks between the isotope label amide I and amide II peaks $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1587, 1547)$

cm^{-1} and its conjugate, have decreased in intensity, indicating that HDX has occurred at Ile-10.

Shown in Figure 3b and 3c are expanded ranges of the spectra from Figure 2, highlighting the Ile-10/amide II cross peaks. To determine the extent of exchange, slices through the pump axes at 1587 cm^{-1} (dashed line) in H_2O and D_2O were compared (Figure 3a). The two slices in Figure 3a point out one cross peak between Ile-10 and the amide II (large-dash boxes in Figure 3a, b, and c) that changes intensity upon deuteration. The blue trace shows a dip in the spectrum near 1547 cm^{-1} , corresponding to the fundamental of the isotope label amide I/amide II cross peak, while the red trace has no resolvable negative peak. Comparing the intensities of these two traces in this region, the cross peak intensity between the isotope labeled amide I and amide II modes decreases by a factor of 4.3 upon HDX. The two slices in Figure 3d are slices through the probe axes at 1587 cm^{-1} of Ile-10 in H_2O and D_2O . The important feature in Figure 3d, enclosed by a small-dash box, is the other cross peak between Ile-10 and the amide II. In Figure 3d at $\omega_{\text{probe}}=1587 \text{ cm}^{-1}$ in H_2O , the cross peak between the amide II and isotope label amide I is well resolved (blue). However, upon addition of D_2O , the intensity of the cross peak decreases by a factor of 2 between the blue and red traces. The other major difference between the blue and red traces in Figure 3d is in the region of $\omega_{\text{pump}} > 1600 \text{ cm}^{-1}$. The only signal in this part of the spectra comes from the overtone of deuterated Arg sidechains; in the blue trace (H_2O), Arg is not deuterated and thus there is very little intensity in this part of the spectrum, while in the red trace (D_2O), Arg is deuterated and the slice goes through the deuterated Arg overtone peak, giving a positive peak above 1600 cm^{-1} . The amide II peak (enclosed with a solid black box in the 2D IR spectra) appears at 1547 cm^{-1} in both H_2O and D_2O . After addition of D_2O and 30 min of exchange time, the amide II peak decreases in intensity by a factor of 3 (slice comparison is not shown). One would expect that the change in intensity of cross peaks above and below the diagonal should be equivalent. However, the cross peaks above the diagonal at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1587, 1547) \text{ cm}^{-1}$ have destructive interference from the diagonal peak of the isotope/Arg combination peak at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1587, 1571) \text{ cm}^{-1}$. One could do multi-peak fits to separate overlapping modes.⁵⁰ However, we have chosen to concentrate on the cross peaks below the diagonal because they have less overlap with the diagonal peaks.

Using the rapid-scan capabilities of our pulse shaper, we measured the kinetics of HDX following the addition of D_2O to a sample that is originally protonated. As explained in the Experimental, the instrumental dead time is less than a fraction of a second and so for all purposes of this paper, the peptides are immediately exposed to D_2O . In addition, since spectra are collected every 0.4 s, the data points are congested in Figure 4 to form one smooth line. Figure 4 shows three kinetics traces: the decay of the intensity of the amide II peak $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1547, 1547) \text{ cm}^{-1}$ (a, blue), the growth of the intensity of the arginine peak $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1607, 1607) \text{ cm}^{-1}$ (b, green), and the decay of the intensity of the amide II/isotope labeled amide I cross peak $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1547, 1588) \text{ cm}^{-1}$ (c, red). Each of these traces shows the change in peak intensity at given pump and probe frequencies immediately after HDX initiation. The kinetics trace of the amide II peak was fit to a biexponential decay function with an offset that separated the exchange rates into two sub-populations of exchangeable amide hydrogen atoms and one population that did not exchange (Equation 1).

$$y = a \exp^{-bx} + c \exp^{-dx} + e \quad (1)$$

The coefficients a , c , and e represent the fraction of residues that exchange with the rate constant $1/b$ (population a), $1/d$ (population c), or do not exchange at all (population e). The coefficients are unitless, while b and d have units of min^{-1} . The fit to the amide II data is

shown in Figure 4a (cyan), and reveals a fast time constant of $0.54 \pm 0.02 \text{ min}^{-1}$ and the slower constant of $0.12 \pm 0.01 \text{ min}^{-1}$ (Table 1). The amide II band is not adequately fit by a single exponential, which indicates that there are a range of HDX timescales in the peptide. In contrast, the kinetics traces of the arginine peak and cross peak were fit to a single exponential decay function (Equation 2) and those fits are shown in Figure 4b (light green) and Figure 4c (magenta), respectively.

$$y = a \exp^{-bx} + e \quad (2)$$

The coefficient a is the fraction of residues that decay with the rate constant $1/b$, and e is the fraction of residues that do not exchange. Variables a and e are unitless and b has units of min^{-1} . The rate constant for exchange of the arginine peak is $1.96 \pm 0.15 \text{ min}^{-1}$ and for the cross peak is $0.36 \pm 0.1 \text{ min}^{-1}$ (Table 1). All fitted variables and decay times are given in Table 1. Kinetics of the cross peak in the upper half of the spectrum are not shown because of the previously described interference with the overtone of the Ile-10/Arg peak.

