Biochimica et Biophysica Acta 806 (1985) 247–254
Elsevier

BBA 41693

Redox characterisation of the Photosystem II electron acceptors. Evidence for two electron carriers between pheophytin and Q

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(Received June 26th, 1984)

Key words: Photosystem II; ESR; Reaction center; Electron transport; (Spinach; *C. Reinhardtii*)

The oxidation reduction potential dependence of low-temperature photochemical reactions associated with the electron acceptors of Photosystem II has been determined in Photosystem II preparations from spinach and *Chlamydomonas reinhardtii*. The redox state of the iron-quinone electron acceptor (Q_H), the pheophytin intermediary electron carrier (I) and the ability to form a spin-polarised reaction centre triplet (^{3}P-680) were measured by electron paramagnetic resonance spectrometry. In redox titrations, Q_H was reduced with $E_m \simeq -10$ mV and I with $E_m \simeq -600$ mV. Illumination at 6 or at 200 K resulted in electron transfer from donors to P-680 to the electron acceptor complex. In oxidised samples photoreduction of Q_H is accompanied by the oxidation of a donor giving rise to signal II_{LT}. When Q_H was chemically reduced, this was no longer seen. Illumination at 200 K then resulted in reduction of I in a small fraction of centres. In the majority (90%) of centres I was photoreduced only following the reduction of a component (Q_L) with $E_m \simeq -300$ mV except that in the presence of pyocyanine this redox step appeared in parallel with the reduction of Q_H. In spinach preparations with intact donor systems slow photochemical reduction of I could be observed at 6 K. Reaction centre triplet formation resulting from the back reaction between I^- and P-680$^+$ was observed only after reduction of another component (U) with $E_m \simeq -430$ mV. It was concluded that four components could accept electrons from P-680 at low temperature, that they are in a single type of reaction centre, and that they most likely function in a sequential electron-transport chain. It was also concluded that there is a very fast electron donation to P-680$^+$ even at 6 K, that two, and possibly three, electrons can be transferred from endogenous electron donors at low temperature and that pyocyanine can act as a nonspecific electron donor at 200 K.

Introduction

The Photosystem II reaction centre in plants and algae catalyses the photochemical formation of the oxidant required for water oxidation in photosynthesis and the transfer of an electron to the plastoquinone pool at a potential close to 0 mV. A number of intermediary membrane-bound electron carriers can be shown to be involved in electron transfer from the excited reaction centre chlorophyll (P-680*) to the plastoquinone pool. A pheophytin ($E_m \approx -600$ mV) can be detected by optical [1] and EPR [2] measurements and two bound quinone acceptors have been characterised [3]. One of these (Q_H) can be detected by EPR and has a spectrum similar to that of the iron-quinone complex in purple photosynthetic bacteria [4–7] with $E_m \approx -10$ mV [8].

In purple bacteria the bound quinone acceptors and the pheophytin interact magnetically with a ferrous iron atom giving rise to characteristic EPR

Abbreviations: E_m, midpoint redox potential; P-680, the reaction centre chlorophyll of Photosystem II.

spectra at cryogenic temperatures [9]. The iron quinone complexes have broad spectra centred near $g = 1.80$. If the pheophytin is reduced while Q_A is in the semiquinone state, the pheophytin radical signal is split to form a 70 gauss wide doublet at temperatures below 8 K. A similar splitting of the pheophytin radical signal is seen in Photosystem II preparations treated in a similar way [2,5,10].

In purple bacteria the formation of a spin-polarised reaction centre triplet can also be observed at cryogenic temperatures. This triplet is formed by a back reaction between P-890$^+$ and reduced pheophytin following illumination at low temperature of samples with Q_A reduced to the semiquinone. A spin-polarised triplet can also be observed in Photosystem II preparations [11].

The effect of the redox states of the various electron acceptors on the observed EPR spectra and photochemistry at low temperature has been extensively characterised in purple bacteria [12–14]. These experiments seem to support a model of the acceptor complex in which electrons are donated directly from the pheophytin to Q_A across a potential gap of 500–600 mV. The identification of similar acceptors in Photosystem II suggests an analogous electron-transfer sequence. However, titrations of the redox dependence of fluorescence and recent EPR measurements suggest a more complex acceptor sequence in Photosystem II. The primary electron acceptor of Photosystem II (Q) was first characterised as a component which quenched chlorophyll fluorescence. Redox titration of this quenching indicated that two components were involved, at least in higher plants [15]. These are termed Q_H ($E_m \simeq 0$ mV) and Q_L ($E_m \simeq -280$ mV). Q_H is also reflected by the absorption change termed C550 and we have recently shown that it is the EPR-detectable iron-quinone acceptor [8]. Rutherford and Mathis [10] determined the redox dependence of the ability to induce the doublet pheophytin radical in spinach particles and found two steps in the titration at potentials similar to those of Q_H and Q_L.

