	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 (1st beamtime)
Beamline: ID26	**Date of experiment**: from: 23.11.2011 to: 29.11.2011	**Date of report**: 16.01.2012 *Received at ESRF:*
Shifts: 18	**Local contact(s)**: Dr. Pieter Glatzel	

Names and affiliations of applicants (* indicates experimentalists):
*Dr. Michael Haumann, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany (LTP spokesperson)
*Dr. Kajsa Havelius, Freie Universität Berlin, Physik, Arnimallee 14, 14195 Berlin, Germany
*Nils Leidel, 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 offers exciting perspectives to gain novel information on molecular structure, electronic configuration, and dynamics of metal centers in biological enzymes and synthetic coordination compounds, to unravel the catalytic mechanism. One focus in this project is the study of hydrogen-forming iron centers in hydrogenase proteins and biomimetic chemical model complexes by XAS/XES (Fig. 1).

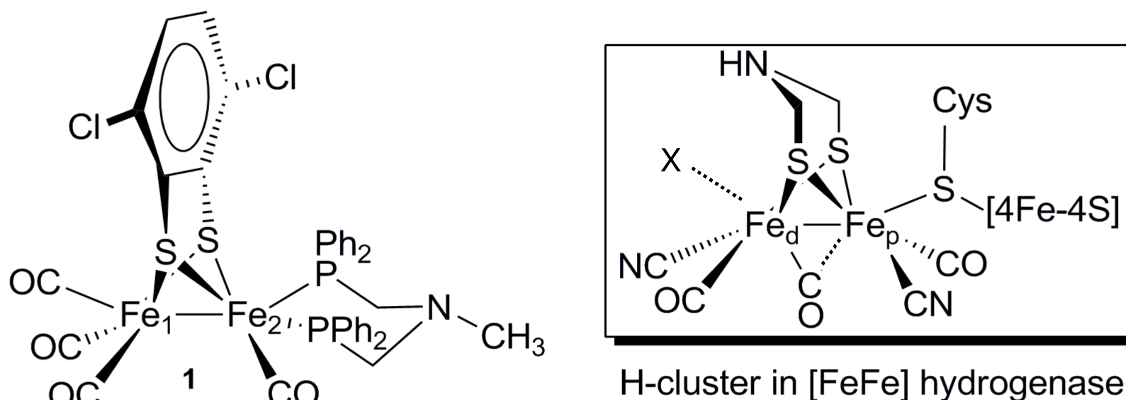

Figure 1: (Left) Structure based on crystallography of the active site H-cluster of FeFe hydrogenase [6]. (Right) Example of a biomimetic model complex for the diiron unit of the hydrogenase iron center, featuring asymmetric ligation of the two iron atoms, which has allowed us to obtain site selective structural and electronic information using XAS/XES [1].

During the first beamtime of this LTP, we studied FeFe-hydrogenase protein from the green alga *Chlamydomonas reinhardtii* by XAS/XES for the first time. A series of FeFe models was studied in addition. In agreement with the milestones formulated in the LTP proposal (milestone 1), the goals were:

(1) to establish XES (Kß emission lines) measurements and Kß-detected XAS for site-selective experiments on dilute protein samples using the Rowland-circle spectrometer at ID26, including radiation damage studies; **(2)** to measure a series of XAS/XES spectra on biomimetic models for the diiron site of hydrogenase proteins.

XAS/XES experiments were suceessfully carried out on a series of FeFe-hydrogenase protein samples, which were active or treated with different inhibitors. In addition, data were obtained for a variety of FeFe site models as obtained from our collaborators. Complete sets of Kß emission spectra (Kß1,3 main lines, Kß2,5 satellite lines), XANES spectra, and in part K-alpha and K-beta detected EXAFS spectra were obtained for these systems. Data analysis is underway, and some of the data was included in a recent publication [1].

The experiments showed that XAS/XES experiments on proteins are well feasible at ID26, radiation damage problems can be handled, and the goals formulated for the 1st beamtime of the LTP were more than reached.

