ESRF	**ESRF Long Term Project:** High-resolution time-resolved XAS/XES on high-valent metal sites in H_2O, O_2, and H_2 activating enzymes	**Experiment number**: SC3218 (5^{th} & 6^{th} beamtime)
Beamline: ID26	**Date of experiment**: from: 15.02.2015 to: 24.02.2015 and 23.06.2015 to: 30.06.2015	**Date of report**: 25.01.2016
Shifts: 2x 18	**Local contact**: Dr. Lucia Amidani	*Received at ESRF:*

Names and affiliations of applicants (* indicates experimentalists):

*Dr. Michael Haumann, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany (LTP spokesperson)

*Peer Schrapers, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany

*Ramona Kositzki, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany

*Dr. Stefan Mebs, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany

*Nils Schuth, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany

Progress Report:

Combining high-resolution and time-resolved X-ray absorption (XAS) and emission (XES) spectroscopy techniques to gain novel information on molecular structure, electronic configuration, and dynamics of metal centers in biological enzymes is the main purpose of this LTP. In this report, we summarize the experiments carried out at ID26 in February and June 2015.

During the February and June beamtimes we focussed on investigations on the NiFe active site of a regulatory hydrogenase (RH) (in collaboration with O. Lenz, TU-Berlin), on the six-iron H-cluster in [FeFe]-hydrogenase mutants (HydA1) (with the group of T. Happe, Uni. Bochum), on the FeFe and MnFe cofactors in a ligand binding oxidase (R2lox) and in the R2 subunit of class-1c type ribonucleotide reductase (with the group of M. Högbom, Uni. Stockholm), on studies of synthetic model complexes for these enzymes (with C. Limberg, HU-Berlin; M. Driess, TU-Berlin; U. Apfel, U. Bochum; S. Ott, U. Uppsala), and on XAS/XES experiments on hemoglobin (HB) and myoglobin (MB) proteins (with A. Hemschemeier, U. Bochum).

The experiments on RH and HydA1 hydrogenases completed and extended our measurements during previous beamtimes in this LTP, so that now complete XAS/XES data sets at Fe and Ni K-edges for wildtype and mutant hydrogenase proteins are available in various functional states. Time-resolved sampling XES experiments were established, which are required to overcome rapid X-ray damage at the cofactors and facilitate collection even of Kß satellite emission lines on highly radiation sensitive and dilute metal systems. Several publications have emerged from these studies and we are presently working on further publications related to the hydrogenase topics (see summary sheet), which summarize the new results. Based on the results for the [FeFe]-hydrogenase, we are writing on a new project proposal to be submitted soon.

The experiments on FeFe or MnFe-cofactor containing enzymes (R2lox, R2) have resulted in extended XAS/XES data sets. These experiments were particularly difficult, due to the extremely fast X-ray photoreduction of the cofactors [2], which was overcome only using time-resolved XAS/XES approaches. We are presently analyzing the data using quantum chemical calculations. These studies have established procedures for determining the protonation state of the cofactors in response to metal oxidation state and pH changes or visible light irraddiation at low temperatures by high-resolution X-ray spectroscopy. Our data have entered a recent publication [1] and further papers are in progress in this group.

The studies on HB and MB proteins were inspired by our previous experiments on spin-crossover systems and hemoglobins [3, 4]. Redox and spin state for HB and MB proteins are still not fully established. We have carried out an extended XAS/XES study for characterizing the temperature dependence and radiation effects on the XAS/XES spectra of the heme cofactors in various states of the proteins. High quality X-ray data and interesting and unexpected effects were obtained, which are presently analyzed using DFT calculations and model systems. We expect that this study will lead to a high-rancking publication soon.

In summary, during the six beamtime periods of this LTP, a large variety of biological and synthetic metal systems was studied by high-resolution and time-resolved XAS/XES methods, aiming at the elucidation of molecular and electronic structures. This has lead to a significant number of publications (see the list), extensive data material, establishing of advanced experimental approaches, and novel insights into the reactivity of biological and chemical transiton metal systems. We have conducted experiments along the line outlined in the project proposal and consider this LTP as highly successful.

