A novel $S=3/2$ EPR signal associated with native Fe-proteins of nitrogenase

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In addition to their $g=1.94$ EPR signal, nitrogenase Fe-proteins from *Azotobacter vinelandii, Azotobacter chroococcum* and *Klebsiella pneumoniae* exhibit a weak EPR signal with $g \simeq 5$. Temperature dependence of the signal was consistent with an $S=3/2$ system with negative zero-field splitting, $D=-5\pm0.7$ cm^{-1}. The $m_s = \pm 3/2$ ground state doublet gives rise to a transition with $g_\parallel^{eff} = 5.90$ and the transition within the excited $m_s = \pm 1/2$ doublet has a split $g_\perp^{eff} = 4.8, 3.4$. Quantitation gave 0.6 to 0.8 spin·mol^{-1} which summed with the spin intensity of the $S=1/2$ $g=1.94$ line to roughly 1 spin/mol. MgATP and MgADP decreased the intensity of the $S=3/2$ signal with no concomitant changes in intensity of the $S=1/2$ signal.

*Nitrogenase   Fe-protein   ESR   Azotobacter vinelandii   Klebsiella pneumoniae
Azotobacter chroococcum*

1. INTRODUCTION

Nitrogenase, the enzyme which catalyses the ATP-dependent reduction of dinitrogen to ammonia, can be readily separated into two redox proteins, the MoFe- and the Fe-protein (reviewed in [1–3]). During enzyme turnover, the Fe-protein transfers electrons to the MoFe-protein in an ATP-dependent reaction. The Fe-proteins are homomeric dimers of relative molecular mass around 64 kDa, and sequence data for 9 Fe-proteins isolated from different organisms show a high degree of conservation [4]. These proteins contain approx. 4 iron and 4 acid-labile sulphide atoms per dimer [1–3] although values significantly higher than these have been reported [5]. However, the consensus view is that these proteins contain a single $[4Fe-4S]^{(2+;1+)}$ cluster which bridges the two subunits.

As isolated, all Fe-proteins exhibit an EPR spec-

Abbreviations: Av₂, Ac₂ and Kp₂, Fe-proteins of nitrogenase from *Azotobacter vinelandii, Azotobacter chroococcum* and *Klebsiella pneumoniae*, respectively

trum with g values at 1.85, 1.94 and 2.06, which integrates to a spin stoichiometry of 0.2 to 0.5 per protein dimer, with most values close to 0.2 [1–3]. The low integration is not due to incomplete reduction of the Fe/S cluster [6]. The suggestion that it arises as a consequence of spin-coupling with an unidentified, rapidly relaxing paramagnet [7], has recently been shown to be incompatible with the temperature and frequency dependence, and power saturation characteristics of the $g = 1.94$ signal [8].

The work presented here shows that highly purified preparation of Fe-proteins from 3 organisms, *Azotobacter vinelandii, Azotobacter chroococcum* and *Klebsiella pneumoniae*, exhibit, in addition to their well-established $g = 1.94$ signal, an EPR signal with g values near 5 due to an Fe/S center in a novel $S = 3/2$ spin state.

During the course of this work, an EPR signal with similar properties was reported for Av₂ [9] and correlated with Mössbauer data obtained for Av₂ in 50% ethylene glycol or 0.4 M urea to show that the mixture of $S = 1/2$ and $S = 3/2$ clusters is roughly half and half.

2. MATERIALS AND METHODS

Nitrogenase components Av_2 and Ac_2 were assayed and purified by standard techniques described for Av_2 [5]. Kp_2 was purified essentially as described in [10] except that dithiothreitol was omitted from buffers used for sample preparation. Samples were concentrated for EPR spectroscopy by absorption and elution from DEAE Sephacryl and subsequently desalted by gel exclusion as described in [5]. Iron was determined colorimetrically with bathophenanthroline disulphonate as described in [11]. Acid labile sulphide was estimated by methylene blue formation using the modified method described in [12]. Protein concentration was estimated by the microbiuret method on samples precipitated with trichloroacetic acid in the presence of deoxycholate [13], using bovine serum albumin as a standard.

EPR measurements and analyses were done as described earlier [8].

3. RESULTS AND DISCUSSION

3.1. *EPR studies on Av_2, Ac_2 and Kp_2: signal characteristics and assignment*

We studied preparations of the Fe protein of nitrogenase by means of X-band EPR spectroscopy to test the prediction [8] that highly purified preparations contain, in addition to the regular [4Fe-4S] cluster, a paramagnet not hitherto detected by EPR spectroscopy.

