Electron Paramagnetic Resonance Study of the Vanadium-Iron Protein of Nitrogenase from *Azotobacter vinelandii*

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Recently, a second nitrogenase was isolated from *Azotobacter chroococcum*[1] and *Azotobacter vinelandii*[2,3] and shown to contain a VFe protein in place of the typical MoFe protein.[4] The low-temperature magnetic circular dichroism (MCD)[5] spectrum and magnetization of this protein (Av1′) from *A. vinelandii* demonstrates[6] the presence of a V–Fe–S center (FeV-co) analogous to the FeMo cofactor (FeMo-co) in the MoFe protein (Av1) but with different electronic and magnetic properties. In order to understand and interpret the MCD spectrum as well as gain a better understanding of the cofactor in V-nitrogenase, it is important to determine the spectroscopic splitting factors (g values) and zero-field splitting parameters of the paramagnetism of Av1′ through a study using electron paramagnetic resonance (EPR) spectroscopy.

The low-temperature EPR spectrum of as-isolated Av1 (Figure 1a) has been interpreted[7,8] to arise from the ground-state Kramer's doublet of an $S = {}^3/_2$ spin system with small rhombicity ($\lambda = 0.053$, where ${}^1/_3 \geq \lambda \geq 0$) and a positive axial zero-field splitting ($D \approx 6.0$ cm^{-1}). The EPR spectrum for Av1′ (Figure 1b) under the same conditions is obviously different, containing both low field inflections at apparent g values of 5.80, 5.40, and 4.34 and high field inflections at 2.04 and 1.93 but lacking the characteristic inflection at 3.7 of Av1 (an inflection at this position, however, is observable[9] in the spectrum of the VFe protein isolated from *A. chroococcum*). The inflections in the spectrum of Av1′ can be rationalized in terms of at least three different spin components. The high field inflections represent one spin component of an axial system with $g_{av} < 2$ and probably correspond to an $S = {}^1/_2$ spin system such as that found in reduced Fe–S clusters.[10] This signal exhibits very strong microwave power saturation at low temperatures (Figure 1 (parts b and c)) and appears to oxidize with thionine[6] more easily (i.e., has an apparent lower reduction midpoint potential) than do the other signals of Av1′. Spin quantitation of this signal shows it to be a minor component with around 0.2 spins per vanadium atom (typically Av1′ analyzes[3] to contain around one V atom per protein). Similarly, the $g = 4.34$ inflection in the low field region of the Av1′ spectrum is also a minor component of the spectrum, and probably corresponds to an adventitious $S = {}^5/_2$ spin system, such as can be found with high spin Fe^{3+}

