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Identification of EPR signals from the states $Q_A^{\cdot-}Q_B^{\cdot-}$ and $Q_B^{\cdot-}$ in Photosystem II from *Phormidium laminosum*

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We have studied the EPR signal in PS II from *Phormidium laminosum* with a *g*-value of 1.66, which we assign to an interaction between the semiquinones of Q_a and Q_b and the non-heme iron. 77 K illumination of samples from dark-poised redox titrations show the rise of the signal has a midpoint potential (E_m) of about +60 mV, and it is lost with an E_m of about −10 mV. Under the same conditions, the rise of the $g = 1.9$ signal from $Q_a^{\cdot-}$-Fe^{2+} in the dark was found to be about +10-mV. The $g = 1.66$ signal can also be formed with a high yield by first illuminating dark-adapted PS II particles at 293 K, followed by a short period of darkness at 273 K and subsequent illumination at 77 K. We have measured the effect on signal yield of varying the period of darkness following 293 K illumination. Over 60% of the maximum signal size is seen after 1 min darkness, and increases further over 2 h. In these samples a signal attributed to $Q_b^{\cdot-}$-Fe^{2+} is seen prior to 77 K illumination. Confirmation of the presence of $Q_b^{\cdot-}$ was obtained by reductant-linked oxidation of the non-haem iron using phenyl-*para*-benzoquinone (PPBQ). Samples treated with the Q_b-analogue tribromotoluquinone (TBTQ) give a modified EPR signal. We propose (i) that Q_b is preserved in PS II preparations from *P. laminosum*; (ii) that Q_b-semiquinone can be readily formed and trapped by freezing; and (iii) the $g = 1.66$ signal arises from a coupling between the primary and secondary plastosemiquinones and the non-haem iron.

Introduction

Photosystem II (PS II) is a multi-subunit complex located in the thylakoid membranes of plants, algae and cyanobacteria. It catalyses light-driven electron transfer from water to plastoquinone. The electron acceptor complex of PS II is composed of a number of cofactors bound to the reaction centre, to which electrons are transferred from the excited state of the special pair chlorophyll, denoted P680*. The immediate acceptor is a pheophytin (I), which is reduced in the picosecond domain by an electron from P680* [1]. I^- rapidly transfers an electron to the first 'stable' acceptor, Q_a, which is reoxidised by the second stable acceptor, Q_b. Q_a and Q_b in PS II are both plastoquinone molecules; however, except in special conditions, Q_a will act only as a single-electron carrier, whereas Q_b undergoes double-reduction and protonation. There is a ferrous iron atom (Fe^{2+}), the function of which is unclear, but which interacts magnetically with the semiquinone forms of Q_a and Q_b. In PS II preparations from a cyanobacterium, *Phormidium laminosum*, a component with a midpoint potential of −250 mV that is not a quinone has also been detected [2,3].

In PS II, several EPR signals arise from the interaction between the primary semiquinone and the non-haem iron ($Q_a^{\cdot-}$-Fe^{2+}). A signal at $g = 1.8$ due to Q_a-semiquinone [4,5] can be detected when bicarbonate has been displaced from its ligation site at or close to the non-heme iron; for example, following displacement of HCO_3^- by formate, or by incubation at pH < 6. This signal is similar to those seen in purple bacteria due to $Q_a^{\cdot-}$-Fe^{2+} and $Q_b^{\cdot-}$-Fe^{2+} [6–8]. A second signal at $g = 1.9$ [9] is observed when bicarbonate is bound at, or close to, the non-haem iron. In PS II from *P. laminosum* we have seen both $g = 1.8$ and $g = 1.9$ signals, and determined their redox potentials at about +25 mV

Abbreviations: TBTQ, tribromotoluquinone; PPBQ, phenyl-*para*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,6-dimethylbenzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EPR, electron paramagnetic resonance; DMSO, dimethyl sulphoxide; PS II, Photosystem II; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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[10–12]. A third EPR signal with $g \approx 1.6$ has also been reported from two cyanobacterial PS II preparations, *Synechococcus* sp. [13] and *P. laminosum* [11].

