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Oxidation-reduction potential dependence of reaction centre triplet formation in the isolated D1/D2/cytochrome *b*-559 photosystem II complex

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Formation of a spin-polarized triplet on illumination at low temperature is thought to be characteristic of charge separation in photosynthetic reaction centres. The formation of a spin-polarized triplet was confirmed in the D1/D2/cytochrome *b*-559 photosystem II reaction centre preparation. The oxidation-reduction potential range in which triplet formation occurs was determined. Triplet formation was observed at potentials between 400 and −530 mV. Loss of triplet formation, at more negative potentials, is suggested to reflect chemical reduction of the pheophytin acceptor. At potentials more oxidised than 400 mV, illumination results in formation of a radical at $g = 2.0025$ with a 0.75 mT linewidth. We propose that this is the result of oxidation of P680 coupled to reduction of an electron acceptor, possibly non-haem iron. The results confirm that none of the other electron acceptors normally found in photosystem II are functional in this preparation.

EPR; Photosynthesis; Primary charge separation; Reaction center; Triplet formation; Photosystem II; Electron transport; (Pea)

1. INTRODUCTION

The polypeptide composition of the purple bacterial reaction centre has been well defined [1]. Two polypeptide subunits, L and M, bind the functional redox components of the reaction centre. The function of the third subunit, H, is not fully understood but its presence stabilizes the normal electron exchange reactions between the quinone acceptors, Q_A and Q_B [2]. The structure of plant reaction centres has been more difficult to determine, with considerable controversy concerning which polypeptides form the PS II reaction centre [3]. This now seems to have been resolved with the isolation of a complex consisting of the D1 and D2 polypeptides [4,5]. This complex also contains the two apoproteins of cytochrome *b*-559 and binds four molecules of chlorophyll *a*, two molecules of pheophytin and one haem of cytochrome *b*-559 [6]. It contains non-haem iron but does not bind plastoquinone [6]. Identification of this complex as the PS II reaction centre was originally based on amino acid sequence comparisons of the D1 and D2 polypeptides with the L and M subunits of the purple bacterial reaction centre [7,8]. This identification has been supported by the observation of the photoreduction of the pheophytin [4,6] and by the detection, using EPR spectroscopy, of a spin-polarized triplet on illumination at low temperature [9]. The triplet had the same zero-field splitting parameters and other properties observed in the reaction centre triplets

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Abbreviations: Chl, chlorophyll; Cyt, cytochrome; D1, *psb*A gene product; D2, *psb*D gene product; P680, primary electron donor of PS II; Pheo, pheophytin; PS, photosystem; Q_A, primary quinone acceptor of PS II; Q_B, secondary quinone acceptor of PS II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine

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in more intact PS II preparations [10]. Subsequent work has demonstrated the formation of a charge-transfer complex in the picosecond time range with a nanosecond lifetime [11,12] and observation of reaction centre chlorophyll oxidation [6].

The D1/D2/cytochrome *b*-559 complex does not contain the Q_A and Q_B quinone electron acceptors, therefore forward electron flow from pheophytin cannot occur and the charge-transfer complex decays in the nanosecond time range with the formation of the spin-polarized triplet of P680. In less purified PS II preparations triplet formation is observed only at low potentials where all other acceptors are reduced or at high potentials when some of the endogenous electron donors are oxidised [13]. Here, we determine the redox dependence of triplet formation in the D1/D2/cytochrome *b*-559 complex isolated from peas in order to investigate the electron acceptor content of the preparation and the redox potential of the pheophytin acceptor.

2. MATERIALS AND METHODS

The D1/D2/cytochrome *b*-559 complex was isolated by solubilizing PS II-enriched membranes in 4% Triton X-100 followed by anion-exchange chromatography on DEAE-Fractogel, using essentially the same procedures as in [6,14]. The complex eluted from the column in 50 mM Tris (pH 7.2) and 0.2% Triton X-100 at approx. 120 mM NaCl. 10% glycerol was added for storage at 77 K. The purity of the samples was judged from the absorption spectrum, chlorophyll/cytochrome *b*-559 ratio and SDS-polyacrylamide gel electrphoresis patterns, according to established criteria [6]. Samples were freeze-thawed no more than twice. For redox titrations the samples were diluted in 100 mM Tricine-HCl (pH 8.0) or glycine-KOH (pH 10.0) to 60–70 μg Chl/ml. A sample for investigation of the $g = 6$ region of the EPR spectrum was concentrated to 650 μg Chl/ml. This was accomplished by centrifuging samples of approx. 130 μg Chl/ml (plus 0.1 M sucrose) in a Beckman TL100 bench-top ultracentrifuge. 0.5-ml samples were centrifuged at 100000 rpm (460000 × *g*) for 60 min at 2°C. This resulted in a gradient of chlorophyll in the tube and about 70% of the chlorophyll was recovered in a loose pellet.

