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 (3^{nd} beamtime)
Beamline: ID26	**Date of experiment**: from: 18.02.2014 to: 25.02.2014	**Date of report**: 16.01.2014
Shifts: 18	**Local contact(s)**: Dr. Pieter Glatzel	*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 will introduce the planned experiments during the upcoming beamtime at ID26 of ESRF in Febraury 2014, and report on the progress in evaluation and publication of data obtained during the previous beamtimes (e.g. the last one in 11/2012, partial covered also in the previous report; we note that we had no beamtime from this LTP in 2013, i.e. we will have the beamtime from the 2^{nd} half round of 2013 in 2/2014 and a further one in 6/2014).

The focus of the previous beamtime and the upcoming one is investigation of 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). Because HydA1 containes only the H-cluster and no further iron-sulfur clusters, it is the ideal system for XAS/XES searching for example for iron-hydride intermediates in the catalytic cycle.

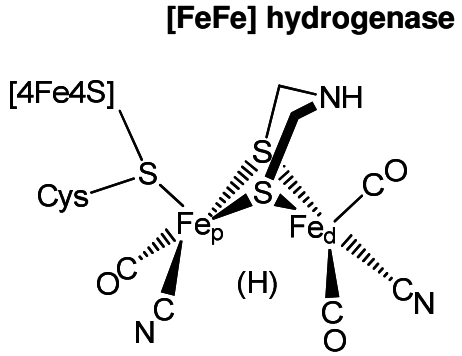

Figure 1: Global structure of the active-site H-cluster of [FeFe]-hydrogenase based on crystallographic and spectroscopic data. (H) denotes protonation at various positions in intermediates in the catalytic cycle. Searching for iron-hydride intermediates is one of the main goals in this LTP.

We have investigated the H-cluster in purified HydA1 proteins in various redox states and supplemented with small molecule reactants like CO, H_2, and O_2 by XAS/XES using Fe Kß-emission detection. Continuation of these experiments is a main topic also in the upcoming beamtime. The goal was to establish site-selective XAS/XES techniques for discrimination between the $[4Fe4S]_H$ and $2Fe_H$ units, making use of the different Kß-emission properties of the low- and high-spin irons in different coordination environments in the two sub-complexes. This has lead to the first direct evidence for formation of iron-hydride formation in the enzyme and to the proposition of a respective reaction cycle scheme.

In the last year, important progress with respect to the in-vitro reconstitution of catalytic activity in apo-HydA1 using synthetic diiron complexes has been obtained [1-2]. Now, apo-HydA1 heterologuously expressed in *E. coli*, which contains only the [4Fe4S] cluster of the H-cluster but not the diiron site, can be reconstituted with synthetic diiron complexes in vitro to obtain full catalytic activity for a complex containing an adt unit, $(SCH_2)_2NH$, bridging the two irons in the diiron site. Similar synthetic complexes have been shown to bind to the [4Fe4S] cluster also. However, these constructs show no activity but may still be active in some redox chemistry. These new approaches now offer the opportunity tu use apo-HydA1 as a "test platform" for studying the binding properties and structural and electronic changes, hydride binding, and oxygen reactions for various synthetic diiron compounds after reconstitution into the enzyme by XAS/XES. This will be one main topic in the upcoming beamtime period. We expect important that new insights into the restraints that govern the recovery of catalytic activity upon diiron complex binding will be obtained.

Experimental: For the experimental procedures, samples, and further details of the 2012 beamtime see the previous report. In the upcoming experiment, [FeFe]-hydrogenase HydA1 protein samples functionally expressed in *C. acetobutylicum*, inactively expressed (apo-protein) in *E. coli*, and, in particular, apo-HydA1 reconstituted with several synthetic diiron complexes will be prepared in various redox states in the group of T. Happe (University of Bochum, Germany). We will also continue our experiments on synthetic iron-hydride complexes, which are synthesized in the groups of M. Darensbourg (USA), M. Driess (Berlin), C. Limberg (Berlin), and F. Gloaguen (France). Besides of this, synthetic complexes showing spin-state transitions will be studied by XAS/XES. On these systems, 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) will be measured using the Rowland-circle spectrometer at ID26 as previously. Data evaluation will involve quantum chemical calculations (DFT) on model structures.

