ESRF	**Experiment title:** Prototypic FeFe cofactors in R2 protein of ribonucleotide reductase studied by NRVS	**Experiment number**: SC3722
Beamline: ID18	**Date of experiment**: from: 13.11.2013 to: 19.11.2013	**Date of report**: 03.03.2014
Shifts: 18	**Local contact(s)**: Dr. Dimitrios Bessas	*Received at ESRF:*

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Report: Prototypic dimetal-carboxylate cofactors (DMC) are typical for the superfamily of ferritin-like enzymes. They catalyze chemically most challenging reactions, which are important in renewable energy and catalysis research, medicine, and the global cell metabolism [1,2]. We have recently been granted a new research project in the Röntgen-Angström-Cluster ("*Dimetal-carboxylate complexes in enzymes and biomimetic materials studied by novel X-ray crystallography and spectroscopy techniques*") funded by the German BMBF and the Swedish VR, which is dedicated to the study of DMC-enzymes by advanced X-ray spectroscopy techniques, and involves 4 groups (Haumann FU-Berlin, Schünemann TU-Kaiserslautern, Högbom U-Stockholm, Ott U-Uppsala). Here we describe the first ^{57}Fe nuclear resonance spectroscopy experiments in this framework on a new DMC-enzyme, which was recently discovered in Stockholm [3].

The new *Geobacillus kaustophilus* ligand-binding oxidase (*Gk*Lox) holds a crystallographically characterized DMC cofactor (Fig. 1), which after metal reconstitution can be of the FeFe, MnFe, or MnMn type. High-valent metal species are formed after reaction of initial M(II) ions with O_2 [3]. Crystal structures have revealed oxygen species bound to the metals, the chemical nature of which is unclear. The goal of the present project was to obtain information from NRS on the origin (from O_2 or water) and the protonation state (OH_x) of metal-bound oxygen species in the FeFe cofactor using ^{57}Fe labelled *Gk*Lox and isotopic substitution (H/D, $^{16/18}O_2$. $H_2{}^{16/18}O$) in the sample preparation. The vibrational dynamics and Mössbauer properties were probed using nuclear inelastic X-ray scattering (NIS; also denoted nuclear resonance vibrational spectroscopy, NRVS) and nuclear forward scattering (NFS) [4] at ID18. *The results prove that isotopic shifts of vibrational bands can be detected. However, the 16-bunch ring filling mode is required for obtaining high-quality NIS spectra on dilute protein samples. We will continue this promising project in future beamtime periods at ID18.*

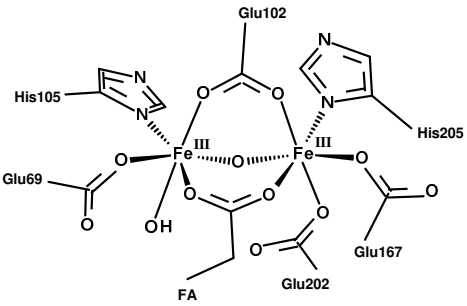

Figure 1: FeFe cofactor in the crystal structure of *Gk*Lox protein [3]. FA denotes a fatty acid ligand. Oxygen species of unclear origin (O_2 or water) and protonation state are ob-served in metal-bridging and terminal positions at the Fe ions. Here the cofactor was labelled to ~100 % with ^{57}Fe for NIS and NFS.

Experimental: $^{57}FeCl_2$ was synthesized from metallic ^{57}Fe powder. Aqueous solutions of $^{57}FeCl_2$ (50 mM) using H_2O, D_2O, and $H_2{}^{18}O$ were prepared, which contained hexaquo-Fe(II) ions. The metal-free apo-GkLox protein was reconstituted with ^{57}Fe in H_2O, D_2O, or $H_2{}^{18}O$, and using $^{16}O_2$ or $^{18}O_2$ as the oxidant to form the Fe(III)Fe(III) cofactor in the laboratory of M. Högbom (U-Stockholm). Protein concentrations were ~2 mM, i.e. samples contained ~4 mM Fe. NIS and NFS measurements were carried out using the set-up at ID18 (~0.5 meV high-resolution monochromator, avalanche photodiode detectors) and a liquid-helium cryostat. Up to 20 NIS scans (a ~45 min) were accumulated on each sample. Partial-vibrational-density-of-state (PVDOS) spectra were generated from raw emission scan data using the software packages available at the beamline.

Results:

(A) NIS and NFS on isotope substituted hexaquo-^{57}Fe. NIS and NFS spectra were obtained for ^{57}Fe(II)(H/D$_2$$^{16/18}$O)$_6$ containing samples (Fig. 2). The NFS spectra revealed the exclusive presence of Fe(II). The NIS spectra showed numerous vibrational bands and significant band shifts due to the isotopic substitutions. Density-functional theory (DFT) calculations are underway for quantitative simulation of the NIS spectra, aiming at the assignment of the vibrational bands to specific iron-ligand vibrational modes.