Discussion

Upon HDX of ovispirin, the amide II peak decreased intensity, as expected. This observation follows previous IR experiments showing that upon deuteration of a protein, the amide II peak intensity decreases and the amide II' peak increases. While the laser bandwidth and center frequency prevented observation of the amide II' peak, the amide II peak decreased by a factor of 3 over 1 h. The decay of the amide II peak is shown in Figure 4a and can be considered the average HDX kinetics for the entire peptide because the amide II mode has contributions from every residue in the peptide. The biexponential fit suggests that 6 residues exchange with a rate of $0.54 \pm 0.02 \text{ min}^{-1}$, 5 residues exchange with a rate of $0.12 \pm 0.01 \text{ min}^{-1}$, and 7 residues do not exchange, although this is only an approximation. Nonetheless, it is consistent with other experimental techniques; when individual residues are not resolved, multistep kinetics are observed.^{18, 24, 51–52} In transmembrane domain proteins, the overall deuterium content has been fit to a triexponential function where the first class of hydrogen atoms exchange rapidly (less than 1 min), the second class exchange slower (on the order of tens of minutes), and the third class virtually never exchanges.^{18, 51} Ovispirin is not a transmembrane peptide and it lies mostly parallel to the membrane surface; however, multiple classes of hydrogen atoms likely exist for this mostly α -helical surface-bound peptide. The residues that lie completely outside the with membrane will likely have the fastest exchange, residues in the most ordered secondary structure deep in the membrane will likely have the slowest exchange, and then residues somewhat exposed to solvent or in a less ordered secondary structure will have intermediate exchange rates. While we do not fit our data to a triexponential function, the fast exchanging hydrogen atoms exchange in $1.9 \pm 0.07 \text{ min}$, the intermediate exchanging hydrogen atoms exchange in $8.3 \pm 0.7 \text{ min}$, and it is likely there is a third, longer exchanging sub-population of amide hydrogen atoms that decay on the 1 h or longer timescale. After 1 h of HDX, the amide II peak has decayed and appears not to be changing, but there is still a peak at the amide II frequency in the 2D IR spectrum (not shown). This suggests that while exchange has become very slow, not every amide II hydrogen atom has exchanged for a deuterium atom.

As described above, upon HDX of Ile-10 ovispirin, an additional peak grows in along the diagonal of the 2D IR spectrum at 1607 cm^{-1} (Figure 2d). This additional peak can be attributed to deuteration of the guanidyl group of Arg sidechains, of which ovispirin has three. Previously reported values of deuterated Arg sidechain frequencies are 1587 cm^{-1} and 1610 cm^{-1} in solution and 1582 cm^{-1} and 1610 cm^{-1} in a micelle.⁵³ The two frequencies derive from degenerate CN stretch modes in the guanidyl group. The Arg peak in Figure 2d derives from one of the degenerate modes, while the other is likely absorbing in the same

position as the Ile-10 peak at 1587 cm^{-1} . Intensity from deuterated Arg is contributing to the intensity of Ile-10 along the diagonal and could impact the scaling factors used to produce the slices in Figure 3. The rapid increase in intensity of the Arg sidechain diagonal peaks shown in Figure 4b suggests that the three Arg residues rapidly exchanged hydrogen atoms for deuterium atoms, in $0.51 \pm 0.04\text{ min}$ with an exchange constant of $1.96 \pm 0.15\text{ min}^{-1}$, and therefore the three Arg residues in ovispirin must be highly solvent exposed. According to structures from ssNMR and 2D IR spectroscopies, the three Arg residues are all solvent exposed; this follows suit with the three Arg residues rapidly undergoing HDX with nearby water molecules.^{39–40} Monitoring HDX of sidechains can provide a way of ensuring the peptide is exposed to D_2O .

