

UVRR studies of fast protein folding initiated by microsecond mixing

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Introduction

The question of how a polypeptide chain can spontaneously fold into a highly ordered three-dimensional structure is of fundamental importance to many areas of biochemistry, molecular biology and biotechnology. The structural and kinetic information of the early events occurring on the sub-millisecond time scale in the folding of a polypeptide along its folding pathway can provide valuable information key to understand how a protein folds. Ultraviolet resonance Raman spectroscopy (UVRR) is a powerful technique to study the aromatic aminoacids tryptophan and tyrosine. Structural and conformational information of these important residues is provided by UVRR: the degree of hydrogen bonding of both tyrosine and tryptophan; the relative orientation of the tryptophan side-chain; and additionally, the degree of solvent exposure of the tryptophan and tyrosine residues can be obtained. Thus the local environment of tryptophan and tyrosine residues can be monitored in detail during the early stages of folding when combined UVRR spectroscopy and ultra-rapid mixing technology are used.

Integration of the microsecond mixing and the UVRR system

The ultra-rapid mixing apparatus we have developed is detailed in Fig. 1. The instrument has a 'T' shape mixer design built within a 45 mm quartz cell. It consists of a 200 μm diameter channel that joints perpendicularly with the 200 x 200 μm observational channel. A stainless steel wire introduced in the 200 x 200 μm inlet channel reduces the cross-section of the channel in order to obtain high flow velocities in the mixing region and confines the mixing chamber to a minimum. Syringe movements are controlled by a PC controlled stepper motor driver capable of working at different linear motor speeds. The mixing cell is mounted beneath the objective of the UVRR system on a precision translation stage. The progress of the mixing reaction is followed downstream from the mixer along the flow direction, which is translated into time according the flow rate and dimensions of the observational channel.

UVRR spectra are obtained with a commercial Raman microscope, a Renishaw RM 1000, adapted for operation with deep UV laser wavelengths (229 and 244 nm laser excitation provided by an intra-cavity frequency-doubled argon ion laser). The laser beam is directed via steering mirrors onto the first Rayleigh rejection filter, this reflects the laser light into the microscope and down on to the mixing cell. Backscattered light from the sample is collected and collimated by the objective, directed through the Rayleigh rejection filter which transmits most of the Raman scattered light on to the diffraction grating before being redirected by a prism to the active surface of the CCD camera.

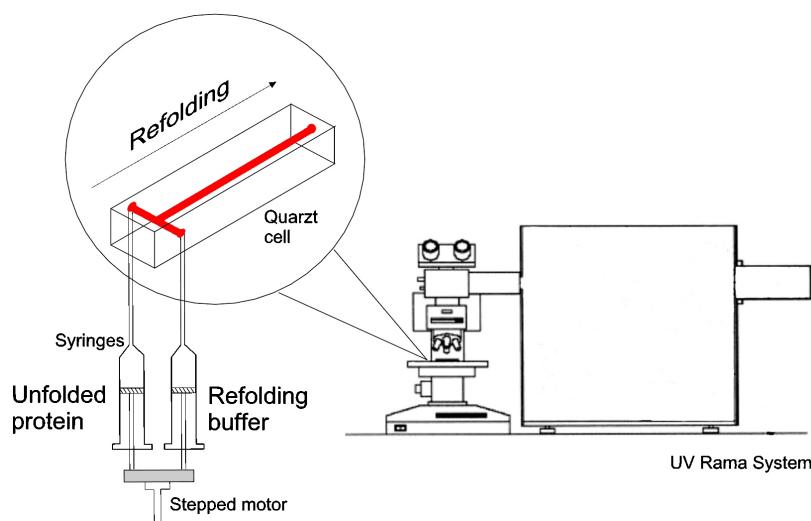


Figure 1: Continuous-flow mixing apparatus and the UVRR system.

