Articles

Nickel L-Edge X-ray Absorption Spectroscopy of *Pyrococcus furiosus* Hydrogenase

J. van Elp,[†] G. Peng,[‡] Z. H. Zhou,[§] M. W. W. Adams,[§] N. Baidya,[∥] P. K. Mascharak,[∥] and S. P. Cramer*,[†,‡]

Energy and Environment Division, Lawrence Berkeley Laboratory, Berkeley, California 94720, Department of Applied Science, University of California, Davis, California 95616, Department of Biochemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, and Department of Chemistry, University of California, Santa Cruz, California 95064

Received June 8, 1994[⊗]

We have investigated the reduced, thionine-treated at 20 °C, and thionine-treated at 80 °C forms of *Pyrococcus furiosus* [NiFe] hydrogenase using L-edge X-ray absorption spectroscopy. At 20 °C, the Ni site is apparently not redox active, since the reduced and 20 °C thionine-treated forms exhibit the same spectra. Results of theoretical simulations as well as comparison with the spectra of model compounds indicate the presence of high-spin Ni(II) in these forms. On the basis of a comparison with the spectral features of model nickel complexes, the nickel site in the hydrogenase appears to be 5- or 6-coordinate. The 80 °C thionine-treated form has a broader Ni L-edge centered at a higher energy, consistent with a charge distribution of at least two holes on the nickel and at least one hole significantly delocalized onto the ligand framework.

Introduction

Hydrogenases are a class of enzymes that catalyze the oxidation and formation of molecular hydrogen. The NiFe hydrogenases contain a single Ni site as well as several different Fe–S clusters.[1,2] The Ni center exhibits a range of EPR signals and magnetic coupling to an Fe–S cluster, and it appears to undergo changes of oxidation states during turnover. The precise structure of the nickel site is still unknown, but spectroscopic evidence suggests that it is 5- or 6-coordinate with at least two sulfur ligands.[3,4] Though several redox schemes with the nickel in different oxidation states have been proposed, the mechanism of the catalytic process remains ambiguous.

The NiFe hydrogenase from *Pyrococcus furiosus*, an organism that grows optimally at 100 °C, is composed of four subunits with total molecular mass of 150 kDa. It contains 30 ± 2 Fe and 0.9 ± 0.2 Ni per enzyme molecule.[5–7] It has unusual properties. EPR measurements on the enzyme show signals characteristic of [2Fe–2S] and $[4Fe–4S]^+$ clusters, both of which disappear upon oxidation of the enzyme at 20 °C.[8] During room-temperature redox titration, no evidence was found for the rhombic nickel EPR signals seen in other Ni hydrogenases.[2] However, when redox titrations are carried out at 70 °C, an EPR signal typical for the form C (g = 2.21, 2.13, 2.01 at an E_m of −320 mV) is observed.[7,8]

The X-ray absorption techniques of Ni K-edge XANES (X-ray absorption near edge structure) and EXAFS (extended X-ray absorption fine structure) have been used to investigate the Ni coordination in [NiFe] hydrogenases.[4,9] The shift of 2 eV in the absorption edge observed with the oxidized and H_2-reduced forms of *Desulfovibrio gigas* hydrogenase led Scott et al.[9] to conclude that a change of oxidation state does occur at the nickel site. In contrast, a recent study[4] on *Thiocapsa roseopersicina* hydrogenase concluded that the nickel site in all redox states of the enzyme is 5- or 6-coordinate and that no significant change in electron density at the nickel site occurs during the redox cycle. Analysis of the EXAFS indicates the presence of 3 ± 1 N/O and 2 ±1 S donor atoms around nickel in this hydrogenase.[4] The K-edge XANES of *T. roseopersicina* hydrogenase is quite similar to that of nickel complexes with $[NiN_3S_2]$ chromophores,[10] a fact that suggests a distorted trigonal bipyramidal structure for the biological nickel site with mixed Ni–N/O and Ni–S ligation. To date, no X-ray absorption data on *P. furiosus* hydrogenase have been published.

