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# Redox study of electron donation to P-680 in Photosystem II

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**Flash-induced absorption changes at 820 nm were studied as a function of redox potential in Tris-extracted Photosystem II oxygen-evolving particles and Triton subchloroplast fraction II particles. The rereduction kinetics of P-680$^+$ in both preparations showed biphasic recovery phases with half-times of 42 and 625 μs at pH 4.5. The magnitude of the 42 μs phase of P-680$^+$ rereduction was strongly dependent on the redox potential of the medium. This absorption transient, attributed to electron donation from $D_1$ (the secondary electron donor in oxygen-inhibited chloroplasts), titrated as a single redox component with a midpoint potential of +240 ± 35 mV. The experimentally determined midpoint potential was found to be independent of pH over the tested range 4.5–6.0. In contrast, the magnitude of the 625 μs phase of P-680$^+$ rereduction was independent of redox potential between +350 and +100 mV. These results are interpreted in terms of a model in which an alternate electron donor with $E_m \approx 240$ mV, termed $D_0$, serves as a rapid donor ($t_{1/2} \leq 2$ μs) to P-680$^+$ in Tris-extracted and Triton-treated Photosystem-II preparations. According to this model, the slower electron donor, $D_1$, is functional only when $D_0$ becomes oxidized.**

## Introduction

According to current understanding, photochemical charge separation in Photosystem II takes place between a primary electron donor P-680 and an electron-acceptor complex composed of pheophytin, an iron atom, and a bound quinone [1]. Upon the absorption of photon of light, P-680 donates an electron to pheophytin, thereby inducing an electrical-charge separation across the thylakoid membrane. This electron is transferred in 200–400 ps from pheophytin to $Q_A$, the bound quinone, and in 600 μs to a two-electron gate known as $Q_B$ [2]. After receiving a second electron, the plastoquinone corresponding to $Q_B$ is released in a time period of about a millisecond to the mobile plastoquinone pool [2].

On the oxidizing side, a secondary donor Z transfers its electron to photooxidized P-680$^+$ in a time domain between 25 and 45 ns [3]. Additionally, the kinetics of electron transport between Z and P-680$^+$ have been demonstrated to be dependent on the flash number [4,5]. When the water-splitting complex in chloroplasts has been inactivated with Tris [6] or $NH_2OH$, the rereduction of photooxidized P-680$^+$ shows biphasic kinetics with half-times of approx. 20–40 μs and 100–800 μs, depending on the preparation [7–10]. Mathis

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Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; Ph, pheophytin intermediate electron acceptor; $Q_A$, primary quinone acceptor; $Q_B$, secondary quinone acceptor; Z, secondary electron donor in oxygen-evolving chloroplasts; $D_1$, secondary electron donor in oxygen-inhibited chloroplasts; Mes, 4-morpholineethanesulfonic acid; $E_m$, midpoint potential; TSF-II, Triton subchloroplast fraction II.

and co-workers interpret the fast phase to represent electron donation from $D_1$, a one-electron, high-potential redox component that is either an accessory donor or a modified state of the physiological donor. The slow phase of P-680$^+$ reduction is usually attributed to a back reaction from $Q_A^-$. An extensive discussion of the donor reactions in Photosystem II has been presented by Bouges-Bocquet [11].

The model that has been proposed to account for the rereduction of P-680$^+$ is shown below:

$$Z \xrightarrow{k_d} P\text{-}680^+ \rightleftharpoons Ph \rightarrow Q_A^- \rightarrow Q_B$$
$$\underset{k_{br}}{\longleftarrow}$$

According to this model, the rereduction of P-680$^+$ can occur by electron transfer from Z or by backreaction from $Q_A^-$. The relative rate of electron donation will depend on the rate constants $k_d$ and $k_{br}$ and on the number of electron equivalents available in Z and $Q_A$. In normal oxygen-evolving chloroplasts electron transfer from the physiological donor Z takes place in less than a microsecond; under these favorable kinetic conditions, little or no backreaction from $Q_A^-$ is likely to take place. $D_1$, the species observed after destruction of the water-splitting site, is considered to be an altered form of Z, the species active in oxygen-evolving chloroplasts. In this regard, $D_1$ has been identified tentatively as a quinone [12,13].

