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On the determination of redox midpoint potential of the primary quinone electron acceptor, Q_A, in Photosystem II

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Abstract

Redox titrations of Q_A, the first quinone electron acceptor, have been performed on Photosystem II (PS II) membranes which were either active or inactive in terms of oxygen evolution. The redox state of Q_A was monitored by measuring the chlorophyll fluorescence yield. When titrations were done at room temperature in the absence of mediators, an E_m value of approx. −80 mV was obtained for active centres and approx. +65 mV for inactive centres. These values confirm earlier reports (Krieger, A. and Weis, E. (1992) Photosynthetica 27, 89–98) in which measurements were made under comparable conditions. In addition, we found that these E_m values were independent of pH from pH 5.5 to pH 7.5, the range of pH over which the O_2-evolving enzyme is stable. To understand better the scattered values for the E_m of Q_A which exist in the literature and to assess the validity of the present values, experiments were performed under a range of different titration conditions. Two main experimental factors were found to have a strong influence on the measured E_m of Q_A. First, the presence of redox mediators at low ambient potentials led to an irreversible shift from the low-potential (active) form to the high-potential (inactive) form. This is attributed to the reduction of the Mn cluster which is thought to remain out of equilibrium when titrations are done without mediators. Secondly, upon freezing of samples poised at low potential a change in the redox state of Q_A occurred, as measured by EPR and fluorescence at low temperature. Freezing and thawing of active PS II at potentials where Q_A is chemically reduced results in an irreversible change in the E_m of Q_A from the low-potential to the high-potential form. This is accompanied by inhibition of oxygen evolution. It is suggested that this effect might also be related to the reduction of the Mn cluster which is, in this case, induced by freeze-thawing in the presence of chemically reduced Q_A^-. Based on these observations, it is suggested that most titrations of Q_A in active PS II that have been reported previously suffer from one or both of these unexpected technical difficulties. Thus, the E_m values obtained at room temperature and without mediators are probably those which should be taken into account in understanding the energetics of PS II.

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

1. Introduction

An understanding of the bioenergetics of electron transfer requires a knowledge of the free energy changes involved in each electron transfer step. Consequently, a great deal of effort has been directed towards measuring the midpoint potentials (E_m) of the redox active components in electron transfer proteins through redox potentiometry [1,2].

In Photosystem II (PS II), the photosystem in plants which oxidises water and reduces plastoquinone [3,4], the E_m values of the electron donor side components involved in normal electron transfer are so oxidising that they are not directly measurable. The electron acceptor side components, however, are in many respects similar to those in the purple bacterial reaction centre, being made up of a pheophytin (Ph) and two quinones (Q_A and Q_B) that are associated with an iron atom [5].

In bacteria, the E_m value of Q_A has been established in many species, at a wide range of pH values [2] and with a very extensive range of artificial quinones in the Q_A site

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; Mes, 4-morpholineethanesulfonic acid; Ph, pheophytin; PS II, Photosystem II; Tyr, tyrosine.

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[6]. The information obtained has been central in understanding the energetics of electron transfer reactions in bacterial reaction centres and has contributed to a more general understanding of electron transfer in proteins [7].

In PS II, redox titrations of Q_A have been reported using material from several species and with a range of different experimental approaches but no consensus has emerged concerning the actual E_m value or its pH dependence (see Refs. [8–34] and Fig. 1). The uncertainties concerning the E_m of Q_A in PS II are sometimes glossed over by researchers in the field (e.g., [5]) who choose to cite an early report [23] in which E_m values and pH dependencies in PS II were reported to be similar to those seen for Q_A in bacterial reaction centres [2]: i.e., a pH-dependent E_m ($E_m \simeq -10$ mV at pH 7) which is presumed to function at the E_m value measured at pH values above the pK of the reduced form ($E_m \simeq -130$ mV above pH 8.9). This assumption is not necessarily justified, since there are many conflicting reports and few explanations for the discrepancies. A survey of the literature values for the E_m of Q_A (Fig. 1) illustrates the level of uncertainty that exists.

Recently, it was reported that the E_m of Q_A was affected by treatments which influenced the electron donor side. In particular, treatments which inhibited O_2 evolution by removal of Ca^{2+} shifted the E_m of Q_A from approx. -120 mV to approx. $+40$ mV [22]. This effect was reversed by reconstitution of the Ca^{2+}. This modulation of the redox potential of Q_A could have important functional relevance [22,35,36].

The validity of the measurements indicating high- and low-potential forms of Q_A was recently questioned based on observations by Johnson et al. [37]. These authors showed that recombination of the charge pair Tyr D^+/Q_A^- gives rise to thermoluminescence at the same temperature (around 50° C) in both active and Ca^{2+}-depleted PS II, an observation that would appear to be inconsistent with the occurrence of a shift in the midpoint potential of Q_A

Given this inconsistency and the uncertainties in the measurements of the E_m of Q_A in the literature, it seemed important to assess the reliability of these new measurements reported in [22] and to relate them to those already in the literature by investigating the factors (intrinsic and artifactual) which could be influencing redox titrations of Q_A in PS II. In this way, we hoped to determine whether the new values should be considered as the true values.