Spin integration of the $g = 1.94$ signal of Av_2, Ac_2 and Kp_2 showed it to be present in substoichiometric concentrations in preparations of all 3 proteins (table 1). However, in addition to the $g = 1.94$ species a rather weak, broad signal around $g = 5$, whose intensity was proportional to protein concentration, was observed in 5 preparations of Av_2 and one each of Ac_2 and Kp_2. The data of table 1 show that for Av_2 both signals disappear upon oxidation with phenazine methosulphate and are restored upon re-reduction with dithionite. Low temperature and high microwave power levels are required to detect the new signal. Fig.1 shows the $T = 4.2$ K spectra of the $g = 4-6$ region for Av_2, Kp_2 and Ac_2. The signals are disturbed by variable amounts of an essentially isotropic signal with $g = 4.3$ that is commonly ascribed to adventitious iron. Aside from

Table 1

Quantitation of the two EPR signals in Av_2, Kp_2, Ac_2

	[S = 3/2]	[S = 1/2]
Av_2	0.8	0.17
Kp_2	0.6	0.40
Ac_2	~1[a]	0.18
Av_2 + urea (0.4 M)	1.0[b]	0.04
Av_2 + ethylene glycol (50% v/v)	0.2	0.40
Av_2 + MgADP (5 mM)	0.6	0.21
Av_2 + .MgATP (5 mM)	0.3	0.20
Av_2 after removal of MgATP	0.8	0.17
Av_2 oxidized with phenazine methosulphate	0	0.01
Av_2 re-reduced by dithionite	0.8	0.25

[a] Overestimate due to the presence of a $g = 4.3$ signal of high intensity

[b] Not corrected for possibly different g values, linewidth or D value

Note: the specific activities (nmol C_2H_2 reduced $\cdot$ min$^{-1} \cdot$ (mg of protein)$^{-1}$), Fe and S^{2-} contents of the Fe-protein samples were: Av_2, 1600, 3, 2.9; Ac_2, 1600, 3.7, 3.7 and Kp_2, 1895, 3.7, 3.7. The residual activities of these preparations due to contaminating MoFe-protein were $Av_2 < 0.001\%$; Ac_2 0.095% and Kp_2 0.16% of their potential activity when assayed with an optimum amount of MoFe-protein

this contamination the signals are very similar for the Fe-protein from the 3 different organisms. They all show an asymmetric peak at $g = 5.9$ and a shoulder and a zero crossing at higher fields.

The shape of the signal is temperature dependent. Upon raising the temperature, the low-field peak at $g = 5.9$ loses intensity relative to the shoulder which ultimately becomes the dominant peak with $g = 4.8$ (see fig.2). Also, the derivative-like feature with zero-crossing at approx. $g = 3.4$ increases in concert with the $g = 4.8$ peak. These features are indicative of a system with $S = 3/2$ and a zero-field interaction whose magnitude is large compared to the Zeeman interaction. In this situation the spin manifold can be described as consisting of two Kramers doublets with $m_s = \pm 1/2$ and $m_s = \pm 3/2$, respectively, which are well separated by, in first order, an axial zero-field splitting of magnitude $2D$. The $m_s = \pm 1/2$ doublet will give rise to an allowed transition ($|\Delta m_s| = 1$) with effective g values of $g_\perp \simeq 4$ and $g_\parallel \simeq 2$. The
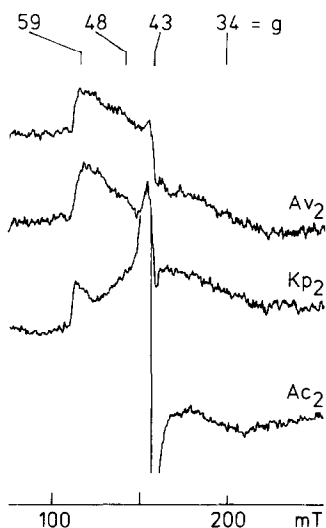
Fig.1. Comparison of the low-temperature EPR spectra of reduced Av_2, Kp_2 and Ac_2 in the $g = 4$–6 region. At $T = 4.2$ K the fractional population of the excited $m_s = \pm 1/2$ doublet ($S = 3/2$, $D = -5$ cm^{-1}) is only a few percent. Thus, the spectra are dominated by the 'forbidden' transition within the ground $m_s = \pm 3/2$ doublet with $g_{\parallel} = 5.9$. The line at $g = 4.3$ is from contaminating Fe. EPR conditions (Bruker ER 200 D): microwave frequency, 9.39 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 20 mW; temperature, 4.2 K.

