Volume 255, number 1, 53-58 FEBS 07563 September 1989

Electron transfer in the isolated photosystem II reaction centre complex

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Received 10 July 1989

We have characterised the electron-transfer properties of the D1/D2/cytochrome *b*-559 complex using EPR spectrometry. The complex can transfer electrons to silicomolybdate and ferricyanide at cryogenic temperatures. In the presence of silicomolybdate or ferricyanide, two chlorophyll cation radicals were observed from $P680^+$ (0.8 mT) and monomeric Chl (1.0 mT). Reduction of silicomolybdate was detected as a 2.7 mT signal at $g = 1.942$. A radical attributed to a tyrosine cation radical (D^+/Z^+) was also observed in a small percentage of centres.

Photosynthesis; Photosystem II; EPR; Triplet state; Tyrosine radical; Reaction center

1. INTRODUCTION

The PS II reaction centre of oxygenic photosynthetic organisms transfers an electron from P680, the reaction centre chlorophyll, to a pheophytin molecule, Phe, and then to two quinones, Q_A and Q_B. Based on homologies with the L and M subunits of the reaction centre of purple photosynthetic bacteria it was argued that these components were bound by a heterodimer of polypeptides termed D1 and D2 [1,2]. Experimental support for this came from the work of Nanba and Satoh [3] who isolated a PS II reaction centre complex for spinach consisting of the D1 and D2 polypeptides and the apoproteins of cytochrome *b*-559. This complex has since then been isolated from other PS II containing species, including pea [4], the alga, *Scenedesmus obliquus* [5] and the cyanobacterium, *Synechocystis* 6803 [6].

The D1/D2/cyt b559 complex isolated from higher plants contains four Chl *a*, one β-carotene and one cyt b559 per two pheophytin *a* molecules [3,7]. In the presence of excess dithionite, illumination leads to the accumulation of reduced pheophytin [3,4,8-10]. At cryogenic temperatures, a light-induced spin-polarised triplet EPR signal can be detected which originates from the radical pair recombination between oxidised $P680^+$ and the reduced pheophytin acceptor, Phe^- [11]. Redox titrations of the spin-polarised triplet EPR signal confirmed the absence of Q_A and Q_B in the isolated D1/D2/cyt b559 complex [12]. However, this study also suggested that the electrons could be transferred beyond pheophytin under oxidising conditions giving a radical assigned to $P680^+$. The optical detection of $P680^+$ has also been possible using silicomolybdate as an artificial electron acceptor [4,13].

Experiments using isotope substitution and site-directed mutagenesis have shown that the electron donors Z and D are probably tyrosine residues located symmetrically on the D1 and D2 polypep-

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Abbreviations: Chl, chlorophyll; cyt, cytochrome; D1, *psb*A gene product; D2, *psb*D gene product; Phe, pheophytin; PS, photosystem; SiMo, silicomolybdate ($SiMo_{12}O_{40}^{4-}$)

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00145793/89/$3.50

tides, respectively [14–17]. Despite this, however, these electron donors have not been detected in the D1/D2/cyt b559 complex [18].

The present work describes EPR experiments which allow a closer analysis of electron transfer from P680 to silicomolybdate and ferricyanide. Experiments carried out at cryogenic temperatures indicate that these artificial electron carriers can accept electrons directly from reduced pheophytin.

2. MATERIALS AND METHODS

The D1/D2/cyt b559 complex was isolated from pea (*Pisum sativum*) by a method based on that of Nanba and Satoh [3] as modified in [8]. Preparations having between 5 and 7 Chl/cyt b559 were used. Deuterated reaction centres were isolated from *S. obliquus* grown on deuterated media (gift from Henry L. Crespi and Marion C. Thurnauer, Argonne National Lab. USA) as in [5].

PS II preparations with 150 Chl/cyt b559 were isolated by the method of Ford and Evans [19] from market spinach (*Spinacea oleracea*).

