Ni L-edge Magnetic Circular Dichroism of Ni Rubredoxin and Reduced NiFe Hydrogenases

Hongxin Wang,[1] Corie Y. Ralston,[2] D. S. Patil,[2] C. Bryant,[1] M. M. Verhagen,[3] M. W. W. Adam,[3] and S. P. Cramer[1,2,*]

1 Department of Applied Science, University of California, Davis, CA 95616
2 Lawrence Berkeley National Laboratory, Berkeley, CA 94720
3 Department of Biochemistry, University of Georgia, Athens, GA 30605

Keywords: NiFe hydrogenase; Ni rubredoxin; Ni enzymes; magnetic circular dichroism; XMCD; sum rules

Abstract

X-ray magnetic circular dichroism (XMCD) measures the inner shell absorption difference between left and right circularly polarized (lcp / rcp) X-rays in the presence of a magnetic field, and provides a direct probe of the spin values localized in the specific metal site. In this study, Ni L-edge XMCD spectra of Ni rubredoxin, and of H_2 reduced *Desulfovibrio desulfuricans, Desulfovibrio baculatus* and *Desulfovibrio gigas* hydrogenases have been measured and analyzed. XMCD multiplets suggest a high spin Ni^{II} while XMCD effects also indicate non-zero Ni spin values for all these enzymatic Ni sites. XMCD sum rule analysis has been carried out to derive the semi-quantitative orbital and spin angular momentum for the Ni sites inside Ni rubredoxin and reduced *D. desulfuricans* hydrogenase.

* To whom the correspondence should be addressed.

Introduction

Hydrogenase is a Ni-containing enzyme that catalyzes the reversible reaction of hydrogen (H_2) formation / consumption..[1-3] It is important not only for its key role in the biological process of respiration, but also for its potential use in bio-energy applications.[4-6] Industrial hydrogen, which is now mainly used as chemical material in refineries and ammonia plants, is equivalent to 1.3% of the primary energy production in this country if burned as fuel.[7] Demand for hydrogen as a clean energy resource will continue to grow because of increasingly restrictive environmental concerns and the need to develop zero-emission vehicles for urban transportation.[8] Understanding the catalytic mechanisms for various hydrogenases is essential in utilizing them for potential bio-H_2 generation and is therefore important for our future energy needs.

NiFe hydrogenase exists in several organisms, all containing one NiFe center for catalysis and several Fe-S clusters for electron transfer. [1-3] For *Desulfovibrio gigas* hydrogenase (as well as several other hydrogenases), the as-isolated (oxidized) 'unready' state produces an EPR-detectable signal and is labeled "Ni-A". Activation of Ni-A with hydrogen (H_2) reduces the Ni to a manifold of various active species, Ni-SI, Ni-C and Ni-R, with alternating silent/active signals. It has been established that the EPR silent Ni-R state has the lowest potential,[9] and that this fully reduced state contains a Ni^{II} ion[1,10], but it is still unclear whether the electronic configuration is low spin (ls-) Ni^{II} [11-14] or high spin (hs-) Ni^{II} [15-17].

The Ni rubredoxin metal center[Huang, 1993 #1130; Huang, 1993 #1027] is structurally similar to Fe rubredoxin [Day, 1992 #1133][Bau, 1998 #1138] except that the Fe is replaced with Ni. It has a single metal site (Ni) with four cysteinyl S ligands. There is no crystal structure data reported for Ni rubredoxin, but by analogy to the crystal structure of Fe rubredoxin[18] and the similar Ni desulforedoxin,[19] the Ni in rubredoxin may have a T_d geometry with the surrounding S. Its smaller molecular size (6 KDa), and similar Ni-S arrangement make Ni rubredoxin an ideal starting system for studying NiFe hydrogenases and other Ni proteins. Oxidized Ni-substituted rubredoxin has already been used as a model to understand the Ni-C EPR spectrum of NiFe-hydrogenases.[20] While the oxidized Ni rubredoxin contains Ni^{III}, its resting form is expected to have an electronic state of hs-Ni^{II}, considering the T_d geometry of the metal cluster.

A Ni^{II} ion can have either a high spin (hs-) state in which two electrons are unpaired while the other six electrons are paired (S=1, Fig. 1a), or a low spin (-ls) state in which all eight

electrons are paired (S=0, Fig. 1a'). For 4-coordinate structures, tetrahedral and square planner complexes typically consist of hs-Ni^{II} and ls-Ni^{II} sites respectively. For 5-coordinate cases, trigonal-bipyramidal complexes (Fig. 1b) are usually hs-Ni^{II} while square-pyramidal (Fig. 1c) complexes tend to contain ls-Ni^{II}. [21,22] However, the electronic spin state for a distorted five coordinate complex is not simple to predict because the geometric difference between even the two extreme 5 coordinate cases (Fig. 1b and c) is small.

