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A NEW EPR-DETECTABLE COMPONENT IN THE UBIQUINOL–CYTOCHROME *c* REDUCTASE SEGMENT OF THE RESPIRATORY CHAIN

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SUMMARY

A new paramagnetic electron acceptor, detectable by the characteristic EPR signal at $g = 1.867$, has been identified. The new paramagnetic species can be detected only below 10 °K. It is reducible by succinate plus ascorbate in the presence of phenazine ethosulphate, and undergoes redox changes parallel with the well-known Rieske iron–sulphur protein monitored at $g = 1.89$ at this temperature. However, it shows a temperature dependence different from that of the Rieske protein.

Extraction of ubiquinone from the mitochondrial membrane results in inhibition of reduction of the new species, and re-incorporation of ubiquinone restores the reduction. It is concluded that the new paramagnetic species is associated with ubiquinol–cytochrome *c* reductase.

INTRODUCTION

Ubiquinol–cytochrome *c* reductase (or Complex III) contains two atoms of iron linked to sulphur per mole of the complex [1]. The iron–sulphur protein is very rapidly reduced by ascorbate or ubiquinol-2 and slowly by succinate. Antimycin has no detectable effect on its reduction by ascorbate, but appreciably inhibits the reduction by succinate. The reduced iron–sulphur protein is readily oxidized by ferricytochrome *c* or ferricyanide, and the oxidation is not inhibited by antimycin. From these observations Rieske et al. [2, 3] suggested that the iron–sulphur protein is closely associated with cytochrome c_1 in the complex, and that it is probably located between the antimycin-sensitive site and cytochrome c_1. These investigators also reported that the reduced iron–sulphur protein shows a prominent EPR signal at $g = 1.90$ when measured at liquid-nitrogen temperature [2, 3]. According to data recently reported [4], the position of the derivative peaks are at $g = 2.026$, 1.887 and 1.809.

In the course of our EPR studies with ubiquinone-depleted pigeon-heart submitochondrial particles [5, 6], it has been found that two iron–sulphur centres moni-

Abbreviation: PES, phenazine ethosulphate.

tored at $g = 1.89$ can be identified on the basis of the sensitivity of their reduction by succinate toward antimycin, and that ubiquinone is required for the reduction of both centres.

Very recently Ohnishi [7] has reported that more than one iron–sulphur centre with the resonance peak at $g = 1.89$ can be identified on the basis of potentiometric titrations. Albracht [8] also reported that the resonance peak at $g = 1.89$ consists of signals from two iron–sulphur centres with different power-saturation behaviour. In the light of our finding and these reports, it is evident that the EPR signal at $g = 1.89$ originates not only from the well-known Rieske iron–sulphur protein*, but also from other paramagnetic electron acceptors present in the succinate–cytochrome *c* reductase segment of the respiratory chain.

The present communication reports a new paramagnetic species with a derivative peak at $g = 1.867$ that undergoes redox changes parallel with that of the Rieske iron–sulphur protein and appears to be present in the same reductase segment of the respiratory chain. A preliminary account has been reported at a recent symposium [9].

METHODS

Ubiquinone-depleted and ubiquinone-incorporated pigeon-heart submitochondrial particles were prepared according to the method described by Ernster et al. [10]. EPR spectra were measured in a Varian E3 EPR spectrometer. The microwave frequency was determined with a Hewlett–Packard frequency counter (5246L) with a frequency converter (5255A). The magnetic field was calibrated with an AEG Magnetfield meter (GA 11-22.2). The details of the experimental conditions are specified in the legend to the corresponding figure. The intensity of a signal was measured by the distance of a peak or a trough in the recorded derivative spectrum from the base line. The position of the signal refers to the trough of the g_y of the first-derivative spectrum.