In the present work, titrations of Q_A were performed in active and Ca^{2+}-depleted PS II. Comparisons were made of redox titrations in which Q_A^- formation was monitored by EPR at low temperature and by fluorescence at room

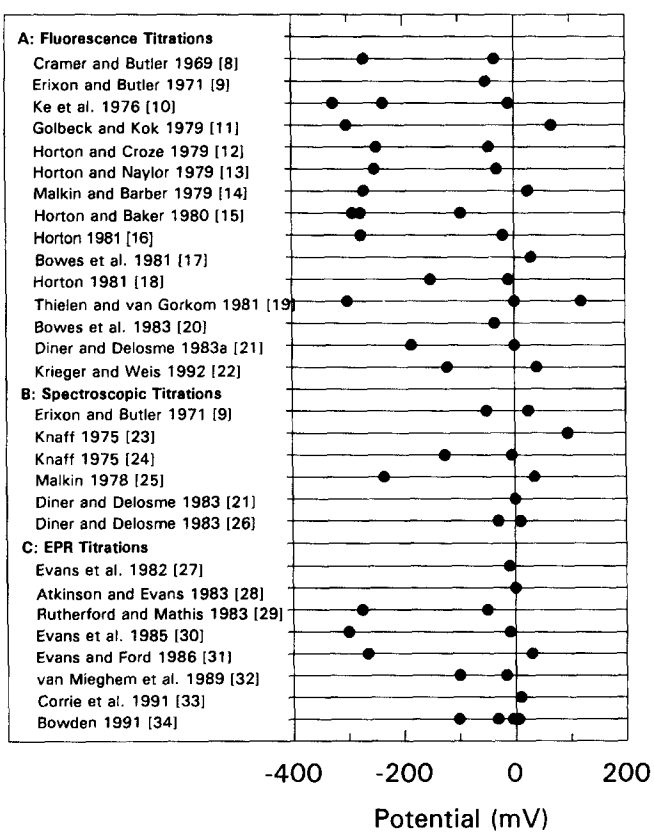

Fig. 1. Midpoint potentials of the Q_A/Q_A^- redox couple reported in the literature. Values shown were measured using different methods in samples from a number of species, under varying conditions (pH, temperature, etc.). In most cases, different combinations of redox mediators were used. In several cases, inhibitors were present or inhibitory treatments were specifically given. In some cases, a dependence of pH or lack thereof was reported. Overall then, the values shown are not necessarily comparable. The figure is shown to illustrate the scatter in reported values.

temperature and in the frozen state. The potential of Q_A was found to be sensitive to experimental factors (the freezing of samples, the presence of mediators) which are encountered in the majority of published redox titrations. The E_m values for Q_A and their pH dependencies which are reported in the present work are proposed to be those with functional relevance in PS II.

2. Materials and methods

PS-II-enriched membranes [38] were prepared from market spinach as described in Ref. [37]. Where samples are described as 'pH-treated' this treatment involved incubation of samples at room temperature for 5 min in light (10–12 $\mu E m^{-2} s^{-1}$) followed by 10 min in darkness in a buffer containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 30 mM glycylglycine (pH 4.2). Samples were then centrifuged and washed in a medium containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 30 mM Mes (pH 6.5).

For redox titrations, samples were diluted to concentrations around 50 (μg Chl) ml^{-1} or, for EPR and some fluorescence measurements, to around 4 (mg Chl) ml^{-1}. In fluorescence titrations, the concentration of the sample did not affect the measurement significantly; however, redox equilibrium was reached more slowly with the higher concentration samples. Samples were placed 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 metre (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. Where measurements were made using redox mediators one of the following mixtures was used: Mixture 1: Varian blue (100 nM; +150 mV), Toluene blue (100 nM; +115 mV), phenazine metasulfate (50 nM; +80 mV), pyocyanine (50 nM; −40 mV) and Nile blue (50 nM; −120 mV), Mixture 2: Indigo tetrasulfonate (100 nM; −46 mV), Indigo disulfonate (100 nM; −125 mV) and Methyl viologen (100 nM; −440 mV).

Fluorescence was measured through the side window of the chamber using a PAM 101 chlorophyll fluorimeter and a fibre optic (Walz, Effeltrich, Germany). Fluorescence was measured using the weak measuring light of the PAM fluorimeter set to 1.6 kHz, as described previously [22]. For measurement of low-temperature fluorescence and EPR, samples were removed from the chamber in the dark using a syringe, placed into appropriate containers and rapidly frozen to liquid nitrogen temperature.

