EPR SIGNALS OF CYTOCHROMES IN SUBCHLOROPLAST PARTICLES

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1. Introduction

Although cytochromes are important components in photosynthetic electron transport, their study in plants by electron paramagnetic resonance (EPR) has been confined to only a few reports. A brief report [1] assigned signals in the $g = 6.0$ region to high-spin forms of cytochrome b_6 and low potential cytochrome *b*-559. A study of low-spin cytochrome signals in the g = 3.0 region [2] revealed signals attributed to cytochrome *f* and low potential cytochrome *b*-559. Photooxidation of the cytochrome *b*-559 was observed at cryogenic temperatures in samples reduced by ascorbate prior to freezing. Information on cytochrome *b*-559 using EPR techniques would be valuable in understanding the role of this cytochrome and its various potential forms in photosystem II and the electron-transport chain of chloroplasts.

Here we investigated both the high- and low-spin cytochrome regions of the EPR spectrum at cryogenic temperatures. This study confirms some of the previous work but also reports new high spin signals, some of which respond to illumination at cryogenic temperatures.

2. Materials and methods

Chloroplasts and digitonin subchloroplast particles were prepared as in [3]. EPR measurements were performed as in [3] with chlorophyll concentrations as given in the legends to the figures. Tris washing of subchloroplast particles was based on the method in [4] and the particles were resuspended at pH 9.0.

3. Results and discussion

3.1. *Low-spin iron*, g = *3.0 region*

When digitonin particles were dark adapted for 20 min before freezing, the EPR spectrum in fig.1a was observed. The optimum conditions for these signals were around 14 K and 10 mW microwave power. Illumination of these samples at cryogenic temperatures produced no significant effects. The

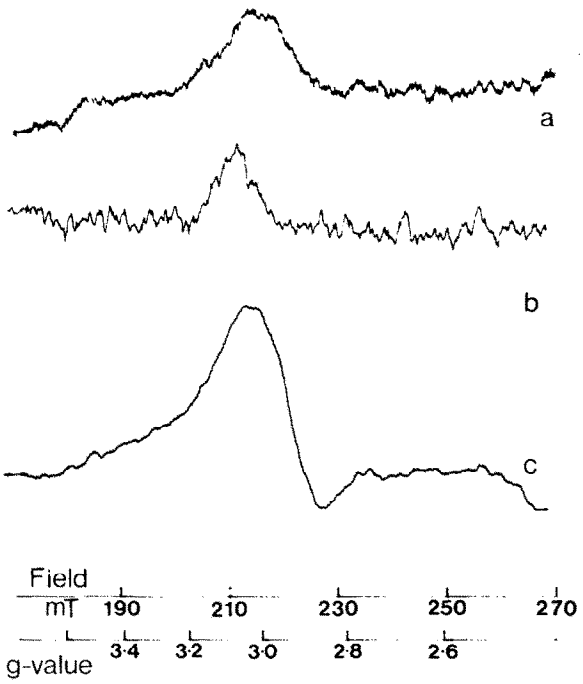

Fig.1.(a) EPR spectrum of digitonin particles, dark-adapted and then frozen: Microwave power, 20 mW; time constant, 0.1 s; scanrate, 25 mT/min; modulation amplitude 1 mT; gain 2.5×10^3; temp. 13.2 K, frequency, 9.076 GHz; chlorophyll, 8 mg/ml. (b) Difference spectrum showing signal induced by illumination at 15 K in digitonin particles, dark-adapted in the presence of 10 mM sodium ascorbate and then frozen. Conditions as above except: temp. 15 K, gain, 10^4; time constant, 1 s. (c) Spectrum of digitonin particles frozen in the dark then cooled from 77–13 K in a field of 400 mT. Conditions as in (a) except: gain, 10^3.

signals at $g = 3.5$ and $g = 3.0$ in fig.1a are probably those attributed to cytochrome *f* and low potential cytochrome *b*-559 in [2]. However, in this digitonin preparation cytochrome *f* was present in lower concentration than in the chloroplasts used in [2].

When digitonin particles were dark-adapted in the presence of ascorbate and dichlorophenolindophenol (DPIP), no signals were observed in the $g = 3.0$ region confirming that the species giving rise to the signals in fig.1a are in the oxidised state. When the digitonin particles were dark-adapted in the presence of benzoquinone only the $g = 3.0$ signal was observed, suggesting a mid-point potential for this signal between benzoquinone and ascorbate, near to that of low potential cytochrome *b*-559.