In this paper we present the results of Ni $L_{2,3}$-edge X-ray absorption spectroscopy (XAS) on the reduced and two thionine-treated (oxidized) forms of *P. furiosus* hydrogenase. The spectra are compared with those of model complexes—[Ni(terpy)(2,4,6-$(i\text{-Pr})_3C_6H_2S)_2$](terpy = 2,2′:6′,2″-terpyridine) with a $[NiN_3S_2]$ center as well as $[Ni(pdtc)_2]^-$ and $[Ni(pdtc)_2]^{2-}$ (pdtc =

* To whom correspondence should be addressed at the University of California, Davis. FAX: 916-752-2444. Phone: 916-752-6153.
[†] Lawrence Berkeley Laboratory.
[‡] University of California, Davis.
[§] University of Georgia.
[∥] University of California, Santa Cruz.
[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1995.

(1) Przybyla, A. E.; Robbins, J.; Menon, N.; Park, M. D., Jr. *FEMS Microbiol. Rev.* **1992**, *88*, 109.
(2) Lancaster, J. R., Jr. *The Bioinorganic Chemistry of Nickel*; VCH Publishers, Inc.: New York, 1988.
(3) Dean, P. E.; Fan, C. L.; Hoffman, B. M. *J. Am. Chem. Soc.* **1994**, *116*, 1033.
(4) Bagyinka, C.; Whitehead, J. P.; Maroney, M. J. *J. Am. Chem. Soc.* **1993**, *115*, 3576–3585.
(5) Bryant, F. O.; Adams, M. W. W. *J. Biol. Chem.* **1989**, *264*, 5070.
(6) Adams, M. W. W. *FEMS Microbiol. Rev.* **1990**, *75*, 219–238.
(7) Adams, W. W. W. *Adv. Inorg. Chem.* **1992**, *38*, 341.
(8) Park, J. B. Ph.D. Thesis, University of Georgia, Athens, GA, 1992.
(9) Scott, R. A.; Wallin, S. A.; Czechowski, M.; DerVartanian, D. V.; LeGall, J.; Peck, H. D., Jr.; Moura, I. *J. Am. Chem. Soc.* **1984**, *106*, 6864.
(10) Baidya, N.; Olmstead, M. M.; Whitehead, J. P.; Bagyinka, C.; Maroney, M. J.; Masharak, P. K. *Inorg. Chem.* **1992**, *31*, 3612.

0020-1669/95/1334-2501$09.00/0 © 1995 American Chemical Society

pyridine-2,6-dithiocarboxylate) with a $[NiN_2S_4]$ ligation. Ni $L_{2,3}$-edge spectroscopy has certain advantages for interpretation over K-edge measurements. The nickel $L_{2,3}$-edge involves fully-allowed L-edge 2p → 3d transitions and has a smaller natural line width compared to the nickel K-edge. $L_{2,3}$-edges are therefore sharper and stronger than the dipole-forbidden K-edge 1s → 3d transitions seen at the foot of the K-edge. In complexes with high symmetry, the L-edge features can be related to ligand field splittings by established theoretical procedures, using atomic multiplet theory with the inclusion of an adjustable ligand field.[11] The positions and splittings of L-edge features are quite sensitive to changes in oxidation states.[11] Finally, $L_{2,3}$-edge branching ratios can distinguish between a high-spin and a low-spin Ni(II) ground state.[11,12] Although magnetic susceptibility measurements can also provide such information such measurements are bulk sensitive, while $L_{2,3}$-edge spectra are element-specific. In this study we exploit the advantages of Ni $L_{2,3}$-edge spectroscopy to address the Ni oxidation state, spin state, and site symmetry in *P. furiosus* [NiFe] hydrogenase.

