Oxidized Nickel-Substituted Rubredoxin as a Model for the Ni-C EPR Signal of NiFe Hydrogenases

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A unifying property of all NiFe hydrogenases is the presence of an EPR signal, termed Ni-C, that appears transiently during reductive titrations and has been attributed to an intermediate species in the catalytic cycle.[1] Typical g values are 2.19, 2.14, and 2.02 in NiFe hydrogenases[1] and 2.23, 2.17, and 2.01 in NiFeSe hydrogenases.[2] This species is light-sensitive, and photolysis at temperatures below 100 K results in quantitative conversion to a more rapidly relaxing resonance (10-fold increase in the half-saturation power, $P_{1/2}$, at 20 K) termed Ni-C*, $g \approx 2.29$, 2.12, 2.04.[3] The rate of photolysis is subject to a large D_2O/H_2O isotope effect and hence has been interpreted in terms of photolytic cleavage of a Ni-H bond.[3a] Although ^{61}Ni-hyperfine splitting ($I = {}^3/_2$) has unambiguously demonstrated that the Ni-C signal is associated with nickel,[4] there is as yet no consensus as to whether this $S = {}^1/_2$ resonance corresponds to a Ni(I) or Ni(III) species.[1] Here we report a striking similarity in the EPR properties of cyanide-bound and as-prepared forms of ferricyanide-oxidized Ni(II)-substituted *Pyrococcus furiosus* rubredoxin (Ni(II)-Rd) with those of the hydrogenase Ni-C and Ni-C* signals, respectively. The results strongly support a Ni(III) assignment for both Ni-C and Ni-C* EPR signals and suggest that the Ni-C signal corresponds to a Ni(III) center with square pyramidal or tetragonally elongated octahedral coordination involving four cysteinyl-S ligands and one equatorially-bound H^-.

P. furiosus Ni(II)-Rd was prepared as previously described and characterized as having tetragonally-elongated tetrahedral cysteinyl-S coordination by the combination of UV/visible/near-IR absorption, variable-temperature MCD, and resonance Raman spectroscopies.[5] Oxidation with ferricyanide results in the appearance of a rapidly relaxing rhombic $S = {}^1/_2$ EPR signal, $g = 2.29$, 2.11, and 2.04, that is observable only below 50 K (Figure 1d).[6] The signal intensity depends on the ferricyanide concentration and the incubation temperature and reaches a maximum of ~0.1 spin/molecule with stoichiometric ferricyanide at room temperature. Although the resonance is broader, probably as a result of heterogeneity in the Ni(III) coordination sphere, the g values are very similar to those of Ni-C* (Figure 1b). Parallel studies of samples reconstituted with ^{61}Ni ($I = {}^3/_2$, 88% enrichment, Oak Ridge National Laboratory) showed pronounced broadening on each of the principal components of the g tensor: 1.2 (fwhm), 1.8 (p-p), and 1.8 (fwhm) mT at $g = 2.29$, 2.11, and 2.04, respectively (data not shown). These EPR properties, i.e. ^{61}Ni-broadening, $g_{av} = 2.15$, and $g_\parallel > g_\perp$, are indicative of Ni(III) with distorted square planar, trigonal bipyramidal, or tetragonally compressed octahedral coordination with the unpaired electron in d_{xy} or $d_{x^2-y^2}$. Very similar EPR spectra have been reported, but not quantified, for ferricyanide-oxidized Ni(II)-Rd from *Desulfovibrio vulgaris*, and the close correspondence to Ni-C* was noted.[8]

Ferricyanide oxidation of *P. furiosus* Ni(II)-Rd in the presence of excess cyanide results in a much sharper, slower relaxing, axial $S = {}^1/_2$ EPR signal, $g_\perp = 2.16$ and $g_\parallel = 2.02$, that is observable without significant broadening up to 150 K (Figure 1c). The spin quantitation depends on the ferricyanide and cyanide concentrations and was maximal at ~0.3 spin/molecule with 10-fold excess cyanide and 2-fold excess ferricyanide. The g

