

Isotope-Edited Amide II Mode: A New Label for Site-Specific Vibrational Spectroscopy

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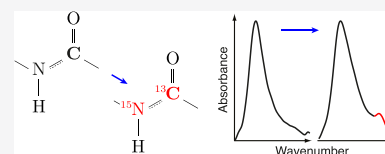
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ABSTRACT: Vibrational spectroscopy is a powerful tool used to analyze biological and chemical samples. However, in proteins, the most predominant peaks that arise from the backbone amide groups overlap one another, hampering site-specific analyses. Isotope editing has provided a robust, noninvasive approach to overcome this hurdle. In particular, the $1\text{-}^{13}\text{C}=\text{O}$ and $1\text{-}^{13}\text{C}=\text{O}$ labels that shift the amide I vibrational mode have enabled 1D- and 2D-IR spectroscopy to characterize proteins with excellent site-specific resolution.

Herein, we expand the vibrational spectroscopy toolkit appreciably by introducing the $1\text{-}^{13}\text{C}=\text{O}$ probe at specific locations along the protein backbone. A new, isotopically edited amide II peak is observed clearly in the spectra despite the presence of unlabeled modes arising from the rest of the protein. The experimentally determined shift of -30 cm^{-1} is reproduced by DFT calculations providing further credence to the mode assignment. Since the amide II mode arises from different elements than the amide I mode, it affords molecular insights that are both distinct and complementary. Moreover, multiple labeling schemes may be used simultaneously, enhancing vibrational spectroscopy's ability to provide detailed molecular insights.



Infrared (IR) spectroscopy measures excited vibrational energy levels of polarizable bonds. Consequently, IR spectroscopy has been used extensively as a tool to investigate numerous different analytes, such as determining the chemical composition of geological¹ and plant samples;² characterizing natural and synthetic polymers;^{3–6} identifying bacterial species through their unique IR spectrum;^{7–9} as well as analyzing biological tissues¹⁰ and fluids to diagnose medical conditions, such as cancer, dementia, and stroke.^{11–13}

IR spectroscopy has also been used to analyze protein structures through peptide bond vibrations. Of particular notice has been the determination of protein secondary structure,^{14–16} orientation,^{17,18} and measurements of hydrogen exchange profiles.¹⁹ However, because the peak-width of individual amide modes is significantly larger than the separation between the distinct peaks, IR spectroscopy has been mostly limited to measuring average protein properties, rather than a detailed structural investigation.

To overcome this inherent limitation, the laboratories of Krimm, Rothschild, Siebert, Gerwert, and others developed isotopic labeling approaches to isolate individual groups in model compounds,²⁰ dipeptides,²¹ and tripeptides.²² Studies were also performed on proteins in which all glycines,²³ aspartates,²⁴ tyrosines,^{25–27} or prolines^{28,29} were uniformly labeled.

In 1991, Tadesse, Nazarbahi, and Walters transformed IR spectroscopy to a tool capable of probing any individual site in proteins, regardless of its sequence.³⁰ The authors introduced $1\text{-}^{13}\text{C}$ labeling that downshifts the amide I vibrational mode (mostly the $\text{C}=\text{O}$ stretch³¹) by ca. 36 cm^{-1} . A further improvement to the isotope-editing of the amide I mode was achieved by double labeling:^{32,33} $^{13}\text{C}=\text{O}$ that shifts the amide

I mode by ca. 64 cm^{-1} . The dual-isotope approach results in near-baseline resolution of the labeled site and is now the preferred probe for IR spectroscopy of proteins.^{34–39} The advent of 2D-IR, having relied on such amide I probes, has revolutionized the capabilities of vibrational spectroscopy.^{40,41} Herein, we aim to expand the vibrational spectroscopy tool kit even further by developing a site-specific, isotope-edited amide II mode.

The amide II vibrational mode is mostly an out-of-phase combination of the N–H in-plane bend and the $\text{C}=\text{N}$ bond stretch.³¹ Consequently, deuteration by Miyazawa and colleagues⁴² has shown to result in a shift to lower frequencies by 92 cm^{-1} . However, it is challenging to employ deuteration for site-specific analyses since the amide deuterium readily exchanges with the bulk solvent, thereby scrambling the unique signal.

