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Thylakoid Photosystem II activity supported by the non-quinone acceptor Q_{400} and an ancillary quinone acceptor Aq

Paul Jursinic [a,b] and Ron Dennenberg [a]

[a] *USDA / ARS *, Northern Regional Research Center, Peoria, IL and* [b] *Botany Department, University of California, Davis, CA (U.S.A.)*

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The reaction center of Photosystem II is photoactive when its primary electron acceptor, Q_a, or one or more of its ancillary electron acceptors are oxidized. In this work two ancillary electron acceptors are distinguished. One of these ancillary acceptors is Q_{400}. It was found to have no photoinduced absorption changes in the ultraviolet and so is not a quinone. An additional ancillary electron acceptor, Aq, was identified. Aq has the following characteristics: it is a quinone based on photoinduced absorption change in the ultraviolet, it has a midpoint potential of 318 mV that is independent of pH in the 6.5–8.0 range, the extent of its oxidation depends on pH and the concentration of ferricyanide, it has no C550 bandshift, and the stoichiometry of Aq: Q_{400}: Q_a is 0.38:0.54:1.00 based on the area above the chlorophyll *a* fluorescence rise curve. When Aq is oxidized it participates in double advancement in transitions of oxygen-evolution S-states. A contribution to double advancement solely by Q_{400} could not be found.

* The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

Abbreviations: Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; DCIP, 2,6-dichloroindophenol; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethyl urea; E_h, redox potential with respect to the standard hydrogen electrode; $E_{m,x}$, midpoint potential at pH *x*; I, pheophytin in the Photosystem II reaction center; Mes, 4-morpholineethanesulfonic acid; OEC, oxygen-evolving complex; P-680, reaction center chlorophyll of Photosystem II; P-700, reaction center chlorophyll of Photosystem I; Tes, 2-{(hydroxy-1,1-bis(hydroxymethyl)methyl]amino}ethanesulfonic acid; Y_2, oxygen yield on flash 2; Y_{ss}, oxygen yield after many flashes; UV, ultraviolet.

Correspondence: P. Jursinic, USDA/ARS, Northern Regional Research Center, 1815 North University Street, Peoria, IL 61604, U.S.A.

Introduction

The reaction center of Photosystem II in its simplest form is schematically written as follows:

P-680 I Q_a Q_b

where P-680 is the reaction center chlorophyll [1,2], I is a pheophytin [3,4], Q_a is a quinone that accepts one electron [5,6], and Q_b is a bound plastoquinone that accepts two electrons from Q_a before exchanging with the plastoquinone pool [7–9]. Also, there is a large population of Photosystem II reaction centers (greater than 40%) that do not have a Q_b, the so-called non-B centers [10,11]. No attempt will be made in this work to differentiate between B- and non-B-type reaction centers.

In addition to the primary acceptor Q_a, a number of ancillary acceptors have been reported. One acceptor identified as X_a is believed to partake in charge separation as indicated by an absorption change at 690 nm, which indicates the formation of P-680$^+$ [12]. The charge separation between P-680 and X_a was unproductive, since advancement in oxygen evolution S-states and generation of membrane potential were not associated with this charge separation [13]. The existence of X_a has been questioned recently because absorption change measurements at 824 nm, which also indicate P-680$^+$ formation, show no evidence of its presence [14].

Another ancillary acceptor, identified as Q_2, quenches Chl *a* fluorescence but does not exhibit UV spectral changes expected of a quinone such as Q_a [15]. Charge separation between P-680 and Q_2 may be coupled to the advancement of oxygen evolution S-states under very high flash intensity [16]. It has been suggested [15] that X_a and Q_2 are identical, since neither gives rise to absorption changes at 550, 320 or 515 nm, which correspond to Q_a reduction (550 and 320 nm) and membrane electric field (515 nm).

At high redox potentials, normally established by ferricyanide, another acceptor, identified as R and now Q_{400}, has been found [17]. When Q_{400} is oxidized, the normalized area above the Chl *a* fluorescence rise curve increases by as much as 100% [17]. Q_{400} is believed to be a single electron acceptor with a midpoint potential at pH 7, $E_{m,7}$, of 400 mV [18]. Charge separation occurs between P-680 and Q_{400} [14], the positive charge is believed to advance oxygen evolution S-states [19,20], and the electron reduces Q_{400}, which has been associated with an absorption change at 325 nm [21]. The reoxidation of Q_{400} by ferricyanide, reportedly, is blocked by DCMU [17], but this DCMU effect is diminished if divalent salts are present [21].

Petrouleas and Diner [22] reported recently that when the redox potential is raised above 400 mV changes occur in EPR signals at $g = 8$, 6.4, and 5.5 and in Mössbauer absorption. To accomodate these new data, they hypothesized that a change occurred in redox state, Fe^{2+}/Fe^{3+}, of iron complexed with the primary Photosystem II quinone, Q_a. Their results [22] identify Q_{400} as iron complexed with Q_a.

