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A FLASH PHOTOLYSIS ESR STUDY OF PHOTOSYSTEM II SIGNAL II_{vf}, THE PHYSIOLOGICAL DONOR TO $P\text{-}680^+$

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SUMMARY

In flash-illuminated, oxygen-evolving spinach chloroplasts and green algae, a free radical transient has been observed with spectral parameters similar to those of Signal II ($g \approx 2.0045$, $\Delta H_{pp} \approx 19$ G). However, in contrast with ESR Signal II, the transient radical does not readily saturate even at microwave power levels of 200 mW. This species is formed most efficiently with "red" illumination ($\lambda < 680$ nm) and occurs stoichiometrically in a 1 : 1 ratio with $P\text{-}700^+$. The Photosystem II transient is formed in less than 100 μs and decays via first-order kinetics with a halftime of 400–900 μs. Additionally, the $t_{\frac{1}{2}}$ for radical decay is temperature independent between 20 and 4 °C; however, below 4 °C the transient signal exhibits Arrhenius behavior with an activation energy of approx. 10 kcal · mol^{-1}. Inhibition of electron transport through Photosystem II by *o*-phenanthroline, 3-(3,4-dichlorophenyl)-1,1-dimethylurea or reduced 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone suppresses the formation of the light-induced transient. At low concentrations (0.2 mM), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone partially inhibits the free radical formation, however, the decay kinetics are unaltered. High concentrations of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (1–5 mM) restore both the transient signal and electron flow through Photosystem II. These findings suggest that this "quinoidal" type ESR transient functions as the physiological donor to the oxidized reaction center chlorophyll, $P\text{-}680^+$.

INTRODUCTION

Current concepts of photosynthetic oxygen evolution in Photosystem II of green plants and algae have been formulated primarily from phenomenological analysis. For example, oxygen yield determinations during a sequence of brief, intense

Abbreviations: Cl_2Ind, 2,6-dichlorophenol indophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; HEPES, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulphonic acid.

light flashes led to the proposal that oxygen evolution is effected by four sequential photoreactions [1, 59]. Similarly, the early fluorescence investigations of Duysens and Sweers [2] introduced the construct that light-induced reduction of the primary electron acceptor (Q) of Photosystem II results in an increase in chlorophyll *a* fluorescence yield [2]. This simple concept is the basis for the steady state and kinetic fluorimetric assay of primary and secondary electron transfer processes in Photosystem II [3, 4].

Despite the success of these indirect methods for monitoring Photosystem II electron transport, the molecular intermediates involved in oxygen production still remain concealed. The major obstacle is imposed by the current paucity of direct probes for Photosystem II intermediates. However, recent spectroscopic refinements have permitted preliminary studies of the components participating in the primary photochemistry of the Photosystem II reaction center [5–10]. These investigations have revealed that a special (possibly dimer) chlorophyll component, *P*-680, serves as the primary electron donor [5–7] and a quinone (possibly a bound plastoquinone) is utilized as the acceptor(s) [8–10]. The light-mediated charge separation occurs in less than 1 μs [11], followed by reduction of the cation *P*-680$^+$ in approx. 35 to 200 μs ($t_{\frac{1}{2}}$) by an enigmatic donor, Z [12, 13]. Additionally the reduced acceptor, Q^-, is reoxidized by a secondary pool (A) of plastoquinone in 200–600 μs [14, 15]. This oxidation-reduction sequence can be schematically represented:

$$ZPQA \xrightarrow{h\nu} ZP^+Q^-A \xrightarrow{35\,\mu s} Z^+PQ^-A \xrightarrow{200\text{–}600\,\mu s} Z^+PQA^-.$$

Although the nature of the redox pair for the primary photochemical act is currently being defined, the identity of the physiological donor, Z, is nebulous. A Photosystem II component, cytochrome *b*-559, has been proposed as an electron donor to the Photosystem II reaction center chlorophyll, since at 77 °K cytochrome *b*-559 serves as a reductant for *P*-680$^+$ [7, 16]. However, at room temperature this hemoprotein does not appear to participate in primary photochemistry or be requisite for O_2 evolution [17, 18]. Recently we have observed a light-induced, rapidly decaying electron spin resonance (ESR) transient having a decay half-time at 20 °C of approx. 700 μs [19]. This species, designated Signal II_{vf}, is sensitive to treatments which lead to destruction of oxygen evolution capability (e.g. Tris washing, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), or heat) and is present in an equimolar ratio to *P*-700. On the basis of these and other data, it has been proposed that this short-lived radical is the donor to *P*-680$^+$ [19]. However, since the lifetime of this ESR transient is similar to that observed for Q^- oxidation [14, 15], the possibility cannot be excluded that the rapid transient arises from the reduced acceptor. In this communication a further characterization of Signal II_{vf} is presented, along with new evidence which supports assignment of this transient to the donor $Z^+\cdot$ rather than Q^-.

