

tionship between the two enzymes can be made from the inhibition, activation, and or simultaneous inhibition-activation studies.

The action of 16 $\alpha$ -methylprogesterone was to inhibit both the high-efficiency 6 $\beta$ - and 16 $\alpha$ -hydroxylase activities. The low-efficiency 16 $\alpha$ -hydroxylase was largely unaffected. Although inhibition of the 6 $\beta$ -hydroxylase was clearly competitive, the exact nature of inhibition for the high-efficiency 16 $\alpha$ -hydroxylase is difficult to identify. This is a result of the interference of such analysis by the low-efficiency 16 $\alpha$ -hydroxylase. Further evidence of this nature is provided by the experiments with 5 $\beta$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol. This compound, a naturally occurring catabolic product of progesterone, has been shown to be the most potent of allosteric effectors studied of the 16 $\alpha$ -hydroxylase activity of P-450 3b, obtained from strain IIIVO/J (Johnson et al., 1983). With the III/J P-450 3b, only the low-efficiency 16 $\alpha$ -hydroxylase appeared to be effected. Again, the precise nature of the activation caused by this compound was difficult to assess because of the simultaneous expression of two distinct 16 $\alpha$ -hydroxylases. However, the combined inhibition-activation study resulted in inhibition of the high-affinity moiety of this activity while stimulating that of the residual low-affinity activity. These results are consistent with the properties of a second enzyme being added to those of the 16 $\alpha$ -hydroxylase expressed in strain B/J. An alternative explanation would require a structural difference in P-450 3b leading to complex negative and positive effector interactions to produce the observed effects by 16 $\alpha$ -methylprogesterone and 5 $\beta$ -pregnane-3 $\beta$ ,20 $\alpha$ -diol on the activity of III/J P-450 3b.

The segregation of 6 $\beta$ H and 6 $\beta$ L phenotypes among inbred rabbits independently of the expression of the P-450 3b cat-

alyzing the 16 $\alpha$ -hydroxylation of progesterone that is stimulated by the pregnanediol leads us to propose two distinct genetic loci encoding P-450 3b. It is likely that a heritable difference between these strains affects one of these loci yielding phenotypic differences in 6 $\beta$ -hydroxylase activity.

**Registry No.** Cytochrome P-450, 9035-51-2; progesterone 6 $\beta$ -hydroxylase, 9082-59-1; progesterone 16 $\alpha$ -hydroxylase, 9082-60-4; progesterone, 57-83-0.

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## Electron Paramagnetic Resonance Spectrum of the Iron Protein of Nitrogenase: Existence of a $g = 4$ Spectral Component and Its Effect on Spin Quantization<sup>†</sup>

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Received April 19, 1985

**ABSTRACT:** The electron paramagnetic resonance (EPR) spectra of 11 separately prepared and highly purified samples of *Azotobacter vinelandii* iron protein, Av<sub>2</sub>, were studied as a function of protein concentration, temperature, and microwave power. Reductive and oxidative EPR titrations of Av<sub>2</sub> demonstrated that the signal height at  $g = 1.94$  and integrated spin intensity (spin per mole of Av<sub>2</sub>) responded precisely and predictably to the degree of oxidation or reduction of Av<sub>2</sub>. When the EPR signals from all 11 samples were integrated in the  $g = 2$  region of the spectrum, spin per mole values near 0.25 were obtained. Low-temperature (below 12 K) and low-power levels (below 0.8 mW) caused significant signal distortion, a phenomenon peculiar to reduced Av<sub>2</sub> and certain of the [Fe<sub>4</sub>S<sub>4</sub>(SR)<sub>4</sub>]<sup>3-</sup> synthetic model compounds. Wide-field scans of Av<sub>2</sub> disclosed the presence of a resonance near  $g = 4$  in all Av<sub>2</sub> samples, and signal integrations that include this low-field resonance significantly increased the spin per mole values (uncorrected for  $g$  value) of Av<sub>2</sub>. Similar effects were observed with Av<sub>2</sub> in the presence of MgATP and for certain [Fe<sub>4</sub>S<sub>4</sub>(SR)<sub>4</sub>]<sup>3-</sup> clusters.

**T**he Fe protein component of the nitrogenase system is a two-subunit protein ( $\alpha_2$ ) containing a single iron-sulfur cluster which is thought to be of the Fe<sub>4</sub>S<sub>4</sub>-type and to be bound by cysteine residues from each of the two subunits (Hausinger

& Howard, 1983). The protein is redox active and has been reported to undergo a well-defined electron-transfer reaction with an  $E_{1/2}$  value of ca. -300 mV vs. NHE (Zumft et al., 1974; Lowe et al., 1980; G. D. Watt, unpublished results). Binding of MgATP to the dithionite-reduced protein lowers the redox potential by ca. 100 mV while concomitantly changing the EPR signal due to the iron-sulfur cluster from a rhombic to an axial form (Lowe et al., 1980; Mortenson &

<sup>†</sup>Contribution No. 872. This work was supported by the U.S. Department of Agriculture Competitive Grants Program, Grants 81-CRCR-1-0675 (J.W.M.) and 82-CRCR-1-1122 (G.D.W.).

