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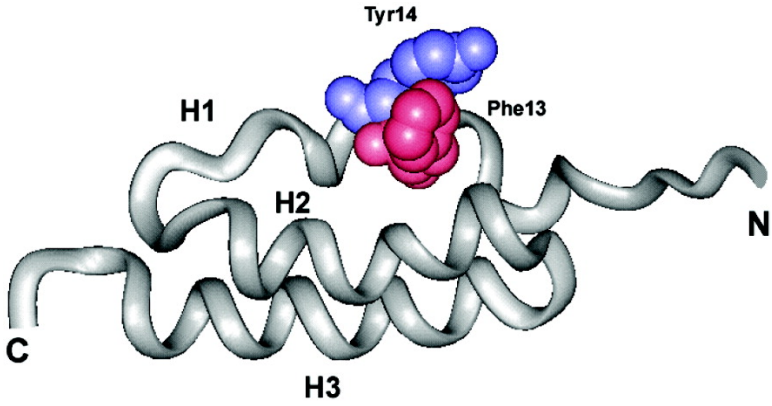

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Experimental Resolution of Early Steps in Protein Folding: Testing Molecular Dynamics Simulations

Dung M. Vu,[†] Eric S. Peterson,[§] and R. Brian Dyer*,[†]

Bioscience Division, MS J586, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, and Chemistry Department, 6600 College Station, Bowdoin College, Brunswick, Maine 04011

Received March 19, 2004; E-mail: bdyer@lanl.gov

Elucidating the molecular details of folding pathways is critical to understanding the mechanisms of protein folding. Molecular dynamics simulations of small, fast-folding proteins are beginning to reveal such details, but experimental tests are required to validate these simulations. A highly effective approach to benchmark molecular dynamics (MD) simulations experimentally is to map transition-state structures using ϕ-value analysis.[1] A complementary approach developed in our laboratories and others is to characterize the earliest intermediates in protein folding using temperature jump (T-jump) and time-resolved spectroscopic methods.[2,3] We have studied the early folding dynamics of the B domain of *Staphylococcal* protein A (BdpA), an ideal model system because of its small size (58 residues) and simple three-helical bundle (H1, H2, and H3) topology. Previous studies found that the folding of BdpA is complete within microseconds,[4,5] a time scale that is accessible to all-atom MD simulations. MD simulations of the folding of BdpA predict a range of folding mechanisms, from hydrophobic collapse[6] to diffusion collision.[4,7] Furthermore, simulations that support initial formation of secondary structure vary in their prediction of the order of formation of the individual helices, depending on the force fields and simulation methods employed.[8−13] Clearly, new experimental data are required to help test and refine the different MD approaches. T-jump IR measurements have shown that fast formation of individual helices is the first step in the folding of BdpA,[5] in agreement with some MD simulations. Unfortunately, the IR experiments do not identify which of the three helices are formed in the fast initial phase. The T-jump fluorescence results reported here demonstrate that H1 is not involved in this fast helix formation step. This observation validates the results of several MD simulations that predict H1 formation only in the final assembly of the helix bundle.

We have probed the relaxation dynamics of BdpA in response to a laser-induced T-jump using time-resolved fluorescence spectroscopy.[2] The fluorescence probe used to monitor the folding dynamics of BdpA is a single tyrosine residue (Tyr14) located in H1. The fluorescence quantum yield of Tyr is sensitive to the local environment of the aromatic ring and hence reports on the protein structure.[14] We have found that T-jump Tyr fluorescence measurements provide a specific probe of the H1 dynamics and thus complement the IR measurements, which probe the dynamics of the entire helical backbone. Together, the fluorescence and IR results provide unique insight into the folding mechanism of BdpA. In addition, this is the first report of the use of Tyr fluorescence to follow fast protein folding events.