We have now measured the redox relationships of the acceptor complex in purified Photosystem II reaction centres from *Chlamydomonas reinhardtii* and in oxygen-evolving preparations from spinach. These experiments indicate that Photosystem II has a much more complex membrane-bound electron-acceptor system than the purple bacterial reaction centre. Two electron carriers which function between the pheophytin and Q_H can be detected. One of these corresponds to the component Q_L detected in fluorescent titration, while the other with $E_m \simeq -430$ mV has not previously been described.

Materials and Methods

Spinach Photosystem II particles with high rates of water oxidation were prepared by triton treatment of washed thylakoid preparations as described previously [16]. In some cases the preparations were stored by freezing in liquid nitrogen without significant loss of activity.

Photosystem II reaction centres were isolated from the mutant of *Chlamydomonas reinhardtii* Str. F54-14, which lacks Photosystem I, essentially by the procedure of Diner and Wollman [17] using polyethylene glycol to concentrate the reaction centres after detergent treatment [8]. Using a chlorophyll concentration of 1.2 mg/ml in the detergent treatment as described by Diner and Wollman gave a variable and in some cases high concentration of light harvesting chlorophyll in the preparation. These preparations were difficult to use for measurements of triplet formation as the signals were very small and as reported previously [5] decreased rapidly during illumination. We modified the preparations using 0.6 mg chlorophyll/ml in the detergent preparations for some of the experiments. This resulted in a preparation, free of light harvesting chlorophyll protein, in which triplet formation could be readily measured and was stable for several minutes after illumination. Formation of the split pheophytin radical signal was unaffected by the high detergent treatment, but the iron-quinone signals were small or absent in this preparation.

Oxidation reduction potential titrations were performed as described previously [18]. Poised samples were stored in liquid nitrogen in the dark until measured. The following compounds were used as redox mediators in the appropriate potential range: dichlorophenol indophenol, thionine, methylene blue, indigotetrasulphonate, pyocyanine, indigodisulphonate, anthroquinone-1,5-di-

sulphonate, phenosafranine, safranine-T, benzyl viologen, methyl viologen and triquat. Titrations were done using two or more mediator concentrations between 10 and 50 μM. Titrations were normally done in the reductive direction and the figures show examples of such titrations. All of the transitions observed were reversible and oxidative titrations showed transitions within the error range of the reductive titrations. Chlorophyll concentrations used in titrations varied between 0.5 and 2.0 mg/ml. Chlorophyll was estimated by the method of Arnon [19].

EPR spectra were recorded using a Jeol FE1X spectrometer with an Oxford Instruments ESR9 cryostat as described previously [18]. Kinetic changes were recorded using an instrument time constant of 800 μs with a Datalab 920 transient recorder and a Datalab 4000B signal averager. A Chromatix tuneable pulsed (600 ns F.W.H.M.) dye laser was used as actinic light source at 660 nm. Continuous illumination was provided by a 1000 W projector filtered through 2 cm of H_2O. Illumination at 200 K was carried out by immersing the samples in a solid CO_2/ethanol bath in an unsilvered dewar and illuminating with the 1000 W projector for 15 min (spinach) or 30 min (*C. reinhardtii*). Maximum signal sizes were attained after 2–5 min except under conditions when pyocyanine was apparently functioning as electron donor. The longer period then required was used routinely as no secondary changes in the spectrum were detected when the illumination period was prolonged.

Results

The results described in this paper depend on measurements of three EPR signals arising from the acceptor system: the $g = 1.82$ signal of Q_H, the pheophytin doublet radical thought to be due to interaction between Q^- and reduced pheophytin, and the signal of the spin-polarised triplet state of P-680. The signals can be observed only at temperatures of 6 K or below. The spectra are shown in Fig. 1.