In this work, we show that Q_{400} has no absorption change in the UV portion of the spectrum and it is not a quinone; an increase in absorption change at 325 nm induced by ferricyanide can be attributed to an ancillary quinone acceptor, Aq, distinct from Q_a or Q_{400}; ferricyanide induced double advancement in the OEC is a result of oxidation of Aq; double advancement of the OEC becomes no larger if both Aq and Q_{400} are oxidized and active in Photosystem II charge separation; reduction of Aq is not associated with a C550 bandshift; based on the area above the Chl *a* fluorescence rise curve the stoichiometry of $Q_a : Aq : Q_{400}$ is 1.00 : 0.38 : 0.54.

Materials and Methods

Broken chloroplasts (thylakoids) were isolated from leaves of dwarf pea seedlings (*Pisum sativum* L. var. Wando) grown in a laboratory growth chamber (16 h day, 25/20°C, 70 W/m^2 light intensity from a combination of cool-white fluorescent lamps and incandescent lamps). The plants were harvested 18–21 days after germination. The isolation procedure was as described previously [23]. Reaction media were as follows: 400 mM sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tes in the pH 7.2–8.5 range and 50 mM Mes in the pH 6–7 range.

Oxygen flash-yield measurements were made with a laboratory-built bare-platinum electrode [24]. Saturating excitation flashes were provided by an unfiltered, EG&G model FX200, xenon, strobe lamp operated with 4.5 J (1500 V with 4 μF capacitance) input energy. Flashes were given at 2 Hz. Signals were detected by a laboratory-built, a.c.-coupled, transimpedance amplifier that had a 2 ms risetime. The sample was incubated in the dark on the electrode surface with the electrode bias off. It was important to avoid electrode bias alteration of the redox poise of the sample during dark incubation. Bias was applied 10 s prior to the measurement. The a.c.-coupling eliminated signal drift that can occur during the first few minutes after application of electrode bias.

Chl *a* fluorescence yield changes were measured with a laboratory-built fluorometer. The actinic source consisted of the following: an incandescent lamp powered by a voltage-regulated d.c. supply, a condensing lens, an electronic shutter (Uniblitz model 23X2A0X5) with a 0.8 ms opening time positioned at the focal point of the lens, and a combination of Corning CS 4–96 and CS 3–71 glass filters. At a right angle to the actinic beam was a United Detector Technology PIN-10DP photodiode shielded with a Corning CS 2–64 glass filter. The photodiode output was amplified by a laboratory-built transimpedance amplifier with a 1 ms risetime. Analog signals were digitized with a Biomation 2805 waveform recorder. Digitized data were transferred to a Hewlett Packard HP87 minicomputer, which had been programmed to determined variable fluorescence as well as area above the rise curve. Variable fluorescence, ΔF, is calculated as $\Delta F = (F_{max} - F_0)/F_0$ where F_0 is the fluorescence level at the beginning of actinic illumination and F_{max} is the maximum level of fluorescence attained during illumination. The area above the fluorescence rise curve is calculated as $A = \int_{t=0}^{t=\infty}(F_{max} - F(t))dt$ where $F(t)$ is the fluorescence level at any time t of illumination.

Solution redox potentials, E_h, were established by adding ferricyanide and ferrocyanide. Different potentials were obtained by changing the concentration of ferrocyanide while maintaining the ferricyanide concentration constant. The potentials were measured with a Cole-Parmer model J-5994-21 combination platinum/reference electrode. Electrode potentials were measured with the millivolt input of a Corning model 125 pH meter. The electrode was calibrated with quinhydrone, which has an $E_{m,7} = 287$ mV [25]. In all of the redox potential experiments samples were allowed to come to equilibrium at each potential for at least 10 min in the dark. All potentials are given with respect to the standard hydrogen electrode.

Photochemically induced absorption changes were measured with a laboratory-built dual-beam spectrophotometer as described previously [21].

Results

Since Q_{400} has now been identified as iron [22] one does not expect a detectable absorption change in the UV portion of the spectra upon photoreduction, during saturating flash excitation. Earlier work [21,26,27] had shown that the addition of ferricyanide to the reaction medium caused an increase in the UV absorption spectrum that was due to oxidation of a quinone acceptor different than Q_a and that was possibly Q_{400}. This additional absorption change at 325 nm is clearly seen in Fig. 1 as a $48 \pm 10\%$ larger signal at $E_h \geq 350$ mV in the presence of ferricyanide (■——■) than in its absence (- - - - -). Also, Fig. 1 shows the redox titration of this additional quinone absorption change at its 325 nm peak. The midpoint potential of the quinone acceptor that is associated with this absorption change is 318 mV and is invariant with pH in the range of 6.5 to 8.0. Clearly, this additional absorption change is not Q_{400} but must be due to another acceptor identified as a quinone with an $E_{m,7} = 318$ mV.

