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Primary Peptide Folding Dynamics Observed with Ultrafast *T*-Jump**

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The folding dynamics of proteins and polypeptides is a complex process involving different time and length scales.[1–4] Among the secondary structural elements the α-helix is the most commonly found configuration with its stability resulting from the unique hydrogen bonding; the C=O group of an amino acid at the position *i* forms a hydrogen bond with the N-H group of another amino acid located at the position *i*+4. The thermodynamic properties of the α-helix are understood in the context of the helix-coil transition, but the dynamics, being of many steps, have a whole range of time scales. The rates have been reported using a variety of experimental methods [for recent reviews see refs. 5–7] including absorption, NMR, Raman, infrared, and circular dichroism. The helix-coil transition was believed to occur on the microsecond timescale, and only recently, by means of fluorescence detection, were the rates measured to be as short as 300 ns,[8–10] prompting the association of the 300 ns results with “ultrafast” dynamics.[9] Theoretical models of helix-coil (polymer type) transitions and molecular dynamics (MD) simulations (see below) have also provided a range of time scales. For example, Schwarz,[11] using Zimm-Bragg nucleation and elongation parameters,[12] estimated the “relaxation time” to be 0.1 microsecond, whereas the time scale in MD simulations of folding (sub-ns to μs) depends on the length and sequence. In order to resolve the primary processes of folding, the dynamics have to be observed with the shortest time resolution possible.

In this contribution, we report the ultrafast folding dynamics of the α-helix with time resolution three orders of magnitude shorter than previously reported with fluorescence detection. The *in situ* measured heating time (3.5 ps), with the ultrafast *T*-jump method,[13] is determined by the water relaxation time of the peptide in pure water, as described below. Earlier, a *T*-jump with time resolution of 70 ps was used to study proteins by heating through dye molecules in the solution.[14] Here, the heating is directly through the water vibrations on the 3.5 ps time scale. We studied the α-helical alanine-based pentapeptide, Ac-W-$(A)_3$-H^+-NH_2 (Wh5), in an acetate buffer at pH = 4.8. Other alanine-based peptides with

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varying lengths, composition, and temperatures were also studied (See SI Figure S2), but the focus here is on the small peptide with 5 residues, as it represents the fundamental nucleus for helix formation without the elongation process being involved. We also performed all-atom MD simulations on the same system in order to compare with the experimental results.

The presence of α-helix for Wh5, with three alanine residues as they have the highest propensity to form helix,[15–17] is evidenced by CD (See below) and also from NMR studies.[9,18] The peptide has been designed to have a single tryptophan residue in position one and a single protonated histidine residue in position five. Tryptophan serves as a fluorescence probe for the local and global structural changes, as its fluorescence is strongly quenched by the nearby protonated histidine in the folded state, and is recovered in the unfolded state.[15] It is worth mentioning that the spatial interaction between tryptophan and nearby protonated histidine contributes to the stability of this helical structure.[15,16] Figure 1 provides the conformations and chemical sequences of the folded and extended structures of the 5 and 21 residue peptides Wh5 and Wh21. These peptides were obtained from California Peptide, CA with greater than 98% purity, characterized as discussed below, and all measurements were made in 20 mM acetate buffer at pH = 4.8.

Figure 2 displays the time resolution achieved in the *T*-jump together with typical results for characterization of the helical structure using far UV circular dichroism; also included are the thermal denaturation curves as a function of temperature. Figure 2A is the observed fluorescence change of free tryptophan in water solution during the *T*-jump (from 296 K → 310 K). The measured data are the circles and the fit to the data is the solid line, giving the overall relaxation time of tryptophan in aqueous solution to be 3.2 ± 0.3 ps. The solvation time of tryptophan is known to be ~1 ps.[19,20] Because tryptophan is heated by the *T*-jump through absorption of the overtone of the OH stretch vibration of water, the hydrogen bond dynamics, which occur in ~5 ps,[21,22] control the energy transfer process and are therefore consistent with the observed 3.2 ps response. In Figure 2B and 2C both the CD and melting curves are provided for the smallest structure studied.