Results:

(1) Publication of data from the LTP.

Since the last report several publications based on the new data from this LTP have appeared or are under review, in addition to our previous LTP-related papers [3-6]. A study in which we elucidate the electronic properties of a hydride-forming diiron model complex has been published as a cover article [7]. A paper on structural changes at an oxygen-activating Mn-Fe cofactor in ribonucleotide reductase, as revealed by XAS and DFT, has appeared in JBC [8]. At the moment, we are assembling a manuscript on systematic comparison of a large number of [FeFe] model complexes (Fig. 2). A comprehensive XAS/XES and DFT investigation on [FeFe]-hydrogenase in the reduced state was published in Chemical Science, which includes the first site-selective XAS measurements using narrow-band Kß-detection on a complex protein-bound metal center [9]. A particular highlight was the first characterization of an iron-hydride intermediate in [FeFe] hydrogenase. These results and complementary data and DFT calculations for various redox states of the enzyme were included in a manuscript submitted to a high-ranking journal in January 2014 [10]. We expect that available unpublished data from the previous beamtimes and data from the upcoming experiments will lead to further high-ranking publications.

(2) Comprehensive study of a series of synthetic diiron compounds.

We have collected XANES and Kß emission spectra for a series of 16 crystallized diiron model compounds for the active-site H-cluster in [FeFe]-hydrogenase (Fig. 2). The main goal was to obtain high-resolution pre-edge absorption (core-to-valence transitions) and Kß-satellite ($Kß^{2,5}$) emission (valence-to-core decay) spectra and to study the spectral changes as function of ligand sphere variations using DFT calculation, for quantitative simulation of the XAS/XES spectra. High-quality spectra were obtained (complete data sets including XAS, XES, and Kß RIXS data). Data analysis is still underway. Preliminary results from DFT have revealed systematic deviations between calculated and experimental spectra, depending on the used DFT approach (functional and basis set combination). This has lead to a calibration of our DFT methods. However, generally good agreement between calculated and experimental spectra was observed. The data facilitate elucidation of systematic changes in the XAS/XES spectra depending on the chemical nature and stoichiometry of the metal ligands and the nature of the bridging dithiolate group.

