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Properties of the Photosystem II electron acceptor complex of *Phormidium laminosum*

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Absorbance changes and EPR signals from Photosystem II preparations of the cyanobacterium *Phormidium laminosum* were measured over a range of redox potentials to investigate the properties of the electron acceptor components. Previous titrations have only been reported for the iron-semiquinone in the presence of formate at $g = 1.8$. We have now titrated both the native $g = 1.9$ and the $g = 1.8$ form. In contrast to spinach, redox titrations of the iron-semiquinone in *P. laminosum* show only one step at potentials close to Q_h, providing further evidence that Q_h is equivalent to Q_a. The redox potential of Q_a is largely unaffected by the absence of bicarbonate. A component that is not an iron-semiquinone and with a redox potential similar to Q_l was detected by EPR and in optical studies. Redox titrations of the split radical signal caused by the interaction of reduced pheophytin (I^-) with the iron-semiquinone showed a low potential step and the nature of this is discussed. Optical experiments also indicated the presence of a component with midpoint potential −400 mV which has previously been reported in spinach.

Introduction

The electron acceptor chain of Photosystem II contains a pheophytin (I) which functions as a short-lived intermediary electron acceptor. It is generally accepted that there are also two iron-quinones (plastoquinone), Q_a and Q_b. Q_a is tightly bound to the reaction centre and is reduced directly by I. Q_b, the secondary quinone acceptor, is also tightly bound when in the semiquinone form [1].

The midpoint potentials of these electron acceptors are not simple to determine. Various methods have yielded a range of values for Q_a. Titration of the fluorescence yield resulted in a two-step curve for reduction of Q_a with a high potential component near 0 mV (Q_h) and a low potential component (Q_l) with E_m near −300 mV [2,3]. Redox titrations of C_{550} [4], low temperature photooxidation of cytochrome b_{559} [5] and of the electrochromic carotenoid bandshift at 518 nm [6] indicate that Q_h is equivalent to Q_a. The nature of Q_l is unknown.

The redox state of components of the Photosystem II electron acceptor complex can be determined directly or indirectly by measurements of the EPR signals arising from the components. The iron-semiquinone, Q_a, can be detected as a signal at $g = 1.9$ in native samples in which bicarbonate is bound, or following treatment to replace bicarbonate by formate, at $g = 1.8$. The $g = 1.8$ signal is similar to that seen in purple bacteria, where there is no bicarbonate binding to the reaction centre. Limited electron transfer through the reaction centre can be induced by illumination at 200 K. If Q_a is already reduced, this procedure results in reduction of the pheophytin, which can be observed either as an increase in a symmetrical 14 G wide radical at g = 2.00, or more characteristically if measurements are made below 10 K, as a split radical reflecting magnetic interaction with the iron-semiquinone. A spin-polarised triplet can be detected on illumination at very low temperature (4–6 K). In *Rhodopseudomonas sphaeroides* this is observed as soon as Q_a is reduced [7]. In higher plant preparations it was only observed following reduction of a component with an E_m of about −420 mV in oxygen-evolving preparations [8]. However, in reaction centres lacking quinone acceptors it was observed at all potentials between +400 and −500 mV [9]. Two steps

Abbreviations: EPR, electron paramagnetic resonance; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulfonic acid; P-680, reaction centre chlorophyll of Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; I, pheophytin; Chl, chlorophyll.

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in reduction of the EPR signal attributed to the iron-semiquinone have also been described in Photosystem II samples that had been formate-washed to remove HCO_3^-, attributed to Q_h and Q_l [10]. Neither of these components appeared to be Q_b. An additional component with midpoint potential around −420 mV was suggested by titration of reaction centre triplet formation.

The redox properties of the electron acceptors can also be investigated by observing the potential dependence of P-680 rereduction following flash excitation in samples in which water oxidation is inhibited. These have also indicated the presence of additional low potential electron acceptors in spinach Photosystem II [11].

Two explanations of these observations have been put forward. Either that all centres have two different types of acceptor (Q_h and Q_l), or that there are two populations of Photosystem II with different redox properties for their respective Q_a acceptors [12,13]. The EPR signal attributed to Q_a differs depending on the presence of bicarbonate and Q_b. Recently it has been suggested that the Q_l and Q_h forms of Q_a result from centres having varying combinations of free or bound bicarbonate or Q_b [11].