Experimental Section

Sample Preparation. *P. furiosus* [NiFe] hydrogenase was purified anaerobically by following published procedures.[5] Samples of the reduced, 20 °C thionine-treated, and 80 °C thionine-treated protein were prepared as partially dehydrated thin films. The films were made in an anaerobic glovebox by placing about 0.1 mL of a 1.0 mM protein solution on a silicon wafer attached to a copper sample holder. Tests on redissolved films showed that at least 90% of activity could be recovered. The model complexes $[Ni(terpy)(2,4,6\text{-}(i\text{-}Pr)_3C_6H_2S)_2]$[14] and $[Ni(pdtc)_2]^-$ and $[Ni(pdtc)_2]^{2-}$ [15] were ground in the glovebox to obtain a fresh surface and placed on double-stick adhesive tape connected to a copper sample holder. The samples were transferred anaerobically from the glovebox to a loadlock. After evacuation of the loadlock, the samples were transferred to the main chamber and mounted on a precooled 10 K cold finger attached to a liquid helium flow cryostat.

Data Collection. The Ni L-edge spectra were recorded using AT&T beamline U4-B at the National Synchrotron Light Source.[16] A refocusing mirror produced an ~1 × 3 mm^2 beam spot. The photon energy resolutions used for the protein samples and Ni model compound were 0.5 and 0.3 eV, respectively. The measurement chamber was maintained at a vacuum in the low 10^{-9} mbar region. The model compound spectra were measured using total electron yield mode, while the protein spectra were recorded using fluorescence detection with a windowless 13-element germanium detector.[17] The fluorescence signal was divided by the incident beam intensity (I_0), which was measured by the total electron yield from a Au-coated grid placed between the sample and monochromator. With fluorescence detection, the nickel fluorescence signal at 850 eV can be electronically resolved from the large protein oxygen Kα background at 525 eV and the strong Fe L fluorescence near 710 eV. The L-edge spectra were calibrated using the total electron yield spectrum of NiF_2, which has an absorption maximum at 852.7 eV.[18] The protein spectra presented represent the sum of approximately 12 25-min scans. To minimize radiation damage, the position of the X-ray beam on the sample was moved every few scans. No evidence was observed for changes over time in the beam.

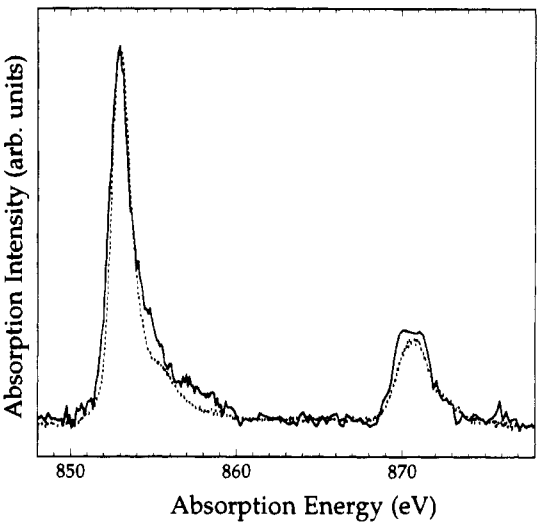

Figure 1. $L_{2,3}$ X-ray absorption spectra of $[Ni(terpy)(2,4,6\text{-}(i\text{-}Pr)_3C_6H_2S)_2]$ (dashed line) and reduced form of Ni hydrogenase from *P. furiosus* (solid line). The spectrum of the reduced form of Ni hydrogenase is the sum of the spectra for reduced and 20 °C thionine-treated forms. To emphasize the 3d absorption spectral weight only, a background was subtracted by fitting a polynomial function to a region in front of the L_3-edge and beyond the L_2-edge.

Results and Discussion

The Ni $L_{2,3}$-edge spectra of the *P. furiosus* hydrogenase and the model complex $[Ni(terpy)(2,4,6\text{-}(i\text{-}Pr)_3C_6H_2S)_2]$ are shown in Figure 1. The Ni in these samples is EPR silent and presumably corresponds to the active, EPR-silent forms of other hydrogenases. Details of the spectroscopic features of Ni $L_{2,3}$-edges, including ligand field atomic multiplet calculations, have already been reported.[11,12,18,19] The spectra are split into an L_3-edge ($2p_{3/2}$) around 853 eV and an L_2-edge ($2p_{1/2}$) around 871 eV because of the 2p spin−orbit coupling. The spectra of the reduced and thionine-treated at 20 °C hydrogenases are identical, and hence only the sum of the two spectra is shown. This spectrum has a sharp L_3-edge with a weak high-energy shoulder and a broad L_2-edge.

