

On the prosthetic group(s) of component II from nitrogenase

EPR of the Fe-protein from *Azotobacter vinelandii*

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The EPR spectrum of the reduced Fe-protein from nitrogenase has been reinvestigated. The dependences on temperature, microwave power, and microwave frequency all suggest that the observed signal represents a magnetically isolated $[4\text{Fe-4S}]^{1+(2+,1+)}$ cluster. Also, the signal can be simulated assuming a simple, *g*-strained $S = \frac{1}{2}$ system. However, the integrated intensity amounts to no more than 0.2 spins per protein molecule. It is, therefore, impossible that Fe-protein preparations contain a single type of $[4\text{Fe-4S}]$ cluster.

Nitrogenase Fe-protein A. vinelandii ESR Spin-spin interaction

1. INTRODUCTION

Nitrogenase catalyzes the ATP-dependent reduction of molecular nitrogen to ammonia in some bacteria and bacteroids. The enzyme complex is composed of two dissociating proteins. Component I or the MoFe-protein, a 220-kDa, $\alpha_2\beta_2$ -tetrameric protein containing iron and molybdenum, is supposed to be the site of N_2 reduction. Component II or the Fe-protein, a 64-kDa homogeneous dimeric protein, provides reducing equivalents to component I in the presence of MgATP [1–3]. Approx. 4 iron atoms and acid-labile sulfur atoms per dimer are repeatedly found in colorimetric analyses of the Fe-protein [1–3]. Cluster extrusion quantitatively yields the $(4\text{Fe}4\text{S}4\phi\text{S})$ core [4]. The EPR *g* values [5], the magnitude and angular dependency of the linear electric field effect on the EPR [6], and the form and magnitude of the magnetic circular dichroism [7] of reduced Fe-protein all indicate the presence of a $[4\text{Fe-4S}]^{1+(2+,1+)}$ ferredoxin structure. It has been proposed that a single cubane is

equally shared by the two identical subunits of the Fe-protein [4,8].

However, shared ligation of a single cluster by different subunits is unprecedented in iron-sulfur biochemistry. Furthermore, several experimental results appear to be not readily compatible with the notion of a single ferredoxin cubane in the Fe-protein. Most importantly, EPR quantitations usually give a value for the spin stoichiometry per protein dimer in the range 0.2–0.5, a typical number being 0.2 [1–3]. Also, recent Fe and S^{2-} determinations in our laboratory have given values significantly higher than 4 per dimer [9]. Finally, the number of electrons transferred by the Fe-protein is uncertain, i.e. one [10–12] vs two electrons [13,14].

In attempts to, at least partly, explain these results, two alternatives for the existence of a single, magnetically isolated $S = \frac{1}{2}$ cubane have been proposed [15,16]: (i) according to Orme-Johnson et al. [15] a low-lying EPR-silent excited state may be thermally populated to the disadvantage of the EPR-active state. The same authors

also suggested [15] that a second paramagnetic center may couple to the spin on the FeS cluster forming an even-spin state without EPR activity, but this idea is in direct conflict with low-temperature Mössbauer spectra [17] that exhibit a broadening consistent with half-integral spin; (ii) Lowe [16] proposed that a second, rapidly relaxing (therefore unobservable) paramagnet is spin-coupled to the FeS cluster, resulting in broadening and decrease of integrated intensity of the FeS EPR signal. More specifically, Braaksma et al. [9] suggested that, at least in Fe-protein with high Fe and S^{2-} content, this rapidly relaxing center is another [4Fe-4S] cluster.

The central virtue that these models share is that they provide an explanation for the low value of the spin stoichiometry. However, these models also implicate unusual behaviour of the observable EPR signal as a function of readily variable external parameters, i.e. temperature, microwave power, microwave frequency. Here we report the results of testing these predictions. Our results limit the possibilities for the prosthetic group(s) in the Fe-protein.