RESULTS

Fig. 1 shows the EPR spectra in the magnetic field near $g = 1.89$, taken at 35 °K (A) and 7 °K (B). In the experiment of Fig. 1, ubiquinone-depleted and ubiquinone-containing (i.e. lyophilized, but not extracted with pentane) pigeon-heart submitochondrial particles were treated with succinate and antimycin in order maximally to reduce electron carriers on the substrate side of the antimycin block in the succinate-cytochrome *c* reductase segment. Subsequently, electron carriers on the oxygen side of the antimycin block were maximally reduced by inducing anaerobiosis in the presence of ascorbate and phenazine ethosulfate (PES). The EPR spectra were taken at various oxidation states, namely, before addition of succinate, after addition of succinate, antimycin and ascorbate plus PES, at anaerobiosis, after a pulse of oxygen and again at anaerobiosis.

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* In accordance with the original description by Rieske et al. [3], the iron–sulphur protein monitored at $g = 1.89$ (maximum of the absorbance) whose reduction by succinate is sensitive to antimycin will be referred to in this communication as the Rieske iron–sulphur protein.

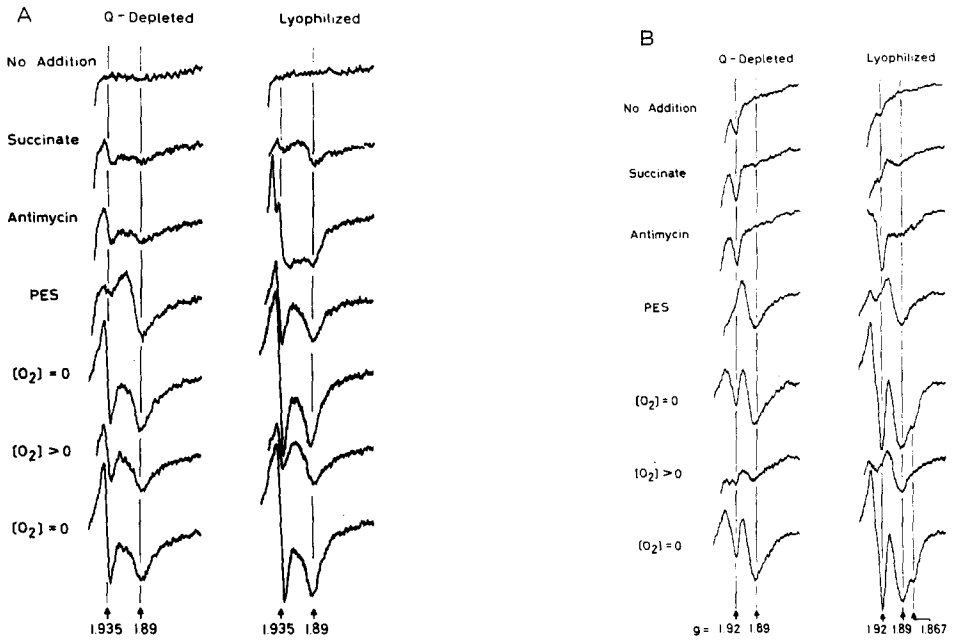

Fig. 1. Electron paramagnetic resonance (EPR) spectra of ubiquinone-depleted and ubiquinone-containing (i.e. lyophilized but not extracted with pentane) pigeon-heart submitochondrial particles, in the magnetic field near $g = 1.89$. From the top to the bottom, traces represent the EPR spectrum taken before the addition of succinate, and after successive additions of succinate (33 mM), antimycin (2 μg/mg protein), ascorbate (0.8 mM) plus PES (33 μM), and after anaerobiosis, addition of a pulse of oxygen and again after anaerobiosis, respectively. (A) The EPR spectra were measured at 35 °K, with microwave power 6.3 mW, modulation amplitude 12.5 G, and receiver gain $4 \cdot 10^5$. (B) The EPR spectra of the same samples were measured at 7 °K, with microwave power 6.3 mW, modulation amplitude 12.5 G, and receiver gain $5 \cdot 10^5$. All the traces were recorded with a scanning time of 4–8 min and a time constant of 0.3 s. The particles were suspended in 167 mM sucrose, 50 mM Tris–acetate buffer (pH 7.4), at a final concentration of 60 mg protein/mg. The concentration of ethanol in all samples was kept constant (5 %). The samples were frozen 45 s after the addition of succinate, 60 s after antimycin, 10 s after PES, 3 min after anaerobiosis and 10 s after a pulse of oxygen, respectively.

