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TWO ELECTRON DONATION SITES FOR EXOGENOUS REDUCTANTS IN CHLOROPLAST PHOTOSYSTEM II

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SUMMARY

Two sites are distinguished for the oxidation of exogenous donors by Photosystem II in non-oxygen evolving chloroplasts. In the presence of lipophilic donors (e.g. phenylenediamine, benzidine, diphenylcarbazide), the rate for Signal IIf rereduction following a flash increases as the concentration of exogenous reductant increases. There is a decrease (20–40 %) in Signal IIf magnitude accompanying donor addition at low ($< 10^{-5}$ M) concentrations, but the extent of the decrease does not change further with increasing donor concentration. Complementary polarographic experiments monitoring donor (phenylenediamine) oxidation show an increase in oxidation rate with increasing donor concentration.

In the presence of the hydrophilic donor, Mn^{2+}, the Signal IIf decay halftime remains constant with increasing Mn^{2+} concentration. However, the flash-induced Signal IIf magnitude progressively decreases with increasing Mn^{2+} concentration.

These results are interpreted in terms of two competing paths for the reduction of $P680^+$. In one path $P680^+$ reduction is accompanied by the appearance of Signal IIf, and lipophilic donors subsequently rereduce the Signal IIf species in a series reaction. This reduction follows pseudo-first order kinetics as a function of donor concentration. In the second path Mn^{2+} reduces $P680^+$ in a parallel reaction that competes with the formation of the Signal IIf species. This results in a decrease in the magnitude of Signal IIf, but no change in its decay time.

INTRODUCTION

The coupling between the reaction center chlorophyll of Photosystem II and the water splitting enzyme is quite labile. A number of treatments have been developed which interrupt this electron transfer reaction without interfering with the primary photochemistry of Photosystem II. Inhibitory treatments which are particularly

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; P680, reaction center chlorophyll of Photosystem II.

effective include mild heating [1], incubation with high concentrations of alkaline Tris buffer [2], hydroxylamine extraction [3, 4], treatment with chaotropic reagents [5] and Mn extraction by exchange with Mg^{2+} [6]. Exogenous reductants become effective electron donors to Photosytem II as oxygen evolution is inhibited. Katoh and San Pietro [1] first utilized ascorbate with heated chloroplasts, and the subsequent studies of Yamashita and Butler [2, 7, 8] established a number of mediators, including phenylenediamine, hydroquinone and benzidine, which increase the efficiency of ascorbate donation to Photosystem II. Mn^{2+} [5, 9], NH_2OH [10] and diphenylcarbazide [11] are also effective electrons donors to Photosystem II in non-oxygen evolving chloroplasts. This subject was reviewed recently by Cheniae [12] and by Trebst [13].

The usefulness of the partial dissection of reactions occurring on the water side of Photosystem II has been exploited in a variety of ways. Yamashita and Butler [8] localized the inhibitory site for heat, tris and aging on the water side of Photosystem II, because exogenous reductants restored both DCMU-sensitive electron flow to $NADP^+$ and fluorescence of variable yield in treated chloroplasts. Giaquinta et al. [14] postulated a *p*-diazonium benzene sulfonate-binding site close to the water-splitting enzyme on the basis of the diphenylcarbazide-mediated restoration of Photosystem II electron flow in *p*-diazonium benzene sulfonate-treated chloroplasts, and Stemler and Govindjee [15] implicated a bicarbonate-binding site by a similar technique. Arntzen et al. [16] studied the lactoperoxidase-catalyzed iodination of chloroplast membranes, and they concluded that a fraction of the iodine labeling occurs directly in the Photosystem II reaction center. Their argument depended partially on the observation that the labeling technique inhibited both oxygen evolution and diphenylcarbazide oxidation. Ort and Izawa [4, 17] and Harth et al. [18] recently showed that the Photosystem II phosphorylation coupling site (Site II) is functional even after inhibition of oxygen evolution by NH_2OH treatment if an exogenous reductant, capable of proton liberation upon oxidation, is provided.

Several sites for electron donation on the water side of Photosystem II have been suggested [19–21]. These sites are implicated on the basis of selective inhibition of donor oxidation by various inhibitors, but the endogenous species involved have not yet been identified chemically. As Trebst [13] has pointed out, however, these results may not reflect distinct sites, but rather may indicate the degree of Photosystem II disruption caused by the various treatments.

Recently, Chen and Wang [6] showed that an increased steady-state amplitude of Signal II accompanies Mn extraction upon exchange with Mg^{2+}. Exogenous reductants suppressed this light-induced Signal II increase, whereas added oxidants had little effect. We reported an analogous effect on Signal II for several of the inhibitory treatments described above and designated this new EPR component Signal IIf [22]. This species is stoichiometric with Signal I (P700), rises with a halftime shorter than 500 μs following a flash and decays with a halftime less than 1 s. The addition of exogenous reductants such as phenylenediamine and hydroquinone decreases the decay time of Signal IIf. At moderate reduction potentials ($E_h < 400$ mV), DCMU inhibits the formation of Signal IIf in preilluminated chloroplasts [23]. On the basis of these results we proposed that the Signal IIf species is Z, the physiological donor to $P680^+$. Upon inhibition of oxygen evolution it serves as a mediator for exogenous reductants.

