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## THE RAPID COMPONENT OF ELECTRON PARAMAGNETIC RESONANCE SIGNAL II: A CANDIDATE FOR THE PHYSIOLOGICAL DONOR TO PHOTOSYSTEM II IN SPINACH CHLOROPLASTS

GERALD T. BABCOCK\* and KENNETH SAUER

*Department of Chemistry and Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, Calif. 94720 (U.S.A.)*

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### SUMMARY

Rapid light-induced transients in EPR Signal IIf ( $F^{\bullet+}$ ) are observed in 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated, Tris-washed chloroplasts until the state F P680  $Q^-$  is reached. In the absence of exogenous redox mediators several flashes are required to saturate this photoinactive state. However, the Signal IIf transient is observed on only the first flash following DCMU addition if an efficient donor to Signal IIf, phenylenediamine or hydroquinone, is present. Complementary polarographic measurements show that under these conditions oxidized phenylenediamine is produced only on the first flash of a series. The DCMU inhibition of Signal IIf can be completely relieved by oxidative titration of a one-electron reductant with  $E'_{0_{8,0}} = +480$  mV. At high reduction potentials the decay time of Signal IIf is constant at about 300 ms, whereas in the absence of DCMU the decay time is longer and increases with increasing reduction potential.

A model is proposed in which  $Q^-$ , the reduced Photosystem II primary acceptor, and D, a one-electron 480 mV donor endogenous to the chloroplast suspension, compete in the reduction of Signal IIf ( $F^{\bullet+}$ ). At high potentials D is oxidized in the dark, and the ( $Q^- + F^{\bullet+}$ ) back reaction regenerates the photoactive F P680  $Q$  state. The electrochemical and kinetic evidence is consistent with the hypothesis that the Signal IIf species, F, is identical with Z, the physiological donor to P680.

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### INTRODUCTION

Three kinetic components of Signal II in  $O_2$ -evolving photosynthetic organisms have been demonstrated recently; in this paper they will be designated Signal IIu, Signal IIs, and Signal IIf. Signal IIu is observed in leaves and chloroplasts in the dark

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

\* Present address: Department of Biochemistry, Rice University, Houston, Texas 77001, U.S.A.

[1] and remains in its free radical state in whole leaves even after several days of dark storage [2]. However, treatments such as aging, heat, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or Tris washing will induce decay of Signal IIu [3–6].

Signal IIs corresponds to the 100 % increase in Signal II magnitude observed upon illumination of dark-adapted chloroplast samples [1, 2]. This light-induced increase proceeds with high quantum efficiency, although the kinetics for both the rise ( $t_{\frac{1}{2}} = 1$  s) and decay ( $t_{\frac{1}{2}} = 4$  h) are sufficiently slow that an integral role for Signal IIs in the transport of electrons from water to NADP can be excluded [1]. Okayama et al. [7] and Lozier and Butler [4] have shown that treatments which disrupt reactions occurring on the water side of Photosystem II accelerate the decay of Signal IIs, as well as that of Signal IIu. Subsequent illumination then restores Signal II to a concentration equal to the sum of Signals IIs and IIu. Esser [5], arguing also from evidence obtained from inhibitor effects, proposed that Signals IIs and IIu may be on a sidepath connecting the water side of Photosystem II with the plastoquinone pool; however, the precise mechanism of Signal II formation is not specified in his model. Flashing light studies in our laboratory [1] on Signal IIs in untreated chloroplasts demonstrated that the formation of this radical involves oxidation of its precursor, F, by the states  $S_2$  and  $S_3$  in the model of Kok et al. [8] for  $O_2$  evolution. These observations, as well as earlier studies with Mn-deficient algae and with mutants lacking the ability to evolve  $O_2$  [9, 10], strongly support a model in which Signals IIs and IIu are associated with reactions occurring on the water side of Photosystem II.

The third component of Signal II, Signal IIf, is observed upon inhibition of  $O_2$  evolution [11]. This species has rapid rise kinetics ( $t_{\frac{1}{2}} \leq 500 \mu\text{s}$ ) and decays with a halftime which decreases as the reduction potential of the chloroplast suspension is decreased. The spin concentration of Signal IIf is equal to the sum of the spin concentrations of Signals IIs and Signal IIu, which are also present in these non- $O_2$  evolving chloroplasts. Signal IIf is only marginally observed in  $O_2$ -evolving broken chloroplasts [11, 12]. However, Warden and Bolton [13] have shown recently that a fast rising ( $t_{\frac{1}{2}} \leq 1$  ms) component of Signal II can be observed in intact chloroplasts prepared as described by Jensen and Bassham [14]. This species is roughly stoichiometric with Signal I and disappears upon osmotic rupture of the chloroplasts.

The EPR spectra of the three kinetic components of Signal II are identical [2, 6, 11], indicating that all three originate from the same chemical species. In this communication this free radical species will be designated  $F^{\bullet+}$ , and the diamagnetic precursor will be designated F.

3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) blocks electron transfer from the primary acceptor, Q, of Photosystem II to the pool of secondary acceptors [15, 16]. As a result,  $Q^-$  accumulates in the light and further electron flow through Photosystem II is inhibited. Previously [11] we showed that in non- $O_2$ -evolving chloroplasts Signal IIf formation is inhibited by DCMU provided  $Q^-$  was generated by illumination prior to the assay for the radical. In the experiments reported in this paper we show that transients in Signal IIf persists until the state F P680  $Q^-$  is obtained. In the presence of suitable electron donors, a single flash is sufficient to saturate this photoinactive state. We also report that the DCMU inhibition of Signal IIf is relieved if sufficiently high reduction potentials ( $E > +480$  mV) are maintained in the chloroplast suspension. Finally we propose a model suggesting that Signal IIf arises from  $Z^{\bullet+}$ , the oxidized physiological donor to P680.

