Experimental Report

Proposal: **1-20-33** **Council:** 10/2012

Title: Feasibility studies of freeze-trapping of structural intermediates inproteins using cryo neutron crystallography on instrument D19

This proposal is a new proposal

Researh Area: Methods and instrumentation

Main proposer: **MOSSOU Estelle**

Experimental Team: BLAKELEY Matthew
ROMOLI Filippo

Local Contact: FORSYTH Trevor
MASON Sax Anton

Samples: Trypsin
Elastase
Lysozyme

Instrument	Req. Days	All. Days	From	To
D19	10	5	29/05/2013	30/05/2013
			30/07/2013	02/08/2013

Abstract:

We propose to use the recently tested D19 cryostream system to investigate the possibility to cryo-trap structural intermediates in enzyme reactions. Although cryo-trapping is a commonly-used technique in X-ray crystallography, as far as neutron crystallography is concerned it would be amongst the first studies of this type. Neutron diffraction would bring crucial insights into such biomolecular processes, allowing hydrogen transfer and protonation states to be observed. Intermediates of systems like Xylose Isomerase have been studied using D19 and allowed the determination of hydrogen transfer mechanism during the reaction. This study was carried out at room temperature and necessitated the use of several compounds representing each stage of the reaction. The development of cryo-trapping in neutron crystallography would open-up huge avenues for future college 8 proposals. We therefore suggest using a series of model enzyme-systems known to grow large crystals suitable for neutron diffraction, to develop a reliable method for cryo-trapping using D19. Given that it is driven by technical issues rather than biological, this proposal clearly falls within the remit of College 1.

Experimental report for ILL proposal 1-20-33

Feasibility studies of freeze-trapping of structural intermediates in proteins using cryo neutron crystallography on instrument D19

E. Mossou[a, b], F. Romoli[c], M.G, Cuypers[a, b], M. Blakeley[b], S.A.Mason[b], S. McSweeney[c], V.T. Forsyth[a, b].
[a] EPSAM/ISTM, Keele University, Staffordshire, ST5 5BG, UK.
[b] ILL, 6 rue Jules Horowitz, 38042 Grenoble, France
[c] ESRF, 6 rue Jules Horowitz, 38042 Grenoble, France

Introduction

On the new D19 diffractometer the success of the experiment 1-20-18 (Cuypers et al) using the cryostream system, collecting data from a macromolecular crystal at atomic resolution, opened new avenues for the development of cryo-cooled experiments. This result gives the opportunity to reach higher resolution limits for neutron macromolecular crystallography and the opportunity to perform structural analysis on reaction intermediates. In order to perform freeze-trapping experiments, the hydrolysis reaction of the amide bond catalyzed by trypsin directly in crystals, was studied through the use of the substrate succinyl-Ala-Ala-Pro-Arg-p-nitro-aniline. The deacylation reaction for trypsin is the rate-limiting step when the substrate is saturating, so under this condition it is possible to trap the enzyme-substrate complex in the acylated state. The compound has been chosen for its high affinity to the active site and the saturating concentration will ensure the presence during the turnover. Soaking and flash freezing protocols were developed to trap the intermediate state before the compound will be removed from the active site through diffusion processes. Data collection has been performed at cryogenic temperature (100-130K) on a 5 mm^3 trypsin and diffraction images were obtained up to 3 Å resolution.

In contrast to previous experiments, where quartz was used to mount crystals, here we developed a novel mounting system that is more suitable to perform data collection using the cryostream. This will ensure a more reproducible approach to perform experiments on D19 diffractometer, a better stability of the crystal under the flux of the cryostream and a sample mount that is almost transparent to neutrons. The novel mount is carbon based and does not contain any material which is prone to significant radioactive activation. Test images were collected at cryogenic temperature (100-130K) on hydrogenated trypsin crystals with a volume of 5 and 8 mm^3, on perdeuterated *Pyrococcus furious* rubredoxin crystal of 1 mm^3 and on hydrogenated lysozyme crystal of 1 mm^3 volume.

Crystals soaking and cryoprotectant preparation prior to cryo freezing

The large hydrogenated trypsin crystals were soaked in deuterated solution of 2.4M $(NH_4)_2SO_4$, 3mM CaCl, TRIS/HCl pD 8.1 buffer (exchanged 3x with D_2O rotative evaporation) and 70 % (w/v) of D6- glucose for cryoprotection. Lysozyme crystal was soaked in deuterated solution of 0.8 M NaCl, 0.1 M $Na/C_2H_3O_2$ pD 4.2 buffer (exchanged 3x with D_2O rotative evaporation) and 30% (v/v) of D8-glycerol for cryoprotection. *Pf.* Rubredoxin cryatal was soaked into a solution of 90% 3,8 M Na/K_2 phosphate buffer (exchanged 3x with D_2O rotative evaporation) and 10% (v/v) D8 glycerol for cryoprotection. No crystals dissolution was observed. Suuccinyl-Ala-Ala-Pro-Arg-p-nitro-aniline was dissolved at a concentration of 3 M in DMSO. The stock solution was diluted in deuterated solution of 2.4M $(NH_4)_2SO_4$, 3mM CaCl, TRIS/HCl pD 8.1 buffer at a final concentration of 30 mM. Soaking was performed with 20 µl of solution for 30 minutes. The crystal was then cryoprotected performing step-wise soaking from a starting concentration of 15% (w/v) D-glucose to a final concentration of 70% (w/v) D-glucose in the