HDX rates of sidechain hydrogen atoms in Arg have rarely been studied; to our knowledge there has been one circular dichroism spectroscopy study of HDX rates of the hydrogen atoms in Arg sidechains in solution⁵⁴ and a few NMR spectroscopy studies that measured the HDX rates of the hydrogen atoms in Arg sidechains in proteins.^{55–56} The Arg sidechain has two types of exchangeable hydrogen atoms, and thus two different exchange rates; however, these rates only differ by an order of magnitude at low pH (~ 2) and are the same order of magnitude at higher pH (>3.5).⁵⁴ At pH ~ 3.5 both exchange rates are $\sim 0.5\text{ sec}^{-1}$, and it can be assumed that the exchange rates increase by an order of magnitude with each increasing pH unit.¹³ At neutral pH, the exchange rate of the sidechain hydrogen atoms can be estimated to be $\sim 0.5 \times 10^4\text{ sec}^{-1}$, which is extremely fast. In protein systems, using NMR spectroscopy, the HDX rates of hydrogen atoms in Arg sidechains has been measured to be $10^2\text{--}10^3\text{ sec}^{-1}$.⁵⁶ Although the HDX rate of Arg in solution has not been measured at neutral pH and thus cannot be directly compared, it must be very fast. Comparing the estimated exchange rate in solution with that measured in ovispirin, the Arg sidechains are protected from HDX in the peptide. It has not been common practice to measure HDX rates of sidechain hydrogen atoms, and thus measure protection factors, likely because exchange happens too quickly for most NMR spectroscopy techniques to capture. However, IR spectroscopy can capture fast HDX rates, and could be used to measure sidechain protection factors.

The most structurally interesting cross peaks in these 2D IR spectra are between the isotope label and amide II peaks, shown in Figure 3 (dashed boxes). By monitoring the intensity of these cross peaks over time, site-specific HDX was monitored in real-time. Amide I/amide II coupling has been shown to be strongest within one peptide unit and has been measured in the model molecule NMA.^{33, 49, 57} The Ile-10/amide II cross peak must then derive from coupling between those two modes on the same amino acid; therefore if the cross peak intensity decreases after addition of D_2O , the amide proton on Ile-10 must undergo HDX. The amide hydrogen atom of Ile-10 exchanged for deuterium with an exchange constant of $0.36 \pm 0.1\text{ min}^{-1}$, corresponding to $2.8 \pm 1\text{ min}$ after deuterated buffer was added to the sample (Figure 4c), and this gives insight into the structure of ovispirin on a membrane. HDX rates are a function of temperature, pH, as well as the residues adjacent to the amide group of interest. The rate of HDX for a variety of peptide sequences at various temperatures and pH has been measured, and attempts to use that data to extrapolate protection factors have been fruitful.⁵⁸ The protection factor at a given residue is a ratio of the rate of HDX of that residue in a solvated unstructured peptide compared to the rate of HDX under experimental conditions such as in a secondary structure or membrane. Theoretically determined HDX rates of Ile-10 were calculated using a program written by Englander, which is derived from a series of papers looking at the rate of HDX under a variety of experimental conditions.^{58–59} Comparing the theoretical rate of exchange at Ile-10 in a random coil ($6.7 \times 10^{-1}\text{ s}^{-1}$) to the experimentally measured exchange rate of 0.36 min^{-1} (Table 1), gives the protection factor of 113. Solvent exposed residues with no secondary structure have typical protection factors of 1, soluble alpha-helices have

protection factors ranging from 2–20⁶⁰, globular proteins with some secondary structure have protection factors of 0.5–2000⁶¹, and membrane bound α -helices have protection factors of 3–2000^{19, 62}. Thus, the protection factor measured for Ile-10 is consistent with that residue being located in an α -helical membrane peptide where the hydrogen bonded secondary structure and amphipathic membrane protect Ile-10 from HDX.

Previous 2D IR spectroscopy measurements and molecular dynamics simulations solved the structure of ovispirin on a membrane; in addition to the three Arg residues being fully solvated, Ile-10 was found to lie in a largely homogeneous electrostatic environment.³⁹ From MD simulations, it was found that Ile-10 resides near a kink in the helix that denatures ovispirin over the next 6 residues. This explains why it is not as greatly protected from solvent as one might imagine being a hydrophobic amino acid in a membrane-bound α -helix.³⁹ Ultimately, kinks cause decreased protection factors, and the effect of kinks in transmembrane peptides has been previously studied.²⁴ A single proline was incorporated into the most hydrophobic region of the peptide to determine what effect this α -helix destabilizer and hydrogen bond breaking residue would have on the rate of HDX, particularly for the residues located in the transmembrane region of the peptide. Overall, the HDX rate of the peptide with the proline mutation was one order of magnitude larger than the non-mutated peptide, showing that the proline kink disrupted the lipid acyl chains enough to allow for faster overall HDX. Although Ile-10 lies in a largely inhomogeneous environment, it is consistent that the kink in ovispirin disrupts the membrane such that HDX is allowed at that residue. Although further experiments could be done to more precisely measure the rate of HDX in random coil ovispirin, we believe this protection factor makes sense for this system. Protection factors on the same order of magnitude have been measured for a 129-residue α -helix/ β -sheet protein and a membrane-bound α -helical peptide.^{62–63}

