# Nanosecond UV Resonance Raman Examination of Initial Steps in α-Helix Secondary Structure Evolution

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The primary sequences of most proteins encode both the structure and the dynamics of the folding process. Unfortunately, it is still impossible to predict secondary and tertiary structures from primary sequences. This is because the complex folding dynamics involve structural evolution over several different time scales.[1−4] Theoretical studies predict α-helix propagation on subnanosecond time scales, while nucleation of secondary structure motifs occurs on ns time scales and tertiary structure forms in the ms time scales. Recently, techniques such as fluorescence and IR spectroscopy have begun to probe protein folding dynamics in ns time scales.[5−10]

In this work we report the first application of ns transient UV resonance Raman spectroscopy (UVRS) to investigate the earliest events in protein structural evolution. We examine the thermal unfolding of the 21 amino acid α-helical peptide $A_5[AAARA]_3A$ (AP), which occurs via two-state kinetics without any observable intermediates.[5,6] We find that the *unfolding* rate constants show Arrhenius-type behavior with an apparent ∼7 kcal/mol barrier with a reciprocal rate constant of ∼200 ns at 37 °C. In contrast, the ∼1.1 $\mu$s *folding* rate constant shows a negative activation barrier. These results support recent protein folding landscape and funnel theories.[1,2]

UVRS excited in the 200 nm spectral region selectively probe protein secondary structure.[11−13] This excitation selectively enhances amide vibrations because it is resonant with the amide backbone electronic transitions.[14−16] These amide Raman bands sensitively depend on secondary structure. We recently determined the UVRS of the pure secondary structure Raman spectra (PSSRS) of the α-helix, $\beta$-sheet and random coil motifs of proteins and demonstrated that these PSSRS can be used to quantitatively determine protein secondary structure.[11]

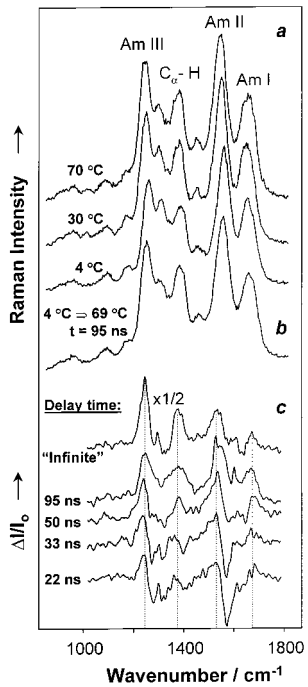


**Figure 1.** (a) Static 204-nm UV resonance Raman spectra of AP (15 mg/mL, pH 7 aqueous solution, no buffer) measured at several different temperatures. (b) Transient UVRS measured 95 ns after a T-jump from 4 to ∼69 °C. (c) Transient difference UVRS of AP initially at 4 °C at several different delay times after a T-jump of ∼33 °C. The static UVRS of AP at 4 °C is subtracted from each of the transient spectra. "Infinite" represents the difference between static UVRS measured at 37 and 4 °C. This static spectrum represents a difference spectrum at an infinite delay time.

The static 204-nm UVRS of AP at high temperature are dominated by the Am I (1655 $cm^{-1}$), Am II (1547 $cm^{-1}$), $C_\alpha$−H bending (1382 $cm^{-1}$), and Am III (1244 $cm^{-1}$) bands (Figure 1a). The relative intensities and frequencies of these bands are very close to those of the random coil PSSRS.[11] We conclude that high-temperature AP is essentially 100% random coil, which is also consistent with CD data. CD and the UVRS measurements indicate a decreasing α-helical content as the temperature increases.

We calculated AP random coil and α-helix basis Raman spectra from the static spectra measured at several different temperatures (Figure 1a) by using CD measurements to determine the secondary structural composition. As a first approximation, we calculated the change in α-helical composition for each T-jump by measuring the average peak heights of the AIII, AII, and $C_\alpha$−H bending bands in the transient difference spectra. We compared these amplitudes to those of the steady-state temperature difference spectra. The UVRS changes between the sample at 4 °C and that measured 95 ns after a T-jump to 69 °C are identical to those expected upon partial unfolding of α-helical AP to the random coil[17] (Figure 1b). Figure 1c shows that the UVRS changes depend on the probe delay time after a T-jump from 4 to 37 °C. The top

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(17) We Raman shifted the ∼3 ns, 1.06 $\mu$m fundamental of a Coherent Inc., Infinity YAG laser to 1.9 $\mu$m (1st $H_2$ Stokes shift) to selectively heat the water solvent. The sample T-jump was independently measured using shifts in the ∼3400 $cm^{-1}$ water Raman band. We probed the peptide structural evolution by exciting the UV Raman amide spectra with delayed 204 nm UV pulses generated by the 5th $H_2$ anti-Stokes shifted frequency of the 3rd harmonic of the same YAG laser. The Raman instrumentation will be described in detail elsewhere. Raman scattering was measured from the surface of a 0.6-mm diameter thermostatically controlled sample solution stream. AP (95% purity) was prepared by the solid-phase peptide synthesis method.

