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EPR STUDIES BY ^{57}Fe ISOTOPIC SUBSTITUTION ON THE NATURE OF AN UNKNOWN ELECTRON ACCEPTOR IN *AZOTOBACTER VINELANDII* PHOSPHORYLATING PARTICLES

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Summary

1. EPR ^{57}Fe isotopic substitution studies provide unequivocal evidence that the $g = 2.011$ signal found in oxidized *Azotobacter vinelandii* phosphorylating particles is due to an iron-containing structure. The broadening constant determined as a result of this electron—nuclear hyperfine interaction was 15.7 G.

2. A similar signal found in a number of iron—sulfur containing proteins was found by quantitative EPR estimations to exist in a variable but substantial concentration when compared to the intensity of the reduced $g = 1.9$ type EPR resonance.

3. Reaction of the phosphorylating particles with excess potassium ferricyanide resulted in an alteration of the initial $g = 2.011$ iron signal resulting in the detection by microwave power studies of at least two different iron species which exhibited major g-values at 1.992 and 2.027.

These EPR studies were undertaken to investigate an intense highly temperature-sensitive resonance in the $g = 2$ region of oxidized *Azotobacter vinelandii* phosphorylating particles [1]. A similar signal has also been reported in oxidized mammalian phosphorylating particles [2] and a number of iron—sulfur proteins [3—6]. Because it was quite unusual to observe that an iron system exhibited the major g-value in the $g = 2$ region and showed signal detection only below 35 °K, it was essential to firmly establish that the resonance was in fact due to iron. This goal was achieved by growth of *A. vinelandii* on

^{57}Fe which has a nuclear spin of 1/2 in place of the natural isotope ^{56}Fe which has no nuclear moment ($I = 0$). As a result of electron—nuclear hyperfine interaction, two hyperfine lines would be expected for an $I = 1/2$ system and in the event of inadequate resolution, only a broadening of the signal relative to that observed for ^{56}Fe would be detected. A preliminary report of this study has been reported elsewhere [7].

A. vinelandii was grown on the standard Burk medium as previously described [1] except that all reagents, including sucrose, were treated with either Chelex 100 (Biorad) or orthophenanthroline to remove trace amounts of ^{56}Fe. In place of ^{56}Fe, ^{57}Fe (obtained as Fe_2O_3 from Oak Ridge National Lab. at 97% enrichment) was dissolved in HCl and neutralized when used in the medium. Six consecutive 1% innoculum transfers of log phase cells grown in ^{57}Fe media were performed before 30 l of *A. vinelandii* were grown on ^{57}Fe. Cell yields were comparable to those obtained on growth with ^{56}Fe. EPR measurements were conducted as previously described [1] and in the temperature range of 12—20 °K utilized an Airco automatic temperature control liquid-helium transfer unit. Further experimental details are recorded in the figure legends.

The preparation of phosphorylating particles and measurement of P/O ratios were carried out as previously described [1]. Phosphorylating particles prepared from ^{57}Fe or ^{56}Fe grown whole cells showed identical substrate turnover numbers and P/O ratios; these values have been previously reported [1].

The phosphorylating particles were reacted with O_2 for 5 min at 0 °C, placed in frequency and intensity-matched EPR quartz tubes of 4 mm outer diameter and stored in liquid nitrogen until measured in the EPR spectrometer. Fig. 1 shows a comparison of oxidized phosphorylating particles prepared from ^{56}Fe and ^{57}Fe grown whole cells. The amplitudes for both signals were adjusted to a similar height and the two spectra were superimposed to illustrate nuclear hyperfine interaction. Although it was not possible to obtain resolution of the expected two hyperfine lines, it is obvious that broadening has occurred in the ^{57}Fe sample when compared to that of the ^{56}Fe sample. After base-line correction, the hyperfine broadening was computed from the line-width at half-maximum intensity at the upper portion of the spectrum. The value obtained was 15.7 G at an incident microwave power measurement of 2 mW. Line broadening was also observed in the lower portion of the ^{57}Fe sample but the magnitude of this broadening was difficult to compute due to interference from another paramagnetic species, possibly cupric copper (cf. ref. 1). At a higher microwave power (10 mW) the broadening was found to decline to 12.8 G due to saturation of the ^{57}Fe species and further decreased to 6.6 G at 30 mW. The broadening observed in the ^{57}Fe sample relative to the ^{56}Fe sample provides unequivocal evidence that electron—nuclear hyperfine interaction has occurred and that the species responsible for the resonance at $g = 2.011$ is definitely an iron-containing structure.

