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Biochimica et Biophysica Acta 1229 (1995) 202–207

BBA Biochimica et Biophysica Acta

A change in the midpoint potential of the quinone Q_A in Photosystem II associated with photoactivation of oxygen evolution

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Received 15 August 1994; revised 17 November 1994; accepted 23 December 1994

Abstract

The effect of photoactivation (the assembly of the Mn cluster involved in oxygen evolution) in Photosystem II (PS II), on the redox midpoint potential of the primary quinone electron acceptor, Q_A, has been investigated. Measurements of the redox state of Q_A were performed using chlorophyll fluorescence. Cells of *Scenedesmus obliquus* were grown in the dark to obtain PS II lacking the oxygen-evolving complex. Growth in the light leads to photoactivation. The midpoint potential of Q_A was shifted, upon photoactivation, from +110 mV to −80 mV. In cells of a low-fluorescence mutant, LF1, that is unable to assemble the oxygen-evolving complex but that has an otherwise normal PS II, the higher potential form of Q_A was found. NH_2OH treatment of spinach PS II, which releases the Mn and thus inactivates the oxygen-evolving complex, causes an upshift of the redox potential of Q_A (Krieger and Weis (1992) Photosynthetica 27, 89–98). Oxygen evolution can be reconstituted by incubation in the light in the presence of $MnCl_2$ and $CaCl_2$. Such photoactivation caused the midpoint potential of Q_A to be shifted back from around +55 mV to lower potentials (−80 mV), typical for active PS II. The above results indicate that the state of the donor side of PS II has a direct influence on the properties of the acceptor side. It is suggested that the change from the high- to the low-potential form of Q_A may represent a mechanism for protection of PS II during the assembly of the O_2-evolving enzyme.

Keywords: Photosynthesis; Photosystem II; Photoinhibition; Fluorescence; Redox titration

1. Introduction

It has been observed that, upon inhibition of photosynthetic oxygen evolution, the midpoint redox potential of the primary quinone acceptor of Photosystem II (PS II), Q_A, measured using the yield of chlorophyll fluorescence, is shifted by approx. 150 mV to a more positive potential [1,2]. This was the case whether inhibition was achieved by destruction of the manganese cluster (using NH_2OH treatment) or by removal of Ca^{2+} (using low pH) [1]. It was suggested that there was a direct link between the activity of the water-splitting complex and the acceptor side of PS II. However, the possibility could not be excluded that the treatments used had a direct effect upon Q_A.

The release of Ca^{2+} from PS II, induced by low pH, was suggested to be of physiological relevance since, during high-intensity illumination, the pH in the thylakoid lumen decreases and may reach levels where oxygen evolution is inhibited [1]. Ca^{2+}-depleted PS II is characterised by a lowered yield of chlorophyll fluorescence and it has been suggested [1] that this process may contribute to the lower level of fluorescence seen in chloroplasts and leaves when they are illuminated with high-intensity light (for a review see [3]). It has been suggested that a shift in the E_m of Q_A may play a role in protecting PS II from damage [1]. However, whether this process occurs in vivo has been questioned, since it is not certain that the pH in the thylakoid lumen is sufficiently low to induce Ca^{2+} release [4,5]. Nevertheless, inactive states of the PS II reaction centre do occur under physiological conditions, in particu-

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenolindophenol; Mes, 4-morholineethanesulfonic acid; Ph, pheophytin; PS II, Photosystem II; Tyr, tyrosine.

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SSDI 0005-2728(95)00003-8

lar during chloroplast development. The PS II holoenzyme can, in some organisms (e.g., the green algae *Scenedesmus*, *Chlamydomonas* and *Chlorella*), be synthesised independently of light, whilst assembly of the functional Mn cluster occurs subsequently and requires illumination (so-called photoactivation) [6]. Even during normal growth in higher plants, it is expected that all PS II centres exist for a certain time in a state in which the Mn-cluster is absent. Reaction centres in which the water splitting complex is inactive are thought to be particularly vulnerable to light-induced damage [7–10]. Charge separation still occurs in such centres, but the normal pathway for reduction of the oxidised primary chlorophyll donor, $P680^+$, is inhibited. $P680^+$ is known to be highly oxidising and may damage the reaction centre ('photoinhibition').