The far-UV CD spectra of Wh5 shows the characteristic signature of an α-helical structure by the presence of double minima around 220 nm and 210 nm. The decrease in molar ellipticity as a function of temperature (266 to 363 K) indicates that the transition from α-helical to random coil conformations extends over tens of degrees. As in other studies already reported in the literature, the evidence for the helix formation is clear in the appearance of the band near 220 nm. We have examined the CD of both the 5 and 21 residue peptides (See Figure 2B and Figure S1) and our spectra are in perfect accord with those reported for short peptides and proteins.[23,24] The fraction of helical structure was estimated from knowledge of the melting temperature and characteristic spectral features of the folded and unfolded species at equilibrium; more details will be provided in our full account of this work. The stability of the α-helical structure is consistent with NMR results, [18] and with the study of small polypeptides containing alanine.[25,26]

The transients describing the folding dynamics of Wh5 are presented in Figure 3. Triggering the peptide system with the ultrafast infrared heating pulse at 1.45 μm shifts the equilibrium toward the unfolded transient structures. The refolding is then followed in time by monitoring the tryptophan quenching fluorescence. Figure 3A–B gives the temporal behavior observed for the Wh5 peptide following the *T*-jump over the time range of −50 ps to 4.8 ns and for several final temperatures. At all temperatures, the experimental data were fitted to two exponential decays, and the corresponding time constants were obtained to be: τ_1 = 850 ± 300 ps and τ_2 = 5.3 ± 1.9 ns at the final temperature of 300 K. At higher temperatures, the rates increase (See Figure. 3): τ_1 = 650 ± 250 ps and τ_2 = 4.7 ± 0.6 ns at 310 K; and τ_1 = 450 ± 150 ps and τ_2 = 3.6 ± 0.5 ns at 323 K. The fast component contributes

about 10–20 % depending on the final temperature, and the error bars on τ_2 are larger because of the short time window (4.8 ns). We have also performed the experiments at higher temperature (330 K) in order to identify the asymptotic level of the recovery (See Figure S3), and thus the quoted error bars are reasonable. The other peptides (four, six and 21 residues) were similarly studied.

The experimental observations indicate the existence of ultrafast dynamics, the fastest relaxation time observed to date in the folding/unfolding process. The rate of nucleation, stabilized by single *i, i* + 4 hydrogen bond without elongation, in the smallest possible α-helical polypeptide depend on temperature: from 450 ps at 323 K to 850 ps at 300 K, and, similarly, the long decay component shortens as the temperature increases (from 5 to 3 ns). Of significance is the fact that, as the temperature rises, the final amplitude change increases (Figure 3), while the amplitude of the fast component decreases. We note that the CD indicates that the peptide forms the helix at equilibrium or at long times.

The folding dynamics involves a landscape of different paths. The peptide may search for the correct hydrogen bonded conformation through intermediates of collapsed structures[27] and different trajectories are involved: those beginning from the unfolded peptides and forming intermediate collapsed structures prior to the helical structure, and those which directly form the helical structure from collapsed structures at shorter length and time scales. Such bifurcation is a general feature of complex molecular reactions with different pathways.[28] To confirm this view we have repeated these experiments for a system of 21 residues which, besides nucleation, also undergoes the helix elongation process. Indeed, the rates found for the 21 residue peptide resemble those of Wh5 when it was partially denatured to have, on average, the Wh5 length characteristics. However, as importantly, the rates in buffer solution for the 21 residue peptide are still similar ($\tau \sim 7$ ns) for the sub-ensemble probed in the time window of 4.8 ns, and are clearly two orders of magnitude shorter than the 300 ns reported before.[9] We note that quenching of the fluorescence may be caused by side chain motions. However, the disparity in time constants in the experiments of 21 and 5 residue peptides (despite the fact that the locations of the tryptophan and histidine are the same in both peptides), together with the MD results, provide the support for excluding such a possibility.

A simple theoretical model involving the rotations of peptide bonds for the transformation from helical to non-helical basins of conformation space (as defined by the Ramachandran plot) predicts folding time constants of about 2 ns at room temperature. This time constant was obtained from knowledge of the water viscosity at different temperatures, and the effective volume of the peptide. The results, which are consistent with the measured τ_2, indicate that the diffusive motion accounts for the rate-limiting step since τ_1 is much smaller than τ_2. Accordingly, the helix nucleation step is an entropically-driven process. However, in order to understand the complete process, including the enthalpic contribution to helix formation, an atomic-scale analysis, as opposed to coarse graining, is required.