## MATERIALS AND METHODS

### *Chloroplasts and reagents*

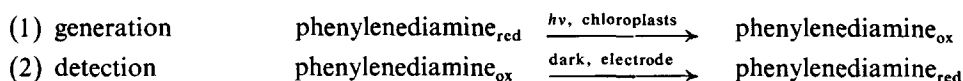
Chloroplasts were isolated from growth chamber spinach as described previously [11]. Tris-washed chloroplasts were prepared by the method of Yamashita and Butler [17] as modified by Blankenship and Sauer [18] and were resuspended in 0.8 M Tris buffer (pH 8.0). EDTA ( $10^{-4}$  M) was added to all samples to suppress the hexaquo  $\text{Mn}^{2+}$  EPR signal invariably present in treated chloroplasts. Control experiments show that Signal IIf is also observed in the absence of EDTA. Chlorophyll concentrations in EPR experiments were between 2 and 4 mg/ml; in experiments in which phenylenediamine or hydroquinone oxidation was followed polarographically, the chlorophyll concentration was 200  $\mu\text{g}/\text{ml}$ .

Spinach ferredoxin and NADP were obtained from Sigma, *o*-phenanthroline from Calbiochem, and DCMU from duPont. The DCMU was recrystallized from methanol and dissolved in 95% ethanol. Ethanol concentration in all experiments was less than 1%. Phenylenediamine and hydroquinone were purified by sublimation. Crude potassium octacyanotungstate (IV) dihydrate,  $\text{K}_4\text{W}(\text{CN})_8 \cdot 2\text{H}_2\text{O}$ , was the kind gift of Dr Richard Malkin. It was purified as described by Heintz [19], and the tetrapositive tungsten salt was oxidized to the pentapositive salt,  $\text{K}_3\text{W}(\text{CN})_8$ , using  $\text{KMnO}_4$  as described by Baadsgaard and Treadwell [20]. Concentration and midpoint potential for the tungstate salt were determined by potentiometric titration with standard ceric sulfate.

### *Light sources, EPR measurements and polarographic detection of electron donor oxidation*

White-light flashes (10  $\mu\text{s}$ ) and continuous white light were obtained from sources as described previously [1]. The Varian E-3 (X-band, 9.5 GHz) EPR spectrometer and signal averaging techniques have also been described [11]. All EPR experiments were carried out at room temperature with a microwave power of 20 mW. Modulation amplitude of 3.2 G for recording spectra was increased to 4.0 G in kinetic experiments to increase the signal-to-noise ratio. The spectrometer scan rate and time constant are noted in figure legends.

Polarographic detection of phenylenediamine or hydroquinone oxidation by Tris-washed chloroplasts was performed using the bare platinum electrode described previously for  $\text{O}_2$  evolution [1]. The flowing electrolyte in these experiments was 0.1 M KCl, 0.01 M phosphate (pH 7.6). Piette et al. [21] and Kolthoff and Lingane [22] have shown that phenylenediamine and hydroquinone are suitable for polarographic study at stationary platinum electrodes. In our system, polarograms for the detection of phenylenediamine and hydroquinone oxidized by Tris-washed chloroplasts show half wave potentials of  $-0.2$  V (vs SCE) and plateau regions between  $-0.3$  and  $-0.5$  V [23]. The experiments described in the text were performed at  $-0.325$  V (vs SCE), which is sufficiently separated from the more negative half-wave potential of  $\text{O}_2$  ( $-0.55$  V in our system) to allow clean separation between phenylenediamine or hydroquinone and  $\text{O}_2$  signals. The reactions occurring upon illumination of Tris-washed chloroplasts in the presence of phenylenediamine (or hydroquinone) may be represented [23] as:



### Potentiometric titrations

Potentiometric titrations of Signal IIf in the presence of DCMU were performed using a Corning Digital 110 pH meter in the potentiometric mode. The electrode system consisted of a platinum electrode and a Corning 476 002 saturated calomel electrode previously calibrated vs saturated quinhydrone [24]. The potential of 2.0-ml aliquots of Tris-washed chloroplasts was adjusted aerobically at 4 °C using various ratios of  $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$  or  $\text{K}_3\text{W}(\text{CN})_8/\text{K}_4\text{W}(\text{CN})_8$ . The total concentration of either redox couple was held constant at 10 mM in all experiments.

Following equilibration at various potentials (approx. 1 min) a 0.3-ml chloroplast aliquot was removed and the flash-induced magnitude of Signal IIf in the absence of DCMU determined. 64 flashes were averaged at each potential. The experiment was then repeated with a second 0.3-ml aliquot to which  $10^{-4}$  M DCMU had been added. At each potential the extent of Signal IIf formation in the presence of DCMU is normalized by dividing by the Signal IIf magnitude in the absence of DCMU. With the ferri/ferrocyanide couple the total change in potential was less than 2 mV in 10 min; with the cyanotungstate (V)/(IV) couple the total change was less than 8 mV in 10 min. All midpoint and redox titration potentials are reported vs the Standard Hydrogen Electrode (S.H.E.).

## RESULTS

### DCMU effects on Signal IIs and IIf

Experiments performed by us [1] and by Esser [5] have shown that DCMU does not inhibit the formation of Signal IIs in dark-adapted, untreated chloroplasts. This is demonstrated in Fig. 1, where we compare the extent of light-induced Signal IIs formation in the absence (a) and presence (b) of  $10^{-4}$  M DCMU. The dark, non-decaying fraction of Signal II shown in Spectrum 1 is Signal IIu. Illumination generates Signal IIs (Spectrum 2) which persists in the dark following illumination (Spectrum 3). DCMU (Fig. 1b) has no effect on this process. As shown previously [11], there is only a slight (<5 %) Signal IIf component observed in these broken, untreated chloroplasts.

The results for Tris-washed chloroplasts are shown in Fig. 1c and d. In the dark (Spectrum 1) Signal IIu magnitude is low since Tris washing destabilizes the normal free-radical state of this species [4]. In the absence of DCMU (Fig. 1c) illumination generates both Signal IIf and IIs (Spectrum 2). In the dark following illumination Signal IIf has decayed leaving only Signal IIs and IIu (Spectrum 3). In the presence of DCMU (Fig. 1d), Signal IIf is not observed in continuous light (Spectrum 2). However, Signal IIs is formed in the presence of this inhibitor, although there is a 20 % decline in total Signal IIs and IIu spin concentration (compare Spectra 3 in Fig. 1c and d). Similar results have been obtained with the inhibitor *o*-phenanthroline.