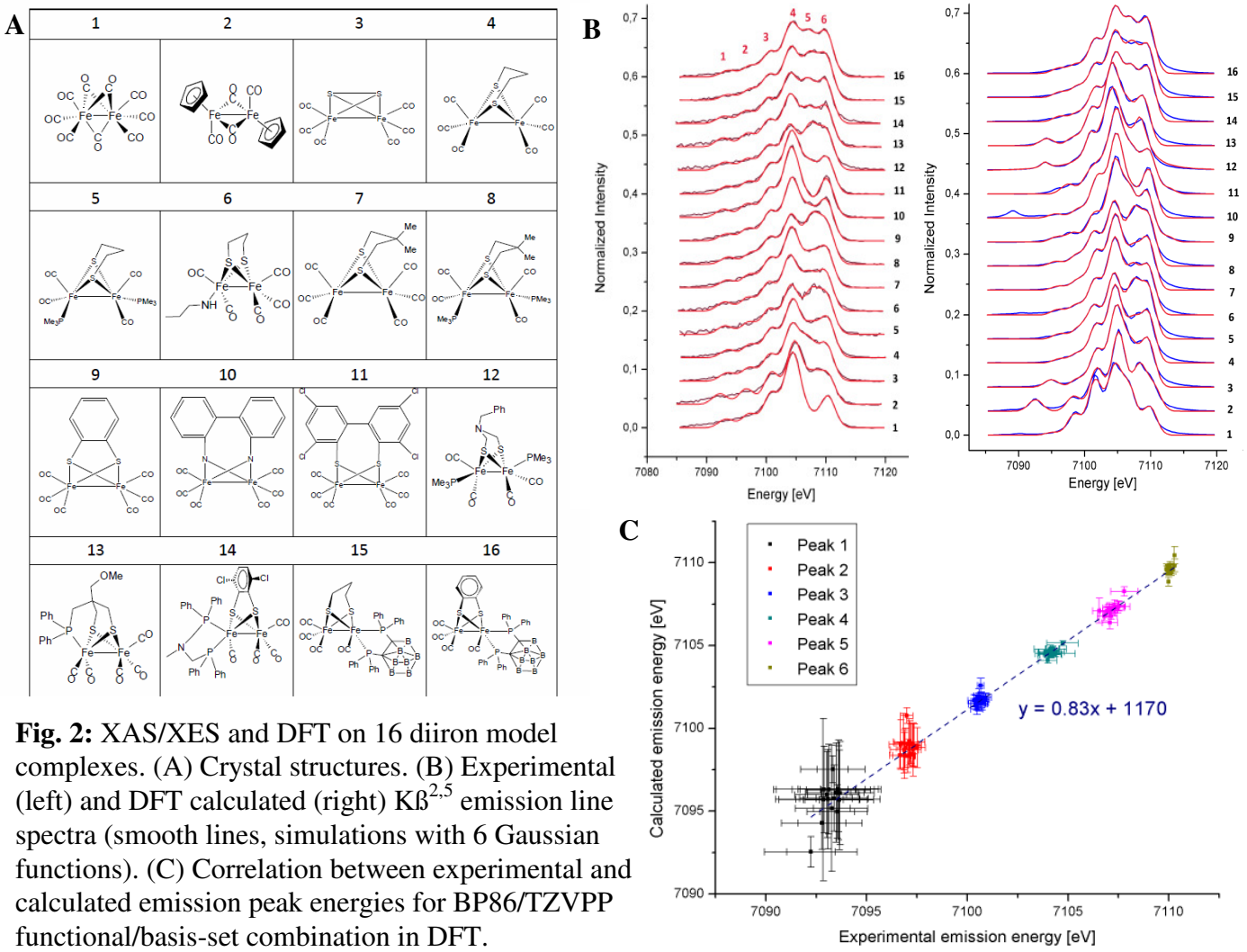

Fig. 2: XAS/XES and DFT on 16 diiron model complexes. (A) Crystal structures. (B) Experimental (left) and DFT calculated (right) Kß^{2,5} emission line spectra (smooth lines, simulations with 6 Gaussian functions). (C) Correlation between experimental and calculated emission peak energies for BP86/TZVPP functional/basis-set combination in DFT.

(3) XAS/XES on hydride-binding model complexes.

We have measured XAS/XES data for eight mononuclear and binuclear crystallized synthetic iron complexes during the previous beamtime (Fig. 3) and this study will be continued in February including further complexes as obtained from our collaboration partners. The goal was to study the sensitivity in particular of the pre-edge absorption and the valence-to-core emission with respect to the formation of iron-hydride bonds and the influence of the spin state, formal oxidation state, and ligation environment on the spectra. The underlying changes in the electronic structure were determined by DFT calculations. This revealed, i.a., the specific power of XAS/XES to determine the spin state and spin coupling for the complexes and the pronounced sensitivity in particular of the core-to-valence absorption for site-selective determination of iron-hydride bond formation [7]. This will be further addressed using complexes, which show spin transitions depending on conditions in the upcoming beamtime. We will also study possible temperature-dependent spin transitions in protein systems.