Line-width broadening due to nuclear hyperfine interaction was also detected in the $g = 6$ region of the oxidized phosphorylating particles grown

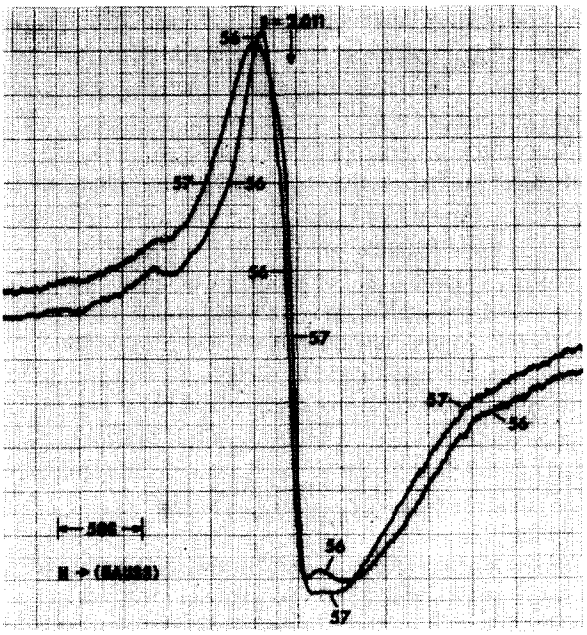


Fig. 1. Comparison of superimposed EPR spectra of ^{56}Fe and ^{57}Fe enriched oxidized phosphorylating particles (119 mg/ml) from *A. vinelandii*. EPR conditions: temperature, 14 °K; frequency, 9.162 GHz; microwave power, 2 mW; scanning rate, 200 G per min; time constant, 0.3 s; modulation amplitude, 2.7 G;

on ^{57}Fe. In this region two non-equivalent heme species have been assigned to cytochrome *d* [8]. These species consist of an axial high-spin ferric heme species (g = 5.86) and a high-spin rhombically-distorted ferric heme species with g-values at 6.20 and 5.48. Due to extensive electron delocalization in the heme systems, the computed hyperfine broadening constants were small. The broadening constant at 20 mW (approximate non-saturating conditions for both the axial and rhombic heme species) for the axial heme system was 5.8 G and for the rhombic heme species 5.2 G.

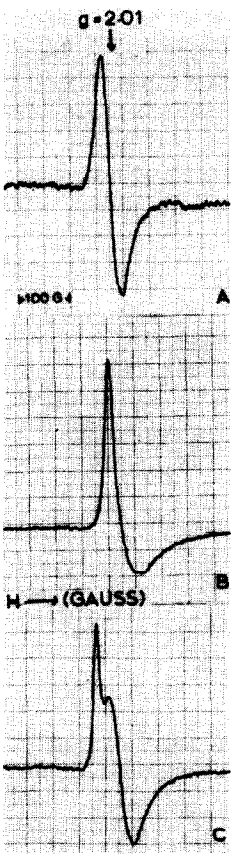
The signal in the g = 2 region of oxidized phosphorylating particles has been definitely established to be due to an iron-containing structure and it is therefore highly likely that a similar resonance in the g = 2 region of oxidized heart mitochondria is also attributable to an iron structure. In this regard Ruzicka and Beinert [9] have recently reported the isolation of an iron—sulfur protein from heart mitochondria exhibiting, in the oxidized state, a resonance at g = 2.01 with virtually identical EPR parameters to the g = 2.011 signal in *A. vinelandii* phosphorylating particles. This protein was reported to show no signals on chemical reduction, contained two iron and sulfide atoms per mole (M_r = 100 000) and the oxidized signal resembled that of the high-potential iron—sulfur protein found in *Chromatium* [10].

