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Temperature Jump and Fast Photochemical Oxidation Probe Submillisecond Protein Folding

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Abstract: We report a new mass-spectrometry-based approach for studying protein-folding dynamics on the submillisecond time scale. The strategy couples a temperature jump with fast photochemical oxidation of proteins (FPOP), whereby folding/unfolding is followed by changes in oxidative modifications by OH radical reactions. Using a flow system containing the protein barstar as a model, we altered the protein's equilibrium conformation by applying the temperature jump and demonstrated that its reactivity with OH free radicals serves as a reporter of the conformational change. Furthermore, we found that the time-dependent increase in mass resulting from free-radical oxidation is a measure of the rate constant for the transition from the unfolded to the first intermediate state. This advance offers the promise that, when extended with mass-spectrometry-based proteomic analysis, the sites and kinetics of folding/unfolding can also be followed on the submillisecond time scale.

The deciphering of protein folding is essential for understanding biological processes and developing therapeutic approaches to misfolding-related diseases.[1−3] Protein folding can be followed in equilibrium experiments, which monitor protein states as a function of temperature or denaturant concentration, and in kinetics experiments, which give a time-dependent conformational change.

Starting in the early 1990s, mass spectrometry (MS) emerged as an effective tool for supporting both thermodynamic and kinetic protein-folding studies. The advantage of modern MS is its ability to measure the extents of protein modifications and pinpoint their locations. For pulsed H/D amide exchange and other pulsed covalent labeling kinetics,[4,5] MS detection can now track folding events down to the millisecond time scale. Protein folding, however, often occurs more rapidly (in microseconds or even less[6]) and has been difficult to access using current MS-based studies.

Here we describe a new approach for the investigation of protein folding. We use two lasers, one to provide a temperature jump (*T* jump) and a second to generate reagents to footprint the consequences of the *T* jump. This is an example of a "pump/probe" experiment,[7,8] but it is distinguished by the use of chemical reactions as the structural probe rather than the usual spectroscopic approaches. To our knowledge, the first irreversible labeling accompanying protein conformational change was demonstrated by Jha and Udgaonkar,[9] who explored protein folding on the millisecond time scale by combining mutagenesis, pulsed thiolabeling, and global protein analysis by MS. Recently, Stocks and Konermann combined rapid mixing and fast photochemical oxidation of proteins (FPOP) to observe protein unfolding[10]/folding[11] intermediates at times in the 10 ms range. Our approach takes advantage of FPOP, developed in our laboratory,[12] whereby pulsed laser photolysis yields hydroxyl radicals that label proteins irreversibly within ∼1 μs.[13] The high speed of this chemical approach now makes feasible an MS-based means of investigating protein folding on that time scale.

We chose barstar (10.3 kDa) as a test protein because it is denatured at 0 °C and folds with a *T* jump; thus, it has been used in many protein-folding studies.[9,14−16] In previous work, refolding of barstar was followed with fluorescence after a laser-based nanosecond *T* jump caused the protein to undergo a thermal folding transition.[15,16] Here we used a similar *T* jump but replaced the low-resolution physical method (fluorescence) with a chemical approach (FPOP) to afford potentially a considerably higher resolution probe of folding.

A Quanta-Ray DCR-2(10) Nd:YAG laser driving a Raman cell (75 cm long, pressurized with 300 psi high-purity H_2 gas) produced a 1900 nm pulse (8−9 ns wide) that was absorbed by flowing H_2O in a tube to cause the *T* jump (∼20 °C) (Figure 1). A 248 nm laser flash from a GAM Laser EX50/250 excimer laser photolyzed endogeneous hydrogen peroxide to give hydroxyl radicals in a 14 ns pulse. A BK Precision 4001A function generator set at 5 Hz provided control of both lasers. With custom-built circuitry, the delay between the Nd:YAG and excimer lasers could be made as short as the radical lifetime in FPOP, which currently is 1 μs.[12,13] The protein solution was prepared by incubating 10 μM barstar in phosphate-buffered saline with 1.5 M GdnCl and 15 mM glutamine for 2 h on ice prior to the two-color experiment in order to ensure that barstar was denatured at the start of the experiment.