We carried out MD simulations of the Wh5 peptide in explicit water with periodic boundary conditions at 311 K, using the CHARMM program (for details see Suppl. Inf.).[29] During the simulations, a folding event is deemed to occur when the peptide comes within 0.5 Å root-mean-squared deviation (RMSD) from the native helical structure for four consecutive ps. The results are presented in Figure 4 for 100 trajectories of 50 ns each and for a total simulation time of 5 μs. The percent helicity over the entire trajectory was 20 %, consistent with the CD results at the same temperature (310 K). It is found that folding involves a range of rates, but with the time scale being of two types: fast annealing to the native structure and slow diffusion to a collapsed structure. This is illustrated by the graph of the maximum RMSD achieved during folding vs. folding time which shows that the fastest

folding events do not reach a completely unfolded structure. Significantly, the frequency histogram of number of folding events vs. folding time shows a biexponential behavior with the timescales in good agreement with those obtained experimentally at the same temperature (5 ns and 370 ps at 310 K). The MD simulations are consistent with those of other (similar) systems. For example, Hummer and co-workers[30,31] examined short Ala-Gly-based peptides, and showed that the formation of the first single helical turn occurs on a time scale ranging from subnanosecond to a few nanoseconds depending on sequence and temperature. Additionally, Tobias *et al*,[32] have carried out MD simulation of a turn-forming pentapeptide in water at room temperature and concluded that the helix structures can form and unform on subnano- and nanosecond time scales.

In conclusion, using our ultrafast *T*-jump methodology for peptides in water solution, with temporal resolution reaching water heating time (3.5 ps), we have observed the fastest elementary events in the folding of secondary structures, hitherto reported to be orders of magnitude smaller in rates. Even in the nucleation of the shortest 5-residue structures the helix-coil transition involves “multi-step” dynamics (bifurcation of trajectories) and the two–state concept in which the peptide molecules are either in the fully folded (helical) or fully unfolded (random coil) conformations cannot describe the observed behavior. We have examined both the 5 and 21 residue dynamics and the dependence of the latter on denaturant. Together with MD simulations we could address issues of folding bifurcation and side chain motions. These findings suggest future studies of the early ultrafast events for other polypeptides and proteins, and, perhaps most significantly, they redefine what is meant by ultrafast dynamics in protein and peptide folding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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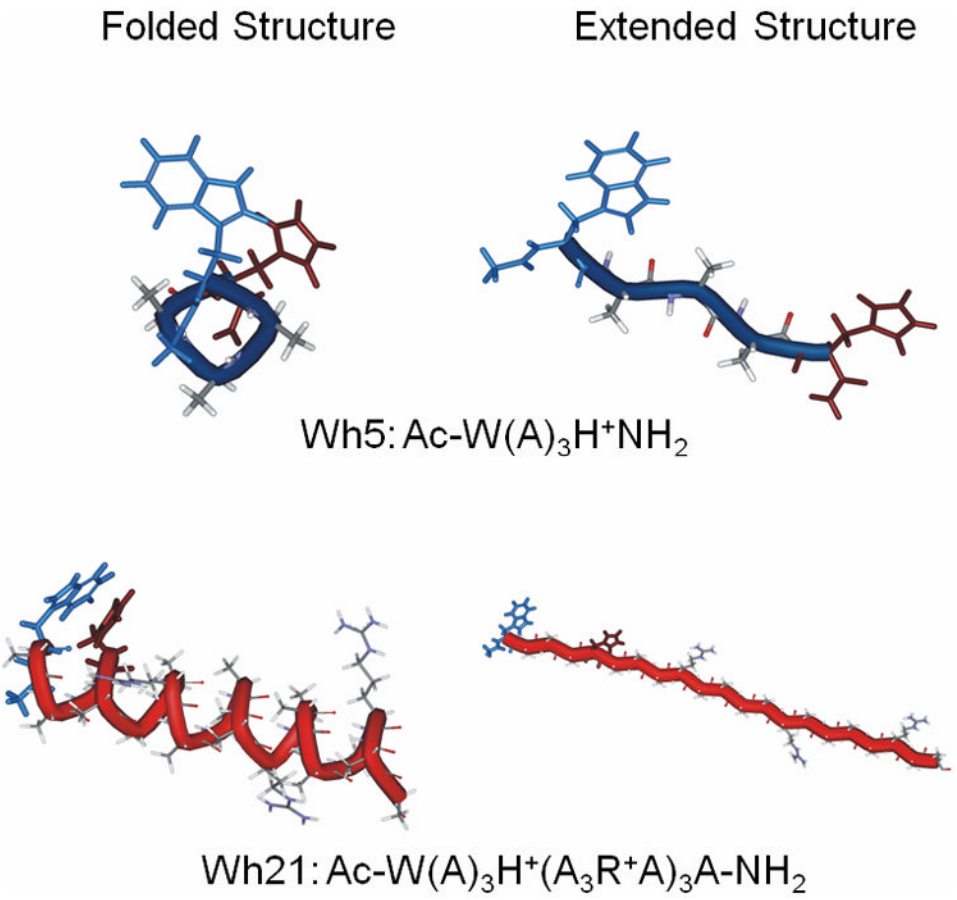

