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A comparison of the absorption changes near 325 nm and chlorophyll *a* fluorescence characteristics of the Photosystem II acceptors Q_a and Q_{400}

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The stoichiometry of chlorophyll/Photosystem II was determined in pea thylakoids. The concentration of Photosystem II was determined by the absorption change at 325 nm. When the 325 nm measurement was made on the first flash in the presence of ferricyanide, the Photosystem II absorption change was found to increase by up to 100% of the same measurement made in the absence of ferricyanide. The increase in absorption change in the presence of various amounts of ferricyanide was found to correlate well with the increase in area above the Chl *a* fluorescence induction curve. Also, the dark recovery of both the 325 nm absorption change and the area above the Chl *a* fluorescence curve are similar and in the order of several minutes. Absorption changes made under repetitive flash excitation showed no increase in signal with the addition of ferricyanide. We conclude that there are two acceptors, Q_a and Q_{400}, for each active oxygen-evolving complex and only Q_a is involved in active electron transport to Photosystem I.

Introduction

Stoichiometry of Photosystem II and I has been measured in green plants by a number of techniques. In general the accepted method for determining the number of Photosystem I reaction centers is the measurement of the absorption change near 700 nm. This absorption change corresponds to the oxidation of P-700, the reaction center Chl of Photosystem I.

The method for determining the number of Photosystem II reaction centers is somewhat less certain, since different experimenters using different techniques have reported values of Chl/Photosystem II that often differ by a factor of 2 or more. In a recent paper Anderson and Melis [1] reported values for Chl/Photosystem II of 297 in thylakoids of spinach. Their values were obtained by the light-induced absorption change method at 320 nm. This absorption change was presumed to correspond to the reduction of Qa, the primary electron acceptor of Photosystem II. However, when values of Chl/Photosystem II were calculated by measuring oxygen yield per flash, Bose et al. [2] found a Chl/Photosystem II ratio of 585 in pea thylakoids. Whitmarsh and Ort [3] measured val-

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; Q_a, primary quinone acceptor of Photosystem II; Q_{400}, secondary acceptor of Photosystem II with a midpoint potential of 400 mV at pH 7; P-700, reaction center Chl of photosystem I; P-680, reaction center Chl of Photosystem II; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]amino}ethanesulfonic acid; $K_3Fe(CN)_6$, potassium ferricyanide.

ues of 630 for Chl/Photosystem II in spinach thylakoids by observing the protons released per flash by the oxidation of water.

In another paper [4] we reported that in pea thylakoids values of 600 for Chl/Photosystem II are obtained when measured by various flash yield techniques. In this paper we have measured the light-induced absorption change at 325 nm and have found Chl/Photosystem II values of 600 in the absence of ferricyanide. This value is similar to the value obtained with the oxygen flash yield measurement. However, when ferricyanide is added to the thylakoid suspension, values of from 300–350 Chl/Photosystem II are obtained, depending on the concentration of ferricyanide added.

In this study we show the existence of a second acceptor at Photosystem II that becomes oxidized only in the presence of ferricyanide. We believe this acceptor is what has previously been designated Q_{400} [5–7]. This acceptor is reduced on the first flash only, since its reoxidation occurs slowly in the dark. We have shown that the increase in absorption at 325 nm with the addition of ferricyanide parallels the increase in area above the Chl *a* fluorescence induction curve. Also, we find that the dark recovery of both the 325 nm absorption change and the area above the Chl *a* fluorescence induction curve are very similar and on the order of several minutes. Based on these experiments, as well as on absorption change measurements that have reached steady-state under flashed actinic light, we conclude that there are two types of Photosystem II electron acceptors. One type, Q_a, is connected to Photosystem I through the plastoquinone pool, and is active in oxygen evolution. The other type, Q_{400}, is unconnected to the plastoquinone pool, and cannot support oxygen evolution. Binding studies with [^{14}C]atrazine are consistent with the view that each quinone is associated with a site for herbicide binding.