X-ray magnetic circular dichroism (XMCD) measures the inner shell absorption difference between left (lcp) and right (rcp) circularly polarized X-rays in the presence of a magnetic field,[23,24] and can provide a direct probe of the spin values localized in the specific metal (*e.g.* Ni) site. For example, there is no XMCD effect for a zero spin species. Further, two important magneto-optical sum rules [23,25-27] allow quantitative predictions to be made about the orbital and spin angular momentum for particular elements in complex samples. In contrast to traditional magnetic measurements such as EPR spectroscopy, X-ray magnetic circular dichroism is element and site specific. In comparison with K-edge XMCD, L-edge XMCD has several additional advantages, including a better spectral multiplet[28-30] and a much larger MCD effect. [Ralston, 1995 #269]

XMCD is the consequence of the selection rules for electric dipole-allowed transitions and the XMCD effect relies on the non-uniform occupation in the energy levels for the initial state. Measurement of an XMCD signal requires a circularly polarized X-ray source, strong magnetic fields (Zeeman split), and low temperatures (Boltzman distribution). In comparison with experiments on ferromagnetic materials,[23,24] extremely high magnetic fields or extremely low temperatures are necessary when trying to magnetize dilute and paramagnetic biological metals.[30] In addition, an ultra-high magnetic field can interfere with a semiconductor X-ray fluorescence detector if the detector is close to the center of magnetic field (sample location), while a low signal-to-noise ratio for the measurements will result when the detector is far from the sample. Therefore, extremely low temperature is usually preferred.[27,31]

In this study, Ni L-edge XMCD for a series of Ni enzymes was investigated using a newly constructed third-generation XMCD apparatus with a superconductor magnet (2 Tesla) and a $^3He/^4He$ dilution refrigerator (reaching 0.5 Kelvin). Ni rubredoxin and H_2 reduced *D. desulfuricans*, *D. baculatus* and *D. gigas* hydrogenases were studied under identical conditions. The XMCD spectra for the Ni rubredoxin and for the H_2 reduced *D. desulfuricans* hydrogenase show a clear high spin Ni^{II} multiplet in the absorption spectra and in the XMCD effects. XMCD

sum rule analysis also shows a significant and comparable $\langle S_Z \rangle$ for these two Ni enzymes, again indicating a high spin state. For H_2 reduced *D. baculatus* and *D. gigas* hydrogenases, although the signal to noise ratio (S/N) is low, the XMCD spectra clearly indicate non-zero XMCD effects.

Experimental Aspects

Sample Preparation As reported in detail previously, *Desulfovibrio* enzymes from bacteria were grown on a sulfate-lactate medium and purified by ion-exchange chromatography.[16,32,33] Hydrogen-reduced *D. desulfuricans*, *D. baculatus* and *D. gigas* hydrogenase samples were prepared by incubation under H_2 for about 8 hours. The film samples for the L-edge XMCD measurement were prepared by syringing a drop (~20 μL) of protein solution onto a gold plated sample holder and allowing it to dry under H_2 atmosphere.[16] The resting form of Ni rubredoxin was prepared in Adam's laboratory (University of Georgia) according to published procedures. [34]

The NiFe hydrogenase activities were estimated by measuring the hydrogen gas evolved in a reaction using methyl viologen as electron carrier and dithionite as reductant.[16] The H_2 reduced *D. gigas* enzyme solution has a specific activity of 500 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$. After re-dissolving the dried material, the measured activity was 450 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$. After the soft X-ray measurements, the re-dissolved sample activity was 400 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$. The measured activities for the H_2 reduced *D. baculatus* hydrogenase were 650 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$, 600 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$, and 500 ± 50 $\mu MH_2 \cdot min^{-1} \cdot mg^{-1}$ for the solution, the re-dissolved partially-dehydrated film, and the re-dissolved film after the soft X-ray measurements respectively.