When samples dark-adapted in the presence of ascorbate and DPIP were illuminated at cryogenic temperatures, a signal appeared irreversibly near $g = 3.0$. Fig.1b shows the difference spectrum obtained by subtracting the spectra obtained before and after illumination. The light-induced signal did not appear at exactly the same *g*-value as the signal in fig.1a and confirmed the report in [2]. It was suggested that different potential forms or species in different environments of cytochrome *b*-559 gave rise to signals at slightly different *g*-values. Optical spectroscopy indicated that some high potential cytochrome *b*-559 was present in digitonin particles although the cytochrome was mainly in the low potential state. The light-induced signal may represent the shoulder seen in fig.1a and may be high potential cytochrome *b*-559 as suggested in [2]. The light-induced signal was also present in digitonin particles Tris-washed and resuspended at pH 9.0. The spectra in fig.1a,b were taken using samples cooled from 77–13 K without an applied magnetic field. This was because in some samples, cooling in the presence of a field of 400 mT or more induced a large (relative to the size of the cytochrome signals) signal which was oriented to the direction of applied field, fig.1c. The occurrence, size and position of the signal varied but usually contained a component near $g = 3.0$. The signal had characteristics similar to those reported in [5] and probably also in [6]. The origin of this signal is unclear but may represent an unusual interaction between cytochromes which enhances the signal. The signals have characteristics of a spin-glass type structure where paramagnetic ions influenced by the magnetic field are frozen in a non-random arrangement at <20 K, giving rise to an oriented signal [7,8].

3.2. *High-spin iron*, g = *6.0 region*

Digitonin particles dark-adapted in the presence of ascorbate and DPIP gave the spectrum shown in fig.2a. Under optimum conditions at <10 K there was a major peak at $g = 6.1$ plus two distinct shoulders on the low-field side. Illumination at cryogenic temperatures had no effect on these signals. The signal not reduced by ascorbate may be due to cytochrome b_6 although the *g*-value does not correspond to those

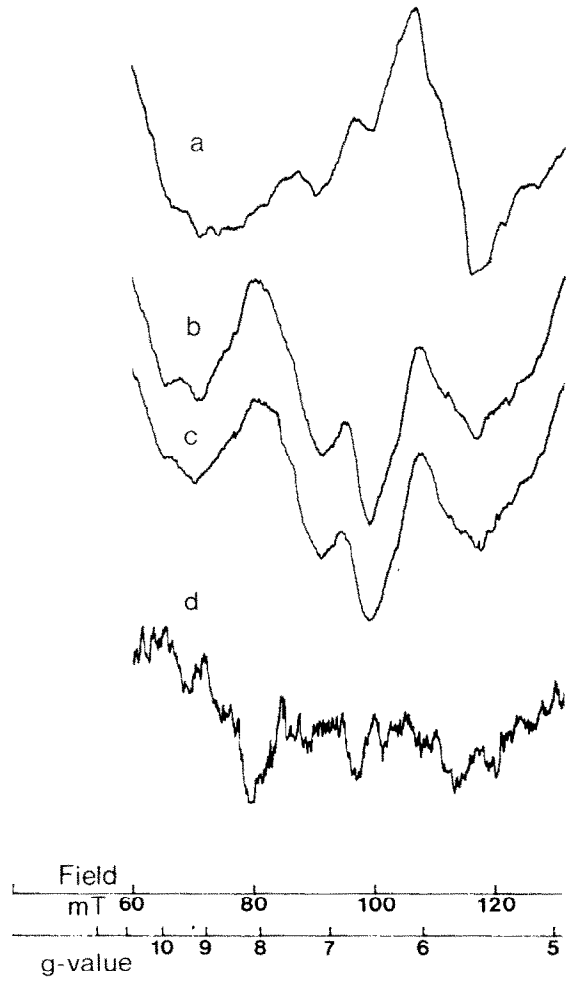

Fig.2.(a) EPR spectrum of digitonin particles dark-adapted in the presence of 10 mM sodium ascorbate and then frozen. Conditions as fig.1a except: time constant, 1s; gain, 5×10^3; temp. 5.3 K. (b) EPR spectrum of digitonin particles dark-adapted and frozen in the presence of 5 mM ferricyanide. Conditions as in (a) except: gain, 6.3×10^3. (c) As (b) after illumination at 5.3 K. (d) Dark after minus dark before illumination difference spectrum (c)–(b) × 3. Conditions as in (b).

given in [1] of $g = 5.66$ and $g = 6.74$. The signal in fig.2a was removed by dithionite.