Conclusions

To conclude, we have shown that it is possible to measure on-the-fly HDX rates in the membrane-bound peptide, ovispirin, using a combination of rapid-scan pulse shaping 2D IR spectroscopy and non-invasive isotope labeling in the amide I mode of one residue in ovispirin. In contrast to linear IR spectroscopy, 2D IR spectroscopy allows one to measure coupled modes through the observation of cross peaks in a 2D IR spectrum. As such, we have monitored the kinetics of cross peaks between the isotope labeled amide I mode at Ile-10 and the amide II mode, which decreases intensity upon HDX. Ten minutes after HDX initiation by addition of deuterated buffer to the sample, the Ile-10/amide II cross peak in the lower half of the 2D IR spectrum decreased by a factor of 2. We calculated a protection factor of 113, which suggests that the Ile-10 amide group undergoes HDX at a rate 100 times slower than it would in solution. Our results are consistent with 2D IR and NMR spectroscopy derived structural models of ovispirin. These results show that 2D IR spectroscopy can be used to obtain site-specific HDX in the same manner as one might use NMR spectroscopy or mass spectrometry, although it can be done on-the-fly for membrane peptides without the need for quenchers or for slowing down the rates by lowering the pH. Our experiments illustrate another possible way in which 2D IR spectroscopy can be used to study protein and peptide structures that we believe will find use during the course of other isotope labeled 2D IR experiments, such as measuring the protection factors for one complete helix turn in ovispirin or measuring the rate of HDX at various time points in the folding process of isotope labeled amylin, the amyloidogenic polypeptide implicated in Type II diabetes.

Acknowledgments

Support for this research was provided by the National Institutes of Health Grant DK79895.

References

1. Bredenbeck J, Helbing J, Nienhaus K, Nienhaus GU, Hamm P. Protein ligand migration mapped by nonequilibrium 2D-IR exchange spectroscopy. *Proceedings of the National Academy of Sciences*. 2007; 104(36):14243–14248.
2. Woutersen S, Mu Y, Stock G, Hamm P. Hydrogen-bond lifetime measured by time-resolved 2D-IR spectroscopy: N-methylacetamide in methanol. *Chem. Phys.* 2001; 266(2–3):137–147.
3. Fayer MD. Dynamics of Liquids Molecules and Proteins Measured with Ultrafast 2D IR Vibrational Echo Chemical Exchange Spectroscopy. *Annu. Rev. Phys. Chem.* 2009; 60(1):21–38. [PubMed: 18851709]
4. Kwak K, Rosenfeld DE, Chung JK, Fayer MD. Solute-Solvent Complex Switching Dynamics of Chloroform between Acetone and Dimethylsulfoxide-Two-Dimensional IR Chemical Exchange Spectroscopy. *The Journal of Physical Chemistry B*. 2008; 112(44):13906–13915. [PubMed: 18855462]
5. Moilanen DE, Wong D, Rosenfeld DE, Fenn EE, Fayer MD. Ion-water hydrogen-bond switching observed with 2D IR vibrational echo chemical exchange spectroscopy. *Proceedings of the National Academy of Sciences*. 2009; 106(2):375–380.
6. Zheng J, Fayer MD. Solute-Solvent Complex Kinetics and Thermodynamics Probed by 2D-IR Vibrational Echo Chemical Exchange Spectroscopy. *The Journal of Physical Chemistry B*. 2008; 112(33):10221–10227. [PubMed: 18665635]
7. Zheng J, Kwak K, Asbury J, Chen X, Piletic IR, Fayer MD. Ultrafast Dynamics of Solute-Solvent Complexation Observed at Thermal Equilibrium in Real Time. *Science*. 2005; 309(5739):1338–1343. [PubMed: 16081697]
8. Zheng J, Kwak K, Chen X, Asbury JB, Fayer MD. Formation and Dissociation of Intra-Intermolecular Hydrogen-Bonded Solute-Solvent Complexes: Chemical Exchange Two-Dimensional Infrared Vibrational Echo Spectroscopy. *J. Am. Chem. Soc.* 2006; 128(9):2977–2987. [PubMed: 16506778]
9. Ghosh A, Remorino A, Tucker MJ, Hochstrasser RM. 2D IR photon echo spectroscopy reveals hydrogen bond dynamics of aromatic nitriles. *Chem. Phys. Lett.* 2009; 469(4–6):325–330. [PubMed: 20622983]
10. Kim YS, Hochstrasser RM. Chemical exchange 2D IR of hydrogen-bond making and breaking. *Proc. Natl. Acad. Sci. U. S.A.* 2005; 102(32):11185–11190. [PubMed: 16040800]
11. Kim YS, Hochstrasser RM. Comparison of Linear and 2D IR Spectra in the Presence of Fast Exchange. *The Journal of Physical Chemistry B*. 2006; 110(17):8531–8534. [PubMed: 16640400]
12. Jeng M-F, Englander SW. Stable submolecular folding units in a non-compact form of cytochrome c. *J. Mol. Biol.* 1991; 221(3):1045–1061. [PubMed: 1658332]
13. Englander SW, Mayne L. Protein Folding Studied Using Hydrogen-Exchange Labeling and Two-Dimensional NMR. *Annu. Rev. Biophys. Biomol. Struct.* 1992; 21(1):243–265. [PubMed: 1525469]
14. Dempsey CE. Hydrogen exchange in peptides and proteins using NMR spectroscopy. *Prog. Nucl. Magn. Reson. Spectrosc.* 2001; 39(2):135–170.
15. Eriksson MA, Härd T, Nilsson L. On the pH dependence of amide proton exchange rates in proteins. *Biophys. J.* 1995; 69(2):329–339. [PubMed: 8527646]
16. Wu Y, Murayama K, Ozaki Y. Two-Dimensional Infrared Spectroscopy and Principle Component Analysis Studies of the Secondary Structure and Kinetics of Hydrogen-Deuterium Exchange of Human Serum Albumin. *The Journal of Physical Chemistry B*. 2001; 105(26):6251–6259.
17. Cotten M, Fu R, Cross TA. Solid-State NMR and Hydrogen-Deuterium Exchange in a Bilayer-Solubilized Peptide: Structural and Mechanistic Implications. *Biophys. J.* 1999; 76(3):1179–1189. [PubMed: 10049303]
18. Demmers JAA, Haverkamp J, Heck AJR, Koeppe RE, Killian JA. Electrospray ionization mass spectrometry as a tool to analyze hydrogen/deuterium exchange kinetics of transmembrane peptides in lipid bilayers. *Proceedings of the National Academy of Sciences*. 2000; 97(7):3189–3194.