It is possible that the additional absorption change at 325 nm reported here in Fig. 1 and earlier [21,26,27] is due to the presence of Q_b^- in our sample. If Q_b^- is present, then upon addition of DCMU, $Q_a\,Q_b^- + DCMU \rightarrow Q_a^- Q_b + DCMU \rightarrow Q_a^- DCMU + Q_b$ will occur [28], which will decrease the amount of photoreducible Q_a. Addition

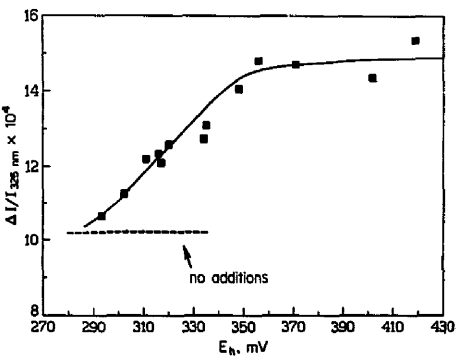


Fig. 1. Flash-induced absorption change at 325 nm as a function of E_h at pH 6.5. The absorption change ($\Delta I/I$) is measured after a single saturating flash given to samples that had been incubated in the dark for at least 10 min at the specified redox potential. The sample concentration was 24.5 μg Chl/ml and 5 μM DCMU was present. Data have been corrected for the cytochrome *f* contribution at 325 nm [27]. The ferrocyanide concentration is kept at 1 mM and the E_h is varied by adding ferricyanide.

of ferricyanide would oxidize Q_b^-, the total amount of Q_a would be photoreducible, and the absorption change at 325 nm would become larger. To rule out this possibility we measured the extent of Q_b^- in our sample.

The amount of Q_b^- can be detected by observing the level of Chl *a* fluorescence yield after DCMU injection [8,10] in samples preilluminated with a series of flashes. Complications due to Q_a^- recombination with oxygen evolution S-states were eliminated by incubation of the thylakoids with 5 mM hydroxylamine [10]. Large cycle-of-two oscillations occur in DCMU-induced fluorescence in our samples that are dark adapted for 10 min (Fig. 2). These large oscillations would not occur [10] if Q_b^- remained in high concentration in our dark-adapted samples. The level of fluorescence induced by DCMU in a dark-adapted sample (0 preillumination flashes) is 0.02 in Fig. 2, which indicates that little Q_b^- is present after 10 min of dark adaptation.

The rate of Q_b^- decay in our samples can be measured by the following method. One preillumination flash is given to generate a maximum amount of Q_b^-. Then after various amounts of time the sample is treated with hydroxylamine and the DCMU induced fluorescence is measured. Fig. 3 indicates that in 10 min Q_b^- decays to 4.4% of its maximum value with a half-time of decay of about 10 s. This is in the same range as the 5 s for half decay found by Lavergne and Étienne [29] and 22 s by Robinson and Crofts [30]. From the data of Figs. 2 and 3 we conclude that in our samples after 10 min dark adaptation only 4.4% of Q_b remains reduced. This amount of Q_b^- cannot account for the 40% increase in absorption change at 325 nm that we observe when ferricyanide is added (Fig. 1 and Refs. 21, 26 and 27).

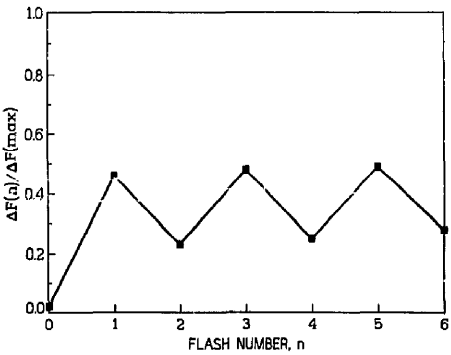


Fig. 2. DCMU-induced Chl *a* fluorescence as a function of preillumination flash number. Measurements were made at a Chl concentration of 10 μg/ml, and DCMU was injected to give a final concentration of 10 μM within 2 s after the final flash. Preillumination flashes were given at 1 Hz. Thylakoids were dark adapted for 10 min and at 10 μg Chl/ml were incubated with 5 mM hydroxylamine for 5 s or more prior to illumination. $\Delta F(n) = (F(n) - F_0)/F_0$ where $F(n)$ is the fluorescence level measured 30 s after DCMU injection to a sample preilluminated with n flashes and F_0 is the fluorescence level measured before a preillumination flash or DCMU injection. $\Delta F_{max} = (F_{max} - F_0)/F_0$ where F_{max} is the fluorescence level measured after the sample had DCMU added and 25 excitation flashes given.

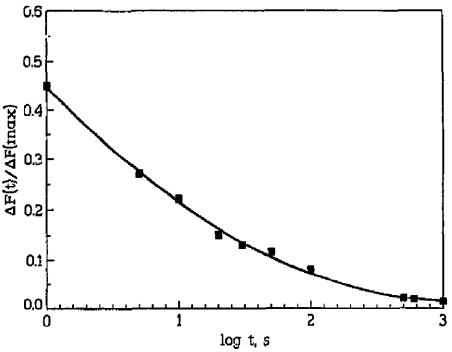


Fig. 3. DCMU-induced Chl *a* fluorescence as a function of time after one preillumination flash. The sample was dark adapted for 10 min and then one saturating flash was given. At various times t after the flash 5 mM hydroxylamine was added to the sample, after 5 s DCMU was added to give a final concentration of 10 μM, and after 30 s the fluorescence $F(t)$ was measured. $\Delta F(t) = (F(t) - F_0)/F_0$ where the variables are as already defined. Other details are as in the legend of Fig. 2.