Similar properties were reported for the $g = 1.6$ signals from the two sources: both were formed by low-temperature photoreduction of Q_a, and treatment with either formate or DCMU prior to illumination suppressed generation of the signal. It was shown in Ref. 13 that illumination above 180 K led not to formation of the $g = 1.6$ signal, but to a mixture of the $g = 1.8$ and $g = 1.9$ signals assigned to $Q_a^{\cdot-}$-Fe^{2+}. This was taken as evidence that the $g = 1.6$ arose from a conformationally excited form of the $Q_a^{\cdot-}$-Fe^{2+} interaction that could not relax at low temperature. We had found [11] that treatments assumed to affect Q_b-semiquinone (DCMU, CCCP and DMBQ) also affected the yield of the $g = 1.6$ signal, and therefore proposed that 77 K photoreduction of Q_a would show a $g = 1.6$ signal only if Q_b-semiquinone was bound at its site.

A $g = 1.8$ EPR signal attributed to $Q_b^{\cdot-}$-Fe^{2+} has been observed in purple bacteria [8]. This signal was titrated and an E_{m8} of +70 mV given to the couple Q_b/Q_b^- in *Rps. viridis* [14]. A signal similar to the $g = 1.9$ arising from the $Q_a^{\cdot-}$-Fe^{2+} has been tentatively assigned to this interaction in PS II [15].

Petrouleas and Diner [16] and Zimmerman and Rutherford [17] have shown that the non-haem Fe^{2+} can be oxidised by analogues of Q_b whose semiquinones have a sufficiently positive redox potential. The EPR signal of the non-haem Fe^{3+} has peaks at $g = 8.1$ and $g = 5.5$ [18] in centres with an empty Q_b site [19]. The peaks are removed following illumination in the range 5–200 K, indicating photoreduction of the iron.

In this paper, we show further evidence that the $g = 1.6$ arises through an interaction of the two semiquinones and the iron. We have investigated the redox properties and the dark-stability of this signal in native preparations; and using tribromotoluquinone (TBTQ), an artificial Q_b analogue [20–22], we have shown induction of high yields of this EPR signal. We also show that exchange of the native Q_b-quinone by phenyl-*para*-benzoquinone (PPBQ) leads to the oxidation of the non-haem iron.

Materials and Methods

P. laminosum was grown at 45°C, in medium D of Castenholtz [23] in 10-litre glass jars and harvested in late log phase. During growth each jar was gassed with 5% CO_2/95% air, and illuminated with a 150 W tungsten filament spot lamp. PS II particles were prepared using a method based on that of Ref. 24. Detergent fractionation of thylakoid fragments at 1 mg Chl ml^{-1}, was performed with 0.5% (w/v) *N*-dodecyl-*N*,*N*-dimethylammonio-3-propanesulphonate (Serva, Heidelberg) for 40 min at 4°C [25]. The sample was centrifuged at $100000 \times g$ for 60 min, and the supernatant containing PS II collected. PS II was concentrated by precipitation with 10% (w/v) poly(ethylene glycol) 6000, and centrifuging at $100000 \times g$ for 20 min. The pellet was resuspended and washed once in 10 mM Hepes, 10 mM $MgCl_2$, 5mM Na_2HPO_4, 25% (v/v) glycerol at pH 7.5 (buffer C); once in buffer C with 10 mM EDTA, and finally again in buffer C before freezing to 77 K. Particles prepared in this way typically evolve oxygen at rates between 1500–2000 μM O_2/mg Chl per h.

Redox titrations for EPR analysis were performed essentially as in Ref. 12. Thionine, methylene blue, Janus Green and indigo tetrasulphonate, all at 20 μM, were used as redox mediators. Samples were frozen in the dark to 77 K. EPR spectra were recorded using a JEOL Fe1X spectrometer with an Oxford Instruments ESR9 cryostat. EPR conditions are given in figure legends.

Samples were illuminated at 77 K in a silvered dewar using a 650 W light source for 10 min. Illumination of identical samples for either 5 min or 10 min at 77 K gave identical signal yields, indicating that the samples are light-saturated. Illumination at room temperature was done in an unsilvered dewar using the same light source for 1 min from a distance of 1 m.

Additions were made in the dark of a 25 mM solution of TBTQ (kindly given to us by Prof. W. Oettmeier) or PPBQ in dimethyl sulphoxide (DMSO) to a final concentration of 625 μM. It should be noted that DMSO is present at a concentration of 2.5% (v/v).

