Time-Resolved Infrared Study of the Helix−Coil Transition Using ^{13}C-Labeled Helical Peptides

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Helices are ubiquitous in proteins. As such, the helix−coil transition has been studied extensively. Seminal works[1] of Schellman, Zimm and Bragg, Lifson and Roig, and Poland and Sheraga form the basis of our understanding of helix thermodynamic stability. Recent studies on the kinetics of the helix−coil transition using either computer simulation[2] or laser-induced *T*-jump method [3−5,17,18] provide new insights into our understanding of the kinetic aspects of helix formation.

It is widely believed that the helix−coil transition proceeds on submillisecond time scales. Recent *T*-jump experiments have shown that this process may actually happen on time scales of tens to hundreds of nanoseconds.[3−5,17,18] For example, Williams et al.[3] measured a 160 ns relaxation event corresponding to a final temperature of 27.4 °C for the Suc-Fs peptide, and Lednev et al.[4] reported a similar relaxation process but with a somewhat longer time constant, that is, 240 ± 60 ns at 37 °C, also for the Fs peptide. Using a different Ala-based peptide, Thompson et al.[5] also observed a process that relaxes with a similar time constant, i.e., 220 ns at 27 °C.

A recent stopped-flow CD study of the helix−coil transition by Clarke et al.,[6] however, suggested that the nucleation process corresponding to helix formation may be much slower than previously thought, on millisecond time scales. Using a 16-residue peptide (AK16), they observed a folding rate constant of 15 s^{-1} at 0 °C. This result is surprising because this rate is considerably slower than that measured in the *T*-jump experiments or by a ^{13}C NMR relaxation method.[7] To explain this discrepancy, Clarke et al. calculated the helix population change using a helix−coil theory and conditions corresponding to the *T*-jump experiment of Williams et al. and concluded that the *T*-jump experiment is totally dominated by perturbing the amount of fraying at the helix termini and the amplitude arising from crossing the nucleation barrier is negligible. They suggested that the *T*-jump experiments were measuring end-fraying which crosses only the propagation barrier. To test this model, we designed and synthesized an Ala-based helical peptide with four ^{13}C-labeled alanines placed at the middle of the peptide sequence. The idea is to use the unique amide I′ band arising from these labeled Ala residues to report on conformational changes associated with only the middle of the peptide and to discriminate against signals due to end-fraying.

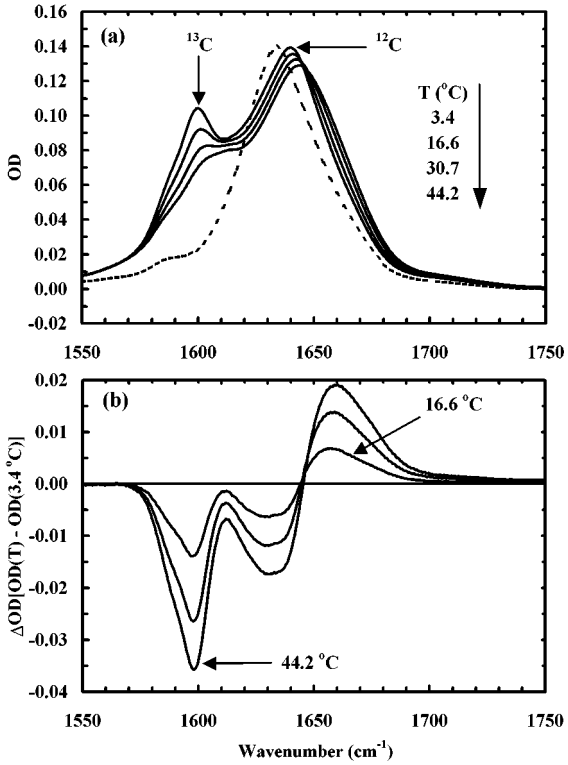

Figure 1. (a) Equilibrium FTIR spectra of the ^{13}C-labeled (solid lines) and unlabeled (dashed line) peptides in D_2O solution measured at 3.4, 16.6, 30.7, and 44.2 °C, respectively, for the labeled peptide, and at 2.9 °C for the unlabeled peptide. (b) Difference FTIR spectra of the labeled peptide generated by subtracting the spectrum collected at 3.4 °C from the spectra collected at higher temperatures.

The amide I′ band of polypeptides (Figure 1) is due mainly to the amide C═O stretch vibration and is sensitive to conformation. As a result, the amide I′ band is commonly used as a global conformational reporter.[8] However, it is often difficult to quantitatively interpret amide I′ bands because they are almost invariably broad and congested with components arising from different structural ensembles. To obtain site-specific structure information, we have employed an isotope-editing approach where several amide ^{12}C═Os were substituted with ^{13}C═Os. Since vibrational transitions are sensitive to isotopic substitution, the amide I′ absorbance of the ^{13}C-labeled carbonyls shifts to lower frequency, ∼40 cm^{-1} apart from that of the unlabeled residues (Figure 1), therefore permitting site-specific studies.[9]

The α-helical peptides, Ac-YGSPEA$_3$KA$_4$KA$_4$r-CONH$_2$, and Ac-YGSPEA$_3$K<u>AAAA</u>KA$_4$r-CONH$_2$, where underlined residues are ^{13}C-labeled, were synthesized based on a derivative of a poly-

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Ala helix originally described by Marqusee and Baldwin[10] with a capping group, D-Arg (r), incorporated at the C terminus.[11] The presence of lysine residues may help to stabilize the helix conformation.[12] Low-temperature far UV CD spectra of these peptides indeed show typical features associated with α-helices. Thermal unfolding of the unlabeled peptide measured by the mean residue ellipticity at 222 nm revealed an apparent two-state helix−coil transition with a thermal melting temperature of ∼16 °C.[13]