### Flashing light studies on Signal IIf in DCMU-treated, Tris-washed chloroplasts

The continuous light illumination for the spectra recorded in Fig. 1d precludes

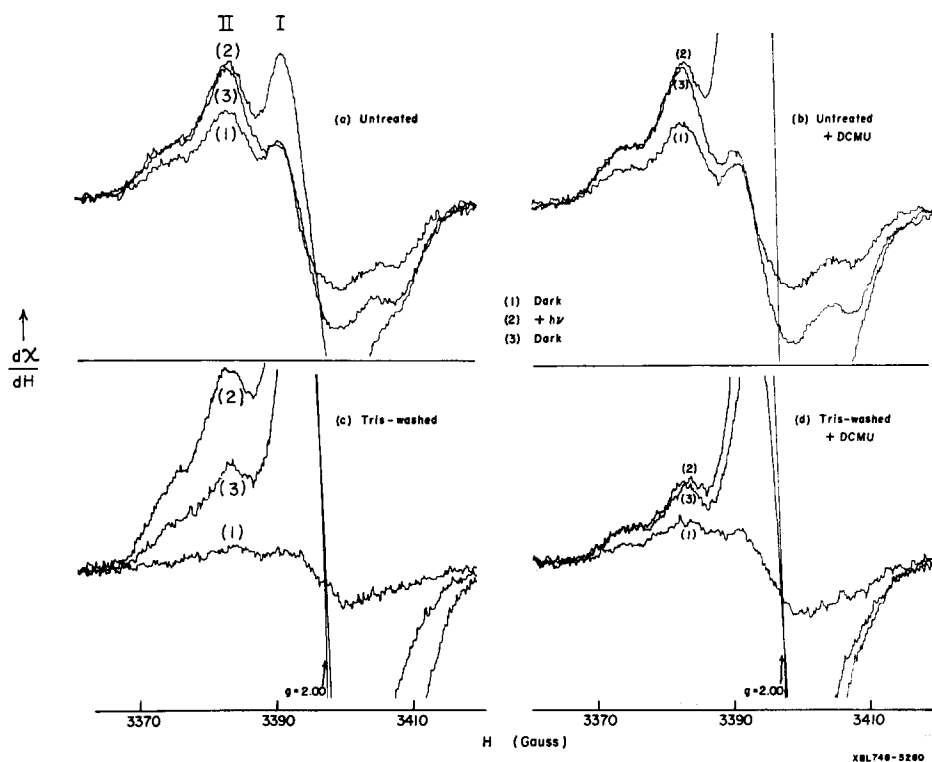
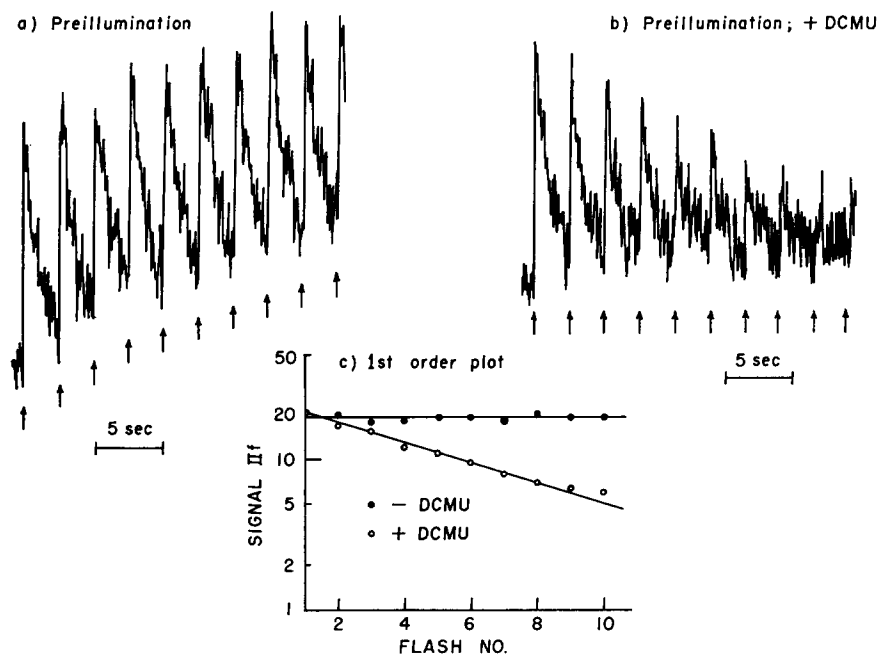


Fig. 1. EPR spectra of Signal II in dark-adapted, untreated (a,b) and Tris-washed (c,d) chloroplasts under various illumination conditions: (1) in the dark prior to illumination, (2) during continuous illumination, and (3) in the dark following illumination. The instrument time constant was 0.3 s with a scan rate of 25 G/min. The dark-adapted chloroplast sample in (b) was identical to that in (a) except that  $10^{-4}$  M DCMU was added; the Tris-washed sample in (d) similarly had  $10^{-4}$  M DCMU added. Chlorophyll concentration in (a,b), 3.6 mg/ml; chlorophyll concentration in (c,d), 2.7 mg/ml.

the observation of any light-induced transients in Signal IIf in DCMU-treated, Tris-washed chloroplasts. Flashing light provides a finer probe, in that Signal IIf can be monitored as a function of flash number during the transition from oxidized Q, at the onset of illumination, to fully reduced Q. The results of this experiment are shown in Fig. 2. Fig. 2a is a control in which we monitored Signal IIf during the first 10 saturating flashes on Tris-washed chloroplasts in the absence of DCMU. Fig. 2b shows the same experiment in the presence of DCMU. The sample in Fig. 2b was illuminated with continuous light prior to DCMU addition to saturate Signal IIs (see below). The first order plot for the data in these two experiments (Fig. 2c) shows that Signal IIf formation on the first flash in a sequence is unaffected by DCMU. However, in the presence of the inhibitor the effectiveness of later flashes declines exponentially with flash number, whereas in the absence of DCMU each subsequent flash is equally effective in generating Signal IIf.

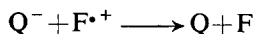
Etienne [25] has observed that several flashes are necessary to exhaust the  $\text{NH}_2\text{OH}$ -oxidizing capability in DCMU-treated, broken chloroplasts. She and also Ducruet and Lavorel [26] have interpreted these results in terms of a model in which



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Fig. 2. Flash-induced response of EPR Signal IIf in preilluminated chloroplasts in the absence (a) and presence (b) of  $10^{-4}$  M DCMU. Instrument time constant, 50 ms with 2.5 s between each saturating flash. Signal IIf was monitored at 3381 G, the low field peak of Signal II in Fig. 1. Each sample was preilluminated with white light for 30 s, and in (b)  $10^{-4}$  M DCMU was added following this preillumination. The response from 10 different chloroplast samples was averaged for each experimental trace shown. In (c) first order plots for the data of Fig. 2a (●) and 2b (○) are shown.