Experimental: Protein samples were provided by our collaborators: [FeFe]-hydrogenase HydA1, T. Happe, U. Bochum, Germany; RH [NiFe]-hydrogenase, O. Lenz, TU-Berlin, Germany; R2lox and R2, M. Högbom, U. Stockholm, Sweden; HB and MB, A. Hemschemeier, U. Bochum, Germany. Synthetic model complexes were provided by the groups of: M. Driess, TU-Berlin; C. Limberg, HU-Berlin; S. Ott, U. Uppsala; U. Apfel, U. Bochum. Significant preparation efforts were spent in these laboratories to provide the large amounts of protein samples (several millilitres) and synthetic complexes (milligrams) that were needed for the XAS/XES experiments. On these systems, Mn, Fe, and Ni XAS/XES data (XANES, EXAFS, Kß and Kß-satellite emission lines for resonant and non-resonant excitation, RIXS plane data, site-selective XAS/XES data, under temperature variation and light irradiation of samples, using time-resolved energy-sampling approaches for data collection) were collected using the Rowland-circle spectrometer at ID26. Data evaluation involved quantum chemical calculations (DFT) using the ORCA and Gaussian programs.

Results:

(1) Publication of data from the LTP.

Since the last report several publications based on the new data from this LTP have appeared [1, 3, 5-7] or are in preparation, in addition to our previous LTP-related papers [2, 8-14]. A comprehensive XAS/XES-DFT study on two spin-crossover complexes of iron has been published [3], our results presented on the XAFS conference in Karlsruhe in 2015 were published in two proceedings, a highlight was a publication on [FeFe]-hydrogenase in Nature Scientific Reports [7], we have contributed to a publication on iron-sulfur clusters in Angewandte Chemie [5], a joint publication on FeFe or MnFe cofactor enzymes has appeared [1], and an extended Kß emission study on the manganese complex of photosynthesis is under review. We are presently working on a number of further publications on the basis of the obtained results.

(2) XAS/XES on the [FeFe]-hydrogenase HydA1.

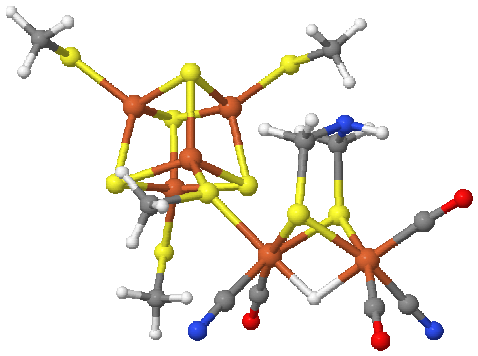

Figure 1: H-cluster cofactor in HydA1 [FeFe]-hydrogenase containing a 4Fe and a 2Fe sub-complex. The enzyme, which was in vitro maturated with a synthetic diiron complex, was studied by site-selective XAS/XES for discrimination of the high- or low-spin iron sub-complexes in various states in wildtype and mutant variants, and in lyophilized samples. In the focus were hydride, oxygen, and carbon monoxide binding at the diiron catalytic site.

We have continued our experiments on the [FeFe]-hydrogenase HydA1 [7-9]. Using in vitro reconstitution of apo-HydA1 protein with a synthetic diiron complex to yield fully active holo-enzyme [15, 16], wildtype and

mutated HydA1 in various states (oxidized, reduced, mutated variants) was studied by XAS/XES at the Fe K-edge, employing our previously established procedures [8, 9, 13] for site-selective discrimination of the four-iron and two-iron sub-complexes of the catalytic cofactor (H-cluster). High-resolution total-fluorescence and narrow-band detected K-edge spectra and Kß emission lines (main and satellite features) were collected, in part selectively for the 4Fe and 2Fe sub-complexes. In particular, we were aiming at discrimination of structural and electronic changes at the diiron site in response to hydride, oxygen, or carbon monoxide binding, on changes in selected mutant variants, and on the effects of lyophilization on the cofactor structure. Data on lyophilized HydA1 have been published recently [7]. We are presently analyzing the new XAS/XES data, using DFT calculations and further spectroscopic methods (e.g. nuclear inelastic scattering).