$m_s = \pm 3/2$ doublet may give rise to a weak, in first order forbidden, transition ($|\Delta m_s| = 3$) with $g_{\parallel} \simeq 6$ and $g_{\perp} \simeq 0$.

We identify the line at $g = 5.9$ with the $g_{\parallel}$ of the transition within the $m_s = \pm 3/2$ doublet and the lines at $g = 4.8$, 3.4 with the $g_{\perp}$ of the transition within the $m_s = \pm 1/2$ doublet split by rhombic distortion. The relative intensities of the lines as a function of temperature (cf. fig.2) indicate the zero-field splitting, $2D$, to be negative. The inset to fig.2 shows the population percentage ($\%N$) of the $|\pm 1/2\rangle$ doublet measured as the product of signal intensity at $g = 4.8$ times the temperature, T, vs T^{-1}. A fit to these data, using the Boltzmann distribution function

$$n_{\pm 1/2} = n_{\pm 3/2}\, \exp(-2D/kT)$$

is optimal for $D = -5 \pm 0.7$ cm^{-1}. The $m_s = \pm 3/2$ doublet is the ground state.

In fig.2 it is also shown that an approximate fit to the $|\Delta m_s| = 1$ spectrum can be obtained assum-
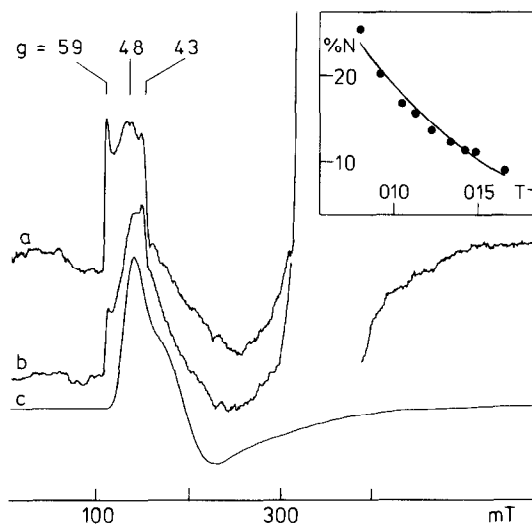

Fig.2. Temperature dependence of the $S = 3/2$ signal from reduced Av_2 and simulation of the transition within the $m_s = \pm 1/2$ doublet. When the temperature is raised from 11 (trace a) to 24 K (trace b) the peak at $g = 4.8$ ($\Delta m_s = 1$) gains intensity relative to the peak at $g = 5.9$ ($\Delta m_s = 3$) indicating the $m_s = \pm 3/2$ doublet to be the ground state ($D < 0$). The inset shows a fit for $D = -5$ cm^{-1} to the population percentage, $\%N$, of the $m_s = \pm 1/2$ doublet measured as temperature-corrected intensities at $g = 4.8$. EPR conditions (Varian E-112): microwave frequency, 9.21 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.25 mT; microwave power, 200 mW; temperatures, 11 and 24 K. Simulation (cf. [14,15]) parameters of trace c: $g_{1,2,3} = 2$, 3.33, 4.78; $\Delta g_{ii} = 0.45$.

ing an effective $s = 1/2$ subsystem broadened by an isotropic g strain (cf. [14,15]).

On the basis of the spin Hamiltonian

$$H = D[S_z^2 - S(S+1)/3] + E(S_x^2 - S_y^2) + g\beta B \cdot S$$

with $|D| = 5$; $|E| = 0.6$; $g = 2$, exact diagonalization of the energy matrix, for the external magnetic field along each of the three principal axes, results in effective g values $g_{\parallel} = 5.9$ for the $m_s = \pm 3/2$ doublet and $g_{x,y} = 4.7$, 3.2 for the $m_s = \pm 1/2$ doublet which compare well with the values of 5.9, 4.8 and 3.4 estimated from the experimental spectra, and also with the values of the 4.8 and 3.3 determined by the $S^{\mathrm{eff}} = 1/2$ simulation of the $|\pm 1/2\rangle$ transition.

