^{57}Fe Q-Band Pulsed ENDOR of the Hetero-Dinuclear Site of Nickel Hydrogenase: Comparison of the NiA, NiB, and NiC States

J. E. Huyett,[†] M. Carepo,[‡] A. Pamplona,[‡] R. Franco,[‡] I. Moura,[‡] J. J. G. Moura,*[,‡] and B. M. Hoffman*[,†]

Department of Chemistry, Northwestern University Evanston, Illinois 60208-3113
Departamento de Química and Centro de Química Fina e Biotecnologia, Faculdade de Ciências e Tecnologia Universidade Nova de Lisboa, 2825 Monte de Caparica, Portugal

Received January 10, 1997
Revised Manuscript Received June 25, 1997

Hydrogenases catalyze perhaps the most fundamental oxidation−reduction process, the activation of dihydrogen according to the reaction: $H_2 \rightleftharpoons 2H^+ + 2e^-$. These are key enzymes in bacterial metabolism, as well as an important link in bioenergetics and fuel production. Although there are hydrogenases that utilize only [Fe−S] centers, those most intensively studied are the [NiFe] and [NiSeFe] enzymes which contain [Fe−S] clusters, but employ a Ni center for catalysis instead.[1−4] A recent X-ray crystallographic analysis of the native *Desulfovibrio gigas* [NiFe] hydrogenase (*Dg* Hase), unexpectedly showed that the active site is not a mononuclear Ni center, as had been believed, but is a hetero-dinuclear, [Ni−X], cluster.[5] The Ni ion is coordinated by four cysteine residues (one being replaced by a selenocysteine in [NiSeFe] hydrogenases[5]), two of which serve as bridging ligands to a second metal ion, X.[5] The second metal ion, which is indicated to be iron by X-ray anomalous dispersion effects and EXAFS studies,[7] is coordinated by three nonprotein ligands,[8] which are indicated by FTIR measurements to be two CN^- ions and one CO molecule.[9] A nonprotein ligand, probably oxygen, is proposed to bridge the two metal ions, completing their coordination sphere (Figure 1).[5]

This unexpected result challenges previous reported spectroscopic studies that failed to reveal any second metal ion and, in particular, did not detect signals from iron, in addition to the iron−sulfur clusters. (i) The Ni center in the as-isolated native enzymes exhibit mixtures of two ($S = {}^1/_2$) EPR signals characteristic of a Ni^{III} ion.[2−4,7] One, denoted NiA ($g = 2.31$, 2.24, 2.02), corresponds to the reversibly inactivated, "unready" state of the enzyme. The second, NiB ($g = 2.33$, 2.15, 2.02), corresponds to the rapidly activated, "ready" state. Reduction

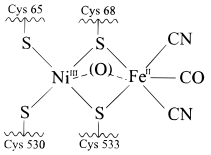

Figure 1. NiFe hetero-dinuclear cluster for NiA. Adapted from Volbeda *et al.*[5a] with X replaced by Fe. The (O) represents a proposed nonprotein bridging ligand as water or hydroxide. Cys530 is replaced by a selenocysteine in [NiSeFe] hydrogenases.[8]

with H_2 generates a new signal, NiC ($g = 2.19$, 2.14, 2.04), that is catalytically active and thought to contain hydride or dihydrogen.[10] EPR measurements *on all three states* give no evidence that the Ni center exhibits the magnetic interactions to be expected from the commonly occurring paramagnetic states of nonheme Fe in biology, Fe(II) ($S = 2$) or Fe(III) ($S = {}^5/_2$),[1,2,4] and the signals from the nickel center showed no ^{57}Fe line broadening. (ii) Detailed ^{57}Fe Mössbauer spectroscopy of *Dg* Hase gave no evidence of an iron ion that was hyperfine-coupled to the spin of the Ni center and, thus, would correspond to X.[11,12] (iii) Finally, to date it has not been possible either to change the redox state of the X ion or to exchange its exogenous ligands.[9] Thus, the assignment of X as iron implies the presence of a hetero-dinuclear [Ni−Fe] cluster with novel properties. The present ^{57}Fe Q-band Mims pulsed electron nuclear double resonance (ENDOR) study of ^{57}Fe-enriched and natural-abundance (^{56}Fe) *Dg* and *D. desulfuricans* (*Dd*) ATCC 27774 hydrogenases has detected a low-spin ($S = 0$) ferrous ion that is hyperfine-coupled to the spin of the Ni center in the NiA state, thereby completing the picture of this center as a novel, trapped valence, [Ni^{III}−Fe^{II}] cluster. This report further uses ^{57}Fe ENDOR to compare the properties of the cluster Fe in the NiA, NiB, and catalytically active NiC states of the [Ni−Fe] cluster.