We have explored the kinetic behavior of Signal IIf following the addition of exogenous reductants using EPR and polarographic techniques. The results support a model involving two sites for Photosystem II-mediated electron donor oxidation. One path includes the Signal IIf species and is accessible from the inner surface of the thylakoid membrane only to hydrophobic donors. The second site is on the outer surface of the membrane, does not include the Signal IIf species and is accessible to hydrophilic donors. A preliminary account of this work has appeared [24].

MATERIALS AND METHODS

Chloroplasts and reagents

Chloroplasts were isolated from growth chamber spinach as described previously [25]. The procedures for Tris-washing, heat treatment and treatment with chaotropic reagents have also been described [22]. EDTA (10^{-4} M) was added to all samples, except those used with Mn^{2+} as donor, to suppress the hexaquo Mn^{2+} EPR signal invariably present in treated chloroplasts. Chlorophyll concentrations in EPR experiments were between 2 and 4 mg/ml, except as noted; in polarographic experiments the chlorophyll concentration was 200 μg/ml. Chlorophyll concentrations were assayed as described in Sun and Sauer [26].

Spinach ferredoxin and NADP were obtained from Sigma; DCMU from duPont. DCMU was recrystallized from methanol and dissolved in 95 % ethanol. Phenylenediamine and hydroquinone were purified by sublimation. Benzidine was converted to the hydrochloride and recrystallized from methanol/acetone as described by Harth et al. [18]. Phenylenediamine, hydroquinone and benzidine were added to chloroplast suspensions as aqueous solutions. Diphenylcarbazide was added as the methanol solution; the methanol concentration was less than 1 % in all chloroplast suspensions and was constant for all diphenylcarbazide concentrations.

Light souces, EPR measurements and polarographic detection of electron donor oxidation

White light flashes (10 μs) and continous white light were obtained from sources as described previously [25]. The Varian E-3 (X-band, 9.5 GHz) EPR spectrometer and signal averaging techniques have also been described [22]. All EPR experiments were carried out at room temperature with microwave power of 20 mW and a modulation amplitude of 4.0 G. Signal IIf amplitude was monitored at the low field maximum of the derivative spectrum for Signal II [27]; at this field there is no Signal I derivative intensity [28]. The experimental protocol and number of passes accumulated in signal averaged experiments are noted in the figure legends.

The techniques and apparatus used for polarographic detection of phenylenediamine oxidation have been described previously [23]. Quantitation of phenylenediamine oxidation per flash was achieved by graphical integration of the area under the current vs time polarographic traces.

RESULTS

Phenylenediamine and hydroquinone donation

Fig. 1 shows the EPR Signal II response in Tris-washed chloroplasts to a train of 10 μs flashes 10 s apart. The non-decaying Signal II component corresponds to

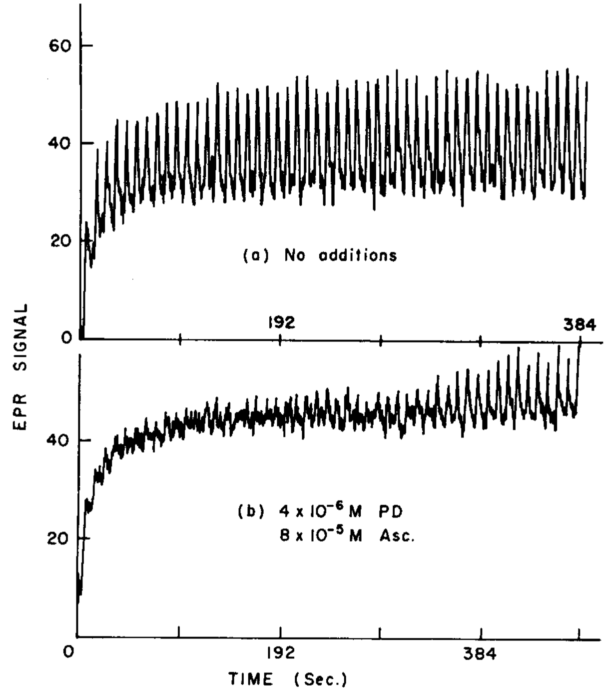

Fig. 1. EPR Signal II response in tris-washed chloroplasts to 10 μs light flashes spaced 10 s apart, (a) with no additions, (b) with 4 · 10^{-6} M phenylenediamine (PD), 8 · 10^{-5} M ascorbate (Asc) added. [NADP], 5 · 10^{-4} M, [Ferredoxin], 20 μg/ml. Instrument time constant, 0.3 s.

Signals IIs and IIu (see ref. 23 for nomenclature), while the superimposed spikes arise from the flash-induced formation and subsequent dark decay of Signal IIf. In the absence of exogenous reductants (Fig. 1a) the Signal IIf decay time is on the order of 1 s. Under these conditions the lifetime of the radical is sufficiently long that it can be observed with the 0.3 s instrument time constant used in this experiment. The addition of the donor system, phenylenediamine/ascorbate, in low concentration (Fig. 1b) suppresses observation of Signal IIf for approximately the first 30 flashes. Upon exhaustion of the donor system, Signal IIf transients appear on subsequent flashes.

Two models can be invoked to explain the results of Fig. 1. In the first, the Signal IIf species is on the pathway between the site of donor oxidation and P680; the addition of the donor decreases the rereduction time so that the radical is no longer observed with the relatively long, 0.3 s, instrument time constant. In the second model, Signal IIf and the exogenous donor compete for Photosystem II-generated oxidizing equivalents. At the donor concentration of Fig. 1b, donor oxidation is favored, consequently inhibiting Signal IIf formation. In the experiments described below, evidence will be presented suggesting that, depending on the specific donor, both cases can be observed.