Experimental: More than 10 FeFe hydrogenase protein samples (Fe concentration 1-5 mM) were obtained from the group of Thomas Happe (University of Bochum, Germany). FeFe hydrogenase was in its reduced or anaerobically oxidized states, treated with the inhibitor carbon monoxide (CO), or incubated for increasing time periods (15 s to 3 h) with the inhibitor O_2. 15 different synthetic model complexes for the FeFe hydrogenase active site were provided by the groups of Marcetta Darensbourg (Texas A&M University, USA), Frederic Gloaguen (University of Brest, France), Andreas Grohmann (Technical University Berlin), and Sascha Ott (Uppsala University, Sweden). XAS/XES experiments were carried out using the Rowland spectrometer at ID26 (Si311 or Si111 excitation monochromator crystals, 5x Ge620 crystals in the emission path, silicon-drift or avalanche photodiode fluorescence detectors). For all samples, complete sets of Kß emission spectra and high-resolution XANES spectra were measured. For selected samples, EXAFS spectra were measured at selected Kß energies using the rapid scan mode of ID26.

Results:

(1) Radiation damage studies. X-ray photoreduction of high-valent metal sites may be a problem in XAS/XES protein studies. A respective study performed by us at ID26 previously on the MnFe and FeFe sites of ribonucleotide reductases, which are also a topic in this LTP, has just been published [2].

In this measuring period, we addressed radiation damage in FeFe hydrogenase protein (Fig. 2). Changes in the Fe K-edge spectrum at increasing X-ray irradiation times under XAS/XES conditions (relatively focused beam) revealed, that for FeFe hydrogenase, using the rapid scan mode of ID26, i.e. EXAFS scans in 10 s or less and samples in the He-cryostat at 20 K, X-ray photoreduction can be avoided even for more oxidized states of the enzyme. This showed that the planned XAS/XES experiments on this system are feasible.

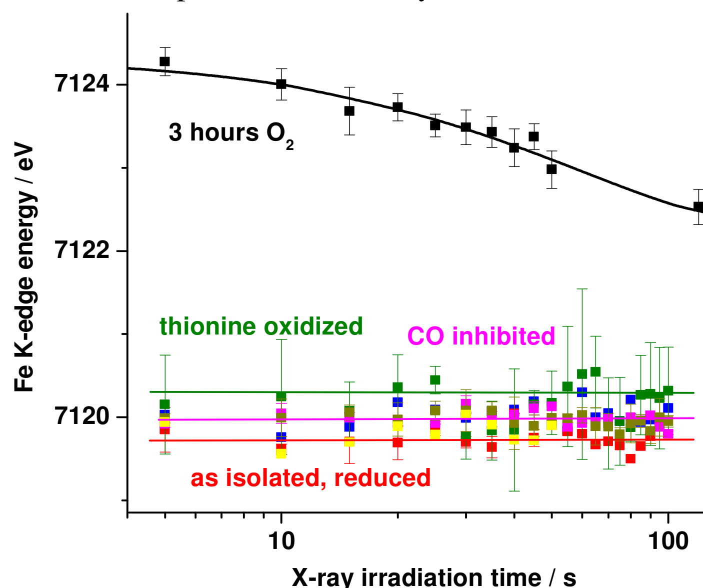

Figure 2: X-ray photoreduction of the FeFe hydrogenase iron cluster. Enzyme was held in the He cryostat at 20 K. Only for enzyme, which was modified with O_2 for a long time (3h), Fe(III) formation and its photoreduction within ~100 s was observed. For other sample conditions, the overall Fe oxidation state remained unchanged This proves that in XAS/XES studies using the Rowland spectrometer and relatively focussed beam and full X-ray flux, radiation damage is avoided using the rapid scan mode of ID26 (i.e. EXAFS scans in 5-10 s).

(2) XAS/XES on FeFe site models. 15 different model compounds for the binuclear unit of the FeFe-hydrogenase active site were studied (see Fig. 3 for examples). Complete sets of Kß emission line spectra were obtained, Kß RIXS plane data were measured for selected compounds (Fig. 4), and XANES spectra were measured for all samples (Fig. 5). The present measurements complement and extend experiments carried out during previous beamtimes at ID26 (reports SC2733, SC2858). The data allow to address spectral changes due to modifications in the ligand environment (terminal and Fe-Fe bridging ligands) and binding of hydride to iron. Using density functional theory calculations of XAS/XES spectra (Kß2,5 emission lines, XAS pre-edge transitions), the electronic structure (molecular orbital contributions and energies, configurations of occupied and empty electronic levels, HOMO-LUMO energy gap) become accessible (Fig. 6), as we have exemplified in a recently submitted manuscript [1]. Good agreement between theoretical and experimental spectra is obtained. General relations between spectroscopic and electronic properties were unraveled for low spin iron compounds [1]. We are currently working on three manuscripts which will

include model data obtained during the present (and previous) beamtimes [3,4,5]. The next step will be a study on FeFe models with protonation at different sites (metal bridging, terminal, secondary ligand sphere).

