Early stages of protein folding studied by sub-millisecond mixing

Adam Drysdale, Hau Lu, Graham Spence, Alastair Smith and Sheena Radford

Introduction

Understanding the mechanisms of protein folding has been one of the key questions in structural biology over the past 30 years and remains one of the most significant challenges in science today. Over the last few years, the folding transitions of various proteins have been studied using a variety of approaches, including stopped flow and quench flow methods, which have led to insights into the conformational properties of partially folded intermediates and rate limiting transition states. However, the key issue in folding - *how the amino acid sequence of a protein encodes its final structure* - remains unresolved. The problem stems in part from the speed of most folding reactions. In general, intermediates containing native-like topology form within the dead time of even the fastest stopped flow spectrometers (2-3 ms), so that the mechanism of their formation cannot be monitored. Major questions remaining in folding are how the amino acid sequence collapses, in some cases to populate relevant folding intermediates, and the nature and importance of these very early stages in protein folding. In Leeds we have constructed and characterised a continuous flow mixer that is able to probe folding reactions with rate constants between 10000 s^{-1} and 250 s^{-1}. In conjunction with stopped flow techniques, this allows us to follow folding reactions over time scales of many orders of magnitude.

Mixer design

Several different mixer designs with varying degrees of complexity have been reported in the literature. We are in the process of constructing and testing several designs but our most successful so far is based on a T-mixer by Takahashi *et al*. The two 'reactants', in this case protein in chemical denaturant and buffer are mixed by impingement at the entrance to a 250 x 250µm flow cell, the subsequent refolding dynamics are observed as a function of time (distance) along the channel (see Fig. 1).

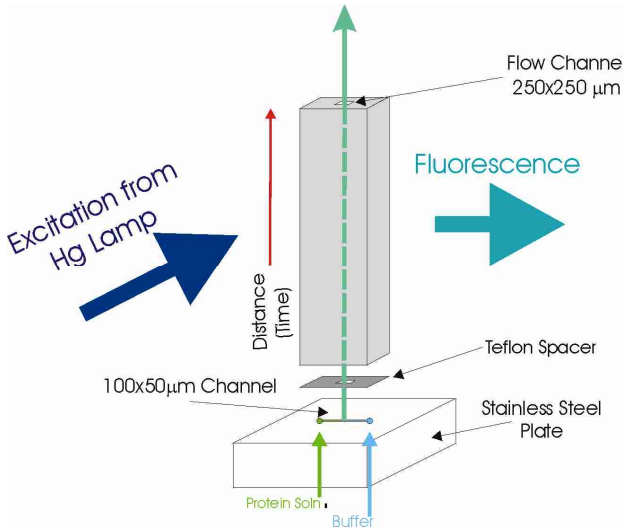

Figure 1 Schematic of the T-Mixer. Protein and buffer solutions are mixed together in a 1:10 ratio and fluorescence intensity is monitored as a function of time (distance) along the flow cell.

The protein folding dynamics after mixing are measured by intrinsic tryptophan fluorescence excited by a UV lamp. This excitation is distributed along approximately 20 mm of the mixing capillary and the fluorescence signal is collected as a single image (see Figure 2) by a CCD camera. Fluorescence emission is selected by either a bandpass filter or a monochromator. Future development of the system using the monochromator will permit the full emission spectrum at each point in the mixing capillary to be recorded rather than simply the intensity of a single wavelength.

We are currently developing several different mixer designs that will, a) have lower back pressure with the same mixing dead time and, b) be much easier to construct and dismantle for cleaning - a major drawback of the Roder design.

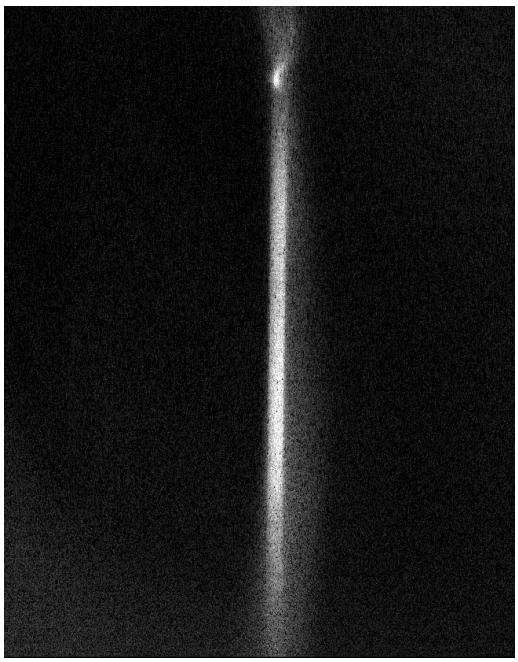

Figure 2 Typical CCD image of the mixing capillary showing NATA fluorescence intensity as a function of time.