Fig. 2 shows a study of the effect of hydroquinone/ascorbate addition on Signal IIf formation and decay in Tris-washed chloroplasts using faster (10 ms) time resolution. With no addition (Fig. 2a), the Signal IIf decay time is 500 ms; with

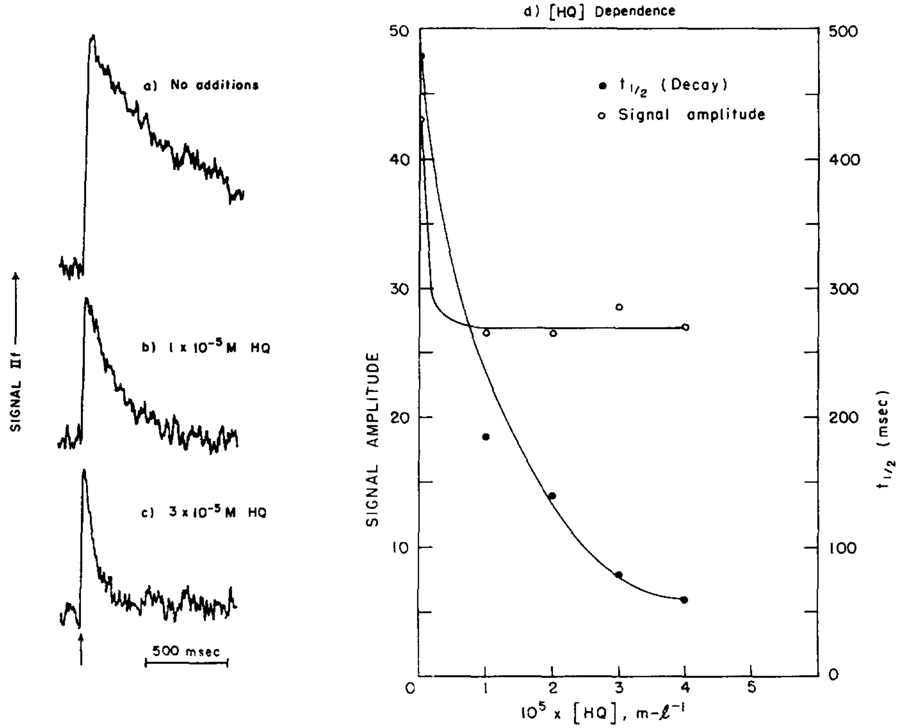

Fig. 2. Effect of hydroquinone (HQ) on EPR Signal IIf response to a single 10 μs light flash. All experiments were performed with tris-washed chloroplasts to which $5 \cdot 10^{-4}$ M NADP, 20 μg ferredoxin/ml had been added, (a) with no further additions, (b) with $1 \cdot 10^{-5}$ M hydroquinone, $2 \cdot 10^{-3}$ M ascorbate added, (c) with $3 \cdot 10^{-5}$ M hydroquinone, $2 \cdot 10^{-3}$ M ascorbate added, (d) hydroquinone concentration dependence for Signal IIf amplitude (○) and decay halftime (●). The vertical scale is the same for the traces in (a), (b) and (c). All experiments are the average of 128 scans, repeated at 6 s intervals, with an instrument time constant of 10 ms.

$1 \cdot 10^{-5}$ M hydroquinone (Fig. 2b), the decay decreases to 200 ms and there is a 35 % decrease in Signal IIf magnitude. At $3 \cdot 10^{-5}$ M hydroquinone (Fig. 2c), the decay time is 75 ms while the Signal IIf magnitude is slightly higher than in Fig. 2b. Fig. 2d summarizes these data along with those obtained with other hydroquinone concentrations. The 35 % decrease in Signal IIf magnitude is observed with all hydroquinone concentrations used, while the decay halftime is inversely proportional to the hydroquinone concentration, decreasing as the hydroquinone concentration increases. In these experiments the ascorbate concentration was fixed at $2 \cdot 10^{-3}$ M for all hydroquinone concentrations. In control experiments in which only the ascorbate was added, the Signal IIf decay time was 250 ms (see below).

We conducted analogous experiments for the phenylenediamine/ascorbate couple. The results are shown in Fig. 3. The chlorophyll concentrations for heated (circles) or Tris-washed (squares) chloroplast samples were adjusted to give equal flash-induced Signal IIf amplitudes prior to phenylenediamine/ascorbate addition. As shown previously [22], under these conditions the Signal IIf decay time is more rapid in the heated chloroplast preparation (140 ms) than in the Tris-washed (500 ms) prep-