XMCD Setup The XMCD apparatus uses a 76 cm split-coil 2 Tesla superconducting magnet in a UHV chamber maintained at ~5×10^{-9} torr.[16,35] The cold finger of a $^3He/^4He$ dilution refrigerator (~ 0.5K) enters the magnet bore from the top of the chamber and the sample is attached to the cold finger at the center of the magnet. A 30-element windowless Ge fluorescence detector[16,27,35] is inserted horizontally between the two coils, perpendicular to the incident X-ray photon beam. The partially-dehydrated film samples were transferred anaerobically to the measurement chamber by a loadlock. The entire chamber is shielded with 4.2K liquid helium and 77K liquid nitrogen. The shield has entrance and exit holes for the

incoming X-ray beam and the outgoing fluorescence emission signal, and an open-close door for sample loading.

The temperature on the cold finger is monitored down to 1.4 K with a Lakeshore DT-470-12A silicon diode. Cold finger temperatures < 4.2 K were measured using a carbon resistance thermometer (Matsushita) and calibrated by the field dependent XMCD effects for Ni^{II} doped MgO crystal.[36] Least squares fitting with a Brillouin function for a J=1 system (Ni^{II}/MgO) gives a temperature of ~0.5K, corresponding to >99% magnetic saturation at 2 Tesla.. Ni enzyme samples were measured under the same condition. To avoid excessive heat input to the dilution fridge via eddy current heating of the copper cold finger, a moderate magnetic field sweep rate (50 g/s) was used. After a magnetic field flip from -2 to 2 Tesla, a minor temperature rise (<100 mK) returned to the baseline temperature within 5 minutes.

XMCD measurements were performed at the Stanford Synchrotron Radiation Laboratory (SSRL) bend magnet beamline 8-2[37,38] using the 1100 l/mm grating. Elliptically polarized X-rays were obtained by moving the first mirror above or below the electron orbit plane, as reported previously.[27,39-42] Based on previous calibrations using ferromagnetic samples[42] and a Ni doped MgO crystal,[36] the optimum XMCD signal was obtained at a beam take off angle corresponding to a circular polarization of 80 ± 5%.[27,36,42] At this take off angle the beam intensity is 75% off its maximum value at the electron orbital plane axis. The entrance and exit slits were both set to 60 μm, and the energy resolution was estimated to be 1.4 eV for circular polarized X-rays.

The signal from the 30 element Ge detector was amplified with 30 Canberra 2026 shaping amplifiers, using 3 μsec shaping time. These pulses were then sent to 8 Canberra 8224 multiplexers to re-organize signals into 8 channels. Peak height analysis of the pulses were performed using 8 Canberra 8715 analogue to digital converters (ADCs). The resulting 8-channel signals were then converted back to a 30 channel histogram (again using 8 multiplexers) and the histogram was stored in memory of a DEC Alpha 3000 computer. One integration region was defined around the Ni L fluorescence signal (F) while the second window was set around the oxygen K fluorescence, used as a measure of the incident intensity (I_0). The incident beam intensity was also independently monitored by the photocurrent measurement from a gold-coated grid in front of the samples. The raw spectra (both F and I_0) were collected as a function of excitation energy and saved for analysis.[16,27]

Data Acquisition / Process Individual scans were taken over the Ni L-edges using 0.2 eV steps at 30 seconds per point integration time for hydrogenase samples and 15 seconds for Ni rubredoxin. One set of scans (20 for each hydrogenase, 16 for Ni rubredoxin) was taken with 'right' circular polarized X-ray. Every two scans, the magnetic field was switched between –2 to 2 Tesla. A second set of scans was then measured with 'left' circular polarized X-ray, again alternating the sign of the magnetic field. As expected, the XMCD effect did reverse with opposite beam polarization or opposite magnetic field. These scans were then classified into two categories, the scans with the 3d electronic spin being parallel to the X-ray helicity ($I^{\uparrow\uparrow}$) and the ones with the 3d spin being antiparallel ($I^{\uparrow\downarrow}$) to the X-ray helicity.[27] The final spectrum is the sum of individual scans for each side.