Evaluation of mixing efficiency

The mixing dead time has been evaluated by using an NATA-NBS quenching reaction. Figure 3 shows the typical NATA fluorescence intensity as a function of time (i.e. distance down the mixing cuvette) for four different NBS concentrations. The NATA fluorescence intensity is normalised against background (water-water) and control (NATA-water) experiments. The point of convergence of the fits (solid lines) corresponds to the time of complete mixing of the reactants, and therefore the dead time can be calculated from the time interval before the first observable data point. In the case of this mixer design the dead time is measured to be ~155 ± 10μs.

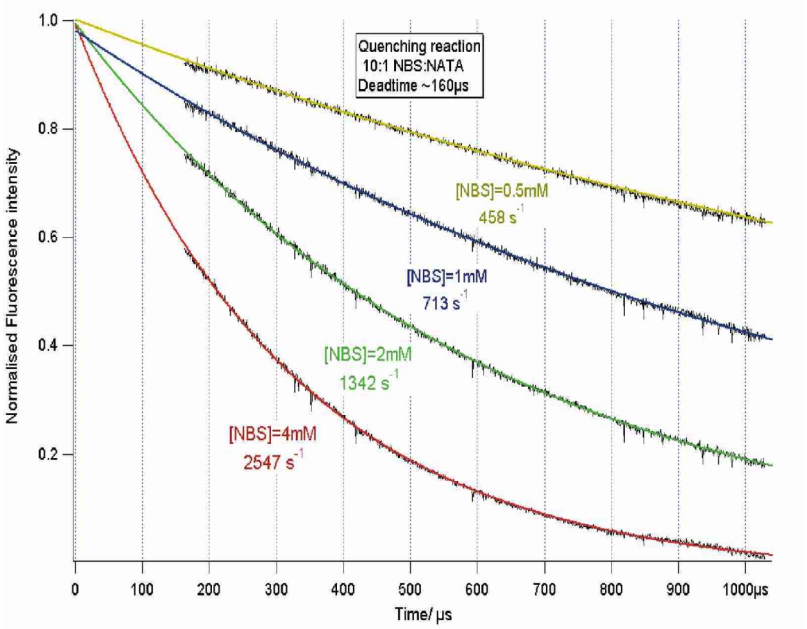

Figure 3 : Dead time calibration of the mixer. (a) Plot of the fluorescence quenching reaction of NATA by NBS at different final NBS concentrations. $[NATA]_{final}$ = 20 μM. (b) The solid line is a fit to the plot of the pseudo-first-order rate constants as a function of NBS concentration. This yields the second-order rate constant for the NATA-NBS reaction of 6.8 $x10^{-5}$ M^{-1} s^{-1}.

The first order rate constant of this quenching reaction is plotted as a function of NBS concentration which yields the second-order rate constant for the NATA-NBS reaction, which in this case is measured to be 6.8 $x10^{-5}$ M^{-1} s^{-1}, in good agreement with the literature value of 7.3 $x10^{-5}$ $M^{-1}s^{-1}$.

Protein system

It is our intention to study first the fast phase of folding of Im7. Im7 is a small four-helix bacterial immunity protein that contains no cysteines or *cis* prolines. Stopped flow experiments have shown that Im7 folds rapidly to its native state (k_{obs} = $300s^{-1}$). The presence of a missing amplitude in the stopped flow data indicates that there is a burst phase and hence

a populated intermediate within the experimental dead time. Without knowing the rate of formation of this intermediate both an on-pathway and off-pathway model can be fit to the data. Capaldi *et al*. showed that with the use of a sub-millisecond mixer it was possible to measure these fast rate constants and hence show that the data fits better to an on-pathway model. We have also prepared various mutants of Im7 with stabilising and destabilising residues being placed at various points in the protein. We therefore plan to perform a detailed sub-millisecond phi analysis of the folding of Im7 in the near future.

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