In the course of studying the flash-induced photooxidation of P-680$^+$ in Triton subchloroplast particles, we noticed that the presence or absence of potassium ferricyanide had a large effect on the magnitude of the absorption change at 820 nm. The TSF-II preparation is nearly devoid of P-700 and yet we found it necessary to include potassium ferricyanide in the reaction mixture to observe the 42 $\mu$s component attributed to the rereduction of P-680$^+$ by $D_1$. Potassium ferricyanide is usually added to eliminate absorption changes due to P-700.

In this work, we investigated the effect of redox potential on the reduction of photooxidized P-680$^+$ in Tris-extracted Photosystem II oxygen evolving preparations and TSF-II particles. We found that the magnitude of the fast phase of P-680$^+$ reduction was strongly dependent on the redox potential of the medium. These results are interpreted in terms of a fast ($t_{1/2} \leq 2$ $\mu$s), alternate electron donor, termed $D_0$, which competes with $D_1$ as the secondary electron donor to P-680$^+$.

## Materials and Methods

Triton Photosystem-II particles were isolated according to the following modification of the TSF-II procedure [14]. Depetiolated spinach leaves (1 kg) were ground for 30 s in a Waring blender with 1200 ml of STK buffer (0.4 M sucrose/0.05 M Tris (pH 7.8)/0.010 M KCl) and filtered through three layers of cheesecloth. The filtrate was centrifuged at 200 × *g* for 2 min to remove large fragments; the supernatant was recentrifuged at 4000 × *g* for 10 min to sediment the chloroplasts. The pellet was resuspended in EDTA-containing buffer (0.020 M Tricine (pH 7.5)/0.015 M NaCl/0.001 M EDTA), incubated for 30 min at 4°C, and centrifuged at 4000 × *g* for 12 min. The pellet was resuspended to 800 $\mu$g/ml chlorophyll in Tricine-$MgCl_2$ buffer (0.020 M Tricine (pH 7.5)/0.010 M $MgCl_2$) and Triton X-100 was added to 0.75% (v/v). After stirring for 30 min at 4°C, the suspension was centrifuged at 14500 × *g* for 20 min and the pellet was resuspended to 600 $\mu$g/ml chlorophyll. Triton X-100 was added to 0.6% (v/v) and the suspension was stirred at 4°C for 30 min. The suspension was centrifuged at 14500 × *g* for 15 min and the TSF-II particles were suspended to 2.5 mg/ml chlorophyll in Tricine buffer (0.020 M, pH 7.5). The particles were stored frozen in 20% glycerol.

Tris-extracted Photosystem-II oxygen-evolving particles were provided by Mr. Kirk Cammarata and Dr. George Cheniae. The oxygen-evolving particles were prepared by treating spinach chloroplasts (1 mg/ml Chl in 15 mM NaCl/5 mM $MgCl_2$/20 mM Mes (pH 6.2)) (incubation buffer) with Triton X-100 (25 mg Triton/mg Chl) for 30 min at 0°C, followed by centrifugation at 25000 × *g* for 30 min. The pellet was resuspended with a camel's hair brush in incubation buffer, centrifuged, and resuspended in the incubation buffer containing 0.4 M sucrose. After assay, the particles were Tris-extracted by the method of Yamashita and Butler [15]. The oxygen-evolving particles showed rates of oxygen evolution from 500 to 600

μmol/ml Chl/h with 300 μM phenylbenzoquinone as acceptor. Subsequent to Tris-extraction, the P-680 content was determined by flash absorption at 820 nm to be one P-680/268 Chl. D-10 particles were prepared according to the method of Boardman [16].

