2018A8017 SACLA BL2

# Probing of Oxygen Induced Intermediate States in Fe Containing Metalloenzymes

In-Sik Kim[a], Joshua Heineman[a], Cindy Pham[a], Hugo Lebrette[b], Vivek Srinivas[b], Juliane John[b], Jos Kamps[c], Patrick Rabe[c], Rahul Bannerjee[d], Asmit Bhowmick[a], Vittal Yachandra[a], Junko Yano[a], Jan Kern[a]

[a] MBIB Division, Lawrence Berkeley National Laboratory, [b] Department of Biochemistry and Biophysics, Stockholm University, [c] Department of Chemistry, Oxford University, [d] Department of Chemistry and Biochemistry, Auburn University

**Abstract**

Metalloenzymes use the environment around the metal site to manage substrate interactions with the metal in order to effect efficient and specific catalysis under ambient conditions. Use of fs X-ray pulses from an XFEL allows to follow such reactions at room temperature (RT) without inherent radiation damage to the active site during the X-ray measurement [1-3]. We developed an approach employing simultaneous X-ray crystallography (XRD) and X-ray Emission (XES) techniques using fs XFEL-pulses [4,5] that provides complementary information: detailed information about changes in the electronic structure of the catalytic metal (XES), and the geometric changes of the metal site and the entire protein (XRD). In this experiment we used fs XFELs-pulses for RT studies of redox active metalloenzymes to capture reaction intermediates and expand time resolved studies of proteins at XFELs beyond photo-activated systems to chemically triggered systems. The main objective of this beam time was to test a novel sample delivery setup that was designed to allow drop on demand sample delivery combined with oxygen gas incubation of the individual sample drops prior to X-ray probing. We intended to test a new geometry, drop-on-wheel, which differs from our previously established drop-on-tape setup [6] and promises easier installation and operation. We included several important metalloenzymes, (ribonucleotide reductase, hydrogenase and methane monooxygenase) in this experiment to test this approach. Initial results from these tests are reported here.

**Keywords:** metalloenzymes, serial crystallography, sample delivery, X-ray emission spectroscopy

## Background and Purpose

The main objective of this beam time was to test a novel sample delivery setup that was designed to allow drop on demand sample delivery combined with oxygen gas incubation of the individual sample drops prior to X-ray probing. In addition, the setup was intended to allow parallel collection of X-ray diffraction and X-ray emission data from the same protein crystals. Establishing a smaller and easier to operate setup instead of the previously tested drop-on-tape approach, would allow for faster deployment and more wide spread use of such a setup. In this experiment we targeted reaction intermediates in the following oxygen activated Fe containing enzymes: 1) the Mn/Fe and Fe/Fe containing class Ic and Ia Ribonucleotide Reductase (RNR); 2) the Methane Mono Oxygenase (MMO); 3) the oxygen-tolerant Membrane-Bound [NiFe] Hydrogenase (MBH); 4) Isopenicilin N Synthase (IPNS). Ribonucleotide reduction catalyzed in RNR is the rate-limiting step in DNA synthesis [7,8]. MMOs are expressed by methanotrophic bacteria and allow them to metabolize from methane. The key reaction cycle intermediate termed compound Q contains a unique binuclear Fe(IV) cluster that reacts with methane to break its $10^5$ kcal $mol^{-1}$ C−H bond and insert one oxygen atom [9]. Hydrogenases like MBH are of interest to $H_2$ production, particularly the small subgroup of the [NiFe] hydrogenases that evolved $O_2$-tolerance [10]. In the IPNS-catalyzed reaction the β-lactam bicyclic ring is formed in a single four-electron oxidation step from a simple tripeptide at a mononuclear Fe but the mechanistic details including intermediate rearrangements of iron, oxygen and primary substrate remains poorly understood (**Fig. 1**) [11].

## Experimental Summary

The new setup installed was specifically designed for the SACLA beam line and was shipped from LBNL and 5 days of setup time before the experiment were spend on assembling and testing the setup. The main component is a solid disk of 120 mm diameter that sits on a high precision spindle to allow exact positioning of the disk (**Fig. 2**). The rim of the disk is covered with a Kapton tape that hangs over the edge of the disk. The sample is applied onto this overhanging part of the Kapton tape using acoustic droplet ejection (ADE)

while the disk is rotating at constant speed. The sample droplet is interrogated by X-rays at 90 degrees from the droplet deposition point and the space in between deposition and probe position can be used for either optical pumping or gas incubation of the droplets.