Thorneley, 1979). The interaction with MgATP also increases the oxygen lability of the protein and enhances its tendency toward cluster destruction by iron-chelating agents (Mortenson & Thorneley, 1979). The exact nature of the reduced  $[\text{Fe}_4\text{S}_4]$  center and how its properties are changed by interaction of the protein with energy-yielding MgATP are important aspects in understanding the role of this protein in biological nitrogen fixation. In particular, the EPR spectrum of this cluster has been, and should continue to be, a useful probe for such studies. Unfortunately, a great deal of confusion exists with regard to this signal and to the  $[\text{Fe}_4\text{S}_4]$  cluster which produces it. For example, numerous attempts have been made to quantitate the spin concentration of the iron protein from various organisms by double integration of the EPR signal (Burgess, 1984; Orme-Johnson et al., 1977). Most reported results give values of ca. 0.20 spin/mol, values well below that expected for a single antiferromagnetically coupled  $[\text{Fe}_4\text{S}_4]^{1+}$  moiety. The only report (Braaksma et al., 1982) of a double integration of the iron protein from *Azotobacter vinelandii* ( $\text{Av}_2$ ) which yielded 1 spin/mol would seem to conflict with other data (Braaksma et al., 1983) from the same laboratory which indicates, contrary to the widely accepted belief, that  $\text{Av}_2$  contains two  $[\text{Fe}_4\text{S}_4]$  centers. It has been suggested that the "normal" low double integration value for  $\text{Av}_2$  might be due both to the existence of a second paramagnetic center which relaxes the main signal-producing center (Lowe et al., 1980; Orme-Johnson et al., 1977) and to the presence of a contaminant (Orme-Johnson et al., 1977). Confirmatory evidence for either of these hypotheses has not been reported.

In order to better understand the role of the iron protein and the nature of the iron-sulfur cluster which it contains, we have begun a systematic study of the EPR spectral properties of this entity. Our preliminary results, some of which appear to shed light on the low double-integration values described above, are reported herein.

#### MATERIALS AND METHODS

The iron protein of nitrogenase from *A. vinelandii*,  $\text{Av}_2$ , was isolated and purified as previously described (Burgess et al., 1980). Protein concentration was determined either from amino acid analysis or optical absorbance measurements using molar absorptivity values of 18.1 and 11.1  $\text{mM}^{-1}$  for the oxidized protein at 365 nm and the reduced protein at 400 nm, respectively (Anderson & Howard, 1984). Iron content and specific activity (Burgess et al., 1980) of the proteins used are indicated in the tables or figure captions. In general, high activity (2500–3300 nmol of  $\text{H}_2$   $\text{min}^{-1}$   $\text{mg}^{-1}$ ) preparations were used except for selected samples low in either activity or iron content used for comparison purposes. Dithionite solutions were prepared from high purity (>98%)  $\text{Na}_2\text{S}_2\text{O}_4$  and standardized optically at 315 nm ( $\epsilon = 8.00 \text{ mM}^{-1}$ ) or coulometrically at –200 mV vs. NHE.

Oxidized  $\text{Av}_2$  was prepared in a Vacuum Atmospheres glovebox ( $\text{O}_2 < 0.1$  ppm) by reaction of dithionite-reduced  $\text{Av}_2$  with excess indigodisulfonate (IDS) or methylene blue (MB) followed by removal of the dyes on an anaerobic G-25 column. The extent of oxidation was determined microcoulometrically (Watt, 1979) or by titration with standardized methylviologen (MV) or  $\text{S}_2\text{O}_4^{2-}$ . Reductant-free, reduced  $\text{Av}_2$  was prepared in a similar way from MV-reduced  $\text{Av}_2$  and the extent of reduction verified by microcoulometry or titration with standardized IDS and MB.