MATERIALS AND METHODS

Broken chloroplasts were prepared as previously described [19]. Intact chloroplasts were isolated according to the method of Jensen and Bassham [21]. Tris-washed chloroplasts were obtained as described by Blankenship and Sauer [22]. *Chlorella pyrenoidosa* were grown in continuous culture and were centrifuged and resuspended in a minimal volume of buffer immediately prior to use. Chlorophyll

content for all samples was 3–4 mg/ml as determined by the method of Arnon [23].

Flash photolysis-electron spin resonance investigations were carried out as previously described [19]. Instrument operating parameters are given in the figure captions. Quantitation of the transient Signal II_{vf} was performed by comparison with Signal I as described previously [19]. Appropriate corrections were applied to compensate for such experimental variations as modulation broadening and differences in microwave power levels or saturation [20]. Variable temperature studies utilized a Varian Model E-257 variable temperature apparatus. Sample temperatures were monitored using a copper-constantan thermocouple.

Techniques and instrumentation for measuring O_2 evolution in continuous light have been described previously [22]. The reaction mixture consisted of 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (pH 7.6), 20 mM NaCl, 1 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM $K_3Fe(CN)_6$ and 1 mM $K_4Fe(CN)_6$. Chlorophyll concentrations were typically 30–40 μg/ml.

Spinach ferredoxin, ascorbic acid (sodium salt) and NADP were obtained from Sigma. DCMU, procured from Dupont, was recrystallized from benzene and dissolved in ethanol. Ethanol concentration in all experiments was less than 1 %. Phenylenediamine and benzoquinone were obtained from J. T. Baker Co. and purified by sublimation. 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was the generous gift of Dr. David Knaff.

RESULTS

Occurrence of Signal II_{vf}

The very fast ESR transient at 3380 G has been observed in broken spinach chloroplasts isolated in a variety of buffer media [19]. If Signal II_{vf} serves as a donor to Photosystem II, then this species should be present in intact chloroplasts and oxygen competent algae as well. Fig. 1b illustrates that indeed Signal II_{vf} is observed at 3381 G in *C. pyrenoidosa*. Signal II_{vf} rises in the time constant of the instrument (100 μs) and decays in approx. 400 μs. Signal I kinetics are presented for comparison in Fig. 1a. Quantitative comparison of Signal II_{vf} with Signal I using data similar to that presented in Fig. 1 indicates that Signal II_{vf}/Signal I = 0.93 ± 0.2. This value is consistent with that observed in broken chloroplasts [19] and suggests that the species giving rise to Signal II_{vf} occurs equimolar with $P\text{-}700^+$; that is, about one spin for every 300–400 chlorophylls.

Similar results obtained with intact, Jensen-Bassham type chloroplasts are shown in Fig. 2. However, Signal II_{vf} decay is superimposed upon a slower kinetic component of Signal II ($t_{\frac{1}{2}}$ approx. 10 s) previously described by Warden and Bolton [24]. The amplitude of Signal II_{vf} is usually twice that of the DCMU-insensitive, slow decaying transient.

Correlation of Signal II_{vf} with oxygen production

Signal II_{vf} has been shown previously to reflect the ability of spinach chloroplasts to evolve oxygen [19]. In chloroplasts treated by Tris washing, Signal II_{vf} as well as oxygen production is inhibited. However, in reactivated Tris-washed chloroplasts, both oxygen evolution and Signal II_{vf} are largely restored [19, 25].

Further evidence for a direct correlation of Signal II_{vf} with electron transport

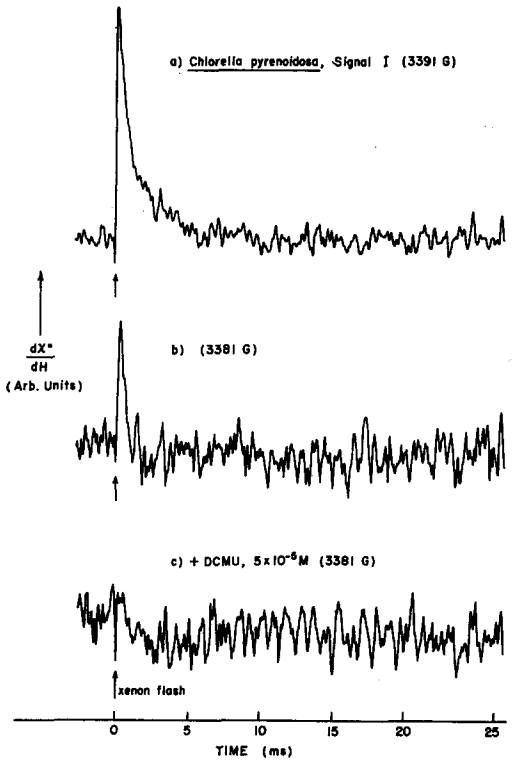

Fig. 1. Flash-induced transient ESR changes in *C. pyrenoidosa* at room temperature: a, Signal I; b, Signal II_{vf} (3381 G); c, *Chlorella* plus $5 \cdot 10^{-5}$ M DCMU (3381 G). Data shown for Signal II_{vf} are the average of 4096 flashes; for Signal I, 1024 flashes. Modulation amplitude, 5 G; microwave power, 50 mW.