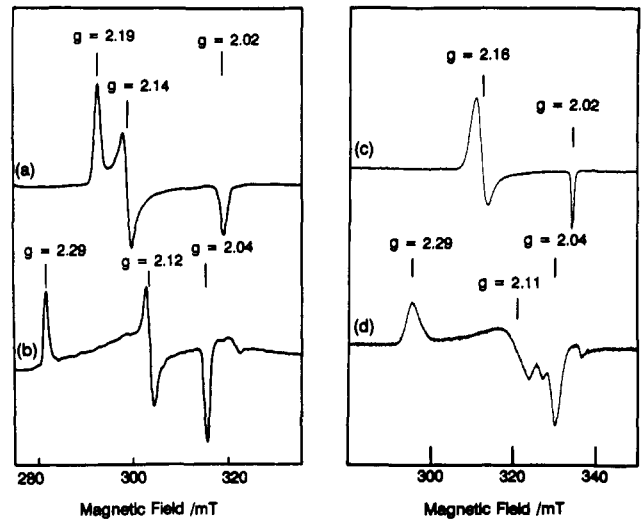

Figure 1. Comparison of the X-band EPR spectra of H_2-reduced *D. gigas* hydrogenase with ferricyanide-oxidized *P. furiosus* Ni(II)-Rd. Left panel: *D. gigas* hydrogenase, 97 μM in 50 mM Tris/HCl, pH 7.6, with 50% (v/v) ethylene glycol, reduced under an atmosphere of H_2 for 24 h at room temperature. Key: (a) The sample frozen in the dark. (b) The sample after photolysis at 70 K with a 200-W xenon-mercury arc lamp at 550 nm for 30 min. The spectra were recorded at 70 K with 1-mW microwave power, 0.63-mT modulation amplitude, and a microwave frequency of 8.98 GHz. Both resonances account for 0.25 ± 0.02 spin/molecule. Adapted from ref 7. Right panel: Ferricyanide-oxidized *P. furiosus* Ni(II)-Rd, 400 μM in 100 mM Tris/HCl, pH 7.8. *Key*: (c) The sample was oxidized with a 2-fold excess of potassium ferricyanide in the presence of a 10-fold excess of sodium cyanide and 55% (v/v) ethylene glycol. The spectrum was recorded at 100 K with 2-mW microwave power, 0.63-mT modulation amplitude, and a microwave frequency of 9.45 GHz. Quantitation under non-saturating conditions indicates that the resonance accounts for 0.3 spin/molecule. (d) The sample oxidized with stoichiometric potassium ferricyanide. The spectrum was recorded at 10 K with 1-mW microwave power, 0.63-mT modulation amplitude, and a microwave frequency of 9.45 GHz. Quantitation under non-saturating conditions indicates that the resonance accounts for 0.1 spin/molecule.

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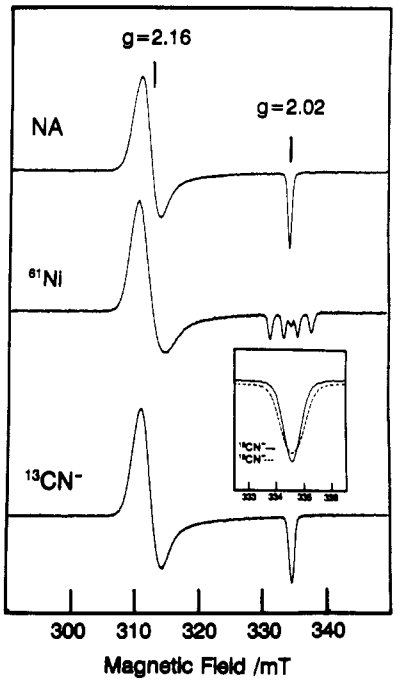

Figure 2. X-band EPR spectra of natural abundance, ^{61}Ni-labeled, and ^{13}CN$^-$-labeled *P. furiosus* Ni(II)–Rd oxidized by ferricyanide in the presence of excess cyanide. The samples and spectrometer conditions are as described in Figure 1. The inset shows an overlay of the $g = 2.02$ features of ^{12}CN$^-$ (solid line) and ^{13}CN$^-$ (dashed line) labeled samples.