We have previously shown that Q_H has $E_m \simeq -10$ mV and that in the detergent preparations used it is pH-independent. Analogy with the results obtained in purple bacteria would suggest

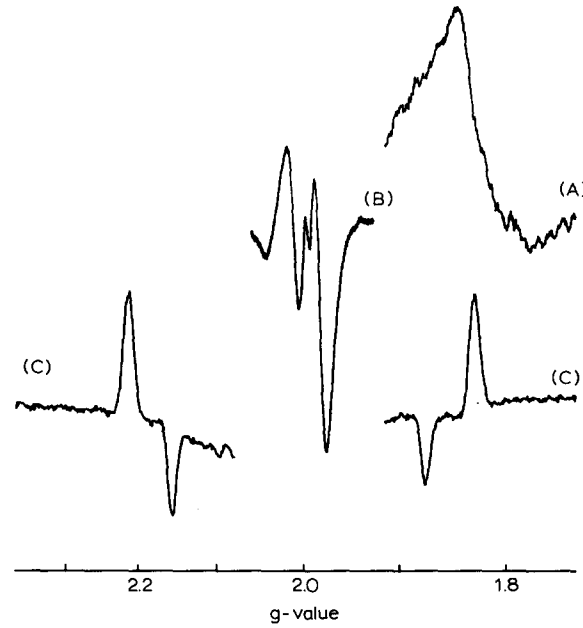

Fig. 1. EPR spectra associated with the electron-acceptor complex of Photosystem II. (A) The iron-quinone electron acceptor Q_H. EPR conditions: microwave power, 25 mW; modulation amplitude, 1 mT; frequency, 9.1 GHz; temperature, 5 K. (B) The split radical doublet assigned to the reduced pheophytin. EPR conditions: microwave power, 50 mW; modulation amplitude, 1 mT; frequency, 9.1 GHz; temperature, 5 K. (C) The reaction centre triplet. EPR conditions: microwave power, 25 μW; modulation amplitude, 1.25 mT; frequency, 9.1 GHz; temperature, 4.5 K. Spectrum recorded under continuous illumination. Spectra were recorded from *C. reinhardtii* particles prepared and reduced as described in the text.

that as Q_H is reduced triplet formation should be observed at 4 K. Furthermore illumination at 200 K should result in reduction of the pheophytin and the appearance of the split radical signal. Fig. 2 shows that split radical formation can indeed occur in parallel with the reduction of Q_H. We have also found that the low temperature (6 K) oxidation of the electron donor, detected as signal II_{LT} [20], is attenuated as Q_H is reduced, although changes in line shape and saturation properties make it difficult to obtain a satisfactory titration curve. There is no induction of the split radical signal following 6 K illumination in the *C. reinhardtii* preparation. At alkaline pH the titration can be extended to lower potentials. Fig. 3 shows a wide-range titration of the intensity of the split radical signal following 200 K illumination. Although a split radical signal is observed following

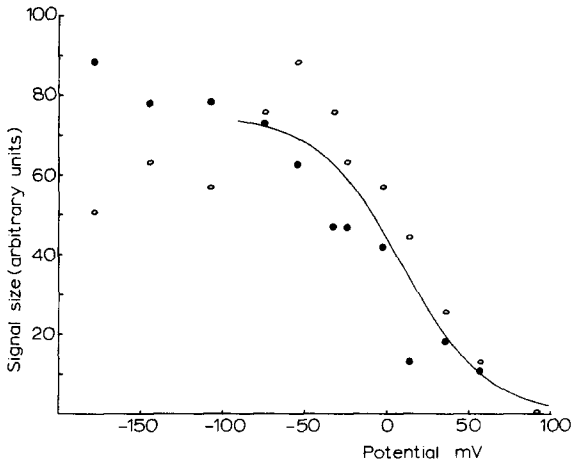

Fig. 2. Redox titration at pH 7.0 of the iron-quinone electron acceptor and the doublet signal induced by 200 K illumination in particles prepared from *C. reinhardtii*. The mediator mixture included pyocyanine. ○, Iron-quinone; ●, doublet. The curves drawn are the theoretical curves for a one-electron transition with $E_m \simeq 0$ mV.

reduction of Q_H, the maximal signal is only seen at lower potential, a second step in the titration being observed at $E_m \simeq -300$ mV. The ability to form the doublet is lost again with $E_m \simeq -520$ mV.