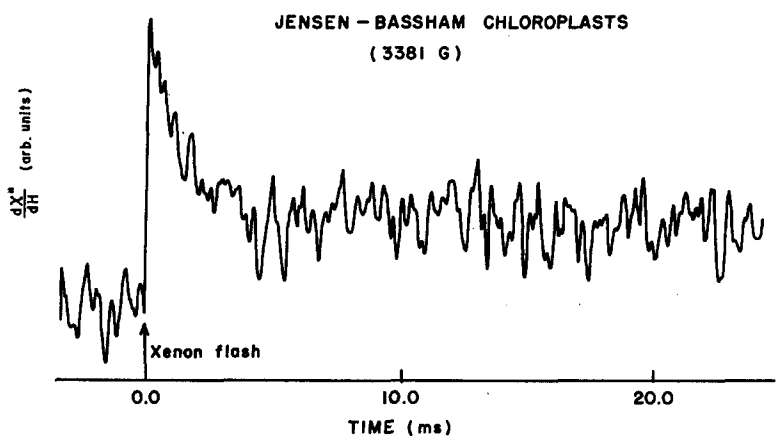

Fig. 2. Flash-induced kinetics for Signal II_{vf} in Jensen-Bassham type chloroplasts. The trace is an average of 2048 repetitive scans. Microwave power, 160 mW; modulation amplitude, 6.3 G.

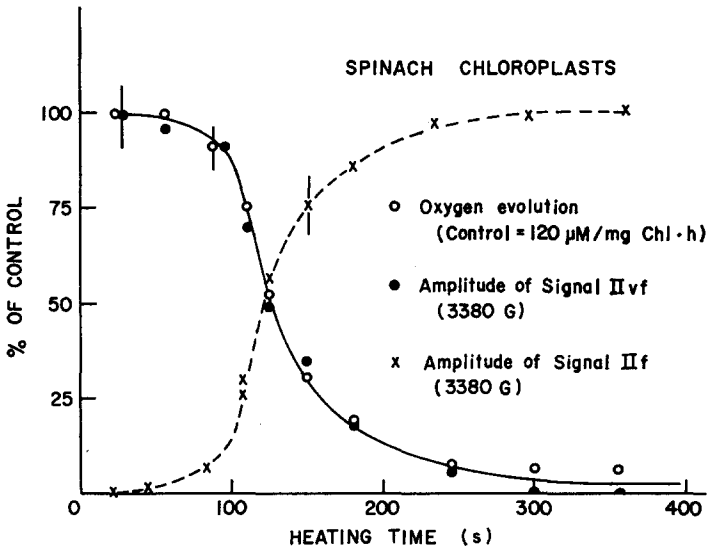

Fig. 3. Amplitude of Signal II_{vf} and Signal II_f and rate of oxygen evolution as a function of chloroplast incubation time at 50 °C. Parameters of Signal II_f measurements: microwave power, 25 mW; modulation amplitude, 6.3 G. ESR parameters for monitoring Signal II_{vf} were the same as in Fig. 2. Each point for the ESR determinations represents the average of 2048 sequential flashes.

from water is presented in Fig. 3. The rate of oxygen evolution and the maximum amplitude of Signal II_{vf} are seen to behave identically with respect to incubation of the chloroplast samples at 50 °C for varying times. After 250 s of heat treatment both the amplitude of Signal II_{vf} and the rate of oxygen production have been suppressed to about 5 % of control. However, as Babcock and Sauer [26] have demonstrated, the slower decaying Signal II_f ($t_{\frac{1}{2}}$ approx. 1 s) is readily observable in the inhibited samples. As illustrated in Fig. 3, we have also observed that the decrease in the amplitude of Signal II_{vf} upon inhibition of oxygen evolution is inversely proportional to the increase in Signal II_f amplitude. These experiments support our assertion that Signal II_f and Signal II_{vf} reflect the same chemical species, presumably the oxidized electron donor $Z^{+}\cdot$.

Wavelength dependence and quantum yield for the formation of Signal II_{vf}

A traditional criterion for the association of a photosynthetic intermediate with one of the photochemical systems has been the observation of the response of the electron transfer component to different wavelengths of actinic illumination. Thus processes which proceed more efficiently in "red" light ($\lambda < 690$ nm) are assigned to Photosystem II; and similarly, processes driven most efficiently by "far-red" light ($\lambda > 690$ nm) are associated with Photosystem I [27, 28]. Fig. 4 illustrates the behavior of Signal II_{vf} when subjected to non-saturating flash illumination with monochromatic light of either 650 or 720 nm. Similar absorbed flash intensities at the two wavelengths are achieved with Balzer neutral density filters. The kinetic traces of Fig. 4 favor assignment of Signal II_{vf} to Photosystem II, since "far-red" light, effective for mediation of Photosystem I reactions, produces no response.