Materials and Methods

Dwarf pea seedlings (*Pisum sativum* L. var. Progress 9) were grown in vermiculite-filled trays in a growth chamber (16 h day; 25/20°C; light intensity 45 W/m^2). Chloroplasts were isolated as previously described [8]. All experiments were run with chloroplasts suspended in reaction medium containing 0.4 M sucrose/50 mM Tes (pH 7.5)/10 mM NaCl/5 mM $MgCl_2$ at 20°C. Total chlorophyll concentration of the chloroplast preparations was determined by the method of Arnon [9] in 80% acetone.

Photochemically induced absorption changes at 705 and 325 nm were measured with a laboratory-built dual-beam spectrophotometer. The sample was held in a 1 cm square quartz-cuvette. Single saturating flashes were provided by a xenon flash-lamp of 3 μs width at half-height. Actinic flashes were filtered through a combination of CS 3-72 and CS 4-96 Corning glass filters. Analytic light was provided through a Bausch and Lomb high intensity monochrometer with a 150 W tungsten-halogen lamp powered by a regulated direct-current supply. For measurements below 300 nm a 45 W deuterium lamp was the light source. The analytic light was passed through either a CS 2-64 or CS 7-54 Corning glass filter to prevent second order light from reaching the detector. An Oriel monochrometer was placed in front of the sample-beam photomultiplier. Except when actinic flashes were delivered at a rate of 2 Hz or greater, a shutter positioned in the analytic beam was opened just prior to the start of the measurement to avoid any large amounts of sample excitation by analytic light. Analog data were digitized by a Biomation model 805 transient recorder, and improvement in signal-to-noise ratio was accomplished by averaging a number of data traces with a Classic 7870 computer from Modular Computer Systems Inc.

Corrections were made for particle flattening [10] to allow light-induced absorption changes to be related to changes in concentration of the photochemically altered component absorbing at a particular wavelength. Correction factors for the measurements reported here were determined by the pigment extraction method of Pulles et al. [11].

Light-induced absorption changes at 705 nm and 325 nm were used to calculate concentrations of P-700, the reaction center chlorophyll of Photosystem I, and Q_a, the primary electron acceptor of Photosystem II, respectively. The extinction coefficient used at 705 nm for P-700 was 64 $mM^{-1} \cdot cm^{-1}$ [12] and at 325 nm was 12 $mM^{-1} \cdot cm^{-1}$ [13].

Chl *a* fluorescence was measured in a conventional fluorimeter. The actinic source was at a right angle with respect to the photodiode (United Detector Technology, PIN 10DP) which was shielded with a Corning CS 2-64 glass filter. The actinic source consisted of an incandescent lamp powered by a voltage-regulated direct-current supply. The lamp emission was focused by a lens and occluded at the focal point by an electronic shutter (Uniblitz model 23X2A0X5), which had an opening time of 0.8 ms. The actinic light was filtered with a Corning CS 4-96 glass filter. The photodiode output was amplified with a laboratory built transimpedance amplifier that had a 1 ms rise-time. A Biomation 2805 waveform recorder was used to digitize the data. Data were transferred to a Hewlett Packard HP-87 computer for analyses and area determination.

Herbicide-binding analysis was previously described [14]. Oxygen yield per flash was determined with a Clark-type electrode. Excitation was provided by two xenon flash lamps that were positioned on opposite sides of the measuring vessel. The flashes were triggered simultaneously and were sufficiently intense to saturate oxygen evolution. Flashes were given at a rate of 5 Hz. From the oxygen yield per flash, the photosynthetic unit size for oxygen production was calculated. This was converted to the Photosystem II concentration by dividing by 4, since on a particular flash only one-fourth of the reaction centers are evolving an oxygen molecule [15].

Results

The absorption change at 325 nm was observed following the first actinic flash in a dark-adapted sample. Fig. 1 shows the absorption change of a sample containing 4 μM DCMU (upper curve) and of a sample containing 500 μM ferricyanide and 4 μM DCMU (lower curve). The ferricyanide was added in the dark and allowed to incubate for 5 min before addition of the DCMU. The introduction of ferricyanide caused the signal to increase by about 70% in this sample. In other samples increases as large as 100% have been observed. There are a number of possible explanations for this ferricyanide-induced increase in the absorption change at 325 nm.