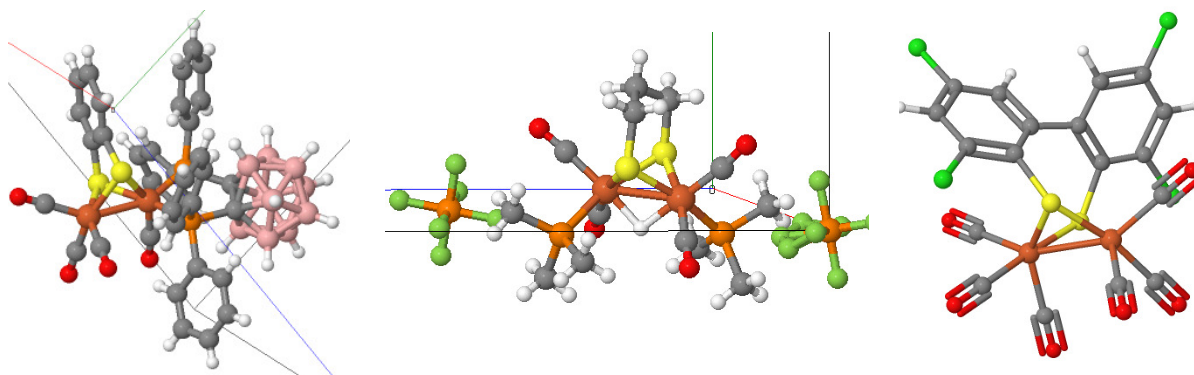

Figure 3: Three examples of the 15 crystallized FeFe site model complexes obtained from our collaboration partners and used for the XAS/XES measurements in this beamtime period. Model structures varied, e.g., in (left) the ligation environment of the two Fe atoms (i.e. symmetric vs. asymmetric ligand spheres), (middle) the presence or absence of a Fe-Fe bridging hydride (H^-), and (right) in the chemical nature of the iron-connecting group. Complete sets of Kß emission line and high-resolution XANES spectra were obtained for the models. Respective data analysis and spectral calculations using DFT are underway.