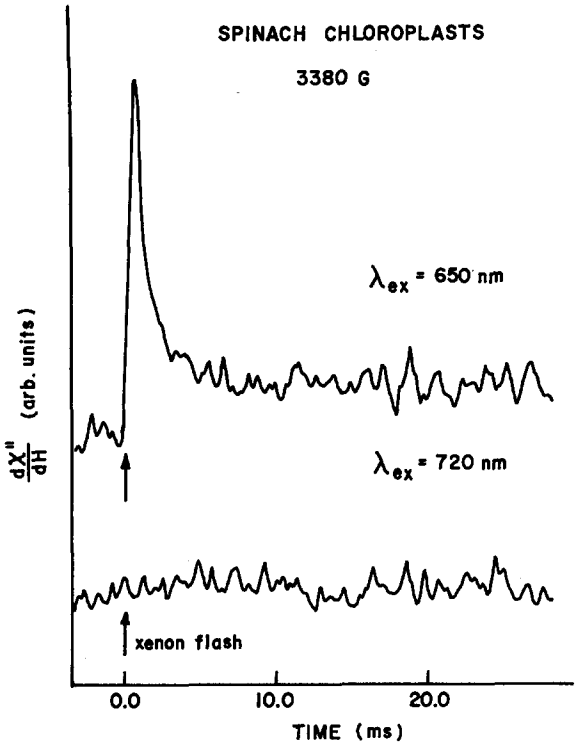

Fig. 4. Comparison of the relative efficiency of red and far red flash illumination for the generation of Signal II_{vf} in spinach chloroplasts. Instrumental conditions were the same as in Fig. 2. Each trace is the average of 4096 events.

The amplitude ratio of Signal II_{vf}/Signal I is always invariant of the intensity of the actinic, broadband xenon flash (data not shown). The parallel response of the two transients to the excitation intensity implies that the quantum yields for the formation of Signal II_{vf} and Signal I are similar. Since the quantum yield for *P*-700 oxidation (Signal I generation) is near unity [29, 30], then a similar value for Signal II_{vf} formation suggests that this step is closely associated with the photochemical apparatus for Photosystem II.

Temperature dependence of Signal II_{vf} formation and decay

The energetics of chemical or physical processes are characterized generally from a study of the temperature dependence of the rate constant for the process (e.g. the Arrhenius relation). To determine whether the formation of Signal II_{vf} is a primary photochemical step, we have charted the temperature dependence for this radical from −180 to 22 °C. Fig. 5 illustrates the kinetic behavior of Signal II_{vf} at four different temperatures: 4, −15 −25 and −43 °C. Between 4 and 22 °C the half-time for radical decay appears to be independent of temperature. However, at temperatures below 0 °C the decay kinetics display a strong dependence on temperature, until at approx. −40 °C there is no observed production of Signal II_{vf}. Continuous illumination of the chloroplast sample at −180 °C revealed no ESR absorption changes

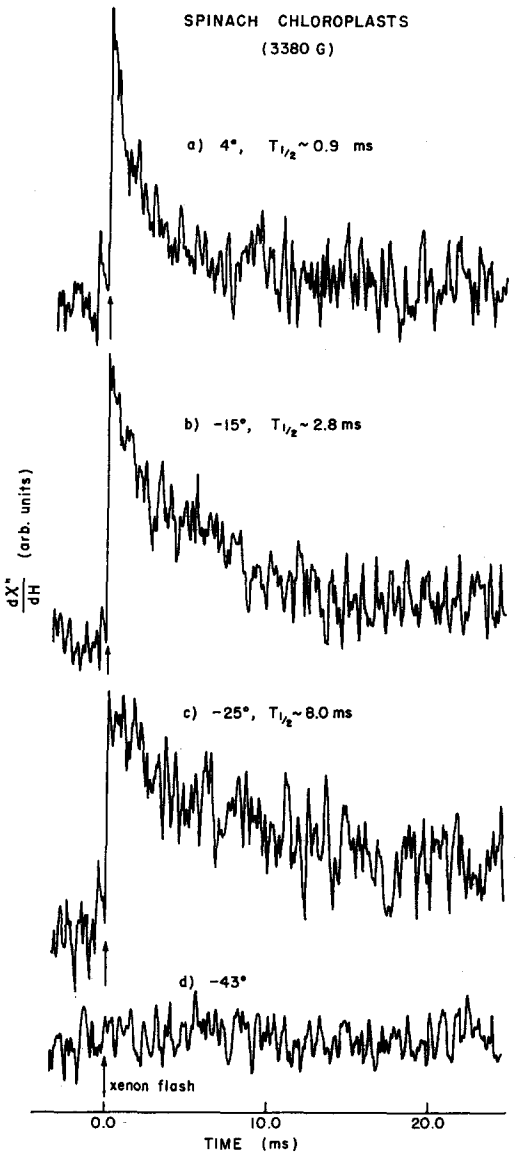

Fig. 5. Time course for the formation and decay of Signal II_{vf} in spinach chloroplasts at various temperatures. Each trace is the average of 2048 scans. Modulation amplitude, 6.3 G; microwave power, 80 mW. Note that no Signal II_{vf} is formed at −40 °C.

other than those resulting from Signal I in the magnetic field region characteristic for Signal II_{vf}. The kinetic traces in Fig. 5 were obtained in the presence of glycerol (55 %, v/v); however, identical results were obtained from samples frozen only in buffer. The glycerol/buffer mixture freezes as a glass, therefore facilitating light absorption by the sample.