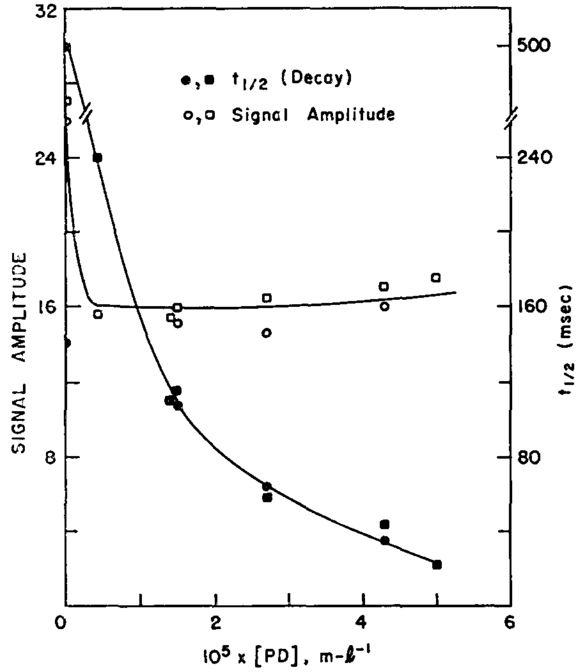

Fig. 3. Effect of phenylenediamine on EPR Signal IIf response to a single 10 μs light flash. Experiments were performed with either heated (circles) or tris-washed chloroplasts (squares) to which $5 \cdot 10^{-4}$ M NADP, 20 μg ferredoxin/ml and $2 \cdot 10^{-3}$ M ascorbate had been added. All experiments are the average of 128 scans, repeated at 5 s intervals. Instrument time constant, 10 ms for decay times greater than 50 ms, 5 ms for decay halftimes less than 50 ms.

aration. For all concentrations of added phenylenediamine, the flash-induced increase in signal IIf is only 60 % of that observed in the absence of exogenous donor. However, as is the case with the hydroquinone/ascorbate couple, increasing phenylenediamine concentrations progressively decrease the Signal IIf decay halftime.

The results obtained with hydroquinone and phenylenediamine, shown in Figs 2 and 3, indicate that these reductants donate electrons through the Signal IIf species to the Photosystem II reaction center. This conclusion is based on the donor concentration sensitivity of the Signal IIf lifetime (see Discussion). The decrease in Signal IIf magnitude observed upon donor addition may correspond to the fact that either donor system only partially restores Photosystem II mediated electron flow. This observation was first made by Cheniae and Martin [29], and in similar experiments we have also observed that phenylenediamine or hydroquinone restores only 50–70 % of the electron flow through Photosystem II following Tris-washing [30].

If phenylenediamine and hydroquinone donate electrons through the Signal IIf species, then the oxidation kinetics for the donors employed should reflect the rate at which the Signal IIf species is reduced. We have recently shown that it is possible to monitor the flash-induced oxidation of hydroquinone or phenylenediamine polarographically [23]. This technique affords good time resolution, and the donor oxidation kinetics can be monitored over a wider range of donor concentrations than in the EPR experiments. The results of a typical experiment with Tris-washed chloroplasts and a phenylenediamine concentration of $1 \cdot 10^{-3}$ M are shown in Fig. 4a. In these experiments the dark time (t_d) between two flashes was varied. The yield of the second (probe) flash measures the number of Photosystem II units which were rereduced by phenylenediamine in the dark time following the first flash and is plotted

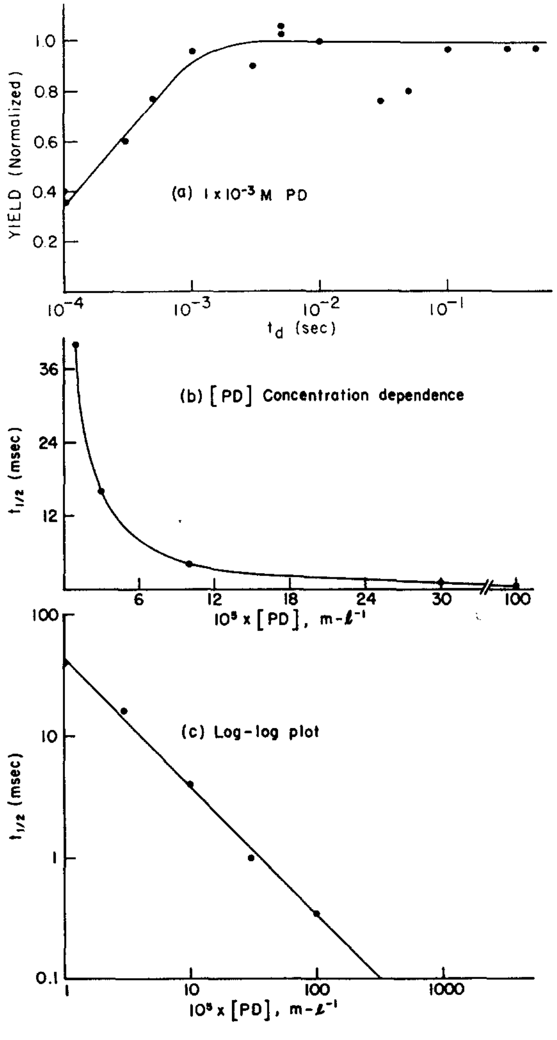

Fig. 4. Polarographic detection of phenylenediamine oxidation in 10 μs flashing light; (a) phenylenediamine concentration, $1 \cdot 10^{-3}$ M; the normalized yield (with respect to the steady-state single flash yield) of phenylenediamine oxidized on the second of two flashes spaced by a dark time, t_d as a function of t_d. In this experiment 50 % of the maximal yield of phenylenediamine oxidized is observed at a dark time of 200 μs. (b) Phenylenediamine concentration dependence for the observed halftime for phenylenediamine oxidation on the second of two closely spaced flashes. (c) Log-log plot for the data in (b). Line drawn with slope 1.0. Polarizing voltage, −0.325 V vs Ag/AgCl; quantitation of phenylenediamine oxidation was carried out as described in Materials and Methods. [NADP], $1 \cdot 10^{-4}$ M; [ferredoxin], 10 μg/ml.

vs t_d. In the experiments in Fig. 4a, the phenylenediamine oxidizing site is completely rereduced within 2 ms following the first flash and 50% reduced within 200 μs. The rereduction halftimes for analogous experiments at other phenylenediamine concentrations are plotted vs. phenylenediamine concentration in Fig. 4b, and in Fig. 4c these same results are presented in a log-log plot (see Discussion). As can be seen in Fig. 4c, the relation between log [phenylenediamine] and log ($t_{\frac{1}{2}}$) is linear with a slope of 1.0 over a 100-fold variation in phenylenediamine concentration.