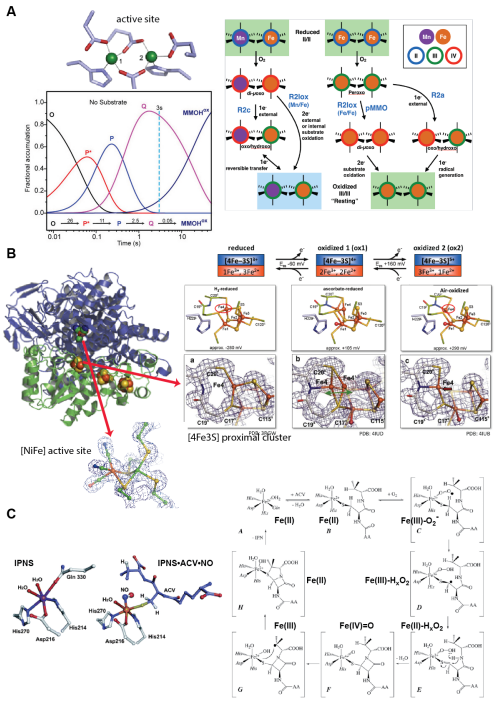


Fig 1. Metalloenzymes used in this experiment. A) Active site of MMO and RNR including a diagram showing the kinetics of the formation of intermediate Q in MM and the different intermediates in RNR FeFe, FeMn, and MnMn systems. B) Structrue of MBH and structural changes observed between the reduced, oxidized and over oxidized form of the proximal FeS cluster in MBH. C) Structure of the active site of IPNS and proposed mechanism.

The incubation time or pump delay can be regulated by adjusting the rotation speed of the disk. Fe Kα X-ray emission was measured at 90 degrees in the horizontal direction. For this purpose, SACLAs own manipulator setup was used to position two cylindrically bend analyzer crystals (250 mm bending radius) at the right position using Van Hamos geometry with the dispersion direction in the vertical and the XES signal was collected on a single element MPCCD that was mounted above the interaction point outside of our He-enclosure. X-ray diffraction was measured downstream using the 8-element MPCCD detector at 30 Hz. This setup allows parallel measurement of both signals from the same sample.

**Results and Discussion**

The enzymes proposed to study where: isopenicilin N synthase (IPNS), the complex of the H and B subunits of methane monooxygenase (MMOH-MMOB) and the R2 subunit of the ribonucleotide reductase (RNR) class 2a (Fe/Fe), class 1b (Mn/Mn) and class 2c (Mn/Fe) (**Fig. 1**).

The Kapton tape was successfully tested and it was found that no serious damage was created on the tape by the XFEL pulses. This was one of the main concerns during the design of the setup and could only be tested using the XFEL beam during this beam time. Stable operation of droplet deposition and droplet probing by X-rays were achieved. Many areas that need significant improvement before routine data collection is possible were identified. These include optimization of the cleaning and drying setup that allows continuous use of the same Kapton tape as well as improvements in sample droplet visualization and alignment procedures (for beam stop, tape position and droplet position). Also, in the next iteration it is planned to modify the He-enclosure of the setup to allow easier access and to bring the interaction point directly into the nominal focus position of the XFEL beam (during this experiment we were slightly behind the focus position leading to a beam size of approximately 10 microns in diameter).

The XES setup using a Ge620 analyzer crystal and a MPCCD detector was succesfully calibrated for the Fe Kα region using 10 mM $FeCl_3$ solutions. Attempts were made to measure Fe Kα from the protein samples but due to problems with cleaning the Kapton tape a constant high Fe background signal was observed that

swamped the signal from the proteins.

The configuration allowed to calibrate the detector-interaction point distance using diffraction measurements from Ag behenate. For this a 2.5 minute data collection run was sufficient and the detector distance was calibrated to be 70.5 mm, allowing collection of diffraction data to up to 1.78 Å resolution on the octal MPCCD. Four different protein crystal samples were successfully ejected and droplets where deposited onto the tape in a reproducible manner using flow rates of 3.3-9 µl/min (droplet volumes of 2-5 nl) at a measurement frequency of 30 Hz. The rotation speed of the kapton disc was 94 mm/s (1/4 turn/s) leading to a spacing between droplets of ~3.1 mm and a travel time of 1 second for the droplets from the deposition region to the X-ray interaction point. XRD data were recorded from four of our samples, with one (IPNS) showing a reasonable protein diffraction hit rate. Problems persisted due to the insufficient removal of debris/dried sample leftovers that lead to large background levels on the detector, making hit identification and indexing very challenging. Evaluation of the IPNS data, that gave the largest number of possible protein hits as estimated from visual inspection of detector images, was attempted but the obtained amount of diffraction was not sufficient to result in a complete data set. A summary of the number of identified hits for each sample is given in Table 1. Please note that these hits include salt and protein crystal hits together and only IPNS showed a low enough salt contamination to actually attempt indexing of these patterns.

