ESRF	**Experiment title:** Time-resolved WAXS studies of the folding/unfolding process in small proteins in solution	**Experiment number**: SC-3144
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Aim of experiment.

The aim of the experiment was to investigate global and local changes during unfolding process triggered by nanoseconds (*ns*) infrared laser flash on several solutions of the small protein Engrailed Homeodomain (EnHD, PDB accession code: 2JWT) both wild-type (WT-EnHD) and mutated ones. In order to enhance the signal to noise ratio, the mutated proteins were labelled with mercury at specific points. An infrared laser flash induced a ns temperatur jump of 8-11°C which caused the proteins in the solution to unfold. In Fig 1, the melting curve of the WT-EnHD is shown. The protein mutants should have a melting temperature lower than the wild type due to instability of the peptide chain. We focused on the time-resolved X-rays scattering measurements in time-scales (μs-ms) relevant for the unfolding process in WT-EnHD. We wanted also to prove the existance of intermediate state between folded and unfolded states and to develop the method of *ns* temperature jump trigger by laser flash.

Experimental

The samples were infused in a quartz capillary and were continuously flowed in both directions during one single set of measurements. The *ns* infrared excitation was delivered by our portable *ns* infrared laser. The dedicated time was split between several samples: WT-EnHD (which took the most of the time), three mutated EnHD mercury labelled on specific cystein sites and one mutated EnHD not labelled. Short time was spent on the measurement of the heating signal on the buffer solution so that such a signal can be filtered out from the protein solution signal. The unfolding process is reversible and X-ray radiation damage is

neglectable (less than 0.1% protein molecules per cycle of pumping) which allowed us to utilize the same sample for several repeats (up to 10 times until protein radiation damage became visible, which was checked by control experiment). The measurements were carried out in the *q*-space between 0 and 2.6 $Å^{-1}$ where signal from both protein and water could be collected. The water signal arising at about 2-2.6 $Å^{-1}$ was used as an internal thermometer to estimate the temperature jumps. In the mutants we noted that after a few minutes of measurements the labelled proteins started to aggregate due to the possible removal of Hg and successively binding between two sulfur of the cystein. Such effect was also visible on the mutated unlabelled protein. Measurements on the WT-EnHD were on the other hand quite succesfull: highlights of the best results, after having filtered out the heating signal, are shown in Figure 2. In the analysis based on the differential scattering and MD simulations we have been able to draw conclusions on the kinetic of the unfolding process in term of volume and radius of gyration of the protein. A manuscript presenting this work is under preparation.

Technical Problems

The infrared laser was able to increase the solution temperature by 8-11°C from the initial temperature (21.5°C). The midpoint temperature, defined as the temperature where fractions of folded and unfolded molecules are equal, is 53°C with major changes in structure laying in equidistant interval of 15°C (see Figure 1). It means that in current conditions we were able to unfold only a few percent of the protein (3-4.5% of excitation). The same temperature jump close to the midpoint would have induced up to 40% of excitation. Unfortunatelly the requested temperature control system (cryostream) could not run more than few minutes of operation due to clogging of the cryo line. So the data was not collected at the optimal temperature but nevertheless we got a sufficient change in scattering to be able to draw conclusions about the folding time and to some extent also the folding path.

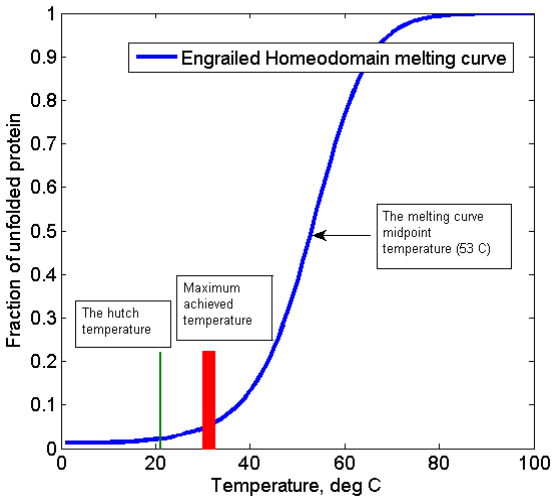

Figure 1: WT-EnHD melting curve with experimental temperatures on the same plot.

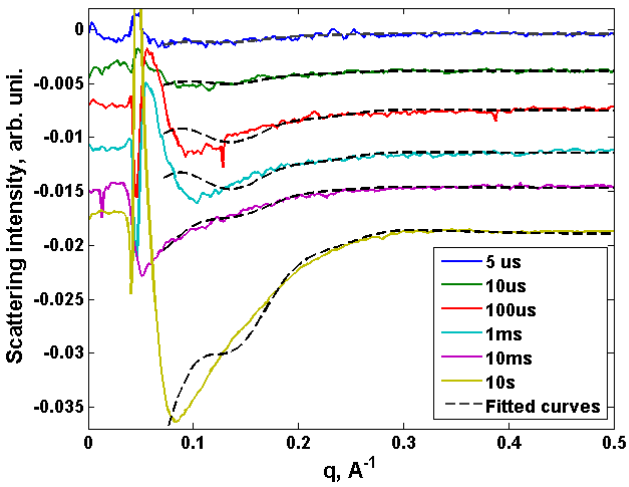

Figure 2: Refined protein differential scattering components.