Redox titrations were carried out as described previously [15] at 5°C. No correction for temperature was applied to the E_m values reported. The following mediators were used in reductive titrations at 20 μM; methyl viologen, benzyl viologen, triquat and tetraquat. Sodium dithionite was used as reductant. In oxidative titrations 20 μM dichlorophenolindophenol and the oxidant, potassium ferricyanide, were the only mediators present. Spectra were recorded under illumination and the height of the low-field peak of the spin-polarized triplet spectrum was taken as an indicator of signal intensity. Because of the difficulty of preparing sufficient material only a small number of titrations could be carried out. The results of all were consistent, but the precision of the mid-point potentials reported is less than in experiments we have reported previously. Four reductive titrations, three at pH 8.0 and one at pH 10.0, and two oxidative titrations were carried out, together with a number of experiments in which samples were oxidised or reduced by addition of appropriate reagents. EPR spectra were recorded using a Jeol FE-1X spectrometer with an Oxford Instruments liquid helium cryostat as in [15].

3. RESULTS AND DISCUSSION

When samples of the preparation, frozen in the dark with sodium ascorbate, were illuminated at liquid helium temperatures the formation of a spin-polarized triplet could be detected by EPR spectrometry (fig.1). The intensity of the signal, relative to that of other PS II preparations [13], reflected the considerable enrichment in reaction centre per unit chlorophyll. The enrichment could not be quantified but the signals were, in fact, several times larger than would have been expected for an equivalent number of reaction centres in PS II-enriched preparations (~200 Chl/reaction centre). This is consistent both with the samples being active reaction centres and their being more efficiently illuminated than in normal PS II-enriched preparations, because of the use of a lower absolute chlorophyll concentration.

The intensity of the spin-polarized triplet signal varied in different preparations. In some, instead of observing the spin-polarized triplet, a $g = 2.0025$ radical was formed irreversibly, following low-temperature illumination (fig.2). This radical was approx. 0.75 mT wide. The *g* value and linewidth are similar to those observed for the bacterial and PS I reaction centre chlorophylls and it seems likely that this signal arises from oxidised P680. Formation of the radical was lost if the sample was reduced by the addition of dithionite before freezing. In contrast, maximum radical formation was observed if the sample was oxidised with potassium ferricyanide before freezing. Low-spin ferric iron signals were seen at $g = 3$ (at 15 K) which were typical of cytochrome *b*-559 [16] and as with the spin-polarized triplet there was a relative enrichment in the size of the signal compared to those seen normally with PS II preparations which contain more chlorophyll per reaction centre. No changes in cytochrome *b*-559 signal were seen on illumination at low temperature. Also, there was no signal II due to the components that have been

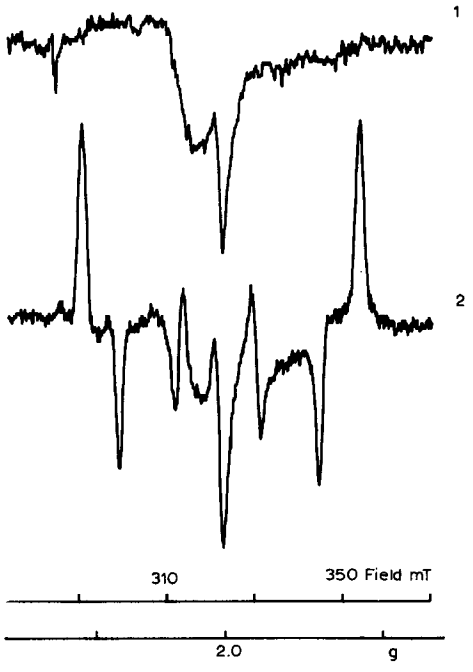

Fig.1. EPR spectrum of the light-induced spin-polarized triplet in the D1/D2/cytochrome *b*-559 preparation. (1) Dark. (2) Light on. The sample containing 60 μg Chl/ml was reduced with 1 mM sodium ascorbate before freezing. EPR conditions: temperature, 4.5 K; frequency, 9.05 GHz; microwave power, 100 μW; modulation amplitude, 1 mT; gain, 2500.