The time resolution of a fast mixing device is determined by the instrumental dead time, which depends critically on the time required to achieve complete mixing of the two reagents, the flow velocity, and the volume between the mixing region and the point of observation. The mixing efficiency was determined with the Raman spectral change caused by protonation of imidazole (Im) to imidazolium (ImH^+). The Raman bands of Im at 1260 and 1326 cm^{-1} are definitely distinguished from those of imidazolium at 1216 and 1457 cm^{-1} . When Im is mixed with citric acid, the UVRR bands of ImH^+ appears at 1457 and 1216 cm^{-1} , and simultaneously, the two strongest Im bands at 1260 and 1326 cm^{-1} became weaker. The kinetic spectra plotted in Fig. 2 show the mixing dead times achieved mixing equal volumes of Im and citric acid at 0.4 and 0.5 ml/s flow rate (Figs. 2A and B respectively), and the dead times observed mixing 1/10 volumes of Im and citric acid at 0.5 and 0.7 ml/s (Figs. 2C and D).

UVRR indicates that the environment of Trp75 is non-native in partially folded variants of Im7* at equilibrium.

In order to study the conformational properties of the on-pathway folding intermediate of Im7* in more detail and, in particular, the origins of the non-native interactions that stabilise this species and give rise to its unusual and characteristic hyper-fluorescence, a series of Im7* variants that closely resemble the kinetic intermediate were designed. The UVRR data presented in Fig. 3 cast new light on the structural properties of the partially folded states of Im7*, particularly in the environment of the single tryptophan residue that gives rise to the unusual fluorescence properties of these species. The UVRR data clearly indicate that the underlying structural changes induced by the mutations in the Im7* variants, L53AI54A, H3G6 and YY, result in an increase in the hydrophobicity of the environment local to Trp75 compared with wild-type Im7*. The increase in fluorescence emission intensity reported for these variants compared with the native state of wild-type Im7* is consistent with these data. Our current time resolved fluorescence measurements together with previous fluorescence quenching data indicate a decrease in the overall solvent accessibility of Trp75 in the variants L53AI54A and YY, whilst the accessibility of Trp75 in the variant H3G6 remains close to that of wild-type Im7*.

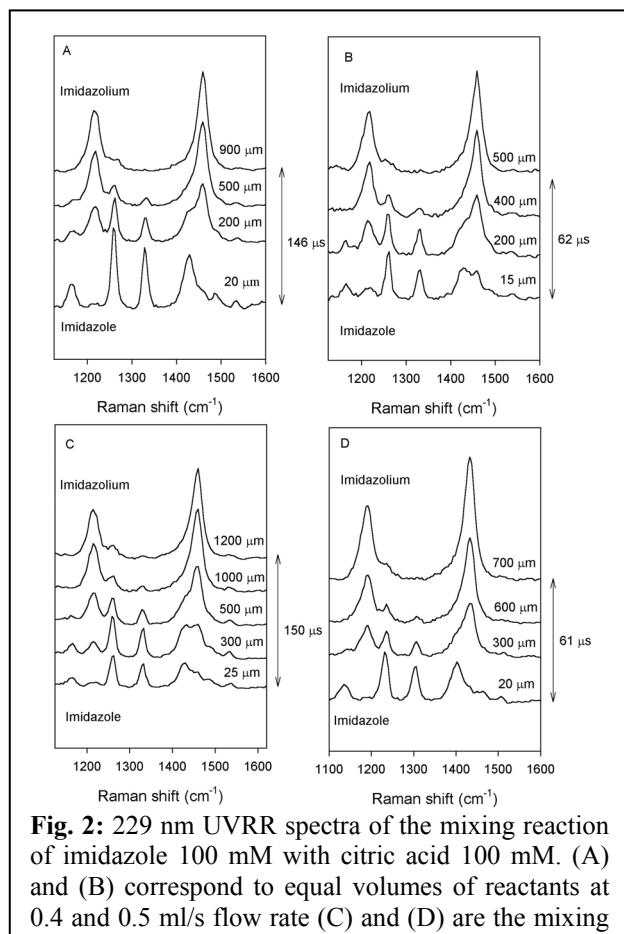


Fig. 2: 229 nm UVRR spectra of the mixing reaction of imidazole 100 mM with citric acid 100 mM. (A) and (B) correspond to equal volumes of reactants at 0.4 and 0.5 ml/s flow rate (C) and (D) are the mixing spectra obtained with 1/10 dilution of Im in citric acid with 0.5 and 0.7 ml/s. Dead times are calculated from the distance between the appearance of imidazole and imidazolium bands and the mean flow velocity of the solution in the observable channel.

