Isotope-Edited Infrared Spectroscopy of Helical Peptides

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Short polypeptides which form stable α helices in aqueous solution are classic models for studying the factors which contribute to helix stability as well as the mechanism of helix formation.[1] The most common tool for characterizing helix content in these peptides is far-UV circular dichroism (CD); however, CD spectra can only give information about the overall helix content of a peptide, not the residue-level distribution of helix content within a peptide. Yet, residue-level information is essential to understanding important questions about helix formation and helix stability, such as the characterization and quantification of end-fraying effects and the impact of end-capping interactions.[2] Nuclear magnetic resonance (NMR)[3] and NMR-monitored amide proton exchange[4] probe conformation at the residue-level; however, parameters such as chemical shift are difficult to calibrate, and the kinetics of the helix−coil transition are too rapid for direct characterization via NMR. Thus, the ideal spectroscopic probe for characterizing model helical peptides would combine the time resolution of an optical measurement such as CD with the residue-level resolution of NMR.

We report that isotope-edited infrared (IR) spectroscopy opens a new window for observing conformation of specific residues in model helical peptides. IR is a powerful tool for probing the secondary structure of polypeptides in the steady state,[5] and transient infrared absorption has been used to observe the kinetics of protein dynamics during folding/unfolding and functional events.[6] The primary spectral feature used in these studies is the amide I′ mode.[7] Due to transition dipole coupling between peptide moieties, the amide I′ mode is very sensitive to the backbone geometry of a polypeptide, and the frequency and intensity of this band are sensitive to protein secondary structure.[5] However, while a conventional Fourier transform IR (FTIR) spectrum gives information about the overall secondary structure content of the polypeptide, it cannot be used to determine conformations of specific local resides, and thus it is no more useful than CD for studying problems such as end-fraying. One approach to increasing the information content of FTIR spectra is to introduce ^{13}C labels into the peptide backbone. Labeling of backbone carbonyls with ^{13}C results in a ∼37 cm^{-1} shift of the amide I′ mode, separating the amide I′ band of ^{13}C-labeled residues from that of the ^{12}C band.[8] Isotope-edited FTIR has been used to probe the structures of particular regions within a protein and to observe conformational changes involved in protein−protein interactions.[9]

We have applied isotope-edited FTIR to probe structural details of an alanine-rich α helical peptide.[10] A series of peptides (L1−L4) were synthesized[11] in which two residues of 1-^{13}C-alanine were incorporated into the sequence (underlined residues are ^{13}C-labeled):

Ac−YAAKAAAAKAAAAKAAH−NH_2 (unlabeled)

Ac−Y<u>AA</u>KAAAAKAAAAKAAH−NH_2 (L1)

Ac−YAAKA<u>AA</u>AKAAAAKAAH−NH_2 (L2)

Ac−YAAKAAAAKA<u>AA</u>AKAAH−NH_2 (L3)

Ac−YAAKAAAAKAAAAK<u>AA</u>H−NH_2 (L4)

FTIR spectra were collected for each peptide in D_2O over the range 0−45 °C (Figure 1).[12] At 0 °C, the unlabeled peptide has an amide I′ band maximum at 1633 cm^{-1}; the band shifts to higher frequency and decreases in intensity as the temperature increases (Figure 1A). The spectra at 0 and 45 °C are consistent with amide I′ bands of short peptides in α helix and random coil conformations, respectively.[5a,6c] In the labeled peptides, a second band appears at ∼1595 cm^{-1} which can be assigned to the ^{13}C amide I′ mode. This ^{13}C amide I′ band also decreases in intensity and shifts to higher frequency as the temperature increases (Figure 1B−E). All of the spectral changes are reversible; the 0 °C spectrum is reproduced upon recooling the sample.

At low temperatures (where the peptides are predominantly helical), the amplitude of the ^{13}C amide I′ bands in the spectra of L1, L2, and L3 are similar, but this band is much smaller in the L4 spectrum, appearing as an unresolved tail to the low-frequency side of the ^{12}C band (Figure 1E). These differences are clearly discerned in the 0 °C labeled − unlabeled difference spectra in Figure 2A; all of the labeled peptides have a positive feature at ∼1596 cm^{-1}, but this feature is significantly smaller in the L4 spectrum. At higher temperatures (where the peptides are

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(7) The amide I mode is predominantly a C═O stretch mode, with small contirbutions from N−H bending. In D_2O, the amide proton in the peptide moiety is exchanged for a deuterium, and the resulting amide I′ mode is shifted to lower frequency. See: Diem, M. *Introduction to Modern Vibrational Spectroscopy*; Wiley: New York, 1993.