Cells of the green alga *Scenedesmus obliquus* can be grown in the dark in a glucose-containing medium. Under such conditions, PS II is assembled normally but the water-splitting complex is not active. Upon transfer to light, photoactivation occurs to give fully functional reaction centres [11–13]. A mutant of *Scenedesmus*, termed LF1, deficient in an enzyme required for processing of the D1 polypeptide is also able to synthesise the PS II holoenzyme but is unable to undergo photoactivation [11–14]. *Scenedesmus* therefore provides an ideal experimental system for studying the effect of photoactivation on the redox properties of Q_A without employing non-physiological treatments. In addition, PS II in vitro that has been treated to remove the manganese cluster (e.g., by NH_2OH) can be reactivated by illumination in the presence of both calcium and manganese chlorides [15,16]. Addition of either salt alone is not sufficient for reactivation [17,18]. We have used both of the above experimental systems to investigate the effect of photoactivation on the midpoint potential of Q_A using chlorophyll fluorescence.

2. Materials and methods

Wild-type and LF1 mutant cells of *Scenedesmus obliquus* were grown in the dark or at a low light intensity (30 $\mu E\,m^{-2}\,s^{-1}$) in enriched medium as described in Ref. [19]. Cells were harvested after 3–4 days and thylakoids were prepared using the method described by Metz and Seibert [20]. PS-II-enriched membranes from market spinach were prepared essentially as in [21] with modifications as described in [22].

Where PS II samples were treated to inhibit O_2 evolution, this involved incubation for 1 h on ice, in the dark, in 5 mM NH_2OH, in a buffer containing 400 mM sucrose, 15 mM NaCl and 50 mM Mes (pH 6.5), followed by two washes in buffer without NH_2OH. Photoactivation was conducted in the same buffer using a final chlorophyll concentration of 250 $\mu g\,ml^{-1}$. Samples were incubated at room temperature and illuminated with white light, using an intensity of 33 $\mu E\,m^{-2}\,s^{-1}$ with the addition of $MnCl_2$ and $CaCl_2$ as indicated. 6 μM DCPIP was used as an electron acceptor [23]; under our conditions other electron acceptors, e.g., *p*-phenylbenzoquinone, were not found to give a better yield of reactivation [23]. Reactivation reached a maximum after 40 min illumination.

For redox titrations, samples were placed, at a concentration of approx. 50 (μg Chl) ml^{-1}, in a Hansatech DW2 oxygen electrode chamber (Hansatech, King's Lynn, UK) and maintained at all times under argon. The redox potential was measured at 15° C by means of a platinum electrode, with a calomel reference electrode (Russell pH, Auchtermuchty, UK) inserted through a seal in the top of the DW2 chamber and connected to a Tacussel pH/millivolt meter (Tacussel, Villeurbanne, France). Measured redox potentials were normalised to the standard hydrogen electrode, calibrating the electrode using saturated quinhydrone (potential = 286 mV at pH 6.5, 25° C). Reductive titrations were performed by gradual addition of sodium dithionite (in 0.5 M Mes, pH 6.5), oxidative titrations by addition of potassium ferricyanide. During titrations, additions of dithionite did not significantly change the pH of the medium. No redox mediators were used because they have been shown to influence redox titrations of Q_A in active PS II, probably by over-reducing the Mn cluster and thereby inactivating the enzyme [2].

Fluorescence was measured through the side window of the chamber using a PAM 101 fluorimeter (Walz, Effeltrich, Germany). Fluorescence was measured using the weak measuring light of the PAM fluorimeter set to 1.6 kHz, as described previously [1].

Fluorescence induction curves were measured using laboratory-built equipment, essentially as described in Ref. [24].