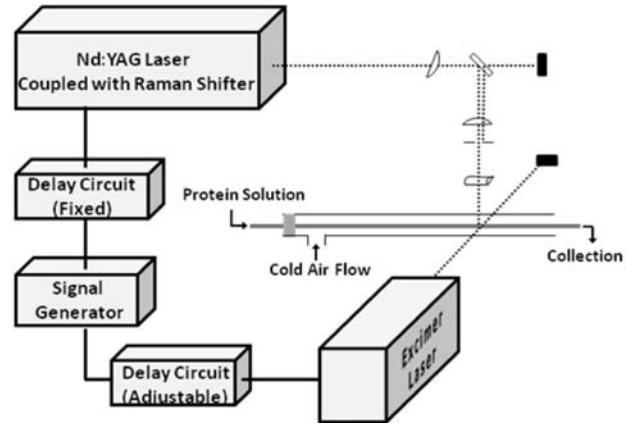

Figure 1. Schematic of the flow system intersected by two laser beams at a window in the tube, as previously described for FPOP.[12] The time between the two laser pulses is adjustable with the "delay circuit" (see the text).

The flowing solution was held at <3 °C using a stream of cold air in an insulating tube enveloping the 150 μm id silica capillary, ensuring that the barstar remained denatured until the *T* jump. After oxidative modification by ·OH, the sample was collected in an Eppendorf tube containing methionine and catalase (to remove

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H_2O_2), desalted with a C18 Zip Tip, and analyzed with a Bruker maXis quadrupole time-of-flight mass spectrometer.

Barstar passes through two intermediate states during its folding.[14] The time to establish equilibration between the unfolded state and the first intermediate state is ~1 ms; for equilibrium between the two intermediate states, ~10−100 ms is required, and for the second intermediate state and the native state to equilibrate, ~100 s is required. We targeted the first transition, which had not previously been resolved using MS methods.

Representative mass spectra of the most abundant charge state of barstar post-FPOP show the clear sensitivity of FPOP to the folding that occurs as a function of the delay between the two laser pulses (Figure 2). As the protein folds, the extent of oxidative modification with ·OH decreases. Each oxidation state is represented by a peak, which is an envelope of isotopic clusters that are resolved here.

Outcomes for other proteins[13] have shown Poisson distributions for the various modified states that are dominated by +16, +32, +48, ..., indicating that the protein exists in a single state during modification. Here we obtained global spectra showing many more modifications, consistent with a denatured protein existing in many states. The amounts of the modified species decreased with an increase in the elapsed time between the heating pulse and the FPOP probe, showing evidence of protein refolding on the <1 ms time scale. The extent of oxidation shown in Figure 2e is greater than that for folded barstar at room temperature (Figure 2f). This indicates that the protein in its first intermediate state possesses a structure that is more solvent-exposed than that of the native state and hence undergoes more extensive oxidative modification. Furthermore, some fraction of the protein may remain unfolded because the heating along the laser beam is not uniform.

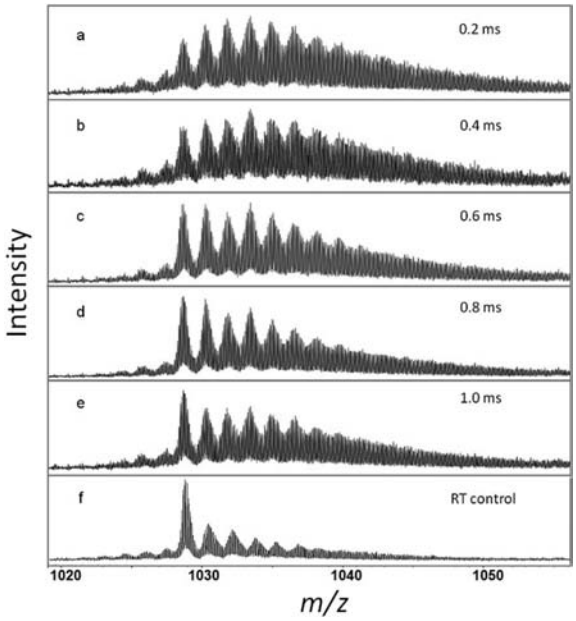

Figure 2. (a−e) Representative mass spectra of the barstar post-FPOP as a function of the time between the heating pulse and the FPOP probe. (f) Mass spectrum of the barstar post-FPOP at room temperature as a control.