A high redox potential produced by ferricyanide allows double turnovers in the Photosystem II reaction center, which under proper conditions can be observed as an increased yield in oxygen after two flashes, Y_2 [19,20]. It has been hypothesized [19,20,31] that under these conditions Q_{400} was oxidized and participated in the multiple charge separation in Photosystem II. However, based on the data presented here the presence of ferricyanide would also oxidize Aq, which could support multiple advancement of the oxygen evolving complex. We wanted to de-

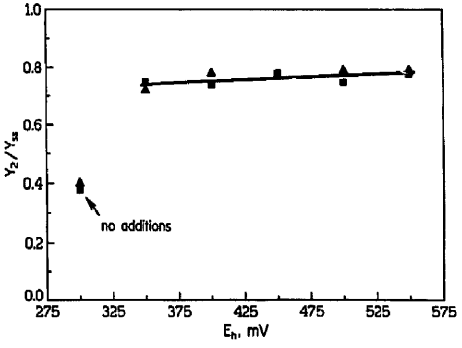


Fig. 4. Oxygen yield on flash two, Y_2, as a function of E_h. The measurement was made at pH 6.5 (■———■) and pH 8 (▲———▲). The ferricyanide concentration is kept constant at 1 mM and the E_h is the varied by adding ferrocyanide. Y_2 was normalized by Y_{ss}, which was calculated as the average of the first eleven flash yields. The sample was incubated in the dark for 10 min or longer at a concentration of 500 μg Chl/ml at the various E_h values.

termine if Aq, Q_{400}, or both could support large Y_2 at high redox potential.

A redox titration of Y_2 is shown in Fig. 4. It is important to note that the ferricyanide concentration was kept constant and the ferrocyanide concentration was varied to obtain the various potentials. At pH 6.5 (■———■) Y_2 is enhanced at all potentials in the 350–550 mV range. This agrees with the hypothesis that Aq supports double advancement in the oxygen-evolving complex. As shown in Fig. 1, at pH 6.5 Aq is fully oxidized in this potential range. Since Q_{400} has an $E_{m,6.5} = 430$ mV and is 95% reduced at $E_h = 350$ mV, the enhanced Y_2 at $E_h = 350$ mV must be supported by Aq. At $E_h = 550$ mV both Aq and Q_{400} are oxidized but Y_2 is no larger. This is likely the consequence of rate limitations in the oxygen-evolving complex. The redox titration could not be carried out below $E_h = 350$ mV. At these potentials, the concentration of ferrocyanide was so large that it interacted directly with the platinum electrode and masked the oxygen signal.

The titration is also shown at pH 8 (▲———▲) in Fig. 4. At this pH both Aq and Q_{400} can be oxidized, since Q_{400} has an $E_{m,8} = 350$ mV. Again, there is no effect of redox potential on the enhancement of Y_2. At $E_h = 550$ mV Q_a, Aq and Q_{400} would all be oxidized. Under these conditions in the presence of DCMU oxygen evolution would be expected if these acceptors had a total capacity for three or more electrons. Oxygen evolution was measured under a series of weak flashes and no oxygen was observed (data not shown). Based on this result the total capacity of these acceptors must be less than three electrons.

The lack of redox titration of enhanced Y_2 shown in Fig. 4 does not agree with an earlier report by Kok and Velthuys [31]. They found that Y_2 apparently titrated with an $E_{m,7} = 400$ mV and a -60 mV/pH dependence. This earlier work altered redox potential by changing the concentration of both ferri- and ferrocyanide. Unfortunately, with this method both the redox potential and the ferricyanide concentration are being altered. Their titration [31] may have demonstrated a dependence on ferricyanide concentration rather than a dependence on redox potential. We tested this possibility by measuring the dependence of Y_2 on ferricyanide concentration at various pH values. In these measurements ferrocyanide was not included and the E_h was measured to be approx. 550 mV at all of the ferricyanide concentrations. These data are shown in Fig. 5. A dependence on ferricyanide concentration is observed, which is modulated by pH.

The results of Fig. 5 corroborate the idea that the earlier titration [31] was not an effect of redox potential but actually an effect of ferricyanide concentration. Also, as the pH is lowered a higher concentration of ferricyanide is needed to cause enhancement of Y_2. Indeed, this pH dependence could be incorrectly interpreted as a positive shift in midpoint potential as the pH is decreased [31].