EPR spectra were recorded at liquid helium temperatures, using a Bruker ESR 200 X-band spectrometer equipped with an Oxford Instruments cryostat. The amplitude of the signal for Q_A^--Fe at either $g = 1.82$ or $g = 1.9$ was estimated as described previously [37]. Room-temperature EPR spectra were recorded using a Bruker ESP 300 spectrophotometer.

Low-temperature fluorescence induction curves were measured using laboratory-built equipment, essentially as described in Ref. [39].

Oxygen evolution was measured with a Hansatech oxygen electrode using 0.5 mM *p*-phenylbenzoquinone and 1 mM potassium ferricyanide as electron acceptors. After poising at certain potentials, samples were washed in pH 6.5 buffer to remove excess dithionite.

3. Results

3.1. Fluorescence titrations at room temperature

The reduction of Q_A forming Q_A^- leads to a high fluorescent state [40]. This can be monitored during the course of a redox titration and the E_m of Q_A can thereby be determined. Fig. 2 shows typical redox titrations of the fluorescence yield from active and Ca^{2+}-depleted PS II membranes. These titrations were done on dilute samples (50 μg Chl/ml) in the absence of redox mediators (see the following section). These titrations fit well to theoretical

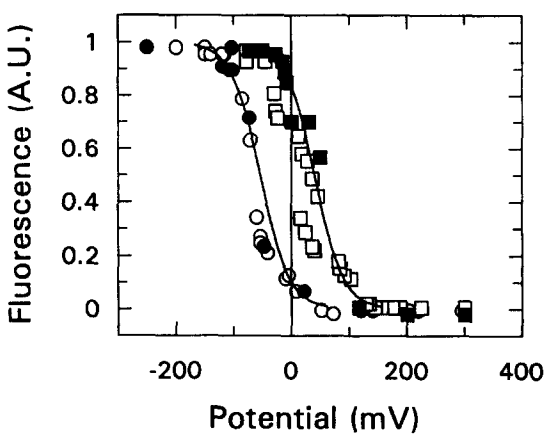

Fig. 2. Typical redox titrations of the yield of chlorophyll fluorescence measured at room temperature in active PS II membranes (circles) and in membranes treated at low pH to inhibit O_2 evolution (squares). Filled symbols, oxidative titration, open symbols, reductive titration. The curves shown represent one-electron Nernst curves fitted to the data. The midpoint potentials of these specific curves are −65 mV and +42 mV for the higher and lower potential, respectively. Note, however, that the mean E_m values, based on multiple repeats of this kind of titration experiment, are −81 mV and +65 mV, repectively (see text).

one-electron Nernst curves and show little or no hysteresis upon comparing titrations in the oxidative or reductive direction. The E_m value for active centres was found to be −81 mV (average of 11 measurements, standard deviation ±16), while the value obtained for inactive samples was +64 mV (average of 13 measurements, S.D. ± 25).

It was observed that the quality of individual titrations was significantly better than the estimated error implies. This reflects variations in the E_m values encountered from titration to titration which we tentatively attribute to electrode performance. It is possible that titrations done without mediators are particularly susceptible to fouling of the electrodes etc. Frequent cleaning and calibration of the electrodes was required. Despite this, many repetitions of the titrations were required to obtain values in which we have reasonable confidence. In addition, when titrations of active and inhibited samples were done on the same day, a difference of around 150 mV was systematically observed despite the variations in the absolute values described above.

These values confirm the earlier report of Krieger and Weis [22], who did redox titrations under comparable conditions. The present results provide a better estimate of the accuracy of this approach. Consistent with the previous observations [22], we observe that the midpoint potential measured in inactive PS II is approx. 150 mV more positive than in active centres.

The pH dependence of the midpoint potential of Q_A under our experimental conditions was investigated between pH 5.5 and pH 7.5. These pH values correspond to the range over which O_2 evolution activity is relatively stable. Table 1 shows data recorded for the midpoint potential of Q_A/Q_A^- at different pH values. No pH dependence is apparent in either active samples or Ca^{2+}-depleted samples.