The fluorescence spectrum of the folded protein at 20 °C has a peak near 312 nm due to the single Tyr fluorescence. The thermal denaturation curve obtained by monitoring the integrated intensity of this peak with temperature shows a linear baseline underlying a single sigmoidal transition; this transition is similar to what is observed with far-UV CD (signal at 222 nm, $T_m = 72.5 \pm 0.1$ °C) and FTIR (buried helix amide I′ IR band at 1652 cm^{-1}, $T_m = 73.3 \pm 1$ °C).[5] The linear baseline dependence of the melt corresponds to the temperature-dependent quantum efficiency of Tyr.[14] The dominant perturbation of the Tyr environment in the sigmoidal transition is most likely the loss of the helical conformation (coupled with the disruption of the tertiary helical bundle). Tyr14 is situated along the side of H1 that packs against H2 and is partially exposed to solvent even in the folded structure.[15] Partial protection of the Tyr14 from solvent is provided by stacking of the aromatic ring against the neighboring Phe13 in the helical conformation. The loss of the helical conformation breaks the Tyr−Phe interaction, increasing the exposure to solvent which decreases the Tyr14 emission intensity and slightly red-shifts the peak emission.

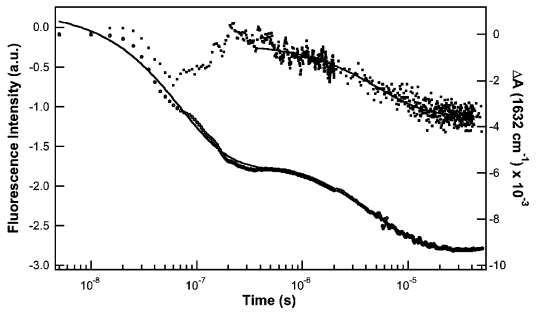

Figure 1. Relaxation kinetics of BdpA following T-jumps probed with Tyr fluorescence, upper trace (56−79 °C) and IR absorbance, lower trace (66−79 °C). The concentration of the protein solution used in the IR and fluorescence experiments was 2−4 mg/mL in phosphate buffer at pH* (uncorrected) 6.75 in D_2O. The solid lines are fits to exponential decays; a single exponential fits the fluorescence transient ($\tau = 5.3 \pm 1$ μs), whereas two distinct phases are observed in the IR transient ($\tau_1 = 70 \pm 10$ ns, $\tau_2 = 5.3 \pm 1$ μs). A cavitation artifact is observed at early time in the fluorescence transient, but the signal returns to baseline prior to the protein response. The fluorescence and IR transient signals are averages of 200 000 and 10 000 laser shots, respectively.

Figure 1 compares the transient fluorescence with the transient IR absorbance monitored at the solvated helix frequency (1632 cm^{-1}) in response to T-jumps to the same final temperature (79 °C). These two probes monitor different aspects of the protein structure and yield very different kinetics responses. The fluorescence and IR T-jump experimental setups have been described elsewhere.[16,17] The magnitude of the T-jump (accuracy, ±1 °C) was calibrated using Trp and D_2O reference solutions held in a separate compartment of the sample cell. In each case, the instantaneous response of the sample to the T-jump (instrument-limited, $\tau \approx 23$ ns), due to the temperature-dependent quantum efficiency of Tyr and IR absorbance of D_2O, respectively, has been subtracted using the transient response of the reference solution. A cavitation artifact is evident at early times in the fluorescence transient, as a consequence of the large T-jump required to generate sufficient signal ($\Delta T = 23$ °C); this artifact also appears in the

[†] Los Alamos National Laboratory.
[§] Bowdoin College.

