EPR Studies on Cytochrome Components in Phosphorylating Particles of *Azotobacter vinelandii*

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Abstract

Oxidized particles of *A. vinelandii* show high-spin ferric signals with an axial and a rhombically distorted component with g-values at 5.94 and 6.24, 5.51, respectively. The signals behave similarly on variation of temperature and/or power and are assigned to cytochrome d. The addition of ligands such as cyanide and carbon monoxide to oxidized particles mainly affects the rhombic component of the signal in the g = 6 region. Prolonged incubation of cyanide with oxidized particles results in the appearance of two new low-spin ferric heme signals at g = 2.99 and at g = 3.23 which are tentatively assigned to low-spin forms of cyanide-liganded cytochrome d. With computer signal-averaging of the EPR spectrum of oxidized particles, the presence of resonances in the g = 3–4 region could be demonstrated. These resonances are assigned to cytochrome b_1 (g-values at 3.68, 3.43), c-type cytochromes (g-values at 3.43, 3.25) and cytochrome a_1, and possibly a low-spin form of a c-type cytochrome (g-value at 3.03). These EPR results represent, to our knowledge, the only such studies reported on the membrane-bound b_1 and c-type cytochromes of a bacterial respiratory-linked phosphorylating electron-transport chain.

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Introduction

Optical spectroscopy has revealed the presence of the cytochromes c_4, c_5, and b_1 and the oxidases d (previously designated as a_2) o, and probably a_1 in *Azotobacter vinelandii* [1–9]. Upon addition of cyanide, the α-absorption band of oxidized cytochrome d disappeared [10], whereas the α-band of cytochrome a_1 was unaffected. In the presence of cyanide, no direct reduction of the heme moiety of cytochrome d was obtained, a property similar to that found for mammalian cytochrome c oxidase [11, 12]. In contrast to cyanide, both carbon monoxide and nitroxide affect the absorption band of reduced cytochrome d [1, 6, 7].

Although extensive EPR studies on the iron–sulfur proteins of *A.vinelandii* have been carried out [13–16], little knowledge is available on the EPR characteristics of the cytochrome in particles of *A. vinelandii* or for that matter on other bacterial membrane-bound cytochromes. EPR studies by Nicholas and co-workers [17] at $77°K$ of oxidized particles showed the presence of high-spin iron resonances at $g = 6$ and $g = 4.3$.

Recently, EPR studies of oxidized particles at $12°K$ have shown the presence of high-spin ferric heme signals in the $g = 6$ region with axial and rhombic symmetry. These signals were tentatively assigned to cytochrome d [18].

Studies on the cytochrome system of the mammalian respiratory chain at lower temperatures ($10–15°K$) have revealed the presence of low-spin heme–iron resonances that could be attributed to b- and c-type cytochromes [19–21]. In order to identify the EPR signals of the b- and c-type cytochromes in *A. vinelandii,* we have studied the EPR characteristics of oxidized particles at $12°K$ and the effect of the classical inhibitor ligands, cyanide and carbon monoxide on the signals.

Methods

Phosphorylating small particles were prepared as described by Pandit-Hovenkamp [22] and stored in 20 mM phosphate buffer (pH 7.2), 0.25 M sucrose, 40 mM KCl at $77°K$. Large particles were prepared according to Jones and Redfearn [23] and stored as described for small particles.

Concentrated particle suspension was oxidized by titration with 100 mM ferricyanide and/or blowing for 5 min with a constant stream of oxygen on a particle suspension, present as a thin layer on the wall of a rotating tube.

EPR spectra were recorded, mainly at $12°K$ on a Varian V4501A or Varian E-3 EPR spectrometer equipped with an Air Products helium-flow system. Other EPR conditions are noted in Figs. 1–3.

Chemicals used (e.g., potassium ferricyanide and potassium cyanide) were of the highest available grade.

Protein concentrations were determined as previously described [18] and final concentrations are given in Figs. 1–3.

