

# Single molecule spectroscopy to probe folding of individual proteins

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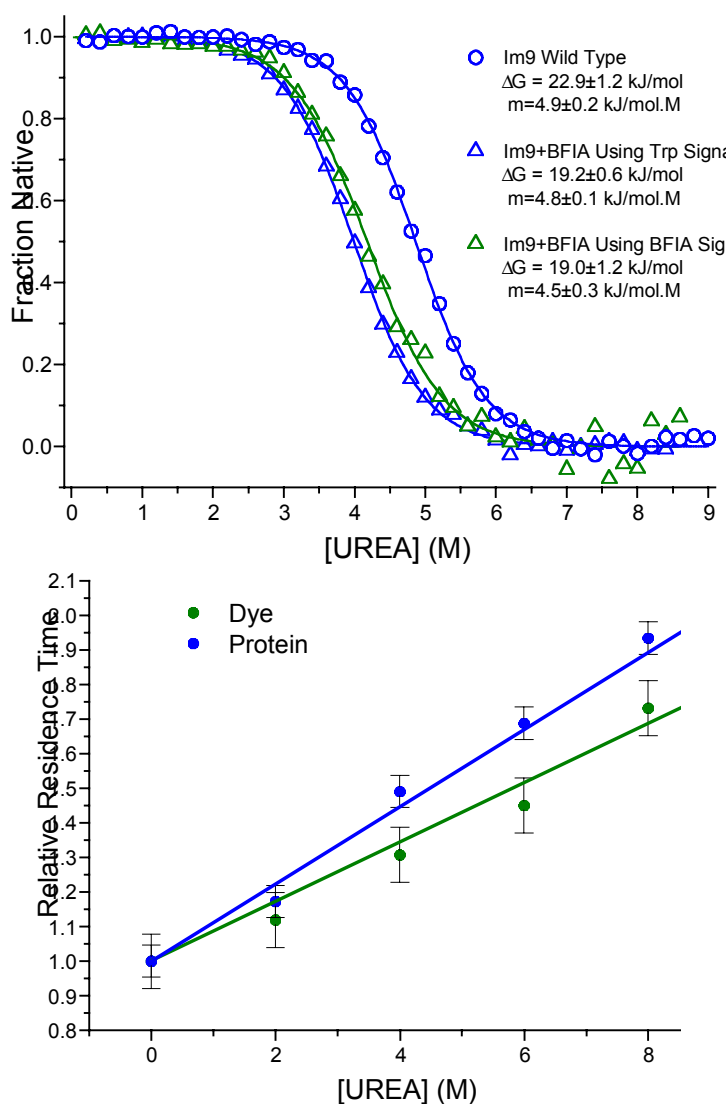
## Introduction

Spectroscopic measurements of bulk sample give only an ensemble-averaged measure of a molecular property. The signal obtained includes a contribution from a variety of different conformers and local environments within the sample. In order to study the heterogeneity of systems it is necessary to remove ensemble effects by examining such systems as single, isolated members. In Leeds we have constructed and characterised an ultra-sensitive inverted fluorescence confocal microscope to study individual protein folding events. Work since last year has concentrated on understanding new and emerging single molecule methods, specifically single molecule fluorescence correlation spectroscopy (smFCS). We have demonstrated our ability detect the fluorescence from individual molecules and carry out this analysis and have begun to apply this method to a well studied protein system which we have labelled with a fluorescent marker.

## Protein system

The most problematical area for the study of single molecule folding is in the choice of system. Labelling with an extrinsic, visible wavelength, fluorescent dye is essential as single molecule methods to detect UV fluorescence from Trp are not yet possible. The specific dual labelling of a large proteins for fluorescence resonance energy transfer (FRET) based studies is a formidable task. To circumvent this problem we have been looking into the possibility of studying a singly labelled protein. Here labelling is more straightforward as a single dye type may easily be attached to a single Cys present in (or mutated into) the protein. We have chosen the small 4-helix protein Im9 which has been extensively studied within the group of Sheena Radford at Leeds. This protein contains a single surface exposed Cys which we have labelled with the fluorescent molecule BODIPY F1 CASE. We have characterised the wild-type and label-mutant using kinetic and equilibrium methods. Figure 1(a) shows 3 equilibrium unfolding curves for Im9.

**Figure 1** Upper panel: the ensemble unfolding of the labelled protein system studied along with data for the wild-type form. Lower panel: single molecule FCS analysis of data for the labelled protein and for a simple dye system.



As can be seen, unfolding followed by the extrinsic dye molecule fluorescence follows that monitored by the intrinsic Trp for the label-mutant, while both are destabilised compared to wild-type unfolding followed by Trp. The dye molecule provides not only a marker of protein position, but also provides a way of monitoring the folding. It is these properties that enable us to exploit single molecule methods for the study of this protein.

### **smFCS**

smFCS measures the rate of diffusion of an analyte in solution. Due to the single molecule nature of the method, it is capable of differentiating between heterogeneous populations in solution as long as these solutions are defined by sufficiently different diffusion coefficients. Figure 1(b) shows the relative diffusion time of a small dye molecule (green) as a function of urea concentration in solution. Here a pseudo-ensemble experiment is conducted where data is from a single molecule sensitive experiment but a model assuming only one species is fit. The relative diffusion time (or residence time) increases as the urea concentration increases. For the dye, the data is fit well by a model accounting for the change in viscosity of the solution (not shown). Also in Figure 1 is the same data for a solution of labelled protein (blue). Two lines showing the maximum and minimum slopes of the data sets are shown as guides to the eye. It can be seen that the rate of change of diffusion time of the protein is greater than for the dye, which we infer to indicate the unfolding of the protein (diffusion time changes faster than the change which would be expected from solely a change of solvent viscosity).

### **Conclusions**

These preliminary results show the potential of the method to resolve the two species (folded residence time is shorter than unfolded), however it also suggests that the change is too small to resolve the two species from a single data set. Early attempts to fit the data to a model for two species developed by others has failed to produce physically meaningful results. However, problems with the model have been discussed elsewhere and we are in the process of re-fitting the data. Indications are that the difference is too small to resolve accurately. Further work is under way to examine the Im9 system under different unfolding conditions (i.e. unfolding in a stronger solvent to try to amplify the hydrodynamic radius difference of the folded and unfolded species). In addition to smFCS other methods which exploit the brightness difference of the protein conformations are being explored.

### **Collaborators**

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### **References**

Gell C., Brockwell D.J., Beddard G.S., Radford S.E., Kalverda A.P. & Smith D.A. (2001) Accurate use of single molecule fluorescence correlation spectroscopy to determine molecular diffusion times. *Single Molecules* **2**, 177-181.

### **Acknowledgements**

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