EPR titrations of  $\text{Av}_2$  were conducted in 3-mm i.d. quartz tubes by using standardized IDS and MB or  $\text{S}_2\text{O}_4^{2-}$  to oxidize or reduce  $\text{Av}_2$  samples, respectively. EPR measurements on these titration samples were carried out at various temperatures

Table I: Characterization and Spectral Double-Integration Values at 12 K for Various Samples of Reduced  $\text{Av}_2$

sample	Fe/mol	nmol of $\text{H}_2$ $\text{min}^{-1}$ $\text{mg}^{-1}$	spin/mol			
			mg/mL	5 mW	80 $\mu\text{W}$	31 $\mu\text{W}$
1	4.79	3088	21.7	0.21	0.18	0.27
2	4.21	2657	14.5	0.21		
3	4.14 <sup>b</sup>	2367	18.0	0.18	0.15	0.25
4	3.72	2630	20.3	0.20		
5	3.60	3116	17.9	0.20	0.18	0.22
6	3.56	1915	17.1	0.20	0.15	
7	3.36	2030	26.9	0.27	0.22	
8	2.97 <sup>b</sup>	2115	21.1	0.23	0.22	
9	3.99	3312	20.6	<i>a</i>	<i>a</i>	<i>a</i>
10	4.31	3107	24.6	<i>a</i>	<i>a</i>	<i>a</i>
11	3.72	2497	27.3	<i>a</i>	<i>a</i>	<i>a</i>

<sup>a</sup> Sample used to acquire data presented in Table II. <sup>b</sup>  $\text{Av}_2$  samples contained  $^{57}\text{Fe}$ .

and power levels. EPR spectra were obtained on a Varian 4502 spectrometer equipped with a Model V4560 100-kHz modulation control unit and an X-band microwave bridge. The EPR samples were cooled with liquid He by using an Oxford Instruments transfer line and Dewar. The spectrometer output was interfaced with an Apple II+ computer for spectral double integration. A reference sample of Cu(II)–EDTA (EDTA/Cu > 10) at either 1.00, 0.500, or 0.100 mM was used to quantitate the spin concentration in  $\text{Av}_2$  samples. The temperature was measured with an iron–gold thermocouple which was placed in the sample position within the Dewar.

#### RESULTS AND DISCUSSION

In order to systematically investigate the EPR spectrum of the iron protein, and in particular to quantitate the spin concentration of this entity, we examined 11 separately prepared samples whose metal content, activity, and protein concentration are given in Table I. Special precautions were taken during protein isolation and sample preparation to eliminate  $\text{O}_2$  exposure, and, for several samples in Table I, the entire preparation was passed through the purification procedure a second time. EPR spectra of these preparations were recorded and double integrated in the  $g = 2$  region at a temperature of 12 K while varying the microwave power from 5 to 0.031 mW. Overall spectral appearance was identical with that previously reported from other laboratories. In earlier EPR studies of this type (Burgess, 1984; Palmer et al., 1972; Orme-Johnson et al., 1977; Yates & Planque, 1975), typically values of ca. 0.2 spin/mol were obtained, and our results (Table I) are in keeping with these findings. The fact that the integrated intensity is constant for the large number of preparations investigated indicates that the low value for spin concentration is not due to the nature of the sample, while the fact that no consistent increase in intensity was observed on lowering the power level likewise provides evidence that saturation is not responsible for this phenomenon. In addition, both integrated signal intensity and signal height measurements were made as a function of protein concentration (2–25 mg/mL) for several of these  $\text{Av}_2$  preparations. Plots of both these parameters vs. protein concentration were linear, eliminating the possibility that concentration-dependent magnetic interactions are responsible for the low values of spin per mole shown in Table I and those previously reported.

The possibility that the observed  $\text{Av}_2$  EPR signal, and its low double-integration value, arises from a contaminant or from an inactive/alterd form of the native protein was examined by the titration studies summarized in Figure 1. In these experiments, dye-oxidized  $\text{Av}_2$  was reduced sequentially with standardized  $\text{S}_2\text{O}_4^{2-}$ , while dithionite-reduced  $\text{Av}_2$  was oxidized sequentially with MB or IDS. The results of these

