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EPR studies on phosphorylating particles from *Azotobacter vinelandii*

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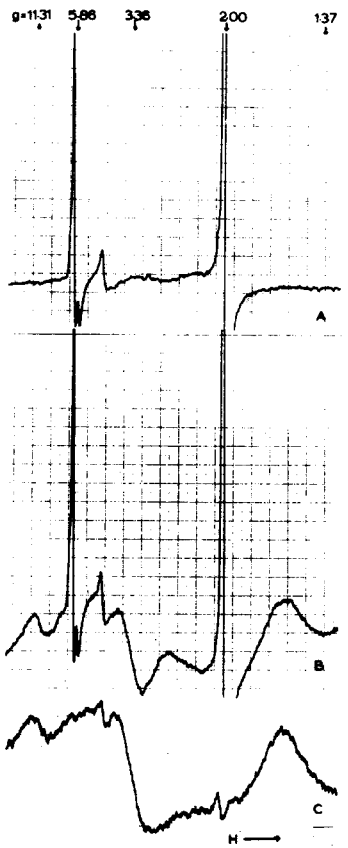
SUMMARY

EPR studies at 12 °K on phosphorylating particles at an intermediate reoxidation level revealed a complex signal (*g*-values of 11.31, 3.36 and 1.37) which was provisionally assigned to non-heme iron. Reoxidation also resulted in a high-spin ferric heme resonance (*g* values at 6.20, 5.86 and 5.48) assigned to a *d*-type hemoprotein. NADH-reduced particles revealed resonances attributable to three different iron–sulfide centers. The third center differed significantly in *g* values from the same center observed in mammalian phosphorylating particles.

EPR studies have been undertaken to establish whether the two different iron–sulfide centers of a low molecular weight (56 500) NADH dehydrogenase[1] containing four iron and labile sulfide atoms per mole isolated from the strict aerobe *Azotobacter vinelandii* are the same centers as in phosphorylating particles, or whether during the purification procedure modifications may have occurred to these centers. The isolated NADH dehydrogenase in addition to the functional iron–sulfide and FMN centers revealed high and unaltered catalytic-center activities in moles NADH per mole FMN (16 510 with menadione and 6260 with potassium ferricyanide) and a typical light-absorption spectrum of an iron-containing flavoprotein. These studies were also undertaken to determine the presence and involvement of heme and other paramagnetic components in the respiratory chain of *A. vinelandii*. A preliminary report of these studies has appeared elsewhere[2].

EPR measurements were carried out as previously described[3] and in the temperature range 12–30 °K utilized an Airco temperature control unit. Further experimental details are recorded in figure legends. Phosphorylating particles were prepared by slight modifications of the procedures of Jones and Redfearn[4] or of Ackrell and Jones[5]. NADH oxidase and ATP

determinations were performed according to Ackrell and Jones. In addition bioluminescent determinations for ATP were carried out according to the method of Knowles and Smith[6]. With NADH as substrate the P/O ratio was usually in the range of 0.6 to 0.7 regardless of the method of ATP determination, and was in reasonable agreement with values reported by others[5,7].



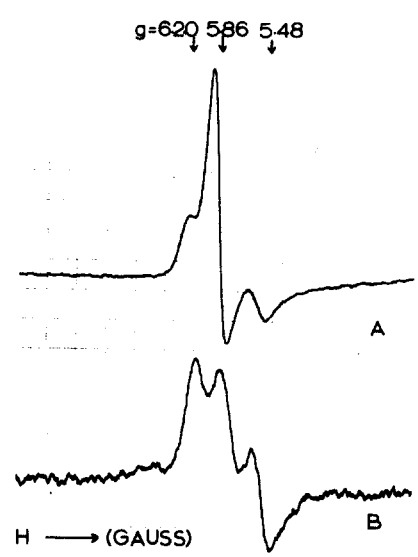


Fig. 1. EPR spectra of phosphorylating particles from *A. vinelandii* and cardiac NADH dehydrogenase. Final concentrations are given for reactants. A, particles (75 mg/ml) in 10 mM piperazine *N,N'*-bis-2-ethane sulphonate (pH 6.4) buffer *plus* 8 mM magnesium acetate after reaction with O_2 for 5 min at 0 °C; frequency, 9.159 GHz, Gain = 125; B, after reaction with 0.7 mM potassium ferricyanide for 5 min at 0 °C, frequency, 9.160 GHz, Gain = 200; C, isolated cardiac NADH dehydrogenase (12.4 mg/ml), *cf.* ref. 10, frequency, 9.174 GHz, Gain = 250. EPR conditions and instrumentation as in ref. 3 and as follows: temperature, 12 °K; scanning rate, 1000 gauss/min; microwave power, 10 mW; modulation amplitude, 5.9 G; time constant, 0.3 s.