3.2. The effect of redox mediators on fluorescence titrations

In most redox titrations of Q_A/Q_A^- in the literature, equilibration was ensured by the addition of a cocktail of redox mediators. Such chemicals may give artifacts in the titration by, for example, preferentially quenching fluorescence in one or other redox state (as was demonstrated in, for example, [12]). In the experiments above, as in some earlier measurements [19,22], no mediators, other than dithionite and ferricyanide, were added. The fit of the data

Table 1
pH dependency of the midpoint redox potential of the Q_A/Q_A^- redox couple, measured using room-temperature chlorophyll fluorescence in active and inactivated PS II membranes in the absence of redox mediators

	pH 5.5	pH 6.5	pH 7.5
Active	−90 mV	−81 ± 26 mV	−90 mV
pH-treated	+60 mV	+64 ± 32 mV	+60 mV

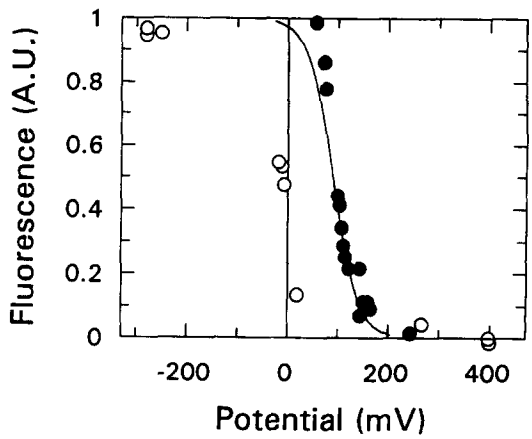

Fig. 3. Typical redox titration of the yield of chlorophyll fluorescence in active PS II membranes in the presence of redox mediators (Varian blue, Toluene blue, phenanzine metasulfate, pyocyanine, nile blue) at low concentrations (see methods). Open symbols, reductive titration, filled symbols, oxidative titration. The reductive titration was performed before the oxidative titration. The curve fitted to the data is a one-electron Nernst curve with an E_m of 85 mV (see text).

to theoretical Nernst curves and the absence of significant hysteresis in the titration indicate that good equilibration was attained under these conditions.

To relate the present titrations to the majority of those reported in the literature, we performed redox titrations of fluorescence in active samples in the presence of mediators (Fig. 3). The mediators were chosen to avoid those known to influence fluorescence and those likely to bind in the binding site of the exchangeable reaction centre quinone, Q_B, i.e., quinone mediators.

In all of our experiments, it was found that addition of mediators (both mixtures 1 and 2, see Materials and methods for details) had significant effects on the titration of Q_A in active samples. In titrations starting at positive potentials then moving the potential to more reducing values by addition of dithionite, the level of fluorescence began to rise at potentials that were consistent with measurements in the absence of mediators but rose more rapidly thereafter in a manner inconsistent with a one-electron Nernst transition. When samples were then re-oxidised, a different, more positive titration curve was obtained. This higher potential curve was found to be reversible and a one-electron Nernst curve was fitted with an E_m of 100 mV (for the data shown), a value close to that seen in titrations of inactive PS II.

It was also found that where the sample had been first incubated with mediators for long periods under mildly reducing conditions (about −150 mV), reversible titrations at potentials consistent with those seen in Ca^{2+}-depleted samples were observed (not shown). In addition, we found that when active samples were incubated for long periods (15 min or more) under very reducing condition (i.e., with an excess of dithionite at pH 7, about −420 mV or lower), even in the absence of mediators, a similar shift in the E_m of Q_A to the high-potential value was observed.

These results are interpreted as follows. The E_m of Q_A in active PS II (i.e., −80 mV), is converted to a high-potential (presumably inactive) form during the course of the redox titration in the presence of mediators. We suggest that a component which remains oxidised and out of equilibrium during redox titrations in the absence of mediators, is reduced when mediators are present (or when incubated for long periods under reducing conditions without mediators). The reduction of this component leads to an irreversible (at least in Nernst terms) change in PS II which is manifest as a shift in the E_m of Q_A to higher potential. We consider it likely that the component in question is the Mn cluster. This will be discussed below.

3.3. Redox titrations of Q_A using EPR

EPR has been used successfully in purple bacteria to titrate Q_A by monitoring the Q_A^-Fe signal directly [41]. In PS II, titrations of the Q_A^-Fe EPR signal have also been reported [27,31–34]. We have seen above, however, that redox mediators and long incubations at low potential lead to a shift in the E_m from the low-potential active form to the high-potential inactive form. Therefore, we attempted to titrate the Q_A^-Fe EPR signal without the use of mediators. Stable and reliable potentials can only be established when samples are more dilute (4 mg Chl/ml) than is optimum for detecting the Q_A^-Fe EPR signals. Confidence in the titrations was obtained by monitoring the fluorescence level of samples during the course of the titrations, prior to extraction of aliquots for EPR. The fluorescence yield under these conditions titrated at potentials consistent with measurements made on more dilute material (not shown).