^{57}Fe-enriched *Dg* and *Dd* Hases samples were prepared[11−15] and examined by Q-band pulsed ENDOR spectroscopy[16] in a spectrometer described earlier.[17] The former enzyme has the majority of the the Ni center in the NiA state, as isolated, and was used to study this state; it was then reduced[18] with H_2 to the NiC state. The latter enzyme is primarily in the NiB form, as isolated, and was used to study this form. In agreement with earlier reports, a careful comparison of the line-widths of the NiA, NiB, and NiC EPR signals obtained from natural-abundance and ^{57}Fe-enriched Hases shows *no* evidence of hyperfine broadening by the ^{57}Fe.

The Q-band Mims three-pulse, stimulated-echo pulsed ENDOR spectra,[19] which cover the frequency range around the ^{57}Fe nuclear Larmor frequency, were taken at numerous fields across the NiA EPR envelope of the natural-abundance and the

[†] Northwestern University.
[‡] Universidade Nova de Lisboa.

(1) 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*, 299−344.
(2) Moura, J. J. G.; Teixeira, M.; Moura, I.; LeGall, J. In *The Bioinorganic Chemistry of Nickel*; Lancaster, J. R., Jr., Ed.; VCH Publishers, Inc.: New York, 1988; Chapter 9, pp 191−226.
(3) Cammack, R.; Fernandez, V.; Hatchikian, E. C. In *Methods in Enzymology, Inorganic Microbial Sulfur Metabolism*; LeGall, J., Peck, H. D., Jr., Eds.; Academic Press Inc.: San Diego, CA, 1994; Vol. 243, Chapter 5, pp 43−67.
(4) Albracht, S. P. J. *Biochim. Biophys. Acta* **1994**, *1188*, 167−204.
(5) (a) Volbeda, A.; Charon, M.-H.; Piras, C.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *Nature* **1995**, *373*, 580−587. (b) Volbeda, A.; Garcin, E.; Piras, C.; de Lacey, A. L.; Fernandez, V. M.; Hatchikian, E. C.; Frey, M.; Fontecilla-Camps, J. C. *J. Am. Chem. Soc.* **1996**, *118*, 12989−1996.
(6) Patil, D. S. In *Methods in Enzymology, Inorganic Sulfur Metabolism*; LeGall, J., Peck, H. D., Jr., Eds.; Academic Press Inc.: San Diego, CA, 1994; Vol. 243, Chapter 6, pp 68−93 and references therein.
(7) Gu, Z.; Dong, J.; Allan, C. B.; Choudhury, S. B.; Franco, R.; Moura, J. J. G.; Moura, I.; LeGall, J.; Przybyla, A. E.; Roseboom, W.; Albracht, S. P. J.; Axley, M. J.; Scott, R. A.; Maroney, M. J. *J. Am. Chem. Soc.* **1996**, *118*, 11155−11165.
(8) Bagley, K. A.; Duin, E. C.; Roseboom, W.; Albracht, S. P. J.; Woodruff, W. H. *Biochemistry* **1995**, *34*, 5527−5535.
(9) Happe, R. P.; Roseboom, W.; Bagley, K. A.; Pierik, A. J.; Albracht, S. P. J. *Nature* **1997**, *385*, 126.
(10) Fan, C.; Teixeira, M.; Moura, J.; Moura, I.; Huynh, B. H.; LeGall, J.; Peck, H. D., Jr.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 20−24.
(11) Teixeira, M.; Moura, I.; Xavier, A. V.; Moura, J. J. G.; LeGall, J.; DerVartanian, D. V.; Peck, H. D., Jr.; Huynh, B. H. *J. Biol. Chem.* **1989**, *264*, 16435−16450.
(12) 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*, 8942−8950.
(13) Krüger, H. J.; Huynh, B. H.; Ljungdahl, P. O.; Xavier, A. V.; DerVartanian, D. V.; Moura, I.; Peck, H. D.; Teixeira, M.; Moura, J. J. G.; LeGall, J. *J. Biol. Chem.* **1982**, *257*, 4620−14623.
(14) Franco, R.; Moura, I.; LeGall, J.; Peck, H. D., Jr.; Huynh, B. H.; Moura, J. J. G. *Biochim. Biophys. Acta* **1993**, *184*, 1256−1260.
(15) These samples were prepared as previously described (refs 11−14) and concentrated to ∼1 mM; glycerol then was added to 30% by volume.
(16) DeRose, V. J.; Hoffman, B. M. In *Methods in Enzymology, Biochemical Spectroscopy*; Sauer, K., Ed.; Academic Press Inc.: San Diego, CA, 1995; Vol. 246, Chapter 23, pp 554−589.
(17) Davoust, C. E.; Doan, P. E.; Hoffman, B. M. *J. Magn. Reson.* **1996**, *A119*, 38−44.
(18) The reduction to NiC was done in the ENDOR tube by blowing H_2 over the solution for 6 h at room temperature and then freezing under liquid N_2.
(19) Gemperle, C.; Schweiger, A. *Chem. Rev.* **1991**, *91*, 1481−1505.