$\text{NH}_2\text{OH}$ , as a donor to Photosystem II, competes only moderately well with a back reaction in the Photosystem II reaction center which generates oxidized Q following a flash. The results of the experiments in Fig. 2b can be explained similarly in terms of a model in which endogenous donors to Signal IIf ( $\text{F}^+$ ) compete inefficiently with  $\text{Q}^-$  in the rereduction of the free radical. Thus, following a flash, the reaction



regenerates Q and the Signal IIf precursor (F) in a sizable fraction of the reaction centers.

We have tested this model in a number of ways. In the experiments of Fig. 2 the chloroplasts were preilluminated, prior to DCMU addition, to saturate Signal IIs. We have repeated these experiments with dark-adapted, Tris-washed chloroplasts in which the Signal IIf and IIs concentration is low prior to illumination. The results of this experiment in the absence (a) and presence (b) of DCMU are shown in Fig. 3. Samples were from the same chloroplast preparation that was used in the experiments in Fig. 2. Under conditions where Signal IIs is generated during the flash sequence (Fig. 3b), transients in Signal IIf are quenched with fewer flashes than if Signal IIs is fully formed prior to flash initiation (Fig. 2b). These experiments also demonstrate a fundamental distinction between Signal IIf and Signal IIs; back reactions between

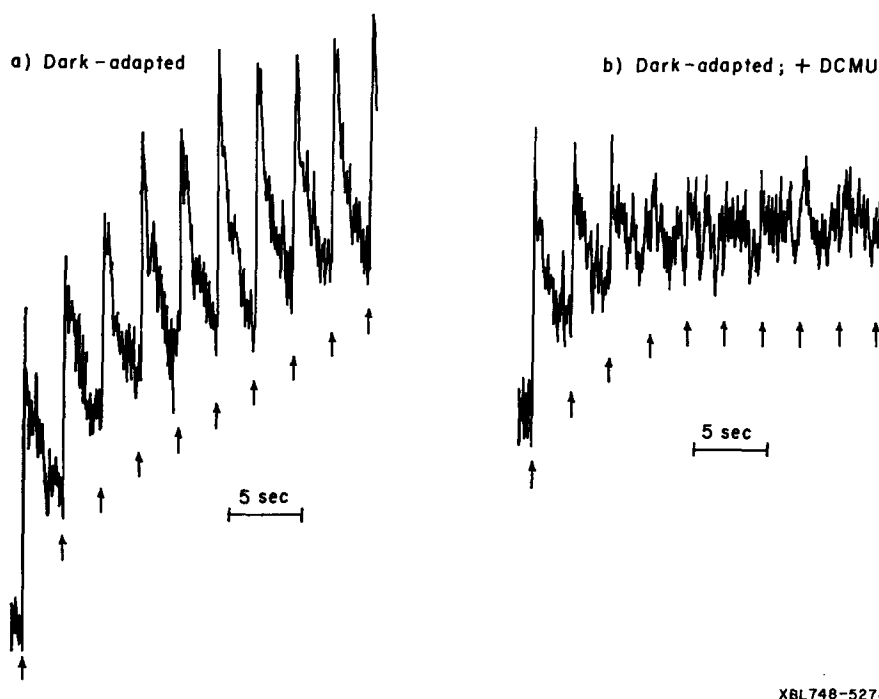
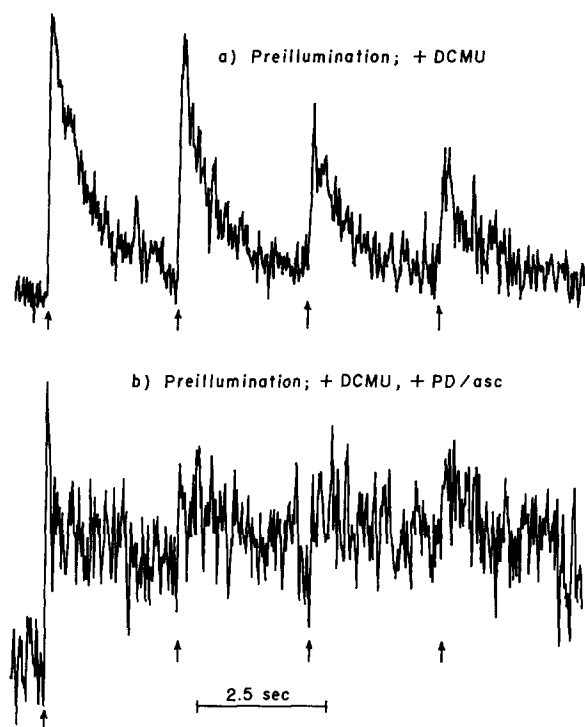


Fig. 3. Repeat of the EPR experiment shown in Fig. 2 except that the chloroplast samples were not preilluminated prior to the flash sequence. Each experimental trace is the average of 10 chloroplast samples.

Signal IIf and  $Q^-$  proceed efficiently, whereas back reactions between  $Q^-$  and Signal IIs proceed only slowly, if at all.

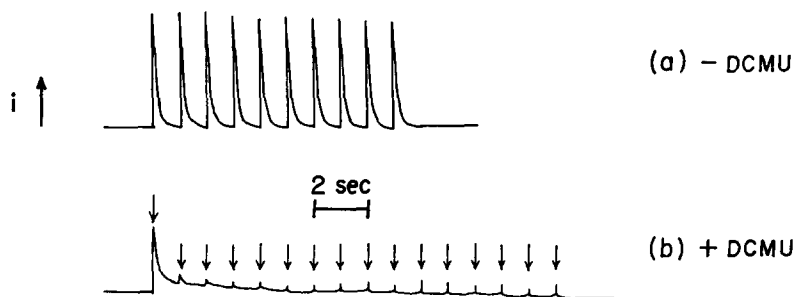
We have shown previously [11] that phenylenediamine is a good donor to Signal IIf and, in the model proposed above, may be able to compete more effectively with the ( $Q^- + F^+$ ) back reaction than endogenous donors in the chloroplast suspension. Fig. 4 shows the results of experiments in which Signal IIf transients were monitored in DCMU-treated, Tris-washed chloroplasts in the absence (a) and presence (b) of 10  $\mu$ M phenylenediamine and 5 mM ascorbate. Fig. 4a shows similar results to those obtained for Fig. 2b, i.e. decreasing effectiveness for each successive flash in Signal IIf generation. In Fig. 4b, with phenylenediamine/ascorbate present, only the first flash generates Signal IIf and the decay for this transient, in agreement with our earlier observation [11], is markedly accelerated compared to Signal IIf transients in the absence of phenylenediamine/ascorbate. The non-decaying Signal II component in Fig. 4b is due to Signal IIs. The flash-induced increase in this component is large, even though the sample was preilluminated immediately prior to DCMU addition. This effect can be attributed to the accelerated Signal IIs decay caused by the donor system [4] during the time between preillumination and initiation of the flash sequence.