Fig. 4 shows titrations of the amplitude of Q_A^-Fe EPR signals. Titrations were performed using active and pH-treated PS II samples. The EPR signals arising from Q_A^-Fe, recorded at various potentials in both active and Ca^{2+}-depleted samples are shown in Fig. 5. The form of the Q_A^-Fe signal differs between the two samples, being centered around $g = 1.9$ in the active sample, as normally observed in untreated PS II, but being centered at $g = 1.82$ in treated samples, similar to the signal that is characteristic of PS II under certain other conditions (e.g. following treatment with formate [42]). The $g = 1.82$ form of the Q_A^-Fe signal is also seen in samples that are depleted of Ca^{2+} by treatment with NaCl/EGTA [43]. The difference in midpoint potential seen in the fluorescence titrations (Fig. 2) is not apparent when Q_A^- was measured using EPR (Fig. 4). The $g = 1.9$ form is small and the size of both Q_A^-Fe signals is highly sensitive to the measuring temperature. Hence, Q_A^- is difficult to quantify accurately in samples at the chlorophyll concentration used. It is important to note that the curves shown in Fig. 4 are not fitted to the data but represent the theoretical one-electron Nernst curves for the mean midpoint potentials measured by fluorescence in active and pH-treated samples. Despite the scatter in the EPR data, it is clear that, both in active and inhibited samples, the appearance of the Q_A^--Fe signal occurs at potentials more consistent with the higher potential transition seen in Fig. 2. In addition, we may conclude that there is no obvious correlation between the E_m of Q_A

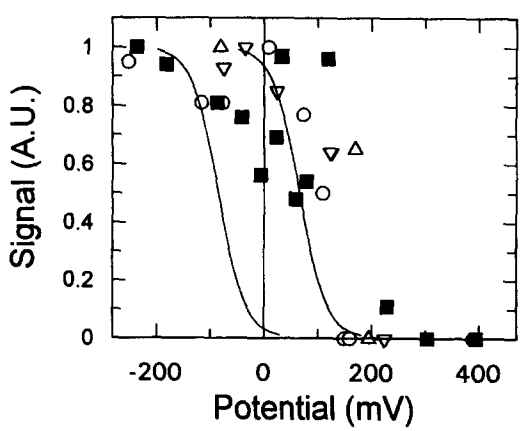

Fig. 4. Redox titrations of Q_A^--Fe EPR signals using active (open symbols) and inactivated (filled symbols) PS II membranes in the presence (circles) or absence (other symbols) of redox mediators as in Fig. 3. Curves shown are not fitted to the data, but represent one-electron Nernst curves corresponding to the average potential recorded for active (−80 mV) and inactive (+65 mV). For the conditions used for EPR measurements see Fig. 5.

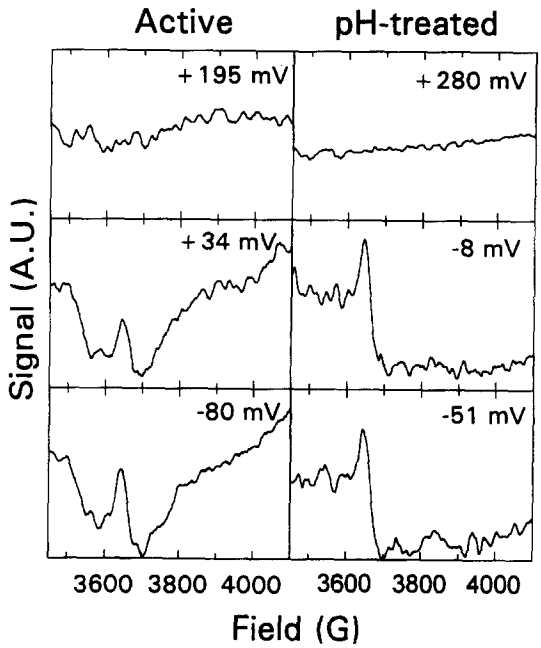

Fig. 5. EPR signals from Q_A^--Fe recorded using active (left) and inactivated (right) PS II membranes poised at the potentials indicated. The broad signal centre around 3525 G in the active samples poised at +34 mV and −80 mV is the so-called $g = 1.9$ form of the Q_A^- Fe signal. The sharper signal with a peak at around 3650 G is the so-called $g = 1.82$ form of Q_A^- Fe. The gain in the left panel is 1.5-times greater than that in the right panel. Instrument settings for measurement of EPR signals as follows: Temperature, 4.6 K; microwave power, 31 mW; modulation amplitude, 22 G; microwave frequency, 9.41 GHz; modulation frequency, 100 kHz.

