Resonant inelastic X-ray scattering on synthetic nickel compounds and Ni-Fe hydrogenase protein

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Abstract. Ni-Fe hydrogenases are proteins catalyzing the oxidative cleavage of dihydrogen ($\text{H}_2$) and proton reduction to $\text{H}_2$ at high turnover rates. Their active site is a heterobimetallic center comprising one Ni and one Fe atom. To understand the function of the site, well resolved structural and electronic information is required. Such information is expected to become accessible by high resolution X-ray absorption and emission techniques, which are rapidly developing at third generation synchrotron radiation sources. We studied a number of synthetic Ni compounds, which mimic relevant features of the Ni site in hydrogenases, and the Ni site in the soluble, NAD-reducing hydrogenase (SH) from the bacterium Ralstonia eutropha by resonant inelastic X-ray scattering (RIXS) using a Rowland-type spectrometer at the ESRF. The SH is particularly interesting because its $\text{H}_2$-cleavage reaction is highly resistant against inhibition by $\text{O}_2$. $\text{K}$α-fluorescence detected RIXS planes in the $1s \rightarrow 3d$ region of the X-ray absorption spectrum were recorded on the protein which allow to extract $\text{L}_3$-edge type spectra. Spectral features of the protein are compared to those of the model compounds.

1. Introduction

The impact of the burning of fossil fuels on the worldwide climate urges the strive for alternative energy resources such as hydrogen. Nature’s hydrogenase ($\text{H}_2$ase) proteins, which perform the reversible production and cleavage of $\text{H}_2$, may offer blueprints for effective synthetic catalysts and for the development of biotechnological applications. One drawback for the use of $\text{H}_2$ases, e.g., in biofuel cells is their usually high sensitivity to inhibition by $\text{O}_2$ [1]. Three major classes of $\text{H}_2$ases are discriminated by their active site metal center, namely Fe-$\text{H}_2$ases with a single Fe ion, Fe-Fe $\text{H}_2$ases housing a binuclear iron site, and Ni-Fe $\text{H}_2$ases with a heterobimetallic center [2]. Interestingly, several Ni-Fe enzymes have been found, which show increased $\text{O}_2$-tolerance [2].

The bacterium Ralstonia eutropha (R.e.) houses even three Ni-Fe $\text{H}_2$ase proteins, which all are $\text{O}_2$-tolerant [2]. In general, the Ni-Fe center is ligated by the thiol groups of four cysteine residues (CysS) and the Fe atom carries two cyanide (CN) and one carbon monoxide (CO) ligand. In particular, the active site of the soluble, NAD-reducing $\text{H}_2$ase (SH) pronouncedly deviates from this situation [3]. In the SH, the Ni and Fe ions seem to bind an additional CN ligand and besides of the sulfur ligands to
Ni, also oxygen ligands are detected by XAS, which possibly stem from sulenate (CysSO) groups [3] (Fig. 1). The unusual Ni (and Fe) coordination is expected to alter the electronic properties of the metal center, thereby influencing its reactivity with oxygen species to increase the O$_2$ tolerance.

**Figure 1**: Structural models of the Ni-Fe site of the O$_2$-tolerant soluble H$_2$ase (SH) of *R.* *e.* [3]. The native site (left) with extra CN ligands at Ni and Fe and XAS-detectable O-ligands at Ni can be converted to a more conventional structure (right) as in O$_2$-inhibited H$_2$ases under various conditions [3].

Synthetic chemistry has generated a considerable number of Fe-Fe and Ni-Fe site mimics. However, their H$_2$-activity usually is still much lower than in H$_2$ases. The comparison of H$_2$ase active sites and model compounds by X-ray spectroscopy may lead to insights into the principles that govern the activity of the proteins and help to find strategies for the synthesis of improved H$_2$ catalysts [4,5].

The high flux of the X-ray beams at undulator beamlines of modern synchrotrons offers new opportunities for high-resolution X-ray spectroscopy in particular on dilute protein samples. Developing techniques such as resonant inelastic X-ray scattering (RIXS) can provide detailed structural and electronic information on the metal centers also in EPR-silent states [6]. Site- and ligand-selective XAS may become feasible. So far, only few RIXS studies on proteins are available.

2. Materials and Methods

SH protein from *R.* *e.* was purified as in [3]. As-isolated SH protein and H$_2$-reduced enzyme obtained after repeated degassing and incubation with H$_2$ gas for 10 min at 20 °C was studied. SH samples (~20 µl) in acrylic glass sample holders for X-ray experiments contained about 1 mM of Ni. Ni compounds were synthesized in our laboratories. Ni salts were purchased from Sigma. Samples of Ni compounds were prepared by dilution with boron nitride and grounding to a fine powder, which was filled into Kapton-covered PVC sample holders. RIXS experiments were performed at beamline ID26 of the ESRF (Grenoble, France). The excitation energy was selected by a Si(111) double-crystal Rowland-circle spectrometer. A total energy bandwidth of 1.3 eV at the Ni K$_{\alpha}$ fluorescence line was achieved using the [533] Bragg reflection of one spherically-bent Si wafer (R = 1 m). An avalanche photodiode served as a detector. RIXS planes were collected by scanning of the excitation energy over the Ni K-edge and of the detected emission energy over the K$_{\alpha}$ line. Samples were kept in a liquid-He cryostat at 20 K. RIXS planes were recorded within ~5 min (one plane per sample spot). Final RIXS data are the average of 8-12 planes. In-house MATLAB programs were used for data analysis.