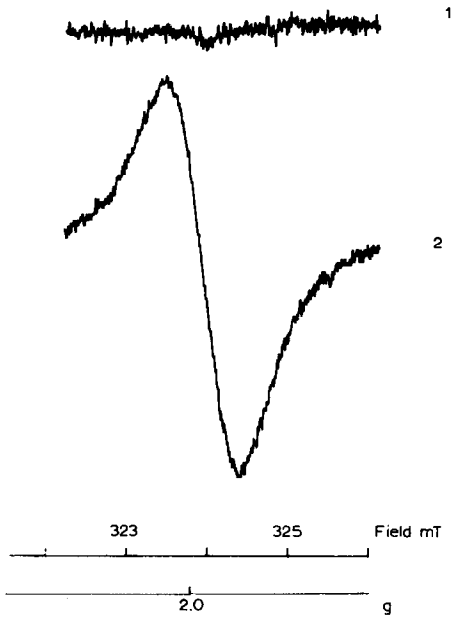

Fig.2. EPR spectrum of the light-induced $g = 2.0025$ signal in the D1/D2/cytochrome *b*-559 preparation. (1) Dark. (2) After illumination. The sample containing 70 μg Chl/ml was poised at 490 mV before freezing. The sample was illuminated at 6 K in the cavity of the EPR spectrometer. The spectrum was recorded with the light off. EPR conditions: temperature, 6 K; frequency, 9.05 GHz; microwave power, 100 μW; modulation amplitude, 0.2 mT; gain, 2500.

designated Z, the electron donor to $P680^+$, and D. The variations in radical and triplet formation suggest that the isolated D1/D2/cytochrome *b*-559 complex may contain an electron acceptor other than pheophytin which is functional at liquid helium temperatures.

We have carried out redox titrations to determine the potential at which oxidation or reduction of the sample results in the loss of triplet formation. In crude PS II preparations triplet formation, seen below -400 mV, is lost at -620 mV [13,17]. This is believed to correspond to reduction of the pheophytin. In the present experiments the extent of triplet formation decreased at approx. -530 mV (fig.3a). Although this potential is 90 mV more oxidised than that seen in more intact preparations it seems likely that it also reflects reduction of the pheophytin. The difference in the observed potential probably reflects the accessibility of the pheophytin to the environment rather than a real difference in potential. The intensity of the triplet signal was constant over a wide range of potentials and there was no loss of triplet formation between -500 and 400 mV. This result suggests that the electron acceptor which were found to function between 0 and -450 mV in the more intact systems [18,19] are lost from this preparation. This is in agreement with analysis showing that no quinones are present [6]. Triplet formation is lost at approx. 450 mV and radical formation appears in parallel with the loss of triplet formation. As discussed above, the radical has properties which suggest that it is $P680^+$. In more intact preparations illumination of oxidised samples results in the appearance of radicals 1.1–1.4 mT wide. These are now usually attributed to secondary donors, damaged Z, carotenoid, or adventitious chlorophyll. It is clear that these are not available in this preparation. It therefore seems likely that the loss of triplet formation at 450 mV reflects the oxidation of a component which can then accept an electron from the pheophytin,