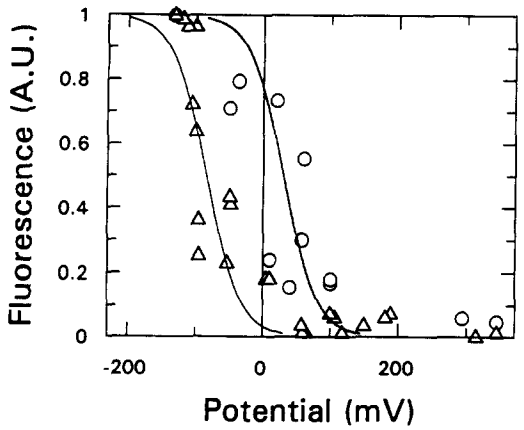

Fig. 6. Redox titration of fluorescence measured at room temperature (triangles) and at 77 K (circles) in active PS II membranes. Each data point was collected using a different sample. Curves shown represent one-electron Nernst curves corresponding to −85 mV and +35 mV.

measured at room temperature and the form of the EPR signal (i.e., $g = 1.82$ vs. $g = 1.9$ forms). The inclusion of redox mediators also had no effect on the apparent redox potential of Q_A as measured by EPR (Fig. 4). The presence of DCMU did not affect the titrations (not shown).

In summary, the low-temperature EPR data indicate that the high-potential form of Q_A seems to be present under all conditions and yet the same sample, when monitored at room temperature by fluorescence, showed the characteristic low-potential/active versus high-potential/inactive phenomenology as described in Fig. 2 and earlier [22]. The most obvious difference between the conditions used for the measurements of the fluorescence and of the EPR was the temperature. It thus seemed possible that freezing of the sample could be affecting the redox titration of Q_A.

3.4. Comparison of fluorescence titrations at room and low temperature

In order to investigate whether freezing has an effect on the redox properties of Q_A, we compared titrations of fluorescence at room and low temperature using active PS II membranes (Fig. 6). As before with active samples, fluorescence at room temperature titrated as a single one-electron component with a midpoint potential of about −80 mV (Fig. 6, triangles). Aliquots poised at various potentials were frozen to 77 K in absolute darkness and fluorescence induction was then recorded (Fig. 6, circles).

Although the scatter in the data make it difficult to estimate accurately a midpoint potential at low temperature, it is clear that the rise in the initial level of fluorescence occurs at more positive potentials than expected from the room-temperature measurements. Here again, it should be noted that the curves shown in Fig. 6 represent theoretical one-electron Nernst curves corresponding to the mean potentials obtained in titrations of room-temperature fluorescence; they are not fitted to the low-temperature data points.

Clearly, the data recorded in active PS II membranes at low temperature fit more closely to the curve expected at room temperature from inhibited samples. From the data in Fig. 6, it appears that the midpoint potential of Q_A/Q_A^- is shifted to more positive values upon freezing and that, as a result, Q_A becomes reduced during freezing in a percentage of centres.

Redox titrations of Q_A measuring fluorescence at room temperature were performed on samples that were frozen and then thawed at a range of potentials. It was found that freshly prepared, non-frozen samples showed redox behaviour which is identical to that seen in samples that had been frozen and thawed at ambient potentials (not shown). However, when samples were frozen under reducing conditions and subsequently thawed, the midpoint potential of Q_A was found to be changed (Fig. 7). The extent of this shift was variable between samples and depended on the specific potential at which the sample was poised prior to freezing. In many cases two waves were seen in the titrations, as in Fig. 7 (squares), where approximately two-thirds of the fluorescence rise is associated with a higher potential wave, the remainder being at a lower potential. The data in Fig. 7 indicate that this freezing-induced change appeared only in those centres where Q_A was reduced.

The phenomena seen in Figs. 6 and 7, whilst both related to effects of low temperature, are clearly different. Measurements performed at low temperature indicate reduction of Q_A at potentials lower than 100 mV, while the damaging effect of freezing and thawing seems to be related to the redox state of Q_A prior to freezing. Thus, at

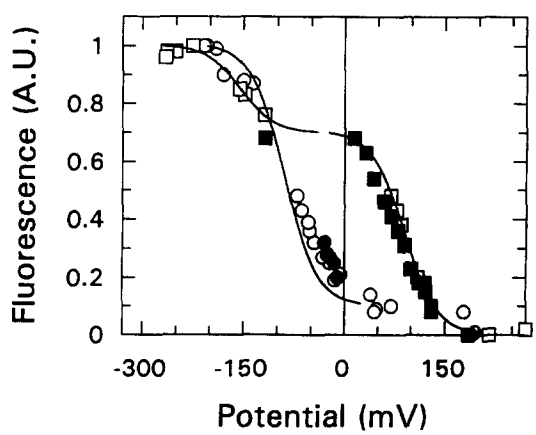

Fig. 7. Redox titrations of fluorescence in active PS II membranes which had been previously frozen and thawed at +170 mV (circles) or at −124 mV (squares). Freeze-thawing was done in darkness. Open symbols, reductive titration, filled symbols, oxidative titration. The curve fitted to the data points shown as circles is a one-electron Nernst curve with an $E_m = -85$ mV. The curve fitted to the majority of the data points shown as squares is a one-electron Nernst curve with an $E_m = +90$ mV, the remaining low-potential points of this titration have been fitted with an arbitary curve.