EPR measurements were performed as in [5]. Illumination at 4°C (400 μEinsteins/m^2 per s) and in the cryostat at cryogenic temperatures, was provided by a 150 W white light source. Signals, except in fig.3, are the average of four spectra. Quantification of EPR radicals was by double integration of the first-derivative signal taken at non-saturating microwave powers using cytochrome *c* as a standard [20]. The scales shown in the figures are approximate and actual linewidths were checked with a manganese standard. Silicomolybdate (SiMo) was purchased from Pfaltz and Bauer and, in the form delivered, was a mixture of Mo(V) and Mo(VI) forms.

3. RESULTS AND DISCUSSION

3.1. *Illumination at cryogenic temperatures*

Fig.1a shows the illuminated minus dark difference spectrum of an untreated sample showing

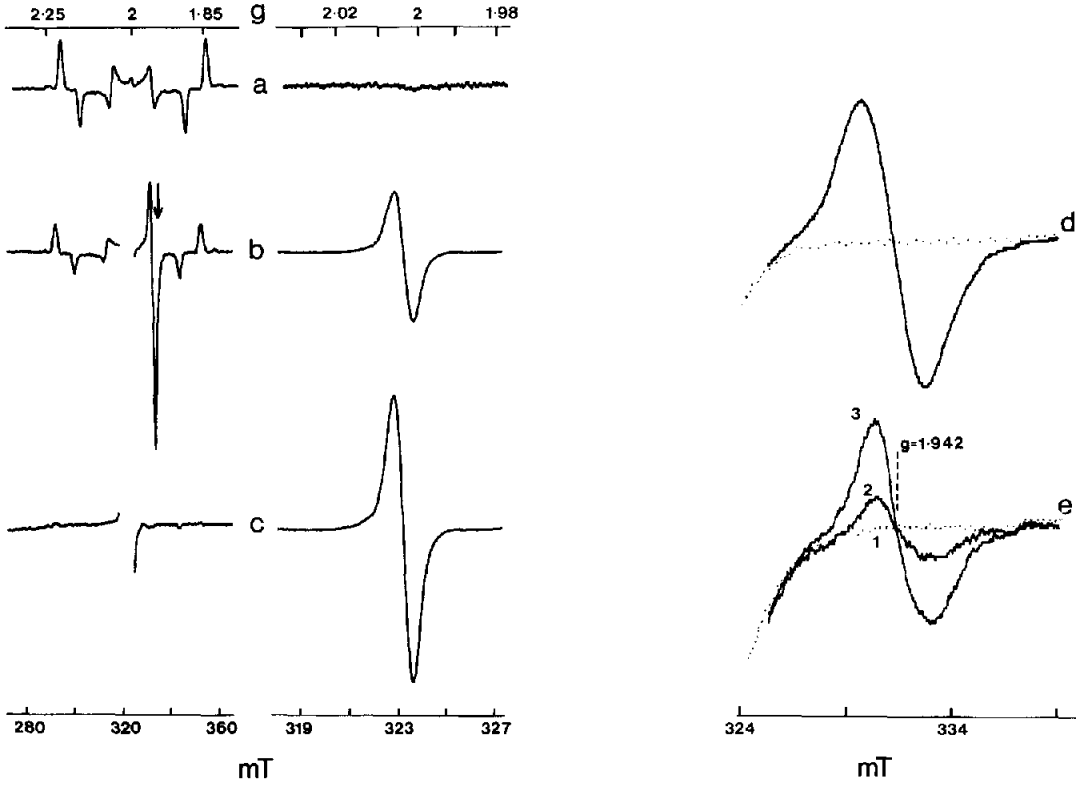

Fig.1. EPR spectra showing the effect of added electron acceptors silicomolybdate and ferricyanide. 4 K illuminated minus 4 K dark difference spectra of the D1/D2/cyt b559 preparation (30 μg Chl/ml). (a) Untreated showing the spin-polarised reaction centre triplet. (b) With 200 μg/ml silicomolybdate added. The signal arising from the reduction of silicomolybdate indicated by an arrow. (c) With 1 mM potassium ferricyanide added. The large $g = 2$ radical observed in (b,c) is shown on the right. (d) Silicomolybdate sample oxidised by ferricyanide (dotted line) and reduced by sodium ascorbate. (e) D1/D2/cyt b559 complex as (b) but with 200 μg/ml silicomolybdate, oxidised by ferricyanide, added. Spectrum (1) (dotted line) dark; (2) dark following illumination; (3) illumination at 5 K. EPR conditions: (a–c) microwave power, 25 μW (left) and 10 μW (right); modulation width, 1.25 mT (left) and 0.2 mT (right); and temperature, 4 K. (d–e) Power, 1 mW; modulation width, 0.25 mT; temperature, 5 K.

the spin-polarised triplet. Addition of 200 μg/ml SiMo prior to freezing caused a reduction in size of the triplet and the appearance of radicals near g = 1.94 and $g = 2$ on illumination at 4 K (fig.1b). Increasing the SiMo concentration further descreased the extent of the triplet (not shown).