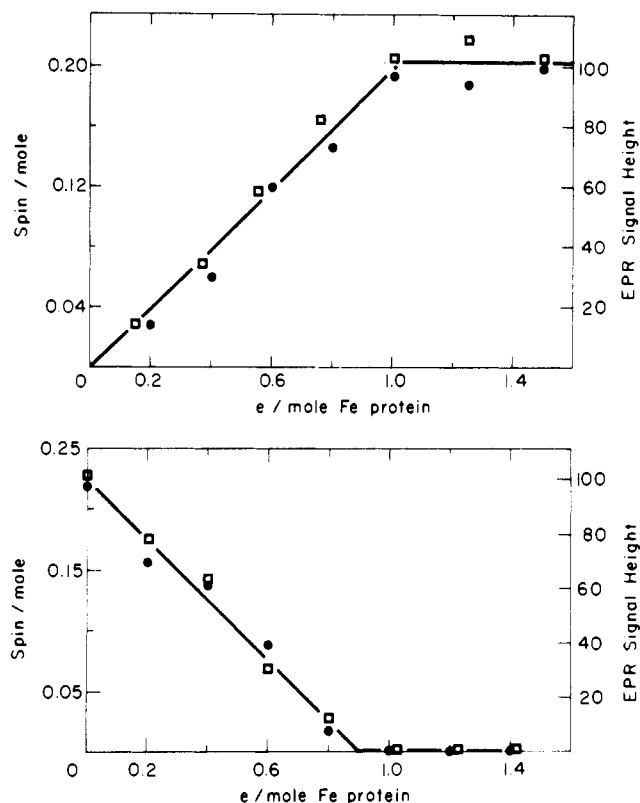


FIGURE 1: EPR titrations of indigodisulfonate-oxidized  $Av_2$  with standardized  $S_2O_4^{2-}$  (upper curve) and methylviologen-reduced  $Av_2$  with methylene blue (lower curve). The reduced protein used in the lower curve was found by microcoulometry to be oxidized by 0.15 electron/mol prior to titrant addition. Sample 9 of Table I was used in these titrations at a concentration of 10 mg/mL. The abscissa is the electrons per mole added to or removed from  $Av_2$ , and the ordinates are spin per mole (■) and percent of EPR signal height at  $g = 1.94$  (●).

studies clearly show that both the doubly integrated spectral intensity and the signal height at  $g = 1.94$  vary linearly with the amount (0–1 equiv) of added reductant or oxidant. It should be noted particularly that a full one-electron equivalent of reagent is required to completely develop or quench the signal, which nevertheless accounts for only ca. 0.25 spin/mol. These results unequivocally establish a strictly-obeyed relationship between the EPR signal intensity and the redox state of  $Av_2$ , a finding which is seemingly inconsistent with the possibility that a small amount of contaminant accounts for the fractional spin observed.

After determining that the low double-integration values for  $Av_2$  are not due to sample preparation, protein concentration, or the presence of a contaminant, we carried out additional experiments to attempt to understand this anomaly. In the course of these investigations, two observations relevant to the EPR spectroscopy of this protein became evident. The first, while not seemingly related to the low spin per mole value associated with  $Av_2$ , indicates that caution should be exercised when spectra of the iron protein are obtained at very low temperatures and power levels. The second discovery is more directly relevant to the integration studies and may explain, at least partially, why these unusually low-spin intensity values have been consistently obtained.

Figure 2 contains the usual presentation of the spectrum of  $Av_2$ , i.e., showing only the  $g = 2$  region, as a function of temperature. The most obvious conclusion from this spectral series is that significant distortion of the  $g = 2$  region of the spectrum begins to occur below 12 K at moderate power (0.8 mW). This effect becomes more evident as both the tem-

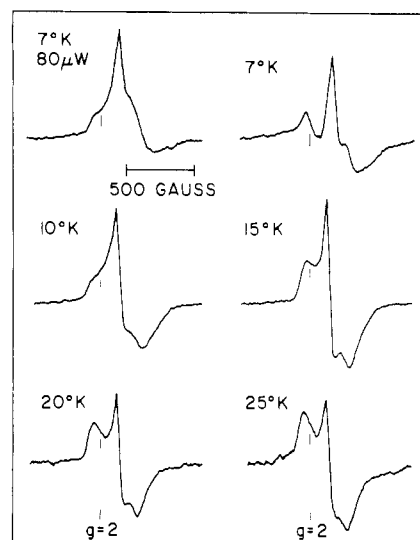


FIGURE 2: EPR spectra of  $Av_2$  (sample 9) in the  $g = 2$  region as a function of temperature at a microwave frequency of 9.15 GHz. The microwave power was 0.08 mW for the indicated 7 K spectrum and 0.80 mW for all other spectra.

perature and power are decreased, so that at 7 K and a low power level of 0.08 mW, most of the derivative signal resides above the middle base line. Because of the conditions necessary for the onset of this phenomenon (low power and temperature), we speculate that it is likely due to dispersion (Randolph, 1972). However, the cause is seemingly less important than the finding that the spectrum of the iron protein can be experimentally subject to these difficulties. Clearly, double integration of derivative spectra that contain unequal areas above and below the base line, e.g., those in Figure 2 at 7 and 10 K cannot be reliable, and thus quantitation of signal intensity at very low temperature and power, conditions that we initially felt might overcome potential saturation problems and result in higher values of spins per mole, cannot be accomplished. To our knowledge, these spectral distortion effects have not been previously reported, although some difficulties in obtaining reliable EPR spectra of the iron protein at low temperature were recently described (Anderson & Howard, 1984) and could be due to the same cause. While we have no theoretical explanation for this apparent dispersion phenomenon at present, it should be noted that the effect is not an instrumental artifact, as evidenced by the fact that a perfectly normal  $S = 3/2$ -type signal of the iron-molybdenum protein of nitrogenase is observed at 5 K and 0.08 mW of power, conditions that produce extreme distortion of the  $Av_2$  signal.