Both hydroquinone and phenylenediamine dissolve in the aqueous phase, whereas the Signal IIf species is membrane bound. In a diffusion-controlled reaction between either reductant and the free radical, we expect the rereduction time of Signal IIf to be dependent upon donor concentration, as is observed, but to be independent of the Signal IIf concentration. We tested this hypothesis by the EPR experiment shown in Fig. 5. We varied the chlorophyll concentration (and consequently the Signal IIf concentration) in a heated chloroplast suspension and measured both Signal IIf amplitude and decay halftime at constant phenylenediamine/ascorbate concentration. The Signal IIf amplitude is linear with the chlorophyll concentration. However, as predicted above, the decay halftime is independent of the Signal IIf concentration.

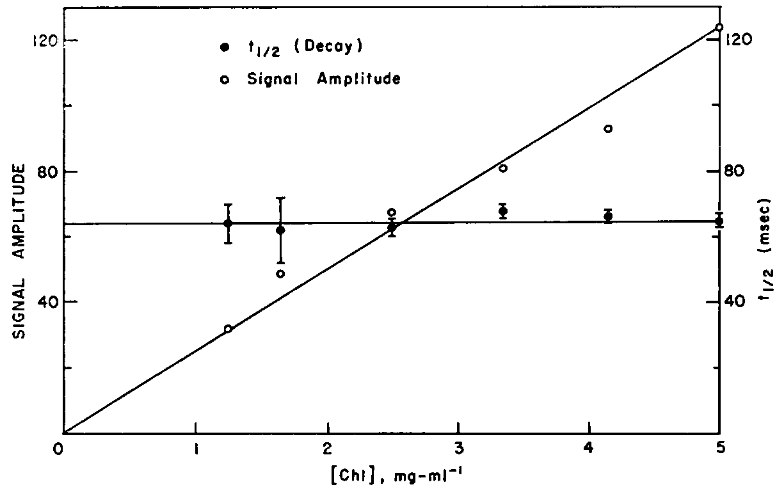

Fig. 5. Effect of chlorophyll concentration in heated chloroplasts on EPR Signal IIf amplitude (○) and decay halftime (●) following a single 10 μs flash. [Phenylenediamine], $2.5 \cdot 10^{-5}$ M; [ascorbate], $4 \cdot 10^{-3}$ M; [NADP], $5 \cdot 10^{-4}$ M; [ferredoxin], 20 μg/ml in all experiments. All experiments are the average of 192 scans, repeated at 2 s intervals, with an instrument time constant of 5 ms.

Mn^{2+} donation

Both hydroquinone and phenylenediamine are uncharged donors and penetrate the thylakoid membrane readily. As shown and briefly discussed above, both donate electrons through the Signal IIf species to the Photosystem II reaction center. The thylakoid membrane is relatively impermeable to Mn^{2+}; Blankenship and Sauer [31] have shown that Mn^{2+} trapped within this membrane diffuses to the outside with a halftime of about 2.5 h. Mn^{2+} is also an efficient donor to Photosystem II in inhib-

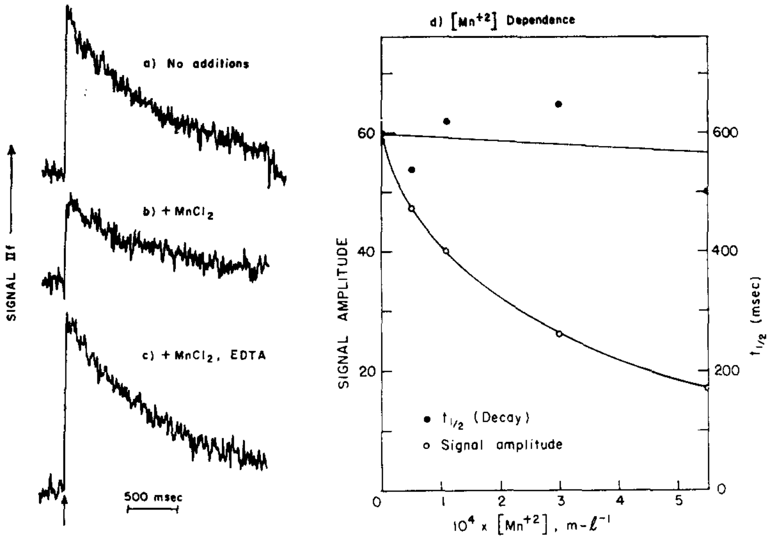

Fig. 6. Effect of $MnCl_2$ on EPR Signal IIf response to a single 10 μs light flash. In (a), (b) and (c) guanidine-washed chloroplasts were used, in (d) tris-washed chloroplasts were used. [NADP], $5 \cdot 10^{-4}$ M; [ferredoxin], 20 μg/ml; in (b) and (c) [$MnCl_2$] was $2.5 \cdot 10^{-4}$ M; in (c) [EDTA] was $1 \cdot 10^{-3}$ M. The vertical scale in experiments (a), (b) and (c) is the same. All experiments are the average of 64 scans, repeated at 8 s intervals, with an instrument time constant of 20 ms.