In contrast to chloroplasts, the low potential acceptor, Q_l, was not observed in fluorescence titrations of Photosystem II preparations from the thermophilic cyanobacterium *Phormidium laminosum* [14,15]. Titrations of C_{550} [16] or the EPR signal of the iron-semiquinone [17] in *Chlamydomonas reinhardtii* only show one step. These preparations may therefore provide a model system to determine the identity of the components of the electron acceptor complex.

Redox titrations using EPR and laser flash absorption spectroscopy of the electron acceptor complex in *P. laminosum* preparations which apparently lack Q_l are reported. Previous redox titrations of the iron-semiquinone by EPR have required the use of formate. We have now been able to titrate the native samples in addition to those with added formate. These confirm the absence of the low potential iron-semiquinone in this organism, but suggest the presence of a component with a similar midpoint potential to Q_l at around −270 mV which may act as an electron acceptor at 200 K and above. The redox potential of Q_a in *P. laminosum* is unaffected by the presence or absence of bicarbonate or Q_b.

Materials and Methods

Growth conditions and preparation of Photosystem II

P. laminosum was grown at 45 °C, in medium D of Castenholz [18] in 10 litre glass jars and harvested in late growth phase. During growth the cultures were gassed with 5% CO_2/95% air, and illuminated with a 150 W tungsten filament spot lamp. Photosystem II particles were prepared using a method based on that of Ref. 15. Detergent fractionation of thylakoid membrane fragments (1 mg · ml^{-1} chlorophyll) was performed with 0.5% (w/v) *N*-dodecyl-*N*, *N*-dimethylammonio-3-propanesulphonate (Serva, Heidelberg) for 40 min at 4 °C [19]. The sample was centrifuged at 100 000 × *g* for 60 min at 4 °C, and the supernatant containing the Photosystem II was removed. This was concentrated by precipitation with 10% (w/v) poly(ethylene glycol) 6000, and centrifuged at 100 00 × *g* for 20 min at 4 °C. The pellet was resuspended and washed twice in 10 mM Hepes, 10 mM $MgCl_2$, 5 mM Na_2HPO_4 and 25% (v/v) glycerol (pH 7.5) (buffer C) and frozen at 77 K until required. Photosystem II particles prepared by this method evolved oxygen at between 1500 to 2000 μM O_2 · mg Chl^{-1} · h^{-1}. When required the oxygen-evolving complex was destroyed by incubating in 0.8 M Tris (pH 8.0) at 0.5 mg · ml^{-1} chlorophyll for 45 min at 4 °C in the dark, and harvested by centrifugation at 100 000 × *g* for 30 min.

Redox titrations for EPR analysis were performed in the dark, with the sample resuspended at 0.8 to 1 mg Chl · ml^{-1} in buffer C at 10 °C as in [19]. The following redox mediators were used at 20 μM: thionine, methylene blue, Janus green, indigotetrasulphonate, phenosafranine, safranine T, benzyl viologen and diquat. Before starting each titration, the sample and mediators were stirred together for 30 min and when required sodium formate (100 mM) was also added and allowed to equilibrate. After this period of equilibration, the potential was between 150 and 200 mV, at values where we have assumed that both Q_a and Q_b are oxidised. The potential and the pH were continuously monitored and adjusted as required with 1% (w/v) sodium dithionite or 0.1 M potassium ferricyanide and 0.1 M sodium hydroxide or hydrochloric acid. Samples were stored in the dark at 77 K until their EPR spectra were recorded using a Jeol FelX spectrometer with an Oxford Instruments ESR9 cryostat.

For optical experiments, samples were resuspended in 5 mM $MgCl_2$, 10% glycerol and either 20 mM Mes (pH 6.0), 50 mM Tris or Tricine (pH 8.0) or 20 mM glycine (pH 10.0). Absorption measurements of P-680 at 820 nm were made using a laser flash spectrophotometer as described in Ref. 20, except that the measuring device was a large area silicon photodiode type UDT 10D (United Detector Technology, from Optilas, U.K.). Samples were excited at 0.5 Hz at 337 nm with a 800 ps flash supplied by a N_2 laser (LN 1000, Photochemical Research Associates, Inc.). For redox titrations a quartz cuvette was built and used as in Ref. 21. The following mediators were used at either 5, 10 or 20 μM: thionine, methylene blue, Janus green, indigotetresulphonate, phenosafranine, safranine T, benzyl viologen, methyl viologen and diquat. The potential was measured using

a microplatinum rod combination electrode (CMMP Pt57, Russell pH Ltd, U.K.). During optical measurements the stirrer was turned off.