The signal at $g = 1.94$ (fig.1b) was shown to be from photoreduced SiMo as it corresponded to the Mo(V) peak ($g = 1.942$) [21] in the spectrum of SiMo chemically reduced by ascorbate (fig.1d). Fig.1e shows the generation of the Mo(V) SiMo EPR signal by illumination at 5 K in a D1/D2/cyt b559 sample. On illumination at 5 K a large 2.7 mT signal at $g = 1.942$ was induced (fig.1e, spectrum 3). This was composed of a reversible and an almost irreversible component (fig.1e, spectrum 2). The 'irreversible' component increased in size depending on the length of illumination, indicating that a multiple-step process was occurring which stabilises the charge separation.

As found previously [12], the addition of 1 mM ferricyanide (fig.1c) removed the ability to generate the spin-polarised triplet and induced the $g = 2$ radical. There were no changes in the $g = 1.95$ region and the EPR signal from ferricyanide was too broad to detect any light-induced changes. The signal at $g = 6$ from oxidised non-haem iron [22] was not observed in any samples. In addition, no light-induced changes were seen in the $g = 3$ region of the EPR spectrum indicative of cytochrome *b*-559 reduction. We conclude therefore that both ferricyanide and SiMo act as electron acceptors at 4 K.

The $g = 2$ radicals formed on illumination (fig.1b,c) have single-line spectra with g values close to 2.0026, characteristic of hydrocarbon cation radicals. These probably arise from electron donors, $P680^+$ or another oxidised species such as monomeric chlorophyll or carotenoid [23,24]. For porphyrin radicals, the major interaction of the unpaired electron is with the magnetic nuclei of the macrocycle protons. To investigate this we prepared reaction centres from *S. obliquus* grown on deuterated media which replaced the large magnetic moment of ^1H by ^2H.

Fig.2 shows that this results in the narrowing of the signal of both reduced pheophytin, Phe^-, accumulated by illumination during freezing in the presence of dithionite (as in [18]) (1.35 to 0.54 mT, fig.2B) and the rapidly reversible $g = 2$ signal seen

