A relationship between the midpoint potential of the primary acceptor and low temperature photochemistry in Photosystem II

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Redox titrations of the photo-induced pheophytin EPR signal in Photosystem II show two transitions which reflect the redox state of Q. The high potential wave ($E_m \simeq -50$ mV) can be photo-induced at 5 K and 77 K. The low potential wave ($E_m \simeq -275$ mV) required illumination at 200 K. This indicates the presence of two kinds of PS-II reaction centres differing in terms of the competence of their donors at low temperature and the E_m-values of their acceptors. Measurements of the semiquinone-iron acceptor also demonstrate functional heterogeneity at low temperature. This is the first observation of the semiquinone-iron acceptor in a non-mutant species.

Pheophytin *Semiquinone–iron complex* *Redox titration* *Photosynthesis* *EPR* *Electron donor*

1. INTRODUCTION

The midpoint potential (E_m) of the classical primary acceptor, Q, in Photosystem II (PS-II) has been measured by several different groups using a variety of methods. In normal chloroplasts and in some subchloroplast preparations, titrations of the increase in fluorescence yield associated with Q reduction exhibited two phases of reduction, corresponding to E_m-values around 0 mV (Q_H) and -300 mV (Q_L) [1–10]. In a Triton subchloroplast preparation [10] and in chloroplasts lacking grana stacks [5] only the Q_L transition was observed, while in a digitonin preparation of *Phorimidium laminosum* only Q_H was observed [11]. Redox titrations of the photo oxidation of cytochrome b_{559} [12,13] and the photo-induction of C-550 change [12] at 77 K in chloroplasts showed a single transition corresponding to Q_H. Chemical induction of the C-550 change at ambient temperature also occurred as a single wave at around 0 mV, however this titration was only possible in digitonin fractionated subchloroplast particles [14]. Very recently a titration of the C-550 change in particles and chloroplasts has been reported with only a single wave at around 0 mV [15,16].

The carotenoid band shift associated with charge separation across the membrane has been titrated at ambient temperature and gives two waves when measured 1 ms after the flash [16,17] but only a single Q_H-type wave when measured at 50 μs after the flash [16].

Recently, 3 new EPR signals have been discovered in the PS-II reaction centre, a split signal due to the reduced Ph acceptor interacting with a semiquinone–iron complex, split Ph^- [18,19], a signal from a spin-polarised triplet state of P-680, ^{3}P-680 [20], and a signal arising from the interaction of the semiquinone form of the primary quinone acceptor with a ferrous iron atom, Q^-Fe [21]. The analogous signals in purple photosynthetic bacteria [22–25] have been titrated and provided good measurements of the E_m of Q [26–31]. In PS-II, redox poising of the ^{3}P-680

Abbreviations: PS-II, photosystem II; PS-I, photosystem I; Ph, intermediate pheophytin acceptor in PS-II; Q Fe, the quinone–iron complex primary acceptor; BPh, the intermediate pheophytin acceptor in purple bacteria

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showed an increase in signal intensity over the range where Q_L was reduced [20], while titration of the Q^-Fe signal showed a monophasic curve corresponding to Q_H [32]. Since both of these measurements were made on detergent-fractionated preparations and, in the latter work, using an extremely labile signal, neither measurement provides much help in explaining the confusing and contradictory fluorescence and optical data.

Here, we have carried out redox titrations in which photo-induction of the split Ph^- signal has been monitored as a probe of the redox state of Q. Since the split Ph^- signal has a significantly greater intensity than either the ^3P-680 or the Q^-Fe signal, the work has been done on unfractionated chloroplasts. The results show an interesting new phenomenon, a functional heterogeneity associated with the Q_H and Q_L transitions, which is most easily explained by the presence of two kinds of centres (rather than by one kind of centre having both a Q_H and a Q_L acceptor) and which also may explain some of the contradictions of earlier measurements.

We also report, for the first time in a non-mutant organism, EPR spectra of Q^-Fe itself and show the presence of functional heterogeneity in its low temperature photo-reduction similar to that described for the split Ph^-.