The relative signal sizes associated with the steps at 0 mV and −300 mV varied extensively and the time of 200 K illumination required to induce the maximum signal at the 0 mV step was long and variable. We have found that the size of signal associated with this step depends on the presence of the redox mediator pyocyanine. Fig. 3 shows a titration in the presence of pyocyanine and Fig. 4 one in its absence. In the absence of pyocyanine the 0 mV step was only 10–15% of the full signal size in either spinach or *C. reinhardtii* preparations. We have found that this effect was not due to changes in the midpoint potential of the iron-quinone electron acceptor induced by pyocyanine. Titrations of the iron-quinone complex with and without pyocyanine result in values for the midpoint potential in the range +50 mV to −50 mV, although the titrations without pyocyanine show poor equilibration and do not fit the Nernst curve well. Experience with Photosystem I [22,23] has shown that exogenous reducing agents, such as ascorbate or dithionite, can act as electron donors at 200 K and, as discussed later, in this case it would seem that the dye is functioning in this way, so that more than one electron acceptor can be reduced by illumination at 200 K.

The potential dependence of split radical formation is essentially the same in *C. reinhardtii* and spinach preparations. However, in the spinach preparations at pH 7–8 the radical can be slowly induced over a period of 30–60 minutes at 6 K as

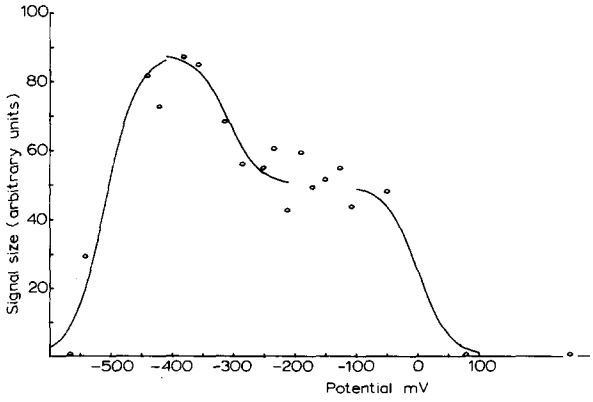

Fig. 3. Redox titration at pH 10.0 of the doublet signal induced by 200 K illumination in *C. reinhardtii* preparations in the presence of pyocyanine. The curves drawn are the theoretical curves for a one-electron transition with $E_m \equiv 10$ mV, −310 mV and −510 mV.

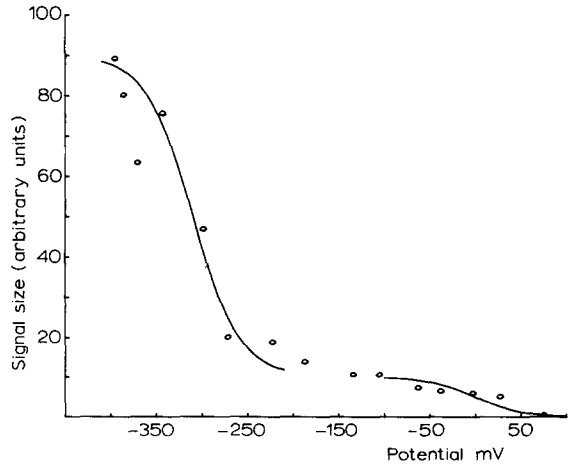

Fig. 4. Redox titration at pH 10.0 of the doublet signal induced by 200 K illumination in *C. reinhardtii* preparations in the absence of pyocyanine. The curves drawn are the theoretical curves for a one-electron transition with $E_m = 10$ mV and −310 mV.

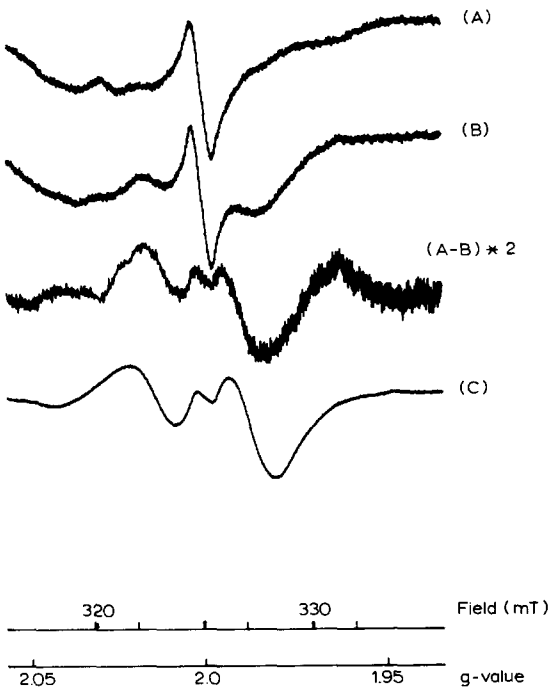

Fig. 5. EPR spectra in the $g = 2.00$ region of oxygen-evolving Photosystem II particles from spinach. The sample from a redox titration was poised at -370 mV and frozen in the dark. (A), dark; (B), light on; (C), after illumination at 200 K. EPR conditions: microwave Power, 50 mW; modulation amplitude, 1 mT; frequency, 9.1 GHz; temperature, 6 K; instrument gain A and B, 1000, C, 100.