2. MATERIALS AND METHODS

Azotobacter vinelandii ATCC 478 was grown in batch cultures of 20 l in a Bioengineering fermentor type LP30. Two types of cells were grown. Cells were harvested during the logarithmic phase (type I) and cells were given an oxygen shock (excess oxygen that inhibits growth). After adaptation cells start to grow again. During this second logarithmic growth cells were harvested and stored at -70°C (type II). Isolation of the nitrogenase and standard nitrogenase activity assays were run as described [9]. Iron was determined colorimetrically with bathophenanthroline disulfonate [18] as described in [9]. The sulfide concentration was estimated according to Fogo and Popowski [19] as modified by Brumby et al. [20]. Protein was estimated with the biuret method with bovine serum albumin as the standard. Two samples isolated from the two types of cells were studied. Fe-protein isolated from the two types of cells had, respectively, a specific activity of 2100 and 2200 $\text{nmol C}_2\text{H}_4 \text{ formed} \cdot \text{min}^{-1} \cdot (\text{mg protein})^{-1}$, an iron content of 3.5 and 5.0, and an S^{2-} content of 4.0 and 5.0 mol per mol Fe-protein (62.8 kDa).

The results presented below pertain to the second sample, however, the results on the first sample were not significantly different.

X-band EPR was measured with a Varian E-112 spectrometer equipped with a home-built He-flow system. The P-band spectrometer, which accepts standard-size X-band samples, has been described [21]. Integrations were performed following [22] using a solution of 10 mM $\text{CuSO}_4/10 \text{ mM HCl}/2 \text{ M NaClO}_4$ as the external standard. The theory and numerical methods of g -strain analysis have recently been described [23,24].

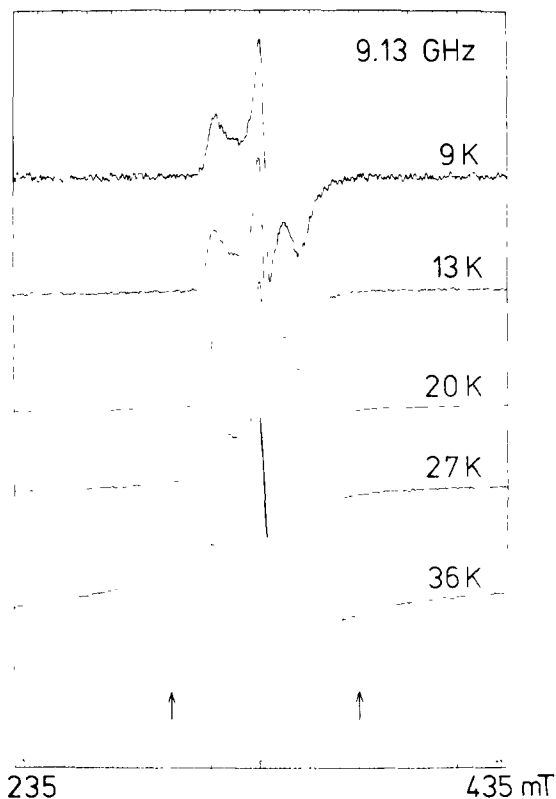


Fig. 1. Dependence on the temperature of the EPR signal of the reduced Fe-protein (11.4 mg/ml) from *A. vinelandii*. The onset of the lifetime broadening above $T = 13 \text{ K}$ is usual for magnetically isolated [4Fe-4S] ferredoxins. The integrated intensity follows Curie's law when the integration is done over the whole spectral trace but not when the integration is cut off at the limits (cf. the arrows) of the unbroader spectrum. EPR conditions: microwave frequency, 9.13 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.63 mT; microwave power (temperature), 0.013 (9), 0.32 (13), 13 (20), 32 (27), 200 (36) mW (K).

3. RESULTS

In fig.1 the EPR signal of reduced Fe-protein from *A. vinelandii* is monitored as a function of the temperature. If not essentially identical, the shape of the low-temperature signal is very similar to those published for earlier preparations of the Fe-protein [25–27]. The shape of the signal is invariant from 9 to 13 K; at 20 K lifetime broadening has set in as evidenced by a relative decrease in amplitude of the sharp, derivative-type feature around g_y , when compared to the amplitude of the absorption-type lines at g_z and g_x . At still higher temperatures the g_z and g_x lines start to broaden until resolution is lost around 36 K, and the signal becomes undetectable above approx. 45 K. This response upon increasing the temperature is standard for a magnetically isolated [4Fe-4S] cluster (cf. [28]). No sign of anomalies due to spin-spin relaxation is found.