Results

Fig. 1 shows EPR spectra of the iron-quinone complex in PS II isolated from *Phormidium laminosum*. A $g = 1.9$ signal is obtained by poising a sample in the dark at −80 mV, Fig. 1a. Fig. 1b shows the result of illuminating this sample at 77 K. A sharp signal with *g*-value of approx. 1.97 can be seen, but there is no other major difference between this spectrum and 1a. Fig. 1c shows a sample poised at +50 mV in darkness. A small $g = 1.9$ signal can be seen at this potential. Illumination of the sample from Fig. 1c at 77 K generates a prominent signal with a *g*-value of 1.66, and an approximate peak-to-trough width of 22 mT Fig. 1d. There is an increase in the size of the $g = 1.9$ signal following low-temperature illumination, and also the formation of the same sharp $g = 1.97$ point seen in Fig. 1b.

We have measured the redox properties of the component giving rise to the $g = 1.66$ signal, by 77 K illumination of samples poised at different potentials in the dark, Fig. 2. It was necessary to take great care to exclude light from these samples, and to allow at least 2 h prior dark-adaption at +350 mV for satisfactory titrations to be obtained from which a value for E_m

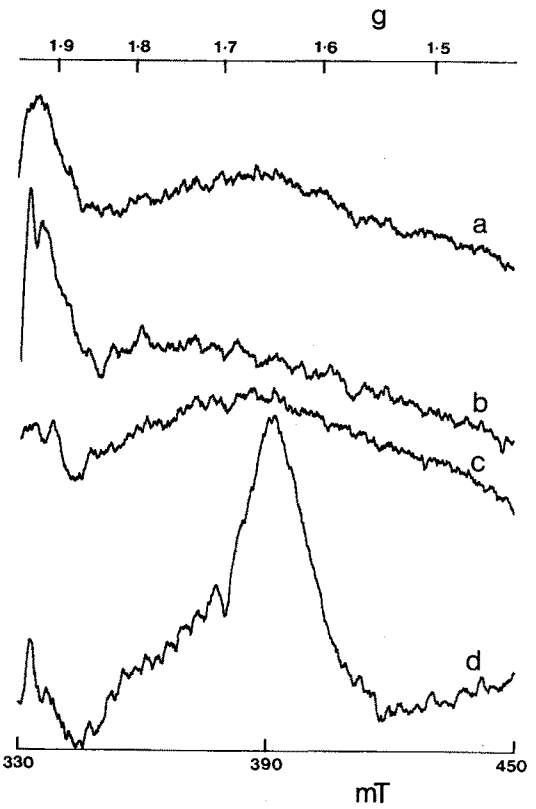

Fig. 1. EPR spectra of the semiquinone-iron region in *P. laminosum* PS II. (a) Sample poised in dark at −80 mV, (b) as (a) following 77 K illumination. Sample poised in dark at +50 mV (c) and (d) as (c) following 77 K illumination. Chlorophyll concentration all 1 mg ml^{-1}. EPR conditions: temperature, 5 K; microwave power, 10 mW; modulation width, 1.25 mT. Spectra shown are averages of three scans.

could be calculated. In samples which had been exposed to light, the $g = 1.66$ signal could be 77-K-photoinduced at high potentials.

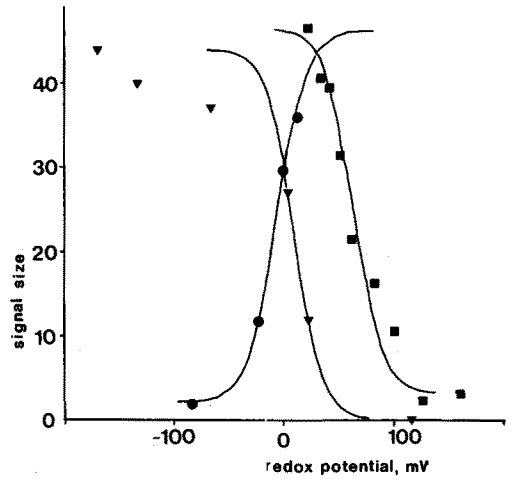

Fig. 2. Reducing redox titration showing variations in intensities of EPR signals from semiquinone-iron components. Squares (■), rise of $g = 1.66$ signal; circles (●), loss of $g = 1.66$ signal; triangles (▼), rise of $g = 1.9$ signal in dark-poised samples. $g = 1.66$ signals were generated by illumination at 77 K. EPR conditions, as Fig. 1.