The results of Fig. 4 suggest that phenylenediamine oxidation should be observed on only the first flash in DCMU-treated, Tris-washed chloroplasts. To test this



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Fig. 4. EPR response of Signal IIf in Tris-washed chloroplasts which were preilluminated prior to DCMU ( $10^{-4}$  M) addition in the absence (a) and presence (b) of  $10 \mu\text{M}$  phenylenediamine, 5 mM ascorbate. Instrument time constant, 20 ms; saturating flashes spaced 2.5 s apart. Each experimental trace shown is the average from 14 samples. Recorder gain in (b) is twice that in (a). Signal IIf intensity monitored at the magnetic field position described in Fig. 2.



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Fig. 5. Polarographic detection of phenylenediamine oxidation in Tris-washed chloroplasts in the absence (a) and presence (b) of  $2 \cdot 10^{-5}$  M DCMU. Phenylenediamine concentration,  $1 \cdot 10^{-4}$  M; saturating flashes spaced 1 s apart; polarizing potential applied to the electrode,  $-0.325$  V (vs S.C.E.). Reduction of oxidized phenylenediamine at the electrode surface results in an electrode current (i) increase which is subsequently amplified and recorded as a positive-going signal.



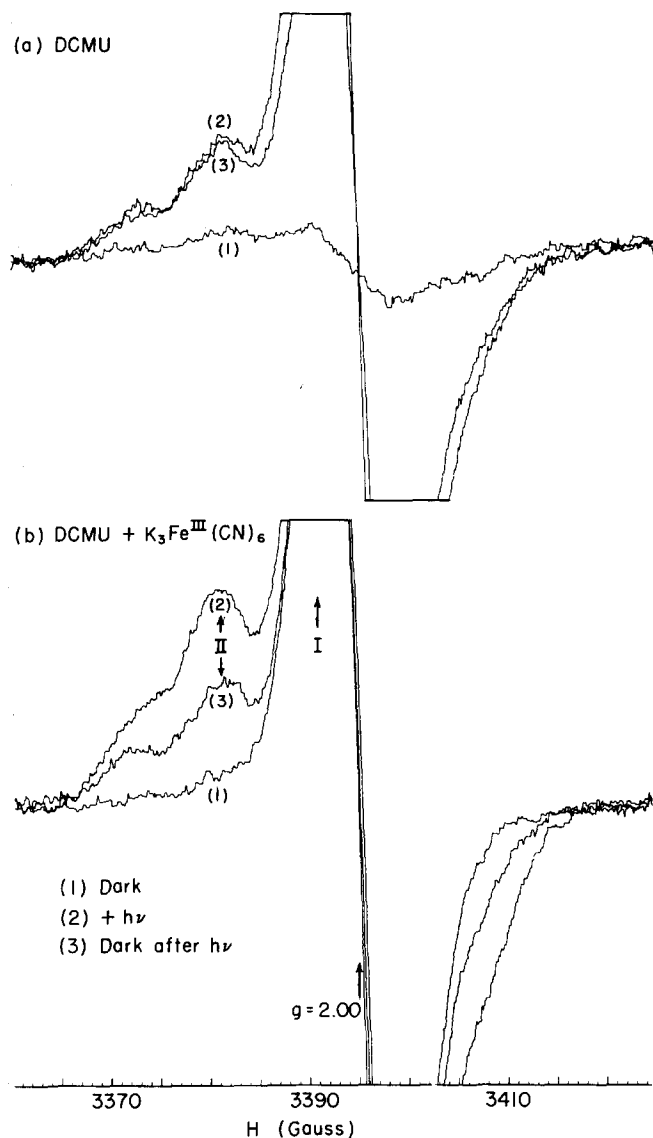
hypothesis we have followed phenylenediamine oxidation in Tris-washed chloroplasts polarographically. Fig. 5a shows the pattern of phenylenediamine oxidation in Tris-washed chloroplasts in the absence of DCMU; each flash is equally effective in oxidizing phenylenediamine. With DCMU added (Fig. 5b), only the first flash generates oxidized donor; phenylenediamine oxidation on all subsequent flashes is inhibited. Therefore both techniques, observation of Signal IIf via EPR and polarographic detection of phenylenediamine oxidation, indicate that, in the presence of an effective donor to Signal IIf, electrons are transferred through Photosystem II on only the first flash following DCMU addition. We have repeated these experiments with hydroquinone, which is also an effective donor to Signal IIf [11], and observed the same effects.

*Removal of DCMU inhibition of Signal IIf generation at high redox potentials*

The results presented above demonstrate that a donor, endogenous to the chloroplast suspension, is able to compete with the  $(Q^- + F^{\bullet+})$  back reaction and, with sufficient flashes, to generate the photo-inactive  $F P680 Q^-$  state. This model predicts that at sufficiently high reduction potentials the oxidation of this donor will occur in the dark and a persistent Signal IIf will be observed even in the presence of DCMU. Fig. 6a is a control in which the DCMU inhibition of Signal IIf is shown; Fig. 6b is an experiment with an identical sample of chloroplasts to which 10 mM  $K_3Fe(CN)_6$  has been added. The addition of the oxidant restores Signal IIf.