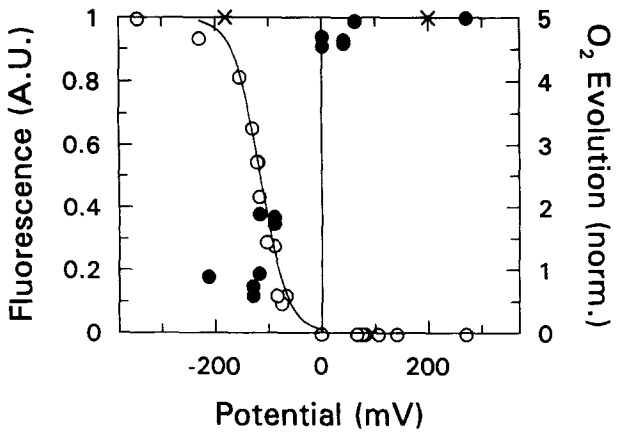

Fig. 8. Redox titration of room-temperature fluorescence (open circles) in active PS II (curve fitted is the one-electron Nernst curve, E_m −85 mV) and measurements of O_2 evolution (filled circles and crosses). O_2 evolution was measured from samples poised at the potentials indicated and then either frozen and thawed in darkness (filled circles) or directly used without freezing and thawing (crosses) to measure O_2 evolution.

potentials between 100 mV and 0 mV the reduction of Q_A, which apparently occurs upon freezing, seems to be reversed upon thawing. In contrast, at lower potentials, those centres which are frozen with Q_A reduced in the equilibrium redox titration, undergo an irreversible change which results in a shift of the E_m of Q_A to high potential.

Despite this complication, the results clearly show that freezing of PS II at low ambient potentials leads to a shift in the measured value for the E_m of Q_A from low potential to a higher potential. The redox titrations of Q_A in active PS II in the literature in which samples were frozen prior to measurement are very likely to be affected by this phenomenon.

3.5. *Effects on O_2 evolution activity*

In previous work, shifts in the midpoint potential of Q_A have been associated with treatments that inhibit oxygen evolution (Ca^{2+}-depletion; NH_2OH-washing). Fig. 8 shows measurements of the rate of oxygen evolution in samples poised at different redox potentials and then frozen and thawed. Freezing of samples under reducing conditions leads to a loss of capacity for oxygen evolution (Fig. 8). The loss of O_2-evolving capacity occurs at potentials below about 0 mV. The percentage of this loss seems to be correlated with the percentage of centres with reduced Q_A Simply poising samples at low potential and then reoxidising them without freezing did not lead to a significant loss of oxygen evolution, except at extremely low potentials (below −400 mV) and following long incubation.

4. Discussion

The results show that the E_m value for Q_A in active PS II is approx. −80 mV, while in PS II in which O_2 evolution is inhibited, the E_m value is shifted to approximately +65 mV. These results confirm earlier measurements made under comparable conditions and give confidence in the E_m values since they are based on a larger number of measurements. In addition, we have extended these measurements over the range of pH values in which the O_2 evolution activity is relatively stable (i.e., pH 5.5–7.5). The measured E_m values in active and inactive PS II were found to be independent of pH.

The method we used to monitor the redox state of Q_A was measurement of the fluorescence yield at room temperature, in the absence of mediators, just as in Krieger and Weis [22]. The reversibility and good fit of the data sets to one-electron Nernst curves testify to the validity of this method. Attempts were made, however, to compare the present method with those used earlier. Several new observations were made which help to rationalise some of the discrepancies and scatter in the E_m values reported in the literature.

4.1. *The effect of redox mediators*

When redox mediators were used with active PS II, a time- and potential-dependent shift of the E_m of Q_A from the low-potential to the high-potential form was observed during the course of the titration. The titration of inactive PS II was unaffected by the presence of the same mediators.

These result are interpreted as indicating that a component within PS II becomes reduced at low ambient potentials in the presence of mediators. The reduction of this component, which is essentially irreversible, results in a shift in the midpoint potential of Q_A to the high-potential form. It is suggested that the component in question remains oxidised, out of equilibrium, during the course of titrations which are done in the absence of mediators. Incubation of samples at very low potentials, even in the absence of mediators, does eventually lead to the reduction of this component and hence to a shift in the E_m of Q_A

The following considerations lead us to suggest that the Mn cluster itself is a likely candidate for the irreversible, reducible component which is responsible for the shift in the E_m of Q_A. First, it is well known that the Mn cluster remains out of equilibrium in the dark-adapted state (S1) and that over-reduction of the Mn cluster leads to irreversible loss of the Mn as Mn^{2+} (e.g., [44]; see [3]). Secondly, it is also known that removal of the Mn from PS II results in the shift of the E_m of Q_A to high potential [22].