Double integration of the  $g = 2$  region of the iron protein EPR spectrum at 15, 20, and 25 K (Figure 2), where no spectral distortion is evident, yielded values of 0.30, 0.28, and 0.23 spin/mol, respectively, values consistent with previously reported determinations. We felt it possible that these low integral values might be due to the presence of another resonance that had been previously undetected in the EPR spectrum of the iron protein. Evidence that additional signals might be present in the iron protein is suggested by early EPR studies of clostridial iron protein ( $Cp_2$ ) (Orme-Johnson et al., 1972; Palmer et al., 1972) that report a resonance near  $g = 4.3$  which was present in varying amounts, depending on the protein preparation. The former authors even report that this signal is split by the presence of MgATP. However, both groups questioned the validity of this signal and attributed it to adventitiously bound iron or to iron from some minority species.

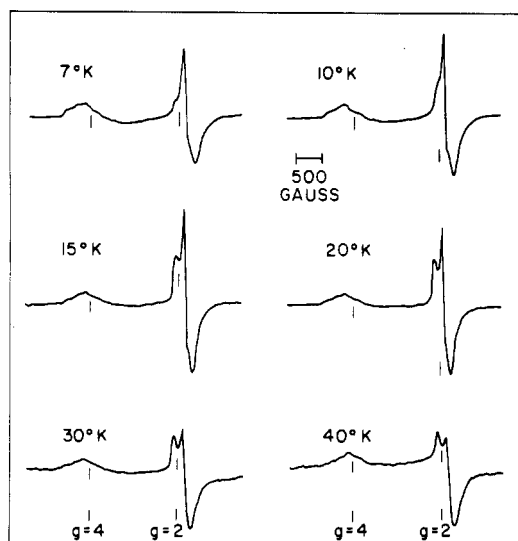


FIGURE 3: EPR spectra of  $\text{Av}_2$  (sample 9) as a function of temperature at a microwave power of 20 mW and microwave frequency of 9.15 GHz.

The presence of contaminating iron or partially destroyed protein in these early  $\text{Cp}_2$  samples of low activity seems quite likely with the attendant consequence that the observed EPR signal near  $g = 4.3$  arises from this cause. However, the possibility that this signal is native to the protein or that a contaminating iron signal obscures part of the native signal must also be considered likely and explored in further detail. We felt that the highly purified, highly active  $\text{Av}_2$  preparations used in this study should be ideally suited to determine if other signals were present, and indeed when wide range scans from ca.  $g = 8$  to  $g = 1.5$  were carried out on these samples, a broad resonance was observed at ca.  $g = 4.4$  in addition to the sharper signal at ca.  $g = 2$ . Figure 3 contains the total EPR spectrum of  $\text{Av}_2$  as a function of temperature at high power and clearly demonstrates the existence of this additional resonance and temperature effects associated with it. The low field component is not an artifact of a particular sample since it was observed at a near constant level for all protein preparations described in Table I. In addition, we are confident that the observed  $g = 4.4$  signal is not a result of  $\text{O}_2$  inactivation because of the care taken during sample preparation and because the  $g = 4.3$  signal that arises from  $\text{O}_2$  inactivation is of a different form (Wang et al., 1984) from that seen in the samples used here. Finally, we conclude that the low-field resonance is not due to contamination by  $\text{Av}_1$  because the signal does not mimic either the qualitative appearance or the exact  $g$  values seen for the  $S = 3/2$  signal of  $\text{Av}_1$ .<sup>1</sup>

As shown in Table II, double integration of the total  $\text{Av}_2$  signal of three different preparations over the wider range yielded values significantly higher than those for integration of the  $g = 2$  region alone. In fact, values as high as 0.84 spin/mol were obtained at high temperature for two of the samples. However, caution should be exercised in accepting these higher values for at least two reasons: (1) Double Integration over such a wide range can result in large errors and uncertainties in the derived value, particularly when the signal to noise ratio is low, as for the above high-temperature spectra. Our experience has shown that definition of the base line of the derivative spectrum (a rather arbitrary decision for noisy spectra) becomes critical for these wide field integrals, and

Table II: Double-Integration Values for Three  $\text{S}_2\text{O}_4^{2-}$ -Reduced  $\text{Av}_2$  Samples (Samples 9–11 in Table I) as a Function of Temperature and Power and Double-Integration Values as a Function of Temperature for  $\text{Av}_2$  in the Presence of 2.5 mM MgATP and for Synthetic  $[\text{Fe}_4\text{S}_4]^{1+}$  Clusters<sup>a</sup>