Results

Phosphorylating particles used in this study showed EPR signals at $12°$K in the oxidized state very similar to those reported by DerVartanian et al. [18], despite a different method of particle preparation and strain of *A. vinelandii*. Both types of phosphorylating particles exhibit a high-spin ferric-heme signal (cf. Fig. 2 of [18]) consisting of a rhombically distorted component with g-values at 6.24 and 5.51 and a predominant axial resonance with the g-value at 5.94. Both signals are strongly temperature-dependent and behave similarly with variation of microwave power and/or temperature. These two high-spin ferric-heme signals are assigned to two heme species of cytochrome *d*. This assignment is based on the fact that large particles as well as particles isolated from urea-grown bacteria both contain lower concentrations of cytochrome *d* than do small particles and show a corresponding lower intensity of the resonances at g = 6. However, in particles of urea-grown *A. vinelandii*, the intensities of the EPR signals of the other cytochromes (below) are hardly suppressed.

The other major cytochrome components (c_4, c_5, b_1, and a_1) present in *A. vinelandii* show characteristic weak low-spin iron resonances which, after computer signal-averaging, reveal g-values at 3.68, 3.43, 3.25, and 3.03 (Fig. 1). Based on the g-values reported for isolated cytochromes c_4 and c_5 [24] and by analogy to the EPR signals of the cytochromes in the mammalian respiratory chain [19, 20] the following tentative assignments have been made: cytochrome b_1, minor contribution to signal at 3.69, major contribution to signal at 3.43; cytochrome c_4, major contribution to signal at 3.25, minor contribution to signal at 3.43; cytochrome c_5, major contribution to signal at 3.25; and cytochrome a_1, major contribution to signal at g = 3.03. It is interesting to note that above $20°$K, considerable broadening of these weak resonances occurs as was observed for the low-spin resonances of the mammalian respiratory system [19–21].

In large particles, the content of cytochrome b_1, as determined from optical absorbance spectra, and especially that of cytochromes c_4 and c_5, is higher than in phosphorylating particles and the higher concentration of these cytochromes can be correlated with increased amplitudes of the signals assigned to cytochromes b_1, c_4, and c_5. The signal at g = 3.43 has been assigned to a mixture of cytochrome b_1 plus c_4. This assignment is based on reduction studies with ascorbate plus
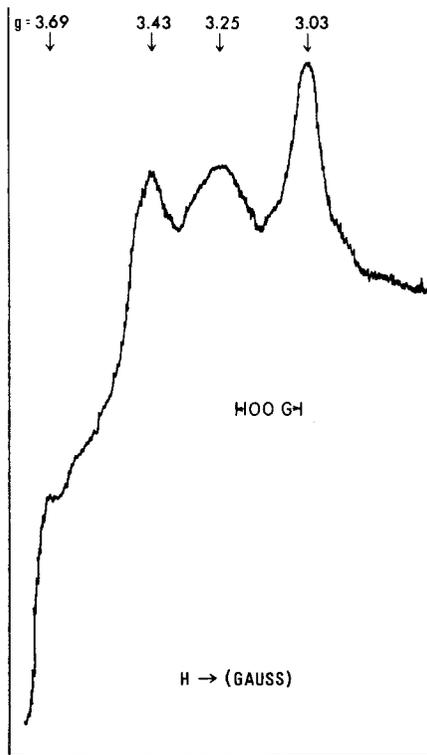
Figure 1. EPR spectrum of oxidized phosphorylating particles from *A. vinelandii.*
The particles (70 mg/ml) were suspended in 40 mM potassium phosphate buffer
(pH 7.2), 250 mM sucrose and 40 mM potassium chloride. EPR spectra were
obtained with a Varian V4501A spectrometer at 12°K, a microwave power of
10 mW, modulation amplitude of 5.9 G and microwave frequency of 9.164 GHz
(frequency-matched EPR tubes). Fifteen spectra were accumulated in a Data General
Corporation Nova computer and averaged. Spectra were recorded with a time
constant of 0.01 sec and scanning rate of 1000 G per min.