3. Results

Fig. 1 shows redox titrations of the chlorophyll fluorescence yield in thylakoids isolated from *Scenedesmus* grown in the dark or in the light and also from the LF1 mutant. In light-grown material (circles), the midpoint potential is approximately −80 mV. In dark-grown cells (triangles) the potential is +110 mV. These values are consistent with measurements on spinach PS II samples, where, previously, we found that the midpoint of Q_A in intact samples is -81 ± 16 mV and is shifted to $+64 \pm 25$ mV after treatment to inhibit oxygen evolution [2]. The data from light-grown LF1 thylakoids (squares) show the same potential as dark-grown wild-type material. In all cases, the data fit reasonably well to theoretical one electron Nernst curves and show little or no hysteresis. There was a small (10%) irreversible increase in the fluorescence level at high potentials which occurred during the course of the titrations in the light-grown wild-type sample. The origin of this effect is not clear and data points at positive potentials have been ignored in estimating the midpoint potential of Q_A.

Light-grown, dark-grown and LF1 mutant *Scenedesmus* cells differed significantly in their fluorescence characteristics (data not shown). The variable fluorescence of light-grown wild-type cells, normalised to the dark level, was 2.5-times greater than in dark-grown. In the LF1 mutant, the variable fluorescence was even lower than in dark-grown cells. Measurements of the kinetics of fluorescence induction, in the absence of DCMU, are 5-fold faster in dark-grown than in light-grown wild-type material, indicating that electron transfer to the plastoquinone is inhibited in dark-grown cells. This is consistent with previous observations from *Chlamydomonas reinhardtii*, where dark-grown cells were found to contain mostly so-called 'non-Q_B-reducing' PS II centres [25]. It seems likely that non-Q_B-reducing centres are equivalent to centres with a shifted midpoint potential of Q_A and this shift could be the sole reason for the impaired electron transfer in non-Q_B-reducing centres.

In Fig. 2 typical redox titrations are shown from NH_2OH-treated PS-II-enriched membranes incubated in the presence of $CaCl_2$ and $MnCl_2$. NH_2OH-treated PS II has the high-potential form of Q_A [1] and this is not affected by addition of calcium and manganese in the dark (midpoint potential: +55 mV for the titration shown). However, incubation in the light in the presence of these salts leads to a shift in the midpoint potential to a low-potential form (−80 mV), as found in active centres. This treatment brings about reactivation of at least some of the oxygen-evolving capacity [16]. Even under optimal conditions, reactivation gave only 40% of the maximal rate of oxygen evolution seen before NH_2OH treatment (not shown), consistent with levels of reactivation seen previously [26]. The inactive centres are probably photodamaged. Callahan et al. [7] observed that 40 min illumination

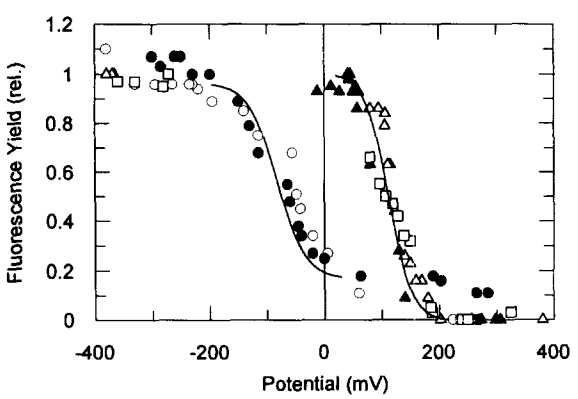

Fig. 1. Redox titrations of chlorophyll fluorescence in thylakoids isolated from light-grown (circles), dark-grown (triangles) wild type and light-grown LFI mutant (squares) of *Scenedesmus obliquus*. Closed symbols indicate oxidative titration points, open symbols, reductive titration. The initial fluorescence level at positive potentials was set to 0 and contributes to 30% of the maximal fluorescence level. The curves shown are one-electron Nernst curves fitted to the data points with E_m values of −80 mV and 110 mV. No mediators were used in these titrations (see [2]).