We have carried out a study in which the fraction of Signal IIf generated in response to a flash was assayed using DCMU-treated, Tris-washed chloroplasts poised at various reduction potentials. Typical data at three different potentials are shown in Fig. 7. On the left side of the figure we show the flash induced response of Signal IIf at each potential in the absence of DCMU. As the potential is increased, the decay time for Signal IIf increases; this will be discussed below. On the right, the results for the same experiment, now in the presence of DCMU, are shown. At  $E = +437$  mV (Fig. 7a) virtually no Signal IIf is observed with DCMU present; at  $E = +487$  mV (Fig. 7b) about half the Signal II intensity is restored, while at  $E = +519$  mV (Fig. 7c) there is virtually no effect of DCMU on the magnitude of Signal IIf formed. It is also seen (compare Figs 7b and 7c) that the decay time for Signal IIf following a flash in the presence of DCMU is not affected by the redox poise of the chloroplast suspension.

Fig. 8 shows the complete redox titration (a) and Nernst plot (b) for Signal IIf generation in DCMU-treated, Tris-washed chloroplasts. In Fig. 7 and also Fig. 8 we have used both the ferri/ferrocyanide ( $E_0 = +0.36$  V) and cyanotungstate (V)/(IV) ( $E_0 = +0.57$  V) [27] redox couples for these titrations. Although there is some scatter in the data obtained with the higher midpoint potential couple (due to difficulty in obtaining redox stability, especially at the lower potentials where buffer capacity is slight; see Materials and Methods), the results obtained with either couple can be reasonably well fit with  $n = 1$ ,  $E'_{o_{s.o}} = +478$  mV. These results indicate that an endogenous, one-electron donor with a midpoint potential of  $+478$  mV is able to compete with the  $(Q^- + F^{\bullet+})$  back reaction and quench Signal IIf generation in Tris-washed chloroplasts; however, upon oxidation of this donor, Signal IIf transients in the presence of DCMU appear.



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Fig. 6. EPR spectra of Signal II in DCMU-treated, Tris-washed chloroplasts in the absence (a) and presence (b) of 10 mM  $K_3Fe^{III}(CN)_6$ . Spectra for the sample in the dark prior to illumination, during illumination, and in the dark following illumination are labeled (1), (2), and (3), respectively. Instrument time constant, 0.3 s; scan rate, 25 G/min.

This conclusion is supported by the data of Fig. 9, which shows the redox potential dependence for the Signal II decay time. In the absence of DCMU, the decay time increases markedly as the redox poise of the chloroplast suspension is increased. For example, at +437 mV in the absence of DCMU,  $t_{\frac{1}{2}} = 500$  ms, whereas at +538 mV,  $t_{\frac{1}{2}} = 1200$  ms. In the presence of DCMU, however, the decay time for

Signal IIf shows only a slight dependence on potential (which may reflect chloroplast damage at higher potentials [28]); for example, at +470 mV,  $t_{\frac{1}{2}} = 300$  ms, while at +575 mV,  $t_{\frac{1}{2}} = 380$  ms. These data demonstrate the two alternative mechanisms

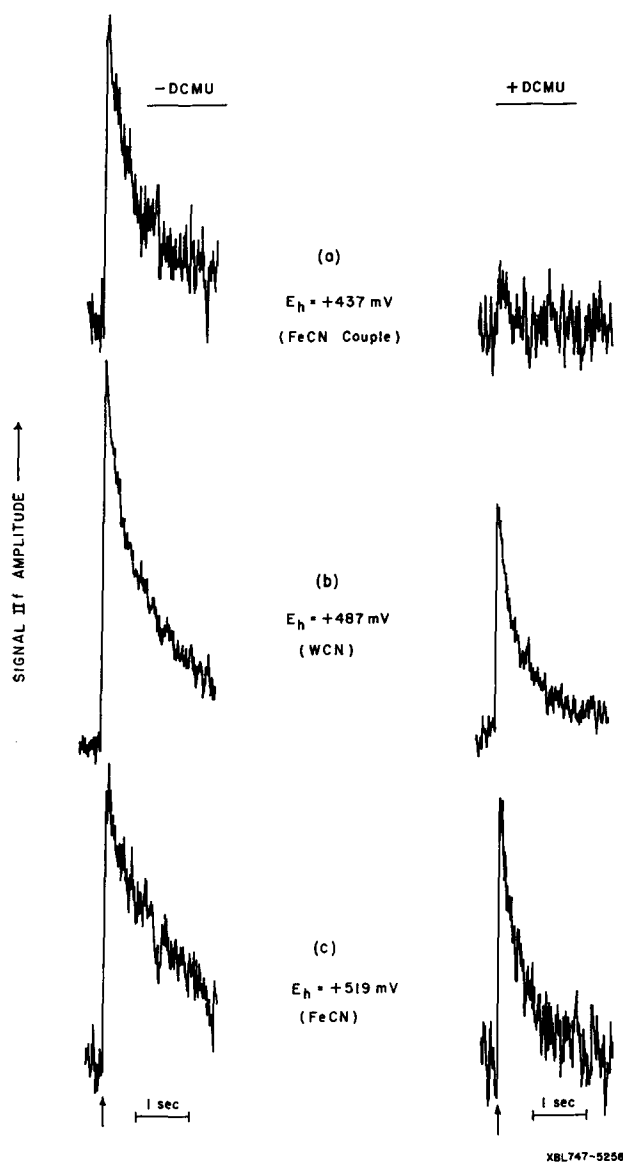
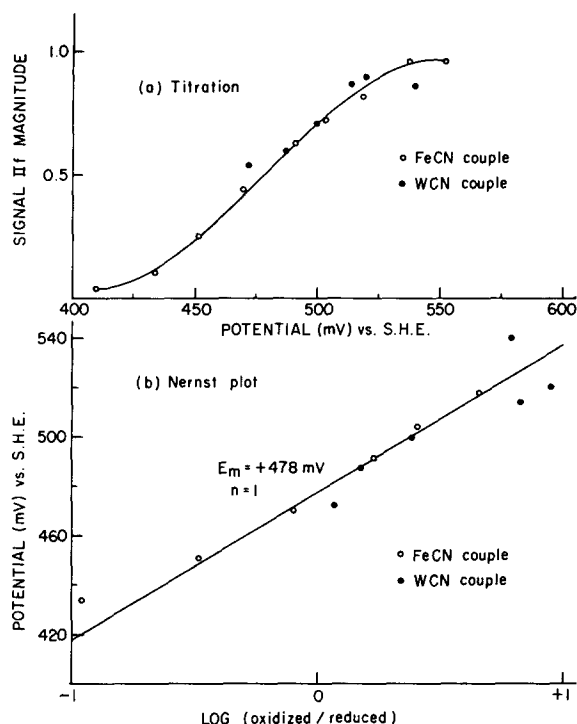
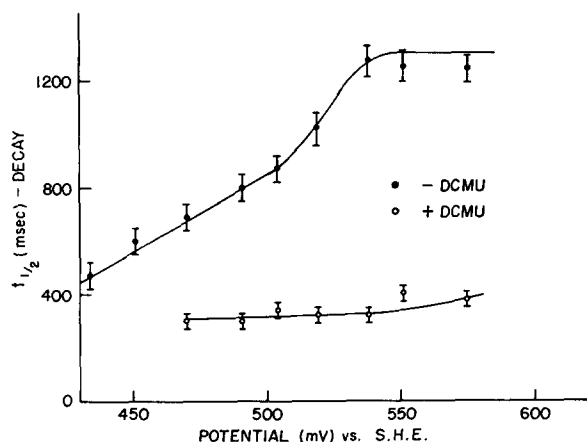