sample	temp (K)	power (mW)	$g = 2^b$ spins/mol	total <sup>b</sup> spins/mol
9	5	5.0	c	c
	10	5.0	0.09	0.33
	20	5.0	0.20	0.56
	30	5.0	0.25	0.83
	5	0.5	c	c
		5.0	c	c
		20.0	c	c
	10	0.5	c	c
		5.0	0.09	0.15
		20.0	0.07	0.16
10	20	0.5	0.30	0.50
		5.0	0.21	0.54
		20.0	0.16	0.34
	30	0.5	0.30	0.71
		5.0	0.23	0.50
		20.0	0.21	0.41
	5	5.0	c	c
	10	5.0	0.12	0.21
	20	5.0	0.24	0.49
	30	5.0	0.33	0.84
9 + 2.5 mM MgATP	5	5.0	0.06	0.11
	10	5.0	0.13	0.24
	20	5.0	0.26	0.46
	30	5.0	0.27	0.68
$[\text{Fe}_4\text{S}_4(\text{SCyH})_4]^{3-}$	3.7	0.05	c	c
		0.5	c	c
	9.3	0.5	0.10	0.15
	20	0.5	0.19	0.36
$[\text{Fe}_4\text{S}_4(\text{SBz})_4]^{3-}$	30	0.5	0.19	0.42
	3.7	0.05	0.22	c
		0.5	0.18	c
	9.3	0.5	0.22	0.22
	20	0.5	0.31	0.36
	30	0.5	0.34	0.35

<sup>a</sup> No correction of the spin concentration for the  $g$  value was made.

<sup>b</sup> Total integration ranged from  $g = 9.0$  to  $g = 1.5$  and  $g = 2$  integration ranged from  $g = 2.5$  to  $g = 1.5$ . <sup>c</sup> Meaningless integral due to dispersion phenomenon.

errors of as much as 50% can result. (2) These signal intensity values do not strictly represent spin concentration, since no correction was applied for the  $g$  values of the unknown and the standard as described by Aasa & Vanngard (1970, 1975). In fact, although the presence of a broad  $g = 4$  resonance is characteristic of an  $S = 3/2$  spin state, the overall spectral appearance is clearly not that of a pure spin system of this type, e.g.,  $\text{Av}_1$  and its cofactor (Burgess, 1984; Orme-Johnson et al., 1977). Rather, the spectra empirically resemble a superposition of  $S = 1/2$  and  $S = 3/2$  systems, a condition which makes correction of signal intensity for  $g$  value difficult. Thus, while it is tempting to postulate that values approaching 1 spin/mol have been found for  $\text{Av}_2$ , this conclusion would seem to us to be inappropriate at this time. Nevertheless, we do conclude that double integration over the wider range to include the  $g = 4.4$  component results in significantly higher and more correct values of spin concentration for this protein. Previous EPR studies of this type that failed to take this low-field resonance into account of necessity would seem to be less accurate than those reported herein.

We have also made a cursory investigation of the EPR spectrum of  $\text{Av}_2$  in the presence of ATP and MgATP as indicated in Figure 4. Interestingly, the former reagent elicits no change in the spectrum of the native protein while the latter, consistent with previous results (Burgess, 1984; Orme-Johnson et al., 1977), causes the  $g = 2$  region of the spectrum to take

<sup>1</sup> While this paper was in press, Lindahl et al. (1985) reported similar resonances near  $g = 5$  in native and urea-containing  $\text{Av}_2$  samples.

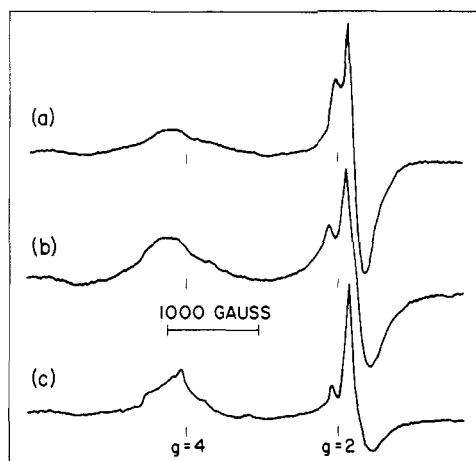


FIGURE 4: EPR spectra of sample 10 (Table I). (a) 0.389 mM  $\text{Av}_2$  in the presence of 2.5 mM MgATP at 19.2 K and 5.0-mW power; (b) 0.389 mM  $\text{Av}_2$  in the presence of 2.5 mM MgATP at 5.0 K and 5.0-mW power; (c) 0.389 mM  $\text{Av}_2$  at 5.0 K and 5.0-mW power. Microwave frequency was 9.15 GHz.