S0002-7863(97)00091-7 CCC: $14.00

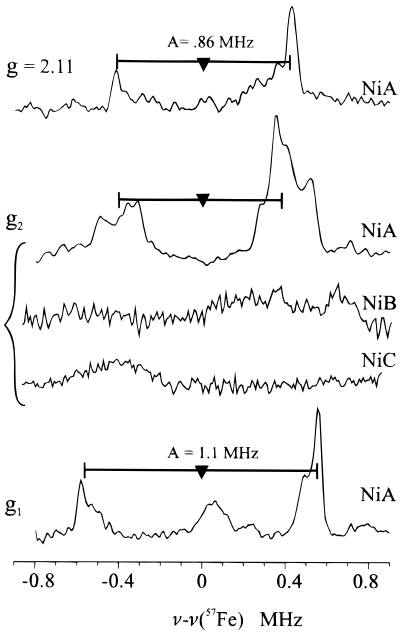

Figure 2. Selected Q-band ^{57}Fe Mims pulsed ENDOR spectra of ^{57}Fe-enriched *Dg* Hase NiA, ^{57}Fe-enriched *Dg* Hase NiC, and ^{57}Fe-enriched *Dd* Hase NiB at g_2. Conditions: microwave frequency for **NiA** 34.685 GHz, **NiB** 34.630 GHz, **NiC** 34.780 GHz, $T = 2$ K, $\pi/2$ pulse width 80 ns, $\tau = 480$ ns, $\tau_{RF} = 60$ μs, repetition rate 25 Hz, 16 shots per point; number of scans for **NiA** $g = 2.11$, 30, $g_2 = 10$, $g_1 = 10$, **NiB** 18, **NiC**, 10. To compare signals from three proteins, the ENDOR intensities have been normalized to Mims proton ENDOR signals taken under the same conditions. The symbol (▼) represents $\nu(^{57}\text{Fe})$ and the brackets represent the hyperfine coupling. The broad low-intensity peaks arise from the ν_- branches of weakly coupled nitrogen.

^{57}Fe-enriched *Dg* Hase. To ensure that the spectra collected are associated *only* with the [Ni−X] dinuclear site, no measurements were made at fields higher than that corresponding to $g \approx 2.11$, since at such fields EPR signals from the [3Fe−4S] cluster are present. As illustrated in Figure 2, at all fields examined, the ENDOR spectra of ^{57}Fe-enriched NiA show a sharp doublet that (i) is centered at the ^{57}Fe Larmor frequency ($\nu_{Fe} = 1.51$ MHz at 11 000 G), (ii) is described by the equation, $\nu_\pm = \nu_{Fe} \pm A(^{57}\text{Fe})/2$, as expected for ^{57}Fe, (iii) is not seen in the ^{56}Fe sample (spectrum not shown). The doublet therefore must arise from a hyperfine-coupled ^{57}Fe. At $g_1 = 2.31$ (Figure 2) the doublet splitting corresponds to an ^{57}Fe hyperfine coupling of $A(^{57}\text{Fe}) \approx 1$ MHz. As the field is increased to $g_2 = 2.24$ (Figure 2), the peaks of the doublet broaden and the hyperfine coupling exhibits a range of values, $A(^{57}\text{Fe}) \approx 0.7-1.3$ MHz. By $g = 2.11$ (Figure 2), the $\nu_\pm$ peaks have again sharpened, and a well-resolved doublet with $A(^{57}\text{Fe}) \approx 0.8$ MHz is observed. The signal does not arise from the ∼10−15% residual NiB because it is not seen at fields near $g = 2.33$, where only NiB contributes, and because it is not seen with a sample of ^{57}Fe isotopically enriched *Dd* Hase (Figure 2) where ∼80% of the dinuclear center is in the NiB state. Reduction[18] of the *D. gigas* Hase by H_2 completely eliminates both the NiA and NiB signals, leaving NiC as the only EPR-detectable state. This sample gave intense ENDOR signals from the strongly coupled exchangeable "hydride" proton, $A(^1\text{H}) = 17$ MHz,[20] previously reported as derivable from H_2, but also showed no ^{57}Fe signals (Figure 2).