Each of the averaged spectra was first corrected for the small energy offset (~ ±0.1 eV) between spectra recorded on opposite polarization based on the test of model compounds.[27,42] To align the polarized absorption spectra for both sides, the spectra were normalized to the background intensities below the L_3-edge. A cubic polynomial was then fit to the raw data in the pre- L_3 and post- L_2 regions to simulate the two-step non-resonant (background) X-ray absorption for the two absorption spectra respectively. The fit non-resonant steps and oxygen K background were then subtracted from the overall spectrum, leaving only the resonant components of the absorption signals. Finally, the spectra were corrected for the fact that the illuminating X-rays were only 80% polarized, by[27]

$$I^{\uparrow\uparrow} = \{(\alpha+1)\cdot I^{\uparrow\uparrow}_m+(\alpha-1)\cdot I^{\uparrow\downarrow}_m)/2\alpha \qquad (1),$$

$$I^{\uparrow\downarrow} = \{(\alpha+1)\cdot I^{\uparrow\downarrow}_m+(\alpha-1)\cdot I^{\uparrow\uparrow}_m)/2\alpha \qquad (2),$$

$$\text{XMCD} = (I^{\uparrow\uparrow}-I^{\uparrow\downarrow})/(I^{\uparrow\uparrow}+I^{\uparrow\downarrow}) = \{(I^{\uparrow\uparrow}_m - I^{\uparrow\downarrow}_m)/(I^{\uparrow\uparrow}_m + I^{\uparrow\downarrow}_m)\}/\alpha \qquad (3),$$

where $I^{\uparrow\uparrow}_m$ and $I^{\uparrow\downarrow}_m$ represent the observed spectra, α is the degree of X-ray polarization, $I^{\uparrow\uparrow}$ and $I^{\uparrow\downarrow}$ are the polarization-corrected intensities, and XMCD is the difference spectrum (also called XMCD spectrum).

Sum Rule Analysis There are two important XMCD sum rules[25-27] which relate the integrated XMCD intensities to element specific projections of the 3d orbital angular momentum $\langle L_z \rangle$, the 3d spin angular momentum $\langle S_z \rangle$, and a magnetic dipole term $\langle T_z \rangle$. For 3d transition metal L-edges, these sum rules are summarized in Equations (4) and (5) respectively.[27]

$$\langle L_z \rangle = -2n_h \cdot \frac{\int_{L_3+L_2} \left[I^{\uparrow\uparrow} - I^{\uparrow\downarrow} \right] d\omega}{\int_{L_3+L_2} \left[I^{\uparrow\uparrow} + I^{0} + I^{\uparrow\downarrow} \right] d\omega} \qquad (4),$$

$$\langle S_z \rangle = -\frac{3n_h}{2} \cdot \frac{\int_{L_3} \left[I^{\uparrow\uparrow} - I^{\uparrow\downarrow} \right] d\omega - 2\int_{L_2} \left[I^{\uparrow\uparrow} - I^{\uparrow\downarrow} \right] d\omega}{\int_{L_3+L_2} \left[I^{\uparrow\uparrow} + I^{0} + I^{\uparrow\downarrow} \right] d\omega} \cdot \left\{ 1 + \frac{7}{2} \frac{\langle T_z \rangle}{\langle S_z \rangle} \right\}^{-1} \qquad (5),$$

Here, n_h represents the number of 3d vacancies in the metal ion, ω is the X-ray frequency, and I^0 refers to the absorption for X-rays linearly polarized along the magnetization direction; as is customary, we approximate this by $(I^{\uparrow\uparrow}+I^{\uparrow\downarrow})/2$. In addition, the $\langle T_z \rangle$ term will be averaged to zero for randomly oriented samples (Ni enzymes).[23] Omitting the $\langle T_z \rangle$ term, approximating I^0 by $(I^{\uparrow\uparrow}+I^{\uparrow\downarrow})/2$ and using the common symbols in the XMCD literature, one can thus re-express the sum rules for 3d transition metal L-edges as (in $\hbar$/atom):[23,27]

$$\frac{\langle L_z \rangle}{n_h} = -\frac{2(A+B)}{3C} \qquad \frac{\langle S_z \rangle}{n_h} = -\frac{A-2B}{2C} \qquad (6),$$

where 'A' and 'B' refer to the integral of the XMCD spectrum over the L_3 and L_2 regions respectively, and 'C' refers to the integral of the spectrum $(I^{\uparrow\uparrow}+I^{\uparrow\downarrow})/2$ over the whole L_3 and L_2 region. As the number of 3d holes in our Ni samples is unknown, orbital and spin angular momentum per unit hole were calculated and compared instead.