P-680 kinetics were monitored at 820 nm with a flash-kinetic spectrophotometer interfaced to a PDP11/23 minicomputer. The 820 nm measuring beam was selected from a 250 W source (Oriel) with interference and bandpass filters. A monochromator was placed after the cuvette to filter out the blue actinic flash and fluorescence artifact. The beam was detected with a PIN-10 Schottky barrier photodiode (United Detector Technology) and amplified with a Model 113 preamplifier (EG &G PARC). The data was recorded with a Biomation Model 805 transient recorder operated in the pretrigger mode and subsequently processed by the minicomputer. In practice, the fluorescence artifact masked the initial absorbance increase at 820 nm and the total absorbance change was determined by extrapolation to the onset of the flash. Actinic flashes were provided by a Photochemical Research Associates 610B xenon flash system (2.5 μs full width at half-maximum).

Flash photolysis was carried out on a 3 ml cuvette containing Photosystem-II particles at a chlorophyll concentration of 20 μg/ml. Where indicated, potassium ferricyanide (1 mM) was added to the cuvette 30 s prior to flash photolysis. Each trace is the average of the specified number of repetitive flashes spaced 10 s apart; however, a one-flash control was performed prior to data collection at each redox potential to check for any anomalies that might result from multiple flashes. The spacing between the flashes was selected to insure the rereduction of $D_1$ before each flash [13,17].

Redox titrations were performed in a specially constructed vessel that contained ports for a silver/platinum measuring electrode and a pH electrode. The sample, redox mediators and titrants were added through a stoppered opening and a steady stream of nitrogen was blown over the surface of the liquid to maintain anaerobic conditions. The system was calibrated with quinhydrone at pH 4.0 and 6.0. Two sets of redox mediators were used: set number 1 contained potassium ferricyanide (+430 mV), *N,N,N'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (+260 mV), 2,6-dichlorophenolindophenol (+217 mV), 1,2-naphthoquinone (+145 mV) and 1,4-naphthoquinone (+60 mV); set number 2 contained potassium ferricyanide (+430 mV), *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride; (+260 mV), 2,5-dimethylbenzoquinone (+180 mV) and 5-hydroxy-1,4-naphthoquinone (+30 mV). Redox titrations were performed routinely with mediator set number 1; key points in the titration were checked either in the absence of mediators or with mediator set number 2 to ensure that the titration was independent of the mediator mix. Mediator sets were prepared as a 10 mM stock solution in ethanol and were added to the reaction mixture to a final concentration of 10 μM each. The redox potential was adjusted chemically by the addition of 10 mM sodium hydrosulfite (prepared in 0.1 M Tris, pH 9.0) or 10 mM potassium ferricyanide. Titrations were performed according to the following experimental protocol. Photosystem-II particles (100 μl at 2.0 mg/ml) and redox mediators (10 μl) were added to 10 ml of 0.1 M Mes buffer (pH 4.5 or 6.0) and stirred in the dark for 3 min under nitrogen. The redox potential was then adjusted by adding small aliquots of potassium ferricyanide or sodium hydrosulfite. The reaction mix was allowed to equilibrate in the dark for 10 min before transfer of 3 ml to the flash-photolysis cuvette.

## Results

*Optical absorption transients at 820 nm*

Flash-induced changes at 820 nm in TSF-II particles were measured routinely in the presence in 1 mM potassium ferricyanide. Fig. 1a shows the P-680 absorption transient at pH 4.5 after 16 repetitive flashes (the amplitude and kinetics of the absorption change in the averaged samples are identical to that seen on a single flash). Upon excitation with an actinic flash, P-680 shows an immediate absorption increase followed by a biphasic decay. In Fig. 1b the logarithm of the absorption change is plotted against time. The slow phase has a half-time of 625 μs and accounts for 28% of the total absorption change. When the slow phase is subtracted from the fast phase, the