We measured the rate constant for equilibration of the folding by using the centroid of all the oxidized states having a 10+ charge, which was the most abundant. The centroid shifted to higher *m*/*z* as the oxidation progressed. There were two parts of the centroid calculation of each mass spectrum. The first part estimated the baseline by taking the average spectral intensity over the interval from *m*/*z* 120/10 to 60/10 less than the theoretical monoisotopic *m*/*z*, where 10 is the magnitude of the charge state; the interval showed no discernible isotopic patterns. The second part used the centroid of the peak area that lay above the baseline in the *m*/*z* interval 7 × 16/10 (the unmodified isotopic pattern plus the first six oxidation states) starting from the theoretical monoisotopic *m*/*z*. The *m*/*z* centroid trend was fit using a single-exponential function including a constant (Figure 3); the fitting utilized the rate constant, amplitude, and constant value as parameters.

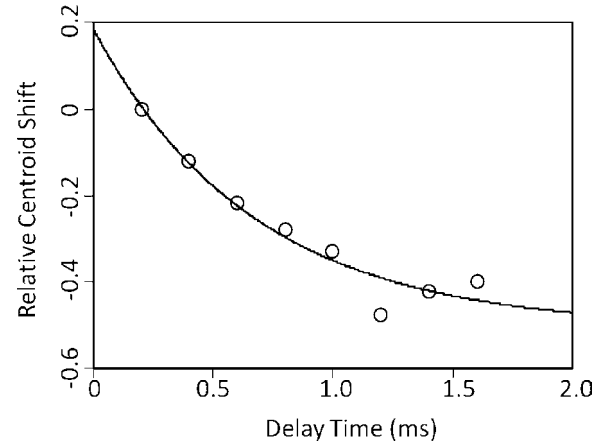

Figure 3. Plot of the normalized centroid mass shift vs the delay time, with a curve obtained by fitting a single-exponential function (solid curve) to the data (○). The fit gives a rate constant of 1.5 ms^{-1}. The standard deviation was estimated to be 0.06 by triplicate measurements at one time point, and the precision of the method was estimated by repeating the kinetics study on another day, which provided a *k* value of 1.0 ms^{-1}.

The rate constant from the fitting was 1.5 ms^{-1}. Because the *m*/*z* centroid trend is dependent on the concentrations of the unfolded and first intermediate states in a way that does not easily reveal the concentration ratio, the trend reveals only the equilibration rate constant. This rate can be compared with the value of 3.10 ms^{-1} for the transition from the unfolded state to the first intermediate state that was measured by Nolting et al.[16] in a *T*-jump experiment with fluorescence detection. The amplitude we found was −0.69, as normalized by the *m*/*z* centroid shift from 0.2 ms to a control obtained from FPOP of the folded barstar at room temperature without the 1900 nm pulse (Figure 2f). This indicates that barstar reached a steady state that is not yet the completely folded state. The advantage of the MS-based approach is that it is general and does not rely on the state of any fluorophore.

In summary, a *T* jump coupled with FPOP allows submillisecond protein-folding kinetics to be followed by a subsequent MS measurement at the global protein level. Although we followed the folding kinetics on the submillisecond time scale for barstar, we suggest that kinetics in the microsecond range can be reached, given the time scales of the two lasers (10 ns for the Nd:YAG laser and 20 ns for the excimer laser) and the quenching time for the hydroxyl radical (<1 μs). An advantage of the approach is the ability to determine modifications and follow kinetics at the amino acid level, especially for large proteins, by using proteolysis and LC/MS/MS. This is the subject of ongoing research. Recently, Hu and Tycko showed that solid-state NMR spectroscopy of frozen states of proteins can also be used to probe regions of proteins that are unfolding.[17]

This MS-based approach has considerable flexibility. Other photochemical processes can be used instead of a *T* jump as a pump, and other labeling reagents (reactive free radicals and radical ions)

can be produced by photolysis and used as the probe. We are currently developing some of these reagent radicals. The molecular mass of the protein is not a severe limitation of the method. Lastly, we suggest that protein−protein and protein−ligand binding dynamics can also be investigated by a two-laser approach wherein the first laser perturbs the thermodynamics and the second uses chemistry to probe the outcome.[7]

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