ESRF	**Experiment title:** NIS on iron centers in [FeFe]-hydrogenase: redox changes and hydride binding	**Experiment number**: SC4151
Beamline: ID18	**Date of experiment**: from: 03.12.2015 to: 10.12.2015	**Date of report**: 29.02.2016
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Report: [FeFe]-hydrogenases are the most active hydrogen (H_2) producing enzymes in nature and therefore of superior interest for renewable energy applications. Their active site is a six-iron center termed H-cluster, consisting of a cubane cluster, $[4Fe4S]_H$, which is cysteine-linked to a diiron complex, $[2Fe]_H$, carrying CO and CN ligands and a protonable dithiolate bridge. Crystallography and spectroscopy have revealed the general structure of the H-cluster. Key questions with respect to its catalytic mechanism are related to the redox states of iron in the two sub-complexes and the binding of substrates (H_2) or inhibitors (CO, O_2).[1-5]

Using nuclear resonance vibrational spectroscopy (NRVS) at ID18, we have studied the H-cluster in the [FeFe]-hydrogenase HydA1, which was selectively labelled with ^{57}Fe at either $[4Fe4S]_H$, $[2Fe]_H$, or both sites. The HydA1 protein contained either only the $[4Fe4S]_H$ complex, which was reconstituted in vitro from inorganic compounds (i.e. using ^{57}Fe or ^{56}Fe), or the $[4Fe4S]_H$-containing protein was functionally reconstituted in vitro with a synthetic diiron complex ($2Fe_{adt}$) in its ^{57}Fe or ^{56}Fe forms, to yield the six-iron H-cluster. These procedures facilitated site-selective NRVS investigations on the two sub-complexes or on the whole H-cluster. The obtained NRVS spectra show that our experimental procedures facilitate quantitative and fully selective ^{57}Fe labeling of the H-cluster, assignment of iron-ligand vibrational modes to Fe-CO and Fe-CN bonds, and thereby discrimination of redox species of the active site.

Experimental: HydA1 apo-protein samples containing $[4Fe4S]_H$ with ^{56}Fe or ^{57}Fe were prepared in the laboratory of T. Happe (Uni. Bochum, Germany). Synthetic ^{56}Fe or ^{57}Fe diiron complexes for reconstitution were prepared in the laboratory of U. Apfel (Uni. Bochum). HydA1 protein was reconstituted with ^{56}Fe or ^{57}Fe and up-concentrated to at least 2 mM protein (8-12 mM iron).[6, 7] Protein samples were prepared in desired states under strictly anaerobic conditions using FTIR spectroscopy control.

NRVS experiments were performed at the nuclear resonance beamline ID18 of ESRF. The heat-load [Si111] and high resolution monochromators provided an energy resolution of ~0.6 meV. NRVS spectra (-20 – 120 meV, ~5 cm^{-1} resolution) and nuclear forward scattering (NFS) spectra were collected using APD detectors. 8-12 h data aquisition per protein sample was sufficient to obtain NRVS spectra of suitable quality. PDOS spectra were calculated from averaged NRVS scan data using the software tools available at the beamline.

Results:

(A) Synthetic $2Fe_{adt}$ complex and apo-HydA1 protein. The synthetic diiron complex $2Fe_{adt}$, which served as starting material for the enzyme reconstitution, was studied in the solid state and in aqueous solution (Fig. 1). The NRVS spectra proved the integrity of the compound under our conditions and revealed changes of the vibrational band structure upon binding of $2Fe_{adt}$ to the $[4Fe4S]_H$ cluster in the [FeFe]-hydrogenase. The apo-HydA1 enzyme containing only the $[4Fe4S]_H$ cluster labelled with ^{57}Fe showed that the vibrational bands of the cubane cluster are well separated from the bands of the diiron site and revealed the quantitative reconstitution of the protein (Fig. 1). NRVS band shifts upon binding of the diiron complex likely are attributed to redox and/or structural changes at $[4Fe4S]_H$ due to, e.g., an altered charge distribution.