TMPD (not shown). As in the case of the mammalian respiratory chain
[19, 20] and isolated complex II [21], the signal at g = 3.43 in particles
of *A. vinelandii* sharpens on reduction due to the persistence of the
low-spin ferric form of cytochrome b_1 and reduction of cytochrome c_4
to the diamagnetic ferrous state.

Reaction of cyanide with oxidized particles

The reaction of oxidized particles with 5 mM cyanide (pH 7.6) results in
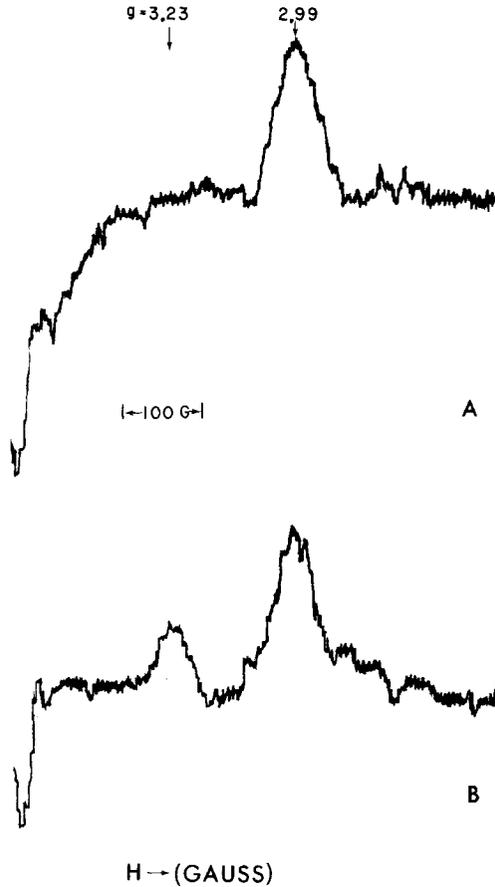the appearance of a new low-spin ferric heme signal (Fig. 2A) at g = 2.99

Figure 2. The effect of potassium cyanide on the EPR spectra of phosphorylating particles. (A) particles (70 mg/ml) were reacted in the presence of oxygen with potassium cyanide (5 mM, pH 7.2) for 20 min at 25°C; (B) as A except that particles were reacted with potassium cyanide (5 mM) for 30 min at 25°C. EPR conditions were as in Fig. 1 except that 19 spectra were accumulated in A and 22 spectra were accumulated in B.

which reaches its maximal intensity in 20–30 min at 25°C. After the reaction with cyanide had occurred for 20 min, a second and new signal of a low-spin cyanide species appears with a g-value of 3.23 (Fig. 2B). These two new low-spin ferric heme signals are unlikely to arise from cyanide-liganded species of cytochromes b_1, c_4, or c_5 and may be attributed to low-spin ferric-heme–cyanide complexes of cytochrome d (cf. [20]).

Figure 3. The effect of carbon monoxide and potassium cyanide on the EPR spectra of phosphorylating particles. (A) oxidized particles (116 mg/ml) in the presence of oxygen. Potassium ferricyanide (10 mM) was added just before the sample was frozen. (B) As A but the oxidized particles were incubated with carbon monoxide for 2 min at 20°C and potassium ferricyanide (10 mM) was added just before the sample was frozen. (C) As A but particles were incubated with 5 mM potassium cyanide (pH 7.2) for 1 h at 20°C and potassium ferricyanide (10 mM) was added just before the sample was frozen. EPR conditions: microwave power, 50 mW; time constant, 0.3 sec; microwave frequency, 9.113 GHz (frequency-matched EPR tubes); temperature, 12°K and scanning rate of 125 G per min; spectra were recorded with a Varian E-3 spectrometer.

