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 (3rd & 4th beamtime)
Beamline: ID26	**Date of experiment**: from: 18.02.2014 to: 25.02.2014 and 24.06.2014 to: 01.07.2014	**Date of report**: 06.01.2015
Shifts: 2x 18	**Local contacts**: Dr. Pieter Glatzel, Dr. Jean-Daniel Cafun	*Received at ESRF:*

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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 2014.

The focus of the February beamtime were investigations on the active site six-iron H-cluster in [FeFe]-hydrogenase (in collaboration with the group of T. Happe, Uni. Bochum, Germany). The [FeFe]-hydrogenase HydA1 from green algae is Nature´s most efficient hydrogen- (H_2) forming enzyme, containing a six-iron complex denoted H-cluster. The H-cluster consits of a canonically [4Fe4S] center, which is cysteine-linked to a binuclear iron unit [$2Fe_H$] (Fig. 1). HydA1 is the ideal system for XAS/XES to characterize iron-hydride intermediates in the catalytic cycle and enzyme functionally reconstituted with synthetic diiron complexes.

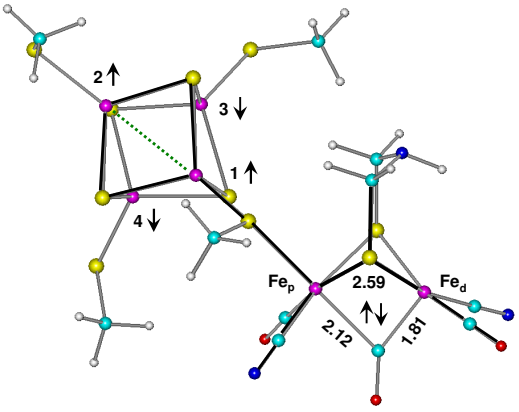


Figure 1: Structural model of the active-site H-cluster of [FeFe]-hydrogenase (HydA1) in the reduced state based on XAS/XES data and DFT including spin orientations (*6*). We have identified an iron-hydride intermediate (*3*) and studied HydA1 enzyme reconstituted with synthetic diiron complexes in this LTP for the first time.

Using site- and spin-selective XAS/XES methods in the Fe Kß-emission region in combination with DFT calculations, we have presented a detailed study on the H-cluster (*6*) and obtained first direct evidence for the formation of an iron-hydride bond in the super-reduced state of the [FeFe]-hydrogenase HydA1 (*3*). These results represent significant progress in the understanding of the reactivity of [FeFe]-hydrogenases and further established site- and spin-selective XAS/XES methods as important tools in metalloenzyme research as recently reviewd by M. Haumann (*5*).

It has been shown that HydA1 can be functionally reconstituted in vitro with synthetic diiron complexes (*1-2*). We have carried out the first XAS/XES study on HydA1 protein reconstituted with three different synthetic dirion complexes in various redox states (Fig. 2). Time-resolved experiments were carried out to track the binding of the diiron complexes to the apo-protein. A large amount of iron absorption and emission spectra of the constructs was collected. These results will lead to several publications in the near future.

