



Submillisecond Time-Resolved Small Angle Scattering from Folding Protein

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I am relatively new to the field of synchrotron science, having cut my teeth in the world of low temperature physics. At temperatures close to absolute zero, time scales are long: one data point comes in every two days or so. I was completely unprepared for life at CHESS, where empty hutches are converted into running experiments on this same time scale.

In the winter of 1998, using Cornell's Nanofabrication Facility, we successfully produced our first synchrotron compatible rapid fluid mixing device. We planned to study protein folding: the process by which a specific, linear chain of amino acids changes shape from a random coil to become biologically active. Folding would be triggered within nanofabricated mixers by rapidly changing the solvent. Small angle X-ray scattering (SAXS) from folding protein would allow us to monitor conformation/shape changes on the submillisecond time scale, more than an order of magnitude faster than any previous SAXS experiment.

With the expert assistance of our colleagues in Bob Austin's group at Princeton, the device was ready for use far ahead of the schedule I had drawn up. Working flow cell in hand, we checked the CHESS schedule: one week of beam remained before a six-week shutdown. We met to discuss our options. The scatter was bound to be weak, we would need to construct a low noise, high-sensitivity system for installation in the D-1 Hutch. In six weeks we could do a good, careful job. A phone call to CHESS provided a twist: there was an unexpected opening at D-line, no user for the impending (last) weekend of beam. Pieces of a small angle beamline located in Sol Gruner's lab were shuttled across campus, to CHESS. The rest was rapidly machined. We designed and built the beamline equipment inside the D-line hutch in just under three days.

This first run was fast and exciting, serving its purpose as our 'feasibility study', as we explored the stability of micron-sized X-ray beams and determined signal strengths from our pico-liter sample volume. By the end of the weekend, we were certain that SAXS could be used to study protein folding within the device. We began to plan the actual folding experiments that would take place during the spring and summer.

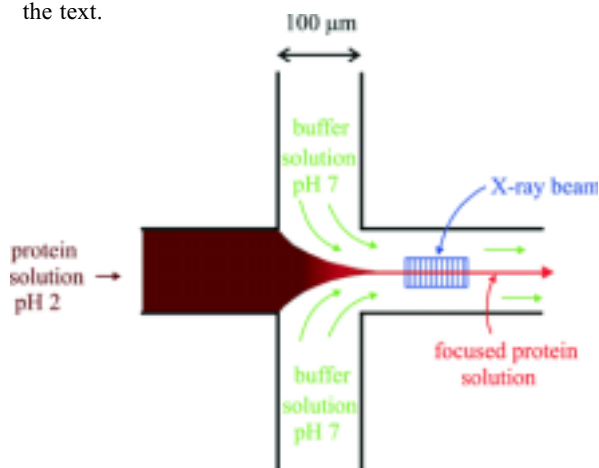
The flow cell

The shape or conformation of a protein is determined by many interactions, including those with the solvent. Proteins assume their 'folded', biologically active or 'native', form under specific solvent conditions. Cytochrome-c, a small protein involved in electron transport, assumes its native structure above pH 3. At lower pH, parts of the protein become charged. Electrostatic repulsion between charges causes the protein to unfold to a random coil. In this state, the protein has lost its functional structure and is referred to as 'denatured'. In cytochrome-c this transition is reversible; the denatured/unfolded protein refolds following an increase in pH.

Small proteins can fold in milliseconds. To effectively study this transition, the pH must change on the microsecond time scale. The design of our flow cell is based on the principle that diffusion can occur on these time scales if the dimensions are kept small. Within narrow (10's of microns) channels, flow is laminar and mixing by diffusion is efficient and rapid. The mixing device takes the form of two narrow, crossed channels etched through the 390 micron thickness of a silicon wafer (Figure 1).

The top and bottom surfaces of the wafer are sealed with 15 micron thick mica windows, leaving a four channel fluid flow device. Unfolded cytochrome-c, at pH 2, is introduced into the inlet (left) channel. Renaturing pH 7 buffer flows into the top and bottom channels at a higher pressure, focusing the protein into a thin stream that shoots out to the right. The pH of the protein stream increases rapidly as small ions/molecules (e.g. protons) diffuse into the surrounding fluid. The larger, macromolecules diffuse much more slowly. The threshold for renaturation is crossed in about 100 microseconds. Any subsequent position in the mixing device corresponds to a fixed time after the pH jump; the device

Figure 1: A cross sectional schematic of the flow cell described in the text.



converts space into time; the proportionality constant is the flow speed. X-rays can be used to sample the diffraction from the protein at any location in the device by translating the sample relative to the beam. A time-resolved picture of a folding protein can be reconstructed by comparing images taken at various positions.