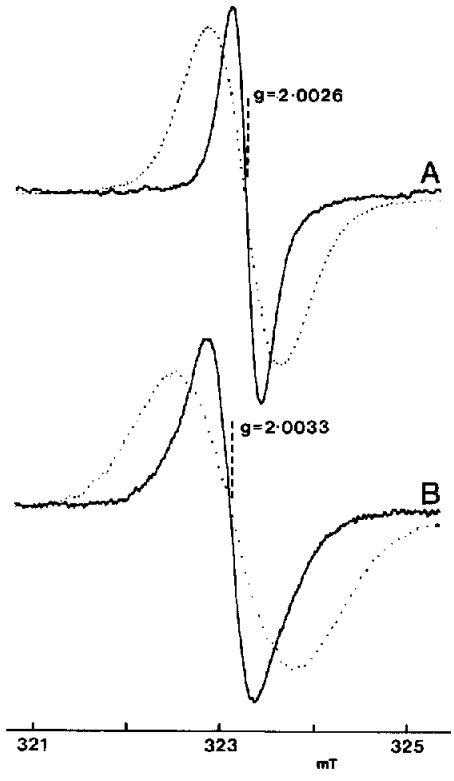

Fig.2. Comparison of the $g = 2$ radicals generated in normal and deuterated D1/D2/cyt b559 samples. 25 μg Chl/ml (dotted lines), normal and (solid lines) deuterated samples. (A) Illuminated at 15 K minus dark after illumination spectra of samples treated with 200 μg/ml SiMo, showing $P680^+$. (B) Illuminated at 277 K minus dark spectra of samples treated with dithionite at pH 10, showing Phe^-. The sizes of individual signals have been changed to give approximately equal amplitudes. Linewidths: (A) solid line, 0.35 mT; dotted line, 0.8 mT; (B) solid line, 0.54 mT; dotted line, 1.35 mT. EPR conditions: microwave power, 10 μW; temperature, 15 K; modulation width, 0.125 mT (solid lines) and 0.2 mT (dotted lines).

on illumination at 15 K, following addition of SiMo (0.85 to 0.35 mT, fig.2A). This is very close to the factor of 2.4 by which the width of the bacteriochlorophyll cation was decreased [25] and is consistent with the signal in fig.2A being due to a chlorophyll cation radical.

Fig.3A,B confirms that in an untreated sample, essentially no $g = 2$ radical was generated by 17 K illumination. Following addition of ferricyanide (fig.3C), the $g = 2$ radical formed on illumination gradually broadened and reached maximum size (approx. 1 spin per 8 Chl) after 5 min. The spectrum was composed of two parts, a rapidly reversi-

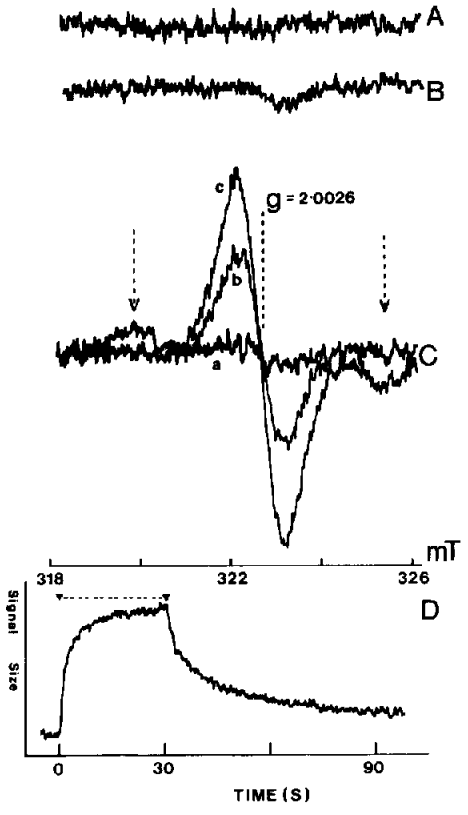

Fig.3. EPR characteristics of electron donation in the D1/D2/cyt b559 complex. 20 μg Chl/ml. (A) Untreated dark-adapted sample. (B) Untreated sample illuminated at 17 K for 5 min. (C) 1 mM ferricyanide-treated sample in the dark (a) and after 2 min (b) and 5 min (c) illumination at 17 K. (D) The time course of changes at $g = 2.006$ during illumination at 17 K of a sample containing 200 μg/ml SiMo. The illumination period (0–30 s) is indicated by the dotted line between the arrows. EPR conditions: microwave power, 50 μW; modulation width, 0.2 mT; temperature, 17 K. The spectra are single rapid scans and are therefore more noisy than other spectra shown.

ble 0.8 mT radical, as seen with SiMo as acceptor in fig.2A, on top of an essentially irreversible 1.0 mT radical. The 1.0 mT radical is characteristic of an oxidised monomeric chlorophyll *a* [24]. It should also be noted that a split radical (arrow, fig.3C) increased in parallel with the 1.0 mT radical and may be due to a radical pair interaction [5].