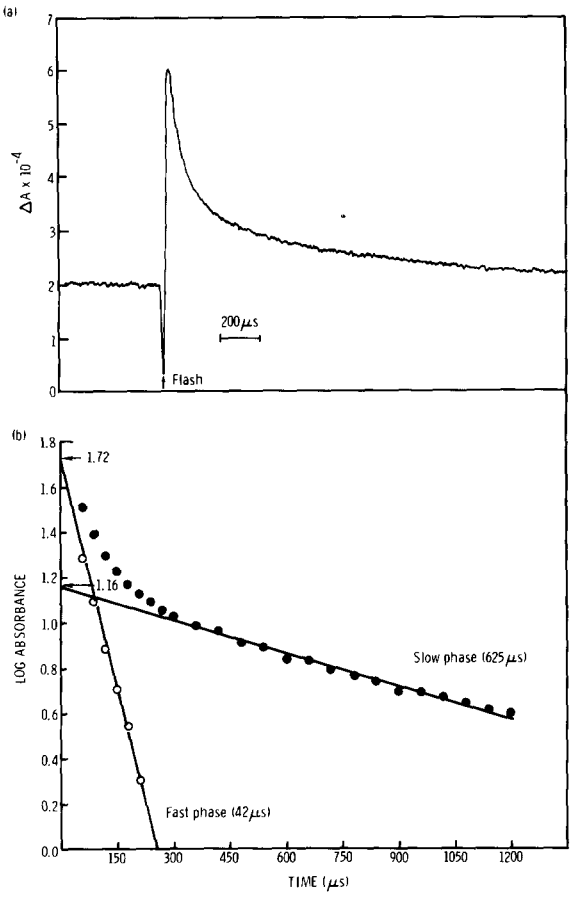


Fig. 1. Kinetics of the rereduction of P-680$^+$ in spinach TSF-II particles following a saturating flash. The particles were suspended in Mes buffer (0.1 M, pH 4.5) at a chlorophyll concentration of 20 μg/ml. (a) Kinetics of the absorption transient at 820 nm in the presence of 10 mM potassium ferricyanide. The figure shown is the average of 16 repetitive flashes. (b) Log plot of the absorption transient at 820 nm. The fast phase was determined by extrapolating the slow phase to the onset of the flash and subtracting the calculated contribution from the total absorption charge.

latter has a half-time of 42 μs and accounts for the remaining 72% of the absorption change. There is no indication of any intermediate or slower phases. When the fast phase is extrapolated to zero time, the total absorption change equals $0.57 \cdot 10^{-3}$ absorbance units; assuming an extinction coefficient of 7000 $M^{-1} \cdot cm^{-1}$ for P-680 at 820 nm [9], the spinach TSF-II particle contains one reaction center per 245 chlorophyll molecules.

There have been significant differences reported in the half-time of the slow phase; values range from 100–200 μs in Tris-washed chloroplasts [3,6,7] to 800–1000 μs in a Photosystem-II reaction-center preparation [8]. Although there is yet no adequate explanation for these variations, we consider it reasonable that the 42 μs component observed in our preparation is due to electron donation from $D_1$ to P-680$^+$, and the 625 μs component is due to either a more distant electron donor than $D_1$ or to the $Q_A^-$ to P-680$^+$ backreaction.

*Redox dependence of P-680 absorption transients*

We have observed that the magnitude of the P-680 transient in Tris-washed chloroplasts and Triton Photosystem-II particles is maximal when an oxidant such as potassium ferricyanide is included in the reaction mixture. The 42 μs component of the absorption change is less than half-maximal in the absence of ferricyanide and completely eliminated in the presence of a small amount of ascorbate. Fig. 2 shows the P-680 absorption transient at pH 4.5 in TSF-II particles poised at several redox values. At a redox potential of +395 mV or higher, the magnitude of the fast phase is maximal (Fig. 2a). Between potentials of +277 and +224 mV, the fast phase is progressively diminished and at +207 mV the fast phase

