Published in final edited form as:

J Am Chem Soc. 2010 November 10; 132(44): 15502–15504. doi:10.1021/ja106518d.

T-jump and Fast Photochemical Oxidation Probe Sub Millisec Protein Folding

Jiawei Chen, Don L. Rempel*, and Michael L. Gross

Department of Chemistry, Washington University, One Brookings Drive, Saint Louis, Missouri 63130

Abstract

We report a new mass spectrometry-based approach to study protein-folding dynamics at the sub millisecond time. The strategy couples 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 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, owing to 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 at sub millisecond times.

Deciphering 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, to 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 extents of protein modification 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 timescale. Protein folding, however, often occurs more rapidly (in microseconds or even less⁶), which has been a difficult range to access in current MS-based studies.

Here we describe a new approach to investigate 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 “pump/probe”^{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 Udgaonkar⁹, who explored protein folding on the msec timescale by combining mutagenesis, pulsed thio-labeling and global protein analysis by MS. Recently, Konermann et al. combined rapid mixing and fast photochemical oxidation of proteins (FPOP) to observe protein unfolding¹⁰/folding¹¹ intermediates at times in the 10 ms range. Our approach takes advantage of FPOP, developed in our laboratory¹², whereby pulsed laser-photolysis yields hydroxyl radicals that

label proteins irreversibly within $\sim 1 \mu\text{s}$ 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 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 T-jump (ns) causes the protein to undergo a thermal unfolding 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 H₂ gas) produced a 1900 nm pulse (8–9 ns wide) that was absorbed by flowing H₂O in a tube to cause the T jump (~ 20 °C) (Fig. 1). A 248 nm laser flash from a GAM Laser EX50/250 excimer laser photolyzed endogenous hydrogen peroxide to give the 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 laser can be as short as the radical life time in FPOP, which currently is $1 \mu\text{s}$ ^{12, 13}. The protein solution was prepared by incubating 10 μM barstar in PBS buffer with 1.5 M GdnCl and 15 mM glutamine for 2 h on ice prior to the two-color experiment to ensure that barstar was denatured at the start of the experiment.

The flowing solution was held at $< 3^\circ\text{C}$ (stream of cold air in an insulating tube enveloping the 150 μm id silica capillary), insuring that barstar remained denatured until the T jump. The sample, after being oxidatively modified by $\cdot\text{OH}$ was collected in an Eppendorf tube containing methionine and catalase (to remove H₂O₂), desalted with a C18 Zip TipTM, and analyzed with a Bruker maXisTM quadrupole TOF mass spectrometer.

Barstar passes through two intermediate states during its folding¹⁴. The time to establish equilibration between the unfolded state and the first intermediate state is ~ 1 ms; between the two intermediate states, ~ 10 – 100 ms and between the second intermediate state and the native state, ~ 100 s. We targeted the first transition, which had not been resolved by 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 (Fig. 2). As the protein folds, the extent of oxidative modification with $\cdot\text{OH}$ decreases. Each oxidation state is represented by a peak, which is an envelope of isotopic clusters that are resolved here.

Outcomes for other proteins¹³ show 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 obtain global spectra that show many more modifications, consistent with a denatured protein existing in many states. The amounts of the modified species decrease with an increase in the time difference between the heat pulse and the FPOP probe, showing evidence of protein refolding on the < 1 ms time scale. The extent of oxidation shown in Fig. 2(e) is greater than that for folded barstar at RT (Fig. 2(f)). This indicates that the protein at its first intermediate state possesses a structure that is more solvent-exposed compared to that of the native state and, hence, undergoes more oxidative modification. Furthermore, some fraction of the protein may remain unfolded because the heating along the laser beam is not uniform.

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 shifts to higher m/z as the oxidation progresses. There were two parts of the m/z 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 discernable isotopic patterns. The second part used the centroid of the peak area that lay above the baseline in the m/z interval $7 \times 16/10$ (the unmodified isotopic pattern plus the first six oxidation states) starting from the theoretical monoisotopic m/z . The m/z centroid trend was fit by a single exponential function including a constant; the fitting utilized the rate constant, amplitude and the constant value as parameters.