(8) Assuming that the amide I′ mode is pure carbonyl stretch undergoing simple harmonic motion, this shift can be estimated using the equation $\nu = (1/2\pi)(\sqrt{k/\mu})$, where μ is the reduced mass of the carbonyl group.

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(10) Sequence based on peptides studied in the following: (a) Armstrong, K. M.; Fairman, R.; Baldwin, R. L. *J. Mol. Biol.* **1993**, *230,* 284−291. (b) Armstrong, K. M.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90,* 11337−113340. The secondary structures of these peptides were determined by measurement of far-UV circular dichroism spectra; the spectra and % helicity are in agreement with data reported by Armstrong and Baldwin. In our experiments, the amide I′ bands of peptide L2 retain the same relative shape when the concentration is varied in the range 1−10 mM, suggesting that no significant peptide−peptide interactions are occurring.

(11) Peptides were synthesized on a Pioneer automated peptide synthesizer (PE Biosystems) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc-1-^{13}C-alanine was purchased from Cambridge Isotopes. Peptides were purified by reverse-phase HPLC; purity was confirmed by analytical reverse-phase HPLC and electrospray mass spectrometry. Residual trifluoracteic acid (TFA) from synthesis was removed by lyophilization.

(12) FTIR spectra were measured on a Mattson Cygnus 1000 FTIR spectrometer at 2-cm^{-1} resolution. For a typical sample, approximately 1 mg of peptide was dissolved in 100 μL of a 0.1% phosphoric acid (pH 3) D_2O buffer. The samples were placed in a variable-temperature cell (Wilmad) with CaF_2 plates and a 50-μm Teflon spacer.

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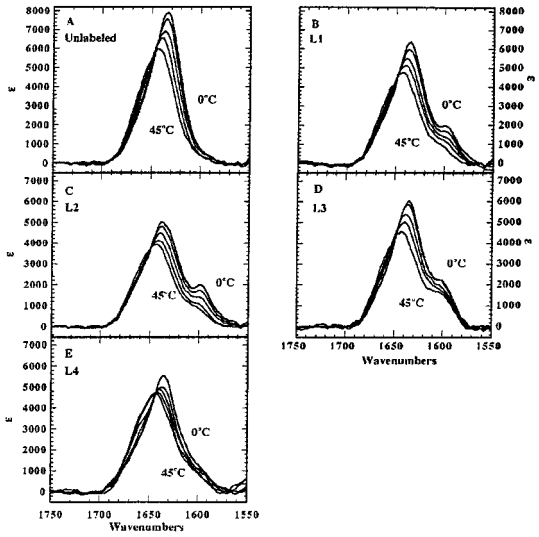

Figure 1. FTIR spectra of the amide I′ region for the series of peptides. Spectra measured at 0, 5, 15, 25, and 45 °C are shown. Peptides were dissolved in a 0.1% phosphoric acid−D_2O buffer (pH 3). Solvent spectra were measured at each temperature and subtracted from the peptide spectra. Temperature-dependent spectral changes were reversible; cooling back to 0 °C after heating to 45 °C regenerates the original 0 °C spectrum. Concentrations of the samples were determined using the UV absorbance of the tyrosine residue ($\epsilon_{275} = 1450\ M^{-1}\ cm^{-1}$; see ref 10); concentrations of the FTIR samples typically fell between 2 and 10 mM. We did not observe concentration-dependent spectral changes in this range.

predominantly random coil), this difference diminishes; the four labeled peptides have similar amplitude of the ^{13}C amide I′ band at 45 °C (Figure 2B). Thus, at 0 °C the conformation of the labeled residues of L4 is different from the conformations of labeled residues within L1, L2, and L3, while at a high temperature the labeled residues in all four peptides have similar conformations.