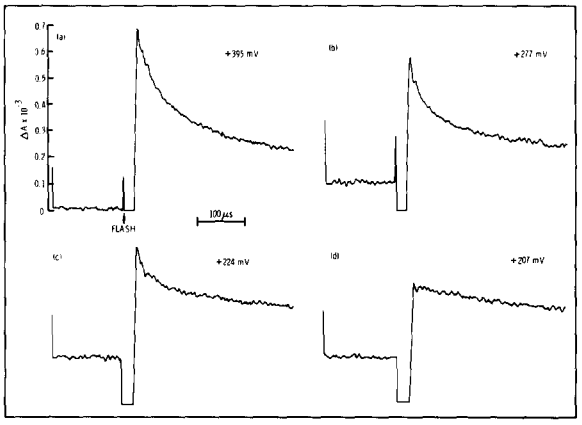


Fig. 2. The effect of redox potential on the amplitude of the 820 nm absorption transient in spinach TSF-II particles. The particles were suspended in Mes buffer (0.1 M, pH 4.5) at a chlorophyll concentration of 20 μg/ml. Redox mediators were set number 1 as specified in Materials and Methods. The potential was established with potassium ferricyanide and sodium dithionite. The traces shown are the average of eight repetitive flashes.

is completely eliminated (Fig. 2b–d). An identical effect of redox potential on the amplitude of the fast phase was found at pH 6.0. Note that the 625 μs phase is still present at +207 mV and that the amplitude of this slow phase does not appear to change throughout the course of the titration. A priori one would expect the extent of P-680 photooxidation to be independent of redox potential in the range of +200 to +300 mV, since $Q_A$ remains oxidized in this region. To account for the disappearance of the fast phase at lower potentials, we must propose either (a) the lifetime of the fast phase is dependent on redox potential or (b) the superposition of a fast reduction phase not detectable within the time response of our apparatus.

The amplitude of the fast phase is plotted as a function of redox potential in Fig. 3a and b for a complete series of titrations. The amplitude of the fast phase at each potential was determined by plotting the logarithm of the absorption change against time and subtracting the contribution of the slow phase (See Fig. 1). At pH 6.0 (Fig. 3a), the fast phase of P-680$^+$ recovery in TSF-II particles has a midpoint potential of +225 mV ± 35 mV. Although the scatter in the data does not allow us to determine if the data represent a one- or two-electron titration, the theoretical curve is shown as a one-electron Nernstian titration. We also performed the titration in Tris-extracted Photosystem-II oxygen-evolving particles; this preparation is used as a control for the TSF-II titration, since it was competent in oxygen-evolution prior to Tris-extraction yet was nearly free of Photosystem I. The midpoint potential was found to be +235 mV ± 30 mV at pH 6.0 (Fig. 3a). At pH 4.5 (Fig. 3b), the fast phase in TSF-II particles showed a midpoint potential of +250 mV ± 25 mV. The half-time of the fast phase at pH 4.5 was shorter at pH 6.0 (32 μs) than at pH 4.5 (42 μs), in agreement with the trend noted by Mathis et al. [10]. The maximum absorption change was 30–35% lower at pH 6.0 than at pH 4.5 and we therefore scaled the relative absorbance changes to the same value for comparative purposes. The scatter in the data below +200 mV (Fig. 3b) is due to the relatively low buffering capacity of the redox mediators below +225 mV at pH 4.5. Both redox titrations indicate that the fast phase has a pH-independent midpoint potential in the region of +225 to +250 mV.