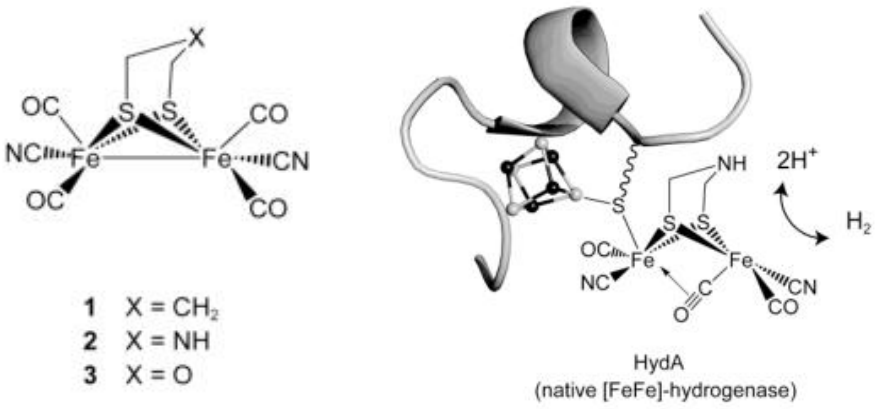


Figure 2: Quantitative in-vitro reconstitution of apo-HydA1 [FeFe]-hydrogenase protein containing only the [4Fe4S] cluster (right) with 3 different synthetic diiron complexes (left) is feasible (*1-2*). We have collected XAS/XES data for the three constructs for the first time to determine differences related to the catalytic activity.

In the June beamtime, we have focused on investigations on nickel systems including a [NiFe]-hydrogenase (in collaboration with the group of O. Lenz, TU-Berlin, Germany), a large series of relevant synthetic model complexes (in collaboration with M. Driess (TU-Berlin, Germany) and C. Limberg (HU-Berlin, Germany)), and a carbon-monoxide-dehydrogenase (CODH) (in collaboration with H. Dobbeck, HU-Berlin, Germany) (*7*) using XAS/XES at the Ni K-edge. A particularly interesting [NiFe]-hydrogenase was studied, the so-called regulatory hydrogenase (RH) which is a hydrogen sensor (*8*) (Fig. 3). Ni absorption and emission spectra in the Kß-region were collected for this enzyme in various redox states for the first time. One goal is to characterize nickel-hydride intermediates in the RH and the CODH by X-ray techniques for the first time. Promising data were collected, which need to be further improved in terms of signal-to-noise ratio. These studies on the RH and other Ni enzymes will be continued in the next beamtime at ID26 in February 2015.

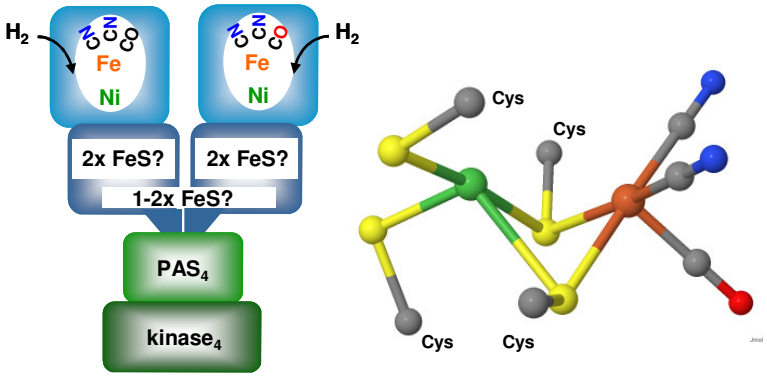


Figure 3: Protein subunit composition (left) and possible structure of the [NiFe] active site of the regulatory hydrogenase (RH) (*5*). We have obtained preliminary Ni XAS/XES data on the RH in 2014 and will continue these experiments in 2015. One goal is in-depth characterization of nickel-hydride states.

In addition, experiments at the Fe K-edge on two classical spin-crossover systems (Fe^{III}dithiocarbamate, $Fe^{II}(Phen)_2(NCS)_2$) were carried out. High-resolution XAS and Kß-XES spectra were collected in a temperature range from 10-300 K monitoring the different types of spin transitions in the complexes. Temperature variation was also applied to various reference compounds and to the [FeFe]-hydrogenase to search for effects on the spin state in the protein. We are presently summarizing these results in a publication.