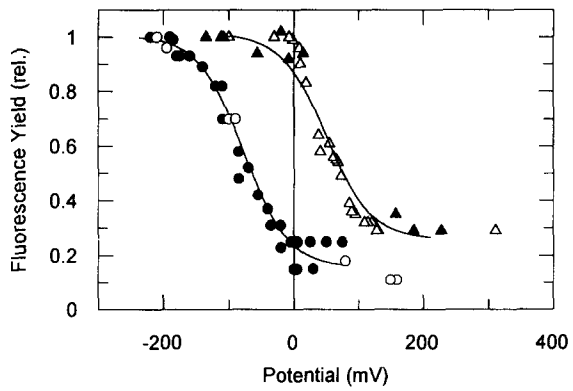

Fig. 2. Redox titrations of fluorescence in NH_2OH-treated PS-II-enriched membranes incubated for 40 min in the presence of 100 μM $MnCl_2$, 50 mM $CaCl_2$ and 6 μM DCPIP either illuminated with 33 $\mu E m^{-2} s^{-1}$ (circles) or in the dark (triangles). Oxidative titrations are represented by filled symbols, reductive titrations by open symbols. The curves shown are one-electron Nernst curves fitted to the data points with E_m values of −80 mV and 55 mV. No redox mediators were used in these experiments (see [2]).

of NH_2OH-treated PS II at 30 $\mu E m^{-2} s^{-1}$ caused a loss of approx. 60% of electron transfer activity measured as the rate of photooxidation of NH_2OH with viologen as acceptor and it is probable that similar damage occurs during our photoactivation treatment.

The fluorescence titration shows only a single, low-potential, component. We explain this as arising from active centres, whilst photodamaged centres presumably do not contribute to the fluorescence titration. This explanation is supported by the observation that the light-induced variable fluorescence in photoactivated samples was lower (about 40%) than in control samples and could not be increased by addition of dithionite. It has been reported that photodamaged PS II shows a quenched level of variable fluorescence that is not reversed by reductants (see, for example, Ref. [27]) and would not, therefore, be detected during titration of Q_A. This is in contrast to the situation where the water-splitting complex is inhibited but the reaction centre is otherwise functional. Under the latter circumstances, variable fluorescence is quenched due to inhibition of electron donation but a high fluorescence state can be regenerated by (chemical) reduction of Q_A (see, for example, Ref. [3]).

In one titration of PS II membranes which had been photoactivated in vitro, a value of −25 mV was obtained rather than −80 mV as shown in Fig. 2. We consider this result as another example of the occasional scatter in the E_m values, despite the lack of scatter in the data points, which was described earlier [2]. This was attributed to electrode malfunction: a problem to which these experiments seem to be particularly prone, probably due to the requirement that no redox mediators be used [2]. Despite this problem, we observed that all PS II samples which were active in oxygen evolution had lower E_m values for Q_A/Q_A^- than was found in nonactive PS II.

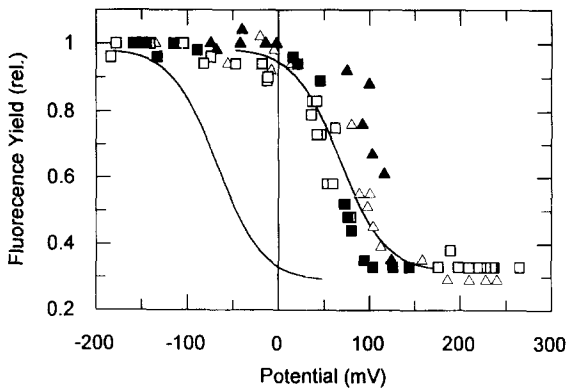

Fig. 3. Redox titrations of fluorescence in NH_2OH-treated PS-II-enriched membranes incubated for 40 min with 33 $\mu Em^{-2}s^{-1}$ light in the presence of 6 μM DCPIP and either 100 μM $MnCl_2$ (triangles) or 50 mM $CaCl_2$ (squares). Oxidative titrations are represented by filled symbols, reductive titrations by open symbols. The curve at high potential is a one-electron Nernst curve fitted to the data points with an E_m value of 70 mV. The curve at low potential is a one-electron Nernst curve with an E_m −80 mV and is taken from Fig. 2 and shown for comparative purposes. No redox mediators were used in these experiments (see [2]).