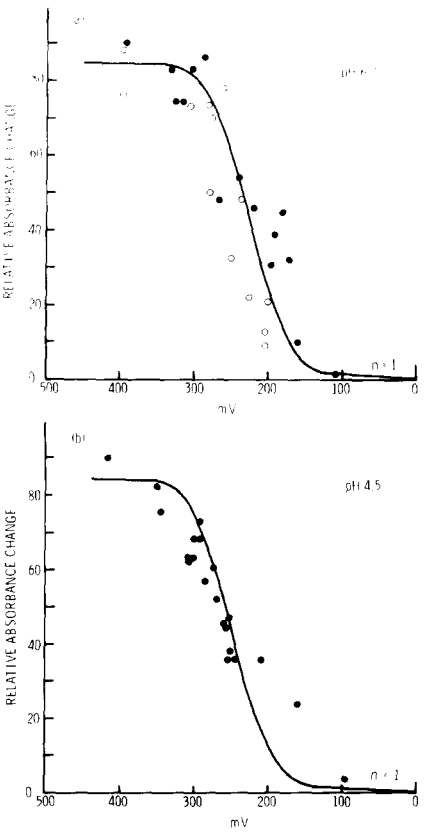


Fig. 3. Redox titration of the amplitude of the fast absorption transient in Tris-extracted Photosystem-II oxygen-evolving particles (○) and TSF-II particles (●) at pH 6.0 (a) and 4.5 (b). The relative absorbance changes are normalized to the same value at both pH units. A fresh sample was used for each determination. A control particle containing 10 mM ferricyanide underwent flash photolysis several times during the titration to ensure that no changes had taken place with time. See Materials and Methods for further details.

In Fig. 4, the half-time of the fast phase of P-680$^+$ recovery is plotted against redox potential for the data shown in Fig. 3a. The data suggest that only the amplitude and not the half-time diminishes as the redox potential becomes increasingly negative. Except for data obtained below +225 mV where very little fast phase remains at pH 6.0 (and hence the error bounds are larger), the half-time does not change when Photosystem-II particles or Tris-washed particles are poised at redox potentials between +210 and +350 mV.

The magnitude of the 625 μs phase of P-680$^+$ recovery in TSF-II particles is plotted against redox potential in Fig. 5. The plot is constructed so

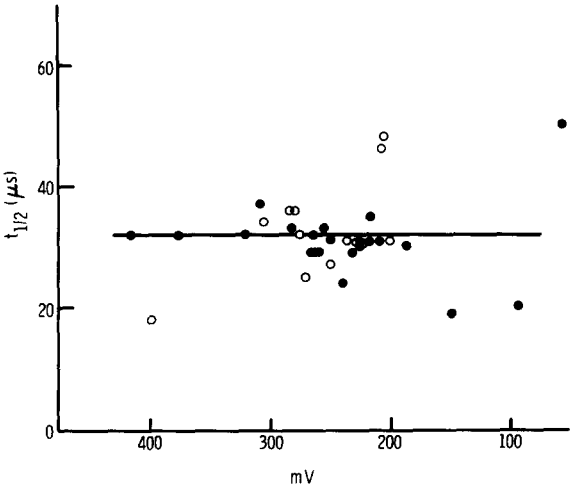


Fig. 4. Half-time of the fast phase plotted against redox potential in Tris-extracted Photosystem-II oxygen-evolving particles (○) and TSF-II particles (●) at pH 6.0. The half-times refer to the fast phase of the absorbance change for the data obtained in Fig. 3a. Experimental conditions are outlined in Fig. 3; see Materials and Methods for further details.

that the magnitude of the slow phase is shown as a fraction of the total absorbance change at the two respective pH values as determined from measurements at high redox potentials ($E_m > 300$ mV). It

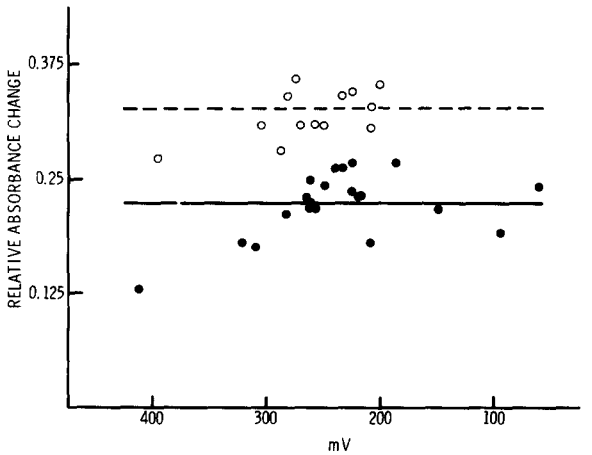


Fig. 5. Amplitude of the slow phase plotted against redox potential in spinach TSF-II particles at pH 4.5 (○) and pH 6.0 (●). The relative absorbance change refers to the amplitude of the slow phase relative to the total absorbance charge for the data obtained in Figs. 3a and b. Experimental conditions are outlined in Fig. 3; see Materials and Methods for further details.