Consideration of the full field dependence of the NiA ^{57}Fe coupling by procedures described elsewhere[16] shows that the hyperfine tensor is roughly isotropic, with a maximum tensor component of ∼1.3 MHz and an isotropic coupling of $a(^{57}\text{Fe}) \approx 1$ MHz. This result is consistent both with the absence of any broadening in the EPR spectrum and the inability of Mössbauer spectroscopy to detect the hyperfine-coupled Fe ion.[11,21] The presence of an isotropic component to the ^{57}Fe hyperfine tensor shows that this iron ion is covalently associated with the NiA center and, thus, is the second metal ion. The large value of the ^{61}Ni hyperfine coupling associated with the NiA signal[22] is consistent with a formal assignment of the Ni to the paramagnetic Ni^{III} ($S = {}^1/_2$) state. The small value of the isotropic hyperfine coupling, $a(^{57}\text{Fe})$, for NiA is incompatible with the assignment of iron as Fe^{II} ($S = 2$ or 1) spin coupled to the Ni^{III}; both the $A(^{57}\text{Fe})$ and the [Ni−Fe] cluster spin of $S = {}^1/_2$ rule out the presence of a spin-coupled, high-spin iron ion. Instead, we assign the iron ion as low-spin (diamagnetic; $S = 0$) ferrous, with the observed coupling stemming from a small degree of delocalization from Ni^{III} within the [NiS_2Fe] core. For comparison to the ∼1 MHz coupling to ^{57}Fe seen here, the isotropic hyperfine coupling to a ^{57}Fe ion in any of its paramagnetic states is commonly more than 20 MHz.[23] Combining the ENDOR result with the X-ray diffraction structure, we conclude that the catalytic center of *Dg* Hase is a [Ni−Fe] cluster that in its NiA state is best described as the trapped-valence, [Ni^{III}−Fe^{II}] center pictured in Figure 1.

The ^{57}Fe ENDOR measurements offer a new probe of the differences between the various states of the Hase catalytic cycle. The IR spectra of the Fe-bound diatomic ligands in the "unready" NiA cluster show little change in frequency (*Dg* Hase[5b]; 1−5 cm^{-1}), or none (*Chromatium vinosum* Hase[8]), upon conversion to the "ready" NiB state and similarly small changes upon conversion to the catalytically active NiC form, indicating that the Fe ion also has the same, ferrous valency in all three states and that any difference at Ni is negligibly propagated to Fe. The close similarity of the $\boldsymbol{g}$ tensors and of the ^{61}Ni hyperfine interactions (∼80 G at g_1)[2] of the NiA and NiB clusters indicate that in both states the Ni is in the trivalent state. Upon conversion of the enzyme to the NiC state, the $\boldsymbol{g}$ tensor changes more substantially, and there is considerable debate about the nature of the valency of Ni and the location of the unpaired spin. However, as illustrated in Figure 2, whereas NiA shows a clear hyperfine interaction with the Fe ion, the NiB and NiC states show no ENDOR signal from ^{57}Fe. This observation for the NiC cluster is particularly surprising given the extensive evidence that the majority of the spin resides on the S, not on the Ni.[7,24] One possible explanation for the difference between NiA, which shows a ^{57}Fe coupling, and NiB, NiC, which do not, is that the nonprotein ligand bridge between Ni and Fe is present in NiA but absent in the other two states (Figure 1). In any event, the present results show that ^{57}Fe ENDOR of the hetero-dinuclear cluster provides a sensitive new probe of perturbations at the Hase active site.

Acknowledgment. This work is supported by the NSF, NIH, and JNICT-PRAXIS. We thank R. Toci from CNRS-Marseille at the Fermentation Plant for bacterial growth. We also thank Professor B. H. Huynh at Emory University, Atlanta, GA, for supplying the ^{57}Fe label and for helpful scientific discussions and Clark Davoust for excellent technical assistance. We also thank Pedro Tavares and Alice Pereira (Emory University) for assisting in the reduction of the *Dg* enzyme to the NiC state.

JA9700919

(20) Fan, C.; Teixeira, M.; Moura, J.; Moura, I.; Huynh, B. H.; Le Gall, J.; Peck, H. D., Jr.; Hoffman, B. M. *J. Am. Chem. Soc.* **1991**, *113*, 20−24.

(21) Surerus, K. K.; Chen, M.; van der Zwann, J. M.; Rusnak, F. M.; Kolk, M.; Duin, E. C.; Albracht, S. P. J.; Münck, E. *Biochemistry* **1994**, *33*, 4980−4993.

(22) Moura, J. J. G.; Moura, I.; Huynh, B. H.; Kruger, H. J.; Teixeira, M.; DuVarney, R. G. M.; DerVartanian, D. V.; Ljungdahl, P. O.; Xavier, A. V.; Peck, H. D.; LeGall, J. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 1388−1393.

(23) Houseman, A. L. P.; Oh, B. H.; Kennedy, M. C.; Fan, C.; Werst, M. M.; Beinert, H.; Marley, J. L.; Hoffman, B. M. *Biochemistry* **1992**, *31*, 2073−2080.

(24) Whitehead, J. P.; Gurbiel, R. J.; Bagyinka, C.; Hoffman, B. M.; Maroney, M. *J. Am. Chem. Soc.* **1993**, *113*, 5629−5235.