The D-line configuration

The D-1 station is fed by bend-magnet radiation. A double bounce multilayer monochromator is used to increase the flux at the expense of bandwidth (1%). The beam is defined by two sets of slits inside the experimental hut. At the sample it is a rectangle: 40 microns tall by 120 microns wide. At an average flow speed of 60 cm/sec for the protein, these conditions result in a time resolution of 200 microseconds per data point. Exposure times vary, but are typically of order 400 seconds for beam (positron) currents of 150 mA. We selected a 1.5 Å X-ray wavelength (λ). A 1k x 1k CCD detector with an active area of 5 cm was located 40 cm downstream of the sample. Under these conditions, we have access to scatter in a range of q ($= 4\pi\sin\theta/\lambda$, 2θ is the scattering angle) from 0.02 to 0.45. Figure 2 shows the inside of the D-1 hut upstream of the sample.

R_g , radius of gyration, as a parameter for theory?

Rapid kinetics of protein folding are frequently studied with optical techniques. Fluorescence experiments on the folding kinetics of cytochrome-c clearly demonstrate a two-step folding process [2]. Time constants can be accurately determined by this technique, but no structural information is available. In contrast, SAXS provides information about the size, shape and compactness of macromolecules, but has not been frequently employed in rapid, kinetic studies because of a low sensitivity to proteins in solution. A determination of these structural properties of folding proteins would allow experimental results to be directly compared to models and simulations.

Statistical mechanical models of protein folding rely on calculations of free energy surfaces that reflect different configurations of the polypeptide chains. One difficulty in connecting theory to experiment has been in finding experimentally accessible parameters for these complex surfaces. The size of the molecule, expressed as its radius of gyration R_g , is easily extracted from SAXS data. At low q , a plot of the log of scattered intensity, I , vs. q^2 , a Guinier plot, yields a straight line with slope equal to $R_g^2/3$. R_g may prove to be a useful coordinate for these free energy surfaces and may allow for direct connection of theory and experiment on the microsecond time scale [1].

Collapse

A related parameter is that of compactness. In its most unfolded state, a protein is an expanded, random coil. In contrast, the folded protein is a compact object, with a density comparable to that of close packed spheres. At what stage in the folding process does collapse to a compact state occur? Does the protein collapse directly into the native state or is rearrangement required? Lattice simulations address these questions with computer models. SAXS can provide experimental data. At angles (or q) larger than those required to determine the radius of gyration, the power law dependence of the scatter indicates the compactness of the macromolecule. The scatter from a compact sphere falls off rapidly, as q^{-4} . The scatter from a long thin object (needle) falls off less rapidly, as q^{-1} . A Kratky Plot, Iq^2 vs. q , provides a qualitative estimate of the compactness of the scatterer. The presence of a peak in the Kratky Plot indicates scatter that falls off more rapidly than q^{-2} : a compact object. The absence of a peak occurs when density variations occur on short length scales, as in an expanded object. Collapse can be directly observed in a time-resolved SAXS experiment. An accurate measure of the time scale for collapse will again allow direct comparison of experimental results with predictions based on models of folding proteins.

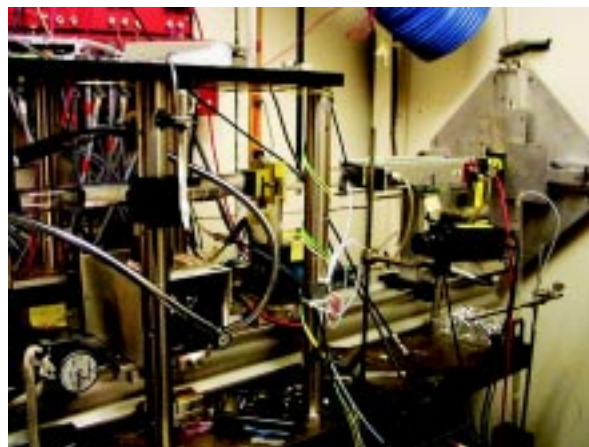


Figure 2: The inside of the D-1 hut upstream of the sample.