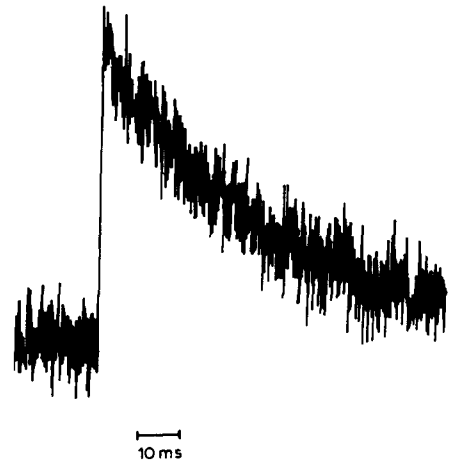

Fig. 6. Kinetics of the decay of the flash-induced doublet spectrum observed in spinach Photosystem II particles prepared as in Fig. 5. Average of 64 flashes. EPR conditions as in Fig. 5 with the spectrometer set to the high field peak of the doublet.

reported by Rutherford and Mathis [10]. Prolonged illumination results in the irreversible formation of the signal; however, following short illumination periods part of the signal is reversible (Fig. 5) and can be induced by flash illumination. The half-time of decay is about 30 ms (Fig. 6). The ability to induce the signal at 6 K was detected only with the -330 mV wave of the titration.

Fig. 7 shows a titration of the redox dependence of triplet formation. Triplet formation appears with $E_m \simeq -430$ mV and is lost again at $E_m \simeq -600$ mV. Rutherford et al. [11] reported that triplet formation in a spinach preparation increased with Q_L reduction. However, they did not carry out a titration, but used samples poised at a few widely spaced potentials and would not have detected that triplet formation actually occurs at a potential about 100 mV more negative than Q_L reduction, as shown in Fig. 7. They reported that triplet formation can be seen in oxidised samples, is lost at approx. 0 mV and reappears at low potential. In our preparation triplet formation in oxidised samples was not observed consistently. In those preparations where it was observed it was decreased by reduction of Q_H and increased again at -430 mV.

The titrations shown in Fig. 3 and 4 were

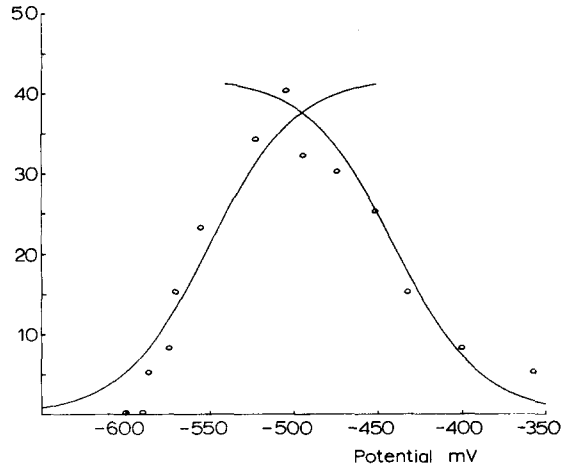

Fig. 7. Redox titration at pH 10.0 of the reaction centre triplet, observed under continuous illumination, of *C. reinhardtii* preparations. The curves drawn are the theoretical curves for a one-electron transition with $E_m = -430$ mV and -570 mV. Ordinate: signal amplitude (arbitrary units)

carried out at pH 10 to allow the lowest potentials to be obtained. However, similar results were obtained at pH 8.0 and 7.0 within the accessible potential range. No pH dependence was observed for any of the transitions, in agreement with our earlier observations that the potential of the iron-quinone is pH-independent in the *C. reinhardtii* preparations. The C550 absorption change also shows a pH-independent midpoint potential in this preparation [21], although it and the fluorescence-detected components Q_H and Q_L do show pH dependence in chloroplast preparations. We have, however, carried out similar titrations of split radical induction on unfractionated *C. reinhardtii* membranes and obtained essentially the same results as in the detergent particles.