Results and Analysis

Ni Rubredoxin We have first examined XMCD for Ni rubredoxin, which has a relatively simple Ni site.[34,43,44] Corrected for the degree of polarization, the Ni L-edge excitation spectra of Ni rubredoxin for the Ni 3d electronic spin being either parallel ($I^{\uparrow\uparrow}$) or antiparallel ($I^{\uparrow\downarrow}$) to the X-ray helicity are shown in Fig. 2a. The L_3 region has a major peak near 853.2 eV and a minor peak near 855.2 eV, exhibiting a typical hs-Ni^{II} multiplet.[15,16,45-47] In the simplest interpretation, the L_3-edge main peak and secondary peak arise from final states with a valence shell hole spin parallel or antiparallel to the core-shell hole spin.[48] The L_2 at 868.5-872.5 eV has a partially resolved doublet structure (peaks at 869.8 and 871.2 eV), again a typical high-spin Ni^{II} feature.[45,47] The spectral multiplets are quite similar for the absorption for both $I^{\uparrow\uparrow}$ and $I^{\uparrow\downarrow}$, although the relative multiplet intensities are different.

The polarization-corrected difference in fluorescence signal ($I^{\uparrow\uparrow} - I^{\uparrow\downarrow}$) - the X-ray magnetic circular dichroism, is shown in Fig. 2a'. At the L_3edge, there is a 32% difference in polarization-corrected absorption intensity between the two L_3-edge main peaks, corresponding to a 16% XMCD effect. At the L_2edge, the XMCD effect is about 24%. The two sub-peaks of the L_3 XMCD spectrum have opposite signs, while the two of the L_2 have the same sign. The bipolar XMCD signal at L_3 is typically a feature of hs-Ni^{II} complexes[36,47] unless there is a strong crystal field distortion (like in D_{2h} or other low symmetries) which could cancel out some of the shoulder features. This effect is not from any anti-ferromagnetic interactions.[49] The bipolar XMCD signal at L_3 has an intensity ratio of 4:1, close to XMCD spectra reported previously for other high spin Ni^{II} systems.[36,47]

In contrast, the Ni L-edge excitation spectra for the 'square-planer' and low spin Ni^{II} models usually show a very different multiplet structure,[Wang, 2000 #236] including singlet L_3/L_2 peaks, a sharper and stronger L_2 and a lower $L_3/(L_3+L_2)$ branching ratio. In a previous study,[Ralston, 2000 #169] the low spin Ni^{II} cluster in the as-isolated *C. thermoaceticum* CO-dehydrogenase (Ct-CODH), another Ni enzyme which catalyzes CO oxidation and acetyl-CoA synthesis,[51,52] also showed a clear low spin Ni^{II} feature as described above for Ni models. In a related experiment, the lcp and rcp excitation spectra for Ct-CODH have also been recorded in the L_3 region normalized to the background intensities prior to the L_3 edge. In contrast to the high spin Ni rubredoxin, the low spin Ni^{II} in Ct-CODH produces no XMCD effect (not shown).

D. d. Hydrogenase Hydrogen (H_2) reduced *D. desulfuricans* hydrogenase exhibits similar Ni absorption (Fig. 2b) and XMCD spectra (Fig. 2b') to the high spin Ni^{II} within Ni rubredoxin discussed above (2a and 2a'). The L_3 edge has a major peak near 853.2 eV and a minor peak (for $I^{\uparrow\downarrow}$) or a shoulder (for $I^{\uparrow\uparrow}$) near 855.0 eV. The L_2 has a barely resolved doublet structure at 868.5-872.5 eV. There is a 27.5% difference in polarization-corrected absorption intensity between the two spectra at L_3, corresponding to ~14% XMCD effect. The XMCD signal at L_3 also exhibits a bipolar multiplet, similar to Ni rubredoxin. The XMCD data off the L_3 and L_2 peak regions were used to calculate the standard deviation σ. This statistical analysis reveals $\pm 2\sigma$ for the XMCD spectra as shown in Fig. 2b' (dotted lines). The major XMCD intensity at L_3 is ten times this error (2σ) while the minor peak (at L_3) is also 2-3 times as big as 2σ.

In comparison with Ni rubredoxin, some differences are worth noting as well. For the *D. desulfuricans* hydrogenase, at L_3, one side ($I^{\uparrow\downarrow}$) of the polarized absorption spectra has a clear secondary peak and the other side shows an unresolved shoulder, while for Ni rubredoxin, both sides have a distinguishable secondary peak. At L_2, the XMCD signal is too weak to be quantified for the *D. desulfuricans* hydrogenase, while it is clear (24%) for Ni rubredoxin. Despite the differences, the overall similarities between the two Ni enzymes are still great. The clear XMCD effect and bipolar XMCD multiplet at L_3 indicates that the Ni sites inside both enzymes are hs-Ni^{II}, not ls-Ni^{II}. A ls-Ni^{II} site has a zero spin value and should be XMCD inactive.