SiMo-containing samples gave a higher proportion of the 0.8 mT reversible signal up to 1 spin per 9 Chl (as shown in fig.2A). The percentage of reversible radical decreased as the length of illumination period and the irreversible radical increased. This correlated with results obtained with the $g = 1.94$ signal due to the reduction of SiMo (fig.1e). The time course of the rise and decay of the signal at g = 2, for an SiMo sample, is shown in fig.3D. Onset of illumination produced an initial rapid rise in signal size followed by a slow increase corresponding to the increase in yield of the irreversible 1.0 mT radical. When the light was turned off two decay phases were observed, a fast and a slow component. The $g = 2$ radicals were removed completely by thawing the sample to 4°C and refreezing in darkness.

The data suggest that in the presence of either SiMo or ferricyanide, $P680^+$ can be formed at 4 K giving rise to the 0.8 mT reversible radical. During illumination electron donation from a monomeric chlorophyll molecule to $P680^+$ occurs, more efficiently with ferricyanide as acceptor, giving rise to the 1.0 mT radical and increasing the stabilisation of the charge separation at cryogenic temperatures. The biphasic recombination with SiMo as acceptor may be indicating recombination between reduced SiMo and $P680^+$ (rapid phase) and between Chl^+ (slow phase), although the involvement of another recombination reaction cannot be ruled out.

The observation that the chlorophyll cation radical assigned to $P680^+$ has a reduced linewidth compared to the monomeric chlorophyll confirms early studies using chloroplasts or crude PS II particles [24,26–29]. In a recent paper, Hoganson and Babcock [30] have shown the width of the $P680^+$ radical, measured kinetically, to be 0.79 mT in the absence of Z^+. The linewidth shown here of 0.8 mT suggests that P680 is undamaged by the isolation procedure. The ease of observation of $P680^+$ in the purified complex makes it a good system for further study of this radical.

Previous authors were undecided about P680 being a monomer or dimer of chlorophyll [24,28,29]. The EPR linewidth of dimeric chlorophyll may be narrowed by $\sqrt{2}$ as compared to the monomer as found in *Rhodobacter sphaeroides* [31]. Unfortunately, variable reduction in linewidth can be caused by environmental factors [32] or asymmetric distribution of charge as in the *Rhodopseudomonas viridis* BChl *b* dimer [33,34]. Therefore, the observed reduction in linewidth of $P680^+$ is not definitive evidence that it is a dimer of chlorophyll. The zero-field splitting parameters of the spin-polarised triplet are characteristic of a

chlorophyll monomer [5,35] but Rutherford [35] has shown that this triplet has an orientation parallel to the membrane plane. Such an orientation would not be expected if P680 were a dimer of chlorophyll comparable with the primary donor of the purple bacterial reaction centre. This indicates, as suggested by Rutherford [36], that the triplet signal detected may not be located on P680 but originates from an accessory chlorophyll monomer. Our results indicate that illumination at low temperatures can indeed generate oxidised monomeric chlorophyll.

3.2. *Illumination at 4°C*

Untreated samples of the D1/D2/cyt b559 complex were also illuminated at 4°C for 15 s and frozen under illumination to 77 K. When examined at 14 K they showed no EPR signals near $g = 2$ either in the dark or on illumination (fig.4a). However, in the presence of SiMo or ferricyanide, illumination of the isolated complex at 4°C for <15 s generated broad EPR signals near $g = 2$ detected at 14 K in the dark (fig.4b). These signals were stable at 77 K but decayed rapidly for the case where the sample was thawed to 4°C. When the sample previously illuminated at 4°C was illuminated at 14 K, the 0.8 mT reversible signal assigned to $P680^+$ was still produced (fig.4b, dotted line) but decreased in amplitude with the length of illumination at 4°C.