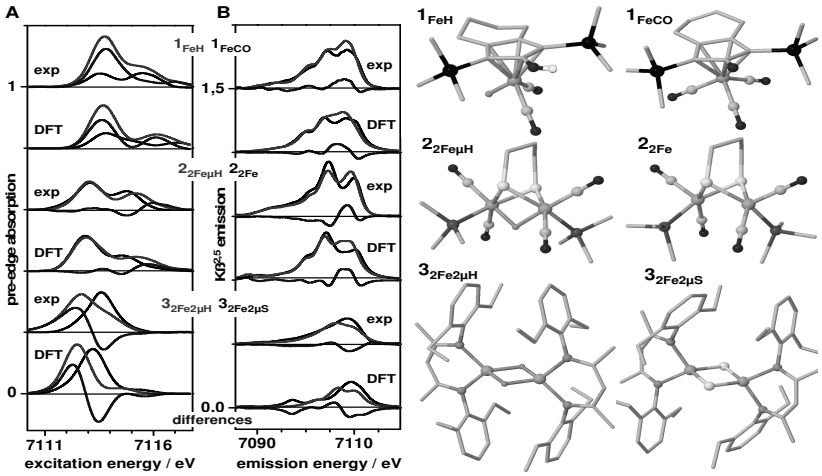

Fig. 3: XAS/XES on iron-hydride model complexes. Experimental (A) pre-edge absorption and Kß^{2,5} emission spectra are compared to DFT calculated spectra in (B). (C) Crystal structures of the complexes.

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

(A) ***Site-selective XAS/XES on HydA1.*** Our recent results on [FeFe]-hydrogenase HydA1 were published in Chem. Sci. [9] and are best summarized by citing from the respective abstract: **"**We utilized the spin-polarization of the iron Kß X-ray fluorescence emission 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 reduced HydA1 protein, 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 $[2Fe]_H$. Iron-ligand bond lengths and intermetal distances in $[2Fe]_H$ and $[4Fe4S]_H$ were resolved, as well as fine structure in the high-spin iron containing cubane. Density functional theory calculations reproduced the X-ray spectral features and assigned the molecular orbital configurations, emphasizing the asymmetric *d*-level degeneracy of the proximal (Fe_p) and distal (Fe_d) low-spin irons in $[2Fe]_H$ in the non-paramagnetic state. This yielded a specific model structure of the H-cluster with a bridging carbon monoxide ligand and an apical open coordination site at Fe_d in $[2Fe]_H$. The small HOMO-LUMO gap (~0.3 eV) enables oxidation and reduction of the active site at similar potentials for reversible H_2 turnover by HydA1, the LUMO spread over $[4Fe4S]_H$ supports its role as an electron transfer relay, and Fe_d carrying the HOMO is prepared for transient hydride binding".

These results represent the first site-selective characterization of a complex metal center in a protein and provide the conceptual and experimental framework for future studies on hydrogenases and other enzymes. Some of the key results are shown in Fig. 4. This work will be continued, using also in-vitro reconstitution of apo-HydA1 with synthetic diiron complexes, in the scheduled beamtime periods (2/2014 and 6/2014).