19. Dempsey CE, Handcock LJ. Hydrogen bond stabilities in membrane-reconstituted alamethicin from amide-resolved hydrogen-exchange measurements. *Biophys. J.* 1996; 70(4):1777–1788. [PubMed: 8785337]
20. Dempsey CE, Butler GS. Helical structure and orientation of melittin in dispersed phospholipid membranes from amide exchange analysis in situ. *Biochemistry.* 1992; 31(48):11973–11977. [PubMed: 1457397]
21. Hoshino M, Katou H, Yamaguchi K-i, Goto Y. Dimethylsulfoxide-quenched hydrogen/deuterium exchange method to study amyloid fibril structure. *Biochimica et Biophysica Acta (BBA) - Biomembranes.* 2007; 1768(8):1886–1899.
22. Hansen RK, Broadhurst RW, Skelton PC, Arkin IT. Hydrogen/deuterium exchange of hydrophobic peptides in model membranes by electrospray ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* 2002; 13(12):1376–1387. [PubMed: 12484457]
23. Zhang A, Qi W, Good TA, Fernandez EJ. Structural Differences between A²(1–40) Intermediate Oligomers and Fibrils Elucidated by Proteolytic Fragmentation and Hydrogen/Deuterium Exchange. *Biophys. J.* 2009; 96(3):1091–1104. [PubMed: 19186145]
24. Demmers JAA, van Duijn E, Haverkamp J, Greathouse DV, Koeppe RE, Heck AJR, Killian JA. Interfacial Positioning and Stability of Transmembrane Peptides in Lipid Bilayers Studied by Combining Hydrogen/Deuterium Exchange and Mass Spectrometry. *J. Biol. Chem.* 2001; 276(37):34501–34508. [PubMed: 11435420]
25. Lanman J, Prevelige PE Jr. High-sensitivity mass spectrometry for imaging subunit interactions: hydrogen/deuterium exchange. *Curr. Opin. Struct. Biol.* 2004; 14(2):181–188. [PubMed: 15093832]
26. Vigano C, Smeyers M, Raussens V, Scheirlinckx F, Ruyschaert JM, Goormaghtigh E. Hydrogen-deuterium exchange in membrane proteins monitored by IR spectroscopy: A new tool to resolve protein structure and dynamics. *Biopolymers.* 2004; 74(1–2):19–26. [PubMed: 15137087]
27. Dzwolak W, Lokszejn A, Smirnovas V. New Insights into the Self-Assembly of Insulin Amyloid Fibrils: An H-D Exchange FT-IR Study[†]. *Biochemistry.* 2006; 45(26):8143–8151. [PubMed: 16800639]
28. Beevers AJ, Kukol A. Secondary structure, orientation, and oligomerization of phospholemman, a cardiac transmembrane protein. *Protein Sci.* 2006; 15(5):1127–1132. [PubMed: 16597826]
29. Ding F-X, Xie H, Arshava B, Becker JM, Naider F. ATR-FTIR Study of the Structure and Orientation of Transmembrane Domains of the *Saccharomyces cerevisiae* α -Mating Factor Receptor in Phospholipids[†]. *Biochemistry.* 2001; 40(30):8945–8954. [PubMed: 11467956]
30. Earnest TN, Herzfeld J, Rothschild KJ. Polarized Fourier transform infrared spectroscopy of bacteriorhodopsin. Transmembrane α helices are resistant to hydrogen/deuterium exchange. *Biophys. J.* 1990; 58(6):1539–1546. [PubMed: 2275968]
31. Ludlam CF, Arkin IT, Liu XM, Rothman MS, Rath P, Aimoto S, Smith SO, Engelman DM, Rothschild KJ. Fourier transform infrared spectroscopy and site-directed isotope labeling as a probe of local secondary structure in the transmembrane domain of phospholamban. *Biophys. J.* 1996; 70(4):1728–1736. [PubMed: 8785331]
32. Krimm, S.; Bandekar, J. Vibrational Spectroscopy and Conformation of Peptides, Polypeptides, and Proteins. In: Anfinsen, CB.; JTE; Frederic, MR., editors. *Adv. Protein Chem.* Vol. 38. Academic Press; 1986. p. 181–364.
33. Maekawa H, Poli MD, Moretto A, Toniolo C, Ge N-H. Toward Detecting the Formation of a Single Helical Turn by 2D IR Cross Peaks between the Amide-I and -II Modes. *The Journal of Physical Chemistry B.* 2009; 113(34):11775–11786. [PubMed: 19642666]
34. Fillaux F, de Lozé C. Structure of matrix isolated N-methylacetamide. *Chem. Phys. Lett.* 1976; 39(3):547–551.
35. DeFlores LP, Ganim Z, Nicodemus RA, Tokmakoff A. Amide I'-II' 2D IR Spectroscopy Provides Enhanced Protein Secondary Structural Sensitivity. *J. Am. Chem. Soc.* 2009; 131(9):3385–3391. [PubMed: 19256572]
36. DeFlores LP, Tokmakoff A. Water Penetration into Protein Secondary Structure Revealed by Hydrogen-Deuterium Exchange Two-Dimensional Infrared Spectroscopy. *J. Am. Chem. Soc.* 2006; 128(51):16520–16521. [PubMed: 17177399]