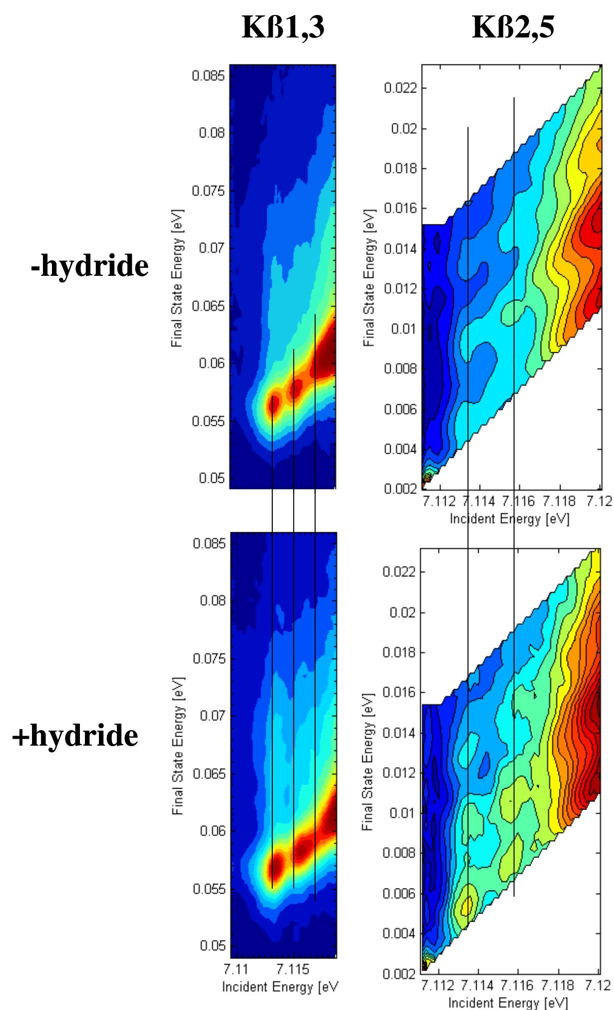

Figure 4: Kß^{1,3} and Kß^{2,5} RIXS plane data (contour plots; i.e. Kß emission spectra for resonant excitation of 1s-3d transitions) for a FeFe model complex w/o a metal-bridging hydride. Clear spectral changes due to hydride binding are observed, which (in the case of the Kß2,5 region) can be interpreted in terms of electronic structure using DFT calculations.

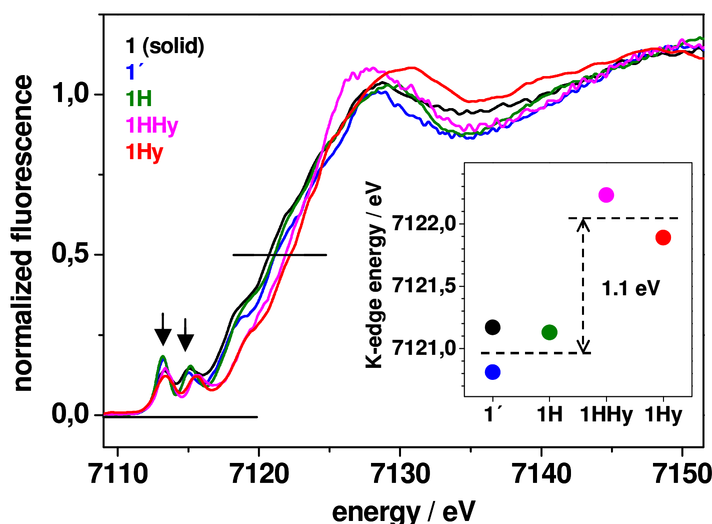

Figure 5: High-resolution XANES spectra (Si311 excitation, Kß1,3 detection) of an FeFe model complex in four different protonation states in MeCN solution and in solid material [1]. Edge energy increases, e.g., are observed for hydride formation.

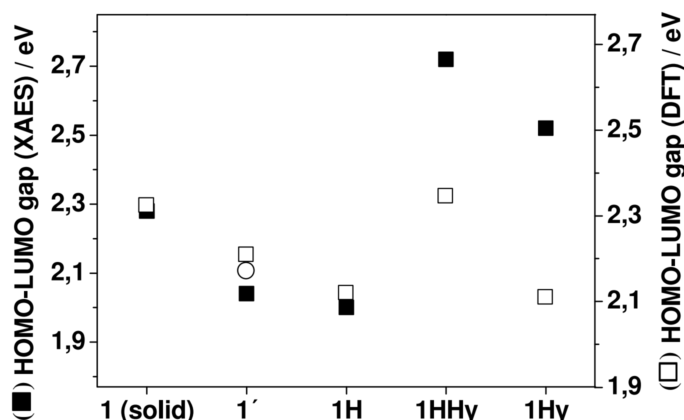

Figure 6: HOMO-LUMO energy diffrences from XAS/XES (right, determined from Kß2,5 emssion features and 1s-3d pre-edge transitions) and DFT calculations (left) for the model complex in Fig. 5 [1].

(3) XAS/XES on FeFe hydrogenase protein.

(a) *High-resolution Kβ emission and XANES data.* Recently, we have published a detailed investigation using conventional XAS on the effects and time course of oxygen (O_2) reactions at the active site of FeFe hydrogenase [6]. Now for the first time, a systematic XAS/XES study on FeFe hydrogenase was performed, focusing on inhibitor effects. Spectra were measured for a series of hydrogenase samples incubated for increasing time periods with O_2. Kß1,3 main line and Kß2,5 satellite lines emission spectra were obtained for the dilute samples (Fig. 7). Emission line energies and spectral changes apparently allow to detect oxidative modifications (Fe oxidation, oxygen binding) as well as CO binding at the hydrogenase metal center. These results in our opinion represent major progress in the field of hydrogenase research, because in principle they allow to directly extract structural and electronic information on inhibitor-modified states of the active site, which can not be obtained, i.e. by EPR or FTIR methods or by conventional XAS. We are currently working on a detailed data analysis using, e.g., DFT calculations on the FeFe hydrogenase active site.

