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Supplementary Material Available: ^1H and ^{13}C NMR spectra of **2** and **3** and ^1H, ^{13}C, and ^{29}Si NMR, H,C COSY NMR, and FT-IR spectra of the intermediate **4** (9 pages). Ordering information is given on any current masthead page.

(12) Biadamantylidene has been reported to react with $^1O_2$ affording the perepoxide intermediate, an oxygen analogue of **7**: Schaap, A. P.; Recher, S. G.; Falker, G. R.; Villasmor, S. R. *J. Am. Chem. Soc.* **1983**, *105*, 1691.

X-ray Absorption Spectroscopic Study of the Reductive Activation of *Thiocapsa roseopersicina* Hydrogenase

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Hydrogenases (H_2ases) are enzymes that catalyze the reversible oxidation of H_2.[1,2] In addition to containing Fe,S clusters, the majority of H_2ases also contain a Ni complex[3] that is redox-active[4] and involves S-donor ligands.[5] The H_2ase isolated from the purple photosynthetic bacterium *Thiocapsa roseopersicina* is a typical Fe,Ni H_2ase.[6] The presence of Ni in Fe,Ni H_2ases is often revealed by $S = {}^1/_2$ EPR signals in the oxidized and catalytically inactive forms of the enzyme (forms A and B), as well as in a reduced and active form (form C).[4,6b] These signals have been assigned to formally Ni(III) and/or Ni(I) centers because of the similarity of the EPR spectra to those of Ni(III) and Ni(I) coordination complexes[4a,b] and the observation of ^{61}Ni hyperfine interactions in spectra obtained from isotopically labeled preparations.[4a,b] These EPR signals have provided the principal spectroscopic probe of the Ni site and have been used to demonstrate the redox activity of the site,[4] the binding of CO (a competitive inhibitor) to Ni,[7] and the interaction of the site with H_2.[8] We report here the results of the first X-ray spectroscopic study of the structural changes in the Ni site accompanying reductive activation of a H_2ase. These studies reveal that the reductive activation of the enzyme is not accompanied by a change in the charge on the Ni or by a major reorganization of the Ni ligand environment.

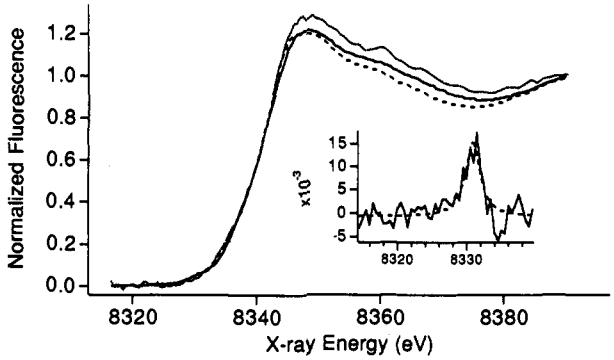

Figure 1. Ni K-edge spectra of *T. roseopersicina* hydrogenase poised in forms A (dashed line), B (solid line), and C (dotted line). Insert: The base line corrected 1s → 3d transition observed for form B (solid line) and a Lorentzian fit (dashed line).