Experimental: Protein samples were provided by our collaborators: [FeFe]-hydrogenase HydA1 protein samples were prepared in the laboratory of T. Happe (U. Bochum, Germany), CODH was prepared in the group of H. Dobbek (HU-Berlin, Germany), RH [NiFe]-hydrogenase was prepared in the group of O. Lenz (TU-Berlin, Germany). *For the XAS/XES experiments particularly large amounts of protein samples (several 100 µl) are necessary, which had become available by improved sample preparation procedures in the laboratories of our collaborators.* Iron model complexes were synthesized in the group of M. Darensbourg (Texas A&M Uni., USA) and by K. Klein (U. Bochum, Germany). Nickel complexes were synthesized in the groups of M. Driess (TU-Berlin) and C. Limberg (HU-Berlin). On these systems, 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) were collected using the Rowland-circle spectrometer at ID26 as previously. Data evaluation involved quantum chemical calculations (DFT) using ORCA and Gaussian.

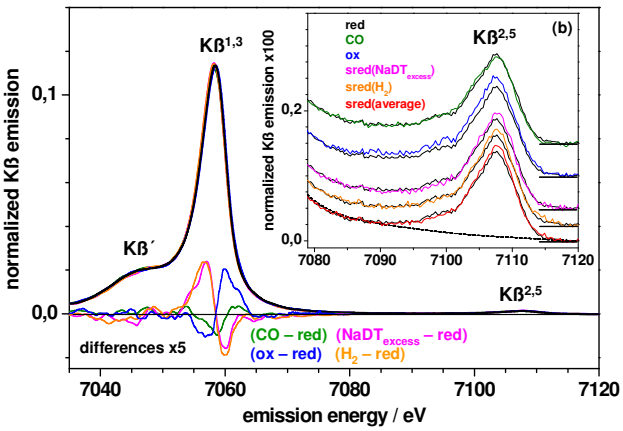
Results:

(1) Publication of data from the LTP.

Since the last report several publications based on the new data from this LTP have appeared (*3, 5-6*) or are in preparation, in addition to our previous LTP-related papers (*9-13*). A comprehensive XAS/XES and DFT investigation on [FeFe]-hydrogenase in the reduced state was published in Chemical Science, including the first site-selective XAS measurements using narrow-band Kß-detection on a complex protein-bound metal center (*6*). A particular highlight was the first characterization of an iron-hydride intermediate in [FeFe] hydrogenase (*3*). A review on X-ray spectroscopy in hydrogenase research is in press (*5*). We expect that the available data from the two beamtimes in 2014 and data from the scheduled experiments in February and June 2015 will soon lead to further high-ranking publications.

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

(A) ***Site-selective XAS/XES on native HydA1.*** Parts of our recent results on [FeFe]-hydrogenase HydA1 expressed in its native and active form in *C. acetobutylicum* have been published recently (*3, 6*). The spin-polarization of the iron Kß X-ray fluorescence emission was employed to perform site-selective X-ray absorption experiments for spectral discrimination of the two sub-complexes, $[4Fe4S]_H$ and $[2Fe]_H$, of the H-cluster in HydA1 protein in various states (oxidized, reduced, super-reduced, CO-inhibited). XANES and EXAFS spectra, Kß emission lines ($3p \rightarrow 1s$ transitions), and core-to-valence (pre-edge) absorption ($1s \rightarrow 3d$) and valence-to-core ($K\beta^{2,5}$) emission ($3d \rightarrow 1s$) spectra were obtained, individually for $[4Fe4S]_H$ and $[2Fe]_H$. Density functional theory calculations reproduced the X-ray spectral features and led to molecular structures and electronic configurations of the H-cluster. In particular, we have obtained first evidence for a iron-hydride bond in the super-reduced state of the H-cluster (Fig. 4), leading to a model of the catalytic cycle (*3*). These results represent the first site-selective characterization of a complex metal center in a protein and provide the conceptual and experimental framework for our future studies on other metalloenzymes.



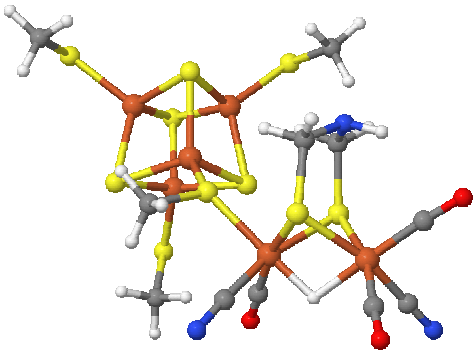


Figure 4: Kß emission spectra of HydA1 [FeFe]-hydrogenase in various states (left) and structural model of the super-reduced H-cluster based on XAS/XES and DFT containing a bridging hydride (right) (*3-4*).