ited chloroplasts [5, 9]. If the Signal IIf species is localized on the inner thylakoid membrane, we expect quite different Mn^{2+} effects on the amplitude and rereduction kinetics of Signal IIf. Typical results from such an experiment with guanidine-washed chloroplasts are shown in Fig. 6. The control with no further additions is shown in Fig. 6a. In Fig. 6b, where $2 \cdot 10^{-4}$M $MnCl_2$ was added to the chloroplast suspension, the Signal IIf amplitude is only half that observed in the absence of Mn^{2+}; however, the decay halftime (750 ms) is unaffected by the addition of the donor. Fig. 6c shows that if Mn^{2+} is chelated by EDTA the Signal IIf behavior, both in magnitude and kinetics, is identical to that observed in the absence of Mn^{2+}. Fig. 6d summarizes the results of analogous experiments, performed with Tris-washed chloroplasts, in which the Signal IIf amplitude and decay time are plotted as a function of Mn^{2+} concentration. These results are reversed from the case in which hydroquinone or phenylenediamine functions as the electron donor; i.e. the Signal IIf decay halftime is constant, whereas the amplitude decreases with increasing Mn^{2+} concentration.

Benzidine, diphenylcarbazide and hydrogen peroxide donation

We have investigated the site of donation for several other Photosystem II reductants. Fig. 7 summarizes results obtained for the donor system benzidine/ascorbate with Tris-washed chloroplasts. A comparison of Figs 7a and 7b shows that upon addition of $2 \cdot 10^{-3}$ M ascorbate alone, the Signal IIf decay halftime is decreased from 1.1. sec to 200 ms. The further addition of $2.5 \cdot 10^{-6}$ M benzidine decreases the decay time to 120 ms (note the change in time scale in Figs 7c and 7d), and doubling the benzidine concentration (Fig. 7d) decreases this time twofold to 60 ms. Fig. 7e presents the dependence of Signal IIf amplitude and decay halftime on benzi-

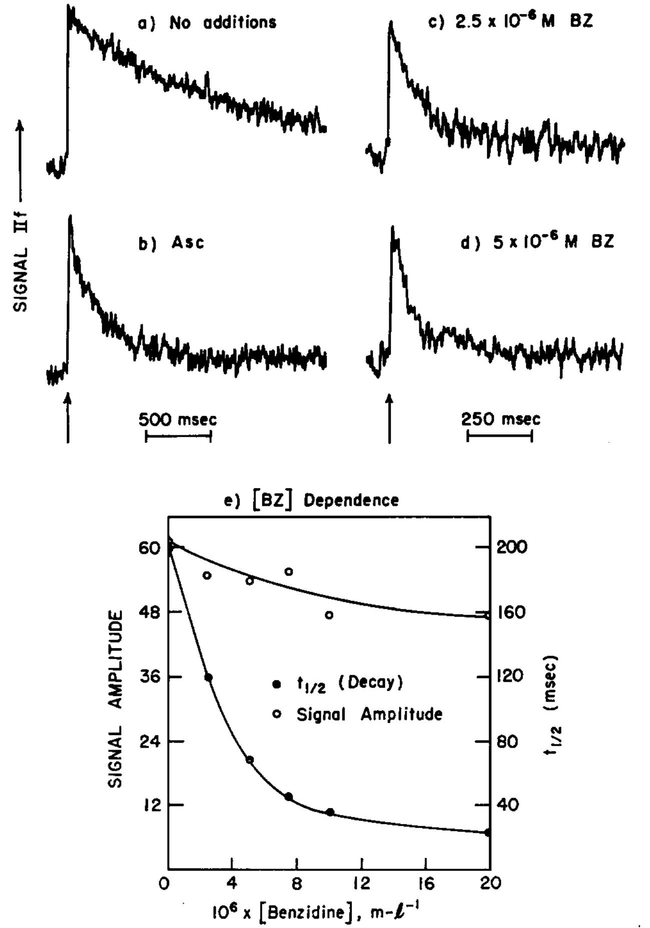

Fig. 7. Effect of benzidine on EPR Signal IIf response to a single 10 μs light flash. All experiments were performed with tris-washed chloroplasts to which $5 \cdot 10^{-4}$ M NADP, 20 μg ferredoxin/ml had been added, (a) with no further additions, (b) with $2 \cdot 10^{-3}$ M ascorbate, (c) with $2.5 \cdot 10^{-6}$ M benzidine, $2 \cdot 10^{-3}$ M ascorbate, (d) with $5 \cdot 10^{-6}$ M benzidine, $2 \cdot 10^{-3}$ M ascorbate, (e) benzidine concentration dependence for Signal IIf amplitude (○) and decay halftime (●). The vertical scales for the traces in (a) –(d) are the same; however, note the time scale change in (c) and (d). All experiments are the average of 128 scans, repeated at 4 s intervals, with an instrument time constant of 10 ms for $t_{\frac{1}{2}}$ greater than 50 ms, or 5 ms for $t_{\frac{1}{2}}$ less than 50 ms.

dine concentration. The plot is quite similar to that presented in Figs 2 and 3 for hydroquinone and phenylenediamine. However, the decline in Signal IIf magnitude is less for benzidine, only 20 % at the highest concentration used, than for the other two donors. Benzidine also appears to be a more efficient donor in that the concentration of benzidine sufficient to give a 60 ms decay halftime is only one-fifth that required for hydroquinone or phenylenediamine.