2. METHODS

Spinach chloroplasts were prepared as in [33], a procedure which includes an EDTA wash to remove hexaquo manganese. Chloroplasts were used fresh or having been frozen at −30°C as pellets for up to 5 days. Redox properties and low temperature behaviour appeared to be the same in both cases. Tris-washing was carried out as follows: chloroplasts pellets were resuspended in 0.8 M Tris (pH 8.2) in room light and were left to incubate at 4°C in darkness for 40 min before being pelleted. Normal and Tris-treated chloroplasts were resuspended in 50 mM MOPS, pH 7.0. Redox poising of EPR samples was carried out using the method described in [34]. The mediators used were as in [2] with the exception of neutral red and with the addition of 5-hydroxy-1,4-naphthoquinone ($E_{m7} \simeq 33$ mV). The mediators used exhibit $n = 2$ transitions and thus do not interfere with measurements of free radicals. Mediators were used at a concentration of 100 μM with the exception of anthraquinone-2-sulphonate which was 200 μM. Titrations were carried out reductively to lower than −400 mV by the addition of sodium dithionite and then oxidatively to +330 mV by the addition of potassium ferricyanide. The final sample was rereduced with excess dithionite to determine if any of the semiquinone interaction had been destroyed during the titration. This measurement indicated that the interaction was more than 75% intact at the end of the titration. The reaction mixture was maintained at 6–9°C throughout the titration.

Subchloroplast granal membranes which were free of PS-I were obtained by Triton digestion as in [35]. The preparation was stored at 77 K in buffer consisting of sucrose (0.4 M), HEPES, pH 7.5 (20 mM), NaCl (15 mM) and $MgCl_2$ (5 mM). EDTA (5 mM) was added to each sample in the EPR tube.

EPR spectra were obtained using a Bruker X band spectrometer fitted with an Oxford Instruments variable temperature liquid helium cryostat and control system. All field and g-values are approximate. Illumination in the cavity was provided with an 800-W projector while illumination at 200 K in an ethanol/solid CO_2 bath was provided by a 250-W projector. EPR conditions (see legends to figures) were chosen so that EPR signals due to PS-I were minimized.

3. RESULTS

Fig.1 shows EPR spectra obtained in normal and Tris-washed chloroplast in the presence of sodium dithionite and the effect of illumination with white light at 200 K. A large split signal attributable to Ph^- interacting with a semiquinone–iron complex was photo-induced in both cases. This signal was first reported after 200 K illumination of PS-II particles and chloroplasts by Klimov and co-workers [18,19].

It can be seen that the only significant difference between the untreated and the Tris-washed chloroplasts is in the radical region where an extra component (a 1.5 mT wide free radical) is photo-induced in untreated chloroplasts. It is possible that this signal reflects the oxidation of a secondary electron donor, the function of which is in-

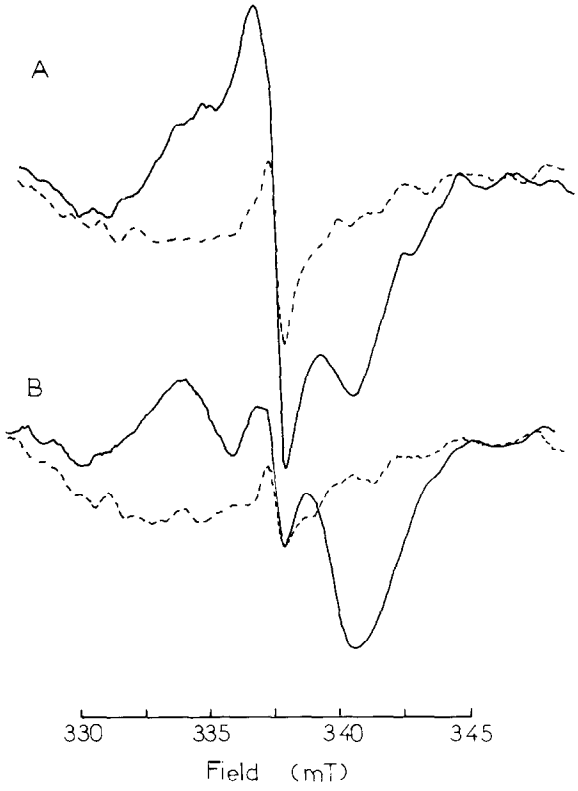

Fig.1. EPR signals photo-induced by 4 min of illumination at 200 K in untreated (A) and Tris-treated (B) chloroplasts. Chlorophyll concentrations were almost identical in both samples (6 mg/ml). Samples were incubated in darkness for 20 min in the presence of sodium dithionite (50 mM) under O_2 free argon gas before being frozen and stored at 77 K. No redox mediators were present in these samples. The broken lines show the EPR spectra recorded in darkness while the solid lines were recorded after 200 K illumination. EPR settings were as follows: temperature 4.8 K, microwave power 35 mW, frequency 9.465 GHz, modulation amplitude 10 gauss (1.0 mT), gain 2.5×10^5.