(B) ***XAS/XES on HydA1 reconstituted with synthetic diiron complexes.*** We have collected full data sets of iron Kß-emission (main and satellite lines) and high-resolution K-edge absorption spectra for apo-HydA1 containing only the [4Fe4S] cluster expressed in *E. coli* and for apo-HydA1 which was reconstituted in vitro with three different synthetic diiron complexes (Fig. 2). All three constructs were studied in three or more redox states, giving more than nine XAS/XES data sets in total. The immense amount of data is currently analyzed, including DFT calculations for different spin configurations of the reconstituted H-cluster constructs. Our data reveal the quantitative incorporation of the diiron complexes into the apo-HydA1 protein. Spectral differences for the three complexes are presently analyzed. Time resolved experiments were performed, in which apo-HydA1 was mixed with the synthetic complexes and XAS/XES spectra were collected following increasing time periods after the mixing event. The goal is to monitor the kinetics of the binding of the synthetic complexes to the cubane cluster in the protein and to determine structural and electronic changes at both complexes due to the chemical bond formation. Furthermore, we hope to learn

about the electronic restraints that account for the different catalytic activities of the three constructs. These data represent the first XAS/XES experiments on in-vitro reconstituted metal complexes in proteins aiming at spin-selectivity. The results will soon lead to new publications.

(3) Spin crossover compounds. XAS/XES facilitates quantitative monitoring of spin changes in metal complexes, but relatively few data on spin-crossover (SC) complexes are available so far. For protein samples, spectroscopic data often are only available at cryogenic temperatures, but spin state changes may occur at ambient conditions where the enzymes are functional. We have carried out a systematic temperature study on two classical SC complexes, Fe^{III}dithiocarbamate and $Fe^{II}(Phen)_2(NCS)_2$ (Fig. 5). Complete sets of iron Kß-emission and K-edge absorption spectra and Kß main line RIXS planes were measured for the two complexes in the 10-300 K temperature range. In addition, several non-SC complexes were studied as reference materials. XAS/XES data as function of the temperature were also obtained for the [FeFe]-hydrogenase HydA1. These experiments are demanding because of the drastically accelerated X-ray photoreduction and damage processes at higher temperatures observed both in synthetic metal complexes and in cofactors in protein samples, which demand strategies for evaluation and avoiding of radiation artifacts. Drastic absorption and emission changes were observed in the SC compounds, which were quantitatively reproduced by DFT calculations. Complementary to our X-ray spectroscopic work, X-ray crystallographic studies were done by our collaborators. These results provide a clear and in many aspects extended model of the electronic and structural changes in the SC compounds. A respective publication is underway. The temperature-dependent spectral changes in the hydrogenase are less well understood at the moment and further work is required to clarify the involvement of spin state changes in the enzyme.