D. b. and D. g Hydrogenases The circular polarized absorption spectra for the H_2 reduced *D baculatus* hydrogenase (Fig. 3a) show similar spectral multiplet structure to those observed for Ni rubredoxin and *D. desulfuricans* hydrogenase, including a major peak at 853.2 eV and a minor secondary L_3 peak at 855.2 eV. The circular polarized absorption spectra for the H_2 reduced *D. gigas* hydrogenase, which are shown in Fig. 3b, also both exhibit a major peak at 853.2 eV and a partially-resolved shoulder at 855.0 eV. These spectra are also similar to the polarized absorption spectra for Ni rubredoxin and *D. desulfuricans*. Although the secondary L_3 shoulders are not resolved, the spectra are typical of a hs-Ni^{II} site. In the L_2 region, the signal-to-noise ratio (S/N) is low for both *D. baculatus.* and *D. gigas* hydrogenase, but both spectra clearly have a broader L_2 than a typical ls-Ni^{II} spectrum.[Wang, 2000 #236]

XMCD spectra for H_2 reduced *D. baculatus* and *D. gigas* hydrogenases are shown in Fig. 3a' and 3b'. Error bars (2σ, dotted lines below and above the XMCD spectra) are calculated with the data pre-L_3 and post-L_2 as described above. Although the S/N ratio is not satisfactory, it is still clear that the XMCD intensities (at L_3) are still about two (for reduced *D. baculatus* hydrogenase) or three (for reduced *D. gigas* hydrogenase) times as large as the size of the error bars (2σ). There is therefore a non-zero XMCD effect and thus a non-zero spin for these species. Further XMCD studies are necessary to determine the bipolar XMCD multiplet at L_3 and the XMCD effect in L_2 for the *D. baculatus* and *D. gigas* hydrogenases.

Sum Rule Results As the number of 3d holes is not accurately known for the Ni enzymes in this study, rather than speculating or assuming an arbitrary number, we calculate and compare the $\langle L_z \rangle$ and $\langle S_z \rangle$ values per Ni(3d) hole instead. For the Ni inside Ni rubredoxin, the functions $(I^{\uparrow\uparrow}+I^{\uparrow\downarrow})/2$ and $(I^{\uparrow\uparrow} - I^{\uparrow\downarrow})$, along with their integrals over the $2p \rightarrow 3d$ regions (A, B and

C, dotted lines) are illustrated in Fig. 4. The values for these integrals are calculated as: A = 3.6±0.1, B = -2.8±0.1, and C = 26.0±0.4 respectively. Substituting these integrals into (6) and assuming n_h=1, we obtain $\langle L_z \rangle$ = 0.02±0.01$\hbar$ /n and $\langle S_z \rangle$ = 0.18±0.02$\hbar$ /n for the Ni within Ni rubredoxin. The "$\hbar$ /n" indicates that the value is for per 3d hole. The error analysis is based on the statistical error plus 10% instrumental error.

Similarly, the functions of $(I^{\uparrow\uparrow}+I^{\uparrow\downarrow})/2$ and $(I^{\uparrow\uparrow}-I^{\uparrow\downarrow})$, along with their integrals over the 2p → 3d regions (A, B and C, dotted lines), for the reduced *D. desulfuricans* hydrogenase are illustrated in Fig. 5. The calculated values for these integrals are: *A* = 3.0±0.2, *B* = -2.2±0.4, and *C* = 26.0±0.6. Again, substituting a value n_h =1 into formula (6), we find the orbital $\langle L_z \rangle$ and spin $\langle S_z \rangle$ angular momentum per 3d hole to be 0.02±0.02$\hbar$ /n and 0.14±0.03$\hbar$ /n, respectively. The error analysis is also based on statistical error plus 10% instrumental error The larger errors in the integrated intensity and in the orbital and spin angular momentum projections $\langle L_z \rangle$ and $\langle S_z \rangle$ are due to the smaller S/N ratio of the overall XMCD spectrum as well as the uncertain XMCD effect at L_2.

Discussion

XMCD is a useful tool for directly probing the localized electron spin states of metal ions within biological molecules, even with a bend magnet beamline. In this study, we have shown that the Ni within Ni rubredoxin exhibits very clear high spin multiplets in the absorption / XMCD spectra and a clear non-zero XMCD effect. All the reduced NiFe hydrogenases studied here also have a non-zero XMCD effect and/or a clear hs-Ni^{II} multiplet in their absorption / XMCD spectra.