The addition of ferricyanide to dark-adapted digitonin particles induced a peak at $g = 8.1$ which as fig.2b shows was now the major peak. A peak at $g = 6.9$ was also present. Upon illumination at <10 K the $g = 8.1$ peak, the $g = 6.9$ peak and a shoulder of the $g = 6.1$ peak at $g = 5.8$ were decreased irreversibly, fig.2c. The difference spectrum obtained by subtraction of 2c from 2b is shown in fig.2d. The signal recovers during storage overnight at 77 K and can then be induced again by illumination at cryogenic temperatures. This was also a property of signal II_{LT}, the signal II species formed upon low temperature illumination [3]. The light-induced signal near $g = 3.0$ did not recover significantly during overnight storage at 77 K.

The signals near $g = 6.00$ probably represent high-spin ferric haem iron in cytochromes and the change on illumination may reflect a partial reduction from the paramagnetic oxidised state. In ferricyanide treated samples P700 the primary donor of photosystem I was oxidised and photosystem I blocked. Therefore the light-induced changes were due to photosystem II reactions and may originate from a photosystem II acceptor. However the spectrum may also arise from magnetic interactions between paramagnetic species and the reduction in signal size on illumination would therefore be caused by a change in this interaction due to oxidation or reduction of photosystem II components which are not directly observed.

The light-induced change was more clearly seen in Tris-washed digitonin particles at pH 9.0 which had been treated with ferricyanide and dark-adapted. Fig.3a shows the spectrum obtained from these samples where the $g = 6.1$ peak was almost absent, leaving the $g = 5.8$ shoulder more prominent. Upon illumination this shoulder was irreversibly reduced in size together with the $g = 8.1$ peak, fig.3b. Fig.3c shows the difference spectrum. No light-induced change in the $g = 6.9$ signal was seen in these samples. The peak at $g = 9.6$ which was present in these samples did not change upon illumination.

The difference in pH between Tris-washed and ordinary digitonin particles could account for the spectral changes, as a similar pH-dependent change occurs in cytochrome *c* oxidase [9]. Alternatively the removal of manganese by Tris washing may affect directly the components giving rise to or influencing

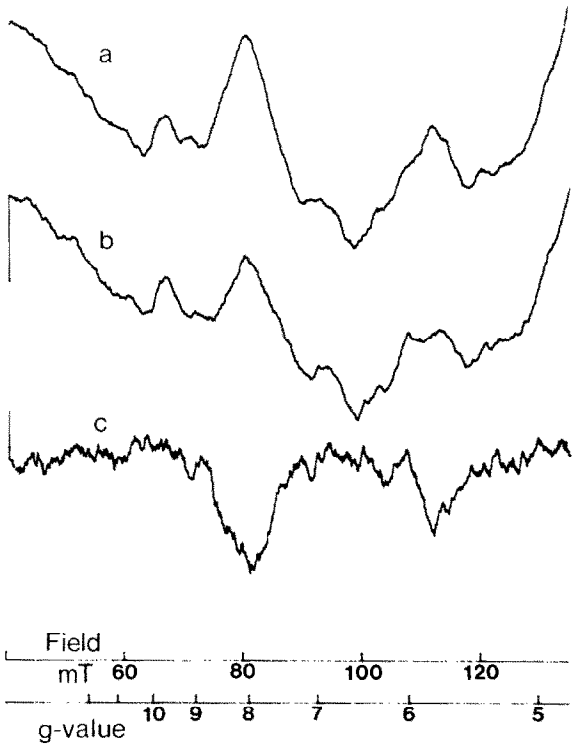

Fig.3. EPR spectra of Tris-washed digitonin particles dark-adapted and frozen in the presence of 5 mM ferricyanide (a) before and (b) after illumination at 5.7 K. (c) Difference spectrum (b)–(a) × 2. Conditions as fig.1a except: time constant, 1 s; gain, 5×10^3; temp. 5.7 K; chlorophyll 6.5 mg/ml.

the signals around $g = 6.00$. Further work to characterise the signals in both the $g = 3.0$ and $g = 6.0$ regions are in progress and will lead to a clearer understanding of the reactions in photosystem II. Further information on the role of the signals reported above and that of signal II_{LT} [3] may result in a scheme for the donor reactions to P680, the primary donor of photosystem II.

Acknowledgements

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