hibited by Tris-treatment. The presence of this signal distorts the split signal thus the majority of the measurements were done with Tris-washed chloroplasts. Illumination at 5 K of reduced chloroplasts (untreated or Tris-treated) resulted in the slow formation of the split Ph^- signal. Approximately 30% of the total split Ph^- signal could be formed at this temperature. This phenomenon has been reported previously in PS-II particles prepared from a PS-I-less mutant of *Chlamydomonas* [36].

Fig.2 shows the results of redox titrations carried out on Tris-washed chloroplasts where the amplitude of the split Ph^- signal formed after illumination at 200 K was monitored. It can be seen that a double-wave titration was obtained with E_m-values close to the reported values for Q_H and Q_L (about -50 mV and -275 mV respectively). The titration is fully reversible and shows no appreciable hysteresis (solid circles, oxidative; open circles, reductive). The points are rather scattered over the range of the high potential wave; this is due to the presence of changes in the radical region occurring at oxidizing potentials which make measurements of the small split signal at potentials higher than -100 mV difficult. The open triangles represent the extent of the split Ph^- signal induced by 20–25 min illumination at 5 K. These measurements were made prior to illumination at 200 K and, due to the long time of illumination re-

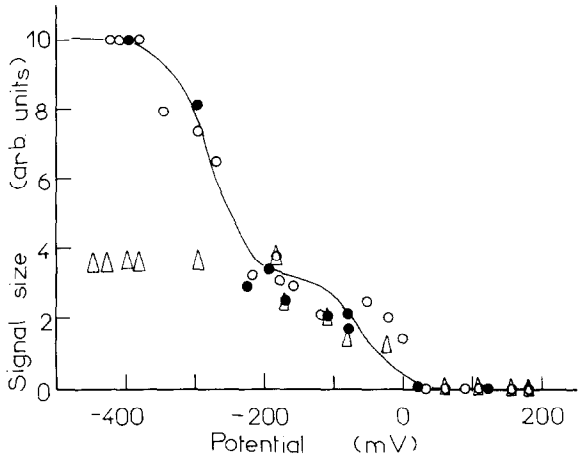

Fig.2. Redox titration of Tris-washed chloroplasts where the extent of the photo-induced split Ph^- signal was measured after 20–25 min illumination at 5 K (triangles) and after 4 min illumination at 200 K (circles). Signal size was measured as the change in signal amplitude at the signal minimum at 340 mT relative to the spectrum recorded in the dark. EPR spectra were recorded as described in the legend to fig.1. The results of two redox titrations are presented and are normalized at the maximum value. Open circles are points obtained titrating in a reductive direction, solid curves are points obtained in an oxidizing direction. Redox potentiometry was as described in the methods. The curves approximate $n = 1$ redox transitions with E_m-values of about -50 mV and -275 mV.

quired, measurements were made on only half of the samples. It can be seen that the signal induced by illumination at 5 K exhibits only the Q_H wave. This phenomenon is further demonstrated in fig.3 and 4.

Fig.3 shows EPR spectra recorded in samples poised at 90 mV, −195 mV and −390 mV. Illumination of the sample poised at 90 mV results in a small increase in the free radical region, probably due, at least in part, to the oxidation of the donor species which gives rise to signal II. Illumination at 5 K of samples poised at −195 and −400 mV results in photo-induction of the split Ph^- signal to almost the same extent and with similar kinetics (fig.4A). Further illumination of these samples at 200 K results in the formation of a large split Ph^- signal in the sample poised at −400 mV while that poised at −195 mV shows virtually no further increase in signal size (fig.3 solid curves and fig.4B). In fig.4B both samples show an apparent increase in signal size with long illumination at 200 K (10 min); this, however, is largely due to a very broad change around $g = 2$ (compare solid curve with the dashed curves in fig.4C for example). This signal is unidentified but may partly correspond to the changes seen after shorter illumination at 200 K in untreated chloroplasts (fig.1). Poising experiments using untreated chloroplasts show almost identical behaviour with respect to potential dependence and temperature dependence (not shown).