Since the discovery of XMCD sum rules by Thole and coworkers a decade ago, there has been extensive examination of these rules on various materials, the results ranging from excellent to poor. However, examination on biological systems is limited due to experimental difficulties. In this study, we have obtained the "sum rule derived" orbital and spin angular momentum localized in the Ni sites with Ni rubredoxin (Fig. 4) and with *D. desulfuricans* NiFe hydrogenase (Fig. 5), and found these two Ni sites have similar "XMCD sum rule analyzed" angular momentum values (*e.g.* $\langle S_z \rangle$ = 0.18±0.03$\hbar$ /n; vs. 0.14±0.03$\hbar$ /n). It once again suggests that Ni rubredoxin is a good starting system to study the electronic states within NiFe hydrogenases. As mentioned above, all the reduced hydrogenases in this study were found to

contain high-spin Ni^{II}. This finding is consistent with recent Ni L-edge results[16,55] but at odds with the conclusions from some previous reports, which have frequently assigned the reduced state as low-spin Ni^{II}.[11,13,56-58] These discrepancies have been discussed and evaluated in a recent publication[16] and will only be briefly repeated here. The previous arguments for ls-Ni^{II} consists primarily of negative results, such as no UV-visible MCD effect for the reduced *C. vinosum* / *D. gigas* / *M. thermoautotrophicum*[58,59] hydrogenases and no parallel mode EPR signal in the form Ni-R of *D. gigas* hydrogenase.[13] The only 'positive' evidence for a ls-Ni^{II} hydrogenase site is the ~2.2 Å[60] average Ni-S bond length found by EXAFS for several Ni-R hydrogenases. It has been argued that this bond length is much closer to the 2.16-2.21 Å range found for low-spin 4-coordinate thiolate complexes than to the 2.39-2.53 Å range found for 6-coordinate thiolate complexes,[11] which usually have hs-Ni^{II}.

However, this EXAFS result is not consistent with the average Ni-S distances from the similar crystal structures (2.415Å for the reduced *D. baculatus*[61] and 2.33Å for the reduced *D. vulgaris*[62]). The crystal structure measured Ni-S values are much longer than ~2.2 Å and could be consistent with hs-Ni^{II}. Furthermore, in the reduced *D. baculatus* hydrogenase structure,[61] the individual Ni-S (and Ni-Se) bond lengths of 2.25, 2.33, and 2.62 Å are too different to be modeled by a single average Ni-S distance in an EXAFS simulation. Recent range-extended Ni EXAFS measurements with an extended k_{max}=16.5[63] have revealed that the Ni-S distances inside the H_2 reduced *D. gigas* hydrogenase are actually split, with an average Ni-S distance of 2.34Å (with 2 Ni-S shells) or 2.37Å (assuming 3 Ni-S shells). These new EXAFS values are significantly longer than that from the shorter k range (k_{max}=12.5) and single Ni-S shell EXAFS analyses (2.20 Å). These new values are in fact close to typical hs-Ni^{II} values. Inferences about the Ni spin state in form R, based on the Ni-S bond length, need to take this longer average bond length into account.[63]

Theoretically, although most early calculations assumed a ls-Ni^{II} for the fully reduced state as a starting point, one of the these *ab initio* calculations[64] illustrated that a hs-Ni^{II} assumption actually yields a closer fit for the measured Ni-S distances and CO, CN vibration frequencies than a ls-Ni^{II} assumption. Another recent density function theory (DFT) calculation for the Ni-R hydrogenase state[17] (based on *D. gigas* hydrogenase information) found that the optimized structures for an unconstrained Ni^{II} geometry yielded nearly equivalent energies for hs-Ni^{II} and ls-Ni^{II} sites. However, they also found that if the Ni-S bonds were constrained to a

geometry similar to the reported crystallographic results, the high-spin state had the lowest energy by about 20 kcal/mol.[17] As illustrated in Fig. 1 (b and c), altering between square-pyramidal and trigonal-bipyramidal Ni^{II} geometries [21,22] requires only small motions of the ligands, but often results in very different Ni spin states. It is possible that slight perturbations of the hydrogenase active site could modulate the 5-cooordinate Ni spin-state. Further studies are required to fully characterize the electronic state of the Ni metal within hydrogenases for all stages of the catalytic cycle.