mother liquor. The crystals were mounted on the vitreous carbon mount and flesh frozen either plunging them directly into the liquid nitrogen or placing them in the cold jet of the cryostream.

Vitreous carbon loop mounting and analysis

The design of the carbon loop is done by AutoCAD and vitreous carbon heads are obtained through the use of a laser cutting machine from 60 µm thick vitreous carbon sheets (SIGRADUR® K). SPINE standard magnetic base is drilled to match the external diameter of a pure aluminium tube at 1.5 mm. The thin (127 µm) aluminium tube (Goodfellow SARL) is cut at the length of 1.5 cm and the profile from one side is shaped like a "V" to ensure a better positioning of the mount. The straight extremity of the aluminium tube is then glued into the adapted goniometer–compatible base using epoxy resin. A vitreous carbon loop is put in position on the top of the "V" shaped extremity of the aluminium tube and epoxy resin is applied carefully inside the tube, as far as possible from the extremity, in such a way that the glue will be at the furthest point from where the crystal will sit and from where the neutron beam will hit the sample. Crystals are easily retrieved thanks to the oval loop shape of the vitreous carbon mount and flesh frozen in liquid nitrogen.

Neutron diffraction images comparing this novel carbon mounts (figure 1a), a thin walled quartz capillary mount (figure 1b) and background air (figure 1c) were collected. The images

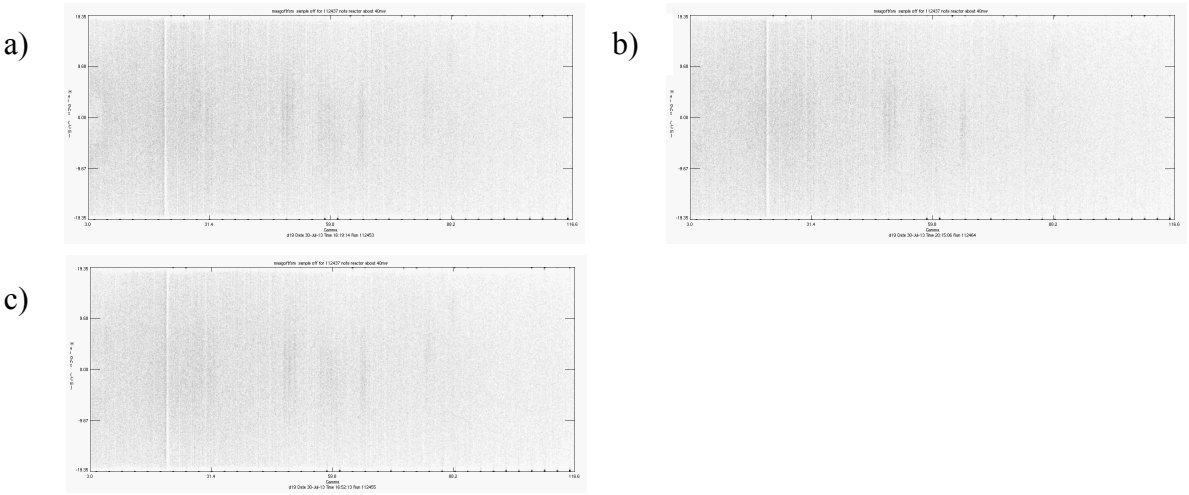

Figure 1 Comparison of neutron diffractograms (2.42 Å wavelength) from a) vitreous carbon loop (100s), b) thin walled (0.01mm) quartz capillary (100s) and c) air (100s).

display that there is no contribution in scattering from the vitreous carbon material, indeed no increase in diffuse scattering, nor Bragg reflections, are observed when the mount is placed and aligned with the neutron beam with respect to air background measured. The neutron diffraction image of a quartz capillary is also similar to the one of the vitreous carbon mount suggesting that the latter could be really comparable, in terms of diffraction properties, to the quartz capillary. The comparison of the scattering properties between vitreous carbon mounts and quartz capillary makes no distinction within the two materials, so assuming that the background scattering contribution remains the same, better handling and reproducibility properties makes the carbon vitreous mount more suitable for cryo-crystallography data collection.