We attribute the differences in the L4 spectrum at 0 °C to helix fraying at the C-terminus. Because the N-terminus of this peptide is capped with an acetyl group, residues at the N-terminus and the center of the peptide are more likely to be helical than residues at the C-terminus.[2,13,14] To support this hypothesis, we have calculated the probability of a residue within this peptide adapting a helical conformation from a partition function for the helix−coil transition generated by a modified Lifson−Roig model;[15] the helix probability as a function of residue position is plotted in Figure 3. Overall, the peptide is predicted to have a helix content of around 70% at 0 °C (close to the value measured by CD); however, the labeled residues in L1, L2, and L3 have a helix probability of 0.7 or greater, and the labeled residues of L4 are predominantly random coil (helix probability below 0.3). The ^{13}C amide I′ amplitudes of L1, L2, L3, and L4 correlate well with predicted helix stability; the ^{13}C amide I′ bands of L1, L2, and L3 have comparable amplitudes (as expected, since the labeled

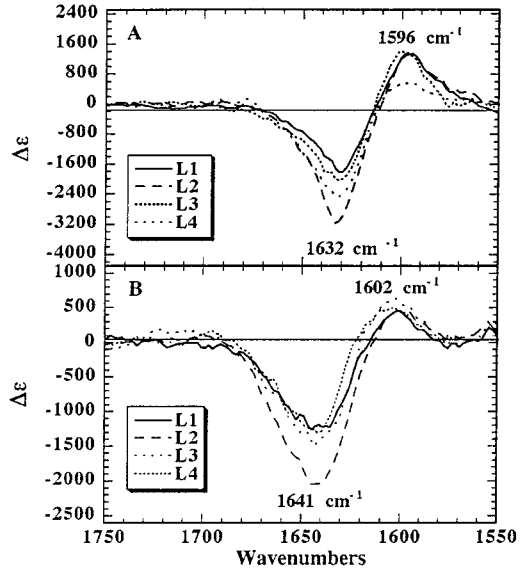

Figure 2. Labeled − unlabeled difference spectra at (A) 0 and (B) 45 °C.

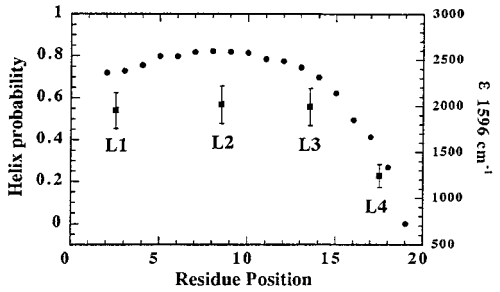

Figure 3. Plot of calculated helix probability versus residue position (left axis, solid circles) and plot of the amplitudes of the ^{13}C amide I′ band versus label position (right axis, solid squares). Calculated helix probabilities were generated from a modified Lifson−Roig calculation using the program Helix2 (ref 15).

residues of each peptide should have comparable helix contents), while in L4 this band amplitude is substantially diminished (reflecting a lower helix content at the C-terminus residues).[16] These observations demonstrate the utility of isotope-edited FTIR of helical peptides for elucidating residue-level variations in helix conformation and stability.

Interestingly, there are also subtle differences in the ^{12}C amide I′ band in the spectra of the four labeled peptides. The most striking difference is observed in L2, which gives rise to a significantly smaller ^{12}C amide I′ band. This is likely due to disruption of transition dipole coupling between ^{12}C amide I′ modes of the helical segments flanking the ^{13}C-labeled residues; further study of the effect of intervening ^{13}C labels on the transition dipole coupling of ^{12}C residues is currently underway.

Isotope-edited FTIR spectroscopy is a valuable technique for observing changes in conformation of specific residues within a peptide or protein, complementing data obtainable via NMR and CD spectroscopy. A series of ^{13}C-labeled peptides may provide a means to observe local unfolding dynamics in temperature-jump/transient IR absorbance. Investigations into these areas and more extensive ^{13}C labeling of peptides are currently underway.

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(16) Martinez and Millhauser have reported that FTIR studies cannot distinguish between 3_{10} and α helical conformation in short, aqueous peptides, both having amide I′ ∼1630 cm^{-1} (ref 5d). Due to ambiguities in the assignment of a 3_{10} amide I′ band, we cannot rule out or confirm the presence of 3_{10} helical residues at the C-terminus in the L4 peptide on the basis of the data presented here. However, vibrational circular dichroism of these labeled peptides may shed more light onto this question. See: Yoder, G.; Polese, A.; Silva, R. A. G. D.; Formaggio, F.; Crisma, M.; Broxterman, Q. B.; Kamphius, J.; Toniolo, C.; Keiderling, T. A. *J. Am. Chem. Soc.* **1997**, *119*, 10278−10285.