Detection of a New Radical and FeMo-Cofactor EPR Signal during Acetylene Reduction by the α-H195Q Mutant of Nitrogenase

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The Mo-dependent nitrogenase of *Azotobacter vinelandii* is a two-component system consisting of iron (Fe) protein and molybdenum−iron (MoFe) protein. In addition to the physiologically relevant conversion of N_2 to NH_3, nitrogenase catalyzes the ATP-dependent reduction of simple, multiple bonded molecules such as C_2H_2, HCN, and HN_3. Substrate reduction is believed to take place at a $MoFe_7S_9$·homocitrate metal cluster called FeMo-cofactor which is contained within the α-subunit of the MoFe protein. During catalysis the Fe protein serves as a specific, MgATP-dependent reductant of the MoFe protein. In its as-isolated form the MoFe protein displays a rhombic $S = 3/2$ EPR signal ($g = 4.3$, 3.6, and 2.0) originating at the FeMo-cofactor. During turnover this signal is diminished by up to 90% to an EPR-silent state. When the potent noncompetitive inhibitor CO is present in the turnover system, two different intense $S = 1/2$ are generated,[1−3] lo-CO ($g = 2.09$, 1.97, 1.93; $P_{CO} = 0.08$ atm) and hi-CO ($g = 2.17$, 2.06, 2.06; $P_{CO} = 0.5$ atm). Recently these signals have been investigated with ENDOR spectroscopy[4−7] and were shown to arise from one or two molecules of CO, respectively, bound to the FeMo-cofactor. Although minor substrate-induced EPR signals have been elicited from the MoFe protein under turnover conditions,[2,8] to date no nitrogenase substrates have been shown to induce strong signals for the wild-type enzyme similar to those observed when CO is present under turnover conditions. Herein, we describe the first report of intense EPR signals, including a radical signal, that are elicited from an altered form of the MoFe protein (α-H195Q) when incubated in the presence of C_2H_2 under turnover conditions.

The α-H195Q mutant form of the MoFe protein was constructed, isolated, and investigated by Kim et al.[9] This altered form of the MoFe protein has glutamine substituted for the α-subunit histidine-195 residue, which is a strictly conserved amino acid within the MoFe protein and is within hydrogen-bonding distance of the FeMo-cofactor.[10−12] The altered protein has received much attention since it previously has been shown that, although its phenotype for reduction of most substrates resembles that of wild-type (acetylene has a nearly identical K_m), it has the unique property that N_2 binds during turnover but is not significantly reduced.[9,13] This mutant MoFe protein also appears to be minimally altered spectroscopically because it exhibits a rhombic $S = 3/2$ EPR signal nearly identical with that found for the wild-type MoFe protein in the as-isolated state. Thus, it was worthwhile to determine if EPR signals arising from enzyme turnover events, previously unobservable in the wild-type MoFe protein, could be observed in this altered protein.

