ESRF	**Experiment title:** [MnFe] and [FeFe] cofactors in a ligand-binding oxidase studied by NRVS	**Experiment number**: LS2475
Beamline: ID18	**Date of experiment**: from: 04.05.2016 to: 10.05.2016	**Date of report**: 01.09.2016
Shifts: 18	**Local contact**: Dr. Alexander Chumakov	*Received at ESRF:*

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Report:

Prototypic dimetal-oxygen cofactors (DMC) perform oxygen-activation reactions in important biological enzymes. This reactivity is among the top-10 challenges of catalysis research.[1] A new class of DMC-enzymes are ligand binding oxidases (lox), which can contain a DMC cofactor of the [FeFe] or [MnFe] type. Both forms are active in O_2 activation, but at altered reactivities. The reasons for the different metallation in the two forms and altered reactivities are widely unclear.[2, 3] Comparative investigation of the ^{57}Fe labelled [FeFe] and [MnFe] cofactors in the lox from the bacterium *Geobacillus kaustophilus* (*Gk*lox) using nuclear resonance vibrational spectroscopy (NRVS) and nuclear forward scattering (NFS) was done here.

The [MnFe] and [FeFe] cofactors can be assembled in vitro into apo-*Gk*lox protein, following a reaction path, which is expected to involve high-valent intermediates of the metal sites,[4] as well as O_2 cleavage and distribution of the products at the cofactor. The in vitro assembly procedure facilitates quantitative labelling of the cofactor with ^{57}Fe for NRVS studies. Crystallographic analysis has shown that the two metal ions are bridged by an oxido group and the metal in site 1 (Mn or Fe) carries a terminal water species.[5] The protonation state of the terminal and bridging oxygen species were unclear. Determining the protonation states in the differently metallated sites was as a major goal of this study.

Experimental:

*Gk*lox protein samples labeled with ^{57}Fe were prepared in the laboratory of M. Högbom (Stockholm University) using established procedures. The [MnFe] or [FeFe] cofactors were reconstituted using $^{16/18}O_2$ incorporation in H/D_2O or $H_2{}^{16/18}O$ solutions for isotopic exchange, follwed by upconcentration to ~3 mM of protein (i.e. 3-6 mM ^{57}Fe). Samples were prepared in the III,III states in wildtype *Gk*lox. Samples were characterized by metal content quantification (TXRF) and biochemical assays prior to NRVS.

^{57}Fe NRVS and NFS experiments were performed at the nuclear resonance beamline ID18 of ESRF. The heat-load monochromator [Si111] and the high resolution monochromators provided an energy resolution of ~0.6 meV. Samples were held in a liquid-He cryostat at ~50 K. NRVS spectra were collected using an APD detector. In parallel, nuclear forward scattering (NFS) spectra were collected. PDOS spectra from NRVS data were interpreted using DFT and QM/MM calculations on cofactor or whole-protein structural models.

Results:

(A) ***NRVS results.*** High-quality NRVS spectra of [MnFe] and [FeFe] cofactors in *Gk*lox were obtained. The spectra of the two cofactors showed significant differences due to structural variations at iron sites 1 and 2 (Fig. 1). In addition, significant shifts of vibrational bands due to H/D and $^{16/18}$O exchange were observed. Quantitative description and analysis of the vibrational spectra was achieved using QM/MM methods. A comprehensive analysis of the (III,III) redox states of the dimetal cofactors including NRVS and XAS/XES data using DFT and QM/MM calculations unambiguously assigned the protonation state of both cofactors as a bridging hydroxide (μOH) and a terminal water ligand (H_2O) at the metal in site 1 (Fig. 1). Our results from NRVS and further X-ray spectroscopy methods have been included in recent publications.[2, 6, 7]

(B) ***NFS results.*** Nuclear forward scattering can reveal the magnetic quadrupole splitting (ΔQ) of the ^{57}Fe nucleus similar to Mössbauer spectroscopy, but has very rarely been applied to protein samples. We have obtained NFS time traces for *Gk*lox and analysed them in a theoretical framework. This has provided ΔQ values in excellent agreement with Mössbauer data and even resolved minor differences in the iron sites in [FeFe] and [MnFe] cofactors due to the superior resolution of the interference method (Fig. 1). These results have further established the method for protein research and were included in a recent publication.[7]