37. Maekawa H, Ge N-H. Comparative Study of Electrostatic Models for the Amide-I and -II Modes: Linear and Two-Dimensional Infrared Spectra. *The Journal of Physical Chemistry B*. 2010; 114(3):1434–1446. [PubMed: 20050636]
38. Rubtsov IV, Wang J, Hochstrasser RM. Dual-frequency 2D-IR spectroscopy heterodyned photon echo of the peptide bond. *Proceedings of the National Academy of Sciences*. 2003; 100(10):5601–5606.
39. Woys AM, Lin Y-S, Reddy AS, Xiong W, de Pablo JJ, Skinner JL, Zanni MT. 2D IR Line Shapes Probe Ovispirin Peptide Conformation and Depth in Lipid Bilayers. *J. Am. Chem. Soc.* 2010; 132(8):2832–2838. [PubMed: 20136132]
40. Yamaguchi S, Huster D, Waring A, Lehrer RI, Kearney W, Tack BF, Hong M. Orientation and Dynamics of an Antimicrobial Peptide in the Lipid Bilayer by Solid-State NMR Spectroscopy. *Biophys. J.* 2001; 81(4):2203–2214. [PubMed: 11566791]
41. Middleton CT, Woys AM, Mukherjee SS, Zanni MT. Residue-specific structural kinetics of proteins through the union of isotope labeling, mid-IR pulse shaping, and coherent 2D IR spectroscopy. *Methods*. In Press Corrected Proof.
42. Torres J, Adams PD, Arkin IT. Use of a New Label $^{13}\text{C}=^{18}\text{O}$ in the Determination of a Structural Model of Phospholamban in a Lipid Bilayer. Spatial Constraints Resolve the Ambiguity Arising from Interpretations of Mutagenesis Data. *J. Mol. Biol.* 2000; 300(4):677–685. [PubMed: 10891262]
43. Torres J, Kukol A, Goodman JM, Arkin IT. Site-specific examination of secondary structure and orientation determination in membrane proteins: The peptidic $^{13}\text{C}=^{18}\text{O}$ group as a novel infrared probe. *Biopolymers*. 2001; 59(6):396–401. [PubMed: 11598874]
44. Shim S-H, Strasfeld DB, Fulmer EC, Zanni MT. Femtosecond pulse shaping directly in the mid-IR using acousto-optic modulation. *Opt. Lett.* 2006; 31(6):838–840. [PubMed: 16544641]
45. Shim S-H, Strasfeld DB, Zanni MT. Generation and characterization of phase and amplitude shaped femtosecond mid-IR pulses. *Opt. Express*. 2006; 14(26):13120–13130. [PubMed: 19532209]
46. Saito H, Shinoda W. Cholesterol Effect on Water Permeability through DPPC and PSM Lipid Bilayers: A Molecular Dynamics Study. *The Journal of Physical Chemistry B*. 2011; 115(51):15241–15250. [PubMed: 22081997]
47. Jansen M, Blume A. A comparative study of diffusive and osmotic water permeation across bilayers composed of phospholipids with different head groups and fatty acyl chains. *Biophys. J.* 1995; 68(3):997–1008. [PubMed: 7756562]
48. Lande MB, Donovan JM, Zeidel ML. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *The Journal of General Physiology*. 1995; 106(1):67–84. [PubMed: 7494139]
49. DeFlores LP, Ganim Z, Ackley SF, Chung HS, Tokmakoff A. The Anharmonic Vibrational Potential and Relaxation Pathways of the Amide I and II Modes of N-Methylacetamide[†]. *The Journal of Physical Chemistry B*. 2006; 110(38):18973–18980. [PubMed: 16986892]
50. Zanni MT, Asplund MC, Hochstrasser RM. Two-dimensional heterodyned and stimulated infrared photon echoes of N-methylacetamide-D. *The Journal of Chemical Physics*. 2001; 114(10):4579–4590.
51. Stelzer W, Poschner BC, Stalz H, Heck AJ, Langosch D. Sequence-Specific Conformational Flexibility of SNARE Transmembrane Helices Probed by Hydrogen/Deuterium Exchange. *Biophys. J.* 2008; 95(3):1326–1335. [PubMed: 18456822]
52. Yamamoto T, Takao T, Izumi S, Gekko K. Site-Specific Hydrogen/Deuterium Exchange Analysis of a Protein by Mass Spectrometry Coupled with Carboxypeptidase Digestion. *Journal of the Mass Spectrometry Society of Japan*. 2007; 55(5):333–338.
53. Ghosh A, Tucker MJ, Hochstrasser RM. Identification of Arginine Residues in Peptides by 2D-IR Echo Spectroscopy. *The Journal of Physical Chemistry A*. 2011; 115(34):9731–9738. [PubMed: 21539337]
54. Takahashi T, Nakanishi M, Tsuboi M. Hydrogen-deuterium exchange study of amino acids and proteins by 200- to 230-nm spectroscopy. *Anal. Biochem.* 1981; 110(1):242–249. [PubMed: 7212267]