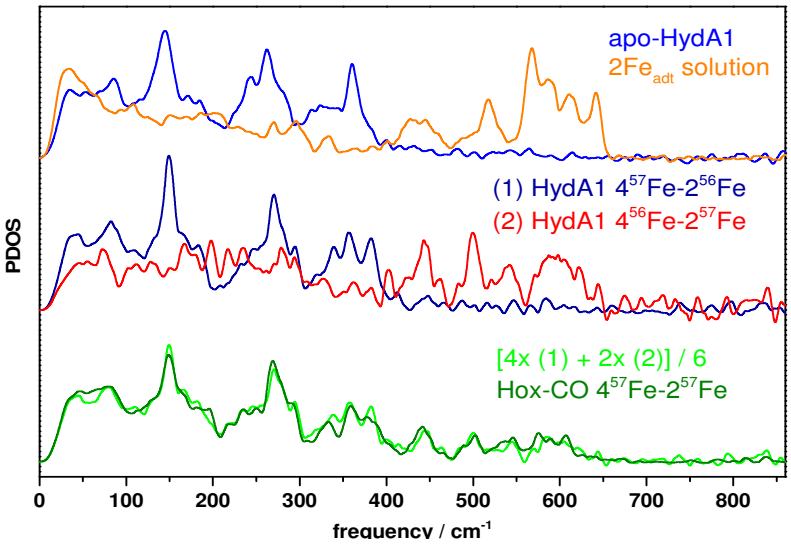

Figure 1: NRVS on [FeFe]-hydrogenase and a synthetic diiron complex. Top: apo-HydA1 with the $[4Fe4S]_H$ sub-complex and $2Fe_{adt}$ in solution. Middle: HydA1 with ^{57}Fe in the 4Fe or 2Fe subcomplexes in H_{ox}-CO state. Bottom: Completely ^{57}Fe H-cluster in HydA1 and weighted sum of spectra. Further spectra of [FeFe]-hydrogenase in other redox states were obtained.

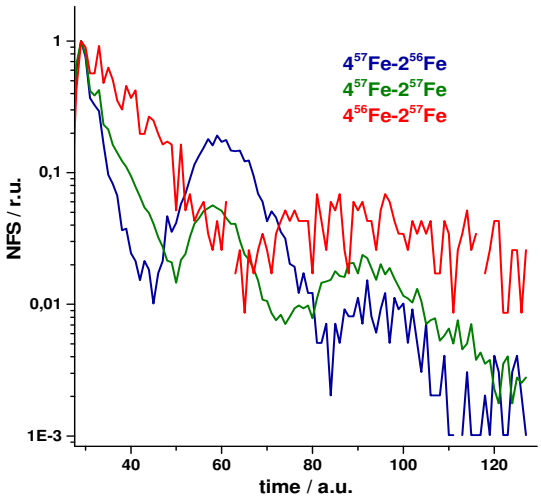

Figure 2: NFS spectra of [FeFe]-hydrogenase with site-selective ^{57}Fe labeling of the H-cluster. Spectra were amplitude-normalized.

(B) ^{57}Fe labelled HydA1 containing the H-cluster. NRVS and NFS spectra were collected for 10 HydA1 samples with ^{57}Fe labelling of the four-iron, diiron, or both sites (Figs. 1, 2). Up to 12 h of data acquisition resulted in spectra with reasonable signal-to-noise ratio. The NFS spectra show frequency changes due to different amounts of Fe(II), Fe(III), or Fe(I) species in the samples. The NRVS spectra show changes in vibrational band frequencies and amplitudes in response to binding of the diiron complex to the [FeFe]-hydrogenase and to the binding of the inhibitor CO to the diiron site. Quantitative reconstitution of the ^{57}Fe labelled diiron complex into apo-HydA1 was suggested by the data. We are presently analysing the data using density functional theory (DFT) calculations on model structures for quantitative interpretation. We aim at a comprehensive description of our NRVS, XAS/XES, and FTIR data on the studied samples with respect to redox states, ligand binding, and vibrational dynamics at the active site. This will lead to improved structural models of the H-cluster in its various redox states in the catalytic cycle of hydrogen formation.

Conclusions: Site-selective ^{57}Fe labeling of the H-cluster of [FeFe]-hydrogenase has facilitated NRVS and NFS experiments that yielded 10 spectra of the [FeFe]-hydrogenase HydA1 in different states. Our methods for in vitro reconstitution of the H-cluster lead to quantitative ^{57}Fe labeling of the samples and intact metal centers. NRVS spectra of good quality were obtained. Data analysis using quantum chemical calculations is underway. This will lead to new insights into the vibrational dynamics of the active site of H_2 formation. We consider this measuring period at ID18 as highly successful. The next steps are population of reduced states of the hydrogenase for studies on iron-hydride intermediates and publication of the present results.

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