Results

In contrast to the results obtained from spinach Photosystem II particles [10], redox titrations of the iron-semiquinone complex in *P. laminosum*, either in the bicarbonate or formate bound states (Fig. 1A), show only one major step (Fig. 2) over the potential range 100 to −300 mV. The midpoint potential of this step corresponds to Q_h in the higher plant titrations. We were unable to observed a second low potential step in these titrations. This is in agreement with the fluorescence titrations of *P. laminosum* [14] where Q_l was not observed. The midpoint potential of the iron-quinone is close to that obtained for Q_a using fluorescence (E_m = +30 mV at pH 7.5 [14]) and is unaffected by the presence of formate. The midpoint potential of the $g = 1.9$ form of Q_a is +27 mV at pH 7.8, whereas the $g = 1.8$ form titrates around 20 mV.

At potentials where Q_h is reduced, changes in the appearance of the $g = 1.9$ signal occur. This is due to the presence of a component that titrates around (−270

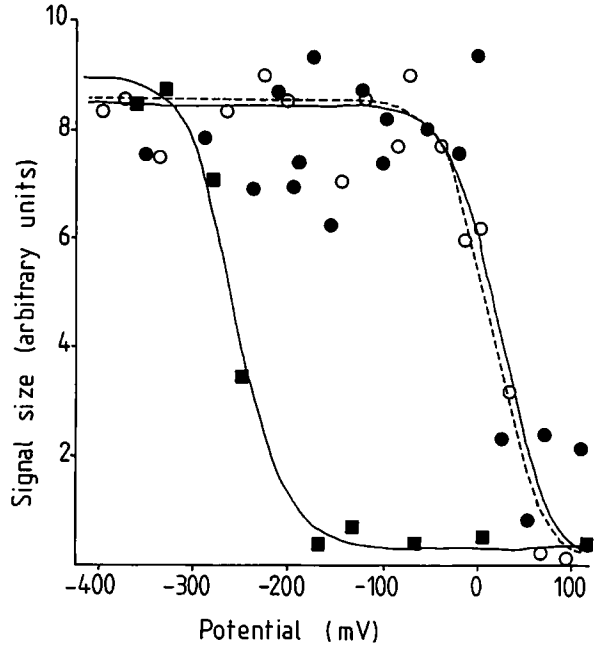

Fig. 2. Redox titrations of *P. laminosum* Photosystem II preparations at pH 7.8. Signal intensity before illumination of the iron-semiquinone signal in the presence ($g = 1.8$, ○) and absence of sodium formate ($g = 1.9$, ●), and the $g = 1.92$ signal (■). The curves drawn are the theoretical curves for one-electron transitions at 20 mV (○), 27 mV (●), and −255 mV (■). EPR conditions as in Fig. 1.

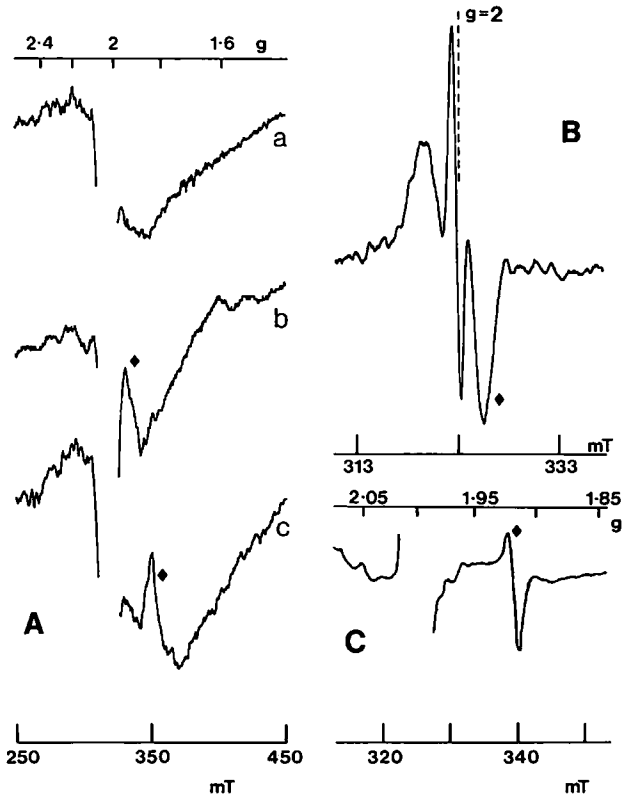

Fig. 1. EPR spectra showing signals from *P. laminosum* photosystem II preparations obtained from redox titrations. (A) Iron-semiquinone Q_a: (a) 100 mV, (b) −100 mV, (c) −100 mV, sample containing 100 mM sodium formate. (B) −100 mV, 200 K illumination minus dark difference spectrum showing doublet EPR signal from $I^- Q_a^- Fe^{2+}$. (C) Sample frozen under illumination showing the $g = 1.92$ and $g = 2.05$ resonances also observed at low redox potentials. Chlorophyll concentration 1 mg·ml^{-1}, EPR conditions: microwave power A and C, 10 mW and B, 25 mW; temperature A and B, 5 K and C, 15 K; modulation width 1.25 mT. The peaks measured are indicated ◆.