The rate constant from the fitting is 1.5 ms^{-1} . Because the m/z centroid trend is dependent on concentrations of the unfolded and the first intermediate states in a way that does not easily reveal the concentration ratio, the trend reveals only the equilibration rate constant. This rate compares with 3.10 ms^{-1} , which was measured by Nolting et al¹⁶, in a T-jump experiment with fluorescence detection for the transition from the unfolded state to the first intermediate state. The amplitude we find is -0.69 , as normalized by the m/z centroid shift from 0.2 ms to a control obtained by FPOP of the folded barstar at RT without the 1900 nm pulse (Fig. 2(f)). 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, T-jump coupled with FPOP allows sub msec protein folding kinetics to be followed by a subsequent MS measurement at the global protein level. Although we followed the folding kinetics at sub msec level for barstar, we suggest that kinetics in the μs range can be reached owing to the time scale 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, which occurs within 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, Tycko and coworkers showed that solid-state NMR of frozen states of proteins can also be used to probe regions of proteins that are unfolding¹⁷.

This MS-based approach has considerable flexibility. Other photochemical processes can be used instead of 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 the two-laser approach wherein the first laser perturbs the thermodynamics and the second uses chemistry to probe the outcome⁷.

Acknowledgments

We acknowledge the generous financial support from the NIH National Centers for Research Resource (Grant P41RR000954 to MLG), Dr. Carl Frieden (WU Biochemistry) for a gift of barstar, and Dr. Dewey Holten (WU Chemistry) for providing the Raman shifter.

References

1. Dobson CM. Nature. 2003; 426:884–890. [PubMed: 14685248]
2. Luheshi LM, Crowther DC, Dobson CM. Curr Opin Chem Biol. 2008; 12:25–31. [PubMed: 18295611]
3. Cohen FE, Kelly JW. Nature. 2003; 426:905–909. [PubMed: 14685252]
4. Kaltashov, IA.; Eyles, SJ. Mass spectrometry in biophysics: conformation and dynamics of biomolecules. John Wiley; Hoboken, N.J: 2005.

5. Uzawa T, Nishimura C, Akiyama S, Ishimori K, Takahashi S, Dyson HJ, Wright PE. *Proc Natl Acad Sci U S A*. 2008; 105:13859–13864. [PubMed: 18779573]
6. Yang WY, Gruebele M. *Nature*. 2003; 423:193–197. [PubMed: 12736690]
7. Callender R, Dyer RB. *Chem Rev*. 2006; 106:3031–3042. [PubMed: 16895316]
8. Kubelka J. *Photochem Photobiol Sci*. 2009; 8:499–512. [PubMed: 19337664]
9. Jha SK, Udgaonkar JB. *J Biol Chem*. 2007; 282:37479–37491. [PubMed: 17959598]
10. Stocks BB, Konermann L. *Anal Chem*. 2009; 81:20–27. [PubMed: 19055350]
11. Stocks BB, Konermann L. *J Mol Biol*. 2010; 398:362–373. [PubMed: 20230834]
12. Hambly DM, Gross ML. *J Am Soc Mass Spectrom*. 2005; 16:2057–2063. [PubMed: 16263307]
13. Gau BC, Sharp JS, Rempel DL, Gross ML. *Anal Chem*. 2009; 81:6563–6571. [PubMed: 20337372]
14. Schreiber G, Fersht AR. *Biochemistry*. 1993; 32:11195–11203. [PubMed: 8218183]
15. Nolting B, Golbik R, Fersht AR. *Proc Natl Acad Sci U S A*. 1995; 92:10668–10672. [PubMed: 7479862]
16. Nolting B, Golbik R, Neira JL, Soler-Gonzalez AS, Schreiber G, Fersht AR. *Proc Natl Acad Sci U S A*. 1997; 94:826–830. [PubMed: 9023341]
17. Hu KN, Tycko R. *Biophys Chem*. 2010; 151:10–21. [PubMed: 20542371]

Figure 1. Schematic of the flow system as intersected by two laser beams at a window in the tube, as previously described for FPOP¹². The time between two pulses is adjustable with the “delay circuit” (see text).

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 RT as a control.

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 (open circles) to give 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 to provide a k of 1.0 ms^{-1} .