With the above characterization of Aq and Q_{400}, redox conditions can be set to vary the oxidation state of these acceptors. At pH 7.8 with $E_h = 300$ mV one expects: 100% Q_a, 33% oxidized Aq, and 11% oxidized Q_{400}; with $E_h = 350$ mV one expects: 100% Q_a, 78% oxidized Aq, and 47% oxidized Q_{400}; and with $E_h = 400$ mV one expects: 100% Q_a, 96% oxidized Aq, and 86% oxidized Q_{400}. This is verified in Fig. 6 by the fluorescence rise curves with DCMU present. As more acceptors become oxidized the rise becomes slower, since it takes longer to photoreduce all of the acceptors. In other words, the area above the

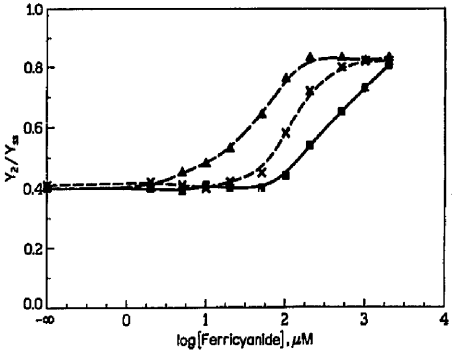


Fig. 5. Oxygen yield on flash two, Y_2, as a function of ferricyanide concentration. The measurement was made at pH 6.5 (■———■), pH 7.2 (×------×), and pH 8 (▲------▲). Ferrocyanide was not added and E_h was maintained at approximately 550 mV. The $-\infty$ point corresponds to no addition of ferricyanide. Other details are as described in the legend of Fig. 4.

rise curve becomes larger as the additional acceptors are oxidized. From the three fluorescence curves of Fig. 6 the area contributions of these acceptors are found. Presuming that the area is proportional to the concentration of the acceptors, the following equations can be written:

$$Q_a + 0.33\ Aq + 0.11\ Q_{400} = \text{area (300 mV)}$$

$$Q_a + 0.78\ Aq + 0.47\ Q_{400} = \text{area (350 mV)}$$

$$Q_a + 0.96\ Aq + 0.86\ Q_{400} = \text{area (400 mV)}$$

where area (mV) is the area above the fluorescence rise curve at the indicated E_h. Solving these equations, the following stoichiometry was found: $Q_a : Ag : Q_{400} = 1.00 : 0.38 \pm 0.18 : 0.54 \pm 0.19$, which is based on an average of 12 measurements with the indicated standard deviation. From the absorption change measurements of Fig. 1 the Q_a:Aq ratio was 1.00:0.48:0.10, assuming the same extinction coefficient for both of these quinones. The determinations of Qa:Aq by fluorescence area and 325 nm absorption change are identical within experimental error.

In view of the relative amounts of these acceptors, when they are all oxidized the total electron capacity would be from 1.92 to 2.02. This agrees with the earlier estimate of less than 3 based on the oxygen evolution data.

A redox titration of the area above the fluorescence curve was done in the 275–400 mV range and is shown in Fig. 7. This titration is done at pH 6.5 to separate the midpoint potentials of Aq and Q_{400}. The titration of Fig. 7 is consistent with

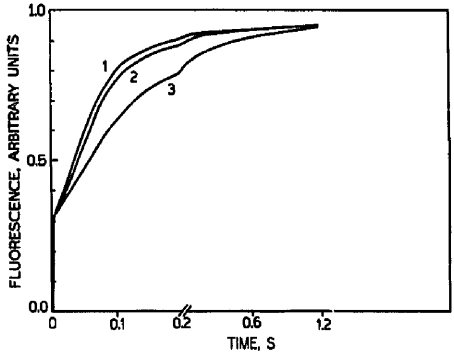


Fig. 6. Rise in Chl *a* fluorescence upon illumination with continuous light. The ferricyanide concentration is 100 μM and the ferrocyanide concentration is varied to set the various redox potentials. The reaction medium was adjusted to pH 7.8. Curve 1 is $E_h = 300$ mV, curve 2 is $E_h = 350$ mV, and curve 3 is $E_h = 400$ mV. The Chl concentration was 10 μg/ml and the sample was dark adapted for 15 min or longer. 20 μM DCMU was added immediately before the measurement.

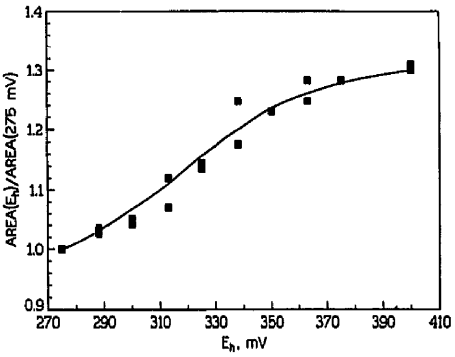


Fig. 7. Area above the Chl *a* fluorescence rise curve as a function of E_h at pH 6.5. The solid line is the theoretically predicted behavior based on Aq having $E_{m,6.5} = 318$ mV and an area contribution that is 40% that of Q_a. Experimental conditions are identical to those in the legend of Fig. 6.

Aq having an $E_{m,6.5} = 318$ mV and an area contribution of 40% of that associated with Q_a. This is in excellent agreement with the 325 nm absorption data of Fig. 1.