It can be seen that, at both 35 and 7 °K, the signal at $g = 1.89$ in ubiquinone-depleted pigeon-heart submitochondrial particles is scarcely increased after the addition of succinate and antimycin. On the other hand, under identical conditions, the signal in lyophilized particles is significantly increased, particularly when measured at 35 °K. Since the appearance of the signal detectable at 35 °K is not sensitive to antimycin, it cannot originate from the Rieske iron–sulphur protein. Upon addition of PES to lyophilized particles, the signal declines when measured at 35 °K, but increases at 7 °K. It increases further after anaerobiosis at both measuring temperatures. The magnitude of increase, however, is much greater when measured at 7 °K than at 35 °K. Thus, it is clear that the signals detectable at 35 and 7 °K, respectively, are not from the same iron–sulphur centre, but from two different species, and that reduction of the centre detectable at 7 °K is inhibited in the presence of antimycin, identifying it with the Rieske iron–sulphur protein [2, 3]. The opposite changes of

signal immediately after the addition of PES measured at 35 °K (decrease) and 7 °K (increase) indicate that, in the presence of antimycin, equilibrium between the two centres is restored by a PES-mediated shunt over the antimycin-sensitive site. At both temperatures, the signal in ubiquinone-depleted particles becomes detectable only after addition of PES, indicating that, in the absence of ubiquinone, succinate cannot reduce either the Rieske iron–sulphur protein or the antimycin-insensitive centre detectable at 35 °K.

Recently, it has been reported [11] that the so-called iron–sulphur Centre 5 of NADH dehydrogenase [12] is associated with the cytochrome bc_1 region, rather than with Complex I. Since the derivative peaks of this centre are at $g = 2.08$ and 1.89, they would interfere with the signal of Rieske iron–sulphur protein at $g = 1.89$. However, since the signals of Centre 5 are nearly completely saturated at 7 °K (the ratio of the height of the signal at $g = 2.08$ to 1.89 is 0.26 at 14 °K and 0.016 at 7 °K under the conditions of our present studies), the interference is very small at this temperature.

At this point it should be also mentioned that submitochondrial particles may be contaminated by small amounts of mitochondrial outer membrane that contains iron–sulphur protein with the derivative peaks at $g = 2.01$, 1.94 and 1.89 [13]. However, interference in our studies with any such contaminating protein can be ruled out, since the iron–sulphur protein in the outer membrane cannot be reduced with succinate.

In lyophilized particles, a new signal at $g = 1.867$ appears upon anaerobiosis. The new signal is detectable when measured at 7 °K, but not at 35 °K, and is absent in ubiquinone-depleted particles. A pulse of oxygen after anaerobiosis causes a decline of the intensity of the signal at $g = 1.867$ as well as that at $g = 1.89$, and when the oxygen is consumed, the intensity increases again. This result demonstrates that the new paramagnetic species undergoes redox changes in the same direction as those of the Rieske iron–sulphur protein.

The signal of oxidized copper [14] of cytochrome *c* oxidase is not detectable in the spectra of anaerobic lyophilized particles measured at a lower magnetic field at either 35 °K (Fig. 2A) or 7 °K (Fig. 2B). On the other hand, strong resonances at g_z from an unidentified electron acceptor around $g = 2.0$, previously reported by Orme-Johnson et al. [15], are still detectable at 7 °K, indicating that the reduction of this electron acceptor in lyophilized particles is much slower than that of copper of the

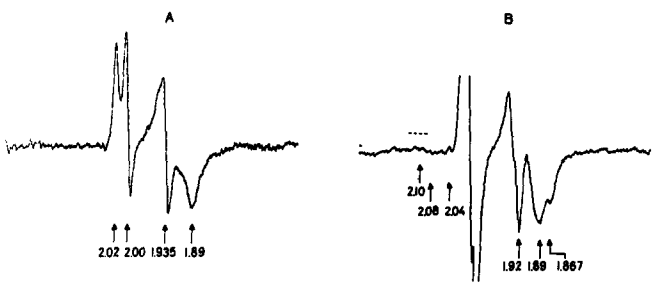

Fig. 2. EPR spectra of anaerobic lyophilized particles shown in Fig. 1 recorded with a wider scan range. (A) EPR spectrum measured at 35 °K and (B) at 7 °K.