A different isotope editing strategy utilizing groups that do not undergo exchange is required to avoid label scrambling. To that end, one might consider employing ^{13}C or ^{15}N . However, previous studies have shown that the individual shifts produced by such labeling strategies are insufficient since they are appreciably smaller than the peak-width of the amide II mode (ca. 23 cm^{-1} , as seen in Figure 2). In particular, ^{13}C labeling

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shifts the amide II peak by 12–13 cm⁻¹, and ¹⁵N labeling shifts the amide II peak by 14–15 cm⁻¹.^{43,44}

One possibility to overcome the small shift of single isotopic substitution is to employ dual labels, as was done with ¹³C and ¹⁸O for the amide I mode.^{32,33} Indeed, a study employing uniform metabolic labeling of yeastolates with ¹³C and ¹⁵N resulted in a single amide II peak shifted by 29 cm⁻¹.⁴⁵ Therefore, we decided to examine if we can observe two amide II peaks simultaneously in the same peptide: one from unlabeled resonances and the other from the ¹³C=15N peptide bond, which serves as the site-specific spectroscopic probe.

As a model system, we used WALP19, a bitopic (single pass), transmembrane, α -helical peptide⁴⁶ reconstituted in lipid vesicles. We measured two variants, both with an identical sequence: the first is an unlabeled WALP19 peptide, and the second is a labeled peptide with the new 1-¹³C=15N label present three times in alternating amide groups, as shown in Figure 1.

GWWLALALALALWLA, L = 1-¹³C, A = ¹⁵N

Figure 1. Sequence of the isotopically labeled WALP19 peptide⁴⁶ used in the study. Red and blue amino acids were labeled with 1-¹³C or ¹⁵N, respectively, generating three doubly labeled peptide bonds.

While future studies may be required to analyze different label distributions, we decided to adopt the approach of Tadesse and co-workers by examining multiple juxtaposed labels.³⁰

The FTIR spectra of the two peptides reconstituted in hydrated lipid bilayers are presented in Figure 2. A comparison between the labeled and unlabeled peptides indicates that the ¹³C=15N labeling impacts both the amide I and amide II vibrational modes.

Both peptides exhibit a similarly pronounced amide I peak centered at 1660 cm⁻¹ indicative of the high-helical content of WALP19.⁴⁶ The 3 cm⁻¹ difference between the two peaks may arise from the partial uncoupling of the amide I mode due to energy differences between labeled and unlabeled groups.⁴⁷ Furthermore, the labeled peptide exhibits an additional amide I peak at 1615 cm⁻¹. As stated above, a shift to a lower frequency of this magnitude results from 1-¹³C=16O labeling and is used to isolate the amide I vibrational mode.³⁰ Finally, we note that both peptides are isotopomeric, and therefore, their structures should be indistinguishable. As such, any differences in IR peak locations or characteristics are expected to arise solely due to the impact of the isotopic labeling on the vibrational modes of the system.

Importantly, the amide II vibrational mode is also affected by the 1-¹³C=15N isotopic labeling. While the principal peak's location, centered at 1545 cm⁻¹, remains constant, isotopic

labeling results in a new mode centered at 1515 cm⁻¹. Fourier self-deconvolution points to the emergence of a new peak at that location due to ¹³C=15N labeling.

To verify that the new peak is indeed an isotopically edited amide II mode, we conducted DFT calculations. Since peptides are too large for such calculations, we conducted calculations of two systems: (i) a single *N*-methylacetamide (NMA), representing one peptide bond, and (ii) three *N*-methylacetamides positioned according to the coordinates of a model helix at the exact location of residues involved in canonical H-bonding: *i*, *i* - 4, and *i* + 4 (see Figure S1). The latter system may be viewed as the smallest molecular mimetic of an H-bonded helical peptide bond.

Three isotopic configurations were used for both the single and triple *N*-methylacetamide systems (see Figure S1): (1) The peptide carbonyl carbon was isotopically labeled with ¹³C. (2) The peptide amide nitrogen was isotopically labeled with ¹⁵N. (3) Both the peptide carbonyl carbon and the peptide amide nitrogen were isotopically labeled (1-¹³C=15N). The results of the frequency calculations of the amide I and amide II modes, shown in Table 1, are in excellent agreement with the experimental measurements. Specifically, the peptide bond's dual-labeling resulted in a calculated shift to lower frequency by 27 and 33.6 cm⁻¹ for the single and triple *N*-methylacetamide systems, respectively. Despite the simplistic nature of the system used in the calculations, the value is very similar to the 30 cm⁻¹ shift that was measured experimentally (Figure 2). Taken together, the calculations confirm the identity of the new peak as a 1-¹³C=15N amide II mode.

This new labeling scheme provides the same advantages as those of amide I labels^{30,33} but for the amide II band. Both of these labels yield an isotope-edited peak that can be examined in the presence of an unlabeled background, facilitating spectroscopic examination of features in a protein in a site-specific manner. Moreover, since the N–H bend is a major component of the amide II mode,³¹ this label can provide us direct information on N–H bond energies, local polar environments, direct site-specific monitoring of H exchange, and the measurement of canonical H-bond strengths.