Previously, it has been seen that both manganese and calcium ions, as well as the presence of an electron acceptor, are necessary for the reconstitution of the oxygen-evolving capacity in NH_2OH-treated PS II [15,16]. In order to distinguish whether both these ions are required for the shift in the potential of Q_A, redox titrations were conducted in the presence of each salt separately (Fig. 3). Incubation of samples in the light with only calcium or manganese chloride was not sufficient to bring about the shift from high- to low-potential Q_A. The only conditions in which this reversal was seen were those under which oxygen evolution could be reconstituted. Whilst in the presence of Mn^{2+} alone the manganese cluster could be assembled, it was only upon addition of Ca^{2+} that this complex became active [17]. Under our conditions, addition of Ca^{2+} in the dark to samples preincubated in the light with Mn^{2+} still gave practically no activity. We were unable, therefore, to determine whether Ca^{2+} is responsible for the shift in the midpoint potential of Q_A during photoactivation. However, in view of earlier work on Ca^{2+}-depleted systems [1], this seems to be the most probable conclusion.

4. Discussion

The data presented in this paper, in combination with earlier data on the effects of damaging the donor-side of PS II, provide strong evidence to support the idea that it is the presence of the active oxygen-evolving complex itself, and hence the structure of the electron donor side of PS II, that determines the midpoint potential of Q_A, rather than an additional effect of the various treatments used. In particular, the presence of a high-potential form of Q_A in the LF1 mutant, which has a normal PS II, grows under normal conditions and lacks only an enzyme necessary for terminal processing of D1 [13,14], strongly indicates that the redox state of Q_A is related to the activity of the oxygen-evolving complex. How the status of the donor side is transmitted to the acceptor side of PS II is not clear; however, this presumably involves long-range structural changes occurring within the protein. As in other cases of conformational changes of proteins, it seems that the effect is correlated with the ligation of a metal ion. A well-studied example for a Ca^{2+}-binding protein is calmodulin, where, upon Ca^{2+} binding, the alpha helicity of the protein changes [28].

Additionally, it is interesting to note that the midpoint potential of cytochrome *b*-559 undergoes a redox shift under the same conditions in which we observed a change of the midpoint potential of Q_A The high-potential form of Q_A seems to be correlated with the occurrence of the low-potential form of cytochrome *b*-559 in *Scenedesmus* prior to photoactivation [11,12,29] and in NH_2OH-treated PS II [30]. A functional relationship, however, between the conversion of the low-potential to the high-potential form of cytochrome *b*-559 and the process of photoactivation was not evident in the work of Tamura and Cheniae [30].

The observation that a high-potential form of Q_A is seen in PS II reaction centres prior to photoactivation may be of physiological importance. The presence of a high-potential form of Q_A in non-activated PS II might be of benefit for several reasons.

(1) In the presence of the high-potential form of Q_A, electron transfer from Q_A^- to Q_B will be inhibited due to a decrease in the equilibrium constant for the reaction $Q_A^-Q_B \leftrightarrow Q_AQ_B^-$. Indeed, inhibition of electron transfer from Q_A^- was observed in PS II prior to photoactivation (see Results and also [25]). In functional PS II, the equilibrium constant for this reaction has been estimated to be around 20 (reviewed in Ref. [31]). This means that, with an $E_m \approx -80$ mV for Q_A/Q_A^-, the functional E_m of the Q_B/Q_B^- couple is close to 0 mV. A shift in the E_m of Q_A to +65 mV would tip the equilibrium towards Q_A, assuming that the E_m of Q_B/Q_B^- remained unchanged. The effect of the inhibition of electron transfer might be expected to be influenced by pH, since the E_m of Q_B/Q_B^- is thought to be pH-dependent, while that of Q_A/Q_A^- is pH-independent. Thus, at lower pH values the electron transfer inhibition would be less marked.