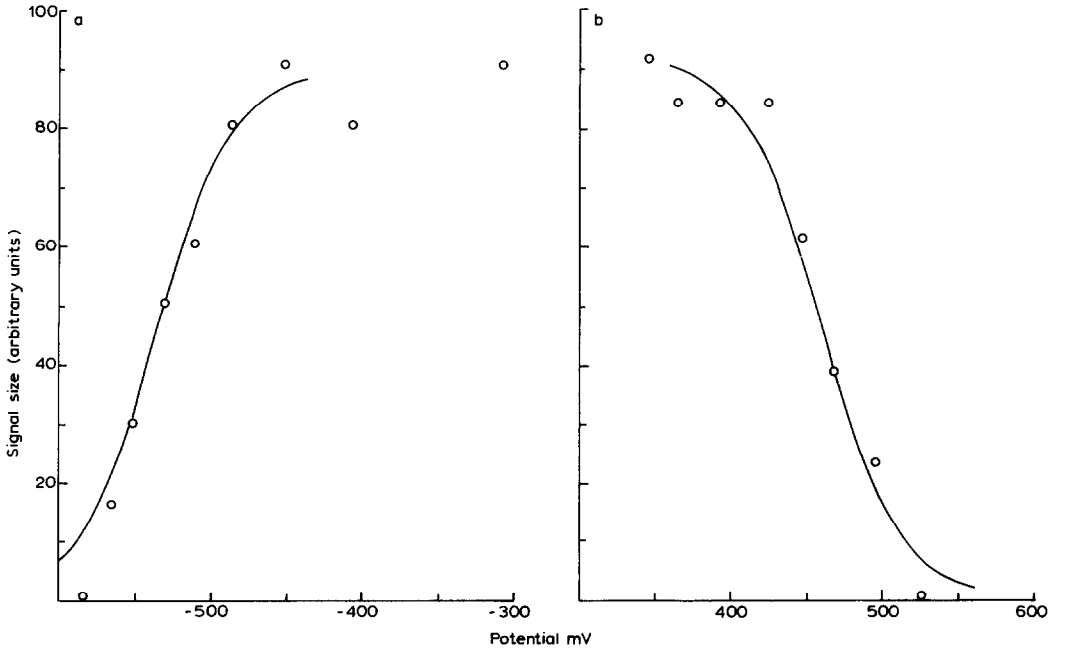

Fig.3. Oxidation-reduction potential titrations of the spin-polarized triplet EPR signal intensity in the D1/D2/cytochrome *b*-559 preparation. (a) Low-potential range. (b) High-potential range. The curves drawn are theoretical curves for a one-electron transition at (a) −535 mV and (b) 460 mV.

preventing the back reaction between reduced $Pheo^-$ and $P680^+$.

The only component which has been shown to have a potential in this range and act as a PS II electron acceptor is the non-haem iron associated with the quinone-binding region [19]. This has EPR signals in the $g = 6–8$ region but the samples used in the redox titrations were too dilute for observation of the signals. We have therefore made a sample with 650 μg Chl/ml. This had signals around $g = 6$ under oxidising conditions (fig.4) which were different from those seen in more intact preparations and we were unable to observe any light-induced change in the signal. Nevertheless, these spectra suggest that the iron is still associated with the preparation but in a modified environment. Although we have not yet been able to demonstrate directly photoreduction of the non-haem iron, we think it is the most likely candidate for the acceptor in our experiments. Diner and Petrouleas [20] proposed that the iron can accept electrons from Q_A, and that it may function as a carrier between Q_A and Q_B. The redox potential of the iron and the analogy with the bacterial system suggest that this is unlikely. Our present experiments indicate that in the absence of the quinone acceptors the iron may be reduced by the pheophytin, but we suggest that its

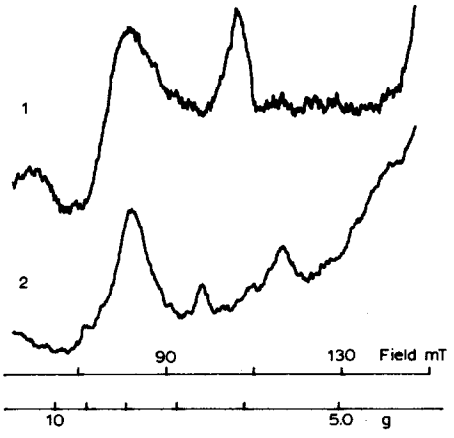

Fig.4. EPR spectra of the $g = 6$ region of (1) the D1/D2/cytochrome *b*-559 preparation, 650 μg Chl/ml. (2) Oxygen-evolving vesicle preparation, isolated by the method of Ford and Evans [21], 4 mg Chl/ml. Both samples were oxidised with 5 mM potassium ferricyanide. EPR conditions: temperature, 4.2 K; frequency, 9.05 GHz; microwave power, 5 mW; modulation amplitude, 1 mT.

function, as in bacteria, is unlikely to be directly involved in electron transfer.

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