3. Results and Discussion

The oxidation and spin states of Ni are reflected in the pre-edge region of XANES spectra due to dipole-forbidden but quadrupole allowed 1s→3d transitions, which may gain dipole intensity by d-p orbital mixing. In 1s2p RIXS, the incident energy (IE) is tuned to 1s→3d$^n$ resonances to populate intermediate 1s3d$^{n+1}$ states and the radiative 2p→1s decay (at emission energies EE) is monitored, leading to the final 2p$^3$3d$^{n+1}$ states at energies TE = IE - EE (transfer energy). The intermediate state in RIXS is the final state in K-edge spectroscopy and the final state may be assumed to be similar to L-edge spectroscopy. RIXS provides high resolution (i) of 1s→3d transitions (at constant EE, CEE), (ii) of 3d orbital energies (at constant TE, CTE), and (iii) of final state energies (at constant IE, CIE).

Figure 2A shows the RIXS plane at the K$_{\alpha}$ emission line of a binuclear Ni compound (5). From such data, CEE, CIE, and CTE line plots were extracted along the dashed lines.

1 For complexes 4 and 5: Manuscript in preparation
In contrast to conventional Ni K-edge spectra the 1s→3d (arrow) and further resonances in the edge rise are well discernable (Fig. 2B), allowing to address changes in the line shape and energy due to the Ni coordination and oxidation state.

**Figure 2:** (A) RIXS plane of Ni complex 5\(^1\) and (B) conventional XANES spectrum (dashes) and CEE line plot (solid line) of 5.

RIXS planes were collected for seven Ni(II) compounds (see Fig. 4) with variations in the number and ratio of more ionic (O,N,Cl) or more covalent (S,P) ligands. Figure 3 shows respective plots for constant final state energy (CTE), due to 1s→3d resonances. The following features are apparent: (i) one main resonance appears for the compounds with octahedral or square-planar Ni coordination by ionic ligands (1-3), but unresolved multiplet structure is visible for compounds with mixed coordination (4-6)\(^1\) and/or with tetrahedral (6,7) or square-pyramidal Ni (4)\(^1\); (ii) the energy of the main resonance varies by ~1 eV for the same Ni(II) oxidation state, with the lines of the compounds with the more covalent S,P ligands being shifted to lower energies.

Figure 4 shows L\(_{3,2}\)-edge type spectra (CIE plots). (i) The absence or presence of multiplet structure resembles that of the 1s→3d resonances; line splitting is observed for tetrahedral and (less pronounced) square pyramidal Ni, but absent for O\(_6\) and square-planar Ni. In part, the line splitting may reflect high-spin Ni(II) character in 6 and 7. (ii) In contrast to the 1s→3d, the main L\(_3\) lines are at higher energies for more covalent Ni.

**Figure 3:** 1s→3d resonances in CTE plots of Ni compounds in Fig. 4. Dashes mark the main line energy.

**Figure 4:** L\(_{3}\)-edge type spectra (CIE plots) of the shown Ni compounds. Right, L\(_3\) and L\(_2\) features at the K\(_{\alpha1}\) and K\(_{\alpha2}\) emission lines; left, magnification of the L\(_3\)-spectra (dashes mark shifts in the line energy).

RIXS data were collected for SH protein in its as-isolated, oxidized state and after incubation with H\(_2\) gas (Fig. 5). The data show a limited signal-to-noise ratio due to the low metal concentration. Weak multiplet structure seems to be present on the 1s→3d (CTE) and L\(_3\)-lines (CIE), in agreement with a mixed S/O Ni coordination, compared to the models. Likely low-spin Ni(II) is present. The line energies are similar, consistent with Ni(II) species under both conditions as also revealed by EPR [3].

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The slight shift to lower energies of the L$_3$-edge in the oxidized SH could reflect higher electron density at the Ni and/or a change in coordination. It is known that an O- ligand (possibly from a hydroperoxide) becomes detached from the Ni upon reduction of the SH [3]. The CEE plot shows a shoulder in the K-edge rise (hardly seen in conventional XANES, Fig. 5). It may indicate a fraction of SH protein with a more conventional Ni-Fe site (Fig. 1, right). Resolved K-edge structure may hence serve to discriminate between site species in heterogeneous protein samples.

Figure 5: K$_{α1}$ RIXS plane of SH protein in the as-isolated oxidized and H$_2$-treated states and respective line plots.

4. Concluding Remarks

RIXS data of a number of Ni model compounds and a hydrogenase protein (SH) were presented. Shifts in the energies and line features of 1s→3d resonances and L$_{3,2}$-edges are observed even for the same Ni(II) oxidation state, depending on the ligand environment of the metal. It was possible to collect RIXS data also for a highly diluted H$_2$ase protein sample. The data are compatible with a mixed-O,-S ligand environment of Ni(II). However, only weak spectral changes were detected after H$_2$-treatment of the protein, possibly due to non-quantitative reaction with H$_2$. Recently, a new XES spectrometer has been built at beamline ID26 of the ESRF, allowing for the use of five analyzer crystals. This instrument will greatly improve the signal-to-noise ratio in measurements on proteins, for future in-depth analysis of structure and electronic configuration of biological metal sites. We are currently exploring RIXS data simulation using a density functional theory (DFT) approach.

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