Titrations performed in the reducing direction show the $g = 1.66$ signal to rise in a single step with a midpoint potential, at pH 8.0 (E_{m8}), of about +60 mV. As the titration proceeds, the signal then decreases in size with a midpoint of about −10 mV. In oxidising titrations, beginning at −100 mV, the signal shows similar behaviour; a rise in the light-induced $g = 1.66$ form is followed by a decrease at higher potentials.

Comparison of the decrease of intensity of the $g = 1.66$ signal formed after 77 K illumination, with the intensity of the $g = 1.9$ signal titrated in the dark is also shown in Fig. 2. The Nernst curve fitted to this titration of the $g = 1.9$ signal has a midpoint at +10 mV, i.e., more negative than the figure we cited in our earlier reference but within the normal error (±15 mV) for this type of titration. The rise of the $g = 1.66$ signal at +60 mV is accompanied by a decrease in the size of a 77 K photoinduced signal at $g = 1.9$. The loss of the $g = 1.66$ signal accompanies the rise of the $g = 1.9$ dark signal at the lower potential. This is considered further in the discussion.

The observation that the $g = 1.66$ signal could in some cases be light-induced at potentials above +100 mV was investigated. Samples were dark-adapted at +350 mV for 4 h to allow equilibration of redox components. Samples were then either frozen in darkness (referred to as 'dark-adapted') or given 1 min saturating illumination at 293 K. The illuminated samples were then returned to darkness, on ice, for varying intervals before freezing to 77 K (referred to as 'relaxed'). A final set of samples were made that were frozen under continuous illumination without an interval of darkness prior to freezing (referred to as 'frozen under illumination'). All of the samples were further illuminated at 77 K for 10 min to cause a single-electron turnover of PS II, to produce Q_a^-. EPR spectra were taken from the samples both before and after this low-temperature illumination.

Spectra from dark-adapted samples show no distinct $g = 1.9$ signal prior to 77 K illumination, indicating little or no reduced Q_a; and after 77 K illumination, they show no large yield of the $g = 1.66$ signal. In samples frozen under illumination, there was observed, prior to 10 min 77 K illumination, a large, broad signal at $g = 1.9$, characteristic of a sample containing Q_a^--Fe^{2+}. 77 K illumination of these samples yields no $g = 1.66$ signal as no further stable electron transfer to the quinone electron acceptors can occur, analogous to the chemically reduced samples seen in the titrations. However, a high yield of the $g = 1.66$ signal was obtained in those samples illuminated at 293 K and allowed a period of darkness prior to freezing. The time-course for this effect, Fig. 3, shows that approx. 60% maximal intensity of the $g = 1.66$ signal is obtained after 1 min, followed by a slow rise in the intensity over the 135 min course. This indicates the involvement of

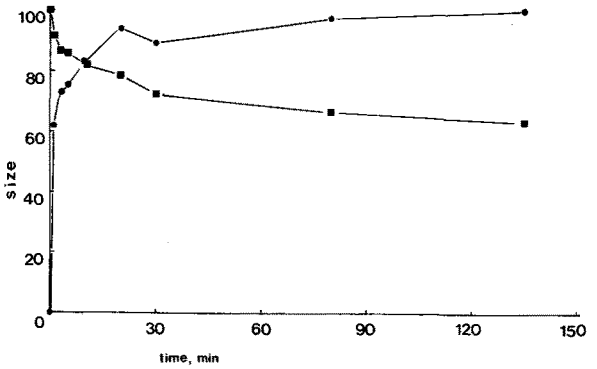

Fig. 3. Variations in the intensities of EPR signals detected following room temperature illumination and dark relaxation. Circles (●), $g = 1.66$ generated by 77 K illumination of relaxed samples; squares (■), signal II_d, measured in the dark.

component(s) additional to $Q_a^{\cdot-}$-Fe^{2+} in formation of the $g = 1.66$ signal.

In the same experiment, the pattern of oxidation of electron donors was monitored, in particular the behaviour of cytochrome b-559 and the tyrosine residue, D (also known as the EPR Signal II_{slow}). This was necessary to determine any possible relation between the intensity of the $g = 1.66$ signal seen after 77 K illumination with availability of functional electron donors at cryogenic temperatures.