The EPR characteristics of the solid-line spectrum in fig.4b suggested that it may arise from two components, a broad and a narrow radical. The latter could be the 1.0 mT chlorophyll radical detected by illumination at 4 K. The broad component resembled the signal from the tyrosine radical (D^+ or Z^+) [14–17]. To identify the broad component of the spectrum, D^+ was induced in an oxygen-evolving PS II-enriched preparation (fig.4c). It is clear that the main features of D^+ can be seen in the spectrum obtained with the D1/D2/cyt b559 complex. Moreover, the estimated g value ($>g = 2.004$) was consistent with this assignment to D^+ or Z^+.

An estimate of the numbers of reaction centres involved in electron transfer from D/Z was made by calibration vs the D^+ signal from the PS II-enriched preparation and by the double integration method [20]. We found that approx. 5% of D1/D2/cyt b559 complexes were involved in the

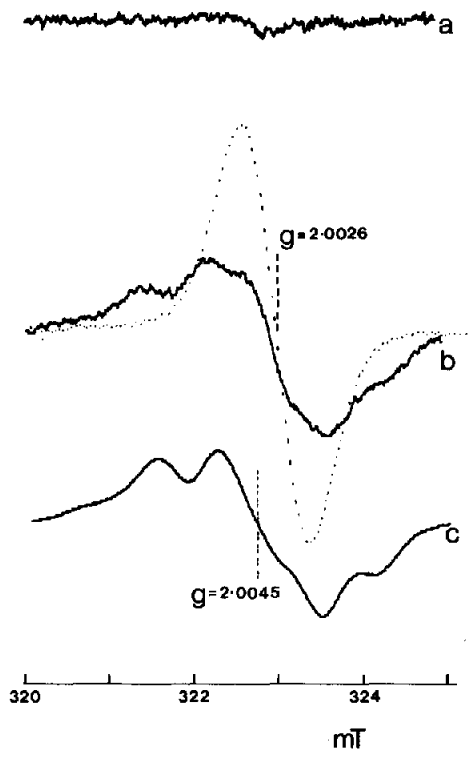

Fig.4. EPR spectra of the radicals produced by illumination at 4°C. 30 μg Chl/ml D1/D2/cyt b559 complex. (a) Illuminated at 4°C for 15 s minus dark spectrum of untreated sample. (b) Solid line, illuminated as (a) minus dark spectrum of sample containing 200 μg/ml silicomolybdate. Dotted line, illuminated at 4 K minus dark after illumination at 4 K spectrum of same sample. (c) Spectrum of D^+ in dark-adapted oxygen-evolving PS II particle (1.2 mg Chl/ml). EPR conditions: microwave power, 25 μW; temperature, 14 K; modulation width, 0.2 mT.

formation of the D^+/Z^+ signal assuming 1 cyt b559 per reaction centre. This low yield of D^+/Z^+ may result from: (i) trapping a small steady state population of D^+ or Z^+ because of a rapid rereduction; (ii) measuring D^+ and/or Z^+ in only a few centres; or (iii) contamination of the preparation by a more intact complex containing extra polypeptides. The latter was ruled out as contaminating polypeptides were not present at the 5% level. The decay of the small signal assigned to the tyrosine electron donors at 4°C in the dark suggests that the stability of D^+ may have been reduced by the exposure of the reaction centre polypeptides during isolation of the complex. However, the direct detection of even some D/Z electron donor in the purified complex provides additional evidence that

Z and D are located on D1 and D2 polypeptides [14–17].

Acknowlegements: We thank the Science and Engineering Research Council and the Agricultural and Food Research Council for financial support. We are especially grateful to Henry Crespi and Marion Thurnauer for providing deuterated algae and for useful comments. We also thank J. De Felice and C.J. Lockett for help with preparative techniques and Professor M.C.W. Evans for helpful discussion.

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