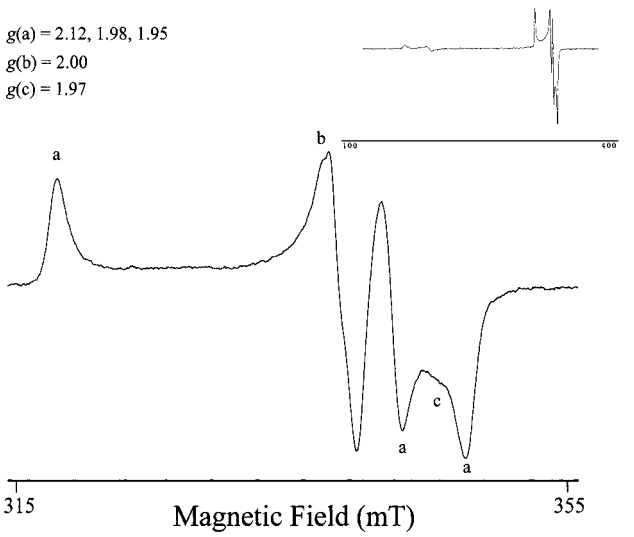

Figure 1. The *g*-2 region of the EPR spectrum of enzymatic turnover for α-H195Q in the presence of acetylene. Inflections originating from the $g = 2.12$ signal ($g = 2.12$, 1.98, 1.95) are marked (a), the $g = 2.00$ signal is marked (b), and the $g = 1.97$ signal is marked (c) The whole spectrum is shown as the insert. Note that the $S = 3/2$ FeMo-cofactor signal is almost completely replaced by the $S = 1/2$ signals. To obtain the high resolution observed in both spectra the modulation amplitude was 0.1 mT, well below the value normally used to record spectra of metal clusters. Experimental conditions: [Fe protein]/[α-H195Q] = [0.020 mM]/[0.100 mM] = 1:5; $[C_2H_2] = 0.1$ atm; [ATP] = 10 mM; $[MgCl_2]$ = 25 mM; $[Na_2S_2O_4]$ = 20 mM; 50 mM TES-KOH, pH 7.4. Spectrometer parameters: microwave frequency = 9.45 GHz; microwave power = 2 mW; modulation amplitude = 0.1 mT; temperature = 4K.

Figure 1 shows the turnover-dependent, acetylene-induced EPR signal of α-H195Q MoFe protein in the *g*-2 region (sample was rapidly frozen in liquid N_2 3 min following initiation of turnover) with the full spectrum included as the insert. This signal (spin integration 0.23 ± 0.02 spins per cofactor) has inflections at $g =$ [2.12, 2.00, 1.98, 1.95] with a minor shoulder at $g = 1.97$ and is not detected when the wild-type enzyme is used under the same conditions. The numerous inflections show that this signal originates from more than one paramagnetic species. To investigate the relationship of these signals, turnover samples were prepared in the presence of C_2H_2 and allowed to incubate at different temperatures (10, 30, and 45 °C) prior to rapid freeze-quench. Figure 2 shows the EPR spectra obtained from samples incubated at 30 and 45 °C illustrating that the $g = 2.00$ inflection has smaller amplitude relative to the $g = 2.12$ inflection at 45 °C compared to 30 °C. Similar changes were observed in turnover samples made at 30 °C compared to those at 10 °C (results not shown) indicating that the $g = 2.00$ and 2.12 inflections represent different species. Temperature-dependent and power-dependency

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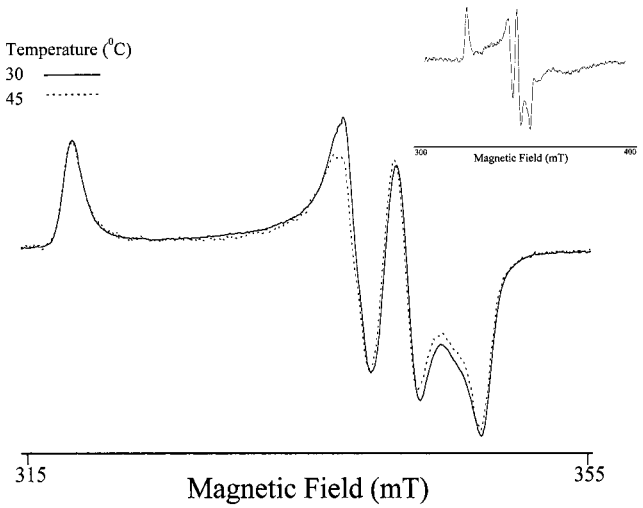

Figure 2. The *g*-2 region of the EPR spectrum of enzymatic turnover for α-H195Q in the presence of acetylene after incubation at $t = 30$ and 45 °C, respectively. Experimental and spectrometer conditions are as described in Figure 1. Insert: The *g*-2 region of the EPR spectrum of enzymatic turnover for α-H195Q in the presence of ethylene (1.0 atm). Experimental and spectrometer conditions are as described in Figure 1.

Table 1. Substrate Isotope Effects on the Line Widths of Individual Inflections

	line width (mT)		
inflection (*g*-factor)	2.12	2.00	1.98
C_2H_2	1.65	1.9	1.7
C_2D_2	1.65	1.7	1.5
$^{13}C_2H_2$	1.8	1.9	1.7

studies (results not shown) further demonstrate that the shoulder at $g = 1.97$ shows behavior that is drastically different from the other signals. These results indicate that there are at least three different signals present in the *g*-2 region. The first (termed *g* 2.12) is a rhombic signal with $g = [2.12, 1.98, 1.95]$. Large deviation from the *g*-factor of the free electron ($g = 2.0023$) is characteristic of an unpaired electron on a transition metal or metal cluster leading to significant spin−orbit coupling. Because the substituted amino acid is located within hydrogen-bonding distance of the cofactor the *g* 2.12 signal most likely originates from the FeMo-cofactor.