values and relaxation properties are very similar to those of Ni–C (Figure 1a), except for the rhombic splitting of the $g_\perp$ component. Moreover, the similarity extends to the magnitude and anisotropy of hyperfine interactions (Figure 2). ^{61}Ni-hyperfine interaction is manifest by broadening of $g_\perp$ ($a_\perp^{Ni} \approx 0.2$ mT) and resolved splitting of $g_\parallel$ ($a_\parallel^{Ni} = 2.2$ mT). Almost identical ^{61}Ni-hyperfine coupling constants have been reported for Ni–C signals in *Methanobacterium thermoautotrophicum* and *Desulfovibrio gigas* hydrogenases, $a_\perp^{Ni} \approx 0.15 - 0.2$ mT[9] and $a_\parallel^{Ni} \approx 2.0$ mT.[4] These EPR properties, i.e. $g_{av} = 2.11$, $g_\perp > g_\parallel$, and highly anisotropic ^{61}Ni-hyperfine with $a_\parallel^{Ni} \gg a_\perp^{Ni}$, are indicative of square pyramidal or tetragonally elongated octahedral Ni(III) species with the unpaired electron in d_{z^2}.[10] The ^{13}CN$^-$-hyperfine interaction is confined to a slight broadening of $g_\parallel$, $a_\parallel^c \approx 0.4$ mT (inset, figure 2), which is consistent with equatorial cyanide coordination.[11,12] Likewise, the small and unresolved ^{1}H-hyperfine interaction on the Ni–C EPR signal can be interpreted in terms of an equatorially coordinated hydride in accord with recent ^{1}H-ENDOR studies.[13] This suggests that cyanide inhibition occurs via cyanide-binding at the hydrogen activation site, and detailed spectroscopic studies of cyanide-inhibited forms of NiFe-hydrogenases are in progress.

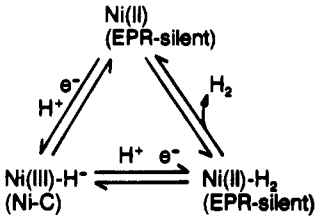

Figure 3. Proposed scheme for the catalytic cycle in Ni-containing hydrogenases.

The EPR results presented add strong support to a Ni(III) assignment for the Ni–C* and Ni–C EPR signals and suggest a close similarity in the nickel coordination environments with those of Ni(III)–Rd and cyanide-bound Ni(III)–Rd, respectively. For Ni(II)–Rd, the nickel is coordinated by four cysteine residues that occur in two Cys–X–X–Cys motifs, one near the N-terminus and the other near the C-terminus.[14] Interestingly an analogous arrangement of conserved cysteines has been found in the N- and C-terminal regions of the large subunit of all NiFe hydrogenases sequenced thus far.[15] The C-terminal Cys–X–X–Cys sequence is clearly implicated in nickel coordination, since selenocysteine, which replaces the first of these cysteines in NiFeSe hydrogenases,[15] is known to coordinate nickel in this class of hydrogenases.[2,16] This work, together with the previous EPR studies of *D. vulgaris* Ni(III)–Rd[8] and the observation of a Ni–C-type EPR signal in a 2:1 disulfhydryl peptide–Ni(III) complex ($g_\perp = 2.16$ and $g_\parallel = 2.02$),[17] raise the possibility that all four of the conserved cysteine residues in the large subunit are involved in coordinating nickel in active NiFe hydrogenases.

These results also permit rationalization of the low levels of hydrogenase activity reported for Ni-substituted rubredoxins[18] and suggest a minimal heterolytic scheme for the catalytic cycle that could be common to both NiFe hydrogenases and Ni-substituted rubredoxins (Figure 3). In this scheme, one electron reduction of the EPR-silent Ni(II) form is coupled with proton addition to give the Ni(III)–H$^-$ species that gives rise to the Ni–C EPR signal. Subsequent one electron reduction and proton addition leads to another EPR-silent species, Ni(II)–H_2, and H_2 evolution regenerates the original Ni(II) form. The lower hydrogenase activity of Ni(II)–Rd is therefore likely to be a consequence of the need to distort and expand the coordination sphere of tetracoordinate Ni(II)–Rd to attain the five- or six-coordinate Ni(III)–H$^-$ or Ni(II)–H_2 forms.

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