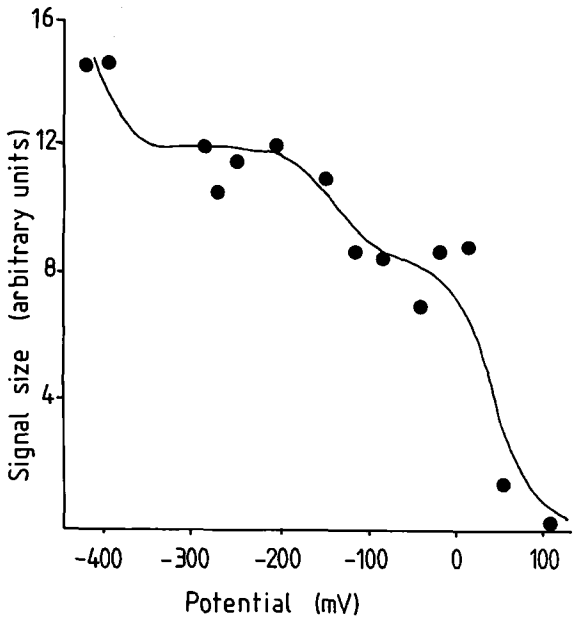

Fig. 3. Redox titration of extent of formation of the split signal (I^- Q_a^- Fe^{2+}) in *P. laminosum* Photosystem II preparations at pH 7.8 in the absence of sodium formate after illumination for 10 min at 200 K. The lines drawn are the theoretical curves for a one-electron transition at 35 mV and −150 mV. EPR conditions as in Fig. 1B.

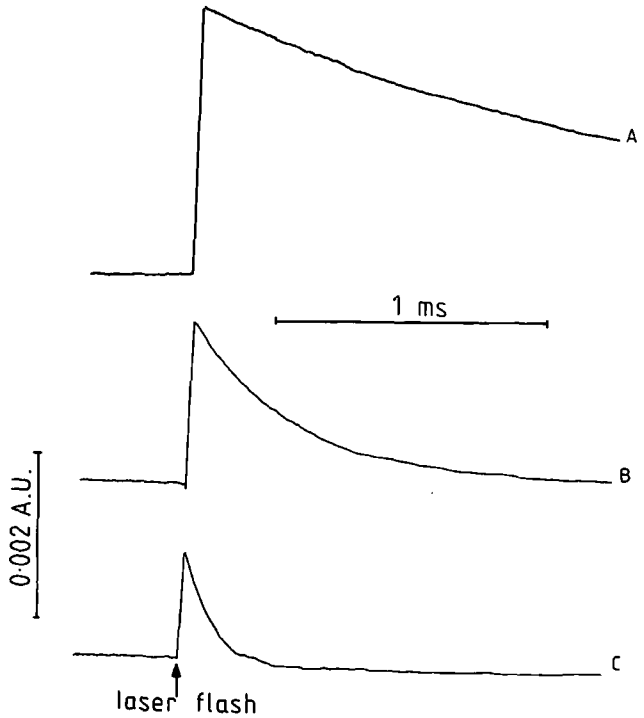

Fig. 4. Absorbance changes at 820 nm in Tris-washed *P. laminosum* Photosystem II at pH 8 in the presence of DCMU (100 μM) at room temperature. Each measurement is the average of 32 flashes at 0.5 Hz using a 337 nm laser flash (800 ps pulse length). The chlorophyll concentration was 7.5 $\mu g \cdot ml^{-1}$, path length 1 cm, and the redox potential was (a) 20 mV, (b) −240 mV and (c) −350 mV.

mV in *P. laminosum*. This $g = 1.92$ signal has been characterised previously and is thought to be an iron-sulphur centre [22,23]. At 15 K this signal at $g = 1.92$ can be observed more clearly (Fig. 1C), and titrates around −255 mV.