The folding experiments: a compact denatured state is identified

Figure 3 shows Kratky plots of the data from the folding experiment of figure 1. The top panel shows data from the left (inlet) channel, the initial state. The increase in Iq^2 at large q indicates that cytochrome-c is an expanded, random coil at pH2. The center panel shows the SAXS pattern from protein just after the pH jump has occurred. Due to the finite extent of the x-ray beam, figure 3b averages over a time interval $t=150-500$ microsecond where $t=0$ is the time at which the pH reaches the renaturation threshold. The appearance of a peak in fig 3b indicates that collapse occurs on this short time scale. Careful analysis and fitting of this curve, the dashed line in the figure, in conjunction with the time constants that were determined by the fluorescence measurements previously described, yields an R_g of 18.1 ± 0.9 Å. This ‘compact denatured’ state has an R_g 30% larger than the native value of 13.9 ± 0.5 Å. [3]. At the end of the exit channel (to the extreme right in figure 1), 10 msec later in time, the scatter from the protein is indistinguishable from that of a much larger sample of native protein: the protein has folded. Data from the larger, static sample is shown as the smoother curve in figure 3c. For cytochrome-c, these experiments show that successful, rapid folding occurs in two steps. Collapse to a compact denatured state is followed by rearrangement to the native/folded state.

Work continues

This work on cytochrome-c demonstrates the potential of this technique for obtaining structural information on short time scales. In the past year, we’ve redesigned the flow cell to provide more rapid, uniform mixing. New feedback elements have been brought on-line at CHESS, in response to our need for small, stable beams. The planned upgrades to the beamlines, notably the addition of G-line, should sharpen our time resolution by two orders of magnitude, and will allow time-resolved SAXS to be performed on the msec time scale.

Over the past two years our experiment has grown more sophisticated. Components are built and assembled long before we are given the ‘keys to the hutch’. Still, I’ve never quite recovered from the first five days I spent on the floor at CHESS. I remember packing the delicate flow cell and its components into the van, preparing to return to our lab. As we left the CHESS parking lot I noticed an empty box on the seat. Panic struck. What had we left behind? I checked the label frantically. The content of the box had not been forgotten, we had simply used it up. The large blue letters on the side of the box read: “duct tape”.

Acknowledgments

I gratefully acknowledge the hard work of all of the collaborators on this experiment: Mark Tate, Nicholas Darnton,

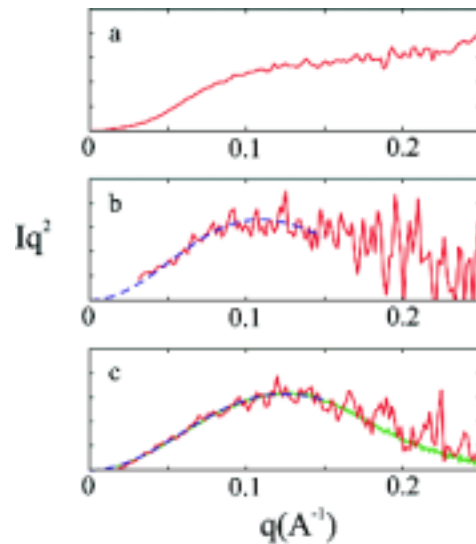


Figure 3: Iq^2 (arbitrary units) vs. q at three different times. a) before the pH jump b) 150-500 microseconds after crossing the renaturation threshold in pH c) 10 msec after the pH jump. The appearance of a peak between a) and b) indicates that the expanded chain has collapsed to a compact structure on this time scale. The shift of the location of the peak between b) and c) indicates rearrangement to the native state (lower R_g). A plot showing the scatter from a much larger (static) sample of native protein is plotted as the smoother curve on top of the data from the device in part c).

James Knight, Sol Gruner, William Eaton, Robert Austin, Martin Novak, the CHESS staff (especially Ernie Fontes), the staff of both the Cornell Nanofabrication Facility and the Developmental Resource for Biophysical Imaging, and Opto-Electronics at Cornell.

Reference

- [1] Eaton, W.A. *Proc. Natl. Acad. Sci.* **96**, 5897-5899 (1999)
- [2] Shastry, M.C.R. and Roder, H. *Nature Structural Biology* **5**, 385-392 (1998)
- [3] Pollack, L., Tate, M.W., Darnton, N.C., Knight, J.B., Gruner, S.M., Eaton, W.A. and Austin, R.H. Compactness of the Denatured State of a Fast-Folding Protein Measured by Submillisecond Small-Angle X-ray Scattering. *Proc. Natl. Acad. Sci.* **96**, 10115-10117 (1999)
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