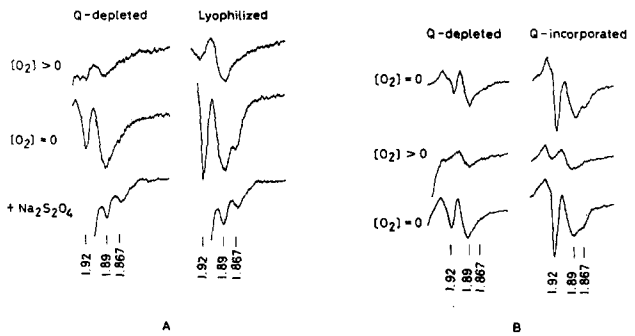

Fig. 3. Inhibition of reduction of the signal at $g = 1.867$ upon extraction of ubiquinone (A) and restoration of the reduction after re-incorporation of ubiquinone (B). (A) The EPR spectra were measured at 7 °K, with microwave power 4 mW and modulation amplitude 12.5 G. The receiver gain for traces in the upper two rows was $5 \cdot 10^5$, and in the third row $1 \cdot 10^5$. (B) The conditions for the measurements were the same as in (A) except that the receiver gain was $1 \cdot 10^5$. Other experimental conditions were similar to those described in Fig. 1.

oxidase. Due to the high noise level, it is difficult to say whether or not a signal at $g = 2.10$, the g_z line of Centre 3, is present in Fig. 3B. At 7 °K the ratio of the intensity of the signal at $g = 2.10$ to that at $g = 1.89$ in dithionite-reduced particles is 0.56 ± 0.02 under the measuring conditions of Fig. 1. On the basis of this ratio and the intensity of the signal at $g = 1.867$ in Fig. 2B (corrected for interference of the signal at $g = 1.89$), the intensity of the signal at $g = 2.10$ derived from Centre 3 can be estimated, on the assumption that the signal at $g = 1.867$ originates entirely from Centre 3. This is shown by the dashed line. Since the observed absorption at $g = 2.10$ is much less than this, it can be safely concluded that Centre 3 is not reduced, and that

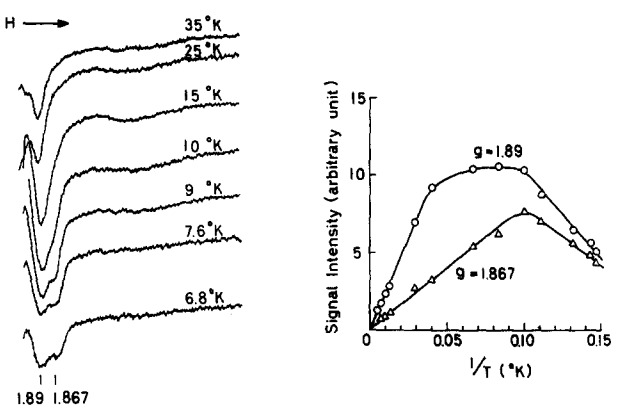

Fig. 4. Comparison of the effect of temperature on the signals at $g = 1.89$ and 1.867. The EPR spectra were obtained with lyophilized pigeon-heart submitochondrial particles at various temperatures, with microwave power 2.25 mW, modulation amplitude 12.5 G, and receiver gain $2 \cdot 10^5$. In expressing the temperature dependence of the two signals (right), each spectrum was resolved into two signals on the assumption that the signal at $g = 1.89$ is a symmetrical peak. The intensity of the signals was then estimated in terms of the height of each signal from the baseline. Other experimental conditions were similar to those described in Fig. 1.

the signal at $g = 1.867$ is not associated with Centre 3. The signal of Centre 5 at $g = 2.08$ [12] is hardly detectable at 7 °K, but reduction of the centre is evident when measured at 14 °K (not shown).