Figure 1.
Folded and extended structures of Wh5 and Wh21 and their amino acid sequence. Tryptophan (light blue) and histidine (dark brown) residues are located in the N- and C-termini of Wh5. The location of tryptophan and histidine in Wh21 is the first and fifth position, respectively.

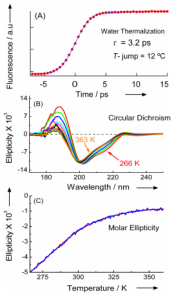

Figure 2.

Time resolution of the water *T*-jump and helicity curves of Wh5. Shown in (A) is the time dependence of the fluorescence intensity change of free tryptophan in water observed during the *T*-jump from 296 K to 310 K. The probe is an ultrafast UV (280 nm) pulse, and the initial heating is through the excitation of the overtone of the OH stretch vibration of water with a near-IR ultrafast pulse centered at 1.45 μm. This process induces a 10 to 15 °C temperature jump with a time constant 3.2 ± 0.3 ps. (B) Recorded are the measured far-UV CD spectra (molar ellipticity/deg cm^2 $dmole^{-1}$) of Wh5 (ranging between 170 nm – 250 nm) as a function of temperatures. (C) Displayed are the temperature denaturation curve (measured at 220 nm) of Wh5 as a function of observed temperature range.

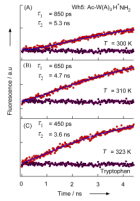

Figure 3.

Transient evolution of peptide following *T*-jump. Shown are the tryptophan fluorescence of Wh5 (20 mM acetate buffer at pH = 4.8) in response to the *T*-jump for final temperatures of 300 (A), 310 (B) and 323 K (C), and over the time window from −50 ps to 4.8 ns. Note that the fluorescence signals are shown with negative amplitude in arbitrary units. Tryptophan was excited using an ultrafast UV pulse at 280 nm, and the fluorescence was monitored in the window of 310 to 500 nm. The points represent experimental data and the solid curves are the best fits with a double-exponential function. For calibration, the experiments were repeated using free tryptophan in aqueous solution under identical conditions and the obtained data (reference) are the unchanging response shown for each transient taken.