The L_3 peak position and the relative height of the L_2-edge are characteristic of a divalent high-spin Ni spectrum.[11] In contrast, a study on the reduced form of *Desulfovibrio baculatus* [FeNiSe] hydrogenase found a diamagnetic low-spin ($S = 0$) ground state for the nickel site.[13] If the *P. furiosus* sample were low-spin, the L_2 feature would be more intense, as discussed by Thole and van der Laan.[12] Furthermore, for low-spin Ni(II), the presence of only a single unoccupied d orbital would give rise to a sharper L_3-edge.[11] Some qualitative conclusions can therefore be drawn immediately from these spectra. Since square planar Ni(II) species invariably contain low-spin Ni, this geometry is clearly excluded. High-spin Ni(II) centers in octahedral (O_h) and distorted tetrahedral (D_{2d}) symmetries exhibit distinctly different shoulders at the high-energy side of the L_3-edge.[11] Thus, the nickel site in *P. furiosus* hydrogenase must have more than four donor atoms in the first coordination sphere and the coordination geometry is not strictly octahedral.

The width of the L_3-edge is also larger than that observed for Ni(II) model complexes with O_h and D_{2d} symmetries. We have used a series of model complexes to correlate the features of the Ni $L_{2,3}$-edges with electronic structure and symmetry.[11] These studies have established that significantly broader L_3-edges are observed with tetragonally distorted high-spin Ni(II)

(11) van Elp, J.; Peng, G.; Searle, B. G.; Mitra-Kirtley, S.; Huang, Y.-H.; Johnson, M. K.; Zhou, Z. H.; Adams, M. W. W.; Maroney, M. J.; Cramer, S. P. *J. Am. Chem. Soc.* **1994**, *116*, 1918.
(12) van der Laan, G.; Thole, B. T.; Sawatzky, G. A.; Verdaguer, M. *Phys. Rev. B* **1988**, *37*, 6587.
(13) Wang, C.-P.; Franco, R.; Moura, J. J. G.; Moura, I.; Day, E. P. *J. Biol. Chem.* **1992**, *267*, 7378.
(14) Baidya, N.; Olmstead, M. M.; Mascharak, P. K. *Inorg. Chem.* **1991**, *30*, 929.
(15) Kruger, H.-J.; Peng, G.; Holm, R. H. *Inorg. Chem.* **1991**, *30*, 734.
(16) Chen, C. T.; Sette, F. *Rev. Sci. Instrum.* **1989**, *60*, 1616.
(17) Cramer, S. P.; Chen, J.; George, S. J.; van Elp, J.; Moore, J.; Tensch, O.; Colaresi, J.; Yocum, M.; Mullins, O. C.; Chen, C. T. *Nucl. Inst. Methods* **1992**, *A319*, 285.
(18) van der Laan, G.; Zaanen, J.; Sawatzky, G. A.; Karnatak, R.; Esteva, J.-M. *Phys. Rev. B* **1986**, *33*, 4253.
(19) van Elp, J.; Searle, B. G.; Sawatzky, G. A.; Sacchi, M. *Solid State Commun.* **1991**, *80*, 67.