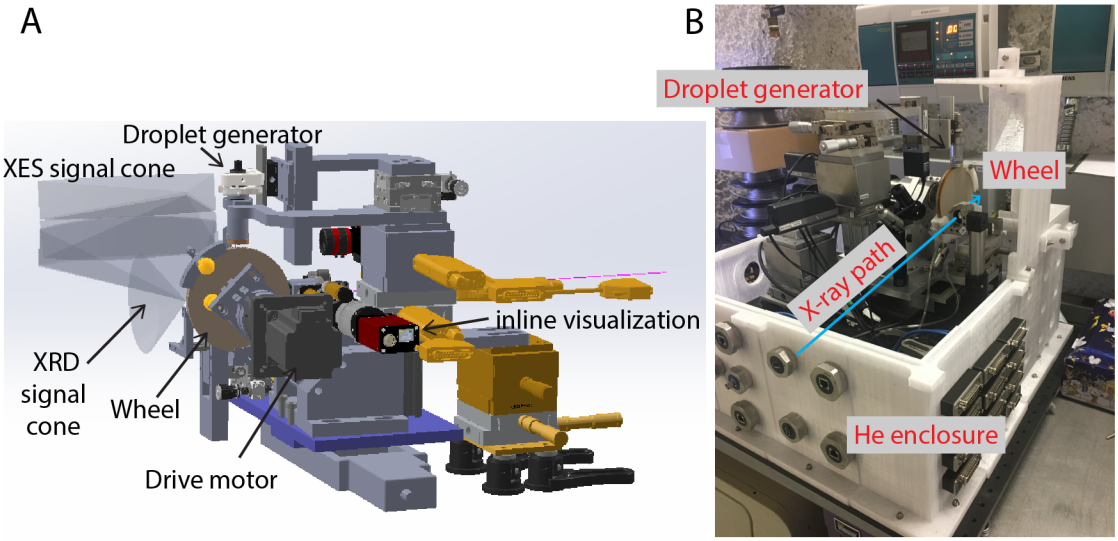


Fig 2. Drop-on-Wheel setup designed for parallel XRD and XES measurement. A) CAD design, B) Overview picture of the actual setup.

| Sample | Run time | Number of hitfinder hits | Hit rate [%] |
|---|---|---|---|
| IPNS | 80 min | 13637 | 10 |
| RNR R2b | 70 min | 28987 | 23 |
| RNR R2c | 130 min | 29042 | 12 |
| sMMO | 65 min | 30785 | 26 |

Table 1. Hit rates for the different samples run during this experiment. Note that hitfinder hits include salt and protein crystal hits.

**Challenges**

The new sample delivery system, tested during this experiment, showed promise but at the same time many shortcomings were discovered that will need to be improved in the next iteration of this setup. The main challenge was that the cleaning procedure for the kapton tape was not optimized, leading to the deposition of large amount of debris on the tape during operation. This made signal evaluation for both the XES and the XRD challenging, due to high Fe backround signal on the XES detector and frequent detection of salt scattering in the forward scattering direction. Another challenge was the alignment and positioning of the setup. The camera included in the setup did not allow for prober in-line viewing and this will need to be improved in the next version. In addition we attempted to operate in a He enclosure but integration of the

beam stop with that enclosure proofed to be challenging and we resorted to running in ambient environment without He present, which lead to increased background scatter. We are designing an improved flight tube/collimator setup currently that will allow operation of this setup in ambient conditions while reducing the air scatter background significantly. We are also working on a new He enclosure that will allow operation of the setup under anaerobic conditions, which is a prerequisite to obtain data for the enzymes we are interested in in their anaerobic active state.

**References**

[1] J. Yano *et al., PNAS,* **102,** 12047 (2005).
[2] M. C. Corbett *et al., Acta Cryst. D.,* **63**, 951 (2007).
[3] K. G. V. Sigfridsson *et al., J. Biol. Chem.,* **288**, 9648 (2013).
[4] R. Alonso-Mori *et al., PNAS*, **109**, 19103 (2012).
[5] J. Kern *et al.*, *Science,* **340**, 491 (2013).
[6] F. D. Fuller *et al.*, *Nature Methods*, **14**, 443 (2017).
[7] B. M. Sjöberg, in Metal Sites in Proteins and Model (Structure and Bonding*,* **88**), Springer, 1997, P. 39.
[8] M. Högbom *et al., Science,* **305**, 245 (2004).
[9] R. Banerjee *et al., Nature*, **518**, 431 (2015).
[10] S. Frielingsdorf *et al., Nat. Chem. Biol.,* **10**, 378 (2014).
[11] P. Rabe *et al*., *Sci. Adv.,* **7**, eabh0250 (2021).

(Received: October 3, 2023; Accepted: November 24, 2023; Published: February XX, 2024)