It has previously been reported [3—6] that an oxidized g = 2 signal

detectable below 35 °K could be observed in a number of iron—sulfur proteins which on chemical or enzymatic reduction revealed $g = 1.9$ type EPR signals. Fig. 2 illustrates the $g = 2$ type signals observed in the oxidized state for the following iron—sulfur proteins: (A) NADH dehydrogenase from *A. vinelandii* which contains 1 mole of FMN and four iron and sulfur atoms per mole (M_r = 56 500) and which on enzymatic reduction shows two different $g = 1.9$ type signals arising from two distinct iron—sulfur centers [4]; (B) nitrate reductase from either *Micrococcus denitrificans* (shown, cf. ref. 3) or *Escherichia coli* (Forget, P. and DerVartanian, D.V., unpublished) which is a molybdenum-containing multi-iron—sulfur protein exhibiting, on enzymatic reduction, at least two different iron—sulfur $g = 1.9$ type EPR resonances; (C) adenylyl sulfate reductase from *Desulfovibrio vulgaris* which is an FAD-multi-iron—sulfur protein which on enzymatic or chemical reduction shows the $g = 1.9$ type resonance [5]. Similar signals can also be detected in the $g = 2$ region of oxidized dissimilatory sulfite reductases (Peck, Jr, H.D., LeGall, J. and DerVartanian, D.V., unpublished) as well as unusual high-potential type iron—sulfur proteins isolated from the sulfate-reducing bacteria (LeGall, J. and DerVartanian, D.V., unpublished).

On the assumption that the signal in the $g = 2$ region arises from a spin = 1/2 system, and using cupric EDTA as the standard, quantitation by double integration of the spin intensity of this signal for the iron—sulfur proteins found in Fig. 2 have been carried out. The amount of spin intensity determined for the $g = 2$ signal of NADH dehydrogenase corresponded to 40% of that found for the $g = 1.9$ type EPR signals. As previously noted [3], the spin concentration determined for the $g = 2$ signal of nitrate reductase from *M. denitrificans* was approximately equimolar with the spin intensity found in the enzymatically reducible $g = 1.9$ type signal. When the latter enzyme was reduced with sodium dithionite which caused the appearance of a second and different iron—sulfur $g = 1.9$ signal, the spin recovery for the $g = 2$ signal now corresponded to 50% of the total reducible iron—sulfur signal intensity. A similar calculation for nitrate reductase from *E. coli* accounted in the $g = 2$ signal for 75% of the total reducible iron—sulfur signal intensity. Likewise such calculations for adenylyl sulfate reductase resulted in a recovery of 43% of the spin intensity found in the $g = 1.9$ type signal. It is clear that although the amount of spin intensity recovered is variable when compared to the intensity of the reduced iron—sulfur signals, the substantial magnitude of spin recovery indicates that the iron species responsible for the $g = 2$ signal is an important constituent of each enzyme.

Fig. 3 shows the effect of 17 mM potassium ferricyanide on oxidized phosphorylating particles from *A. vinelandii*. Altering the redox potential in a more positive direction results in a change of the initial $g = 2.011$ signal (Fig. 3A) to a different resonance form (Fig. 3B). Microwave power saturation studies clearly indicate that this latter signal is composed of at least two unlike iron species (Fig. 3C) based on differing saturation behavior found on increasing microwave power. The *g*-values of these two species were deter-



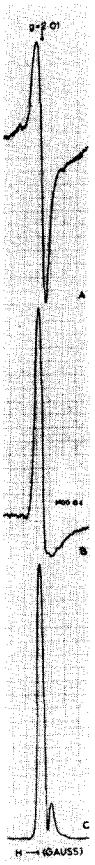


Fig. 2. EPR spectra of the oxidized states for the following iron—sulfur containing proteins: (A) NADH dehydrogenase (16 mg/ml) from *A. vinelandii*. EPR conditions: 400 G/min; time constant, 0.3 s; modulation amplitude, 5.9 G; frequency, 9,230 GHz; temperature, 15 °K; microwave power, 3 mW, from ref. 4. (B) Nitrate reductase A (8 mg/ml) from *M. denitrificans*. EPR conditions as in A except frequency was 9.239 GHz, from ref. 3. (C) Adenylyl sulfate reductase (15 mg/ml) from *D. vulgaris*. EPR conditions as in A except temperature was 17 °K and frequency 9.246 GHz, from ref. 5.