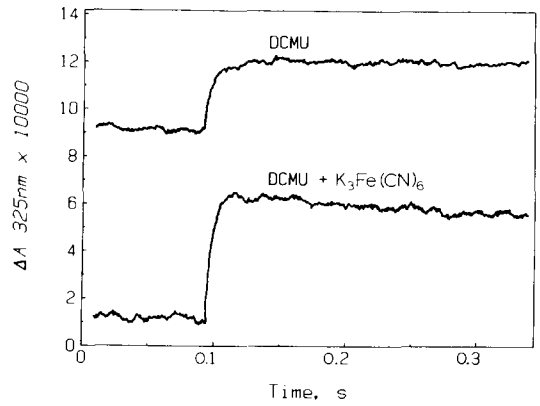

Fig. 1. Absorption change at 325 nm in pea thylakoids after a single flash of saturating intensity. The samples were measured at a Chl concentration of 25 μg/ml. These signals were an average of 49 measurements. The electronic rise-time was 20 ms. The sample represented by the upper trace was dark adapted for 10 min at 20°C before the addition of 4 μM DCMU. The sample represented by the bottom trace had 500 μM ferricyanide added 5 min prior to the addition of 4 μM DCMU.

In a dark-adapted sample with DCMU present, a single flash will cause charge separation to occur at Photosystem II and I. At Photosystem II oxygen S-states will turn over and Qa will be reduced. At Photosystem I, P-700 will be photooxidized and then reduced as charge passes through the intersystem carriers (plastoquinone, cytochrome, and plastocyanin). The absorption change at 325 nm is a composite of absorption changes associated with all these charge carriers. Based on the difference spectrum of Van Gorkom [13] it is believed that Q_a is the major contributor to the absorption change at 325 nm. By inhibiting the Photosystem II charge separation, with the preillumination of a sample containing DCMU and hydroxylamine, the contribution from Photosystem I and the associated charge carriers was determined [4,16]. This contribution was found to be 18% of the control, but in the opposite direction. Thus, if the addition of ferricyanide oxidized P-700 as well as the intersystem charge carriers and eliminated their absorption changes at 325 nm, this would only increase the 325 nm signal by 18%. The 70–100% increase observed here (see Fig. 1) must have another explanation.

It is known that incubation in the dark with ferricyanide oxidizes an additional acceptor associ-

ated with Photosystem II [5]. This acceptor has been designated as Q_{400}, since it has a 400 mV midpoint potential at pH 7 [5,6]. Under normal conditions, in the absence of ferricyanide, the redox potential of the reaction medium will be well below 400 mV and Q_{400} will be reduced and inoperative. However, when oxidized it can be detected as an increase in area above the Chl *a* fluorescence transient [5–7], and it can participate in multiple charge-separations at Photosystem II [7,17]. It seemed likely that Q_{400} was oxidized in our sample in the presence of ferricyanide, and was therefore responsible for the increased absorption change seen in Fig. 1.

In order to determine if there was a relationship between the increase in the 325 nm signal in the presence of ferricyanide and additional PS II electron acceptors, we plotted both the increase in absorption change at 325 nm and the increase in the area over the Chl *a* fluorescence induction curve as a function of ferricyanide concentration in Fig. 2. The data have been normalized by dividing each data point by the value of the measurement in the absence of ferricyanide. The two data curves show good agreement in their dependence on ferricyanide concentration.