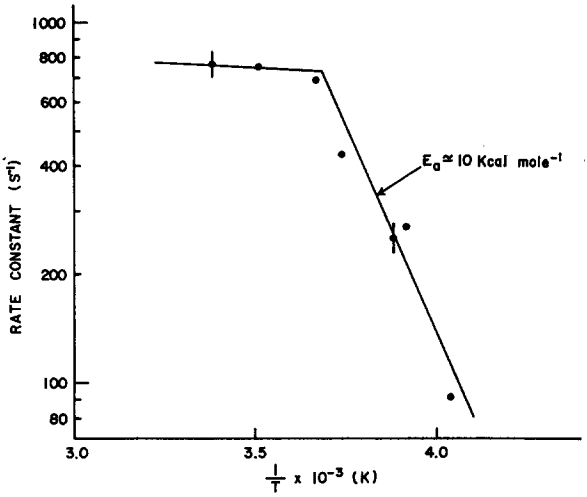

Fig. 6. Arrhenius plot of the logarithm of the first-order decay rate constant of Signal II_{vf} vs T^{-1}. Each point is the average of 2048 flashes. Instrumental conditions are given in Fig. 5.

The temperature dependence of the decay kinetics for Signal II_{vf} is presented in an Arrhenius format in Fig. 6. The Arrhenius plot reveals a discontinuity in slope at approx. −5 °C. Below this temperature the slope of the plot yields an activation energy for Signal II_{vf} decay of approx. 10 kcal/mol. However, above −5 °C, the temperature insensitivity of the curve suggests an energy of activation of approx. 0 kcal/mol. This lack of activation energy for the electron transfer is characteristic for quantum mechanical tunneling [31].

Effect of redox agents on Signal II_{vf}

Oxidizing and reducing agents, possessing well defined midpoint potentials, have proven invaluable for charting the role of electron carriers in biological electron transport chains [32]. Early investigations by Duysens and Sweers [2] indicated that the primary acceptor, Q, could be reduced either by light or by the addition of the reductant $Na_2S_2O_4$. Elegant redox titrations of the fluorescence yield by Cramer and Butler [33] and later by Erixon and Butler [34] further established that the Photosystem II acceptor possesses a midpoint potential of +25 mV. More recent studies have demonstrated that even under extremely oxidizing conditions [35], the reaction center is capable of sustaining electron transport.

In view of the current concept of redox interactions between components of Photosystem II, it is desirable to ascertain the potentiometric response of Signal II_{vf}. The kinetic behavior of the transient ESR signal in the presence of oxidants or reductants is depicted in Fig. 7. Of the reductants utilized, only dithionite is effective in quenching the transient. This behavior is consistent with the observation that dithionite chemically reduces Q, thus inhibiting net electron transport through Photosystem II. Ascorbate cannot reduce Q or the secondary acceptor pool A [36], and this species has no effect on Signal II_{vf}. Similarly the oxidant ferricyanide (in the range of 1–10 mM) produces little alteration in Signal II_{vf} kinetics.

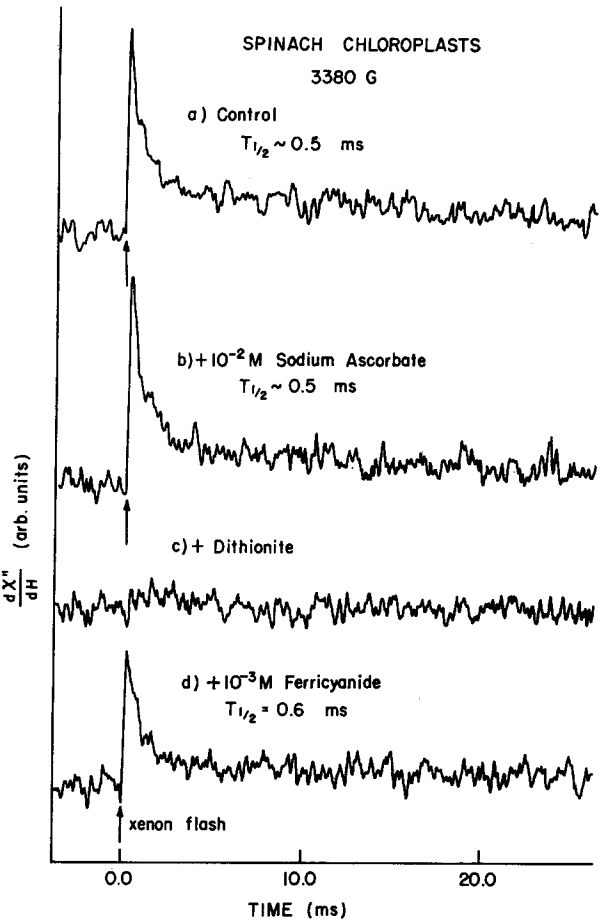

Fig. 7. Formation and decay kinetics for Signal II_{vf} in the presence of various reductants and the oxidant ferricyanide. Modulation amplitude, 6.3 G; microwave power, 150 mW. Each trace is the average of 2048 scans.