(3) X-ray spectroscopy on NiFe-hydrogenase.

Nickel is believed to be the actual catalytic site for hydrogen conversion in [NiFe]-hydrogenases and involved in cofactor modification related to oxygen tolerance [6]. Completing and extending our previous experiments, an extended XAS/XES study was carried out on the regulatory hydrogenase (RH). This enzyme is special because it shows only two prominent cofactor states, one of which (Ni-C) is expected to contain a Ni-Fe bridging hydride. Data at the Ni K-edge were collected for the complete multimeric enzyme complex, as well as for a variant with a truncated protein chain. These experiments were particularly difficult because of rapid X-ray photoreduction of the Ni(III) ion. Therefore, time-resolved energy-sampling experiments were necessary to collect in particular Kß satellite emission lines in an efficient and safe way. This has established the feasibility of such experiments for collection of emission spectra with reasonable signal-to-noise ratio for radiation-sensitive samples down to a metal concentration of ~1 mM. Now complete XAS/XES data sets are available for the RH enzyme, which show significant spectral changes in response to redox changes at the cofactor and visible light irradiation at cryogenic temperatures. Data analysis is underway and a respective publication in preparation. These studies on the [NiFe]-hydrogenase have paved the way for a new joint project proposal, which is currently designed for the German Research Council and will involve extensive XAS/XES studies on [NiFe]-hydrogenases.

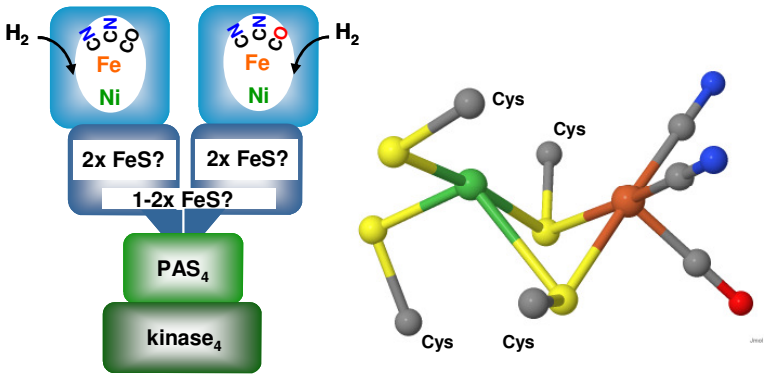

Figure 2: Protein subunit composition (left) and possible structure of the [NiFe] active site (right of the regulatory hydrogenase (RH). We have obtained complete Ni XAS/XES data (XANES, EXAFS, Kß emission lines) on the RH in this LTP. One goal was characterization of nickel-hydride states in response to redox changes at the cofactor.

(3) XAS/XES on ligand binding oxidases (R2lox) and R2 proteins.

The class of dimetal-carboxylate enzymes comprises R2-type proteins such as ligand binding oxidases and the R2 subunit of ribonucleotide reductases and the dimetal cofactors can be of the FeFe or MnFe types. Structural and electronic differences in the two cofactors species need to be elucidated for understanding the oxygen activation and redox chemistry at the cofactors. We have carried out extensive XAS/XES studies at the Fe and Mn K-edges (absorption and Kß emission data collection) on two R2lox and two R2 type enzymes. Extremely rapid X-ray photoreduction of the cofactors even in the Fe/Mn(III)Fe(III) oxidation state, which is further accelerated in higher valence states, complicated the experiments. Therefore, only time-resolved energy-sampling approaches for emission lines or rapid-scan techniques for absorption spectra have facilitated collection of data for the high-valent enzyme states. Now we have complete XAS/XES data sets for one R2lox enzyme and selected data for the other enzymes available. In addition, low-temperature light-irradiation, as well as pH studies were carried out, for modification of the protonation state of the cofactors.

Combination of DFT calculations and XAS/XES data analysis has unambiguously assigned the protonation and redox states of the cofactors and revealed differences between the FeFe and MnFe types. These results show that collection of high-quality XAS/XES data is feasible using time-resolved approaches and further established X-ray spectroscopy as a tool for determining protonation dynamics at transition metal cofactors. The results have in part been published [1, 11] and further publications are underway.