The split radical signal (the pheophytin doublet radical thought to be due to interaction between the semiquinone and reduced pheophytin) that is observed following 200 K illumination of untreated *P. laminosum* or with 500 μM DCMU [23] is shown in Fig. 1B. The split signal is very small and narrowed in the presence of formate and we have been unable to titrate it. However, the formation of the split signal in the absence of formate occurs in parallel to the reduction of Q_h in untreated samples with a midpoint potential of about +35 mV at pH 8.0 (Fig. 3). A small second step is observed that accounts for 20 to 25% of the maximum signal. The potential of this second step is difficult to measure but seems to vary between −150 to −250 mV. No change in the iron-semiquinone signal was observed, although, since the signals are small, it is not possible to state that there is no small change. However, this is clearly different to the situation in higher plant preparations where extensive formation of the split radical signal is seen only after reduction of the Q_l component.

Optical experiments

The decay of P-680$^+$ cation following laser flash illumination in Tris-washed samples at 20 mV (pH 8.0) is shown in Fig. 4. Only one component is observed with a half-time of 2 to 3 ms. At about 150 mV, the

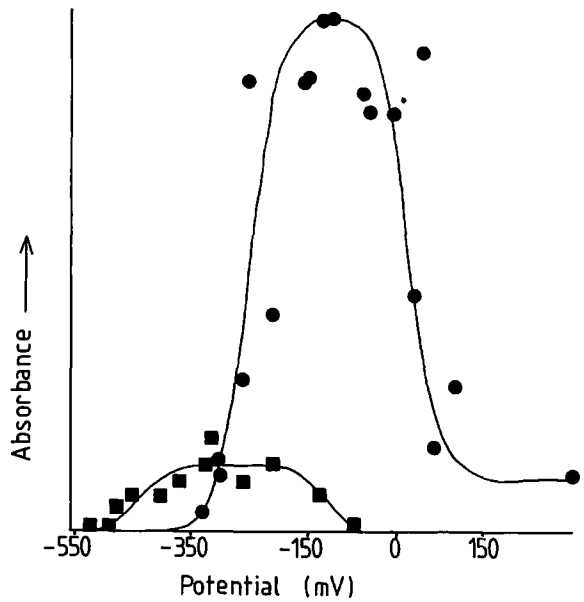

Fig. 5. Redox titration of absorbance changes at 820 nm in Tris-washed Photosystem II particles at pH 8 in the presence of DCMU (100 μM). Decay kinetics were fitted to absorbance transients as described in Materials and Methods. Half-time of decay 2 to 3 ms (●), 200 μs (■). Each measurement is the average of 32 flashes using a flash rate of 0.5 Hz at 337 nm (pulse length 800 ps). Theoretical Nernst curves ($n = 1$) are indicated with $E_m = 20$ mV and −250 mV for the ms component and $E_m = -150$ and −400 for the μs component.

signal is too small to be resolved easily but as the potential is lowered the size of the signal increases, as shown in the redox titration in Fig. 5. The midpoint of the increase in signal size at pH 8.0 is about 0 mV and is changed in the presence of 100 μM DCMU to about +25 mV as was also observed by fluorescence measurements [14]. Titration of the ms component in the absence of redox mediators shows essentially the same results, confirming that the effect is not due to mediator redox changes. The appearance of the ms component parallels the reduction of Q_a in EPR and fluorescence titrations.

In the EPR titrations, a component with a midpoint potential at about −250 mV was observed. However, the amount of this component varied and it was not seen in all preparations of *P. laminosum*. These still show high rates of oxygen evolution so that the $g = 1.92$ component does not appear to have a essential role in electron transfer from water to the quinone pool. In most experiments the loss of the ms component takes place at approx. −250 mV. However, this was also variable, in some experiments some of the ms component is lost at around −100 mV and in one experiment all of the ms component is lost at this potential. The midpoint potential of about −250 mV at pH 8.0 for the loss of the ms kinetics is at values close to the second step in the split signal, and the $g = 1.92$ component, suggesting that the ms kinetics may arise from this component.

The appearance of a component with a half-time of about 200 μs occurs (Fig 4) with a midpoint potential of about −150 mV (Fig. 5) although this value is difficult to determine as the signals are small. This component is lost as the potential is lowered with an E_m of about −400 mV at pH 8.0 and appears to correspond with the electron acceptor, U, observed in redox titrations of triplet yield in spinach Photosystem II [8] and suggests that an additional acceptor may function at potentials in between Q_h and I in *P. laminosum*. The existence of a low potential acceptor in *P. laminosum* has been inferred previously, since the spin-polarised reaction centre triplet can only be observed when the samples are reduced with excess dithionite at pH 10. No triplet, or only a very small signal, was observed at potentials where only Q_a was reduced [23].