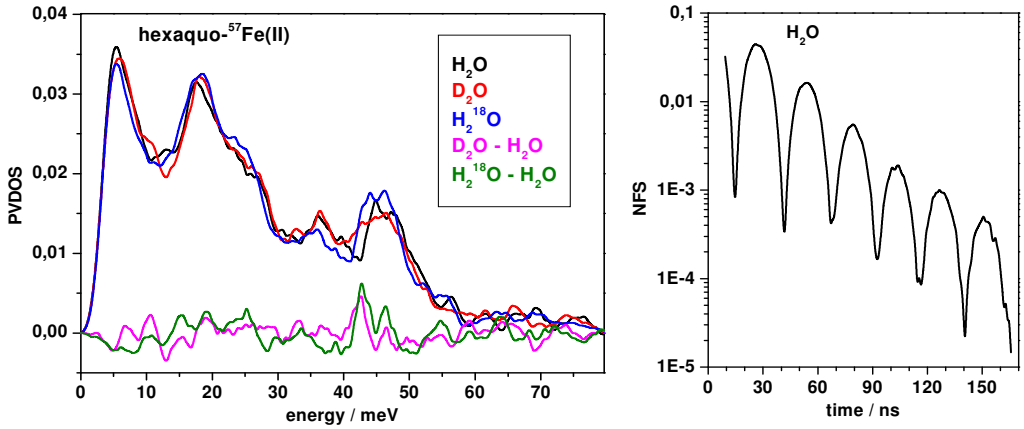

Figure 2: Vibrational NIS and NFS spectra for hexaquo-^{57}Fe(II). Left: resolved band shifts in PVDOS spectra due to isotopic substitution (see difference spectra). Right: NFS spectrum revealing only Fe(II) in the solution samples. DFT calculations on the PVDOS for band assignment are underway.

(B) NIS and NFS on GkLox protein. The Fe(III)Fe(III) cofactor was studied by NIS and NFS in ~100 % ^{57}Fe labeled *Gk*Lox samples (Fig. 3). Spectra were measured for samples prepared with H_2O, D_2O, $H_2{}^{18}O$, or $^{18}O_2$. Useful spectra were obtained after ~20 scans (~15 h). However, the signal-to-noise ratio that could be achieved in the limited beamtime was low, which precluded straightforward assignment of isotope shifts of vibrational bands. *The storage ring was operated in the 7/8 filling mode (200 mA), so that only 2 bunches could be used for the NIS measurements. For obtaining high-quality NIS spectra of relatively dilute protein samples, the 16-bunch filling mode (100 mA) is definitely required, which would increase the S/N ratio by a factor of at least 5 and diminish the measuring time per protein sample to less than one shift (8 h).* We have applied for a further beamtime at ID18 in the 2nd round of 2014 for continuing this promising project. DFT calculations on the PVDOS spectra of *Gk*Lox protein for band assignment are underway in our laboratories.

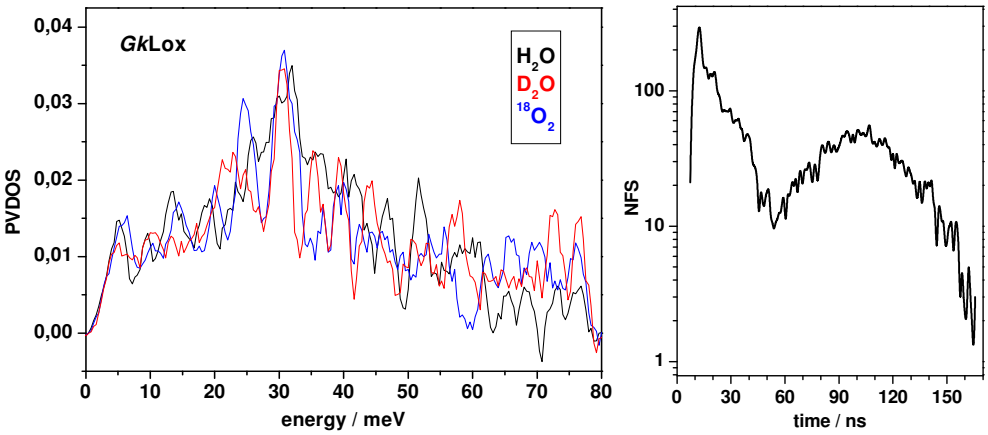

Figure 3: NIS spectra (left) and NFS data of Fe(III) (right) for *Gk*Lox protein. NIS spectra were smoothed for display. The global spectra are similar to published data for another DMC enzyme [5]. The limited S/N ratio presumably precludes assignement of isotopic shifts. This can be improved using the 16-bunch mode of ESRF. DFT on the PVDOS is underway.

Conclusions: NIS and NFS spectra were obtained for hexaquo-^{57}Fe(II) and ^{57}Fe-labelled *Gk*Lox protein in samples with isotopic substitutions for the first time. We show that H/D and $^{16/18}$O isotopic shifts can be resolved by NIS. ***For protein measurements, the 16-bunch filling mode of the ESRF is necessary to obtain high-quality NIS data in reasonably short measuring periods. Further improvements, e.g., of the cryostat set-up will be implemented in close collaboration with the beamline scientists.*** We aim at continuation of this promising project on *Gk*Lox protein and other DMC-enzymes in future measuring periods at ID18.

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