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reference data (i.e., it is independent of the protein sample) and has a characteristic time response. Although the early time region of the fluorescence data is partially obscured by this artifact, the signal returns to the baseline before the microsecond kinetics phase, indicating that no nanosecond kinetics phase is present in the fluorescence response. The fluorescence transient is well fit by a single-exponential process with a relaxation time of 5.3 ± 1 μs. In contrast, the IR transient absorbance clearly displays two distinct relaxation processes. The IR solvated helix band (1632 cm^{-1}) reports on the hydrophilic side of the helical bundle as well as the individual solvated helices.[5] The microsecond relaxation process ($\tau = 5.3 \pm 1$ μs) corresponds to the relaxation of the tertiary helical bundle, whereas the faster relaxation process ($\tau = 70 \pm 10$ ns) corresponds to the helix−coil transition of the individual helices.[5] Numerous studies by our group and others have shown that the relaxation of helices in peptides and proteins has a characteristic rate ($\sim 10^7$ s^{-1}).[5,17−22] Thus, the helix−coil relaxation transition is detected by IR absorbance at 1632 cm^{-1} but is missed by Tyr fluorescence.

The results of this study give further insight into the folding mechanism of BdpA. Different relaxation behaviors for BdpA are observed in response to fast laser-induced T-jumps, depending on whether the protein is probed with IR or fluorescence spectroscopy. While the microsecond relaxation dynamics of the helical bundle are observed by both of these probes, only the IR probe reports on the ~70 ns helix−coil transition. There are two plausible hypotheses to explain the absence of the fast helix−coil dynamics in the fluorescence measurements. One possibility is that the Tyr fluorescence is not sensitive to the helix−coil transition, and consequently the kinetics follow only the helical bundle formation. The second possibility is that the Tyr fluorescence is sensitive to the helix−coil transition, but the rapid initial formation of individual helices does not involve H1. In the second model, H1 is formed later (in the microsecond kinetics phase) by docking with one or both of the other helices.

The weight of evidence favors the latter hypothesis, that the formation of H1 occurs concomitantly with the docking to (and stabilization by) the other two helices to form the helical bundle. Support for this model is based on several factors: (1) In the NMR structure of BdpA, solvent protection of Tyr occurs primarily through stacking of the aromatic rings of the adjacent Phe13 and Tyr14, not from interaction with side chains from the other helices in the bundle.[15] Thus, the Tyr fluorescence should be quite sensitive to the helix−coil transition, which removes these interactions and causes the observed decrease in fluorescence. (2) T-jump studies of a small alanine peptide show that an extrinsic fluorescence probe is sensitive to the helix−coil transition, even when no specific quenching interaction is present in the folded state,[23] further evidence that the Tyr fluorescence should also track the helix−coil transition for H1. (3) Experimental studies with peptide fragments of BdpA found that H1 was the least stable, whereas H3 was determined to be the most stable,[24] consistent with the model that H1 only forms when stabilized by the bundle. (4) Experimental ϕ-value analysis of BdpA conducted by Fersht and colleagues shows that H1 is weakly structured in the transition state, whereas the central H2 is nearly fully formed.[25]

Thus, the evidence supports a folding model for BdpA in which H2 and/or H3 is formed early (the nanosecond IR kinetics phase), whereas H1 is formed late, concomitant with the formation of the helical bundle (the microsecond IR and fluorescence kinetics phase). These results lend credence to a number of MD simulations of the folding mechanism of BdpA.[9−13,26] Some of these studies suggest that H3 forms first, followed by the formation of an H2−H3 microdomain and the final docking of H1 to this microdomain.[9−12,26] In an MD *unfolding* simulation, Alonso and Daggett observed the reverse order of events and concluded that the stability of H1 is associated only with its tertiary interactions with the other helices.[9] Garcia and Onuchic also observed early formation of H3, although they find substantial formation of H1 stabilized by interactions with H2 in the transition state.[13] While these studies differ in the details of the folding mechanism predicted for BdpA, the consensus is that H1 is formed only in the final assembly of the helical bundle.

In summary, we have used T-jump Tyr fluorescence as a novel probe of early folding dynamics to study the folding mechanism of a fast-folding helical protein, BdpA. Our results confirm the predictions of some MD simulations that the formation of H1 occurs very late in the folding of this protein, concurrent with the formation of the tertiary helical bundle.

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