It was found that in a dark-adapted sample D^+ was present at 60% of the maximal level which could be induced by freezing under illumination. Following 293 K illumination, the level of D^+ is thus initially maximal, and relaxes to the 'dark' level over the 135 min course of the experiment, reflecting the re-reduction of this component, Fig. 3. Therefore the amounts of D in a dark-adapted sample and in the 135 min relaxed sample prior to 77 K illumination are almost equal. However, it is observed that the yield of $g = 1.66$ signal following 77 K illumination is maximal in the 135 min-relaxed sample, and only 15% in the dark-adapted sample.

Following 77 K illumination of PS II, it was seen that the oxidation of D approached maximal levels in all samples. The photooxidation of D at 77 K or below is unusual in dark adapted samples of isolated PS II, but has previously been noted in chloroplasts [26]. It should be emphasised that this represents a different degree of photooxidation of D in each sample, since over the course of the dark relaxation following 293 K illumination there is a time-dependent decrease of D^+, and consequently a different amount available for re-oxidation at 77 K. In the dark-adapted sample, where oxidation of D at 77 K was greatest, there was no corresponding large increase of the $g = 1.66$ signal. From this, it is concluded that there is no simple relation between the size of the $g = 1.66$ signal and the presence of reduced D.

The EPR spectra of cytochromes at $g = 3.05$ revealed no differences between samples dark-adapted, frozen under illumination or relaxed (data not shown).

In PS II from *P. laminosum* we propose that $Q_b^{\cdot-}$ is present in the dark following the 293 K illumination/dark adaption protocol described earlier in the paper, and we have therefore investigated the effect of adding the high-potential quinone PPBQ on the EPR spectra of both the semiquinone and non-haem iron species. The results are shown in Figs. 4 and 5.

In a 20 min relaxed sample with no additions, there are no features near $g = 6$ attributable to the oxidised non-haem iron, Fig. 4a. This result indicates that native $Q_b^{\cdot-}$, which is present in this relaxed sample, is not itself capable of oxidising the non-haem iron. However, addition in the dark of 625 μM PPBQ to a 20 min relaxed sample results in the oxidation of the non-haem iron, giving the spectrum shown in Fig. 4b, with signals at $g = 8.7$, $g = 6.9$ and $g = 5.1$. The central peak at $g = 6.9$ has been attributed to the ferric non-haem iron where centres bind oxidised quinone at the Q_b-site [19]. By comparison of the $g = 8.7$ peak heights, we have estimated that these signals represent approx. 50% of the ferricyanide-oxidisable non-haem iron. This result confirms the presence of $Q_b^{\cdot-}$ in the relaxed sample; we

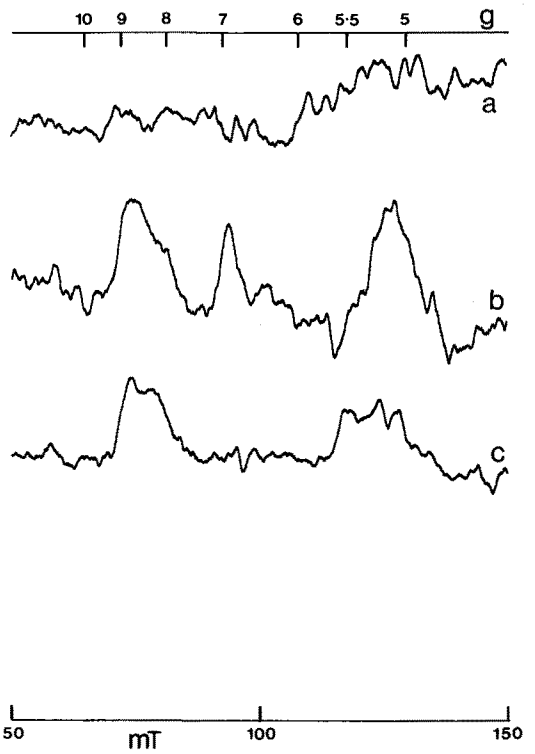

Fig. 4. EPR spectra of the $g = 6$ non-haem iron region of the spectrum following room temperature illumination and dark relaxation. Chlorophyll concentration: 2 mg ml^{-1}. (a) 77 K illuminated-minus-dark of relaxed sample, with no additions. (b) difference spectrum showing effect of addition of 625 μM PPBQ to a relaxed sample. (c) dark-minus-77-K-illuminated difference spectrum of the PPBQ-treated sample. EPR conditions: temperature, 4.5 K; microwave power, 5 mW; modulation width, 1.25 mT.

envisage an exchange at the Q_b-site of the native $Q_b^{\cdot-}$ for $PPBQ^{\cdot-}$, which is then able to oxidise the non-haem iron. Illumination of this sample at 77 K photoreduces the iron, giving rise to the observed dark-minus-illuminated difference spectrum shown in Fig. 4c. The loss of only the peaks at $g = 8.7$ and $g = 5.1$ contradicts the assignment of the $g = 6.9$ peak to the oxidised non-haem iron and will be investigated further. This peak also does not fit easily with the parameters used for simulation of the $g = 8.1$ and $g = 5.5$ resonances [19].