As shown in Fig. 3A, the signal at $g = 1.867$ appears after addition of dithionite to ubiquinone-depleted particles. The intensity of this signal in ubiquinone-depleted particles is the same as that in lyophilized particles. As shown in Fig. 3B, re-incorporation of ubiquinone into the depleted particles restores the appearance of the signal at $g = 1.867$ on anaerobiosis with succinate plus ascorbate as electron donors. The signal disappears on aeration.

Fig. 4 illustrates the effect of temperature on the intensity of the signals at $g = 1.89$ and 1.867. It can be clearly seen that, upon decreasing the temperature below 10 °K, the sharp signal at $g = 1.89$ and the broad signal at higher field (approximately $g = 1.81$) become saturated, so that the signal at $g = 1.867$ becomes well resolved from that at $g = 1.89$. The plot of the intensity of the two signals as a reciprocal function of the measured temperature demonstrates that the two signals exhibit a different temperature dependence. In view of the small difference in g values, it is unlikely that the different relaxation behaviour is due to g-anisotropy. Thus, it is probable that the signals belong to different centres.

DISCUSSION

Several possibilities can be considered for the origin of the new paramagnetic resonance signal at $g = 1.867$.

First, since the lyophilization of submitochondrial particles in the presence of 0.15 M KCl results in extensive structural re-arrangement of the mitochondrial membrane [16], the appearance of the signal at $g = 1.867$ could be attributable to modified (or dislocated) Rieske iron–sulphur protein. An argument against this possibility is provided by the observation that the signal at $g = 1.867$ is also present in phosphorylating ATP–Mg particles derived from pigeon-heart or bovine-heart mitochondria (unpublished observations). Thus, the signal originates from an electron acceptor present in the intact mitochondrial membrane.

Secondly, the possibility that the signal at $g = 1.867$ is from the low-spin ferric haem ($g = 2.6$, 2.2 and 1.87) of incompletely reduced cytochrome c oxidase [17] can be ruled out by the finding that the EPR spectra of anaerobic particles shown in Fig. 2 did not show the signal of oxidized copper. Moreover, the spectrum shown in Fig. 3A indicates that the signal at $g = 1.867$ becomes detectable after the addition of dithionite to ubiquinone-depleted particles, which would reduce cytochrome c oxidase completely.

Thirdly, Beinert [18] reported that the mid-point potential of the iron–sulphur Centre 3 of NADH dehydrogenase is higher than that of Centre 2. Thus, the possibility exists that Centre 3 could be reduced by succinate in the presence of PES which would directly shunt electrons between succinate dehydrogenase and Centre 3, thereby contributing to the resonance signal at $g = 1.867$. This seems very unlikely, because the experiments shown in Fig. 2 clearly demonstrate that the signal at $g = 1.867$ requires ubiquinone for its reduction, even in the presence of PES. Moreover, the weak signal at $g = 2.10$ in the spectrum shown in Fig. 2B shows that little reduction of Centre 3 takes place under the experimental conditions used, so that the signal at

$g = 1.867$ cannot be assigned to a derivative peak of Centre 3.

The precise location of the component responsible for the signal at $g = 1.867$ and its functional role in the respiratory chain have yet to be clarified. The inhibition of its reduction upon extraction of ubiquinone or on the addition of antimycin suggests that the component is probably located on the oxygen side of the antimycin block in the ubiquinol–cytochrome *c* reductase segment of the respiratory chain. In addition, the cyclic redox behaviour of the signal at $g = 1.867$ comparable with that of the signal at $g = 1.89$ measured at 7 °K (cf. Figs 1B and 2B) may indicate that the new paramagnetic species is closely associated with the Rieske iron–sulphur protein, and that it participates, directly or indirectly, in the electron transfer in the respiratory chain.

The identification of an iron–sulphur centre with a resonance signal at $g = 1.89$ detectable at 35 °K and reducible by succinate in the presence of antimycin has raised an interesting question concerning the extent to which the signal from the Rieske iron–sulphur protein would contribute at $g = 1.89$, when measured at 77 °K. This remains uncertain until the EPR characteristics of all the paramagnetic centres contributing the signal at $g = 1.89$ are described.

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