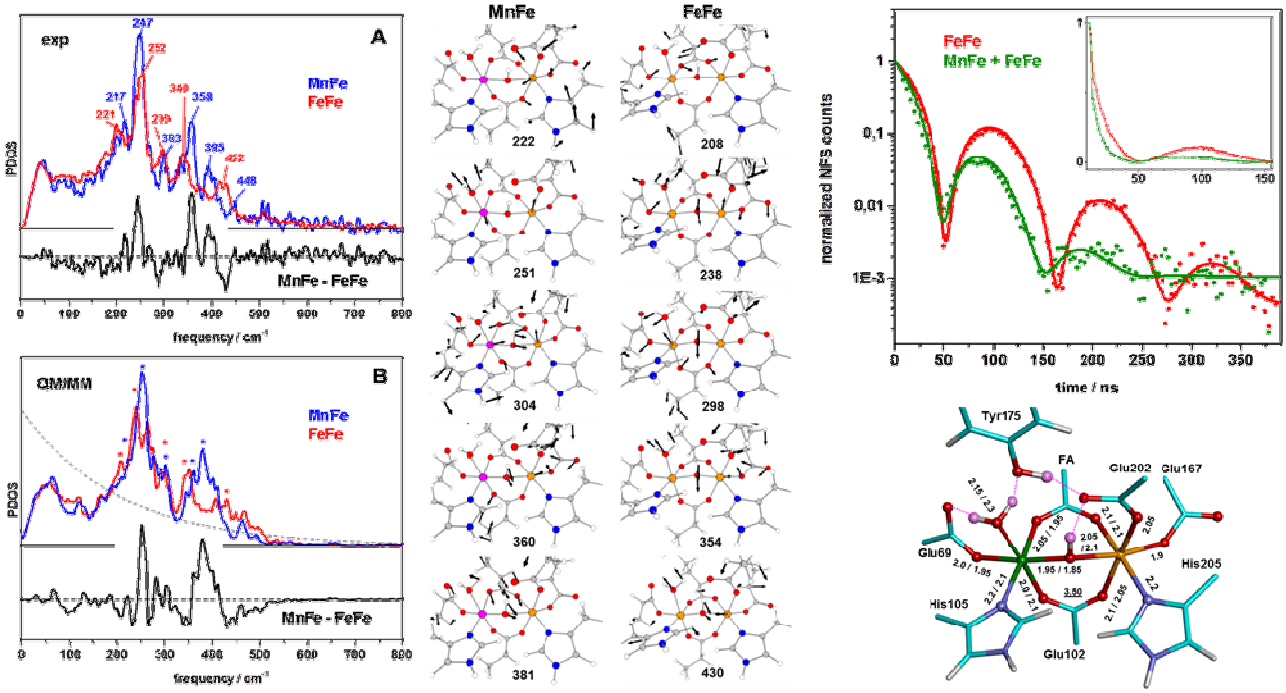

Fig. 1: NRVS and NFS on *Gk*lox proteins. Left: NRVS (PDOS) spectra of [MnFe] and [FeFe] cofactors and differences (top, experimental data; bottom, QM/MM simulations). Right, top: experimental NFS time traces (dots) and simulations (lines). Right, bottom: structural model of the dimetal cofactor with protonation and hydrogen-bonding patterns assigned on basis of NRVS and XAS/XES data and DFT and QM/MM. These results have recently been published.[2, 6, 7] A further publication on isotope labeling and mutations is pending.

Conclusions:

We consider the beamtime as very successful. High-quality NRVS and NFS data were obtained for *Gk*lox (and for samples of a [FeFe]-hydrogenase and model complexes). We have established quantitative analysis of the spectra using quantum chemical and further theoretical approaches. This revealed the vibrational modes of the sites and lead to unambiguous assignment of protonation states in [MnFe] and [FeFe] cofactors.

We thank A. Chumakov and R. Rüffer at ID18 for excellent support and the BMBF for funding in the Röntgen-Angström Cluster.

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