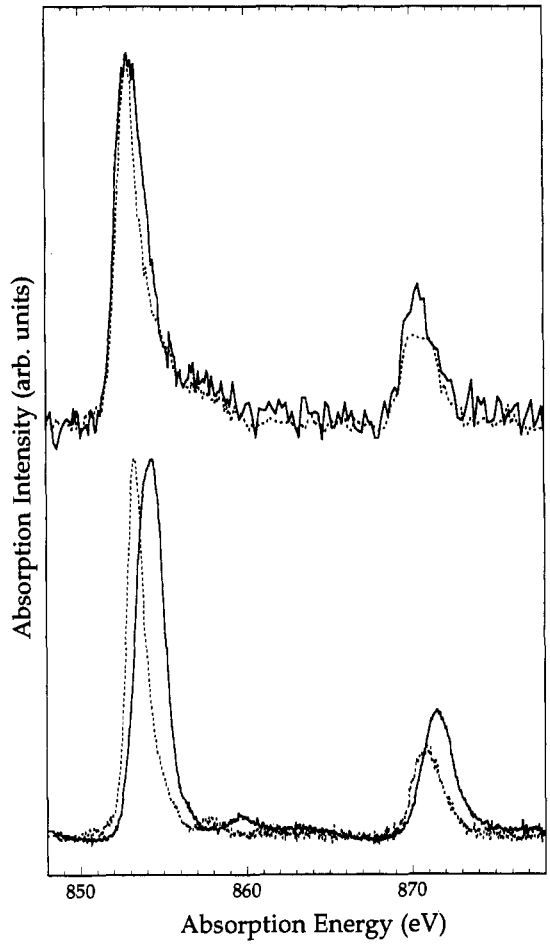

Figure 2. Top: $L_{2,3}$ X-ray absorption spectra of the 80 °C thionine-oxidized form (solid line) of Ni hydrogenase and reduced form (dashed line) of Ni hydrogenase, the same one as above. Bottom: $L_{2,3}$ X-ray absorption spectra of $[Ni(pdtc)_2]^-$ (dashed line) and $[Ni(pdtc)_2]^{2-}$ (solid line). To emphasize the 3d absorption spectral weight only, a background was subtracted by fitting a polynomial function to a region in front of the L_3-edge and beyond the L_2-edge.

complexes with approximate D_{4h} symmetry, such as Ni(cyclam)-Cl_2. In these complexes, the empty e_g orbitals are split, and a double-peaked structure in the main part of the L_3-edge is observed. The width of the L_3-edge in *P. furiosus* hydrogenase suggests a small splitting of a few hundred millielectronvolts of these e_g orbitals. This could arise from a 5-coordinate (square pyramidal or trigonal pyramidal) or distorted 6-coordinate geometry. These geometries would be consistent with the ones indicated for other hydrogenases.[4] Although we have not indicted error bars for the geometrical constraints, candidate models for the hydrogenase Ni site must have sufficiently low symmetry to split the two half-filled d orbitals but sufficiently weak splitting (<500 meV) to avoid spectroscopic resolution.

The peak positions and relative intensities for the [Ni(terpy)-(2,4,6-(i-Pr)$_3$C$_6$H$_2$S)$_2$] model compound spectrum are quite similar to the features in the *P. furiosus* hydrogenase spectrum. However, some minor differences exist. In the spectrum of the model complex, the shoulder on the higher energy side of the L_3-edge is weaker in intensity and appears at a slightly higher energy. Also, the L_2 region is a single peak, while the enzyme L_2 almost splits into a doublet. Further work is required to elucidate the factors that give rise to these subtle differences.

The L-edge spectrum of the *P. furiosus* hydrogenase shows changes upon treatment with thionine at 80 °C (Figure 2). Previous K-edge experiments with *T. roseopersicina* hydrogenase showed very small changes with oxidation,[4] while significant shifts were observed for Ni K-edges in *D. gigas* hydrogenase.[9] The intensity of the shoulder at the high-energy side of the L_3-edge increases, though the edge threshold does not shift. Also, the relative height of the L_2-edge increases to a significant extent and the shape of the peak is altered. The changes observed at the L_3-edge upon thionine treatment at 80 °C are comparable to those seen in relatively covalent Ni systems upon oxidation. For comparison, the $[Ni(pdtc)_2]^-$ and $[Ni(pdtc)_2]^{2-}$ $L_{2,3}$-edges are included in Figure 2. As with the enzyme spectrum, the edge features broaden upon oxidation of the complex, but no additional peaks are observed. In contrast, in the Ni(cyclam) system, oxidation of the complex results in a double-peak L_3-edge with a very different appearance.[11] Previous work by this group has shown that ligand field atomic multiplet calculations reproduce the spectral feature of Ni(II) complexes of high symmetries quite well.[11] However, such calculations do not reproduce the features of the $L_{2,3}$-edge spectra of so-called Ni(III) complexes, because the ionic calculations for d^7 Ni(III) predict multiplet features at the low-energy sides of the L_3- and L_2-edges which are not usually observed.[20] The absence of such features appears to indicate that some formally Ni(III) species are better described as Ni(II) plus a hole on the ligand framework.