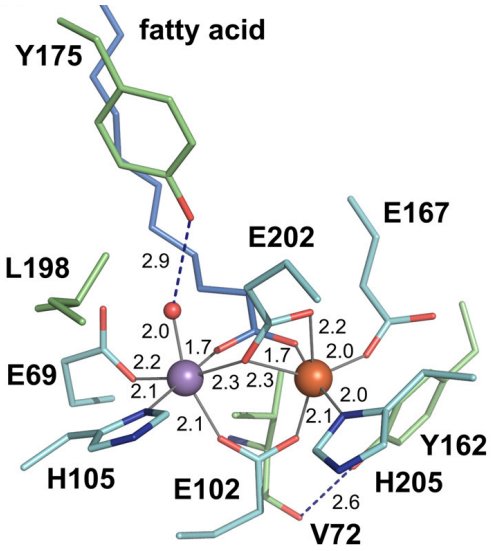

Figure 3: Crystal structure of the MnFe cofactor in a ligand binding oxidase [1]. We have studied FeFe and MnFe cofactors in this type of R2-like enzymes (ligand binding oxidases and R2 subunit of ribonucleotide reductase) in proteins from several organisms. XAS/XES data were collected for the III,III state of the cofactors and higher valence levels. Time-resolved collection of XES spectra and rapid-scan XAS is necessary to overcome rapid X-ray photoreduction of the cofactors. Our experiments have established, e.g., the protonation state of the cofactors and revealed differences between the metallation species.

(4) High-resolution X-ray spectroscopy on hemoglobin (HB) and myoglobin (MB).

Application of XAS/XES techniques to heme proteins may be expected to clarify the picture with respect to the long-standing discussion on spin and oxidation states of the iron center. Inspired by our previous work on synthetic spin-crossover systems [3], we carried out an extended XAS/XES study at the Fe K-edge on HB and MB in the reduced state and with iron-bound O_2 or CO molecules. Complete data sets were collected on more than 100 samples with ~10 mM iron each. In particular, we studied the temperature-dependence of K-edge absorption and Kß emission spectra and the effects of X-ray exposure on the metal sites. Surprisingly, at high temperatures (~250 K), but even at 10 K for certain enzyme states, rapid X-ray induced modifications were observed, which impacts on crystallographic results for these systems. Therefore, time-resolved X-ray spectroscopy experiments had to be carried out to obtain spectra of the unperturbed states. Pronounced spectral differences between the enzymes, as well as between the cofactors species were observed, which are currently analyzed using comparison with synthetic heme reference complexes showing spin and redox state variations (studied by us in parallel to the enzymes) and quantum chemical calculations. We feel that these data will lead to a high-ranking publication.

(5) Spin-crossover studied by XAS/XES.