Summary

In this study, Ni L-edge X-ray magnetic circular dichroism (XMCD) for Ni rubredoxin and for H_2 reduced *D. desulfuricans*, *D. baculatus* and *D. gigas* hydrogenases has been measured and studied for the first time. The results illustrate that XMCD is a useful tool for probing the localized electron spin of biological metal sites. The Ni sites inside Ni rubredoxin and inside the H_2 reduced *D. desulfuricans* hydrogenase show a clear hs-Ni^{II} multiplet in their absorption and XMCD spectra. XMCD sum rule analysis also shows that the reduced *D. desulfuricans* hydrogenase has a significant and comparable $\langle S_Z \rangle$ with that for Ni rubredoxin, again indicating a hs-Ni^{II} site. For *D. baculatus* and *D. gigas* hydrogenase, although the signal to noise ratio (S/N) is not satisfactory for sum rule analysis, the XMCD spectra still indicate an XMCD active species. These results suggest that the Ni site in Ni rubredoxin and in all the reduced NiFe hydrogenases in this study are high spin Ni^{II}.

The development of elliptically polarized undulator (EPU) magnetic beamlines (*e.g.* ALS beamline 4.0.2[53,54]) provides several orders of magnitude higher photon flux. This higher flux, in combination with up to 100% circular polarization and better energy and beam position stability, makes it possible to observe smaller XMCD effects in even more dilute metalloenzymes. The ability of fast and frequent flip of the X-ray beam's helicity enables a point-to-point XMCD spectrum. The preliminary results here, combined with the availability of 3rd generation synchrotron radiation sources and more advanced beamlines, points to a promising future for biological XMCD experiments. XMCD studies on various biological metal sites are currently underway in this laboratory, using advanced 'magnetic spectroscopy beamtime'[53,54] at the ALS and better data-acquisition electronics.

Acknowledgments This research was supported by the National Institutes of Health (GM-44380), the National Science Foundation (BIR-9317942 and BIR-9105323), and by the Department of Energy, Office of Health and Environmental Research. The Stanford Synchrotron Radiation Laboratory is supported by the Department of Energy, Office of Basic Energy Sciences.

Figure Captions

Figure 1 The 3d energy diagram (a) and corresponding structure of 5-coordinate triangle-bipyramidal (b) and 5-coordinate square-pyramidal (c) complexes.

Figure 2 Polarization-normalized, background-subtracted Ni fluorescence excitation spectra for Ni rubredoxin (a), taken with photon angular momentum in parallel (solid line) and in antiparallel (dashed line) to Ni 3d electronic spin. Polarization corrected difference spectrum ($I^{\uparrow\uparrow}$-$I^{\uparrow\downarrow}$) or XMCD spectrum for Ni rubredoxin (a'). Normalized Ni excitation spectra for the reduced *D. d* hydrogenase (b), taken with photon angular momentum in parallel (solid line) and in antiparallel (dashed line) to Ni 3d electronic spin. XMCD spectrum ($I^{\uparrow\uparrow}$-$I^{\uparrow\downarrow}$) for the reduced *D. d* hydrogenase (b'). The thin dotted lines above and below the spectrum (b') are the error bars $\pm 2\sigma$ for the XMCD spectrum.

Figure 3 Polarization-normalized, background-subtracted Ni fluorescence excitation spectra for Ni in hydrogen (H_2) reduced *D. baculatus* (a) and *D. gigas* (b) hydrogenases, taken with photon angular momentum parallel (solid lines) or antiparallel (dashed lines) to Ni 3d spin. Beneath each polarized absorption spectra are the polarization corrected XMCD spectra for H_2 reduced *D. baculatus* (a') and *D. gigas* (b') hydrogenases. The thin dotted lines above and below the XMCD spectra are the error bars $\pm 2\sigma$.

Figure 4 The averaged excitation spectra of ($I^{\uparrow\uparrow}$+$I^{\uparrow\downarrow}$)/2 (a, solid line) and the XMCD spectra of ($I^{\uparrow\uparrow}$ – $I^{\uparrow\downarrow}$) (b, solid line) for Ni rubredoxin. The dotted lines beneath are the integrated curves for A, B and C/2 respectively (see text for details).

Figure 5 The averaged excitation spectra of ($I^{\uparrow\uparrow}$+$I^{\uparrow\downarrow}$)/2 (a, solid line) and the XMCD spectra of ($I^{\uparrow\uparrow}$ – $I^{\uparrow\downarrow}$) (b, solid line) for the reduced *D. d* hydrogenase. The dotted lines beneath are the integrated curves for A, B and C/2 respectively (see text for details).

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