Previous work has suggested that the second integral of the signal is unusually low because 'the very long tails are not fully included' [16]. The two arrows at the bottom of fig.1 indicate the approximate limits of a spectrum, taken at $T = 18$ K, originally published by Smith et al. [26], and later used for computer analysis [16]. To test this suggestion we have integrated the spectra of fig.1 within the arrows and compared this to wide-scan (i.e. 200 mT) integrations. The results are given in table 1. The values show that it is indeed likely that some intensity is overlooked under the conditions used by Smith et al. and Lower [16,26]. However,

Table 1

Integrated EPR intensity of the reduced Fe-protein from *A. vinelandii* as a function of field limits and temperature

Temperature (K)	Spin concentration ^a	
	For whole scan	With truncated wings
9	0.19	0.19
13	0.16	0.17
20	0.20	0.14
27	0.16	0.11
36	0.18	0.04

^a mol spins per mol Fe-protein (62.8 kDa)

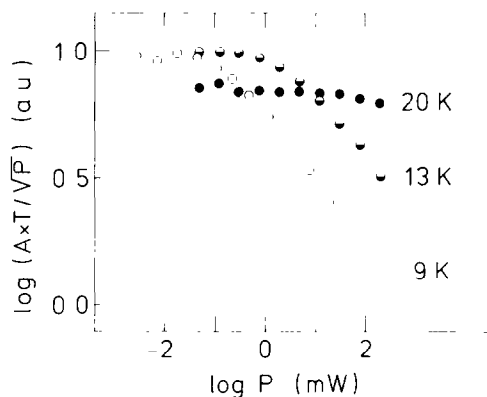


Fig.2. Dependence on the microwave power of the EPR signal of the reduced Fe-protein from *A. vinelandii*. The intensity was measured as the peak-to-peak amplitude (A) of the derivative line at g_y as a function of the power (P) and the temperature (T). The quantity on the ordinate should be a constant for an unsaturated, unbroadened signal. The saturation behavior found is usual for magnetically isolated [4Fe-4S] ferredoxins. EPR conditions were as in fig.1.

this slight underestimation is not nearly enough to account for a substoichiometric spin concentration. When integrated over a sufficiently large field range, the second integral is inversely proportional to the temperature (i.e. Curie's law). In other words, the temperature-corrected spectral intensity is essentially a constant; this is to be expected for an isolated $S = 1/2$ system. Still, only 0.2 electron equivalents are found per molecule of Fe-protein. At low temperatures (9–13 K) the spectrum has no significant intensity outside the field range marked by the two arrows.

A [4Fe-4S] cluster in interaction with a system that relaxes so fast that it cannot be detected by EPR should be much harder to saturate than an isolated cluster. Fig.2 shows the intensity of the g_y lines as a function of incident microwave power. In contrast to prediction, saturation at $T = 9$ K sets in at a power as low as approx. 20 μ W. Rupp et al. have reported ranges of power-saturation data for FeS proteins at temperatures from 13 to 21 K (cf. fig.2 in [28]), and our data at 13 and 20 K fall nicely within the range for magnetically isolated [4Fe-4S] clusters. Again, no anomalies due to spin-spin relaxation are observed.

If the spectrum of fig.1 contains information on exchange or magnetic dipole-dipole interaction,

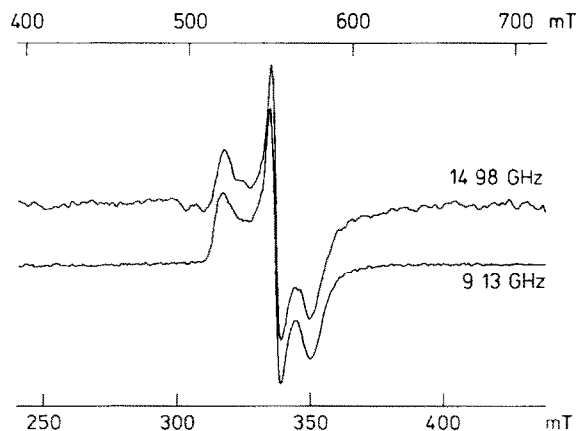


Fig.3. Dependence on the microwave frequency of the EPR signal of the reduced Fe-protein from *A. vinelandii*. The spectrum was measured in X-band (the lower trace is identical to the 13 K spectrum of fig.1) and in P-band (the upper trace and upper field axis). The two spectra were linearly replotted on the same g -value scale. It is characteristic for a magnetically isolated $S = \frac{1}{2}$ system that the two traces become identical under this operation. P-band EPR conditions: microwave frequency, 14.98 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; microwave power, 0.13 mW; temperature, 13 K.