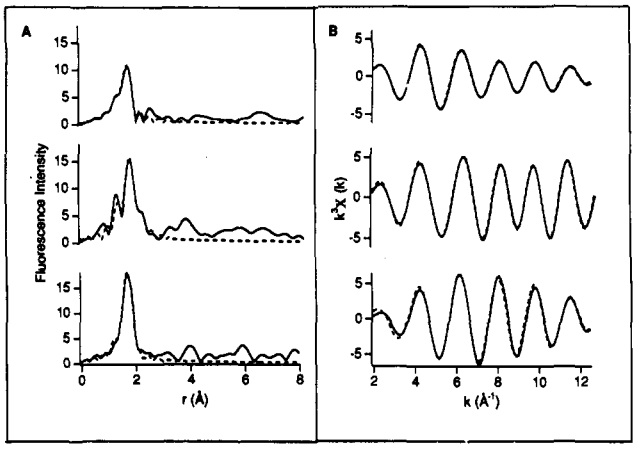

Figure 2. Ni K-edge EXAFS spectra obtained from *T. roseopersicina* hydrogenase poised in forms A (top), B (middle), and C (bottom). (A) Fourier transformed EXAFS spectra (uncorrected for phase shift, solid lines) and first coordination sphere fits (dashed lines). (B) Fourier filtered first coordination sphere EXAFS spectra (back-transform window = 1.1-2.3 Å, solid lines) and fits (dashed lines). Fits shown: Form A, (3) Ni-N 1.99 Å, $(10^3)\Delta\sigma^2 = 11.8$ Å^2; (2) Ni-S 2.20 Å, $(10^3)\Delta\sigma^2 = 0.2$ Å^2; (1) Ni-S 2.40 Å, $(10^3)\Delta\sigma^2 = 2.5$ Å^2, $R = 0.10$. Form B, (2) Ni-N 1.93 Å, $(10^3)\Delta\sigma^2 = -2.7$ Å^2; (3) Ni-S 2.24 Å, $(10^3)\Delta\sigma^2 = -1.3$ Å^2; (1) Ni-S 2.50 Å, $(10^3)\Delta\sigma^2 = -2.9$ Å^2, $R = 0.24$. Form C, (3) N 2.06 Å, $(10^3)\Delta\sigma^2 = -6.4$ Å^2; (2) Ni-S 2.21 Å, $(10^3)\Delta\sigma^2 = 1.6$ Å^2, $R = 0.34$.

T. roseopersicina was cultured and the H_2ase isolated and assayed as previously described.[5b,9] The enzyme was poised in forms A and B by using a minor modification of the procedure described by van der Zwaan et al.[7] X-ray absorption spectra were obtained on frozen solutions (77 K) prepared in 20 mM Tris·HCl (pH 8) buffer containing 20% glycerol that had Ni concentrations of 0.8 mM. X-ray fluorescence data were collected at beam line X9A at the National Synchrotron Light Source (2.53 GeV, ca. 110-200 mA) employing a monochromator and Si[111] crystals (resolution ca. 1 eV) and a 13-element Ge X-ray fluorescence detector. Energy calibrations were performed by using Ni foil

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(1) Adams, M. W. W.; Mortenson, L. E.; Chen, J.-S. *Biochim. Biophys. Acta* **1981**, *594*, 105.
(2) Vignais, P. M.; Colbeau, A.; Willison, J. C.; Jouanneau, Y. *Adv. Microb. Physiol.* **1985**, *26*, 155.
(3) Fauque, G.; Peck, H. D., Jr.; Moura, J. J. G.; Huynh, B. H.; Berlier, Y.; DerVartanian, D. V.; Teixeira, M.; Przybyla, A. E.; Lespinat, P. A.; Moura, I.; LeGall, J. *FEMS Microbiol. Rev.* **1988**, *54*, 299.
(4) (a) Cammack, R.; Fernandez, V. M.; Schneider, K. In *The Bioinorganic Chemistry of Nickel*; Lancaster, J. R., Ed.; VCH Publishers: New York, 1988; Chapter 8. (b) Moura, J. J. G.; Teixeira, M.; Moura, I.; LeGall, J. *Ibid.*; Chapter 9. (c) Patil, D. S.; Moura, J. J.; He, S. H.; Teixeira, M.; Prickril, B. C.; DerVartanian, D. V.; Peck, H. J.; LeGall, J.; Huynh, B. H. *J. Biol. Chem.* **1988**, *263*, 18732. (d) Huynh, B. H.; Patil, D. S.; Moura, I.; Teixeira, M.; Moura, J. J.; DerVartanian, D. V.; Czechowski, M. H.; Prickril, B. C.; Peck, H. J.; LeGall, J. *J. Biol. Chem.* **1987**, *262*, 795. (e) Teixeira, M.; Moura, I.; Fauque, G.; Czechowski, M.; Berlier, Y.; Lespinat, P. A.; Le, G. J.; Xavier, A. V.; Moura, J. J. *Biochimie* **1986**, *68*, 75. (f) Zorin, N. A. *Biochimie* **1986**, *68*, 97.
(5) (a) Maroney, M. J.; Colpas, G. J.; Bagyinka, C. *J. Am. Chem. Soc.* **1990**, *112*, 7067. (b) Maroney, M. J.; Colpas, G. J.; Bagyinka, C.; Baidya, N.; Mascharak, P. K. *J. Am. Chem. Soc.* **1991**, *113*, 3962. (c) 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. (d) Scott, R. A.; Czechowski, M.; DerVartanian, D. V.; LeGall, J.; Peck, H. D., Jr.; Moura, I. *Rev. Port. Quim.* **1985**, *27*, 67. (e) Lindahl, P. A.; Kojima, N.; Hausinger, R. P.; Fox, J. A.; Teo, B. K.; Walsh, C. T.; Orme-Johnson, W. H. *J. Am. Chem. Soc.* **1984**, *106*, 3062. (f) Eidsness, M. K.; Sullivan, R. J.; Scott, R. A. In *The Bioinorganic Chemistry of Nickel*; Lancaster, J. R., Ed.; VCH Publishers: New York, 1988; Chapter 4. (g) Eidsness, M. K.; Scott, R. A.; Prickril, B. C.; DerVartanian, D. V.; LeGall, J.; Moura, I.; Moura, J. J. G.; Peck, H. D., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 147. (h) Albracht, S. P. J.; Kröger, A.; van der Zwaan, J. W.; Unden, G.; Böcher, R.; Mell, H.; Fontijn, R. D. *Biochim. Biophys. Acta* **1986**, *874*, 116.
(6) (a) Kovacs, K. L.; Tigyi, G.; Thanh, L. T.; Lakatos, S.; Kiss, Z., Bagyinka, C. *J. Biol. Chem.* **1991**, *266*, 947. (b) Cammack, R.; Bagyinka, C.; Kovacs, K. L. *Eur. J. Biochem.* **1989**, *182*, 357.
(7) van der Zwaan, J. W.; Coremans, J. M. C. C.; Bouwens, E. C. M.; Albracht, S. P. J. *Biochim. Biophys. Acta* **1990**, *1041*, 101.
(8) Fan, C.; Teixeira, M.; Moura, J.; Moura, I.; Huynh, B. H.; Le, G. J.; Peck, H. D. J.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 20.
(9) (a) Kovacs, K. L.; Tigyi, G.; Alfonz, H. *Prep. Biochem.* **1985**, *15*, 321. (b) Bagyinka, C.; Zorin, N. A.; Kovacs, K. L. *Anal. Biochem.* **1984**, *142*, 7.