Effect of Photosystem II electron transport inhibition on Signal II_{vf}

As has been shown above and in our previous communication, inhibition of oxygen evolution by Tris washing or heat treatment results in a retardation of the decay of Signal II_{vf}. Similarly addition of DCMU or *o*-phenanthroline inhibits the formation of this rapid transient, a result consistent with the assignment of Signal II_{vf} to Photosystem II (Fig. 1).

Reduced DBMIB introduced by Trebst et al. [37] serves as an inhibitor to Photosystem II electron flow, acting at the level of plastoquinone. Although this plastoquinone antagonist permits the oxidation of Q^- by the A pool (plastoquinone), the subsequent reoxidation of A is prevented [38]. Thus under continuous or high repetitive flash illumination the plastoquinone pool becomes reduced with the consequent reduction of Q and inhibition of net electron transport. In the presence of 1 mM reduced DBMIB we note a 60 % reduction in the amplitude of Signal II_{vf}, however,

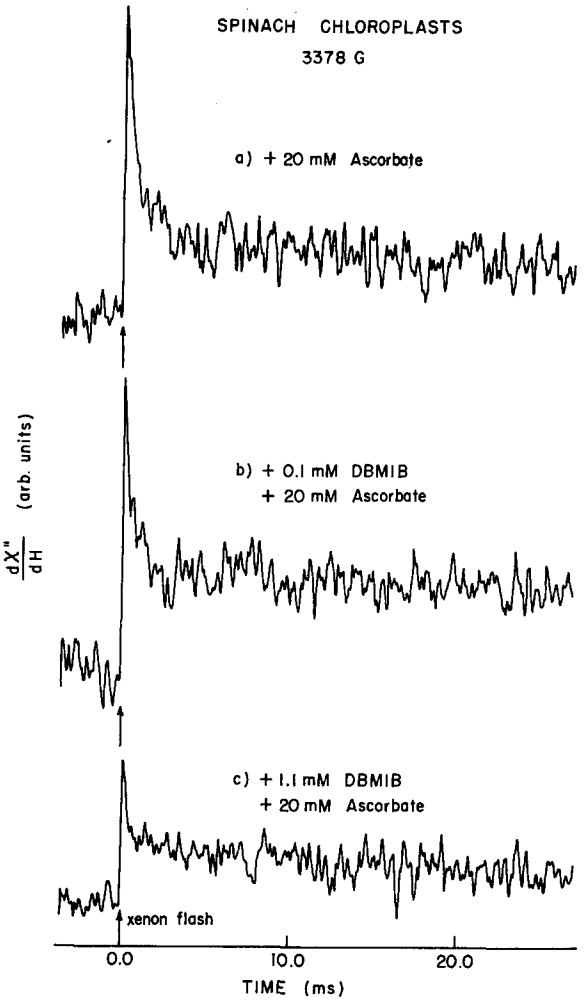

Fig. 8. Decay kinetics for Signal II_{vf} in spinach chloroplasts in the presence of two different concentrations of reduced DBMIB. Trace (a) is the control sample. Instrumental parameters are given in Fig. 7.

the decay kinetics for this species is unaltered (Fig. 8). The implications of this observation are delineated in Discussion.

DBMIB in its oxidized form at low concentrations inhibits Photosystem II electron flow [38]. However, at higher concentrations this quinone behaves as an electron acceptor, restoring most of the electron transport capability of the inhibited chloroplasts [39, 40]. This effect has also been confirmed in Signal II_{vf} formation. At low concentration of DBMIB (0.2 mM) the maximum transient amplitude of the intermediate is reduced to approx. 60 % of control (data not shown). Addition of oxidized phenylenediamine or benzoquinone, both lipophilic electron acceptors for Q^-, restores the transient amplitude of Signal II_{vf}. However, at higher DBMIB

concentrations (approx. 1 mM), Signal II_{vf} is formed to almost the same extent as in control samples. Similar to the behavior of reduced DBMIB, the presence of the oxidized inhibitor has no noticeable effect upon the formation or decay kinetics of the radical. It is worth noting that at high concentrations of DBMIB flash illumination leads to formation of the semiquinone, which then decays very slowly (> 10 s). Thus it is doubtful that reduced DBMIB serves as a direct donor to $P\text{-}700^+$, which decays in approx. 1 s in these samples [41].

Hg^{2+} treatment (approx. 1 mM) has been reported by Radmer and Kok [54] to act similarly to reduced DBMIB in that Hg^{2+} inhibits electron transport from the A pool to Photosystem I. This inhibition, presumably at plastocyanin [55], can be readily reversed by utilizing ferricyanide as an electron acceptor. After incubation of chloroplasts with 1 mM $HgCl_2$ for 30 min at 0 °C, the amplitude of Signal II_{vf} was reduced by 70 % compared to control samples. Restoration of the transient amplitude to 90 % of control could be effected by addition of 1 mM $Fe(CN)_6^{3-}$. In contrast to amplitude inhibition by $HgCl_2$ the decay time for Signal II_{vf} remained invariant.