In Fig. 5 are shown changes to the spectra arising from semiquinone-iron components, occurring in parallel with those from the non-haem iron shown in Fig. 4. Fig. 5a shows the prominent signal at $g = 1.66$ obtained after 77 K illumination of a 20-min relaxed sample. There are no changes in either the $g = 1.9$ or $g = 1.8$ regions, suggesting that following low-temperature illumination almost all centres are in the state $Q_a^{-}Q_b^{\cdot-}$. Therefore, prior to 77 K illumination it may be possible to see a signal arising from $Q_b^{\cdot-}$-Fe^{2+} alone. A weak, broad signal, close to $g = 1.7$, was observed, which was removed by incubation with PPBQ, Fig. 5b and c. The spectrum shown in Fig. 5b is the difference between the 20-min-relaxed sample and the dark, PPBQ-treated

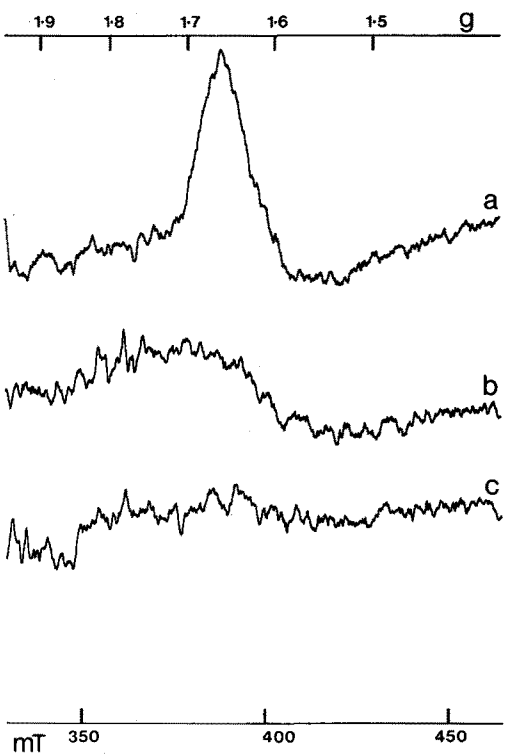

Fig. 5. Spectra arising from iron-semiquinone components occurring in parallel with those in Fig. 4. (a) 77 K illumination-minus-dark difference spectrum of a relaxed sample. (b) difference spectrum showing the effect of addition of 625 μM PPBQ to a relaxed sample. (c) dark-minus-77 K-illuminated difference spectrum of the PPBQ-treated sample. Chlorophyll concentration: 2 mg ml^{-1}. EPR conditions as in Fig 1.