Fig. 7. Flash-induced Signal IIf EPR response at various reduction potentials in Tris-washed chloroplasts in the absence (left side of figure) and presence (right) of  $10^{-4}$  M DCMU. The ferri/ferrocyanide couple (FeCN) was used to obtain potentials of +437 mV (a) and +519 mV (c); the cyanotungstate (V)/(IV) couple (WCN) was used to obtain the +487 mV potential in (b). The same chloroplast preparation was used for the experiments in (a) and (c); a second preparation was used for (b). Instrument time constant, 10 ms; each trace is the average of 64 Signal IIf flash responses. Signal IIf response was monitored at the field position described in Fig. 2.



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Fig. 8. Redox titration data (a) and Nernst plot (b) for the magnitude of EPR Signal II<sub>f</sub> flash response in DCMU-treated, Tris-washed chloroplasts. Each point on the titration curve in (a) is the average of 64 scans, as in Fig. 7, and normalization was carried out as described in Materials and Methods. In (b) the ratio [oxidized]/[reduced], corresponds to Sig II<sub>f</sub>/(Sig II<sub>f</sub>(max) - Sig II<sub>f</sub>) where Sig II<sub>f</sub> is the Signal II<sub>f</sub> formation at the measured potential and Sig II<sub>f</sub>(max) is the Signal II<sub>f</sub> formation at  $E = +528 \text{ mV}$ . Data obtained with both the ferri/ferrocyanide couple (○) and cyanotungstate (V)/(IV) couple (●) are shown.



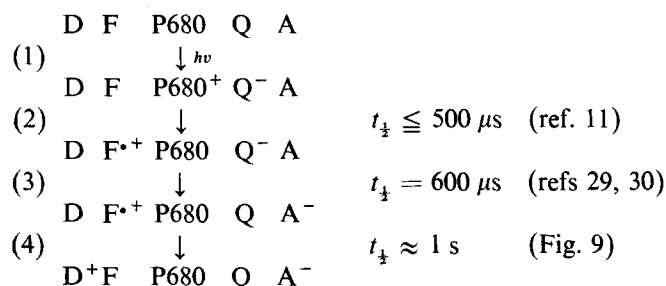
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Fig. 9. Reduction-potential dependence of the decay half-time for Signal II<sub>f</sub> transients following a flash in the absence (●) and presence (○) of DCMU ( $10^{-4} \text{ M}$ ). Only the ferri/ferrocyanide couple was used in this experiment, although analogous results have been obtained with the cyanotungstate (V)/(IV) couple. Instrument time constant 10 ms; each experiment is the average of 64 scans; Signal II<sub>f</sub> monitored as described in Fig. 2.

by which Signal IIf is reduced. In the absence of DCMU, rereduction occurs principally by endogenous donors in the chloroplast suspension. The rate of this reaction depends on the concentration of these reductants. Since this concentration decreases upon going to higher potentials, the lifetime of Signal IIf is increased. In the presence of DCMU, the  $Q^-$  species is available to back react with Signal IIf via a reaction whose rate is redox potential insensitive. At low reduction potentials, the endogenous donor concentration is high and rereduction of Signal IIf by this species is favored, thus quenching Signal IIf transients. However, at higher potentials the endogenous donor concentration is low and the  $(Q^- + F^{•+})$  back reaction proceeds more efficiently. Under these conditions, persistent Signal IIf transients are observed.

## DISCUSSION

Previously we demonstrated that inhibition of  $O_2$  evolution by treatments which act on the water side of Photosystem II allows the observation of rapid and reversible light induced transients in a Signal II component which we have designated Signal IIf [11]. We proposed that Signal IIf is generated by reactions occurring at the Photosystem II reaction center, which can be schematically represented as



where D represents endogenous reductants in the chloroplast suspension; F is the (diamagnetic) precursor to  $F^{•+}$ , the Signal IIf free radical; P680 is the Photosystem II reaction center chlorophyll; Q is the primary acceptor and A is the large acceptor pool on the reducing side of Photosystem II. Reaction 1 corresponds to primary charge separation, Reaction 2 to the generation of Signal IIf ( $F^{•+}$ ), Reaction 3 to reoxidation of  $Q^-$ , and Reaction 4 to the rereduction of Signal IIf by endogenous reductants.

In the experiments reported here we have carried out an analysis of flash-induced Signal IIf transients in DCMU-treated, Tris-washed chloroplasts. Tris washing was used as the inhibitory technique because this method has been well characterized and because the Signal IIf decay times are generally longer in these washed chloroplasts than, for example, in heat-treated chloroplasts [11]. When we have repeated analogous experiments with heated (51 °C, 150 s) or chaotropic agent-washed chloroplasts [31], we found results similar to those observed with Tris-washed chloroplasts.

In the presence of DCMU, only Reaction 3 above is inhibited, and our proposed model predicts that Signal IIf transients will be observed until the photoinactive state,  $F P680 Q^-$ , is produced. The results of Fig. 2 show that this predicted result is observed. The exponentially decreasing magnitude of Signal IIf with flash number

shown in Fig. 2b indicates that either of two mechanisms is possible: (a) the acceptor pool in the presence of DCMU is large, and more than a single equivalent per reaction center must be transferred from F to fill it, or (b) the acceptor pool is filled by each flash, but a back reaction between  $Q^-$  and Signal IIf occurs rapidly enough to compete with the relatively inefficient reduction of  $F^{*+}$  by D. The results with the exogenous donor, phenylenediamine (Fig. 4), and observation of Signal IIs (Fig. 3) show that model (b) is the better hypothesis.