Fig. 3. EPR spectra of phosphorylating particles from *A. vinelandii*. (A) Oxidized particles (59 mg/ml), EPR conditions as in Fig. 2A except that frequency was 9.162 GHz, and Gain = 100; (B) oxidized particles plus 17 mM potassium ferricyanide, EPR conditions and Gain as in Fig. 3A except frequency at 9.164 GHz; (C) as B but at microwave power of 10 mW, frequency of 9.163 GHz and Gain of 40.

mined to be at 1.992 and 2.027. Palmer et al. [11] noted that addition of ferricyanide at different concentrations resulted in complex changes in signals found in the $g = 2$ region of molybdoferredoxin from the nitrogenase complex of *Clostridium pasteurianum*. Cammack [12] reported that the high-potential iron-sulfur protein from *Chromatium* which reveals only a $g = 2$ signal in the oxidized state and no signals in the reduced state could yield a ferredoxin-type signal, i.e. revealing a $g = 1.9$ type signal in the reduced state, by treatment of the iron—sulfur protein with dimethylsulfoxide under anaerobic conditions followed by reduction with sodium dithionite.

A critical structural orientation within the iron—sulfur centers of both the high-potential and ferredoxin-type iron—sulfur proteins appears to be a prerequisite for the detection of paramagnetic states in either the oxidized or reduced states. Alterations of this orientation by drastic changes in the redox potential (e.g. high ferricyanide concentration as in Figs 3B and C) or solvent environment as in the studies of Cammack [12] result in significant and dramatic changes in paramagnetic behavior. As in the case of the ferredoxin-type iron—sulfur centers which participate in biological reactions exhibiting a wide range of redox potentials (approx. −420 mV—+420 mV), it is tempting to postulate that the high-potential iron—sulfur centers, characterized by the temperature-sensitive $g = 2$ oxidized paramagnetic iron species, and involved in a number of diverse biological functions, may display a similar range of redox potentials. In this light it is suggested that these high-potential iron—sulfur containing systems should be considered either as a subclassification under ferredoxins or as true ferredoxins differing only by an unknown finite orientation in the iron—sulfur cluster structure.

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References

1 DerVartanian, D.V., Iburg, L.K and Morgan, T.V. (1973) Biochim. Biophys. Acta 305, 173—178
2 Orme-Johnson, N.R., Orme-Johnson, W.H., Hansen, R.E., Beinert, H. and Hatefi, Y. (1971) Biochem. Biophys. Res. Commun. 44, 446—452
3 Forget, P. and DerVartanian, D.V. (1972) Biochim. Biophys. Acta 256, 600—606
4 DerVartanian, D.V. (1972) Z. Naturforsch. 276, 1082—1084
5 Peck, Jr, H.D., Bramlett, R. and DerVartanian, D.V. (1972) Z. Naturforsch. 276, 1084—1086
6 DerVartanian, D.V. (1973) J. Magn. Resonance 10, 170—178
7 DerVartanian, D.V., Morgan, T.V. and Brantner, R.V. (1974) Fed. Proc., in the press
8 Kauffman, H.F., DerVartanian, D.V. and VanGelder, B.F. (1974) Ned. Fed. Vergadering, in the press
9 Ruzicka, F.J. and Beinert, H. (1974) Fed. Proc., in the press
10 Palmer, G., Brintzinger, H., Estabrook, R.W. and Sands, R.H. (1967) Magnetic Resonance in Biological Systems (Ehrenberg, A., Malmstrom, B.G. and Vanngard, T., eds), pp. 159—171, Pergamon Press, New York
11 Palmer, G., Multani, J.S., Cretney, W.C., Zumft, W.G. and Mortenson, L.E. (1972) Arch. Biochem. Biophys. 153, 325—332
12 Cammack, R. (1973) Biochem. Biophys. Res. Commun. 54, 548—554