Fig. 8 presents a similar plot for the diphenylcarbazide donor system, which is often used in non-oxygen evolving systems because diphenylcarbazide does not reduce such acceptors as 2,6-dichlorophenolindophenol (DCIP) in the dark [32]. The results

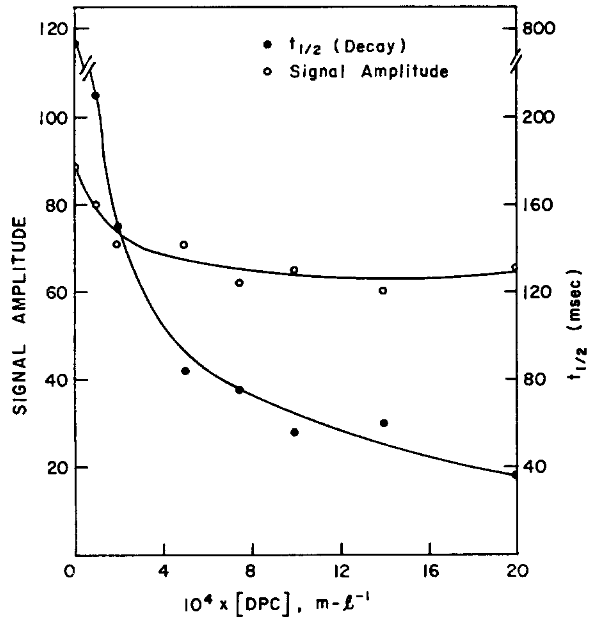

Fig. 8. Diphenylcarbazide concentration dependence for the amplitude (○) and decay halftime (●) for Signal IIf response in Tris-washed chloroplasts to a single 10 μs flash. Other conditions as in Fig. 7e.

of Honeycutt and Krogmann [19] using phenylmercuric acetate treatment and of Shneyour [21] using a mutant alga suggested that the diphenylcarbazide donor site is distinct from that observed for phenylenediamine or hydroquinone. However, our data show that in Tris-washed chloroplasts diphenylcarbazide affects the Signal IIf parameters in a manner analogous to the other two donors; i.e. little effect on Signal IIf magnitude but a dramatic decrease in the decay halftime with increasing concentration. As observed by Vernon and Shaw [11], diphenylcarbazide requires about 10-fold higher concentrations than phenylenediamine or hydroquinone to operate efficiently as a Photosystem II donor. We have also tested H_2O_2 as a reductant for Photosystem II, and (data not shown) it also appears to donate electrons via the Signal IIf pathway.

DISCUSSION

Recently we have shown that the Signal IIf species, which we have described in detail in chloroplast systems inhibited on the water side of Photosystem II, can also be observed in untreated, oxygen-evolving chloroplasts (see Blankenship, et al., reference 37). In the untreated chloroplasts the kinetic characteristics of the Signal IIf species lead us to postulate its identification with Z, the immediate physiological donor to the Photosystem II reaction center chlorophyll. The experiments described in this communication allow us to characterize the reaction between Z and $P680^+$ and to gain insight into the location of both Z and P680 in the membrane.

For chloroplasts with oxygen evolution inhibited by treatments which act on the water side of Photosystem II, we postulate that the following reactions occur upon illumination in the presence of various Photosystem II electron donors:

$$\mathrm{P680} \xrightarrow{h\nu} \mathrm{P680^+} \quad (1)$$

$$\mathrm{P680^+ + Mn^{2+}} \longrightarrow \mathrm{P680 + Mn^{3+}} \quad (2a)$$

$$\mathrm{P680^+ + Z} \longrightarrow \mathrm{P680 + Z\cdot^+} \quad (2b)$$

$$\mathrm{Z\cdot^+ + D} \longrightarrow \mathrm{Z + D^+} \quad (3)$$

where P680 is the Photosystem II reaction center chlorophyll, Z is the diamagnetic precursor to the Signal IIf free radical, $Z\cdot^+$ and D represents an exogenous lipophilic donor. Reaction 1 corresponds to primary charge separation in the Photosytem II reaction center. Reactions 2a and 2b correspond to alternate pathways for $P680^+$ reduction: in 2a Mn^{2+} is present in the chloroplast suspension and rereduces the reaction center chlorophyll; while in 2b the Signal IIf species, Z, is the $P680^+$ reductant. Reaction 3 is the rereduction of $Z\cdot^+$, formed via Reaction 2b, by exogenous electron donors.

The competition between Mn^{2+} and Z as $P680^+$ reductants, represented by Eq 2a and 2b, is controlled by the Mn^{2+} concentration, because the concentrations of $P680^+$ and Z are fixed. Thus, as the Mn^{2+} concentration is increased, Reaction 2a is favored and Signal IIf ($Z\cdot^+$) formation is progressively inhibited. However, since Mn^{2+} has no effect on the rate of $Z\cdot^+$ rereduction by endogenous donors [23], the Signal IIf decay time remains constant for all Mn^{2+} concentrations until radical formation is completely inhibited. These are essentially the results reported in Fig. 6.