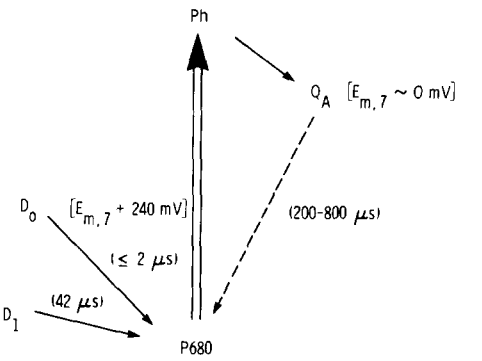


Fig. 6. Model for the Photosystem-II reaction center based on kinetic and thermodynamic properties of secondary electron donors and acceptors. $D_0$ is the proposed +240 mV component that, under reducing conditions, outcompetes $D_1$ in Tris-extracted Photosystem-II oxygen-evolving particles and triton Photosystem-II particles.

is clear that the slow 820 nm transient is not particularly sensitive to redox potential between values of +100 and +350 mV. The slow phase, however, accounts for a larger fraction of the total absorbance change at pH 6.0 (32%) than at pH 4.5 (21%). We suggest that the slow phase is due to a population of P-680 that is not connected to an efficient secondary electron-donor system.

## Discussion

We propose that in the Photosystem-II preparations described here, an alternate electron donor with $E_m \approx 240$ mV, termed $D_0$, competes with $D_1$ as a reaction partner with P-680$^+$. According to this model (Fig. 6), $D_0$ would outcompete $D_1$ as a donor to Photosystem-II at potentials lower than +240 mV (i.e., in the absence of potassium ferricyanide). This rapid donation ($t_{1/2} \leq 2$ μs) would result in the elimination of P-680$^+$ transients in those centers connected to an efficient secondary electron-donor system. The existence of the 625 μs decay component is indicative of a population of Photosystem-II reaction centers that are disconnected from the secondary donor pool. Thus centers which lack $D_1$ are also deficient in $D_0$ (see Fig. 5) and reduction of P-680$^+$ can occur only via a more distant electron donor or by charge recombination with the reduced quinone acceptor.

We performed these titrations with the expectation that the redox behavior of P-680 would reflect the chemical titration of $Q_A$. However, the experimentally-determined midpoint potential of the fast P-680 transient is several hundred millivolts too positive to be accounted for by this acceptor. Nearly every determination of the midpoint potential of $Q_A$ in chloroplasts, as determined by titration of the fluorescence rise curve, shows an $E_m$ near 0 V at pH 7.0 (for the high-potential fluorescence quencher: $E_{m,7.0} = -35$ mV [18]; $E_{m,7.8} = -45$ mV [19]; $E_{m,7.6} = +25$ mV [20]; $E_{m,7.2} = +68$ mV [21]). In Triton subchloroplast particles, the value is similar ($E_{m,7.8} = +48$ mV [19]), which is consistent with the potential observed for C550 and fluorescence yield changes in broken chloroplasts ($E_{m,7.0} = +25$ mV [22]) and C550 changes in Triton Photosystem-II particles ($E_{m,7.8} = +45$ mV [23]). Based on this evidence, we conclude that the redox titration of the fast phase of P-680 rereduction does not represent the titration of $Q_A$ in Triton Photosystem-II preparations.

