Ultraviolet resonance Raman spectroscopy in protein folding investigated by microsecond mixing

Iñigo Rafael Rodriguez, Alastair Smith and Sheena Radford.

Introduction

The problem of understanding how a protein folds from an extended polymer chain in solution to a functional three dimensional structure is one of the key challenges in structural molecular biology today. One of the barriers to understanding this process has been the time scale on which experiments could be performed. Mixing of two solutions has traditionally been used to initiate refolding and conventional instruments have dead times of the order 2-3 milliseconds, within which time most folding reactions have already begun. In Leeds we have constructed and characterised a continuous flow mixer that is able to probe folding reactions *by fluorescence* with rate constants between 10000 s^{-1} and 250 s^{-1}. Whilst fluorescence spectroscopy is able to provide kinetic rates of folding, it is not, in general, able to provide detailed structural information about conformational changes. In the Department of Physics and Astronomy in the University of Leeds we are therefore developing an ultraviolet resonance Raman (UVRR) probe to be combined with the mixing technology we have developed. There is almost no published UVRR data on protein folding on a sub-millisecond time scale, but the combination of this powerful technique with rapid mixing is capable of providing a detailed picture of the conformational changes during folding.

UVRR and ultra-rapid mixing

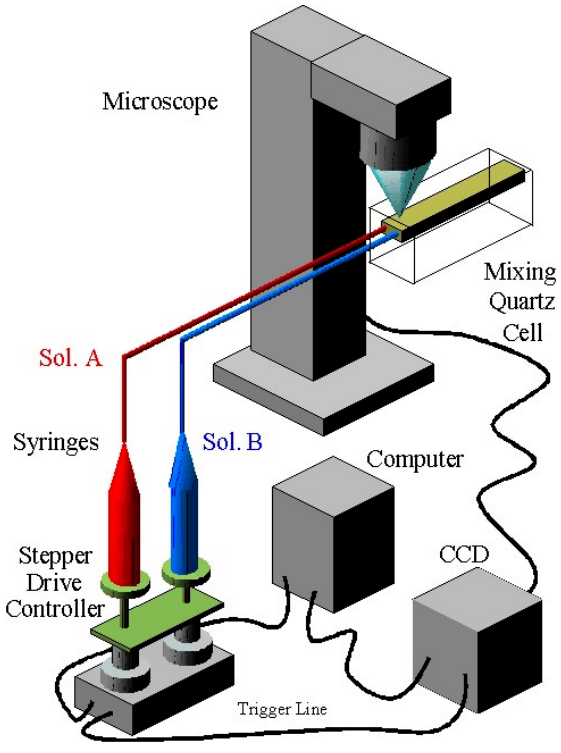

Fig. 1 The experimental setup.

Infrared spectroscopy and ultra-rapid mixing can provide information on the secondary structural elements within the protein as it undergoes folding. UVRR spectroscopy can provide similar information without the same level of interference from water. In addition, at the ultraviolet wavelength that we are using (244 nm), we avoid fluorescence from natural chromophores (tyrosine, tryptophan and phenyalanine) and we obtain information about the non-covalent interactions of the aromatic residues tyrosine and tryptophan in the protein. The technique therefore is most suited to proteins with single (or very few) such residues.

A schematic of the apparatus is shown above. Pneumatically driven syringes deliver the denatured protein solution and the buffer via HPLC plumbing to the T-mixing plate. Then the completely mixed solution is injected into the 250µm quartz flow cell, which is illuminated by the beam laser through the microscope. The UVRR spectra are collected by the microscope objective and steered to a commercial UV Raman spectrometer (Renishaw plc UV Raman System). The UVRR mixing experiment captures spectra from single points along the mixing capillary (see schematic opposite). Different points along the capillary probe different times after the initial mixing of the denatured protein and the buffer. With high resolution position of the capillary we are able to obtain UVRR spectra from points along the folding pathway that are separated by a few tens of microseconds.

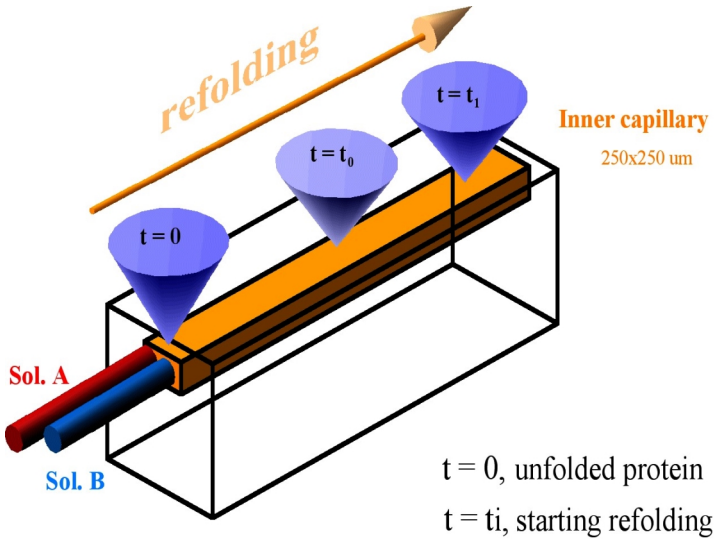

Fig. 2 After rapid mixing, the refolding protein flows down a narrow capillary. Thus, the distance along the capillary represents the time after mixing as a function of flow speed.

The protein systems

Horse heart cytochrome c, horse myoglobin and the immunity protein, Im7 are the proteins chosen for this study.

Cytochrome c and myoglobin will provide an excellent test of the conformational and temporal resolution of our instrument. They are well-characterised proteins that have been studied extensively, and which are available commercially at low cost. They are therefore ideal model systems of protein folding for developing our apparatus. Cytochrome c contains only one Trp residue and four Tyr residues and myoglobin contains two Trp and two Tyr residues making these two proteins ideal candidates for developing UVRR for monitoring protein folding.

Im7 adopts a simple fold that can be considered as a distorted antiparallel four helix bundle and contains a single Trp residue, which is located in the core of the native protein. Ultra-rapid mixing and UVRR will provide detailed structural information to compliment the fluorescence kinetic data from the mixing apparatus that are already being studied. UV Raman analysis of the burst phase of Im7 will allow probing of the ground state properties of the single Trp and will allow the nature of the solvent exposure of the Trp residue to be directly probed during the burst phase.

Acknowledgements

We would like to thank the BBSRC for funding.