Fig. 2. EPR spectra of oxygenated phosphorylating particles from *A. vinelandii* and bisulfite reductase from *D. gigas*. A, as Fig. 1A; B, isolated bisulfite reductase (67 mg/ml), frequency, 9.149 GHz. EPR conditions as Fig. 1 except microwave power was 10 mW and scanning rate was 400 gauss/min.

The phosphorylating particles are isolated in a partially reduced state as judged by the detection of the following absorptions: a signal at $g = 2.01$ which is frequently ascribed to a ferric form of iron, a weak signal at $g = 5.86$ attributable to a high-spin ferric form of a hemoprotein and reduced iron–sulfide resonances at $g = 1.93$ and 1.89. Reaction of the isolated particles with oxygen (Fig. 1A) results in a 5-fold increase of the $g = 2.01$ signal, a complete loss of the reduced iron–sulfide signals and a 15-fold increase in the signal at $g = 5.86$. Arbitrarily assigned g values of the ferric heme resonance can be now seen to occur at $g = 6.20$, 5.86 and 5.48. As Fig.2 reveals this latter high-spin ferric heme resonance is almost superimposable in g values and highly distorted line shape with that of the ferric high-spin signal observed with bisulfite reductase from the sulfate-reducer *Desulfovibrio gigas* (Lee, J.-P., Peck, Jr, H.D. and DerVartanian, D.V., unpublished). This latter protein has been recently identified as a *d*-type hemoprotein[8]. Based on the striking similarity of EPR signal with that observed for bisulfite reductase, the detection (by computer signal averaging) and therefore accountability of ferric EPR resonances (not shown) due to cytochromes b_1 and c_4 *plus* c_5, the light-absorption changes noted in the oxidized (maximum at 648 nm) and reduced states (maximum at 631 nm) from this study and from the reflectance measurements previously reported by Beinert *et al.*[9], the observed EPR resonance at $g = 5.86$ is attributed to a respiratory-chain *d*-type oxidase. This assignment represents then the first direct EPR evidence for the presence of cytochrome *d* in *A. vinelandii.*

Titration of the isolated particles with increasing amounts of the oxidant ferricyanide results in a loss of the initially present reduced iron–sulfide signals, and increases in both the $g = 2.01$ and 5.86 signals, which at high ferricyanide concentrations, reach a maximum intensity equivalent to that obtained by oxygenation of the particles. However, at an intermediate state of reoxidation in the presence of ferricyanide, there appears a complex signal with g values at 11.31, 3.36 and 1.37 (Fig. 1B) which on further oxidation with increasing amounts of ferricyanide completely disappears. This complex signal is also absent in the completely oxygenated particles (Fig. 1A). Because a particular preparation of isolated particles may be present at a slightly different reduction level, each preparation must be individually titrated with ferricyanide to achieve maximal development of this complex resonance. As observed in Fig. 1C virtually the same complex signal was observed[10] in isolated cardiac NADH dehydrogenase prepared by Baugh and King[11]. It had been previously assumed that the signals at $g = 3.36$ and 1.37 were not related to the low-field resonance but the studies with *A. vinelandii* particles clearly show that all three signals appear and disappear in unison with respect to oxidation cycling as well as applied incident microwave power and temperature variation. Since the cardiac NADH dehydrogenase contains as the major form of iron, 28 non-heme iron (and labile sulfide) atoms per mole, the complex signal at $g = 11.31$, 3.36 and 1.37 may be provisionally ascribed to non-heme iron but of unknown spin state. The oxidized state of iron–sulfide proteins has been reported to be diamagnetic (*cf.* ref. 12) and therefore the detection of intense paramagnetism in the isolated dehydrogenase, as well as with the phosphorylating particles from *A. vinelandii,* is not an easily resolvable problem. In order to explain an unusual resonance in molybdoferredoxin from the nitrogenase system of *Clostridium pasteurianum,*

Palmer *et al.*[12] proposed that exchange coupling from neighboring iron atoms resulted in a net paramagnetic ground state. A similar proposal may be invoked in the detection of the $g = 11$ type complex signal since a large number of iron atoms are clearly present in the cardiac NADH dehydrogenase and presumably also in the respiratory particles of *A. vinelandii*. Further studies are in progress to ascertain the nature of the $g = 11$ type signal in terms of spin state and enzymatic redox function. In any case to our knowledge this EPR signal has not been previously reported in studies dealing with respiratory systems.

