# Experimental report

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**Proposal:** 8-01-458 **Council:** 4/2015

**Title:** Direct observation of hydronium and Zundel ions in proteins by neutron crystallography: a reverse labelling approach.

**Research area:** Biology

**This proposal is a new proposal**


**Main proposer:** Maxime CUYPERS

**Experimental team:** Trevor FORSYTH
Juliette DEVOS

**Local contacts:** Trevor FORSYTH
Sax Anton MASON
Estelle MOSSOU


**Samples:** Perdeuterated rubredoxin

| Instrument | Requested days | Allocated days | From | To |
|---|---|---|---|---|
| D19 | 16 | 11 | 19/05/2016 | 01/06/2016 |


## Abstract:

We request beamtime on D19 for cryogenic and ambient temperature neutron crystallographic studies of perdeuterated rubredoxin (Rb) in hydrogenated water. Previous neutron work on perdeuterated rubredoxin has revealed, at atomic resolution, a remarkable network of ordered water that includes hydronium ions. These observations prompted ultra-high resolution studies on D19 using cryo-cooled samples at 100K. The results, which were obtained to a resolution of 0.88A, were striking: the visibility of the water-hydronium network was greatly enhanced, and exotic species such as Zundel ions [seen as D5O2]+ were observed. Given the significance of these results we wish to carry out a crucial crystallographic verification of this network by reverse labelling the water around the protein with H2O. The key aspect of this study is that the hydrogen atoms will appear as negative peaks in the Fourier maps and allow an unambiguous verification of the water, hydrogen, hydronium and Zundel features that have been found using perdeuterated protein hydrated with D2O. This experiment will also allow an ultra-high resolution analysis of the subtle differences between H and D analogues of these species.

# Experimental report for ILL proposal 8-01-458

## Direct observation of hydronium and Zundel ions in proteins by neutron crystallography: a reverse labelling approach.

M.G. Cuypers[a,b], S.A. Mason[b], E. Mossou[b], J. Devos[b], E.P. Mitchell[c], M. Haertlein [b], V.T. Forsyth[a,b].

a) EPSAM/ISTM, Keele University, Staffordshire, ST5 5BG, UK. b) ILL, 6 rue Jules Horowitz, 38043 Grenoble, France. c) ESRF, 6 rue Jules Horowitz, 38042 Grenoble, France.

### Introduction

The precedent experiments 1-20-18, 8-01-389 and 1-20-16 provided outstanding results leading to the discovery of new structural and biological features for the first time on both the reduced and oxidised-Fe forms of *Pf* rubredoxin, namely hydronium ions and carboxylic deuterons. The ultra high resolution (better than 0.59 Å, pdb 5NW3) diffraction data obtained by X-ray at 100 K as well as the outstanding data obtained by neutron crystallography at 100K (0.59 Å resolution, deposited pdb 5NVT) motivated the neutron diffraction experiment at cryogenic temperature for the $H_2O$ exchanged crystal of this experiment, given the established capability of the D19 diffractometer to acquire high quality with this protein system.

### Results

We have successfully collected atomic resolution data on 7 $mm^3$ crystals of perdeuterated *Pf.* rubredoxin soaked in $H_2O$ on D19 at cryogenic temperature (100 K) in both reduced and oxidised-Fe crystal forms, respectively. A new methodology for crystal mounting shows several technical advantages while combining the use of a cryo cooling device with neutron diffraction.

The large *Pf.* rubredoxin crystals were soaked into a solution of 90% 3,8 M (final concentration 3,4 M) Na/K2 phosphate buffer (in H2O) and 10% Hydrogenated glycerol. No crystal dissolution was observed.

The crystal was then fished onto a Carboloop (see Figure 1) and placed on the cryostream regulated at 100K. (See experiment report 1-20-18 for a more complete description of the cryofreezing method.)

### Data collection and analysis

Neutron data collection was performed successfully on a 100K cryo cooled 7 mm3 perdeuterated oxidised Fe form *Pf* rubredoxin crystal on D19 for 10.5 days at 1.46 Å wavelength. Full neutron dataset was recorded using step scans of 0.07° with an exposure time of 92s per step, yielding data to 1,00 Å resolution. A complete dataset is available and is currently under refinement. The refinement proves challenging but very rich in solvent based unique species.