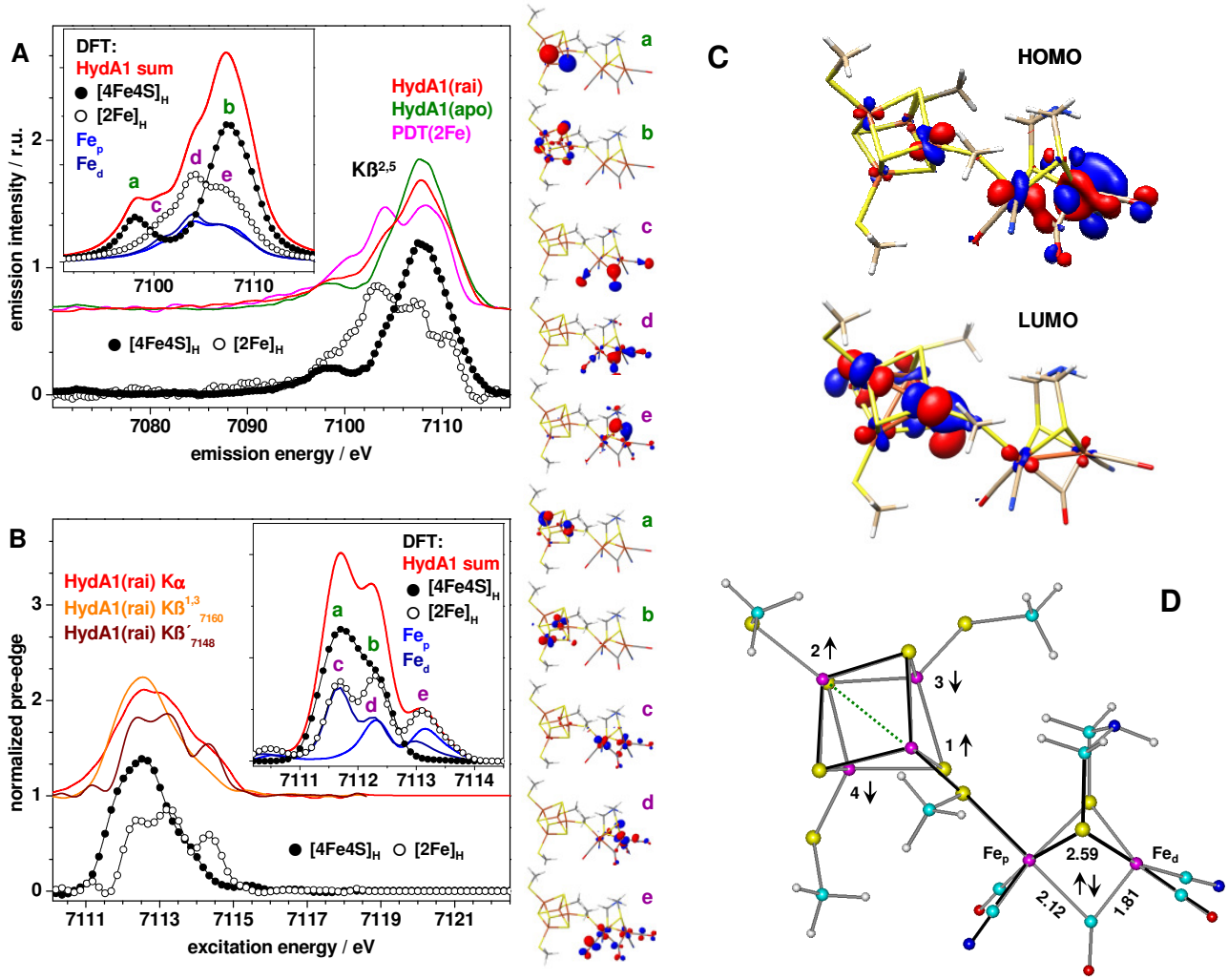

Fig. 4: Site-selective XAS/XES on [FeFe]-hydrogenase HydA1 [9]. (A) and (B), experimental and DFT calculated $K\beta^{2,5}$ emission (top) and pre-edge absorption (bottom) spectra for reduced HydA1 for the [4Fe4S] and [2Fe] sub-complexes and (right), involved occupied (top) and unoccupied (bottom) molecular orbitals. (C) HOMO and LUMO configurations. (D) Model structure from DFT including spin orientations (arrows).