0002-7863/91/1513-6288$02.50/0

spectra. Previously published model compound data were used in analyzing the protein EXAFS data over the k range 2-12.5, following the procedure previously employed in the analysis of data from form C.[5b] EPR spectra taken before and after exposure to synchrotron radiation did not reveal any changes in these samples during data collection and were used to ascertain the "purity" of the samples. The form A sample had 80% of the enzyme poised in form A, while the form B sample contained 85% of the enzyme poised in form B. The X-ray absorption spectra reported have not been corrected for sample composition, since performing this correction did not lead to significantly different results. Edge spectra monitored as a function of exposure time did not show any significant changes.

Nickel K-edge X-ray absorption spectra obtained for H_2ase poised in forms A, B, and C are compared in Figure 1. Forms A and B show small but discernable peaks at 8331 eV, which are assigned to 1s → 3d transitions.[5f,10] The absence of a peak or shoulder near 8338 eV in both spectra and the small relative areas of the 1s → 3d peaks (0.010 (5) eV and 0.020 (5) eV, respectively) indicate that the Ni sites in both complexes are six-coordinate.[5f,10a] No 1s → 3d peak is observed in the spectrum obtained from the 0.3 mM sample of form C, although an examination of the edge structure is most consistent with a site composed of five non-hydrogen ligands in a distorted trigonal-bipyramidal arrangement.[10a] The reason that no preedge feature is observed may lie in the increased noise apparent in the spectrum obtained from the more dilute sample of form C.

The most striking result shown in Figure 1 is that within the precision of the experiment (±0.2 eV) the edge energies of the three forms do not change. The edge energy obtained from a number of Ni complexes in various oxidation states has been shown to reflect the charge on the Ni center.[10a,11] The observation that the edge energies in forms A, B, and C do not vary indicates that the electron density on the Ni center does not change during reductive activation.

The charge residing on a metal center in a complex is determined by the oxidation state of the metal and the ability of the ligands to reduce the charge on the metal through covalent interactions. The possible effect of changes in the Ni ligand environment that might occur during reductive activation can be addressed by using EXAFS analysis. Figure 2 shows EXAFS spectra of the Ni center in forms A, B, and C. Fits of the Fourier filtered spectra from forms A and B were obtained by analyzing all possible combinations of N(O) and S(Cl) donors with a coordination number of six, as determined from the edge analysis (vide supra), and are compared with the results obtained previously for form C. In all three cases there are 2 ± 1 S(Cl) donors at ca. 2.2 Å and 3 ± 1 N(O) donors at ca. 2.0 Å. The largest difference arises from the presence of long Ni-S(Cl) interactions in form A (Ni-S(Cl) = 2.40 Å) and in form B (Ni-S(Cl) = 2.50 Å). The absence of a long Ni-S interaction in an active form of the enzyme (form C) suggests that it is one possible site of protonation in the heterolytic cleavage of H_2.