55. Thakur A, Chandra K, Dubey A, D'Silva P, Atreya HS. Rapid Characterization of Hydrogen Exchange in Proteins. *Angew. Chem.* 2013; 125(9):2500–2503.
56. Segawa T, Kateb F, Duma L, Bodenhausen G, Pelupessy P. Exchange Rate Constants of Invisible Protons in Proteins Determined by NMR Spectroscopy. *ChemBioChem.* 2008; 9(4):537–542. [PubMed: 18247446]
57. Hayashi T, Mukamel S. Vibrational-Exciton Couplings for the Amide I, II, III, and A Modes of Peptides. *The Journal of Physical Chemistry B.* 2007; 111(37):11032–11046. [PubMed: 17725341]
58. Bai Y, Milne JS, Mayne L, Englander SW. Primary structure effects on peptide group hydrogen exchange. *Proteins: Struct., Funct., Bioinf.* 1993; 17(1):75–86.
59. Connelly GP, Bai Y, Jeng M-F, Englander SW. Isotope effects in peptide group hydrogen exchange. *Proteins: Struct., Funct., Bioinf.* 1993; 17(1):87–92.
60. Rohl CA, Baldwin RL. Exchange Kinetics of Individual Amide Protons in ¹⁵N-Labeled Helical Peptides Measured by Isotope-Edited NMR. *Biochemistry.* 1994; 33(25):7760–7767. [PubMed: 8011641]
61. Kuroda Y, Endo S, Nagayama K, Wada A. Stability of α -helices in a molten globule state of cytochrome c by hydrogen-deuterium exchange and two-dimensional NMR spectroscopy. *J. Mol. Biol.* 1995; 247(4):682–688. [PubMed: 7723023]
62. Halsall A, Dempsey CE. Intrinsic helical propensities and stable secondary structure in a membrane-bound fragment (S4) of the shaker potassium channel. *J. Mol. Biol.* 1999; 293(4):901–915. [PubMed: 10543975]
63. Lacroix E, Bruix M, López-Hernández E, Serrano L, Rico M. Amide hydrogen exchange and internal dynamics the chemotactic protein CheY from *Escherichia coli*. *J. Mol. Biol.* 1997; 271(3): 472–487. [PubMed: 9268672]

Figure 1.