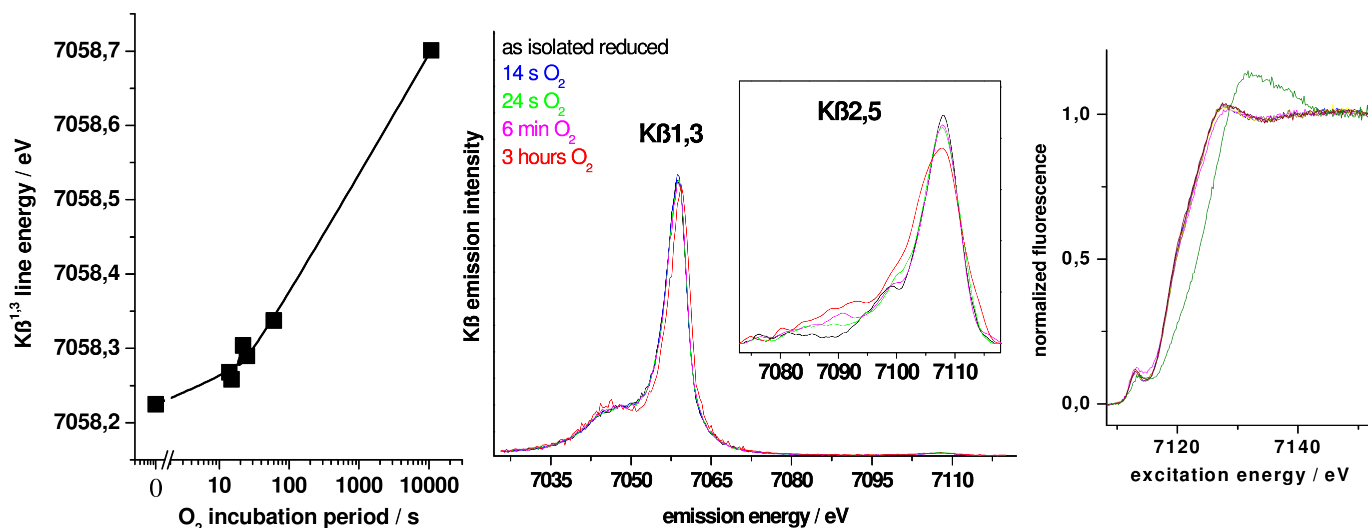

Figure 7: The first Kß emission spectra of the H-cluster in FeFe hydrogenase protein (middle) and respective $Kß^{1,3}$ line energies (left) and (right) XANES spectra of protein samples after increasing incubation with O_2. Spectral changes are related to modifications at the iron cluster due to O_2 binding and reactions [6]. We are presently performing DFT calculations on the H-cluster to simulate the $Kß^{2,5}$ spectra.

(b) *Site-selective EXAFS studies.* In principle, narrow-band Kß-detection of XAS spectra allows for site- and spin-selective measurements. For the FeFe hydrogenase system, in particular those Fe ions of the six iron cluster showing the higher spin state should contribute preferentially in the Kß´ emission region. Selectivity may also be obtained in the Kß1,3 region, in which the low-spin CN/CO-coordinated iron atoms of the diiron site may contribute at different energies than the high-spin iron of the 4Fe4S cluster. In the present measuring period, we have confirmed with model systems (mixtures of low-spin $Fe_2(CO)_9$ with iron-carbamate or high-spin Fe_2O_3) using Kß-detected EXAFS that high site-selectivity in principle can be obtained (data not shown).

EXAFS spectra of FeFe hydrogenase were obtained using Kß´ or Kß1,3 detection (emission energy resolution of ~1 eV) at selected emission energies (Fig. 8). We note that such experiments for proteins so far have very rarely (we do not know any example) been performed. Our data show that site-selective XAS/XES experiments on protein in principle are feasible. However, these experiments proved to be extremely difficult due to the very low count rates and Compton scattering background contributions. Accordingly, averaging of several hundred EXAFS rapid scans was necessary for a reasonable signal-to-noise ratio. We were able to obtain several EXAFS spectra of FeFe hydrogenase, which show differences, depending on the detection energy (Fig. 8). A detailed analysis of the spin- and site-selective features in the EXAFS data is underway. We consider these data as preliminary and will continue this study in the next beamtime period. These experiments are highly promising and hopefully will unambiguously reveal the primary site of O_2 inhibitor modification in the hydrogenase.