Discussion

These experiments provide information about the electron-acceptor complex of Photosystem II and indirectly about the electron-donor complex. Five redox-dependent changes can be detected, but the identification of the components involved is difficult as four of them are measured indirectly. We had expected the results to be analogous to those obtained in purple bacteria, as the components which can be directly detected are apparently the same. The results are, however, much more complicated than in purple bacteria and the analogy may in fact be misleading.

If the changes detected in these titrations are considered in redox potential order, the first is the reduction of the iron-quinone at about 0 mV. This is paralleled by the loss of photoinduction of a $g = 2.00$ radical of the electron donor, signal II_{LT}, and also of the spin-polarised triplet seen under oxidising conditions. This potential corresponds to Q_H in fluorescence titrations. Thielen and Van Gorkom [24] have suggested that Q_H is an artefact caused by the presence of mediators, and that an acceptor at +100 mV is associated with a pool of PS II complexes, termed β-centres, which are distinguished by chlorophyll fluorescence induction measurements. We cannot obtain titrations without mediators because the iron-quinone equilibrates very poorly with the medium in the absence of mediators. Pyocyanine affects some of the results we obtain but not the measured potential of the iron-quinone. Our results do not conclusively allow us to distinguish whether Q_H might be a component of β-centres. However, our results show that the acceptor is present in preparations of spinach grana which would only be expected to contain α-centres, and, as discussed below, our experiments suggest that the acceptor is in the same reaction centre as the −300 mV acceptor (Q_L).

The second change which can be detected is the appearance of the split radical signal following 200 K illumination. In the absence of pyocyanine 90% of this signal appears with E_m around −300 mV. This signal is thought to reflect the photo-reduction of the pheophytin and the signal is split at very low temperatures due to magnetic interaction with an iron atom and a semiquinone anion. We have been unable to detect an EPR signal associated with a component with $E_m = -300$ mV directly. When pyocyanine is present, a large part of the split radical signal appears in parallel with the reduction of the iron-quinone Q_H. We propose that this is because pyocyanine can act as an electron donor at 200 K, allowing an additional electron to pass through the reaction centre. When this occurs, 200 K illumination would result in reduction of the pheophytin apparently in parallel with the reduction of the 0 mV iron-quinone component. This shift suggest that the 0 mV (Q_H) and −300 mV (Q_L) components are sequentially reduced in the same reaction centre. It could be suggested that the −300 mV step also reflects the reduction of a mediator which acts as an electron donor. We have carried out titrations with different mediator combinations with no effect on the −300 mV step in the titrations. We conclude therefore that the development of the ability to induce the split radical signal around −300 mV reflects the reduction of a component of the electron-acceptor complex. A low-potential step in the titration of the induction of the split radical signal in *Rhodopseudomonas viridis* was interpreted [14] as being due to changes in the interaction with the iron atom after double reduction of the secondary quinone acceptor, Q_B. This is unlikely to be the explanation in Photosystem II as the *C. reinhardtii* preparation does not contain the secondary quinone [17]. The ability to form the split radical is lost at a more oxidised potential ($\simeq -530$ mV)

than that at which the pheophytin is reduced. It seems likely that this disappearance reflects the double reduction of the quinone giving rise to the interaction. We have been unable to measure this directly, as the iron-quinone cannot be detected at the alkaline pH required for this titration.

In purple bacteria the ability to form the spin-polarised reaction centre triplet on 6 K illumination parallels the reduction of the primary quinone acceptor [13]. This is not the case in Photosystem II. The reaction centre triplet appears as a component with $E_m \simeq -430$ mV is reduced, and is lost again around -600 mV. The -600 mV change reflects the reduction of the pheophytin, in agreement with the measurements of Rutherford et al. [11] and Klimov et al. [1]. The increase in triplet yield at -430 mV represents the reduction of an acceptor which normally takes electrons from the pheophytin. If there is an acceptor with $E_m \simeq -430$ mV, two electrons must be transferred through the reaction centre to induce split radical formation with $E_m \simeq -300$ mV. This would be possible, even in kinetic experiments with short flash illumination, if on the initial illumination an electron is transferred from P-680 to the -430 mV component and the P-680$^+$ is rereduced by a fast low-temperature donor. Continuing illumination or subsequent flashes would then result in reduction of the pheophytin. This charge separation would be expected to have a very short lifetime (less than 10 ns [25]). However, the observation that in a small proportion of centres (approx. 1%) pheophytin reduction can be observed at 6 K as a reversible process with $t_{1/2} \simeq 30$ ms and that reduced pheophytin slowly accumulates irreversibly at 6 K suggests that the fast donor is rereduced by secondary donors and can then compete with the back reaction from the pheophytin to P-680$^+$. The very slow back reaction time for the reversibly induced signal indicates that the back reaction is between the pheophytin and a secondary donor to P-680.