After illumination, both Q_a and Q_{400} are completely reduced but become reoxidized in the dark. In Fig. 3 we have plotted the increase in both the absorption change at 325 nm and the area over the Chl *a* fluorescence curve in the presence of ferricyanide as a function of the dark time after preillumination. Again, the data have been normalized by dividing each point by the value of the absorption change or the Chl *a* fluorescence area in the presence of DCMU only. The kinetics of recovery of both ferricyanide effects are the same, with half times of about 120 s. This recovery in area above the Chl *a* fluorescence curve does not agree with Ikegami and Katoh [5] who reported little or no reoxidation of Q_{400} if DCMU were present. We noted that their buffer contained no $MgCl_2$, while ours normally had 5 mM $MgCl_2$. The dark recovery experiment was also done in buffer containing no $MgCl_2$ (■——■) in Fig. 3. Under these conditions an extremely slow recovery was observed. This result seems to be related to work by Itoh [18,19] who demonstrated that Qa was more rapidly oxidized by ferricyanide when $MgCl_2$ was present. It was hypothesized that Mg^{2+}

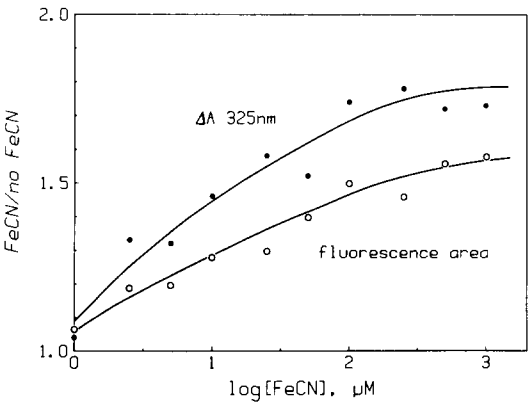

Fig. 2. Effect of varying ferricyanide concentration on (○) the area above the Chl *a* fluorescence curve and (●) the absorption change at 325 nm. The area above the fluorescence curve was normalized by dividing by the difference between the maximum fluorescence level, F_m, and the minimum level, F_0. Both fluorescence and absorption change data are expressed as the ratio of the measurement with ferricyanide to the measurement without ferricyanide. The sample Chl concentration was 25 μg/ml. The sample was incubated for 10 min in the dark at 20°C at the various ferricyanide concentrations. After incubation, DCMU was added to a final concentration of 4 μM. The absorption change measurement was an average of 49 acquisitions.

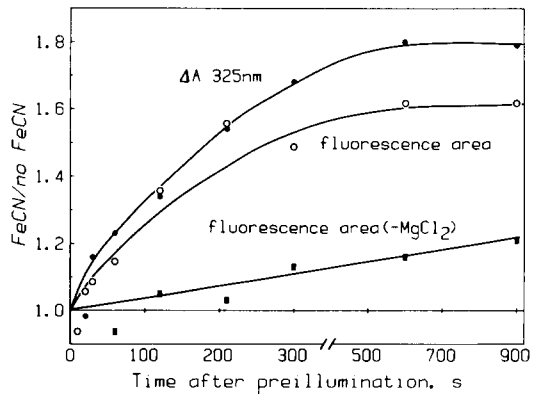

Fig. 3. The effect of time after preillumination on (○) the area above the Chl *a* fluorescence curve and (●) the absorption change at 325 nm, using standard buffer containing 5 mM $MgCl_2$. The recovery of fluorescence area was also measured in buffer without $MgCl_2$ (■). Both fluorescence and absorption change data are expressed as the ratio of the measurement with 500 μM ferricyanide as a function of dark time after preillumination to the measurement without ferricyanide. The absorption change measurements were an average of 10 acquisitions. All other measurement details are the same as in Fig. 2.

shielded the negative surface charge of the thylakoid membrane and allowed ferricyanide to approach the membrane surface more readily. Our data (Fig. 3) indicate that this same principle holds for the oxidation of Q_{400} by ferricyanide.

Based on the data of Fig. 3 the recovery of Q_{400} is on the order of minutes. If multiple flashes were given rapidly, Q_{400} would be reduced on the first flash and would be inoperative on subsequent flashes due to its slow reoxidation. If our hypothesis is correct, that Q_{400} is responsible for the additional absorption change at 325 nm when ferricyanide is present (Fig. 1), then this ferricyanide induced change should disappear under multiple-flash excitation.