The second signal (termed *g* 2.00) is narrow, nearly isotropic with $g = 2.00$, characteristic of radicals which exhibit small *g* anisotropy due to minor spin−orbit coupling. The presence of this signal is significant because it is the first observation of a radical in a nitrogenase turnover sample. There are three possibilities for the origin of the radical species: (1) a C_2H_2 reduction intermediate, (2) an amino acid, perhaps one that is located along the electron-transfer path between the Fe protein docking site and the FeMo-cofactor, and (3) the homocitrate molecule that provides the bidentate ligands to the Mo atom of the FeMo-cofactor. The third signal in Figure 1 is a minor shoulder at $g = 1.97$. The origin of this inflection is not clear and will not be further discussed.

To investigate the possible association of C_2H_2 with either of the first two signals, turnover samples were prepared with $^{13}C_2H_2$ or C_2D_2 as substrate. The line widths of individual inflections are listed in Table 1 and reveal small yet reproducible isotope effects. An overall general trend is evident; the line widths of the *g* 2.12 signal (i.e., $g = 1.98$ inflection) observed in C_2D_2 turnover samples is narrower than what is observed for C_2H_2 turnover samples. Since the magnetogyric ratio for a deuteron is smaller (∼1/6) than for a proton, the EPR signals from species containing C_2D_2 adducts should be narrower than when C_2H_2 is the substrate. This result is consistent with a broadening of the *g* = 2.12 inflection observed in $^{13}C_2H_2$ turnover samples is slightly broader than that observed for C_2H_2 turnover samples (Supporting Information). These data suggest the *g* 2.12 signal arises from an C_2H_2 adduct(s) bound to the FeMo-cofactor during enzymatic turnover.

While an acetylene-induced signal has not been reported previously, a weak axial $S = 1/2$ signal ($g = [2.125, 2.000, 2.000]$; 0.017 spins per cofactor) has been detected[2] in turnover samples of nitrogenase from *Klebsiella pneumoniae* in the presence of ethylene (1.0 atm; signal maximizes at 29 K and 20 mW microwave power). This signal is not observed in turnover samples under identical conditions using either wild-type or α-H195Q nitrogenase from *A. vinelandii*. However, reducing the temperature to 4 K on the α-H195Q sample reveals a weak signal (Figure 2 insert) nearly identical with that observed in the presence of acetylene (Figure 1), strongly suggesting that the *g* 2.12 signal arises from ethylene bound to the FeMo-cofactor.

The possible identity of the *g* 2.00 signal can be further refined. If this signal arose from an intermediate radical of reduced acetylene, the spin density would be localized on the carbon of the radical and a large isotope-induced change in the EPR signal would be expected with either $^{13}C_2H_2$ or C_2D_2. Only very small changes are observed (Table 1) which may originate from changes in the underlying *g* 2.12 signal. In summary, the results suggest that the *g* 2.12 signal arises from C_2H_4 bound at the FeMo-cofactor while the *g* 2.00 signal is most associated with either an amino acid or homocitrate radical species that is generated during enzymatic turnover.

All three signals are observed at C_2H_2 concentrations as low as 0.001 atm and the amplitude ratio of the individual inflections remain unchanged. In other words, there are no hi-/lo-C_2H_2 signals analogous to the aforementioned hi-CO and lo-CO CO signals. A plot of signal amplitude vs C_2H_2 at low concentrations results in a sigmoidal curve suggesting cooperative binding of more than one C_2H_2. This is consistent with the works of Davis et al.[3] and Shen et al.,[14] who have provided evidence for more than one C_2H_2 binding site within the MoFe protein. Finally, similar to the formation of hi-CO under low flux conditions,[15] all three acetylene-induced EPR signals appear at low electron flux. Increasing the component ratio resulted in an overall increase in the final amplitude of the EPR signals without a major change in the amplitude ratios of the individual inflection. Thus, the three species contributing to the spectrum in Figure 1 are simultaneously generated in approximately the same ratio regardless of substrate concentration or electron flux suggesting that one of the species is not a mechanistic precursor of another.

In summary, strong EPR signals, including a radical signal, are elicited from an altered form of the nitrogenase MoFe protein when incubated under turnover conditions in the presence of C_2H_2. The identification of such signals under these conditions is an important advance in our attempt to determine where and how substrates become bound to the active site during nitrogenase turnover, and to determine how the FeMo-cofactor polypeptide environment contributes to that process.

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Supporting Information Available: Figure 3 showing the g = 2.12 inflection of enzymatic turnover for α-H195Q (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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