As the redox potential of the -430 mV component was characterised in samples treated with high-detergent concentrations, it would be possible that it reflected a damaged form of the -300 mV acceptor. This is unlikely as we have made measurements of triplet formation and split radical induction in the same samples, and there is clearly a 100 mV difference between them. Triplet formation has also been seen in samples with the iron-quinone oxidised. It seems likely that in these oxidised samples low temperature illumination results in the irreversible oxidation of the fast donor detected as signal II_{LT}. P-680$^+$ is then no longer reduced by the fast donor and a detectable proportion of P-680$^+$ pheophytin pairs decay through the back reaction from the pheophytin via the triplet state of P-680.

These experiments suggest that there are four membrane-bound electron acceptors which can function at low temperatures in Photosystem II. They also show that two, and in some situations three, electrons can be transferred through the reaction centre from electron donors at low temperature. In samples with damaged donor systems (alkaline pH or high detergent treatment) this electron transfer occurs only at higher temperatures. A number of chloroplast components are known to undergo photooxidation at very low temperatures, including cytochrome *b*-559 and signal II_{LT}, and the S_1 state of the water-oxidising enzyme at 200 K and these could all provide electrons to reduce the first donor to P-680$^+$. Electron donation at 200 K may be nonspecific as exogenous donors such as pyocyanine may also function, although very slowly.

The interpretation of these experiments is difficult; but the sequential appearance of the observed changes in redox titrations and the fact that when an additional electron donor is available the apparent sequence is moved on one stage suggests to us that the components are all in the same reaction centre. The results do not show whether the acceptors or donors are arranged in a linear chain or in a branched system. We would propose that a linear chain with acceptors spaced at about 150–200 mV intervals is likely to be the most favourable arrangement and that the organisation of electron acceptors in Photosystem II is similar to Photosystem I [22], rather than the purple bacteria where a single-step electron transfer over 500 mV is thought to occur. Fig. 8 shows a diagrammatic representation of the organisation of the Photosystem II reaction centre.

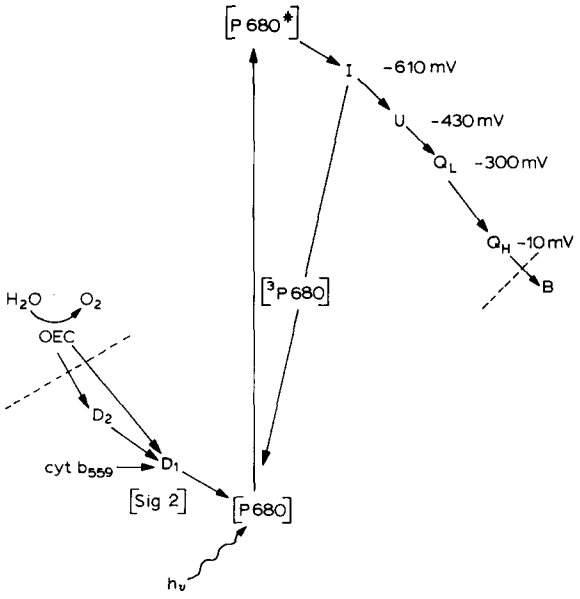

Fig. 8. Diagrammatic representation of the electron-transfer pathway of the Photosystem II reaction centre. P-680: the reaction centre chlorophyll; P-680*: excited singlet state of the reaction centre chlorophyll; ^{3}P-680: triplet state of the reaction centre chlorophyll; D_1, D_2: donors to the reaction centre chlorophyll; I: pheophytin intermediary electron carrier; U, Q_L: unidentified electron carriers; Q_H: the iron-quinone electron acceptor; B: secondary quinone acceptor; OEC: oxygen-evolving complex.

Acknowledgements

This research was supported in part by grants from SERC and the Royal Society. We are grateful to Ms. K. Perks and Ms. L. Tilling for technical assistance.

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