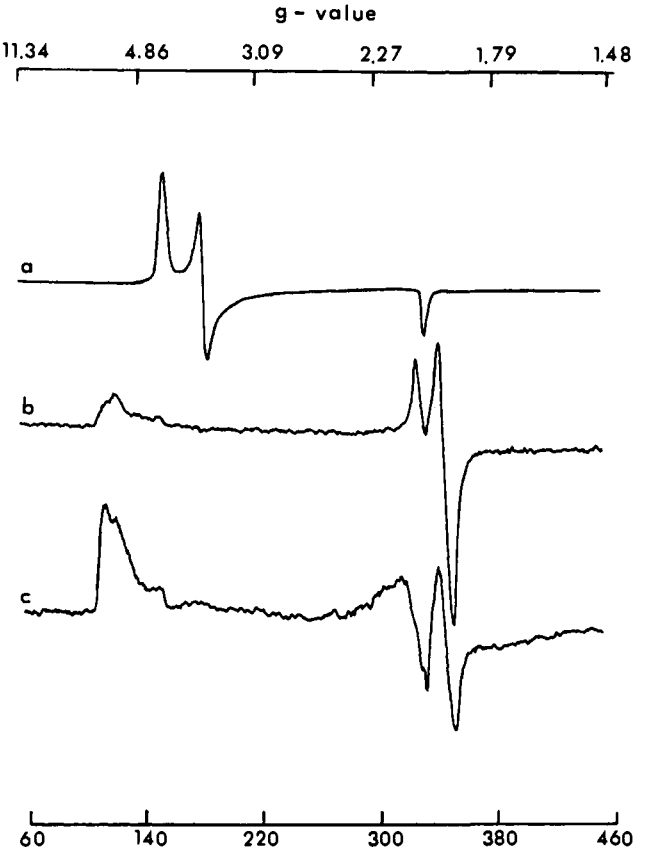

Figure 1. Low-temperature EPR spectra of Av1 (a) and Av1′ (b and c) in 25 mM Tris buffer, pH 7.4, containing 0.25 M NaCl and 2 mM dithionite. Conditions: (a) protein concentration, 21 mg mL^{-1}; specific activity, 1500 nmol C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; temperature, 5.5 K; microwave power, 50 μW; modulation amplitude, 0.5 mT; frequency, 9.32 GHz; gain, 4×10^4; (b) protein concentration, 25 mg mL^{-1}; specific activity, 200 nmol C_2H_2 reduced min^{-1} (mg of protein)$^{-1}$; temperature, 5.5 K; microwave power, 0.2 mW; modulation amplitude, 2 mT; frequency, 9.32 GHz; gain, 5×10^5; (c) shows the effect of low temperature and high power on the $g = 2$ signal with the same conditions as in (b) except temperature, 3.5 K. The origin of the broadening of spectrum in the $g = 2$ region in (c) is, as yet, unknown. It should be noted that the concentrations of the $S = {}^3/_2$ signals in the spectra Av1 and Av1′ are approximately one spin per Mo atom and V atom, respectively. The great difference in the intensities of these signals in the spectra of these two proteins, as can be seen in (a) and (b), is probably due, in part, to the greater spectral width of the Av1′ signal. Proteins used were identical with those described in ref 6.

The remaining two inflections at 5.80 and 5.40 correspond to the third component of Av1′. These inflections are not commonly observed in biological samples but, like the paramagnetism of Av1, can be associated with an $S = {}^3/_2$ spin system. In general, the sets of principal g' values (for $S = {}^3/_2$) corresponding to the two Kramer's doublets (labeled 1 and 2) can be expressed[11] as

$$g_{1x}' = g_0[1 + (1 - 3\lambda)/(1 + 3\lambda^2)^{1/2}]$$
$$g_{1y}' = g_0[(1 + 3\lambda)/(1 + 3\lambda^2)^{1/2} + 1]$$
$$g_{1z}' = g_0[2/(1 + 3\lambda^2)^{1/2} - 1]$$
$$g_{2x}' = g_0[1 - (1 - 3\lambda)/(1 + 3\lambda^2)^{1/2}]$$
$$g_{2y}' = g_0[(1 + 3\lambda)/(1 + 3\lambda^2)^{1/2} - 1]$$
$$g_{2z}' = g_0[2/(1 + 3\lambda^2)^{1/2} + 1] \quad (1)$$

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(5) Abbreviations: Av1, MoFe protein of conventional nitrogenase from *Azotobacter vinelandii*; Av1′, VFe protein of alternative nitrogenase from *Azotobacter vinelandii*; EPR, electron paramagnetic resonance; FeMo-co, iron–molybdenum cofactor from conventional nitrogenase; FeV-co, iron–vanadium cofactor from alternative nitrogenase; MCD, magnetic circular dichroism; T, Tesla.
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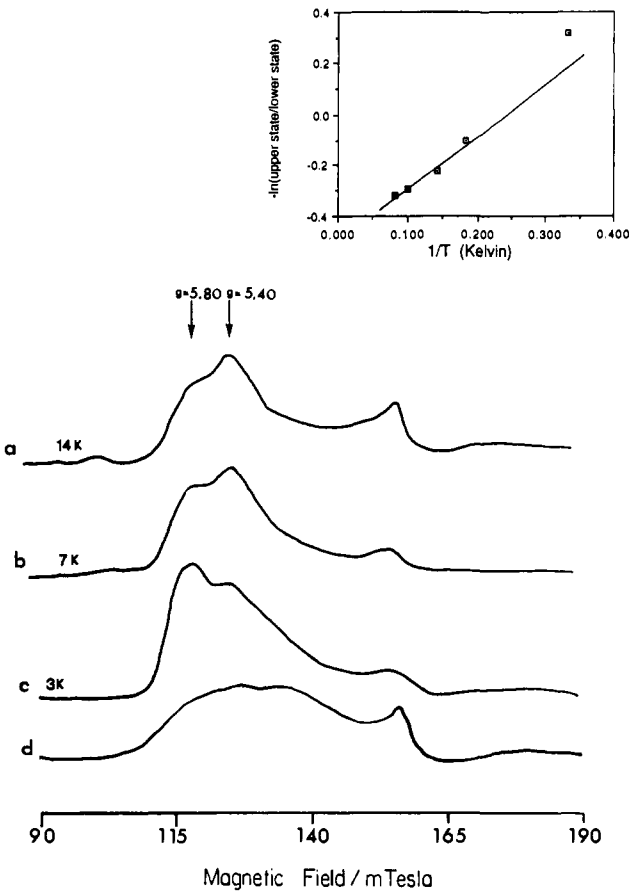

