ESRF	**Experiment title:** NIS on hydrogen formation in [FeFe]-hydrogenase: iron-hydride intermediates	**Experiment number**: LS2746
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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 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 cofactor protonation states as related, e.g., to the binding of hydride species at the iron centers. We have summarized these issues in a recent review.[1]

Using nuclear resonance X-ray scattering (NRVS and NFS) at ID18, we 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 on several states of the H-cluster, which were selectively enriched, revealed the site-selective protonation and reduction of the cofactor and clearly supports our earlier formulated, but controversially debated, model of the catalytic reaction cycle.[1-4]

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).[5, 6] Protein samples were prepared in desired states under strictly anaerobic conditions using FTIR spectroscopy control. In addition, several samples of an earlier studied oxidase protein with a ^{57}Fe labeled FeFe or MnFe cofactor were studied.[7, 8]

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) NRVS and NFS on [FeFe]-hydrogenase. NRVS and NFS spectra were collected for ca. 10 HydA1 samples with ^{57}Fe labelling (Fig. 1). Up to 12 h of data acquisition resulted in spectra with reasonable signal-to-noise ratio. The NRVS spectra show frequency changes due to different cofactor state populations in the samples (as verified by FTIR). The NFS spectra provided Mössbauer parameters. Data analysis using crystal structures and QM/MM calculations assigned the vibrational modes and the redox and protonation states of the H-cluster in the various states (Fig. 1). These recently published results supported selective protonation at the four-iron cluster in the Hred′ state and of the diiron site in the Hred state holding a hydride of the cofactor, in agreement with our model of the catalytic cycle.[2]

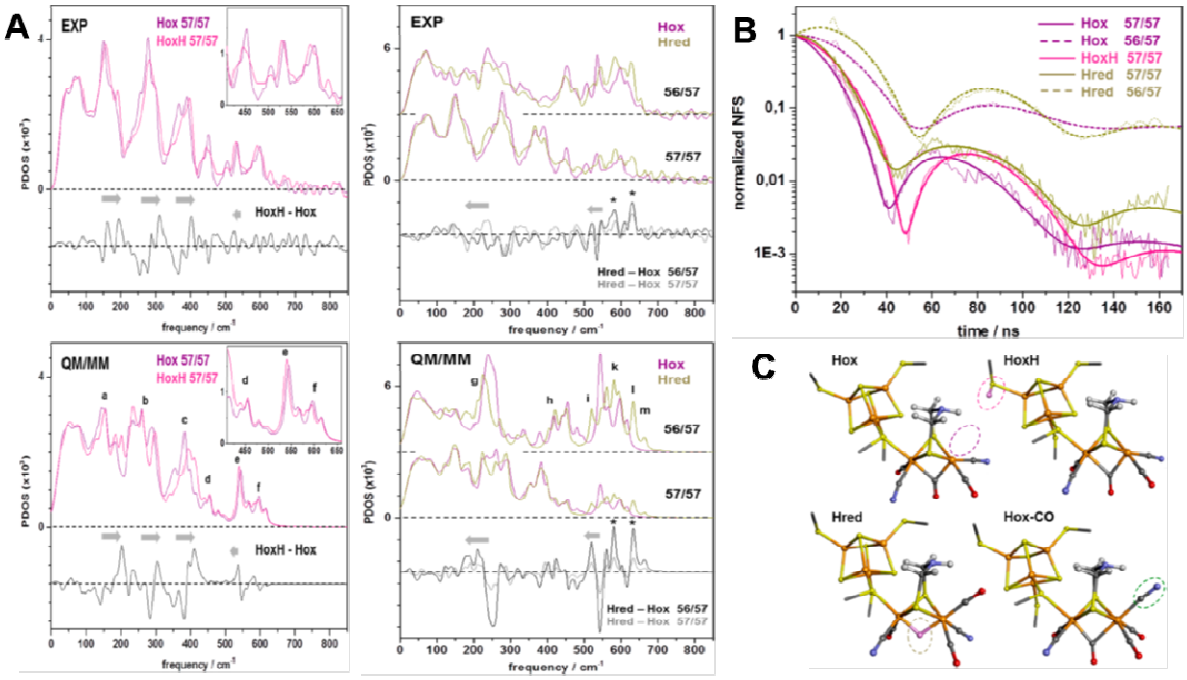

Fig. 1: NRS on [FeFe]-hydrogenase. High-quality sets of NRVS spectra (A) and NFS time traces (B) were collected. QM/MM analysis revealed the site-selective protonation at the H-cluster in the different redox states. The data collected during this beamtime were included in a recent publication.[2]

(B) NRVS and NFS on an oxidase protein. Several protein samples of an oxidase (R2lox) with a MnFe or FeFe cofactor were studied with isotopic labelling (H/D, $^{16/}$18O) in continuation of earlier work. We obtained high-quality data, which were included in a recent publication.[7]

Conclusions: Site-selective ^{57}Fe labeling of the H-cluster of [FeFe]-hydrogenase has facilitated NRVS and NFS experiments that yielded NRVS spectra and NFS data of the [FeFe]-hydrogenase HydA1 in different states. The results have clarified the differential protonation at the cofactor and shed further light on the catalytic mechanism as described in a recent publication.[2] We furthermore completed an isotope-exchange study on an oxidase protein as shown in a recent paper.[7] We consider this measuring period at ID18 as particularly successful because high-quality data were obtained. The next step is characterization of high-valent iron species in oxygen reactions of enzymes and model compounds by NRS.

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