Schematic for how 2D IR spectroscopy and isotope labeling provide site-specific HDX. (a) A protonated peptide unit with a single $^{13}\text{C}^{18}\text{O}$ isotope label and (b) the corresponding 2D IR spectrum for the entire protein. (c) The corresponding protein and (d) 2D IR spectrum after HDX has deuterated the isotope labeled peptide unit. Peaks always appear in pairs in 2D IR spectra (blue is for fundamental transitions and red is for overtone and combination band transitions). The large amide I peaks have contributions from all of the unlabeled residues in the protein while the $^{13}\text{C}^{18}\text{O}$ amide I peaks are much smaller because there is only 1 labeled residue. Cross peaks between the unlabeled amide I and amide II are shown, which are typical of all proteins that are not fully deuterated. In (b) cross peaks also appear between the $^{13}\text{C}^{18}\text{O}$ label and the amide II because the labeled residue is protonated. In (d), these cross peaks are absent because the labeled residue is deuterated and the amide II and its cross peaks are also weaker (shown shaded) because some, but not all, of the protein has exchanged. The above scenario is for a residue that exchanges faster than the average.

Figure 2.

(a) Diagonal slice through the 2D IR spectrum below. (b) 2D IR spectrum of Ile-10 ovispirin in H₂O. Spectrum (b) shows the unlabeled amide I, isotope labeled amide I, and amide II modes along the diagonal. Cross peaks between the amide I and amide II modes are highlighted with small-dash boxes. Cross peaks between Ile-10 and amide II modes are highlighted with solid boxes. (c) Diagonal slice through the 2D IR spectrum below. (d) 2D IR spectrum of Ile-10 ovispirin in D₂O. Spectrum (d) shows the unlabeled amide I, isotope labeled amide I, and amide II modes along the diagonal. Also along the diagonal, enclosed with a large-dash box, is the peak arising from the deuterated sidechain on Arg at 1607

cm^{-1} . Cross peaks between the amide I and amide II modes are highlighted with small-dash boxes. Cross peaks between Ile-10 and amide II modes are highlighted with solid boxes.

Figure 3.

(a) Slices through the pump axis at 1595 cm^{-1} (blue, dashed, H_2O) and 1596 cm^{-1} (red, dashed, D_2O). (b) 2D IR spectrum of Ile-10 labeled ovipirin in H_2O . The dashed blue line at $\omega_{\text{pump}}=1587\text{ cm}^{-1}$ designates the pump slice shown in blue in (a) and the solid blue line at $\omega_{\text{probe}}=1587\text{ cm}^{-1}$ designates the probe slice shown in blue in (d). (c) 2D IR spectrum of Ile-10 labeled ovipirin in D_2O . The dashed red line at $\omega_{\text{pump}}=1587\text{ cm}^{-1}$ designates the pump slice shown in red in (a) and the solid red line at $\omega_{\text{probe}}=1547\text{ cm}^{-1}$ designates the probe slice shown in red in (d). (d) Slices through the probe axis at 1587 cm^{-1} (blue, H_2O) and 1587 cm^{-1} (red, D_2O). All solid black boxes enclose amide II peaks on the diagonal of 2D IR spectra. All large-dash boxes enclose cross peaks between isotope labeled amide I and amide II modes in the upper part of the 2D IR spectra. All small-dash boxes enclose cross peaks between isotope labeled amide I and amide II modes in the lower part of the 2D IR spectra.

Figure 4.

(a) Decay of the intensity at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1547, 1547) \text{ cm}^{-1}$ immediately following addition of D_2O to the sample (blue). Biexponential fit (cyan). (b) Rise of the intensity at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1607, 1607) \text{ cm}^{-1}$ immediately following addition of D_2O to the sample (dark green). Single exponential fit (light green). (c) Decay of the intensity at $(\omega_{\text{pump}}, \omega_{\text{probe}}) = (1547, 1588) \text{ cm}^{-1}$ immediately following addition of D_2O to the sample (red). Single exponential fit (magenta).

Table 1

Coefficients of the biexponential fit (Equation 1) to the amide II trace and single exponential fit (Equation 2) to the arginine trace and the isotope label amide I/amide II cross peak trace shown in Figure 4. Decay times (T) are also given.

Peak	frequency (cm ⁻¹) (ω_{pump} , ω_{probe})	a	b (min ⁻¹)	T (min)	c	d (min ⁻¹)	T (min)	e
Amide II	1547, 1547	0.75 ± 0.02	0.54 ± 0.02	1.9 ± 0.07	0.57 ± 0.02	0.12 ± 0.01	8.3 ± 0.7	0.82 ± 0.03
Arginine	1607, 1607	1.18 ± 0.063	1.96 ± 0.15	0.51 ± 0.04				0
Cross peak	1547, 1588	0.17 ± 0.028	0.36 ± 0.1	2.8 ± 1				0.15 ± 0.02