UVRR is known to be a sensitive probe of the solvent accessible surface area of tyrosine and tryptophan residues in proteins - the lower the area exposed, the greater the Raman band intensities are expected to be. The UVRR data indicate an increased hydrophobicity local to the indole ring in all of these variants. The UVRR studies presented here, therefore, indicate that a newly formed hydrophobic environment is a shared property of all of the variants of Im7* studied and reveal that subtle changes local to the environment of these residues can lead to significant changes in their fluorescence properties. These data substantiate the results of ϕ -value analysis by providing direct evidence that the environment local to Trp75 in partially folded Im7* is both non-native and hydrophobic in nature.

Future Work

We will now examine the evolution of the intermediate populated transiently during folding of wild-type Im7* combining UVRR spectroscopy with ultra-rapid mixing. Structural and environmental properties concerning the side chains of the aromatic residues tryptophan and tyrosine will be obtained from this intermediate and compared with our present UVRR data at equilibrium.

Publications

Rodriguez-Mendieta, I. R., Spence, G. R., Gell, C., Radford, S. E. and Smith, D. A. (2005) Ultraviolet resonance Raman studies reveal the environment of tryptophan and tyrosine residues in the native and partially folded states of the E Colicin-binding immunity protein Im7. *Biochemistry*. In press.

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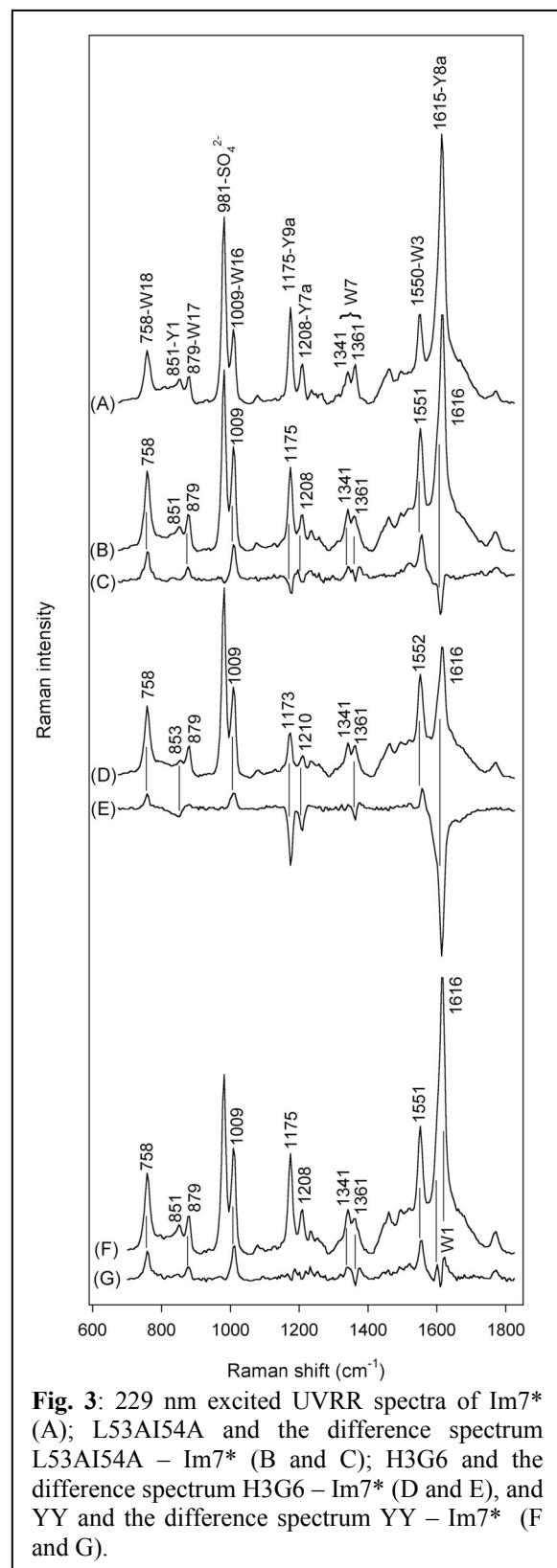


Fig. 3: 229 nm excited UVRR spectra of Im7* (A); L53AI54A and the difference spectrum L53AI54A – Im7* (B and C); H3G6 and the difference spectrum H3G6 – Im7* (D and E), and YY and the difference spectrum YY – Im7* (F and G).