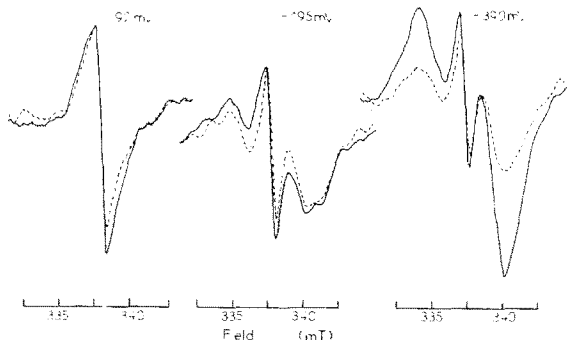

Fig.3. Redox potential dependence of photo-induction of split Ph^- by illumination at 5 K (broken lines) and 200 K (solid lines). Dotted curves are the spectra recorded in darkness. Tris-washed chloroplasts were redox poised, as described in the methods, at the potentials marked above each set of spectra on the figure. Illumination at 5 K (broken lines) was carried out for 20 min before samples were further illuminated at 200 K for 4 min (solid lines). EPR settings were as described in fig.1.

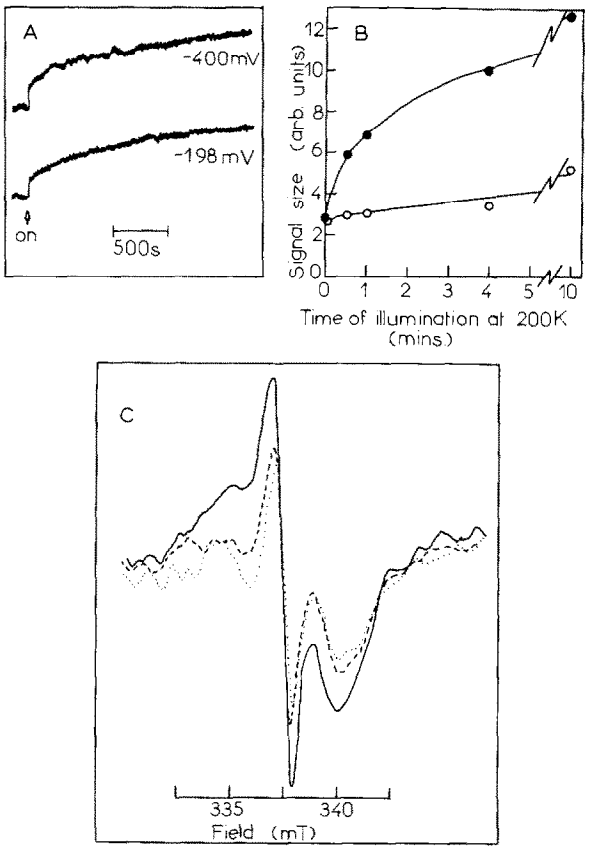

Fig.4. (A) The kinetics of low temperature photo-induction of the split Ph^- signal at 5 K. EPR spectrometer was set at the field value of the signal maximum. The upper trace was recorded using a sample poised at −400 mV, the lower trace was recorded using a sample poised at −198 mV; (B) The effect of 200 K illumination upon samples poised at −400 mV (solid) and −198 mV (open). In both samples illumination at 5 K has already been carried out; (C) The effect of long illumination at 200 K upon the shape of the EPR spectrum in Tris-washed chloroplasts. The sample was poised at −198 mV. The dotted line was recorded after illumination at 5 K for 30 min (i.e., zero time of illumination at 200 K), the broken line is after 1 min of illumination at 200 K, and the solid line is after 10 min illumination at 200 K. EPR conditions were as described in fig.1.

Attempts were made to monitor the Q^-Fe signal directly in chloroplasts but its weak intensity did not allow it to be distinguished from the noise.