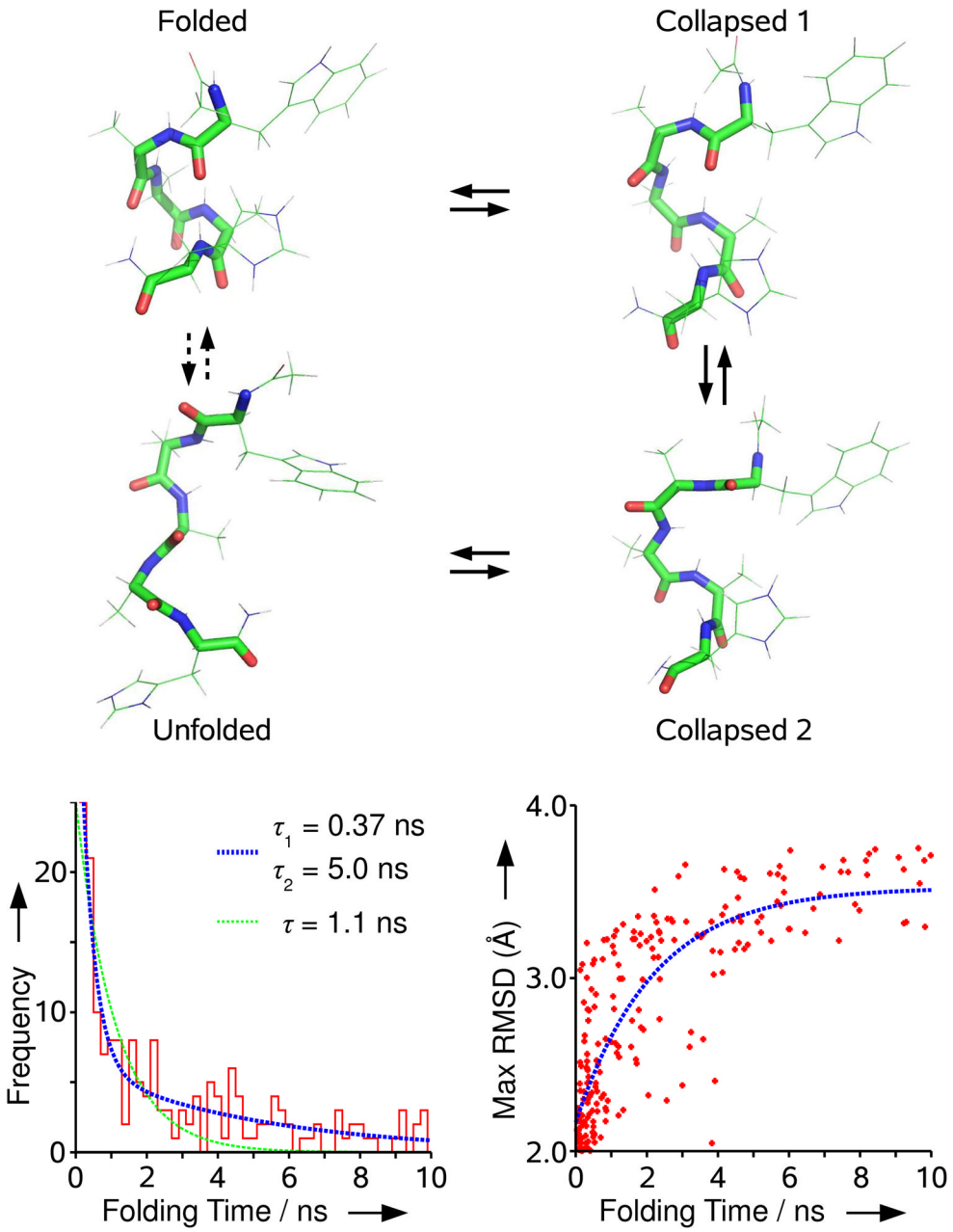

Figure 4.

Folding statistics from molecular dynamics trajectories. One hundred separate explicit-solvent MD trajectories of 50 ns each were carried out at 311 K and 1 atm pressure. Top: Snapshots taken from the MD simulations depicting reversible (un)folding between the helical, collapsed, and unfolded structures. Many of the fast refolding events involve, first, the breaking of the hydrogen bond and small deformations of the helical backbone, followed by relaxation of the structure back to the helical form. Bottom left: folding frequency (histogram counts of folding events) as a function of folding times showing that folding times range from sub-ns to many ns. The slow decay in the frequency of longer folding times indicates that there is more than a single time scale for folding. When fitted with a double exponential (blue), the mean-squared error was half that of the single exponential fit

(green). Significantly, the values of the two fitted timescales are in good agreement with those measured experimentally at the same temperature. Bottom right: the maximum RMSD between the native helix and the folding peptide as a function of folding time is plotted for each folding event. The shorter folding times correspond to refolding of collapsed or native-like structures with smaller RMSD, while longer timescales correspond to folding from a completely unfolded structure with larger RMSD.