Spin $S = 3/2$ systems are unusual in biological systems and since the foregoing is also an adequate

first-order description for the X-band EPR from the 'M'-cluster in reduced MoFe-protein from nitrogenase or from the isolated FeMo-cofactor [16,17], it is a pertinent question as to whether the signals of figs 1 and 2 might not arise from contaminating MoFe-protein, be it native, denatured, or modified in some other way. Five observations argue against this notion: the level of contaminating MoFe-protein in our preparations of Fe-proteins was extremely low, either undetectable (Av$_2$ and Ac$_2$) or less than 1% (Kp$_2$) on visual inspection of strained SDS gels; the residual activity due to contaminating MoFe-protein was at most 0.15% that of their potential activity when fully complemented with MoFe-protein (see table 1); the g values of the new spin 3/2 signal are distinctly different from those exhibited by MoFe-protein; the zero field splitting parameter is of the opposite sign; and finally quantitation (see section 3.2) indicates a significant spin concentration to be associated with this center.

3.2. Quantitation of the S = 3/2 signal

The $S = 3/2$ signal has been quantified in two different ways: (i) comparison of the low-field half of the $g = 4.8$ line with the second integral of a CuII standard making use of the Aasa-Vänngård formula for isolated single peaks [18]; (ii) comparison of the amplitude of the simulation of fig.2 with the amplitude of a g-strain simulation of the $S = 1/2$ signal [8] from the same sample, where the number of spins in the experimental $S = 1/2$ signal is known by double integration vs a CuII standard. Applied to a sample of Av$_2$, methods i and ii both gave 0.8 spins. The similarity between these determinations is remarkable in view of the number and character of the assumptions and uncertainties involved in these equations. This result lends credit to the notion that the $S = 3/2$ and the $S = 1/2$ signal add up to approximately one electron per dimer of Fe-protein containing approx. 4 Fe atoms.

The result of the quantitations appears as the first entry in table 1, and this value was used as the reference to quantitate the $S = 3/2$ spectra from Ac$_2$ and Kp$_2$ by comparison with their signal intensities at $g = 4.8$. Taken together, the $S = 3/2$ and $S = 1/2$ spin integration approximates to 1 e$^-$ per dimer and accounts for the low spin integration of preparations of Fe-protein on the basis of their $g =$ 1.94 signal [1–3] but which now amounts to 1 e$^-$ per 4 Fe atoms. However, we feel that in view of the uncertainty as to the structure of the cluster giving rise to the novel EPR signal, the finding of approximately 1 e$^-$ per 4 Fe atoms is insufficient to conclude that it arises from a single [4Fe-4S] ferredoxin-type cluster. Indeed, the spacing of the 5 invariant cysteine residues that each subunit of Fe proteins contain shows no homology with the cysteinyl spacing that characterises the ligands to Fe/S centers in ferredoxins (cf. [4]).

3.3. Solvent dependence of electronic states

We have previously reported that in 50% (v/v) ethylene glycol the spin integration of the $S = 1/2$ EPR signal is increased [19]. Table 1 shows that under these conditions, which do not result in loss of activity, the $S = 3/2$ signal is diminished which may suggest that spin-state changes are induced by solvent perturbation. The effect of ethylene glycol is of significance for the interpretation of magnetic circular dichroism studies on Fe-proteins [6]. Since samples are prepared under conditions which cause the loss of the $S = 3/2$ EPR signal, MCD spectra have been performed until now on samples which exhibit only the $S = 1/2$ spin state.

3.4. Effect of MgATP and MgADP on the S = 3/2 signal

The addition of MgATP, and to lesser extent MgADP, to Av$_2$ or Kp$_2$ results in a decrease of $S = 3/2$ signal without a concomitant increase in intensity of the $S = 1/2$ signal (table 1); this effect was reversible since removal of the MgATP by gel filtration restored the $S = 3/2$ signal. These observations are difficult to reconcile with an ATP-induced conformational change resulting in a conversion of spin-state from $S = 3/2$ to $S = 1/2$. We looked for, but failed to detect, EPR signals which would characterise higher spin states. It has been suggested [9] that the effect of ethylene glycol in bleaching the $S = 3/2$ signal with enhancement in the intensity of the $S = 1/2$ signal (see also table 1) could be explained by a solvent effect on the spin state of a single Fe/S center. The effect of MgATP we observe is clearly different. Since no increase in absorbance in the visible region accompanies loss of the $S = 3/2$ signal, it is unlikely to be due to oxidation, and its restoration when MgATP is removed is not consistent with its being due to in-

stability of the Fe/S cluster. Further work is necessary to resolve this problem and to clarify the nature of the Fe/S centers present in Fe-proteins of nitrogenase.

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