Reductive activation of H_2ase by H_2 has been monitored by EPR.[4b] The disappearance of the EPR signals associated with forms A and B, followed by the appearance of the signal associated with form C and its eventual disappearance under continued exposure to H_2, has been interpreted in terms of a number of schemes based on Ni-centered redox chemistry.[4a,b,12] These schemes employ formal Ni oxidation states from III to 0 to interpret the appearance and disappearance of the EPR signals. On the basis of XAS studies of complexes with well-established metal-centered redox chemistry, edge shifts of 2-3 eV and 1-2 eV would be expected for the oxidations of Ni(I) to Ni(II) and Ni(II) to Ni(III), respectively.[5f,10a,13] In the absence of major changes in the ligand environment,[10a] the lack of a significant shift in the edge energy observed between oxidized and reduced forms is a strong indication that no real change in oxidation state of the Ni occurs during reductive activation of the H_2ase. Further, no change in the Ni-S bond length associated with two S-donor ligands present in all three forms of the enzyme is detected. This is in contrast to a decrease of 0.14 Å in the average Ni-S distance upon oxidation of a six-coordinate Ni(II) model complex to Ni(III).[14] Thus, mechanisms invoking different oxidation states for forms A, B, and C and implying significant changes in electron density at Ni are inconsistent with these XAS results.

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(10) (a) Colpas, G. C.; Maroney, M. J.; Bagyinka, C.; Kumar, M.; Willis, W. S.; Suib, S. L.; Baidya, N.; Mascharak, P. K. *Inorg. Chem.* **1991**, *30*, 920. (b) Schulman, R. G.; Yafet, Y.; Eisenburger, P.; Blumberg, W. E. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 1384. (c) Hahn, J. E.; Scott, R. A.; Hodgson, K. O.; Doniach, S.; Desjardins, S. E.; Soloman, E. I. *Chem. Phys. Lett.* **1982**, *88*, 595.

(11) (a) Sarode, P. R.; Ramasesha, S.; Madhusudan, W. H.; Rao, C. N. R. *J. Phys. C* **1979**, *12*, 2439. (b) Suchet, J. P.; Bailly, F. *Ann. Chim. (Paris)* **1965**, *10*, 517. (c) Manthiram, A.; Sarode, P. R.; Madhusudan, W. H.; Gopalakrishnan, J.; Rao, C. N. R. *J. Phys. Chem.* **1980**, *84*, 2200.

(12) (a) van der Zwaan, J.; Albracht, S. P.; Fontijn, R. D.; Slater, E. C. *FEBS Lett.* **1985**, *179*, 271. (b) Teixeira, M.; Moura, I.; Xavier, A. V.; Huynh, B. H.; DerVartanian, D. V.; Peck, H. D., Jr.; LeGall, J.; Moura, J. J. G. *J. Biol. Chem.* **1985**, *260*, 8942. (c) Moura, J. J. G.; Teixeira, M.; Moura, I. *Pure Appl. Chem.* **1989**, 915.

(13) (a) Furenlid, L. R.; Renner, M. W.; Fajer, J. *J. Am. Chem. Soc.* **1990**, *112*, 8987. (b) Furenlid, L. R.; Renner, M. W.; Szalda, D. J.; Fujita, E. *J. Am. Chem. Soc.* **1991**, *113*, 113.

(14) Krüger, H.-J.; Holm, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 2955.

Novel Diferric Radical Intermediate Responsible for Tyrosyl Radical Formation in Assembly of the Cofactor of Ribonucleotide Reductase[1]

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Ribonucleotide reductases (RNRs) play an essential role in DNA biosynthesis, catalyzing the conversion of NDPs to dNDPs.[2,3] *Escherichia coli* RNR, the prototype for the mammalian and herpes viral reductases, is composed of two subunits, B1 and B2. The homodimeric B1 subunit binds both the NDP substrates and the dNTP and ATP allosteric effectors and contains the cysteine residues which are essential for substrate reduction. The B2 subunit, also a homodimer, contains the dinuclear iron cluster-tyrosyl radical cofactor which is required for activity. The mechanism of assembly of this unusual cofactor in B2 has been the subject of ongoing investigation in several laboratories.[4-7]

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(2) Stubbe, J. *J. Biol. Chem.* **1990**, *265*, 5329-5332.

(3) Reichard, P.; Ehrenberg, A. *Science* **1983**, *221*, 514-519.

(4) Fontecave, M.; Gerez, C.; Atta, M.; Jëunet, A. *Biochem. Biophys. Res. Commun.* **1990**, *168*, 659-664.

(5) Sahlin, M.; Sjöberg, B.-M.; Backes, G.; Loehr, T.; Sanders-Loehr, J. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 813-818.

(6) Ochiai, E. I.; Mann, G. J.; Gräslund, A.; Thelander, L. *J. Biol. Chem.* **1990**, *265*, 15758-15761.

0002-7863/91/1513-6289$02.50/0