Figure 2. Effect of temperature and ethylene glycol on the $S = {}^3/_2$ spin signals of Av1′ spectrum. Buffer, protein concentration and specific activities as in Figure 1. Conditions: (a) temperature, 14 K; microwave power, 10 mW; modulation amplitude, 1 mT; frequency, 9.30 GHz; gain, 3.5×10^6; (b) temperature, 7 K; microwave power, 10 mW; modulation amplitude, 1 mT; frequency, 9.30 GHz; gain, 2.5×10^6; (c) temperature, 3 K; microwave power, 0.2 mW; modulation amplitude, 2 mT; frequency, 9.30 GHz; gain, 5×10^5; (d) conditions identical with those of figure (b) except solvent contains 50% ethylene glycol. Insert: plot of the negative natural logarithm of the ratio of the relative amplitudes of $g = 5.40$ to the $g = 5.80$ inflections versus the reciprocal of the absolute temperature. Inflection observed around 100 mT in (a) is not consistently observed in the Av1′ spectrum and, therefore, probably is exogenous to the Av1′ paramagnetism.

With use of $\lambda = 0.26$ and $g_0 = 2.06$ in eq 1 for Av1′, the predicted g' values are $g_{1x}' = 2.46$, $g_{1y}' = 5.40$, $g_{1z}' = 1.69$ for the first level and $g_{2x}' = 1.66$ and $g_{2y}' = 1.29$, $g_{2z}' = 5.80$ for the second. Obviously, the observed 5.80 and 5.40 inflections of Av1′ correspond to g_{2z}' and g_{1y}', respectively, while the remaining four inflections are undetectable, possibly due to g strain broadening.[12,13]

Since the two low field inflections represent different energy levels of the same $S = {}^3/_2$ spin system, their relative amplitudes must be coupled and temperature dependent. Figure 2 shows the low field region of Av1′ recorded at different temperatures, clearly demonstrating the temperature dependence of the shape of the spectrum and indicating that the intensity of the 5.8 signal increases with decreasing temperature and, therefore, implying that this signal corresponds to the ground-state level. For this inflection to correspond to the ground state, the axial zero-field splitting parameter, D, must be negative (as compared to a positive D for Av1). Furthermore, by plotting the natural logarithm of the ratio of the amplitudes of the two low field inflections against the reciprocal of the absolute temperature (insert in Figure 2), the magnitude of D can be calculated to be -0.74 cm^{-1}. This value obviously is much smaller than that determined[6,7] for the Av1 (≈ 6 cm^{-1}) and is consistent with the greater nesting observed in the magnetization of this paramagnetic cluster in Av1′ compared to that in Av1 as determined by MCD spectroscopy.[6]

Since the results recently reported on the MCD spectrum and magnetization of Av1′ were performed with the protein in a 50% ethylene glycol glass, it is important to determine whether this medium significantly perturbs the environment of the paramagnetic cluster. Figure 2d shows the spectrum of Av1′ recorded in 25 mM Tris·HCl buffer, pH 7.4, with 50% ethylene glycol. In the presence of ethylene glycol, the two low field inflections corresponding to the $S = {}^3/_2$ spin system are both significantly broadened and slightly shifted to higher fields. Due to this extensive broadening, no obvious g' values can be extracted from the spectrum. Spin integration[14] shows a slight increase in intensity of the $S = {}^3/_2$ system in the presence of ethylene glycol (0.94 spins per V atom in 50% ethylene glycol versus 0.89 spins per V atom in buffer), although the uncertainty in these integrations, especially in the presence of ethylene glycol where specific g' values are difficult to assign, may make this difference insignificant. Within experimental error, no significant change was observed in the integrated intensity of the axial $S = {}^1/_2$ signal at 2.04 and 1.93 in the two media.

In summary, the VFe protein of alternative nitrogenase, like the MoFe protein of the conventional nitrogenase, possesses a paramagnetism, part of which can be attributed to an $S = {}^3/_2$ spin system. The major differences between the two proteins is, first of all, the presence of an $S = {}^1/_2$ axial signal possibly associated with a reduced Fe–S cluster in Av1′ and, secondly, the much higher rhombicity and smaller (as well as negative) axial zero-field splitting parameter in the $S = {}^3/_2$ signal of the VFe protein. At present, it is not known whether these latter differences arise from the substitution of V for Mo in FeMo-co, differences in the proteins holding these cofactors or the production of an entirely new type of cofactor in FeV-co.

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Exceptional Efficacy of Some New Lacunar Dioxygen Carriers Based on Schiff Bases Derived from β-Diketones and Triamines

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Dioxygen carriers continue to attract intense interest because of their immense promise in the many applications dependent on gas-phase dioxygen separations and because of the need to understand a variety of O_2 adducts in living systems.[1-4] The *performance parameters* and structure-parameter relationships that determine the efficacy of O_2 carriers are not thoroughly understood, but the greatest difficulties are associated with the fact that all dioxygen carriers are oxidized by O_2. The design of more efficacious oxygen carriers is a fascinating challenge.

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