then the spectral shape should change upon varying the microwave frequency. In fig.3 we compare the low-temperature, unsaturated spectra of reduced Fe-protein at microwave frequencies of 9 and 15 GHz. Within experimental noise, no differences are detectable. This observation suggests that the spectrum comes from a magnetically isolated $S = \frac{1}{2}$ system, broadened essentially only by g strain. No sign of any interaction to a second spin system is observed.

In the absence of interaction it should be possible to generate numerically the EPR signal on the basis of a mathematical description of g strain in randomly distributed $S = \frac{1}{2}$ systems [23]. The result of extensive minimization (following [24]) is given in fig.4. Some slight misfits remain. A less satisfactory fit was obtained in a simulation on the basis of two interacting spins [16]. However, the two analyses are not readily comparable, since the author in [16] did not attempt an exhaustive minimization. For example, he assumed the absence of anisotropy in the second spin, since 'detailed studies of these effects produce many am-

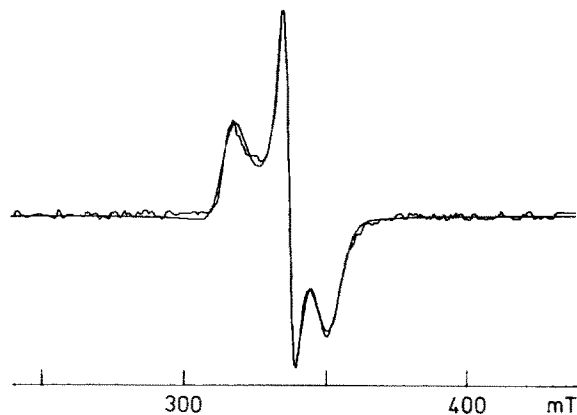


Fig.4. Computer simulation of the EPR signal of the reduced Fe-protein from *A. vinelandii*. A 256-point digitization of the upper trace of fig.1 was simulated assuming a magnetically isolated $S = \frac{1}{2}$ system subject to g strain. The quality of the fit, as compared to a previous simulation, favors this model over the previous model of a system subject to spin-spin interaction. Simulation parameters: $g_{1,2,3} = 1.854, 1.936, 2.063$; $\Delta g_{11,22,33,12,13,23} = 0.024, 0.010, -0.030, 0.004, -0.005, 0.001$; residual broadening, 0.006. See [23,24] for details on the simulation procedure.

biguities' (cf. [16]). The point in question is that it is not necessary to go beyond the simplest possible assumption of an isolated $S = \frac{1}{2}$ system in order to fit the spectrum.

4. DISCUSSION

The integrated intensity of the EPR signal from the Fe-protein follows Curie's law. This observation strongly argues against the population of an EPR-silent low-lying excited state. The power-saturation characteristics of the EPR signal are standard for [4Fe-4S] ferredoxins. Its dependence on frequency is characteristic for a simple $S = \frac{1}{2}$ system. A better, or at least equally satisfactory, simulation is obtained when assuming a magnetically isolated, g -strain broadened $S = \frac{1}{2}$ system than assuming two interacting spins. The data presented here argue against the existence of spin-spin interaction; they are fully consistent with the notion that the EPR signal stems from a regular ferredoxin cubane.

We feel that we have proven the models, outlined in section 1, to be incorrect. However, the EPR signal does integrate to about 0.2 electron per

dimeric Fe-protein. In other words, some 20% of the Fe-protein molecules have a $[4\text{Fe-4S}]^{1+}$ cluster. Therefore, the common notion that the Fe-protein contains one ferredoxin cubane as its single prosthetic group per 64 kDa dimer cannot possibly be correct. Either there are multiple spin systems in some of the protein molecules or some of the clusters have a ground state with half-integer spin not equal to one-half. Only these combinations will simultaneously explain the paramagnetism in the Mössbauer spectroscopy and the absence of an EPR signal. Further studies are required to establish the nature of the prosthetic group(s) in the active Fe-protein of nitrogenase.

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