In the presence of lipophilic donors the reaction sequence is 1, 2b and 3: $Z\cdot^+$ is formed upon reduction of $P680^+$ and is subsequently rereduced by the exogenous reductant. The rate of reaction 3 is determined by the donor concentration. We can describe this dependence mathematically as follows:

$$-\frac{d[Z\cdot^+]}{dt} = k_3[Z\cdot^+][D] \quad (4)$$

where $-d[Z\cdot^+]/dt$ is the rereduction rate for Signal IIf, and k_3 is the rate constant for the reaction. Representing $-d[Z\cdot^+]/dt$ with r we can express the rates r_1 and r_2 at donor concentrations $[D]_1$ and $[D]_2$ as

$$r_1 = k_3[Z\cdot^+][D]_1 \quad (5)$$

$$r_2 = k_3[Z\cdot^+][D]_2 \quad (6)$$

Since $k_3[Z\cdot^+]$ is constant at a fixed chlorophyll concentration and light intensity and the reaction rates, r_1 and r_2 are inversely proportional to the Signal IIf reduction half-times, we can write

$$(t_{\frac{1}{2}})_1/(t_{\frac{1}{2}})_2 = \frac{[D]_2}{[D]_1} \quad (7)$$

or

$$(t_{\frac{1}{2}})_1\ [D]_1 = (t_{\frac{1}{2}})_2\ [D]_2 = \text{constant} \tag{8}$$

and finally

$$\log\ (t_{\frac{1}{2}}) + \log\ [D] = \text{constant.} \tag{9}$$

Eq 8 predicts that a plot of donor concentration vs decay halftime is hyperbolic and that, for example, doubling the donor concentration will decrease the $t_{\frac{1}{2}}$ by a factor of two. This is the observed result for the donors hydroquinone, phenylenediamine, benzidine and diphenylcarbazide, as shown in Figs 2d, 3, 7e and 8, respectively. Eq 9 predicts that a plot of the log of Signal IIf reduction halftime, or alternatively of log donor oxidation halftime, vs log donor concentration will be linear. We have shown (Fig. 4c) that this relation holds for the polarographic data with phenylenediamine as the electron donor.

Based on their studies of lactoperoxidase-catalyzed iodination of chloroplast membranes, Arntzen et al. [16] suggested that the reaction center of Photosystem II is localized on the outer surface of the thylakoid membrane. Blankenship and Sauer [31] have shown that the chloroplast membrane is relatively impermeable to Mn^{2+}. Thus, exogenous Mn^{2+} has a higher concentration on the outside of the thylakoid membrane and is a more efficient donor to an exterior site. Therefore, both the location of the reaction center chlorophyll and the membrane impermeability of Mn^{2+} indicate that oxidation of exogenous Mn^{2+} occurs on the outer surface of the thylakoid membrane.

The Signal IIf pathway is utilized by lipophilic donors. Recently Harth et al. [18] and Ort and Izawa [4, 17] showed that Site II phosphorylation accompanies the oxidation of lipophilic donors provided that proton liberation accompanies their oxidation. In these systems the donor oxidation and proton releasing site was proposed to be localized on the inner thylakoid membrane surface. The data presented in this communication associate the Signal IIf species with this path, and the simplest explanation places $Z^{\cdot +}$ at this energy coupling site. However, we cannot eliminate the possibility of intermediate electron carriers (for example, cytochrome b_{559} [33] between the actual site of donor oxidation and $Z^{\cdot +}$. A definitive study of cytochrome b_{559} redox reactions at room temperature in flashing light with fast time resolution could resolve this question.

The information about the localization of Photosytem II components can be used to add spatial detail to the pebble-mosaic model [34] of the chloroplast thylakoid membrane structure. The proposed location of several of the basic components with respect to the inside and outside surfaces of the thylakoid membranes is illustrated in Fig.9. The Photosystem II reaction center, P680 Q is localized toward the outer surface of the thylakoid membrane. The evidence for this location of P680 and its identification as the Mn^{2+} oxidizing site was discussed above. Trebst [13] recently summarized the evidence for an exterior location of Q. We also use Trebst's assignment [13] for the location of plastoquinone on the acceptor side of Photosystem II. The physiological donor to P680, Z, is localized on the inner surface of the thylakoid membrane and is the oxidation site for exogenous lipophilic donors in non-oxygen evolving chloroplasts. The arrangement of Q on the outside and Z on the inside of the thylakoid membrane is also in agreement with the conclusion of Fowler and Kok [35] based on observations of light-induced electric field formation. Finally, we place the water

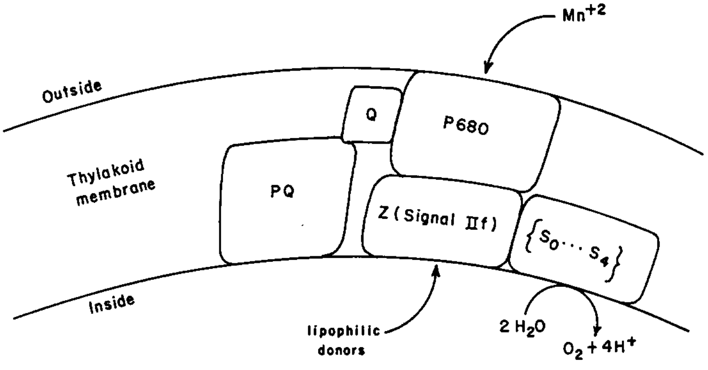

Fig. 9. Proposed model for the location of components and reactions occurring in Photosystem II. Details described in text.

oxidation site, $\{S_0 \ldots S_4\}$, on the inner surface based on the recent observations by Fowler and Kok [36]. Their results suggest that proton release accompanying water oxidation occurs to the inside of the thylakoid.

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