Another possible method for detecting the presence of Aq would be the C550 bandshift of pheophytin that is associated with the photoreduction of Q_a [6,32]. If the quinone Aq has a pheophytin associated with it as does Q_a, then photoreduction of Aq would be expected to give rise to a C550 bandshift. Many absorption changes occur in the 540–560 nm range besides that of the C550 bandshift; electrochromism, cytochrome b_6, cytochrome f and P-700. These coincident absorption changes are eliminated by the following methods and protocol: electrochromism is dissipated by the inclusion of gramicidin [33], cytochrome b_6 is oxidized by ferricyanide [34], and cytochrome f and P-700 are irreversibly oxidized by illumination in the presence of DCMU and ferricyanide [32,35]. Fig. 8 shows the C550 bandshift as the difference in the flash-induced absorption change at 551 minus 541 nm. Under these experimental conditions, at 30 s after illumination Q_a^- is 95% reoxidized (determined by Chl a fluorescence decay data not shown) and Aq^- is 20% reoxidized [21] and at 10 min after illumination both Q_a^- and Aq^- are 100% reoxidized. The upper trace (10 min dark adaptation) and the middle trace (30 s dark adaptation) in Fig. 8 are identical within experimental error. Thus, a change from 20%–100% oxidized (photoreducible) Aq gives rise to no additional C550 bandshift within experimental error. The C550 bandshift is only related to Q_a photoreduction.

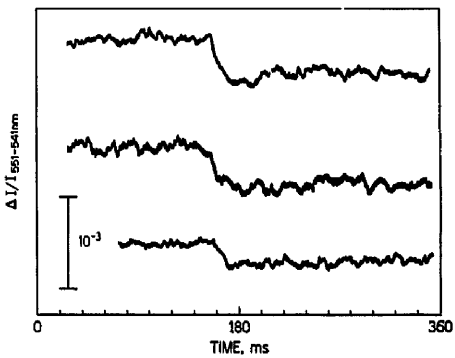


Fig. 8. The difference in the absorption change at 551 nm minus 541 nm induced by a saturating xenon flash in pea thylakoids. The sample contained 27 μM Chl, 500 μM ferricyanide, 0.5 μM gramicidin, and 5 μM DCMU in pH 7.5 reaction medium in a 1 cm pathlength cuvette. The upper trace was measured 10 min after preillumination with continuous light, the middle trace was measured 30 s after preillumination, and the lower trace included 4 mM hydroxylamine and was measured 2 min after preillumination. All traces are an average of 16 experiments at each wavelength. The flattening correction at 550 nm was 1.08.

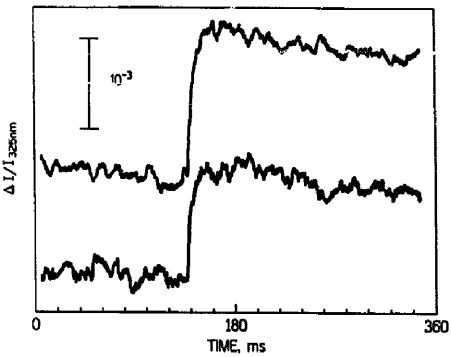


Fig. 9. Absorption change at 325 nm induced by a saturating xenon flash in pea thylakoids. The sample contained 27 μM Chl, 500 μM ferricyanide, 0.5 μM gramicidin, and 5 μM DCMU in pH 7.5 reaction medium in a 1 cm pathlength cuvette. The upper trace was measured 10 min and the lower trace 30 s after preillumination with continuous light. The upper trace is an average of 16 measurements and the lower trace is an average of 9 measurements. The flattening correction at 325 nm was 1.68.

The oxidation of Aq^- under these conditions can be demonstrated by measuring the absorption change at 325 nm and is shown in Fig. 9. The signal from the 10 min dark-adapted sample (Q_a + Aq) is larger than in the 30 s dark-adapted sample (Q_a + 20% Aq).

The absorption change in Fig. 8 can be used to quantify the Q_a in this sample. While precautions were taken to eliminate electrochromic, P-700, and cytochrome contributions to the C550 bandshift in Fig. 8, other undefined contributions persisted. This is shown in the bottom trace of Fig. 8 in a sample treated with hydroxylamine and preilluminated to irreversibly reduce Q_a and block Photosystem II charge separation [36,37]. Correcting for this residual $\Delta abs_{551-541\ nm}$, the bandshift for Q_a was obtained. Two differential extinc-

tion coefficients have been published for the C550 bandshift: 2.2 $mM^{-1} \cdot cm^{-1}$ [38] and 5.2 $mM^{-1} \cdot cm^{-1}$ [39]. Applying these differential extinction coefficients to the data of Fig. 8, the concentration of Q_a is 2.21 mmol/mol Chl and 0.94 mmol/mol Chl, respectively.

The absorption changes in Fig. 9 can be used to quantify the amount of Q_a and Aq in this sample. Using the same extinction coefficient, 12 $mM^{-1} \cdot cm^{-1}$ [6], for Q_a and Aq, the concentration of Q_a is 2.26 mmol per mol Chl and Aq is 1.25 mmol per mol Chl. This value for Q_a concentration is in good agreement with the 2.21 mmol per mol Chl value from the C550 bandshift. Based on this agreement, we favor the Van Gorkom et al. [38] value for the differential extinction coefficient of C550.

Discussion

The principal conclusion of this study is that in addition to Q_a two ancillary acceptors, Q_{400} and Aq, can be involved in Photosystem II photochemistry. Identification of Q_{400} and Aq was made possible by a careful analysis of the pattern of oxygen yields with flash excitation, flash-induced absorbance changes at 325 nm, and Chl *a* fluorescence. These experimental parameters were measured under various conditions of pH, E_h and ferricyanide concentration.