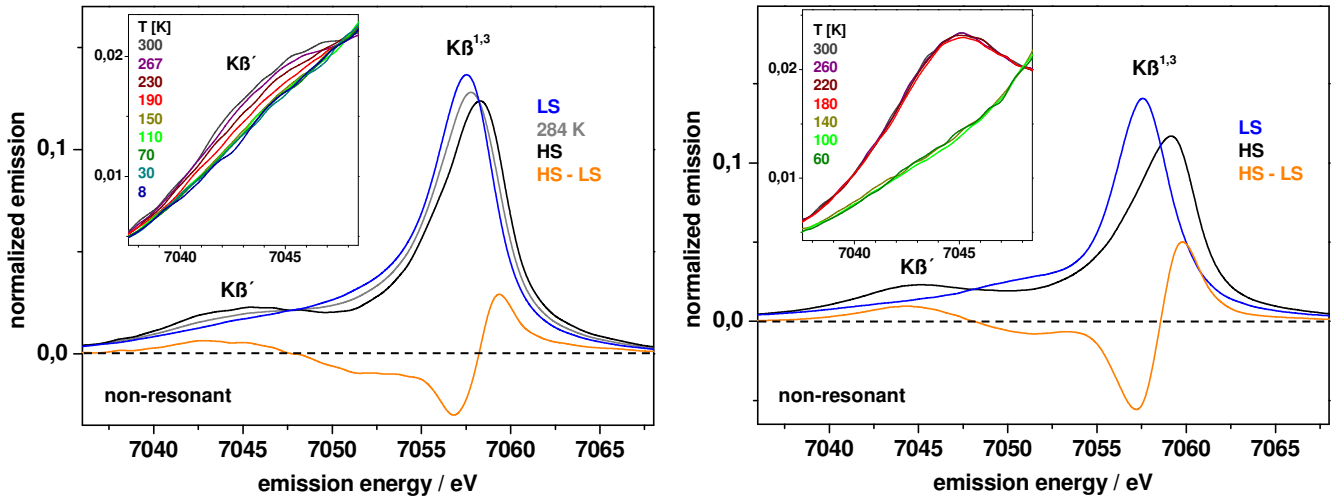


Figure 5: Kß emission spectra of two classic spin-crossover (SC) compounds as function of the temperature. Left: Fe^{III}dithiocarbamate with gradual SC; right, $Fe^{II}(Phen)_2(SCN)_2$ with abrupt SC; LS and HS denote spectra for pure low- or high-spin species (S. Mebs & M. Haumann, ms in preparation).

(4) Ni compounds. Nickel is involved in the active sites of many enzymes involved for example in hydrogen and carbon-oxide (CO_x) conversion. Prominent examples are [NiFe]-hydrogenases and carbon-monoxide-dehydrogenases (CODH) (Fig. 3). An important aspect of their reactions is the postulated formation of nickel-hydride intermediates in the catalytic cycle. Such intermediates are difficult to detect by many spectroscopic methods. XAS/XES provides a viable tool to detect and characterize metal-hydride intermediates. However, X-ray spectral changes due to Ni-hydride bond formation have not been studied so far.

We have collected sets of nickel K-edge absorption and Kß main and satellite lines emission spectra, as well as Kß RIXS data, for 14 synthetic Ni complexes with different metal ligation motifs and oxidation state, including models with Ni-hydride bonds (Fig. 6). These data will serve as benchmarks for DFT calculations and comparison with the enzyme data. We are working on a respective publication.

The regulatory hydrogenase (RH) is a particularly interesting [NiFe]-hydrogenase because it functions as a hydrogen sensor in the cell. We have collected XAS/XES data (Kß main line emission and K-edge absorption) for RH protein in the oxidized state and in the H_2-induced reduced state. In addition, spectra were

collected for RH samples illuminated at cryogenic temperatures, which is believed to affect hydride binding to the nickel. These experiments turned out to be extremely material consuming due to the rapid X-ray photoreduction of the higher valence state of nickel in the enzyme and the low metal concentration (~1 mM) in the samples. Pronounced spectral changes were observed in the preliminary K-edge data (Fig. 6). However, the signal-to-noise ratio of the spectra still is too low to allow for quantitative analysis. Furthermore, Kß valence-.to-core emission spectra, which according to our DFT calculations should be very decisive, could not be obtained yet due to sample and beamtime limitations. These spectra will be collected in the upcoming measuring periods in 2015. We are convinced that these studies on [NiFe]-hydrogenase and other nickel enzymes will soon provide new insights into the sophisticated H_2 and CO_x chemistry at the dedicated protein-bound metal complexes.