on a more axial appearance. As shown in Figure 4, a full spectral scan from  $g = 8$  to  $g = 1.5$  for  $\text{Av}_2$  in the presence of 2.5 mM MgATP contains a broad resonance similar to that observed for  $\text{Av}_2$  alone, and the results of double integration of the spectrum in both the  $g = 2$  region and over the wider range as a function of temperature at fixed power are presented in Table II. These data are similar to that observed for  $\text{Av}_2$  alone, with significantly higher spin concentrations resulting from the wider range integration, but with the values still not accounting for the expected 1 spin/mol. Although the wide range of values seems to show a linear increase in intensity with increasing temperature, it should be remembered that the value of 0.68 spin/mol at 30 K is subject to error as pointed out above. The relative constancy of the more accurate and reproducible  $g = 2$  region integrals at 20 and 30 K for the  $\text{Av}_2/\text{MgATP}$  system indicates that the overall increase may not be meaningful. The dispersion phenomenon described above for  $\text{Av}_2$  was also observed in the  $\text{Av}_2/\text{MgATP}$  system, but in general the effect was less evident in the presence of MgATP under the same conditions of temperature and power. This finding is demonstrated by the fact that a meaningful double integral could be obtained for the  $\text{Av}_2/\text{MgATP}$  system, but not for the native protein alone, at 5 K and 5.0 mW (Table II), and also by representative spectra presented in Figure 4.

At the present time, we have no complete or totally convincing explanation for the complex spectral appearance of  $\text{Av}_2$ . Perhaps most relevant to the understanding of this behavior are the magnetic properties of salts of the  $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{3+}$  ions, the chemically synthesized analogues of the  $[\text{Fe}_4\text{S}_4]^{1+}$  center in the protein (Laskowski et al., 1979). In the solid state, certain of these species exhibit EPR and magnetic susceptibility data consistent with an  $S = 1/2$  ground state while others appear to be characterized by an  $S = 3/2$  ground state on the basis of these magnetic parameters, including resonances at ca.  $g = 4$  in their EPR spectra. This dichotomy is postulated to be due to the existence of tetragonal ( $S = 1/2$ ) and nontetragonal ( $S = 3/2$ )  $\text{Fe}_4\text{S}_4$  core structures in the solid state depending on the nature of the thiolate and the cation associated with the trianion. In frozen organic solvents, however, all of the  $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{3-}$  species, where R was a substituted phenyl or benzyl group, exhibited EPR spectra containing only a  $g = 2$  resonance and no significant absorption in the  $g = 4$  region, indicating that the above structural differences do not persist in solution (Laskowski et

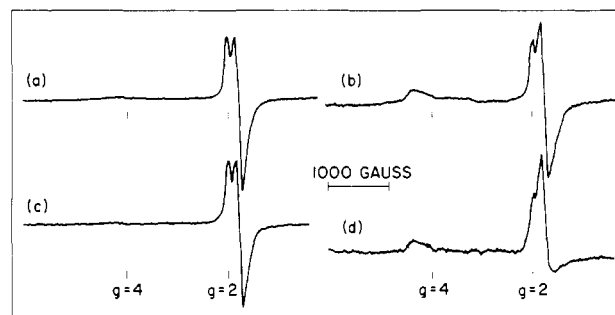


FIGURE 5: EPR spectra of (a)  $[\text{Fe}_4\text{S}_4(\text{SBz})_4]^{3+}$  ( $\text{Bz} = \text{CH}_2\text{C}_6\text{H}_5$ ) at 12.5 K and 0.50-mW power, (b)  $[\text{Fe}_4\text{S}_4(\text{SCyh})_4]^{3+}$  ( $\text{Cyh} = \text{C}_6\text{H}_{11}$ ) at 12.5 K and 0.50-mW power, (c)  $[\text{Fe}_4\text{S}_4(\text{SBz})_4]^{3+}$  at 3.7 K and 0.05-mW power, and (d)  $[\text{Fe}_4\text{S}_4(\text{SCyh})_4]^{3+}$  at 3.7 K and 0.05-mW power. Samples were 1.0 mM in dimethylformamide (DMF). Microwave frequency was 9.15 GHz.

al., 1979). In connection with our studies on the EPR spectrum of  $\text{Av}_2$ , we have now obtained data on several of these trianions that contain purely aliphatic thiolate residues and find that these species exhibit frozen-solution EPR spectra which are subject to all of the anomalies of the spectrum of the protein, i.e., low double integration values, broad  $g = 4$  resonances, and apparent dispersion phenomena at low temperature and power. The EPR spectral properties of the benzylthiolato and cyclohexylthiolato trianions are compared in Table II and Figure 5. Interestingly, even though the benzyl derivative shows no  $g = 4$  resonance or apparent dispersion as seen for the cyclohexyl species, nevertheless, it does show anomalously low double-integral values. Further experiments are anticipated to study the EPR spectral properties of these synthetic clusters as a probe for the biological system and to carry out a study of the magnetic susceptibility of oxidized and reduced  $\text{Av}_2$  so that its magnetic properties may be directly compared with those established for the synthetic analogues. It can be noted at this time, however, that the data obtained for the trianions indicate that the  $\text{Av}_2$  spectrum is perhaps not as surprising as we initially believed and may be a relatively general phenomenon in synthetic and protein systems containing  $[\text{Fe}_4\text{S}_4]^{1+}$  clusters.