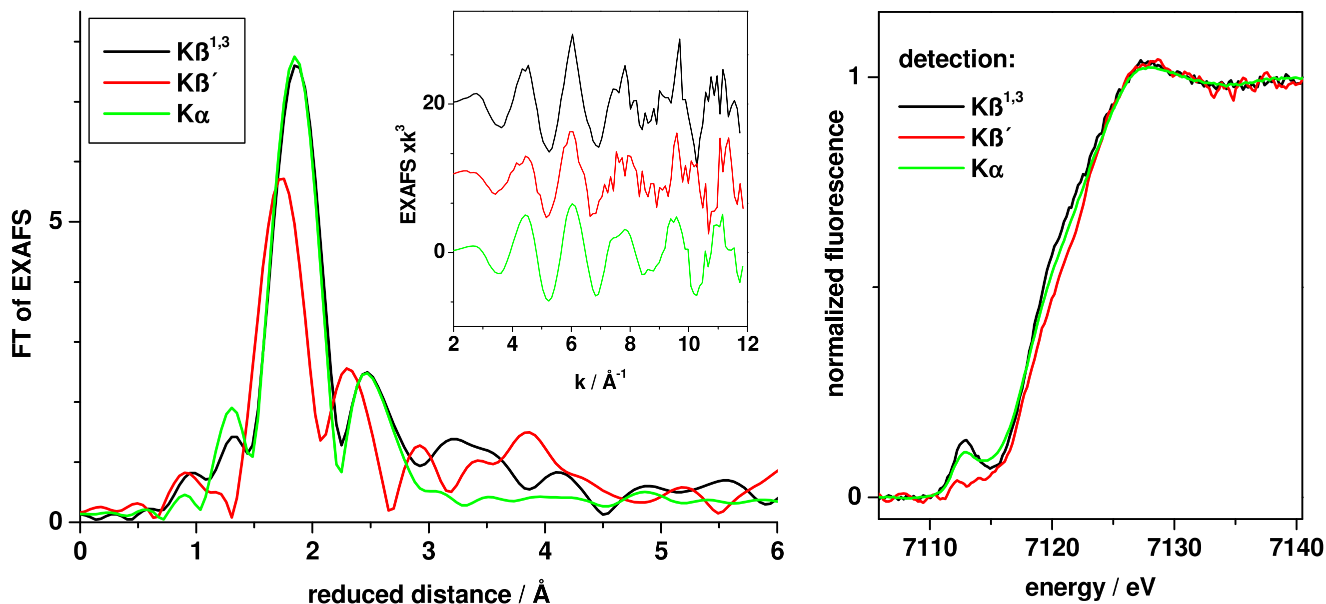

Figure 8: (Left) EXAFS spectra of as-isolated FeFe hydrogenase protein derived using narrow-band (~1 eV) partial X-ray fluorescence detection in the $Kß^{1,3}$ and Kß´ spectral regions or total fluorescence (mostly Kα) detection. Data represent the average of at least 200 rapid scan (10 s) traces each. Pronounced differences in the FT spectra reflect spin- and site-selectivity of the EXAFS data, i.e. varying contributions from the diiron site (low spin) and the 4Fe4S part (high spin) of the H-cluster to the spectra. (Right) Respective XANES spectra; spin selectivity is clearly reflected in the pre-edge region. Similar data sets were obtained for 6 hydrogenase samples incubated for increasing time periods with O_2 and with the inhibitor CO.

In summary, we consider the current XAS/XES results on the FeFe hydrogenase as very promising and we believe that they will be publishable [7]. Further experiments along this line will be performed in the next beamtime.

(4) Problems encountered.

Technical problems. Due to technical developments at the beamline, the previously feasible simultaneous scan of 2 undulators during rapid EXAFS (undulator gap) scans was not feasible in this measuring period. Due to this problem, the emission countrate in EXAFS measurements was lower than expected, leading to a loss of signal quality. After fixing of this problem, we believe that data of even higher quality can be obtained for the protein systems. We note that all other components of the beamline worked without any problems, due to excellent support by the beamline scientists and technical staff, so that the beamtime could be used highly efficiently and data were obtained for all samples as planned.