Data collection and analysis

Neutron data collection was successfully performed on D19 diffractometer at 2.42 Å wavelength, on three different crystalline model samples. A 5 and 8 mm^3 trypsin crystals, with and without soaking of succinyl-Ala-Ala-Pro-Arg-p-nitro-aniline respectively, were mounted on the novel carbon loop pins and collected at 100K. Images were acquired for 1000s and showed maximum diffraction resolution around 3 Å (Figure 2). A trypsin crystal was collected also at

room temperature, showing again a resolution of about 3 Å. A lysozyme crystal of 1 mm^3 was mounted on a carbon loop and cryostream frozen. Images were collected for 1000s and diffraction resolution was 3.4 Å. A perdeuterated *Pf* rubredoxin crystal of 1 mm^3 was also mounted on the carbon loops and frozen in the cryostream jet. Images were acquired for 1000s and resolution was observed until the right edge of the detector at 1.38 Å. Depending on the amount of liquid present on the mount when the crystal was frozen the presence of ice rings could be observed hence the removal of the excess of liquid is preferred (figure 3).

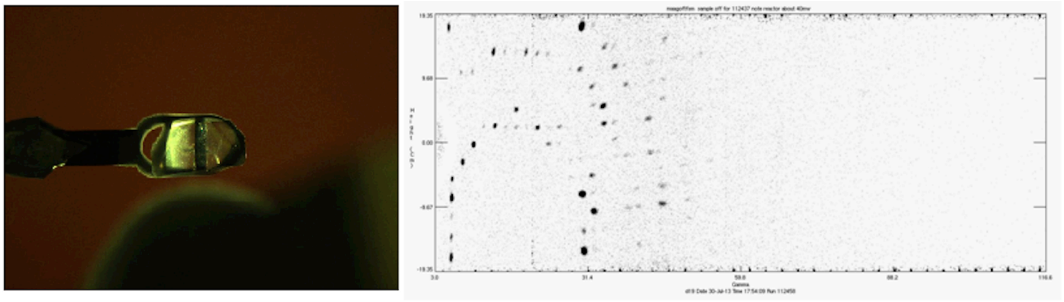

Figure 2 A 5 mm3 trypsin crystal mounted on a vitreous carbon pin and corresponding neutron diffraction image. Data collection was performed at 100 K on D19 single crystal diffractometer at ILL with at wavelength of 2.42 Å for a 1000s acquisition time

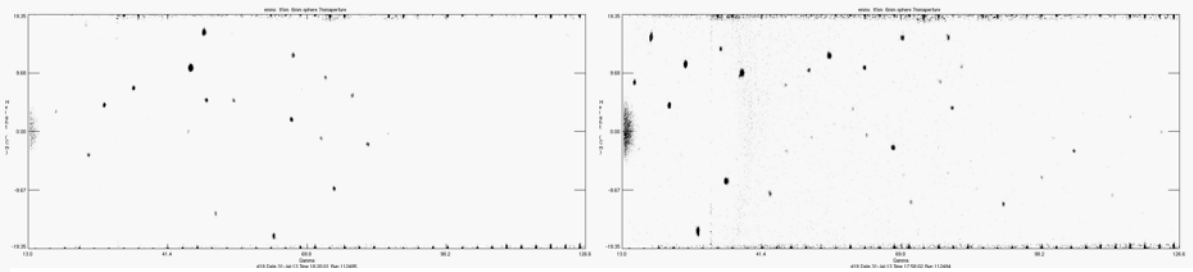

Figure 3 Neutron diffraction images from a perdeuterated rubredoxin crystal collected on a vitreous carbon neutron compatible mount at 100 k on D19 at wavelength of 2.42 Å for a 1000s. (left) the diffraction pattern do not present any icerings because the crystal was dried with a tissue before freezing. (right) the presence of icerinngs is detected when the excess of liquid is not removed.

Conclusions

The development of the new carbon based system for retrieving and collecting macromolecular crystals on D19 instrument provided to be extremely suitable for performing experiments at cryotemperature. The neutron transparency and the stability of the mounting system allowed us to collect data from several different systems without problem of background noise, without ice formation and with high reproducibility. The production of other loops will give the opportunity to use these mounts also on other systems and perform more experiments at cryotemperature, helping to solve the problem of crystal handling. Moreover they are compatible with X-ray data collection, allowing joint neutron and X-rays studies to be performed. We have collected images on three different macromolecular crystalline systems, allowing us to test this material using different conditions. Future experiments will include the use of this mounting system to test other crystals and collect entire data sets at cryotemperature using protocols to freeze-trap reaction intermediates that were developed.

NB. Please note that only 2 and half days of the 5 that were allocated for this experiment were used. The remaining 2 and half days could be rescheduled during the next reactor cycle to perform further experiment as described above.