With the data for the thionine-oxidized (at 80 °C) form of *P. furiosus* hydrogenase, one can rule out certain electronic structures as possibilities for the oxidized nickel site. For example, the Ni $L_{2,3}$-edges of the ferricyanide-oxidized form of the Ni-substituted *P. furiosus* rubredoxin have been reported.[11] Although this system has EPR properties strikingly similar to those of the form C signal of hydrogenases, the Ni $L_{2,3}$-edges are not that similar, and we find that this is not a model for the oxidized nickel site in *P. furiosus* hydrogenase. On the other hand, this might be expected as this form of the hydrogenase is EPR-silent. Another structure that can be immediately ruled out is Ni(I). A d^9 initial configuration would give a sharp L-edge, because of the d^{10} filled shell in the final state. At the other extreme, an ionic Ni(III) site would have low-energy multiplet-induced shoulders, which are not observed. Finally, the broad features of the spectrum are incompatible with a simple ionic, noninteracting Ni(II) site, since pure Ni(II) systems invariably display sharper peaks. One possibility is a trivalent nickel species with significant delocalization of the third hole onto the ligand framework. Recently, oxidative transformations of model nickel complexes with alkane thiolates afforded products in which the sulfur centers are oxidized instead of nickel.[23] It is therefore possible that, in the thionine-oxidized at 80 °C form of *P. furiosus* hydrogenase, the nickel site is not pure Ni(III) and the third hole is significantly delocalized onto the ligands around the metal center. Additional calculations are needed to test this model.

Summary

The spectral features of the $L_{2,3}$-edge spectrum of the nickel site of *P. furiosus* hydrogenase, isolated in the presence of dithionite, are consistent with a high-spin Ni(II) species. The coordination geometry around nickel is neither octahedral,

(20) van Elp, J. Ph.D. Thesis, University of Groningen, 1990.
(21) Kuiper, P.; Kruizinga, G.; Ghijsen, J.; Sawatzky, G. A.; Verweij, H. *Phys. Rev. Lett.* **1989**, *62*, 221.
(22) van Elp, J.; Eskes, H.; Kuiper, P.; Sawatzky, G. A. *Phys. Rev. B* **1992**, *45*, 1612.
(23) Mirza, S. A.; Pressler, M. A.; Kumar, M.; Day, R. O.; Whitehead, J. P.; Maroney, M. J. *Inorg. Chem.* **1993**, *32*, 977.

tetrahedral, nor square planar.[24] The thionine-treated (at 80 °C) form of the enzyme consists of a more oxidized species with significant charge delocalized onto the ligands. This work also demonstrates that Ni $L_{2,3}$-edge spectroscopy is a valuable technique for characterizing nickel-containing enzymes, which can provide information complementary to K-edge spectroscopy.

Acknowledgment. We wish to thank M. J. Maroney for the Ni−pdtc samples and H.-J. Lin for his help during the experiments at the U4-B beamline. This work was supported by the National Science Foundation through Grant DMB-9011583 (to M.W.W.A.), the Petroleum Research Fund (to P.K.M.), the National Institutes of Health through Grant GM-44380 (to S.P.C.), and the Department of Energy, Office of Health and Environmental Research (to S.P.C.). The Center for Metalloenzyme Studies at the University of Georgia is funded by a grant from the National Science Foundation (DIR-9014281), and the National Synchrotron Light Source is supported by the Department of Energy, Office of Basic Energy Sciences.

IC9406594

(24) A recent crystal structure of *D. gigas* hydrogenase suggests distorted $Ni(cys)_4X$ coordination: Volbeda, A.; Charon, M.-H.; Piras, C.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *Nature* **1995**, *373*, 580.