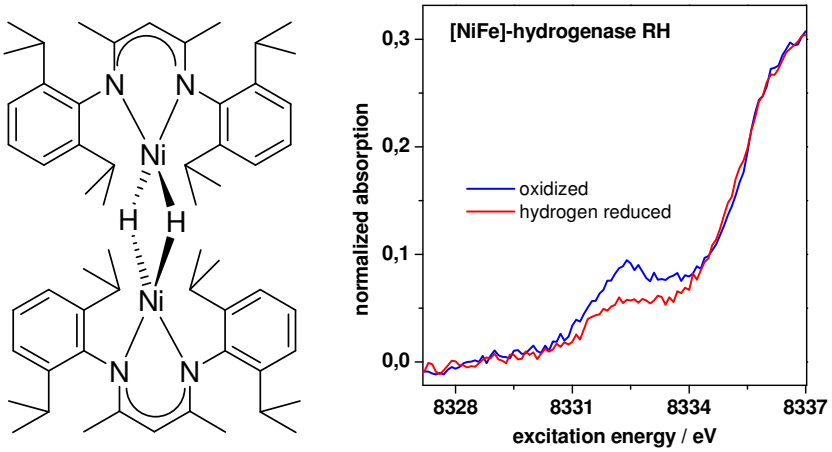


Figure 6: Nickel systems studied by XAS/XES. Left: example out of our serries of synthetic complexes showing nickel-hydride bonds. Right: preliminary Ni Kß^{1,3}-emission detected pre-edge absorption spectra of regulatory [NiFe]-hydrogenase protein in two states. Experiments at the Ni K-edge on enzymes and models will be continued in February 2015.

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

Reconstitution of proteins with synthetic cofactors for new functions is a rapidly developing field. Our XAS/XES results on [FeFe]-hydrogenase reconstituted with synthetic diiron complexes have shown that X-ray spectroscopy is a powerful tool to characterize such systems (Noth, Kositzki, et al., and Haumann, Happe, ms in preparation). We are in the process of preparing a new grant application in 2015 at the Deutsche Forschungsgemeinschaft (DFG) together with our colleagues from Bochum (Germany) on this topic.

(6) Technical development and resources.

Technical improvements. We have discussed about dispersive XES with the ID26 staff and performed test experiments at SLS in December 2014. They showed that dispersive XES likely will not work with dilute protein samples, at least in the valence-to-core emission region, using currently available hybrid-pixel detectors, because of insufficient background counts suppression. Therefore, the use of a scanning XES spectrometer as at ID26 for protein measurements seemingly is mandatory (at a conventional X-ray source). A further significant improvement in terms of minimization of deadtime and increase of experimental efficiency of the XES spectrometer at ID26 would be implementation of a continuous scanning mode.

Resources for the project. A new postdoc (S. Mebs) and a Ph.D. student (N. Schuth) were hired in the Haumann group in 2014 (funded by the BMBF), who will participate in the beamtimes in 2015 of this LTP. Extended financial resources are available from new funded projects of the Haumann group, e.g. for purchasing of equipment for use at the ID26 beamline. We are discussing this with P. Glatzel at ID26.

Conclusions:

In the two beamtime periods at ID26 in 2014, a large amount of very promising iron and nickel XAS/XES data on [FeFe] and [NiFe] hydrogenase proteins, synthetic Fe and Ni model complexes, and iron spin-crossover compounds were collected. Time-resolved and temperature-dependent X-ray experiments were carried out. Important new insight has been obtained into hydrogen chemistry in hydrogenases, which has been published (*3, 5-6*). The analysis of the data soon will lead to further publications. Further goals as formulated in the milestones of the proposal were reached, in particular with respect to characterization of metal-hydride intermediates in proteins. Dedicated work in the LTP will now be continued on the basis of secured funding in the Haumann group and in new collaborative projects. The next step will be time-resolved

XAS/XES on [NiFe]-hydrogenase and [MnFe]-oxidase enzymes, to collect core-to-valence absorption and valence-to-core emission spectra on these highly radiation sensitive systems. We expect that further significant research progress will be obtained in this LTP.

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