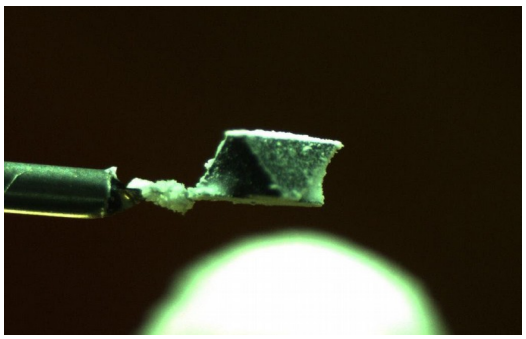

**Fig1. The actual crystal of Rubredoxin back exchanged in D2O and yielding neutron diffraction data to 1.00A d-spacing resolution.**

Table 1. Table of the neutron data collection characteristics and statistics obtained from SCALA. The protein crystal sample is perdeuterated. Overall values for the selected resolution ranges are presented. Values in parentheses are for the highest resolution shell.

| **Sample oxidation state** | **Oxidised (Fe3+)** |
|---|---|
| Time/frame (s) (monitor count (n°/s)) | 92 (10000) |
| Space group | $P2_1\ 2_1\ 2_1$ |
| Unit cell (Å) a | 33.62 |
| b | 34.53 |
| c | 43.07 |
| Resolution range (Å) | 24.04-1.00 |
| Rmerge | 0.074 (0.566) |
| R r.i.m. (or R m.e.a.s.) | 0.088 (0.673) |
| R p.i.m. | 0.046 (0.347) |
| CC (1/2) | 0.999 (0.705) |
| Mn $I/\sigma(I)$ | 7.4 (1.5) |
| Completeness (%) | 94.7 (86.6) |
| Multiplicity | 3.2 (2.8) |
| Number unique reflections | 26296 (3450) |

Rmerge = (S(I-<I>) / S(I); where I is the intensity measured for a given reflection, <I> is the average intensity for multiple measurements of this reflection.
Rr.i.m. = (Σ[N/(N-1)]1/2 Σ|I-<I>|) / Σ(I); where N is the redundancy.
Rp.i.m. = (Σ[1/(N-1)]1/2 Σ|I-<I>|) / Σ(I).
Rwork = Σ||Fobs|-|Fcalc|| / Σ|Fobs|; where Fobs and Fcalc are the observed and calculated structure factor amplitudes, respectively, for 95 % of the reflection data used in refinement.
Rfree = Σ||Fobs|-|Fcalc|| / Σ|Fobs|; for 5% of the reflection data excluded during the refinement.

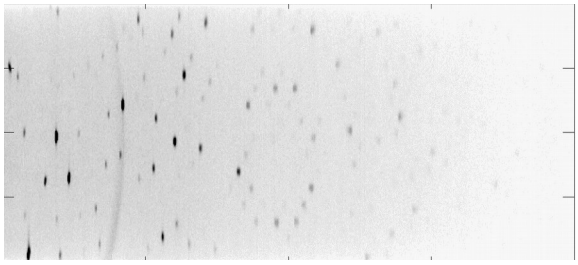

Figure 2. Neutron diffraction patterns at 1.46 Å wavelength from the oxidised form crystal with $H_2O$ solvent cryo-frozen crystals (1000s accumulation) showing diffraction diffracts to a resolution better than 1.00 Å (0.83 Å right edge resolution). Note: The partial ring visible (left quarter on image in Fig. c) is an artefact from the beamstop instead of an ice ring.

This experiment is part of a set of where we obtained the highest resolutions ever described to date for a neutron crystallographic study on any such protein experiment, and demonstrates the serious gains possible for monochromatic studies of perdeuterated proteins at cryogenic temperature.

**Conclusions**

This is an unprecedented successful experiment on large perdeuterated protein crystals bringing new results and informations about the organisation of the solvent around this redox relevant protein adding to the information available with previously observed differences in both the reduced and oxidised states at both 295K and 100K.

The relative simplicity of the method can easily be applied to other protein systems, be they capable to be back echanged in $H_2O$, which is expected to often be the case at least for the solvent surrounding the protein.

An unprecedented cryocooling experiment on large perdeuterated protein crystals is presented and we bring innovation to the methodology of large crystal mounting for neutron diffraction experiments. Not all large protein crystals are as crack-resistant as rubredoxin crystals or tolerant to $H_2O$ back exchange (reverse labelling). Our new crystal mounting protocol has been used successfully for the 3$^{rd}$ time and proven reliable in terms of stability and reproductibility. The resolution capabilities of the new detector of D19 has been successfully evaluated by collecting data at cryogenic temperature using a cryostream system.

A manuscript describing the work methodology and results is about to be submitted together with the outstanding $D_2O$ soaked data at 100K, as it reveals unique cations present in solvents.

We wish to continue this proposal with the neutron diffraction experiment of the back exchanged crystal of perdeuterated rubredoxin in $H_2O$ at ambient temperature. The whole allocated beam time has been used for the 100K experiment. There are a number of interesting points about the hydronium (Hyd 1, Cuypers *et al.*, 2013) nearby Leu51 in the oxidised form only and what was previously proposed to be an imidic acid tautomerisation equilibrium and appears absent in other redox states of the protein.