The involvement of a fast electron donor is further strengthened by the finding that only the fast phase of the $P\text{-}680^+$ absorption transient in TSF-II particles titrates with a midpoint potential of +240 mV. We found no effect of redox potential on the slow phase up to a potential of +100 mV. The fast phase of $P\text{-}680^+$ recovery arises from $D_1$, the electron donor to $P\text{-}680^+$ found in functionally disrupted (Tris-washed, $NH_2OH$-treated) chloroplasts. If the slow phase of the absorption transient is due to the backreaction between $P\text{-}680^+$ and $Q_A^-$ in reaction centers, and if $Q_A$ had been chemically reduced, we might have expected the slow and fast phases to cotitrate with a midpoint potential near 0 mV.

Finally, we need to address the possibility that TMPD is the fast donor to $P\text{-}680^+$, since the midpoint potential of TMPD is similar to the $E_m$ of $D_0$. We consider donation by TMPD unlikely for the following reasons. First, TMPD's midpoint potential is pH-dependent over the range 4–6.5 (its $pK_a$ is 6.5). Hence, at pH 4.3, the $E_m$ will be about +360 mV. Second, the +240 mV midpoint potential of $D_0$ was found to be pH-independent over the range 4.5–6.0. Finally, ascorbate alone is sufficient to eliminate the fast phase of $P\text{-}680^+$ rereduction in TSF-II particles.

One added complication in the above analysis is the possibility of a double hit phenomenon in Photosystem II induced by a xenon flash. Given recovery kinetics of 30 ns for the reduction of $P\text{-}680^+$ in oxygen-evolving chloroplasts, double-hits may occur during a 2–4 $\mu$s flash pulse. Renger and co-worker [24] and other [25,26] have provided evidence for a redox component ($X_a$) which acts as a further electron acceptor of electrons from P-680 if $Q_A$ (X320) is blocked in the reduced state. $Q_A$ can be reoxidized by two routes: (i) a 600 $\mu$s reoxidation by the plastoquinone pool [27], and (ii) a 200 $\mu$s backreaction with $P\text{-}680^+$ [28,29]. The latter reaction will only occur in the absence of the normal 30 ns rereduction of $P\text{-}680^+$ by Z (in oxygen-evolving chloroplasts) or by the 42 $\mu$s rereduction of $P\text{-}680^+$ by $D_1$ (in Tris-treated chloroplasts). Since the Triton-treated particles used in this study were devoid of oxygen evolution, the majority of $P\text{-}680^+$ was rereduced by $D_1$. Thus, photooxidized $P\text{-}680^+$ would require at least 42 $\mu$s to become half-rereduced in the Triton-treated preparation and the 2–4 $\mu$s flash would not be expected to produce a significant number of double turnovers in Photosystem II. Based on this line of reasoning, we conclude that the +240 mV component does not represent the titration of $X_a$.

It is interesting to note that Horton and Croze [30] found that the high-potential form of cytochrome *b*-559 ($E_{m,7.8} = +383$ mV) is converted to an intermediate-potential form ($E_{m,7.8} = +240$ mV) by hydroxylamine treatment of chloroplasts. This +240 mV species of cytochrome *b*-559 is associated with chloroplasts that cannot oxidize water but can still function using alternative donors to Photosystem II. Further conversion to the low-potential form ($E_{m,7.8} = +90$ mV) resulted in 80–90% inhibition of Photosystem-II activity from artificial donors. Both TSF-II particles and Tris-extracted oxygen-evolving particles are unable to oxidize water but can sustain electron flow from artificial donors. The precedence for a +240 mV component of Photosystem II in inhibited preparations is suggestive that $D_0$ in our terminology might be cytochrome *b*-559. Additional support for this assignment is suggested by the observation that cytochrome *b*-559 can be photooxidized by $P\text{-}680^+$ at cryogenic temperatures, thus signifying a close association of the two redox components

[31,32]. Whatever the identity of $D_0$, our data indicate that its potential is too reducing to serve in the direct sequence of charge accumulation that results in oxygen evolution.

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