Characteristics of the ESR spectrum of Signal II_{vf}

The spectrum of Signal II_{vf} closely resembles Signal II as observed at low

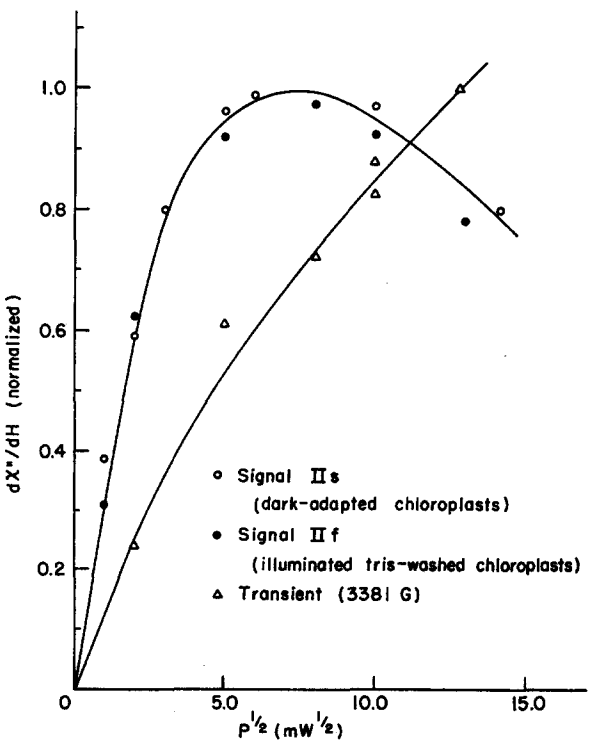

Fig. 9. Power saturation profiles for Signal II (○), Signal II_f (●) and Signal II_{vf} (△) in spinach chloroplasts. Signal amplitudes were determined at the low field derivative maximum (3380 G) and each point for the Signal II_{vf} profile represents the average of 2048 flashes. Modulation amplitude, 6.3 G.

resolution [19, 24, 42]*. The peak-to-peak linewidth of the transient signal is approx. 19 G. Based upon the cross-over point of the transient spectrum, the g-factor for Signal II_{vf} is calculated to be 2.0045 ± 0.0005, using perylene cation as the g-factor standard [43].

The microwave-power saturation profile of a radical, usually plotted as signal amplitude vs (power) $^{\frac{1}{2}}$, is a sensitive probe of the environment of the unpaired electron. Signal II as recently reported by Warden and Bolton [24] saturates at microwave power levels above 25 mW. Signal II_{vf}, on the other hand, is only partially saturated at 200 mW (Fig. 9). The power saturation curves of Signal II and Signal II_f (Tris-washed chloroplasts) are presented in Fig. 9 for comparison with that for Signal II_{vf}. These data indicate that although the relaxation environments are similar for Signal II and Signal II_f, Signal II_{vf} possesses a shorter effective spin-lattice relaxation time (T_1) and is therefore more difficult to power saturate.

Transition metal and rare earth paramagnets have been utilized recently as dipolar probes for membrane- or protein-bound electron transfer components [44]. Leigh [45] in an analysis of the interaction of isolated spin pairs has demonstrated

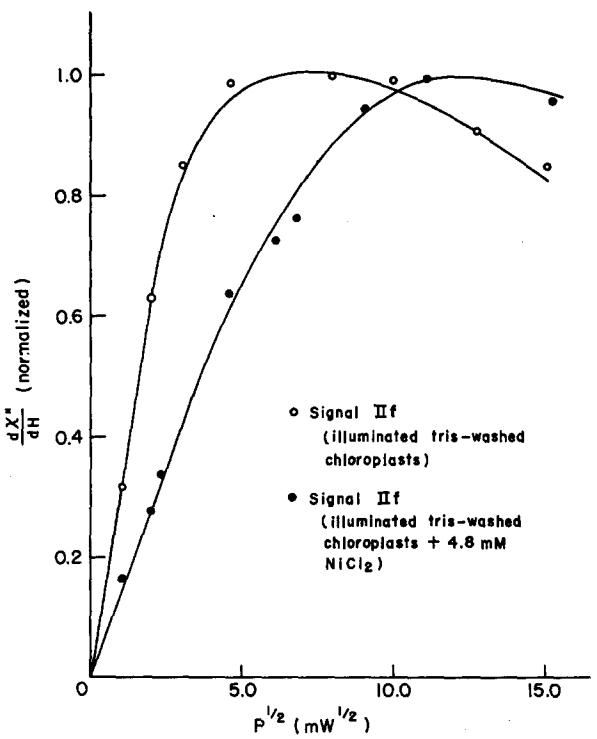

Fig. 10. Power saturation profiles for Signal II_f in the presence (●) and absence (○) of 4.8 mM $NiCl_2$. Signal amplitudes at the low field derivative maximum were determined during illumination.

* See Fig. 2 in ref. 19.

that dipolar interaction between a free radical and a paramagnetic ion probe can produce a reduction in signal amplitude of the radical without appreciable line broadening.