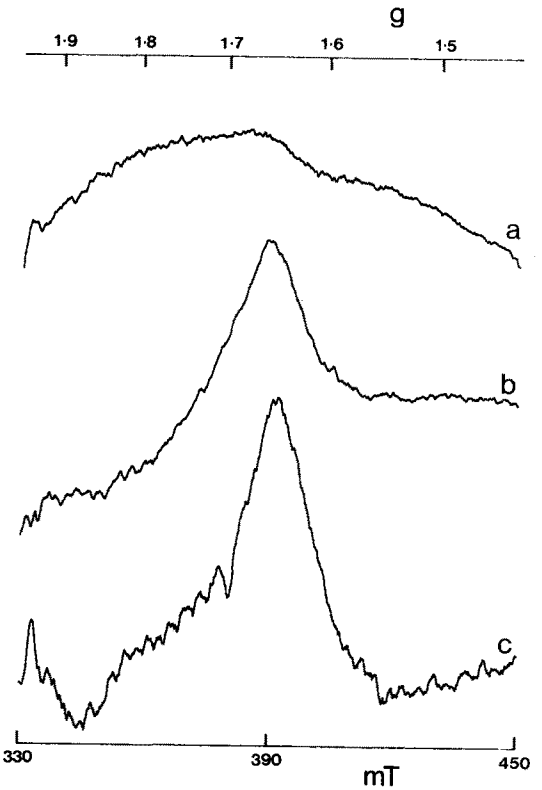

Fig. 6. EPR spectra of *P. laminosum* PS II treated with 625 μM TBTQ. (a) TBTQ-treated sample in darkness; (b), as (a) following 77 K illumination; (c) shows the $g = 1.66$ signal generated in native sample poised at +50 mV. Chlorophyll concentration: 1 mg ml^{-1}. EPR conditions as in Fig. 1.

sample. This broad signal is different from the signal at $g = 1.9$ previously ascribed to $Q_b^{\cdot-}$-Fe^{2+} [15]. Finally, 77 K illumination of the sample treated with PPBQ does not give rise to changes in the EPR spectrum in the iron-semiquinone region, consistent with a removal of $Q_b^{\cdot-}$ and presence of non-haem Fe^{3+} as the electron acceptor.

The effect of 77 K illumination of a sample treated with 625 μM TBTQ is shown in Fig. 6. Fig. 6a shows the spectrum from a sample dark-adapted with TBTQ. The broad resonance near $g = 1.7$, which we attribute to $Q_b^{\cdot-}$-Fe^{2+}, was present. Following 77 K illumination we observe a signal similar to the $g = 1.66$ signal seen in native samples, except that the peak occurs at $g = 1.67$, and that the peak-to-trough width of 17 mT is narrower.

The induction of the $g = 1.67$ after dark-adaption and following 293 K illumination is different from that in untreated samples. In particular, a greater fraction of $g = 1.67$ signal (80%) was seen to be induced following 77 K illumination of the dark-adapted samples, compared with those that had been illuminated at 293 K; whereas in untreated samples this fraction is approx. 15%. Freezing under illumination again gives essentially no yield of $g = 1.67$ signal. The addition of TBTQ almost completely abolishes the dark-stable Signal II

(D^+) in these samples, as reported previously in Ref. 22. Instead, we see an asymmetrical, 1.975 mT wide signal with $g = 2.008$ (not shown), that presumably arises from TBTQ-semiquinone. Following 77 K illumination there is a reoxidation of EPR Signal $II_{(slow)}$.

Discussion

Titration of the $g = 1.66$ signal in native samples shows a component which in reducing titrations rises with E_{m8} of about +60 mV. Accepting our earlier proposal that this signal mirrors the presence of $Q_b^{\cdot-}$ at its site, this figure would represent the first direct determination of a value for the redox couple $Q_b/Q_b^{\cdot-}$ in PS II. An assumption we have made is that the paramagnetic species, $Q_b^{\cdot-}$, will have a greater effect on the spectrum of $Q_a^{\cdot-}$-Fe^{2+} than the uncharged quinone and quinol forms. In the purple bacterial reaction centre, the effect of this interaction between Qb-semiquinone and $Q_a^{\cdot-}$-Fe^{2+} is to attenuate both signals [8]. However, it is proposed here that this state in PS II, if induced at low temperatures, has a substantially altered EPR spectrum. There is no reason to suppose that this interaction should be identical in the two analogous systems, since (1) no $g = 1.9$ signal is observed in the purple bacterial systems concerned and (2) no $Q_b^{\cdot-}$-Fe^{2+} interaction giving a purple bacterial-type $g = 1.8$ signal has been observed in untreated PS II, although a signal attributed to $Q_b^{\cdot-}$-Fe^{2+} has been observed [27]. Bicarbonate exerts a major influence on the lineshape of the $Q_a^{\cdot-}$-Fe^{2+} signal [28], and is also required for generation of the $g = 1.66$ signal, as previously shown by its absence in samples treated with formate [11,13]. The results when TBTQ binds lend more support to this explanation, as discussed below.