A measurement was made of the steady-state absorption change at 325 nm, using actinic flashes delivered at 5 Hz. Fig. 4 shows the absorption change at 325 nm for pea thylakoids with methyl viologen as the electron acceptor (upper curve). The bottom curve has ferricyanide as the electron acceptor. No DCMU was used in the steady-state experiments. It should be noted that the amplitude of the absorption change was essentially the same when either methyl viologen or ferricyanide was used as the electron acceptor, and both measurements gave approximately the same Chl/Photosystem II value as the first flash experiment in the presence of DCMU only (Fig. 1). It is apparent from the above data that the increase in signal size seen with ferricyanide is peculiar to the first flash only. It is interesting to note the change in signal decay kinetics. The half-time of decay for methyl viologen is about 0.5 ms, while that of the ferricyanide samples is about 1.3 ms. We have as yet no explanation for this change in kinetics with alteration of the acceptor.

It has been suggested by a number of workers that the absorption change at 325 nm in untreated thylakoids is a composite signal made up of changes at both the donor and acceptor sides of Photosystem II [20–22]. In order to see what effect the donor side of Photosystem II had on the 325 nm absorption change under steady-state flashed light, we treated thylakoids with hydroxylamine. Hydroxylamine is known to be an effective electron donor at P-680 and to block normal electron flow between P-680 and the donor side of Photosystem II. In this way any absorption change due to S-states or charge carriers on the donor side of Photosystem II can be eliminated. Fig. 5 shows the steady-state absorption changes at 325 nm for thylakoids in a control sample and a hydroxylamine-treated sample containing 50 μM methyl viologen as the electron acceptor. The data show a decrease of about 15% in the hydroxylamine treated sample. This 15% is the contribution to the 325 nm

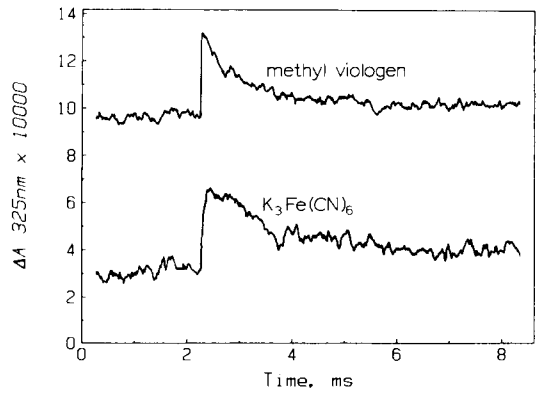

Fig. 4. Absorption change at 325 nm in pea thylakoids after multiple saturating flashes given at 5 Hz. The samples were measured at a chlorophyll concentration of 25 μg/ml. These signals were an average of 600 measurements. The electronic rise-time was 100 μs. The upper trace had 50 μM methyl viologen as the electron acceptor. The bottom trace had 500 μM ferricyanide as the electron acceptor.

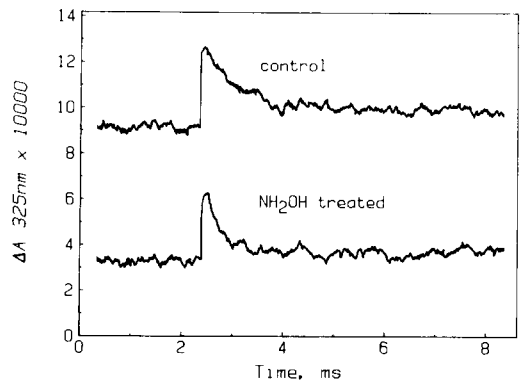

Fig. 5. Absorption change at 325 nm in pea thylakoids after multiple saturating flashes given at 5 Hz. All samples contained 25 μg Chl/ml. These signals were an average of 600 measurements. The sample was changed after 200 data aquisitions. The electronic rise-time was 100 μs. The upper trace had 50 μM methyl viologen as the electron acceptor. The bottom trace had 50 μM methyl viologen as the electron acceptor and was incubated with 3 mM hydroxylamine for 10 min in the dark at 20°C to stop Photosystem II donor transitions.

change from the donor side of P-680. An identical 15% contribution has been observed in Tris-treated chloroplasts [4]. As already discussed, contributions due to Photosystem I and intersystem charge carriers have been determined to be 18%, but in the opposite direction as the control for first flash measurements [4,16]. Similar type experiments could not be done with ferricyanide present or under steady-state flash conditions, so we have presumed the same contribution percentages apply in these cases. Since contributions due to donor- and acceptor-side carriers are essentially equal and of opposite direction the 325 nm absorption change can be used directly (uncorrected) when making stoichiometry calculations.