However using granal membranes containing no PS-I prepared by Triton digestion $^{35}Q^{-}Fe$ signals could be observed. Fig.5A is a spectrum recorded in a dark oxidized sample when no $Q^{-}Fe$ is expected to be present. Fig.5B,C and D show spectra of the same sample after illumination at 5 K, 77 K and 200 K, respectively. Approximately 30% of the signal was stably photo-reduced at 5 K and 77 K while 100% of the signal was formed with illumination at 200 K. Fig.5E shows the EPR spectrum generated by reduction of a similar sample with sodium dithionite prior to freezing in the dark. The extent of the chemically induced signal is similar to that induced by illumination at 200 K. These spectra exhibit the low field resonance characteristic of the analogous signal in bacteria. The position of this deflection is slightly different depending upon whether Q Fe is chemically or photochemically reduced (g = 1.70 and g = 1.67, respectively). Attempts to titrate the $Q^{-}Fe$ have so far been unsuccessful due to the high concentration of chlorophyll required and the lability of the signal in the presence of some mediators.

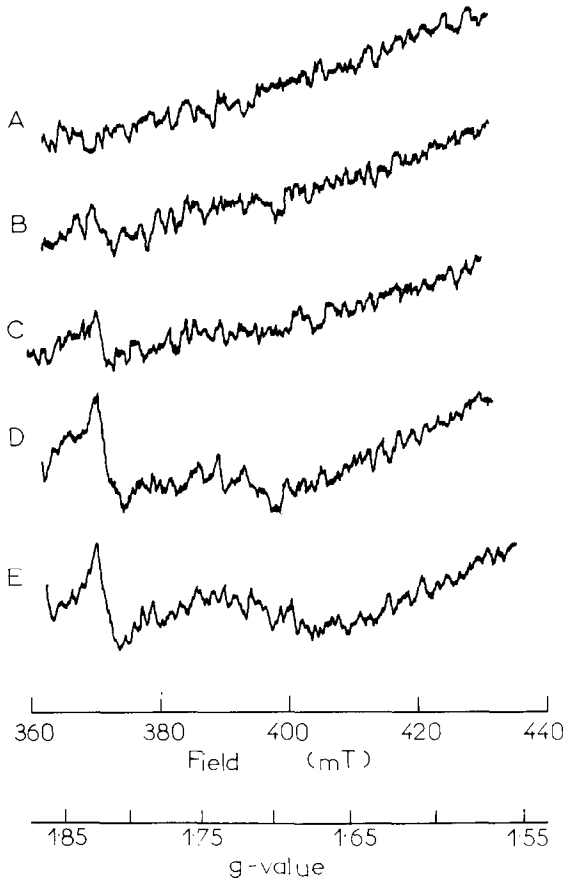

Fig.5. Photochemical and chemical reduction of the semiquinone-iron acceptor $Q^{-}Fe$ in PS-II membranes: (A) is a sample (about 10 mg Chl/ml) frozen in the dark in the presence of dichlorodicyanobenzoquinone (10 mM); (B) after illumination for 10 min at 5 K; (C) after illumination for 10 min at 77 K; (D) after illumination for 4 min at 200 K; (E) is a similar sample frozen in the dark in the presence of sodium dithionite (50 mM). No mediators were present. EPR settings were as follows: modulation 1.0 mT; frequency 9.465 GHz; power 12.5 mW; temperature 4.8 K. Gain 1×10^{6}.

4. DISCUSSION

The redox titrations of chloroplasts monitoring phototrapping of split Ph^{-} at 200 K result in a characteristic stepped curve with E_m-values at pH 7.0 in the region of −50 mV and −275 mV. These values correspond well with those obtained for the increase in fluorescence as Q undergoes reduction [1–10]. Analogous EPR experiments in photosynthetic bacteria have demonstrated that the amplitude of the photo-induced split BPh^{-} signal is a direct reflection of the amount of $Q^{-}Fe$ present before illumination [26,27]. Thus, although potentially complicated due to the possible involvement of multiple donors and acceptors, it seems that photochemistry at 200 K in PS-II can be interpreted in fashion analogous to that in photosynthetic bacteria [22], i.e.,

above 0 mV
D P-680 Ph Q Fe $\xrightarrow{h\nu}$
D P^{+}-680 Ph $Q^{-}Fe$ $\longrightarrow$
$D^{+}P$-680 Ph $Q^{-}Fe$
no split signal

under reducing conditions
D P-680 Ph $Q^{-}Fe$ $\xrightarrow{h\nu}$
D P^{+}-680 $Ph^{-}Q^{-}Fe$ $\longrightarrow$
$D^{+}P$-680 $Ph^{-}Q^{-}Fe$
split signal