g= 6.24 5.94   5.51

H→ (Gauss)
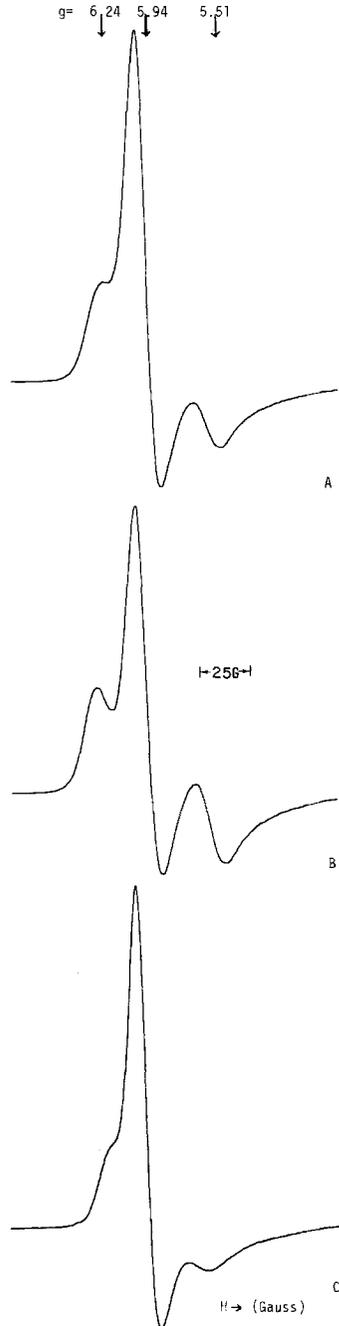
Effect of carbon monoxide and cyanide on the g = 6 signals of oxidized particles

The reaction of carbon monoxide with particles previously oxidized with ferricyanide results in a major effect in the g = 6 region (Fig. 3B) when compared to ferricyanide-oxidized particles in the absence of added ligand (Fig. 3A). There is a decrease of signal intensity in the axial component of approximately 20%, and although there is only a slight increase in the rhombic component, this latter species shows a significant increase in the degree of rhombicity, resulting in an apparent 6 G increase in the peak-to-peak width of the rhombic species. This is in contrast to the effect of cyanide (Fig. 3C) which causes a considerable decrease in the rhombic component and in the degree of rhombicity (9 G in the peak-to-peak width of the rhombic species), and does not cause a decline or other change in the axial species.

Discussion

The complex signal of the high-spin ferric-heme iron at g = 6, showing axial as well as rhombic components, can be interpreted either as occurring from two different hem d moietes or being derived from two conformations of cytochrome d in equilibrium [25]. Ligands such as cyanide and carbon monoxide mainly affect the rhombic component with only minor changes in the axial resonance. It is interesting to note that the changes which occur with the rhombic component of the g = 6 signal after the addition of cyanide are reflected in substantial changes in the optical properties of the 648 nm band of oxidized cytochrome d [10].

The low-spin signals in the region between g = 2 and g = 4, although similar to those observed in the mammalian system [19, 20], show an additional resonance stemming from cytochromes c_4 and c_5. In the mammalian respiratory system the peak at g = 3.03 has been attributed to a mixture of low-spin ferric forms arising from cytochrome c plus the heme of cytochrome c oxidase. Since the reported g-values for isolated cytochromes c_4 or c_5 [24] show no g-values as low as g = 3.03, it is likely that in the *Azotobacter* respiratory system this signal arises from a low-spin ferric form of cytochrome a_1, although we cannot rule out the possibility that other cytochrome components also absorb in this region. This study has shown that the low-spin ferric forms of cytochrome a_1, b_1, and c_4, plus c_5 in particles of *A. vinelandii* are readily detected. Further studies are in progress to ascertain the role of these cytochromes in the mechanism of energy conservation as found in bacterial membranes.

Acknowledgments

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