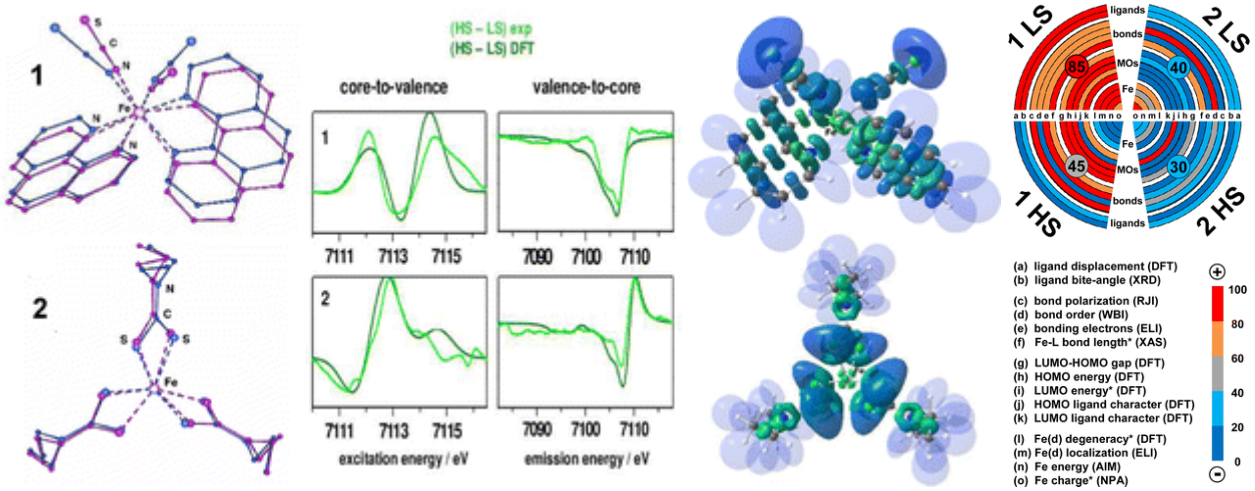

Figure 4: Spin-crossover studied by XAS/XES [3]. Two molecular SCO complexes were compared, using for example high-resolution XAS/XES spectroscopy and DFT calculations (left), which resulted in a detailed picture of geometric and electronic changes in the complexes upon SCO (right).

We have collected extensive XAS/XES data over a wide temperature range for two spin-crossover complexes of iron in the course of this LTP and a respective publication has appeared recently [3]. These results have further established high-resolution X-ray spectroscopy as a viable tool for characterization of the spin state and associated electronic and molecular structure changes of transition metal systems.

(6) New projects and funding related to this LTP.

Our XAS/XES studies have contributed to establish procedures for in vitro reconstitution of [FeFe]-hydrogenase HydA1 with synthetic diiron site analogues, for time-resolved energy-sampling XES as a tool for X-ray spectroscopy on dilute proteins such as [NiFe]-hydrogenases, as well as for in vitro assembly of the FeFe or MnFe cofactors in R2-type enzymes for X-ray spectroscopy. These developments have contributed to progress in several ongoing funded research projects and led to new collaborative project proposals to be submitted in 2016. The outcome of this LTP is highly useful to emphasize the importance of X-ray spectroscopy as a viable tool for studies on transition metal systems.

(7) Technical development and resources.

We have established time-resolved energy-sampling XES as a method for studying highly radiation-sensitive metal cofactors in higher valence states. A recent study on the manganese complex of photosystem II has shown that this method yields Kß main line spectra with good signal contrast (ms submitted). Our studies on hydrogenases and R2-enzymes show that the method is applicable to protein samples with only ~1 mM metal. The energy-sampling approach is feasible at a third-generation undulator beamline like ID26 and presumably superior in many cases, compared, i.e., to FEL approaches. The XAS/XES spectrometer at ID26 may be further improved for minimization of deadtime and increase of experimental efficiency by implementation of a continuous emission scanning mode. We have purchased new Si analyzer crystals and a Ketek detector (ca. 38 kEUR) in 2015, for general user application at ID26. A new postdoc (S. Mebs) and Ph.D. student (N. Schuth) in the Haumann group participated in the 2015 beamtimes and were trained in the X-ray experiments.

Conclusions:

In the two beamtime periods at ID26 in 2015, a large number of high-quality XAS/XES data on [FeFe] and [NiFe] hydrogenases, FeFe and MnFe cofactors in R2lox and R2 enzymes, hemoglobin and myoglobin proteins, and synthetic Fe, Mn, and Ni model complexes were collected. Time-resolved energy-sampling XES and rapid-scan XAS experiments were carried out, using temperature variation and light-irradiation of samples. These experiments complemented and extended the data series collected during this LTP, so that complete XAS/XES data sets have become available, for most of the studied systems for the first time. Data analysis will lead to further publications soon. Most of the goals as formulated in the milestones of the proposal were reached within the 6 beamtimes of this LTP, in particular with respect to characterization of metal-hydride intermediates in proteins and establishing time-resolved XAS/XES experiments on high-valent cofactors. The studies in the LTP have led to important insights into the structure and function of metalloproteins centrally involved in biological small molecule activation. New collaborative research projects are now being designed based on the obtained results. We consider this LTP as highly successful.

Acknowledgements

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