(In PS-II, D may be one of several different endogenous donors and, under some conditions, the ultimate electron donor may be dithionite.) Thus, in PS-II, the extent of the split Ph^{-} signal reflects the redox state of Q. In normal chloroplasts the positive charge may be located on a component

different to that in Tris-washed chloroplasts (see [37]) and thus might give rise to the extra 1.5 mT wide signal in the radical region in untreated chloroplasts (fig.1). This, however, does not allow a second donation that would result in Ph^- reduction at oxidized potentials.

Authors in [36] reported that in some PS-II centres split Ph^- could be formed by illuminating at 5 K while in other centres split Ph^- could only be stably formed by illumination at 200 K. These results are confirmed in this paper but it is also demonstrated that low temperature phototrapping of Ph^- is largely associated with Q_H whilst 200 K phototrapping of Ph^- is associated with Q_L.

There have been several different hypotheses proposed to explain the double wave in Q titrations in PS-II:

(i) The presence of Q_H and Q_L acceptor in each reaction centre (i.e., [4,9]);

(ii) An equilibrium effect between Q and Q_B, the secondary quinone acceptor

$$(\text{i.e., } Q\,Q_B \xrightarrow[0\text{ mV}]{} Q\,Q_B^{2-} \rightleftharpoons Q^-Q_B^- \xrightarrow[-300\text{ mV}]{} Q^-Q_B^{2-}) \qquad [7]$$

and

(iii) The presence of two kinds of centres, some having Q_H, others Q_L [2,5,9].

Clearly, the last of these possibilities most simply explains the results presented here. Two kinds of reaction centres can be envisaged:

(i) D P-680 Ph Q_H which has a high potential quinone and a donor which functions at 5 K;

(ii) D′ P-680 Ph Q_L which has a low potential quinone and a donor functional only at higher temperatures.

This idea is further supported by the observation made here (fig.5) and in [36] that only a fraction of Q^-Fe can be photo-reduced at 5 K or 77 K while all of it can be photo-reduced at 200 K.

From this it would be predicted that a direct titration of Q^-Fe in chloroplasts should give a double wave in contrast to the single wave reported for this component in digiton particles [32]. Extending this idea further it seems possible that titrations of photo-induced changes of cytochrome b_{-559} and C-550 carried out at 77 K would only measure Q_H type centres. This could explain the absence of a Q_L step in [12] and [13]. This hypothesis can be tested by redox poising experiments looking at C-550, or Q^-Fe after illuminating at 77 K and 200 K.

A brief comparison of the results reported here with those from photosynthetic bacteria can be made. Although double-wave titrations are not the rule [38], there are occasional reports of the phenomenon for reductions of the Q/Q^- in bacteria. Authors in [39] observed such a pattern in *R. rubrum* when monitoring P-870 photooxidation at room temperature and authors in [27], doing experiments analogous to those reported here, observed a two-step rise in the amplitude of the split Ph^- signal and of the Q^-Fe signal in *Rps. viridis*.

It seems possible that, where results obtained on the acceptor side of PS-II appear to be different from those generally accepted in bacteria, this reflects gaps in our knowledge of bacterial photochemistry rather than a breakdown of the analogy between the two systems.

Although the significance of the presence of two kinds of PS-II reaction centre is difficult to judge, it may be possible to relate this kind of heterogeneity to other data in the literature. Measurements of secondary electron acceptors in PS-II indicate a heterogeneity, in that some centres have an oscillating secondary quinone while others do not [40,41]. Since the oscillations of the secondary quinone can be almost completely inverted by pre-illumination at 77 K [42] it is tempting to correlate the low-temperature, Q_H centres with those possessing an oscillating secondary quinone while the 200 K, Q_L centres may be equated with those lacking an oscillating secondary quinone.

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