Discussion

EPR spectroscopy and laser flash kinetic spectroscopy at room temperature were used to investigate the electron acceptors in Photosystem II of the cyanobacterium *P. laminosum*. This is the first report of the titration of the native $g = 1.9$ iron-semiquinone. In contrast to the results obtained in spinach [8], but in agreement to those obtained by fluorescence measurements in *P. laminosum* [14], only one step was observed in redox titrations of both the $g = 1.9$ and 1.8 EPR signals attributed to the iron-semiquinone. The midpoint potential of this step was around 20 mV equivalent to Q_h, confirming the absence of a low potential iron-quinone type quencher, Q_l [14].

Formate treatment has been shown to inhibit electron flow at the acceptor side of Photosystem II [24]. This is not due to changes in the midpoint potential of Q_a, as the E_m of the $g = 1.8$ and $g = 1.9$ forms of Q_a were similar at 20 and 25 mV, respectively, at pH 7.8. Fluorescence titrations have also shown that the potential of Q_l is unaffected by formate, although Q_h was not detected in these experiments [25]. Optical experiments also indicate that the loss of Q_b has no major effects on the redox potential of Q_a.

Although a low potential iron-semiquinone was not observed in EPR titrations of *P. laminosum*, a component with midpoint potential −250 mV at pH 8, at similar values to Q_l was observed in both EPR titrations (at $g = 1.92$) and in optical titrations of the rereduction of P-680 following a laser flash. Upon reduction of Q_h, P-680 was reduced with a half-time of 2 to 3 ms. These decay kinetics were lost as the potential was lowered with a midpoint potential of about −250 mV, at similar values to the $g = 1.92$ component. The nature of this component is not known, but it may be an iron-sulphur centre [26]. Although, as observed in the optical experiments, it may function as an electron acceptor above 200 K, the $g = 1.92$ component is not photoreducible at lower temperatures [22]. This component does not appear to be essential in the pathway of electron flow from water to the quinone pool, as Photosystem II preparations in which it is absent still show high rates of oxygen evolution [23]. The $g = 1.92$ component does not appear to act as a quencher of fluorescence in Photosystem II as it was not detected in Ref. 14.

The redox titration of the split signal also indicates that the potential of Q_a is about 30 mV as 70% of the maximum signal observed above −400 mV occurs with this step. Although a second step was not observed in titrations of the iron-semiquinone, a small step of rise in amplitude occurred in the appearance of the split signal at lower potentials (around −150 to −200 mV). The split signal arises from a complex interaction between I and Q_a-Fe, its size and shape are very sensitive to the environment, e.g., it is much smaller and narrowed in the presence of formate so that we were not able to titrate it. Therefore it is difficult to determine the nature of the second step. It may arise from the existence of an additional electron acceptor with redox potential between Q_h and I [8], or from a change in the environment affecting either I or Q_h as another component becomes reduced. In some titrations the second step in the split signal parallels the reduction of the $g = 1.92$ component and the loss of the ms component in the optical experiments. The size of the split signal may be

sensitive to this change. The simplest explanation of these results would be that when Q_a is reduced, electrons are transferred to the $g = 1.92$ component which functions as a "bypass" in the electron transfer system to prevent accumulation of I^- and photoinhibition. At low temperatures, the mixed effects seen in the I^- titration may reflect either inefficient electron transfer to the $g = 1.92$ component or variable amounts of this component in different preparations. Experiments where the $g = 1.92$ component is present in defined amounts would be required to investigate this. The double reduction of Q_b, at low potential in centres in which Q_b is unprotonated, could also give rise to the second step in the split signal as the interaction between Q_a^- and Q_b^- may prevent its appearance.

The presence of a component with midpoint potential -400 mV was also observed in optical titrations of *P. laminosum*. This potential is similar to that of component U in the electron acceptor complex of spinach Photosystem II detected by redox titration of the reaction centre triplet. Van Mieghem et al., have recently shown that this results from double reduction of Q_a [27]. Previous work on *P. laminosum* demonstrated that the samples had to be reduced with dithionite at pH 10 before triplet was observed [8]. Titrations of the formation of triplet need to be carried out in *P. laminosum* to confirm that the μs component observed in optical titrations is equivalent to component U and is caused by double reduction of Q_a.

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