The nature of the intense signal at $g = 2.01$ in the fully oxidized particles (Fig. 3A) remains obscure as to origin, spin state or biological significance. It is also present in mammalian submitochondrial preparations[13], is concentrated in the cytochrome b–c_1 region and responds to chemical or enzymatic redox cycling. It is very likely due to a ferric form of iron since it has been observed in the oxidized state of

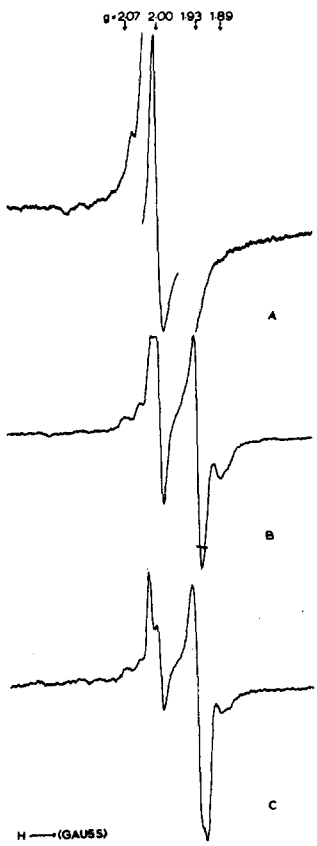


Fig. 3. EPR spectra of phosphorylating particles from *A. vinelandii*. A, as Fig. 1A, $g = 2.01$ signal was at Gain = 20 and remainder of signal at Gain = 250, temperature, 12 °K; B, after reduction with 3 mM NADH for 5 min at 0 °C, Gain = 125, temperature, 18 °K; frequency, 9.162 GHz; C, as B but at Gain = 200 and temperature of 27 °K. EPR conditions as in Fig. 2 except microwave power was 2 mW in B and C.

a number of homogenous iron proteins containing functional iron–sulfide centers (*cf.* refs 1, 14). To date the signal has not been reported in any iron–sulfide protein containing less than 4 iron atoms per mole. Quantitation of the signal on the assumption of a doublet system results in highly variable recoveries of spin intensity in these iron proteins. In one instance (nitrate reductase A from *Micrococcus denitrificans*), the recovered spin intensity is of the order of the enzymatically reduced iron-sulfide center[3]. However, a similar spin recovery has not been observed in other iron proteins (*cf.* ref. 1) where the spin intensity is often considerably less or greater than the intensity of the iron–sulfide centers. The signal is largely absent in the region of the mammalian NADH or succinate dehydrogenase systems but is present in the low molecular weight NADH dehydrogenase derived from *A. vinelandii.* Its intensity is however also less than that of the reduced iron–sulfide centers. Fig. 3A also reveals the present of a small cupric signal at $g = 2.06$ with apparent hyperfine lines resolvable at approximately $g = 2.11$, 2.17 and 2.23. This rather weak signal is probably due to a copper contaminant.

Figs. 3B and 3C show the reduced iron–sulfide centers of the phosphorylating particles under anaerobic conditions (helium) after reduction with excess NADH. It was not possible to achieve a similar degree of reduction under initially aerobic conditions although NADH was in excess and sufficient time was allowed to achieve anaerobiosis. Reduction with NADH also caused the disappearance of both the $g = 2.01$ and ferric high-spin *d* resonances implicating the involvement of both types of iron in the respiratory chain.

The observed iron–sulfide resonances were resolved into three distinct centers with different *g* values by suitable variation of temperature and incident microwave power. Figs 3B and 3C show the effect of temperature on resolution of the three iron–sulfide centers. The *g* values of the observed centers were as follows: Center 1, $g = 2.02$, 1.93; Center 2, $g = 2.04$, 1.93. A third iron–sulfide center revealed *g* values at $g = 2.07$, 1.89, and 1.88. This latter center differed significantly in *g* values from those observed in the mammalian particles[13], indicating that either this latter center is a different iron–sulfide center or that in phosphorylating particles from *A. vinelandii* Center 3 has altered *g* values.

It is clear from the above *g* values that the isolated low molecular weight NADH dehydrogenase has retained Centers 1 and 2 which have been reported[15] to display the largest difference in redox potential. A third center was not observed in the dehydrogenase either on reduction with NADH or sodium dithionite. It is interesting to note that the *A. vinelandii* NADH dehydrogenase does not appear to have reconstitutive activities which is in contrast to the cardiac NADH dehydrogenase. This latter enzyme with its large number of iron atoms has been reported to show reconstitutive activity with the respiratory chain under appropriate conditions. It has been suggested[16] earlier that the additional iron–sulfide centers observed in respiratory enzymes such as NADH and succinate dehydrogenase may have a bearing on reconstitutive properties where for example these additional centers could serve as an electron link with the respiratory chain.

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