Table I gives the photosystem stoichiometry of pea thylakoids obtained by several different Photosystem II measurements. All of the 325 nm absorption change measurements except ΔA 325 nm (1st) (DCMU + $K_3Fe(CN)_6$) give Chl/Photosystem II values of about 600. The oxygen flash yield experiment gives a somewhat similar value of 650. It should be noted that for the absorption change at 325 nm on the first flash with DCMU and ferricyanide present the Chl/Photosystem II ratio is approx. one-half that found under other conditions. The Chl/Photosystem II value of 355 is essentially the same as that obtained from atrazine binding measurements. Herbicide binding such as the atrazine measurements carried out here give a value for the number of binding sites in a given amount of Chl. The binding site is thought to be the 32 kDa protein of Photosystem II. Thus there appears to be one binding site for each Q_a and Q_{400}.

TABLE I

STOICHIOMETRY OF PHOTOSYSTEM II AND I IN PEA THYLAKOIDS

Photosystem ratios (PS II/PS I) are given for a Chl/PS I ratio of 825 as determined by ΔA_{705nm}. Based on five repeat measurements the standard deviation for ΔA_{325nm} was ±10%, for atrazine binding ±15%, and for oxygen yield ±5%. The flattening correction for ΔA_{325nm} was 1.60. ss, steady state; 1st, first flash.

Method	Chl/PS II	PS II/PS I
ΔA_{325nm} (ss) (methyl viologen)	583	1.42
ΔA_{325nm} (ss) ($K_3Fe(CN)_6$)	594	1.39
ΔA_{325nm} (1st) (DCMU)	598	1.38
ΔA_{325nm} (1st) (DCMU + $K_3Fe(CN)_6$)	355	2.32
Atrazine binding	367	2.25
Oxygen yield	650	1.27

To confirm the identity of the light-induced absorption changes at 325 nm, we measured a light-minus-dark difference spectrum for pea thylakoids containing DCMU (Q_a spectra) and for pea thylakoids containing both DCMU and ferricyanide (Q_a plus Q_{400} spectra). Fig. 6 shows the reduced-minus-oxidized spectrum near 325 nm. Both spectra have maxima at 325 nm and minima at 275 nm with isobestic points near 288 and 253 nm. These spectra have many features in common with the semiquinone anion-minus quinone difference spectra of Bensasson and Land [23]. We have plotted in Fig. 6 (solid line) the difference spectra for Q_{400}, which is calculated from the difference between the (DCMU + $K_3Fe(CN)_6$) and (DCMU) curves. This spectrum, which has a maximum at 315 nm, a minimum at 276 nm and isobestic points near 290 and 253 nm, also has features in common with the semiquinone-anion-minus-quinone difference spectra [23].

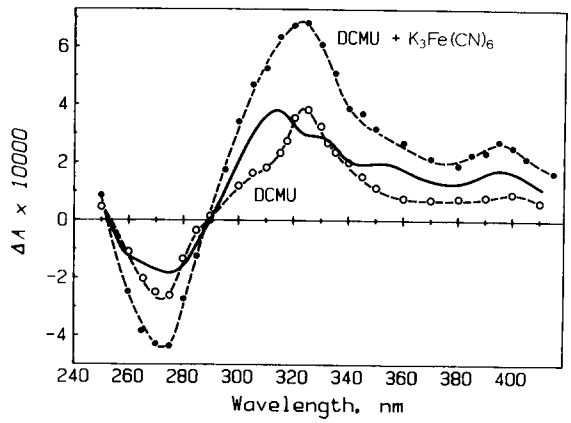

Fig. 6. Light-induced absorption changes of pea thylakoids as a function of wavelength. The sample represented by solid points contained 4 μM DCMU and 500 μM ferricyanide. The sample represented by open points contained 4 μM DCMU and was obtained by giving a group of four flashes. Absorption changes after the 1st flash were assumed to be contributions from components other than Q_a and were thus added or subtracted depending on their direction. These multiple flash components were very small at 325 nm but became significant at wavelengths where P-700 absorptions are large [12]. All samples contained 25 μg Chl/ml. Each point was the average of at least 36 acquisitions.