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curve, which displays the static UVR difference spectrum between AP samples at 37 and 4 °C, shows peaks which result from the melting of AP α-helices to random coil peptides. At the shortest delay times, we see small features, which result from the known downshifts of the Am II and Am III bands with temperature.[18] We only observe random coil formation at longer delay times. However, even at 95 ns the difference spectral features have not evolved to the magnitude of those in the static temperature difference UVRS (labeled "infinite" (in Figure 1c). This conclusion is consistent with that of an IR study of a similar peptide by Williams et al.,[5] which also demonstrated spectral changes involving only two species. It should be noted, however, that the transient 95 ns difference spectrum differs somewhat from the "infinite" static AP temperature difference spectrum between 37 and 4 °C. We are examining these differences to obtain further information on these intermediate species.

Assuming monoexponential kinetics and by using infinite delay time data from steady-state Raman difference measurements, we calculate a relaxation time of 180 ± 60 ns for a 33 °C T-jump and determined 120 ± 50 and 70 ± 30 ns relaxation times for 43 and 65 °C T-jumps. Monoexponential relaxation kinetics were previously observed for similar ala peptides.[5]

If protein unfolding involves only a simple two-state reaction we can calculate the folding and unfolding rate constants and the activation barriers from our measured temperature-dependent equilibrium constants and the relaxation rates. The relaxation kinetics are determined by the sum of the rate constants, $k_h$ and $k_c$, for α-helix formation and melting, respectively. Using the equilibrium constant, $K = k_h/k_c$, obtained from CD measurements, we calculate the reciprocal of the α-helix melting rate constants of 210 ± 60, 130 ± 50, and 70 ± 30 ns at 37, 47, and 69 °C, respectively. In contrast, the reciprocal of the α-helix formation rate constant was estimated as $1.1^{+1.0}_{-0.3}$ μs at 37 °C.

We find that the temperature dependence of the α-helix melting rate constant shows Arrhenius-type behavior, with a 7 ± 2 kcal/mol activation energy (Figure 2). Assuming a two-state model, we can calculate the temperature dependence of α-helix formation: $k_h = Kk_c$ (Figure 2). We find that $k_h$ decreases as the temperature increases with an apparent negative activation energy of −7 ± 3 kcal/mol. This clearly indicates a failure of our use of transition state theory in our two-state modeling of AP thermal unfolding. A weak anti-Arrhenius behavior ($E_a = -1$ kcal/mol) was also recently observed in results from a fluorescence spectral T-jump measurement of a model β-hairpin peptide.[19,20]

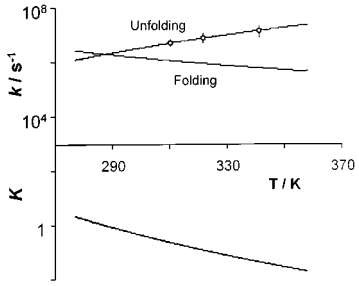


**Figure 2.** Temperature dependence of the AP folding and unfolding rate constants, *k*, and the α-helix−random coil equilibrium constant, *K*.

We believe that these results, which are the first measurements of the activation barriers for the earliest stages in α-helical folding and unfolding, reflect the complexity of the protein-folding mechanism. Although monoexponential relaxation is expected for simple systems, macromolecules utilize much more complex multidimensional reaction coordinates. This concept has recently been discussed in the energy landscape and protein-folding funnel theories, which propose that the folding and unfolding rates are determined by competition between the landscape energy surface slope and the landscape roughness along paths connecting the native and unfolded protein structures.[3,4] Obviously, our temperature-dependent rate constants sense this energy landscape complexity. The slower folding kinetics and their unusual temperature dependence signals a folding bottleneck which may involve α-helix nucleation. In fact, the recent 2D lattice model by Dill et al.[1,2] predicts Arrhenius behavior for protein unfolding and an anti-Arrhenius behavior (negative apparent activation energy) for protein folding around the heat denaturation temperature region.

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