It had been shown previously [21,26,27] that an additional absorption change at 325 nm induced by ferricyanide was due to photoreduction of a quinone and only occurred on the first flash after about 5 min dark adaptation. It was suggested that this quinone was Q_{400}. We know that this additional 325 nm absorption change is associated with a quinone that has an $E_{m,7} = 318$ mV (Fig. 1). Accordingly, we now hypothesize the existence of a quinone, Aq, different from Q_a or Q_{400}. The oxidation and photoreduction of Aq account for the additional 325 nm absorption change. Our earlier identification of Q_{400} with this additional 325 nm absorption change was incorrect. Q_{400} is not a quinone, which is consistent with the conclusion of Petrouleas and Diner [22] that it is iron complexed with Q_a.

The additional absorption change at 325 nm induced by ferricyanide is not due to the presence of Q_b^- in our samples. Based on measurements of Chl *a* fluorescence (Figs. 2 and 3) we estimate that after the usual 10 min dark adaptation less than 4.4% Q_b^- remains in our samples. The Q_b^- argument is also unlikely for the following reason. If the presence of Q_b^- reduced the absorption change at 325 nm, then only in the presence of ferricyanide would Q_b^- be oxidized and the absorption change due to 100% of Q_a be observed. However, we observed [21,26,27] that in the presence of ferricyanide the absorption change at 325 nm was 40–70% greater than estimates of Q_a concentration based on flash yields of oxygen evolution, proton evolution, and DCIP reduction. The additional 325 nm absorption change on the first flash in the presence of ferricyanide is explained by ferricyanide oxidation of an ancillary acceptor, Aq.

Double advancement in oxygen evolution had been associated with the oxidation of Q_{400} [19,20,31]. However, data in this work indicate that Aq also can support double advancement in oxygen evolution. At pH 6.5 and $E_h = 350$ mV (Fig. 4) Aq is oxidized while Q_{400} is not, but Y_2 is still enhanced. At pH 8 enhancement of Y_2 is unaffected by E_h between 350 and 550 mV, a range where Aq remains oxidized but Q_{400} is shifted between oxidized and reduced states.

A decision cannot be made about the ability of Q_{400} to support double advancement in oxygen evolution. The experimental conditions for increased Y_2 (Figs. 4 and 5) oxidize Aq or both Aq and Q_{400}, but never Q_{400} alone. At high pH and high E_h both Aq and Q_{400} are oxidized, but the enhancement of Y_2 does not become measurably larger (Fig. 4). This could mean that Q_{400} is unable to support additional enhancement in Y_2 or that there are kinetic limitations in the oxygen-evolution mechanism. We favor the latter explanation because earlier work [20] demonstrated that 300 ns laser flashes could cause multiple charge-separations in the Photosystem II reaction center without consequential enhancement of Y_2.

Table I presents the various properties of Q_{400} and Aq as determined in this work and from previous work. Scheme I is a suggested reaction scheme for Photosystem II that shows Aq and Q_{400} under various experimental conditions prior to illumination. In agreement with earlier sugges-

TABLE I

VARIOUS PROPERTIES OF THE ANCILLARY ELECTRON ACCEPTORS OF PHOTOSYSTEM II

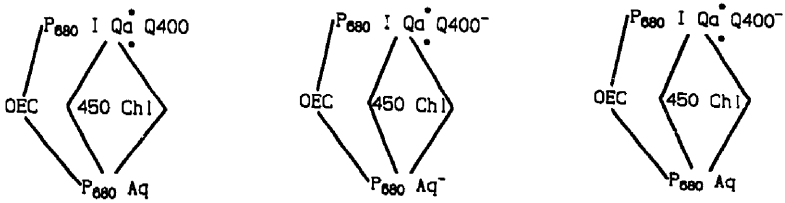
Acceptor	$E_{m,7}$ (mV)	Increased 325 nm absorption change	Increase in Chl fluorescence area	Increased Y_2/Y_{ss}	C550
Q_{400}	400 [18]	no (Fig. 1)	yes (Fig. 6)	? (Fig. 4)	no [46]
Aq	318 (Figs. 1 and 7)	yes (Figs. 1 and 9)	yes (Fig. 6)	yes (Fig. 4)	no (Fig. 8)

tions [21,26,27], the main idea is that two reaction centers exist, P-680 Q_a and P-680 Aq. P-680 Q_a is connected to an active OEC, the plastoquinone pool and Photosystem I, and an aggregate of 450 Chl [27] that act as antenna pigments. There are two herbicide binding sites for every Photosystem II center that is active in oxygen evolution [26,40]. P-680 Aq is similar to P-680 Q_a except that it is unconnected to the plastoquinone pool and Photosystem I. After a single saturating flash all acceptors become reduced. Aq^- and Q^-_{400} are reoxidized on the time scale of minutes [21] and so are not active in photochemistry under steady state conditions, i.e., in continuous light or after the first flash. This is a key point for understanding disagreements in the literature. Samples incubated with ferricyanide in the dark will have Q_a and Aq oxidized (Scheme IB and IC). Upon illumination after dark adaptation both quinones will become reduced and the absorption change at 325 nm will be larger than it is under steady-state conditions [27]. This larger absorption change reflects both P-680 Q_a, which is active under steady-state conditions, and P-680 Aq, which is only active under first illumination but inactive under steady-state conditions. Unfortunately, the uncorrected absorption change at 325 nm, which reflects both P-680 Q_a and P-680 Aq, has been interpreted [41,42] as a measurement of only P-680 Q_a. The absorption change at 325 nm has also been measured in samples that contain ferricyanide but are only dark adapted for 1 min [39]. Under these conditions photoreduced Aq will only be 30% reoxidized between illuminations, since its