If the E_m of Q_B were also positively shifted in inactive PS II, then the inhibition of electron transfer out of the reaction centre would still be expected, since the high-potential E_m of Q_A is close to or even higher than the functional E_m of the plastoquinone pool (N.B. the pH-dependent $E_{m7} \approx 120$ mV; it is expected to decrease by 59 mV per pH unit as the pH is raised and the stromal pH is likely to be higher than pH 7, see [31]).

A consequence of the inhibition of electron transfer resulting from the high potential of Q_A, is that charge

recombination of $Tyr_Z^+Q_A^-$ will compete with forward electron transfer. This is likely to be beneficial for PS II. If normal electron transfer out of the reaction centre were allowed to occur, multiple photochemical charge separations, in the absence of the water splitting complex, would generate long-lived, oxidising species (in particular $P680^+$). Such species are thought to cause damage to the reaction centre [9].

(2) During photoactivation, the Mn^{2+} ions are thought to be oxidised by Tyr_Z^+ in a series of reactions, at least one of which has a low quantum yield (see, for example, [26,30]). It is expected that the efficiency of manganese oxidation will be related to the lifetime of the Tyr_Z^+. The upshift of the midpoint potential of Q_A^- might result in a slower $Tyr_Z^+Q_A^-$ recombination reaction. It is, however, difficult to predict the effect of the shift in the E_m of Q_A/Q_A^- on this recombination reaction, since many of the relevant parameters remain unknown in PS II. If, as we suggest below, there is a change in the reaction pathway for $P680^+Q_A^-$ recombination, it seems quite possible that a slight deceleration occurs. The reported kinetics for $Tyr_Z^+Q_A^-$ recombination in Tris-washed material, in which the Mn is removed, is $t_{1/2} = 120$ ms [32].

(3) Charge recombination at physiological temperatures in active PS II reaction centres (with Q_A in the low-potential form) is thought to occur via reformation of the $P680^+Ph^-$ state which recombines to give a high yield of P680 triplet [33]. It has been suggested that charge recombination in the intact enzyme under physiological conditions might lead to triplet-mediated formation of singlet oxygen which is responsible for protein damage [34].

It has been observed in the bacterial reaction centre that the pathway of the recombination reaction for $P^+Q_A^-$ is determined by the free energy difference (ΔG) between $P^+Q_A^-$ and P^+Ph^- [35–38]. When the free energy difference is greater than 400 meV, the recombination occurs via a direct route. In contrast, when the $\Delta G < 400$ meV, the back-reaction takes place via a route in which a P^+Ph^- state is formed [36]. The high potential of Q_A seen in non-photoactivated and inhibited PS II is expected to result in a larger ΔG between $P680^+Q_A^-$ and $P680^+Ph^-$ than in active samples. It is thus possible that the change in the E_m of Q_A could result in a change in the route of the $P680^+Q_A^-$ recombination reaction: being indirect, via the $P680^+Ph^-$ state, in active centres, but direct in inactive centres. If this hypothesis is correct, then the upshift of the potential of Q_A in PS II could have a very significant protective effect.

It should be possible to test the validity of this idea by considerations of thermodynamic and kinetic data already published in the literature. From electroluminescence experiments the free energy between the $P680^+Ph^-$ and the $P680^+Q_A^-$ states has been estimated to be 330 meV [39] in oxygen-evolving PS II (see also [33] for a similar estimate based on other thermodynamic and kinetic arguments) [3]. This is consistent with an estimate of the activation energy for the $P680^+Q_A^-$ recombination reaction (given in [33] from data in [40]) in active PS II. From the free energy difference between $P680^+Ph^-$ and $P680^+Q_A^-$ ($\Delta G \approx 330$ meV [39]), and the forward electron tranfer rate for electron transfer from Ph^- to Q_A ($t_{1/2} = 300$ ps; see, for example, Ref. [46]), one can use the the relationships, $\Delta G = -RT\ln K$ and $K = k_{forward}/k_{back}$, to estimate the equilibrium constant, K, and back-reaction rate for the $P680^+Ph^- \leftrightarrow P680^+Q_A^-$ reaction in active PS II. The following values are obtained: $K \approx 4 \cdot 10^5$ and $k_{back} \approx 5.7 \cdot 10^3\ s^{-1}$ ($t_{1/2} \approx 120\ \mu s$). The actual rate of recombination of $P680^+Q_A^-$ at ambient potentials is unknown, since the extremely efficient donor, Tyr_Z, is present in the functional enzyme. However, a rate which is equal to or slower than 120 μs seems reasonable (see [33,47]) and would be consistent with the occurence of recombination via $P680^+Ph^-$.