The pattern of the redox titration curve of the $g = 1.66$ signal compared with that of the $g = 1.9$ signal supports the dependency on $Q_b^{\cdot-}$-Fe^{2+}. It is important that the $g = 1.66$ signal cannot ordinarily be generated at potentials in the region of +350 mV. If the $g = 1.66$ signal is dependent on an involvement of the fully-oxidised Q_b-quinone, or if it is independent of Q_b altogether, we might expect to see the $g = 1.66$ signal induced at this potential following 77 K-photoreduction of Q_a. However, we do not see the $g = 1.66$ signal generated under these conditions. Therefore, a situation is envisaged at these higher potentials where Q_b is either bound in its fully-oxidised quinone form or not bound at all, and where photoreduction of Q_a at 77 K gives rise to the $g = 1.9$ instead. Progressive reduction of Q_b to Q_b-semiquinone is then reflected by generation of the $g = 1.66$ signal after low-temperature illumination.

The loss of ability to generate the signal with E_{m8} of −10 mV can be explained in one of two ways. (1) As Q_a is chemically reduced, which can be seen by measuring the rise of the $g = 1.9$ signal in the dark, further photoreduction is not possible. Because the $g = 1.66$ signal can only be generated by low temperature photoreduction it is not seen. (2) Double reduction of Q_b might also be expected to lead to a loss of the signal. It is reasonable to suppose that the midpoint potential of the $Q_a/Q_a^{\cdot-}$ couple should be more negative than those of both the $Q_b/Q_b^{\cdot-}$ and $Q_b^{\cdot-}/Q_b^{2-}$ couples, and consequently Q_a should be reduced after the second reduction of Q_b. However, the closeness of the redox potentials means that in titrations there will be substantial mixing of populations. Support for this would come from an observation of the loss of the signal separate from the rise in the dark $g = 1.9$ signal, but our present results do not allow us completely to distinguish the two. The loss of the signal due to second reduction might show a pH-dependence, but experiments to show this have proved inconclusive.

In calculating these midpoint values of +60 mV and −10 mV, another assumption we have made is that the maximum signal size seen in the experiment is also the true maximum. If this is not so, then the true values will differ from those measured: the value +60 mV would be changed to a less positive value. This does not affect the interpretation of the results, which depends more on the relative behaviour of the different redox curves than on the absolute values of their midpoint potentials.

The dependence of the $g = 1.66$ signal in samples poised at +350 mV on a pre-illumination at room temperature, followed by the illumination at 77 K is clearly inconsistent with its being due solely to Q_a Illumination of a dark-adapted sample at 77 K does not by itself lead to maximum formation of the signal, even though under these conditions Q_a is fully oxidised. Only 15% of the maximum $g = 1.66$ signal is generated if the sample is not pre-illuminated at room temperature. The explanation for this observation is that saturating illumination at room temperature will tend to reduce the electron acceptors. Therefore, no $g = 1.66$ is generated when samples frozen-under-illumination are illuminated at 77 K. However, after 1 min relaxation in the dark to allow decay of $Q_a^{\cdot-}$, a large yield of the $g = 1.66$ signal can be induced.

There is previous evidence that TBTQ binds to a site at or near the Q_b-binding site of PS II [23]. One of the suggested modes of action was that the compound binds in the form of a stable semiquinone. The observation that on 77 K illumination a $g = 1.67$ signal, similar to the native $g = 1.66$ signal, can be seen is extremely significant from the point of view of identifying the $g = 1.66$ signal as the interaction between the two semiquinones coupled to the iron.

The observed oxidation of the non-haem iron by exchange of the native Q_b-semiquinone with the more oxidising semiquinone of PPBQ is further evidence for

the presence of $Q_b^{\cdot-}$ in these particles following the illumination regime described.

In summary, we propose that generation of the $g = 1.66$ signal requires the following conditions: (i) oxidised Qa bound at its site; (ii) an illumination temperature that allows only single-electron transfer to Q_a; (iii) a need for Q_b-semiquinone to be bound at its site. We conclude that the $g = 1.66$ signal then arises from an interaction between $Q_a^{\cdot-}$, $Q_b^{\cdot-}$ and Fe^{2+}, when the primary quinone is photoreduced at low temperature. Titration of this signal gives a midpoint potential for the couple $Q_b/Q_b^{\cdot-}$ of +60 mV. The results may also suggest that the $Q_b^{\cdot-}/Q_b^{2-}$ couple has a midpoint potential of around 0 mV, although the present results do not conclusively show this. Future work will aim to clarify this by means of pH titrations of the signal.

We have also detected a signal in the $g = 1.6$ region in the EPR spectrum from higher plant PS II, also arising from the interaction of Q_a and Q_b semiquinones. This will be the subject of a future publication (Bowden et al., unpublished data).

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