## CONCLUSIONS

The magnetic properties of the iron protein component of the *Azotobacter vinelandii* nitrogenase system are more complex than previously thought. Our results show the presence of a heretofore unreported, broad  $g = 4$  spectral component which, when included in double-integration determinations, results in a significant increase in the total spin parameter (but still does not account for the expected total spin density) for this protein over that previously reported where only the  $g = 2$  spectral region was considered and when anomalously low spin per mole values were found. The  $g = 4$  resonance may be due to at least to partial population of an  $S = 3/2$  ground state, with past (Laskowski et al., 1979) and present studies of the magnetic properties of synthetic analogues for the  $[\text{Fe}_4\text{S}_4]^{1+}$  core of the protein supportive of this hypothesis. In addition, the EPR spectrum of  $\text{Av}_2$  was found to be distorted at low temperature and microwave power, conditions that suggest that dispersion is responsible for this effect and indicate that caution is in order when examining the magnetic properties of this protein.

## ACKNOWLEDGMENTS

We are grateful to Professor R. H. Holm and co-workers of Harvard University for generously supplying the  $[\text{Fe}_4\text{S}_4(\text{SR})_4]^{3-}$  species and to Professor R. B. Frankel of MIT and

Professor Holm for helpful discussions. We thank Dr. W. E. Newton for insight into the apparent dispersion phenomenon reported.

**Registry No.** MgATP, 1476-84-2;  $[\text{Fe}_4\text{S}_4(\text{SCyH})_4]^{3-}$ , 98652-71-2;  $[\text{Fe}_4\text{S}_4(\text{SBz})_4]^{3-}$ , 63138-11-4; nitrogenase, 9013-04-1.

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## Purification and Properties of an $\text{O}_2^{\cdot-}$ -Generating Oxidase from Bovine Polymorphonuclear Neutrophils<sup>†</sup>

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Received May 16, 1985

**ABSTRACT:** A membrane-associated  $\text{O}_2^{\cdot-}$ -generating oxidase has been purified from activated bovine polymorphonuclear neutrophils (PMN). The oxidase was extracted with Triton X-100 from a PMN membrane fraction largely devoid of lysosomal granules. The Triton extract was purified by a series of steps, including ion-exchange chromatography on DE-52 cellulose, gel filtration on Sephadex G-200, and isoelectric focusing. The  $\text{O}_2^{\cdot-}$ -generating oxidase activity was assayed as a superoxide dismutase inhibitable cytochrome *c* reductase. The activity of the purified enzyme was strictly dependent on NADPH as electron donor. The purification factor with respect to the phorbol myristate acetate activated PMN was 75, and the recovery was about 6%. The reactivity of the purified oxidase was increased by 3-4-fold after incubation with asolectin. The minimum molecular weight of the oxidase, deduced from migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was  $65\,000 \pm 3000$ . The optimum pH of the oxidase was 7.5, its  $K_{M,\text{NADPH}}$  was  $\approx 30\ \mu\text{M}$ , and its isoelectric point was at pH 5.0. The enzyme was inhibited by low concentrations of mersalyl (half-inhibition  $\approx 10\ \mu\text{M}$ ) and Cibacron Blue (half-inhibition  $< 10\ \mu\text{M}$ ). It was insensitive to 1 mM cyanide. Rapid loss of activity occurred at 0-2 °C, concomitantly with a decrease in sensitivity to superoxide dismutase: both activity and sensitivity to superoxide dismutase could be restored by addition of asolectin. The purified oxidase contained no spectrophotometrically detectable cytochrome *b*, and enzymatic assay failed to detect FAD in oxidase preparations subjected to heat treatment or trypsin digestion.

**P**olymorphonuclear neutrophils (PMN)<sup>1</sup> respond to a number of membrane stimulants by a sharp increase in superoxide

( $\text{O}_2^{\cdot-}$ ) production. The  $\text{O}_2$  uptake corresponding to the respiratory burst is catalyzed by a cyanide-resistant NADPH

<sup>†</sup> This work was supported by grants from CNRS/U.A. 529 (Centre National de la Recherche Scientifique), INSERM/U.191 (Institut National de la Santé et de la Recherche Médicale), and Faculté de Médecine, Université Scientifique et Médicale, of Grenoble.

<sup>1</sup> Abbreviations: PMN, polymorphonuclear neutrophils; PMSF, phenylmethanesulfonyl fluoride; PMA, phorbol myristate acetate; SOD, superoxide dismutase; LAPAO, (laurylamido)-*N,N*-dimethylpropylamine oxide; EDTA, ethylenediaminetetraacetic acid; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.