Phenylenediamine-oxidizing capability is exhausted on a single flash in Tris-washed chloroplasts treated with DCMU (Figs 4 and 5). This demonstrates conclusively that the site of this action of DCMU is located on the acceptor side of Photosystem II and that the photoinactive state produced by DCMU treatment in Tris-washed chloroplasts is  $F\ P680\ Q^-$ . Our observation on phenylenediamine oxidation is in marked contrast with a recent report by Etienne [25], who found that multiple flashes are required to exhaust  $NH_2OH$ -oxidizing capability in DCMU-treated  $NH_2OH$ -extracted chloroplasts. However, we have found that there are at least two distinct sites for electron donation on the water side of Photosystem II, one involving donation through the Signal IIf species and the other at a second non-Signal IIf site (Babcock, G. T. and Sauer, K., submitted). For example, phenylenediamine and hydroquinone are donors to Signal IIf, whereas  $Mn^{2+}$  donates through the second site. We are currently exploring the behavior of Signal IIf in  $NH_2OH$ -extracted chloroplasts and also attempting to determine the site of  $NH_2OH$  donation in inhibited chloroplasts.

The behavior of Signal IIf in DCMU-treated, Tris-washed chloroplasts at high potential is consistent with the model presented above. Upon oxidation of D, competition with the ( $Q^- + F^{*+}$ ) back reaction is eliminated, and Signal IIf transients in the presence of DCMU are observed. The redox titration reveals that D is a one-electron donor with a midpoint potential of +480 mV. In performing redox titrations it is necessary to establish that the redox characteristics of the component titrated are independent of the specific mediator used in the titrations. For example, if  $K_4Fe(CN)_6$  is able to reduce Signal IIf directly, then the midpoint potential in a titration in which only the ferri/ferrocyanide couple is used might reflect the redox properties of this couple rather than those of a specific component in the chloroplast suspension. However, the results of Fig. 8 show that we observe the same midpoint potential for D using either the ferri/ferrocyanide couple or the cyanotungstate (V)/(IV) couple. Since the midpoint potentials of these two couples differ by 200 mV [27], these results demonstrate that the true potential of D is +480 mV ( $n = 1$ ). A component with identical redox properties has been titrated by Bearden and Malkin [32]. They characterized this component as a secondary donor to the Photosystem II reaction center chlorophyll at low temperatures. The results of Butler et al. [33] and Vermeglio and Mathis [34] also suggest the existence of a secondary donor to P680, with a potential somewhat higher than that of cytochrome  $b_{559}$  ( $E'_0 = +375$  mV) [35], although neither study characterized its reduction potential.

Our results allow us to characterize D in more detail. All previous work has been done at low temperatures [32–34], whereas the results reported here were obtained at 20 °C, indicating that this donor is also functional at physiological temperatures. Fig. 7 and particularly Fig. 9, which show that in the absence of DCMU the decay time for Signal IIf increases as D becomes more oxidized, suggest that at room

temperature this reductant donates electrons through Signal IIf to  $P680^+$ . Our results also show that D is present in relatively high concentrations, since all of the flashing light results have been obtained by signal averaging Signal IIf transients under repetitive flashes. Finally, we showed earlier [11] that the Signal IIf decay time could be lengthened by washing the chloroplast preparation, which suggests that D can be solubilized. Thus the picture emerges that an endogenous one-electron reductant, present in relatively high concentration and with a midpoint potential of  $+480$  mV, functions as a moderately efficient electron donor to Signal IIf. In the absence of DCMU and exogenous donors, D is the principal Signal IIf reductant, since the lifetime of  $Q^-$  ( $600 \mu s$ ) [29, 30] is two to three orders of magnitude shorter than its back reaction time with  $F^{\bullet+}$  (300 ms, Fig. 9). When DCMU is present this back reaction occurs more readily, because the lifetime of  $Q^-$  has been extended by this inhibitor. At low reduction potentials D and  $Q^-$  compete as reductants for Signal IIf; however, Signal IIf transients are eventually quenched since reaction with D leads to the photo-inactive  $F P680 Q^-$  state. At higher reduction potential D becomes oxidized, the ( $Q^- + F^{\bullet+}$ ) back reaction predominates, and DCMU inhibition of Signal IIf transients is relieved.

In our previous communication [11] on Signal IIf we favored a model in which this free radical species represented a second electron donor pathway to  $P680^+$ , which was activated as  $O_2$  evolution was inhibited. This model was constructed in analogy with the alternate electron donors to ubiquinone, succinate dehydrogenase and NADH dehydrogenase, in mitochondria [36]. However, a simpler model is also compatible with our results: the Signal IIf precursor (F) is Z, the immediate physiological donor to  $P680^+$ . In  $O_2$  evolving chloroplasts, both the oxidation ( $t_{\frac{1}{2}} = 35 \mu s$ ) [37] and rereduction of Z by the water-splitting enzyme ( $t_{\frac{1}{2}} \leq 600 \mu s$ ) [8, 38] are too rapid to be observed via EPR. Only upon inhibition of  $O_2$  evolution does it remain in its paramagnetic state for a sufficient time to be observed. The properties of Signal IIf described earlier [11], i.e. rapid rise kinetics, high quantum efficiency for formation and concentration, are consistent with this hypothesis. The results reported here also support the identification of Signal IIf with  $Z^+$ . We have demonstrated that Signal IIf has a high midpoint potential, since it is reduced by a species which has a midpoint potential of  $+480$  mV. Furthermore, we have observed no decrease in the flash-induced increase in Signal IIf magnitude at external potentials of chloroplast suspensions as high as  $+575$  mV (Figs 8 and 9), and even under these conditions Signal IIf is rereduced with a halftime on the order of 1 s. Finally, we have shown that Signal IIf is capable of a back reaction with the reduced primary acceptor  $Q^-$ , a reaction which has been recently proposed for  $Z^+$  [25, 26]. Therefore both the kinetic and concentration parameters demonstrated for Signal IIf in our previous communication [11] and the electrochemical properties described here are consistent with the identification of Signal IIf ( $F^{\bullet+}$ ) with  $Z^+$ . However, we are continuing to test both this model and the one presented previously [11].

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