Perhaps even more importantly, the new label could allow us to simultaneously look at two separate sites: the 1-¹³C=18O amide I peak and the 1-¹³C=15N amide II peak. In addition, since the transition dipole moments of both modes are appreciably different from one another,³¹ orientational studies of both labels would provide excellent constraints for structural analyses. One may even envisage a single peptide group that is triply labeled: ¹⁵N=13C=18O, generating two new isotopically edited peaks.

It is noteworthy that, as opposed to the 1-¹³C=18O amide I label, which is not yet commercially available,^{33,48} the 1-¹³C=15N

Table 1. Calculated and Measured Spectral Shifts^a

label	amide I shift (–cm ⁻¹)			amide II shift (–cm ⁻¹)		
	DFT		FTIR	DFT		FTIR
	NMA	triple NMA		NMA	triple NMA	
1- ¹³ C	43	39	36–40 ^{30,32,43,44}	11	19	12–13 ^{43,44}
¹⁵ N	0	5	0–1 ^{43–45}	17	15	14–17 ^{43–45}
1- ¹³ C=15N	44	48	46, ^b 39 ⁴⁵	27	34	30, ^b 29 ⁴⁵

^aSpectral shifts calculated by DFT or measured experimentally by FTIR of the amide I and amide II peaks that result from the isotopic labeling schemes seen in Figure S1. Two systems were used in the DFT calculations: a single *N*-methylacetamide (NMA) or three H-bonded *N*-methylacetamides (for details, see the text and Figure S1). The shifts are calculated relative to the same system without any isotopic labels.

^bExperimental results obtained in the current study.

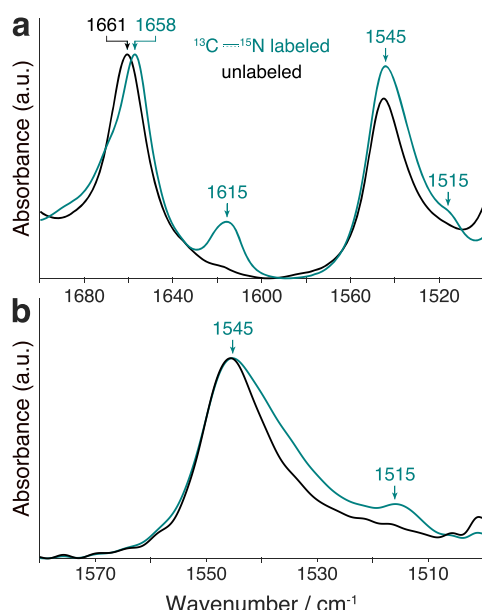


Figure 2. FTIR spectra of WALP peptides in hydrated 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine lipid bilayers. Spectra of $1\text{-}^{13}\text{C}\text{-}^{15}\text{N}$ labeled and unlabeled peptides are depicted in teal and black, respectively. (a) Spectra in the amide I and amide II regions; (b) Fourier self-deconvoluted spectra in the region of the amide II mode.

label can easily be purchased by combining two readily available amino acids, one with $1\text{-}^{13}\text{C}$ and the following with ^{15}N . Insertion into larger proteins may be possible through developments in peptide ligation^{49–51} and perhaps even utilizing expanded genetic code technologies.⁵²

Future studies will be needed to establish the behavior of the new $1\text{-}^{13}\text{C}\text{-}^{15}\text{N}$ label when placed in different secondary structural elements. For example, does the site-specific amide II probe broaden extensively in a random coil setting like the amide I group?⁵³ The stability of the WALP peptide in lipid bilayers precludes such experiments.⁵⁴ Similarly, do adjacent labels behave differently in comparison to individual sites? The presence or absence of vibrational coupling may impact the location of such peaks.⁴⁷

Vibrations arising from side-chain groups may overlap the $1\text{-}^{13}\text{C}\text{-}^{15}\text{N}$ peak. In particular, one should mention tyrosine and tryptophan side-chains that resonate around 1510 cm^{-1} (see the following comprehensive review⁵⁵). The above notwithstanding, the $1\text{-}^{13}\text{C}\text{-}^{15}\text{N}$ label is clearly visible in the peptide used in the current study, despite the fact that it contains four tryptophans. Finally, difference spectroscopy can always be employed when spectral resolution is an issue, even though it was not required in the current system.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpclett.1c01073>.

Detailed description of the methods and a figure depicting the different configurations used in the DFT calculations (PDF)

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Notes

The authors declare no competing financial interest.

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