We conclude that redox titrations of Q_A in PS II that is active for O_2 evolution, are probably not feasible when mediators are used. The combination of low potentials and good redox mediators results in over-reduction and loss of the Mn cluster, forming inactive PS II which exhibits a high potential Q_A. In the literature there are cases were the characteristics of the hysteresis reported can be nicely explained in this way (e.g., [9,10].

4.2. The effects of freezing and freeze-thawing

When redox titrations of active PS II, which were performed at room temperature (in the absence of mediators), were measured in the frozen state, either by EPR or by fluorescence, the low-potential E_m for Q_A was not observed even though it was observed in the same samples prior to freezing. The origin of this effect is not clear. Possible origins include a cooling-induced shift in the E_m of Q_A or in the equilibrium between Q_A and a reduced form of Q_B. This effect appears to be reversed upon thawing for samples poised in the range 0 to 100 mV.

By contrast, freezing and thawing of active samples in which (low-potential) Q_A is reduced, results in an irreversible shift of the E_m to the high-potential form. This is accompanied by the loss of oxygen evolution. Again the origin of this effect is not clear but the loss of activity and appearance of a Mn^{2+} EPR signal, albeit in substoichiometric amounts (not shown), indicate that the effect might be at the level of the Mn cluster. We might speculate that freezing of the sample in the presence of Q_A^- at low ambient potential results in an overreduction of the Mn cluster by some kind of temperature-dependent reverse electron transfer process. Such a process may be considered to be a cooling-induced, Q_A^--mediated equilibration of the Mn cluster with the ambient potential.

Whatever the mechanism of the freezing and freeze-thawing effects, it is clear from our observations that redox titrations of Q_A in intact PS II, if measured at low temperature, are greatly affected by freezing-induced artifacts.

Overall, we conclude that the majority of redox titrations of Q_A reported in the literature are likely to be influenced by the mediator-induced and/or freezing-induced artifacts described above. This is only true for PS II which is purported to be active in terms of oxygen evolution. In principle, centres which are inactive (Tris-washed membranes, etc. (e.g., [29,32])) should not be susceptible to these artifacts. If the shift from the low-potential/active form to the high-potential/inactive form were a systematic consequence of the experimental conditions used in the majority of published redox titrations of Q_A, then we would expect the reported E_m values in all PS II material to tend towards the high-potential inactive form reported here for Q_A. Uncertainties concerning species variation, partial transitions between the low- and high-potential forms, other possible mediator effects and complications with the normalisation of redox electrodes make direct comparisons between reports in the literature difficult; however, the majority of values are found to fall above the range measured here for the low-potential form of Q_A.

A survey of the literature shows many reports of a very low-potential wave in titrations of Q_A [8,10–16,19,25,29–31]. This is sometimes referred to as Q_L In the present work, this wave of the titration was not present. We are therefore unable to add any useful comment to the debate on its possible origins. Understanding of the 'Q_L' phenomenology will have to await future work specifically aimed at addressing this question.

At this point it may be worth making a remark which is aimed at reconciling recent apparently contradictory observations made by the authors of the present work concerning the E_m of Q_A [22,35,37]. As mentioned in the introduction, Johnson et al. [37] recently showed that recombination of the charge pair Tyr D^+/Q_A^- gives rise to thermoluminescence at the same temperature (around 50° C) in both active and Ca^{2+}-depleted PS II, an observation that would appear to be inconsistent with the occurrence of a shift in the midpoint potential of Q_A (as pointed out by those authors). However, oxygen evolution, which is known to be sensitive to high temperatures [45], may be inactivated during the thermoluminescence experiment. If so, the same inactive high-potential form of Q_A^- would be involved in recombination with $TyrD^+$ whatever the status of the enzyme prior to the measurement. This explanation, if correct, could apply to other high-temperature ($\simeq$ 50° C) thermoluminescence peaks reported in PS II.

In conclusion, we consider that estimates of the E_m of Q_A in oxygen-evolving PS II can be measured only in the absence of redox mediators and without freezing of the samples. The pH-independent E_m value of -80 mV which is obtained under these conditions is thus the value that should be taken into account when considering the energetics of electron transport in PS II. Discussions of the energetic requirements for electron transfer from Q_A to the plastoquinone pool [46] suggest that the E_m of Q_A must be at least 80 mV lower than that of plastoquinone (30 mV at pH 8.3 [19]). Our measurement of the low-potential form of Q_A clearly fills this requirement. The pH-independent E_m value of $+65$ mV obtained for inactive PS II is also relevant to specific conditions encountered by PS II in vivo, i.e., prior to photoactivation [36] and possibly during conditions of PS II down-regulation [22,47]. It is expected that with an E_m of $+65$ mV, electron transfer to plastoquinone will be severely impaired. Measurements of fluorescence induction suggest that this is indeed the case (not shown). The functional relevance of the modulation of the E_m of Q_A is discussed elsewhere [36].

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