In inactive PS II, however, the free energy difference between the $P680^+Ph^-$ and the $P680^+Q_A^-$ states is increased by 145 meV to 475 meV, due to the shift in the E_m of Q_A. Thus the equilibrium constant and back-reaction rate become $K \approx 1.1 \cdot 10^8$ and $k_{back} \approx 2.1 \cdot 10^1$ ($t_{1/2} \approx 35$ ms), assuming the forward electron transfer rate to be unchanged. In inactive centres, the $P^+Q_A^-$ recombination reaction occurs in 200 μs–1 ms [48,49], a value which is inconsistent with a pathway involving Ph^-. Furthermore, the activation energy for $P^+Q_A^-$ recombination in inactive centres has been estimated to be 140–160 meV [48], a value which is too small to allow for a back-reaction involving Ph^-, since the activation energy should include the free energy difference between $P680^+Q_A^-$ and $P680^+Ph^-$ (i.e., 475 meV, assuming the E_m for Ph/Ph^- is the same in active and inactive centres).

To test the suggestion made here that the back reaction pathway may change from a direct to an indirect route upon activation of O_2 evolution, measurements of the temperature dependence of the $P680^+Q_A^-$ recombination reactions and the luminescence yield in active and inactive

[3] We note that if the measured E_m for Ph/Ph^- (-610 mV [41,42]) is used to estimate the free energy difference between $P680^+Ph^-$ and $P680^+Q_A^-$, the values obtained are much too big to allow for a back reaction via Ph^-, even in active centres ($\Delta G \approx 525$ meV). There are several possible reasons for the free energy difference being overestimated when the equilibrium E_m value of Ph/Ph^- is used. (a) The E_m value for Ph/Ph^- was measured at high pH in reaction centres which were modified by double reduction of Q_A ([41,42], see also [43] and [44]). (b) In the $P680^+Ph^-$ radical pair, the presence of $P680^+$ might effectively increase the E_m of Ph/Ph^-, (Brettel, K., personal communication). (c) In the bacterial reaction centre, the P^+Ph^- state involved in the indirect pathway for $P^+Q_A^-$ recombination is a 'relaxed' state which is around 200 meV below the P^+Ph^- state which is formed initially in forward electron transfer [38,45]. The equivalent state in PS II may also be 'relaxed'. We consider, then, that the estimate for the ΔG between $P680^+Ph^-$ and $P680^+Q_A^-$ measured by electroluminescence (i.e., 330 meV [39]) to be more valid than that determined from the redox titrations of Ph/Ph^-.

PS II should be made. The hypothesis predicts that the luminescence yield in inactive PS II would be much smaller than in active PS II.

In conclusion, we consider that the high-potential form of Q_A in non-photoactivated PS II may result in electron transfer properties which allow a compromise between the contrasting requirements existing during photoactivation: the need to minimise photodamage while maximising Mn^{2+} photooxidation.

Acknowledgements

We would like to thank J.R. Bowyer (Royal Holloway College, London) for useful discussions, helpful suggestions and for kindly providing wild-type and LF1 cultures of *Scenedesmus*, A. Seidler for his help in growing the cultures, E. Weis (University of Münster), K. Brettel, P. Mathis, T. Mattioli, J. Sturgis and P. van Vliet for useful discussions and A. Boussac for discussions and for the critical reading of the manuscript. G.N.J. and A.K. were supported by EEC Human Capital and Mobility fellowships.

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