A
Control conditions:
no additions or
$0\ mV < E_h < 290\ mV$

B
$E_h = 350$ mV
pH = 6.5

C
$E_h = 550$ mV
pH = 8.0



	Oxidized acceptors	Number of Chl per active P-680	Number of Chl per photoreducible quinone	Y_2/Y_{ss}	325 nm absorption change	ESR signal $g = 8$
A	Q_a	450	450	0.4	small	small
B	Q_a, Aq	300	300	0.8	large	small
C	Q_a, Aq, Q_{400}	300	300	0.8	large	large

Scheme I. Reaction scheme for Photosystem II under different experimental conditions.
The * represents a herbicide binding site. The stoichiometry of Q_a : Aq : Q_{400} is 1.00 : 0.48 : 0.54. The oxidation of Q_{400} is associated with an ESR $g = 8$ signal [46]. See Discussion section for other details.

half-time of reoxidation is approx. 2 min [21]. Measurement of P-680 Q_a by 325 nm absorption change would be incorrect by an amount equal to the 325 nm absorption change contribution from the 30% of Aq^- that is reoxidized in the 1 min dark time between illuminations.

The model in Scheme I is consistent with the finding [22] that under subsaturating flash conditions the 325 nm absorption change decreased when Q_{400} was oxidized. Under the conditions used [22], Q_a and Aq would both be oxidized and contribute to the 325 nm absorption change upon photoreduction. Since subsaturating flashes were used, Q_a and Aq photoreduction would be decreased as Q_{400} became oxidized and therefore able to compete for the photoreduction capacity of the flash. This would be observed as a decrease in 325 nm absorption change, since Q_a and Aq are quinones but Q_{400} is not. If saturating flashes are used, as in this work (Figs. 1 and 9), then oxidation of Q_{400} has no effect on the amplitude of the 325 nm absorption change.

Another difference between P-680 Q_a and P-680 Aq is that P-680 Q_a has pheophytin while P-680 Aq does not. The C550 bandshift is believed to be a shift in the absorption spectra of a pheophytin associated with the photoreduction of Q_a [6,32]. Since the photoreduction of Aq does not generate a C550 bandshift (Figs. 8 and 9), we suggest that P-680 Aq does not have pheophytin.

An additional reason to believe that the P-680 Aq reaction center does not contain pheophytin is based on measurements of Chl *a* fluorescence. The data of Fig. 6 indicate that oxidation and reduction of Aq has no effect on the F_0 (initial level) or F_{max} (final level) of the rise of Chl *a* fluorescence, during continuous illumination. This means that P-680 Aq does not modulate the intensity of Chl *a* fluorescence. One theory of variable fluorescence maintains that it arises from charge recombination [43] as follows: $P\text{-}680^+ I^- Q_a^- \rightarrow P\text{-}680^* I Q_a^-$ where I is pheophytin and P-680* is the excited singlet state of the reaction center Chl. So, the lack of a variable fluorescence component modulated by the redox state of Aq we suggest means that the Aq reaction center does not have pheophytin. The change in area above the fluorescence rise curve observed in Figs. 6 and 7 with redox state of Aq is a result of P-680 Q_a and P-680 Aq sharing antenna ensembles (see Scheme I). As Aq^- becomes oxidized, quanta are used for the P-680 Aq photoreaction in competition with the P-680 Q_a photoreaction. Therefore, the rise from F_0 to F_{max}, which is controlled by Q_a reduction, takes longer and the area above the fluorescence rise curve increases.

In recent work [44] we demonstrated that at pH 6.5 in the presence of DCBQ and ferricyanide, flashes of 5 μs or longer duration gave up to 35% enhanced flash yields of oxygen but no enhancement in $\Delta abs_{325\ nm}$. This enhancement was shown to be a consequence of double turnovers of Photosystem II during the flash emission of 5 μs or longer. Enhancement of proton flash yield under similar conditions was interpreted to be evidence for inactive Photosystem II reaction centers [45]. This inactive center hypothesis was rejected when the enhancement was shown to be dependent upon flash duration [44]. A model was proposed [44] in which DCBQ and ferricyanide were suggested to oxidize Q_{400} in less than 50 ms, the flash period. Q_{400} was indicated to be the acceptor that supported the double turnovers in Photosystem II. Aq or any other quinone, even Q_a, cannot be the acceptor that supports these double turnovers since as a quinone an enhanced $\Delta abs_{325\ nm}$ would be generated. However, this was not observed [44].

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