Temperature-dependent infrared spectra[14] in the amide I′ region (Figure 1a) show that the labeled peptide exhibits two major bands at ∼1600 and ∼1640 cm^{-1}, respectively, whereas the unlabeled peptide has only one major band (dashed line), centered at ∼1634 cm^{-1}. Thus, it can be concluded that the 1600 cm^{-1} band is due to the ^{13}C-labeled residues, in agreement with previous studies.[9] The ^{12}C band of the labeled peptide is at somewhat a higher frequency, that is, 1640 cm^{-1}, compared to that of the unlabeled peptide, presumably due to the higher percentage of the disordered conformations, whose amide I′ band peaks at ∼1646 cm^{-1},[15] seen by the unlabeled residues. As indicated by the FTIR difference spectra (Figure 1b), the amide I′ band loses intensity as temperature increases, and the concurrent formation of a new spectral feature at the higher energy side. The negative feature, centered at ∼1598 cm^{-1}, is due to the loss of helical structures reported by the ^{13}C-labeled residues. Thus, the relaxation kinetics of the helical conformation involving only those labeled residues, which in this case are at the middle of the peptide sequence, following a *T*-jump,[16] can be studied by probing this specific spectral feature. It is worth to note that another negative feature, centered at ∼1632 cm^{-1}, which is due to the melting of unlabeled helical residues, is diminished by the positive signals arising from the labeled residues that assume disordered conformations.

The relaxation kinetics (Figure 2) following a *T*-jump[17] show marked difference between those probed at different frequencies. For example, the relaxation kinetics obtained at 1630 cm^{-1}, which probe conformational changes reported by the unlabeled carbonyls, exhibit a dominant instantaneous component (>60%) that is estimated to be faster than the response time of the detection system (∼10 ns) and likely correspond to end-fraying,[18] whereas the relaxation kinetics obtained at 1600 cm^{-1}, where the signals are dominated by the labeled alanines, exhibit nonexponential kinetics that can be described by the following equation: $\Delta OD(t) = A[1 - 0.31 \exp(-t/\tau_1) - 0.16 \exp(-t/\tau_2) - 0.53 \exp(-t/\tau_3)]$, with $\tau_1 < 10$ ns, $\tau_2 = 38 \pm 10$ ns, $\tau_3 = 230 \pm 40$ ns, and $A = -0.0117$, respectively. The relaxation kinetics obtained with the unlabeled peptide at 1600 cm^{-1} show similar behavior as those probed at 1630 cm^{-1} with the labeled peptide, only with smaller amplitudes, indicating that the signal probed at 1600 cm^{-1} for the labeled peptide contains only a small (<10%) contribution from the remaining ^{12}C carbonyls. Together, these results show that the 230 ns component, which is similar to those observed with other peptides,[3−5] does not arise from end-fraying and should correspond to transitions that cross the nucleation barrier.

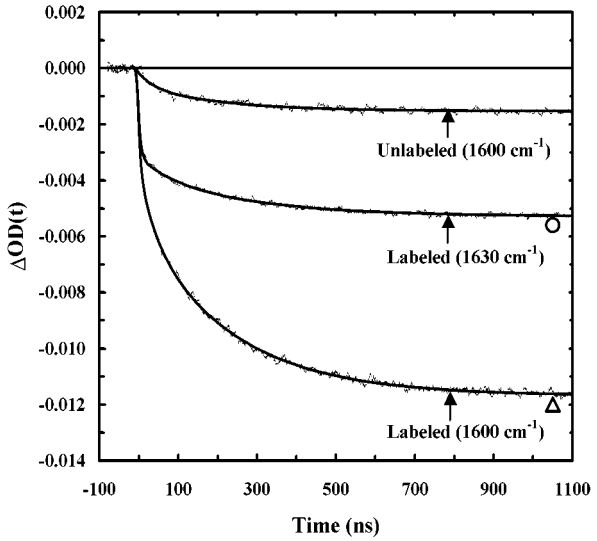

Figure 2. Relaxations obtained at different probing frequencies, as indicated in the plot, for both labeled and unlabeled peptides. Note that the signal of the unlabeled peptide has been scaled to reflect the difference in concentration. The T-jump was 11.5 ± 1 °C, from 9.5 to 21 °C. The smooth lines are fits to a multiexponential function convolved with the instrument response function that was determined by fitting the D_2O signal to a Gaussian function (fwhm = 10.4 ns). Symbols O and Δ represent the equilibrium amplitudes, i.e., ΔOD at infinity time, for the labeled peptide at 1630 and 1600 cm^{-1}, respectively. The good agreement between the kinetic and equilibrium amplitudes indicates that there are no kinetic events present on longer time scales (within our experimental uncertainties).

A surprising result is the multiexponential relaxation kinetics. Although nonexponential helix−coil transitions have been suggested by theoretical studies[18,19] and observed for nonbiological helices,[20] our results show unambiguously that the helix−coil transition is complex and does not follow a two-state model. Several reasons could lead to deviations from two-state kinetics, such as intermediates or multiple pathways or both. A diffusive search through conformational space can also lead to nonexponential kinetics.[20] Distinguishing these possibilities is certainly not easy, and more work needs to be carried out to understand in detail the helix−coil transition.

In conclusion, this study provides strong evidence supporting the picture that the nucleation process is fast, on nanosecond time scales. The ability to provide both fast time resolution and a site-specific probe, as shown by this study, makes the combination of isotope-editing technique and time-resolved infrared spectroscopy an ideal approach for studying secondary structure formation as well as fast events in folding.

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