Discussion

The addition of ferricyanide to chloroplasts causes an increase in the area above the Chl *a* fluorescence rise curve. Since the area above the curve normalized by dividing by the variable fluorescence is proportional to the size of the acceptor pool, it suggests that the presence of ferricyanide causes the oxidation of a high-potential second acceptor called Q_{400} [5,6]. The presence of a second acceptor at P-680 induced by ferricyanide incubation has also been reported by Jursinic [7] and Velthuys and Kok [17]. They found that ferricyanide increased the oxygen yield on flash number two, and this has been interpreted as an increase in the double advancement in S-states only following the first flash.

In the data presented here we demonstrate that the increase in absorption change at 325 nm in the presence of ferricyanide (Fig. 1) correlates well with the increase in area above the Chl *a* fluorescence induction curve as a function of ferricyanide concentration (Fig. 2). This correlation between Chl *a* fluorescence area and the absorption change at 325 nm is also seen in the dark recovery following preillumination (Fig. 3). From this data we conclude that the increase in absorption at 325 nm in the presence of ferricyanide is in fact a manifestation of the acceptor Q_{400}.

Since the dark recovery of the ferricyanide oxidized acceptor Q_{400} is on the order of 5–10 min (Fig. 3), it is reasonable to expect that it will be observed only on the first flash following dark adaption. This is observed in Fig. 4 where flashes are delivered at 5 Hz and the 325 nm signal is the same size with or without ferricyanide. Q_{400} under these rapid flash conditions does not have time to become reoxidized in the dark, whereas Q_a is reoxidized in 1–2 ms [24].

The spectra generated in Fig. 6 suggest that both Q_a (open circles) and Q_{400} (solid line) are quinones, since both show similarity to the quinone difference spectra of Bensasson and Land [23]. The suggestion that Q_{400} is a quinone is also consistent with the fact that the atrazine binding data show a greater number of binding sites than prediced by oxygen flash yield (Table I, lines 5 and 6) and that the number of atrazine binding sites is close to the number of $Q_a + Q_{400}$ (Table I, lines 4 and 5). Both Q_a and Q_{400} appear to have binding sites for atrazine with similar affinity.

For every active oxygen evolving system we find one Q_a and one Q_{400}. We propose the following two models that are consistent with this observation:

M	Z	P-680	Ph	Q_a *	Q_{400} *	(1)
M	Z	P-680	Ph	Q_a *		
M′	Z	P-680	Ph	Q_{400} *		(2)

where M is an active and M′ an inactive oxygen evolving complex, Z is the reaction center electron donor, * the atrazine binding site and Ph is pheophytin. In the series model (1) a single P-680 reaction center is connected to both Q_a and Q_{400}. The parallel model (2) has an oxygen-evolving complex for each reaction center P-680, but only about half the reaction centers are connected to the electron acceptor Q_a. The other reaction centers are connected to Q_{400}. In both models under normal conditions only Q_a is available for charge separation. Q_{400} is available for charge separation only when it is oxidized in the dark by ferricyanide or some other high potential oxidant. Its subsequent reduction by light can be observed by either Chl *a* fluorescence induction or absorption change at 325 nm. Since reoxidation takes place slowly, Q_{400} does not play a role in electron transport during steady-state flashed light or continuous light. Its function in the Photosystem II reaction center complex remains a question for further experimentation.