(B) Hydride binding in HydA1. Whether and at which position hydride species bind to the H-cluster in the course of the H_2-forming reaction cycle is one of the most important questions in hydrogenase research. We used XAS/XES on the H-cluster in HydA1 [FeFe]-hydrogenase to address this question. An extraordinary large amount of data has been collected on different redox states of the enzyme (see also the previous report). Comparison of experimental and DFT calculated spectra has revealed formation of an iron-hydride intermediate for the first time in the [FeFe]-hydrogenase. These results have recently been submitted to a high-ranking journal [10]. For confidentiality reasons, we can not show these data here, but will do so in the next report after acceptance of our manuscript. We consider our results as a breakthrough in the characterization of [FeFe]-hydrogenase, which provides new insight into the reaction cycle of hydrogen formation and offers new ways for in-depths characterization of natively expressed and in-vitro reconstituted enzyme These experiments will be continued in the upcoming beamtime periods, using in particular HydA1 reconstituted with synthetic diiron complexes. These results represent a highlight of research in this LTP.

(5) New funding obtained for future research related to this LTP.

We have recently been granted a new collaborative research project for 4 years in the German-Swedish Röntgen-Angström Cluster funded by the German BMBF and the Swedish VR, involving two German groups working on X-ray spectroscopy (Haumann, Berlin; Schünemann, Kaiserslautern) and two Swedish groups (Ott, Uppsala; Högbom, Stockholm), entitled **"**Novel X-ray crystallography and spectroscopy techniques to unravel dimetal-carboxylate catalysis in enzymes and biomimetic materials." A central topic in this project is the characterization of oxygen-activating enzymes by XAS/XES including time-resolved methods. Part of this work will be carried out in the frame of this LTP using extended manpower and financial resources (also for upgrading of equipment at the involved ESRF beamlines ID26 and ID18). Success in the highly competetive call in part was based on the good results obtained so far in this LTP, which we consider as encouraging evidence for the novelty and originality of this work.

In summary, we consider our work in this LTP as highly successful. Experimentally highly demanding site-selective XAS/XES data on HydA1 was implemented for the first time. Extensive investigations were carried out on synthetic model complexes. New insight into iron-hydride systems has been obtained and hydride species were characterized in hydrogenase by XAS/XES for the first time. These results provide the conceptual and experimental framework for site-selective studies on heterogeneous materials. We will now extend these studies towards semi-synthetic enzyme systems and, e.g., nickel enzymes active in CO_x conversion (e.g. within collaborations in the Berlin Cluster of Excellence "Unifying Concepts in Catalysis" unicat). We expect that further interesting results from this LTP will soon become available.

(6) Problems solved.

Technical improvements. The new capabilities at the ID26 beamline for simultaneous monochromator and rapid gap-scans of the three undulators for maximal X-ray flux have greatly improved the efficiency of data collection on our dilute samples and this will be a particular advantage also in the upcoming beamtimes. We will also discuss the options for dispersive XES with the ID26 Staff on site in February, i.e. for purchasing additional equipment from the new grants of the Haumann group. We have upgraded our PC cluster for DFT at Berlin so that more efficient calculation of XAS/XES spectra also on the basis of large model structures is now feasible. This will accelerate data analysis in the future.

Resources for the project. The Haumann group has hired a new Ph.D. student (funded by the DFG), who will perform work in the LTP in the upcoming beamtimes. A postdoc and a Ph.D. student will be hired in Spring 2014 (funded by the BMBF), who will participate in future beamtimes of this LTP. Extended financial resources are now available from the new funded projects of the Haumann group, e.g. for purchasing of equipment for use at the involved ESRF beamlines.

Conclusions:

In the previous beamtime periods of the LTP, a large amount of important new data on the [FeFe] hydrogenase HydA1 and on a large variety of iron model complexes has been collected. Further technical developments at the beamline have lead to improved data quality and more efficient data collection for the dilute protein samples. Further goals as formulated in the milestones section of the proposal were reached, in particular with respect to hydride binding in [FeFe]-hydrogenase, which we consider as a particular scientific highlight. For the first time, a detailed study on site-selective XAS/XES on a protein was successfully conduced. These research directions will be further pursued in the two scheduled beamtimes in 2014. Dedicated work in the LTP will now be continued on the basis of secured funding in the group of M. Haumann involving two new Ph.D. students and a postdoc. We expect that further significant research progress will be obtained in this LTP.

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