Funding of the project. Auxiliary funding for the group of M. Haumann in the framework of this LTP (e.g. for a mirror and MCA unit at ID26) has been requested at the German Research Council (Deutsche Forschungsgemeinschaft, DFG). Unfortunately, this proposal has been rejected in December 2011. However, the final statement of the Scientific Evaluation Committee (Fachkollegium) of the DFG was that “the proposal is very interesting and good, and the Fachkollegium would like to see it being funded”, but it did not pass the DFG senate, in which only ~30 % of proposals are approved. Accordingly, the chances in the next proposal round of the DFG are high and we plan to submit a revised version by the end of March 2012. We note that funding of the student (N. Leidel) who is doing practical work in the project is ensured until the end of 2012 and in addition two postdocs (P. Chernev, K. Havelius) in the group of M. Haumann are engaged in the work. Smaller equipment for improving the beamline setup can be purchased from current funds of M. Haumann if required.

Conclusions:

In the first beamtime period of this LTP, the goals formulated in the milestones section of the proposal were reached and we thus consider it as highly successful. We have established that XAS/XES experiments, including narrow-band Kß-detection site-selective EXAFS, are feasible on dilute protein (FeFe hydrogenase) samples. Radiation damage problems can be overcome. Such experiments so far have not been performed on any other protein system. The first Kß-emission spectra and high-resolution XANES spectra on native and inhibitor treated FeFe hydrogenase samples were obtained, which, in combination with DFT calculations, will reveal structural and electronic changes at the active site. An immense amount of data on FeFe model compounds was obtained, which will be included in several respective publications underway.

In summary, we believe that this LTP is highly fruitful and we are on a good way in reaching its goals. We intensely seek for auxiliary funding to finance a new student starting in 2013 in the group of M. Haumann and additional equipment at the beamline ID26. We therefore hope that the remaining beamtime periods of the LTP will be granted by the ESRF committee.

Acknowledgement

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References:

[1] N. Leidel, P. Chernev, K. Havelius, S. Ezzaher, S. Ott, M. Haumann, Site-selective X-ray spectroscopy on an asymmetric model complex of the [FeFe] hydrogenase active site, Inorg. Chem., submitted (2011).

[2] N. Leidel, A. Popovic-Bijelic, K. Havelius, P. Chernev, N. Voevodskaya, A. Gräslund, M. Haumann, High-valence [MnFe] and [FeFe] sites in ribonucleotide reductases, Biochim. Biophys. Acta, in press, doi:10.1016/j.bbabio.2011.12.008 (2011).

[3] N. Leidel, K. Havelius, P. Chernev, M. Haumann, Valence-to-core transitions in a series of diiron-carbonyl model complexes of the FeFe hydrogenase active site studied by X-ray emission spectroscopy, manuscript in preparation.

[4] N. Leidel, P. Chernev, K. Havelius, L. Schwartz, S. Ott, M. Haumann, A model complex for the FeFe hydrogenase active site studied by XAS/XES and DFT: rotational isomers in solution, protonation and hydride binding, and the electronic structure, manuscript in preparation.

[5] P. Chernev, C.-H. Hsieh, N. Leidel, K. Havelius, M. Darensbourg, M. Haumann, Hydride binding in a FeFe hydrogenase model: a high-resolution XAS/XES and DFT study, manuscript in preparation.

[6] C. Lambertz, N. Leidel, K. Havelius, P. Chernev, J. Noth, M. Winkler, T. Happe, M. Haumann, O_2 reactions at the six-iron active site (H-cluster) of [FeFe]-hydrogenase, J. Biol. Chem. 286, 40614-40623 (2011);
I. Czech, S. Stripp, O. Sanganas, N. Leidel, T. Happe, M. Haumann, The FeFe hydrogenase maturation protein HydF contains a H-cluster like [4Fe4S]-2Fe site, FEBS lett. 585, 225-230 (2011).

[7] K. Havelius, C. Lambertz, P. Chernev, N. Leidel, T. Happe, M. Haumann, XAS/XES on the H-cluster in FeFe hydrogenase, manuscript in preparation.