When Q_{400} is oxidized it participates in Photosystem II charge separation [5–7] and under proper conditions can give rise to double advancement of oxygen S-states [7,17]. The series model accomodates this observation without difficulty since the re-reduction (Z P-680$^+ \rightarrow Z^+$ P-680) takes place in about 23 ns on the first flash [25]. Thus P-680 has ample time to become re-reduced during the 3 μs flash, and therefore is able to undergo a second charge separation during the single flash. Based on this model the stoichiometry ratios expected in our plant material for P-680/Qa/Q_{400}/Chl is 1 : 1 : 1 : 600. Eckert et al. [26] have measured P-680 under repetitive flash conditions in spinach thylakoids and found P-680/Chl is 1 : 360. Their sample material was quite different from ours, and

apparently had a much smaller antenna system, since their oxygen flash yields gave 360 Chl per PS II. Their data was consistent with the series model with one P-680 for every active oxygen-evolving complex (however, see below).

In order to explain double advancements of S-states [7,17], the parallel model requires that positive charge generated at the Q_{400} reaction center on the first flash be shared with the adjacent Q_a reaction center. This type of charge sharing by reaction centers has been suggested before for oxygen evolving centers partially inhibited by DCMU or given subsaturating flashes [27]. We therefore hypothesize that the oxygen-evolving complex of the Q_{400} center, M', is inactive and unable to use any positive charge generated at its reaction center. On subsequent flashes Q_{400} will be reduced and the reaction center will be unable to accept quanta. These quanta will be dissipated as heat or transferred to neighboring Q_a centers where charge separation is possible.

The parallel model is consistent with some recent findings by Murata et al. [28] where measurements of P-680, oxygen-evolving complex, Mn and Chl were found to be in the ratio of 1 : 1 : 4 : 220 in Photosystem II particles of spinach. In a similar Photosystem II particle preparation Lam et al. [29] found a ratio for Q (measured in the presence of ferricyanide) to Chl of 1 : 230. Thus when both Qa and Q_{400} are measured (dark adapted sample + ferricyanide) there is a 1 : 1 : 1 ratio between Q, P-680 and the total oxygen evolving complex (active + inactive).

The measurements of Eckert et al. [26] seem to support the series model. However, under their repetitive flash conditions Q_{400} would be reduced and $P\text{-}680^+$ Ph^- Q^-_{400} would be produced after every flash. If recombination between $P\text{-}680^+$ and Ph^- occurs in 2–4 ns [30] then Eckert et al. [26] would have missed the component of $P\text{-}680^+$ Ph^- Q^-_{400} decay. Indeed, they observed a $P\text{-}680^+$ decay component with a half-time of less than 10 ns, but were unable to carry out any quantitative measurements on it. Thus the $P\text{-}680^+$ decay data are consistent with either model (1) or (2). Further experimentation will be required to determine which is the correct model.

The results reported here bring to light the importance of defining the method of measuring Photosystem II when discussing photosystem stoichiometry. In terms of functional electron transport it is important to use oxygen evolution or either the absorption change at 325 nm after a single flash without ferricyanide or the absorption change after many flashes for determining Photosystem II concentration. The Photosystem II/Photosystem I stoichiometries based on atrazine binding or 325 nm absorption changes in the presence of ferricyanide give additional information about Photosystem II structure, i.e., the existence of a secondary acceptor Q_{400}.

Our results are in agreement with Whitmarsh and Ort [3] in that they measure values for Chl/Photosystem II of about 630 by flash yield techniques, but values of only 290 by absorption change measurements at 323 nm in the presence of 1 mM ferricyanide. However, they speculate that the absorption change technique is in error because of underlying assumptions made about the amount of correction for particle flattening, and that donor side reactions at 325 nm have not been subtracted. The data presented in this paper indicate, however, that the absorption change at 325 nm made in the presence of ferricyanide leads to erroneous conclusions about Chl/PS II because the existence of the secondary acceptor Q_{400} has previously been overlooked [1,3,22]. In our opinion previous calculations of Photosystem II concentration, Photosystem II/I stoichiometry, Photosystem II_α and II_β antenna sizes [1,31–36] based on the absorption change at 325 nm with ferricyanide present need to be reexamined in order to account for non-functional reaction centers in steady-state electron transport.

We believe that our new techniques of measuring the absorption change at 325 nm in the absence of ferricyanide or under steady-state flashed light leads to an accurate and reliable estimate of active Photosystem II concentration.

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