Tris washing blocks electron transport on the water side of Photosystem II, resulting in an inhibition of oxygen evolution and a release of manganese from the thylakoid membrane [22]. This inhibition of physiological electron transport from water is reflected by the disappearance of Signal II_{vf} and the activation of Signal II_f [19].

The considerations above suggest that presence of bound manganese in oxygen competent Photosystem II reaction centers might facilitate the relaxation of Signal II_{vf} through dipolar perturbation. Removal of the bound manganese through Tris treatment or incubation with 0.5 M $MgCl_2$ would eliminate the paramagnetic interactions, resulting in a more easily saturable transient radical, as illustrated in Fig. 9. Conversely this hypothesis predicts that incubation of Tris- or magnesium-treated samples with high concentrations of $NiCl_2$, a small, highly efficient paramagnetic relaxer, should induce an alteration in the Signal II_{vf} power saturation curve similar to that observed in untreated samples. Verification of this prediction is illustrated in Fig. 10. Incubation of Tris-washed chloroplasts with 4.8 mM $NiCl_2$ results in a shift of the saturation curve toward higher power levels than observed in the control Tris-washed sample. The magnitude of the induced saturation curve shift depends on the mobility of the paramagnetic probe into the thylakoid, the distance between the free radical (Signal II_f) and the probe and relative values of the T_1's for the two interacting spins. The data of Fig. 10 qualitatively support the assertion that the power saturation characteristics of Signal II_{vf} arise from dipolar relaxation by a transition metal (manganese?) located in close proximity to the species giving rise to Signal II_{vf}.

DISCUSSION

The assignment of Signal II_{vf} to either the primary acceptor or the donor to $P\text{-}680^+$ can be attempted by comparison of the known properties of these redox intermediates to those for the ESR transient. The application of fluorimetric [3, 4, 46, 47] and spectroscopic [4] techniques have provided the following characteristics for the Photosystem II acceptor: (1) The primary acceptor is provisionally a quinoidal component which is reduced during illumination, even at cryogenic temperatures [4]. (2) The oxidation of the photoreduced acceptor by the plastoquinone pool ($t_{\frac{1}{2}}$ approx. 200–600 μs) is inhibited by DCMU [2]. (3) The oxidation of the acceptor is highly temperature dependent with the step $Q^-A \rightarrow QA^-$ exhibiting a Q_{10} of 3.7 [48]. Thus at 0 °C a $t_{\frac{1}{2}}$ of approx. 3 ms is observed for the electron transfer to the A pool [48]. At temperatures below approx. −30 °C reoxidation of Q presumably proceeds by a charge recombination with oxidized intermediates on the water side of Photosystem II [48, 49].

Characterization of the donor, Z, has not been feasible, due to the lack of spectroscopic probes for this high potential reductant. However, determinations of oxygen yield during a sequence of flashes suggest that the half-time for Z^+ reduction by the O_2-producing complex lies in the region of 0.6–1 ms [1, 50]. Furthermore the kinetics for Z oxidation have been determined indirectly by Gläser et al. [12] to be

approx. 35 μs. In view of our ignorance of the donor to $P\text{-}680^+$, our discussion of the role of Signal II_{vf} will concentrate on the similarities and differences between this ESR transient and the primary acceptor Q.

Like Q, Signal II_{vf} is formed most efficiently in "red" (System II) light and exhibits a high quantum yield. Signal II_{vf} occurs at the level of approx. 1 : 300–400 chlorophyll, a concentration similar to that reported by Pulles et al. [10] for a plastoquinone component and by Gläser et al. [12] for *P*-680. Signal II_{vf} decays with a $t_{\frac{1}{2}}$ similar to that reported for Q^- oxidation and possibly for Z^+ reduction [14, 15]. Whereas Q^- oxidation exhibits multiple component kinetics ($t_{\frac{1}{2}} \approx 200$ μs, ≈ 2 ms, 20 ms) [11, 14, 15], Signal II_{vf} is monophasic within experimental error with a decay halflife in the range of 400–900 μs. In the presence of reduced 2,6-dichlorophenol indophenol (Cl_2Ind) (1 mM) or oxidized phenylenediamine the halflife for the fast ESR signal is generally on the order of 900–1000 μs; however, with ferricyanide or ferredoxin/$NADP^+$ as acceptors the decay time is approx. 300–600 μs. The variation of decay time with added acceptors is intriguing, particularly in the light of the report by Bouges-Bocquet [50] that reduced Cl_2Ind enhances the accumulation of S_0 during dark adaptation. The decay of Signal II_{vf} may be therefore a sensitive indicator of the dark ratio of S_0/S_1. Doubtless, further investigation is needed to ascertain if the decay time of the ESR transient is modified during a flash sequence driving the cyclical transition from S_0 to S_4.

Signal II_{vf} is not formed at 77 °K as evidenced by lack of amplitude changes in the 3381 G region of the ESR spectrum during continuous illumination. However, under similar conditions Q becomes reduced, resulting in an enhanced fluorescence yield. Similarly, Q^- reoxidation exhibits strong temperature dependence in the range of 0–20 °C; however, over this temperature region Signal II_{vf} kinetics are invariable within experimental error. Only below approx. −5 °C does Signal II_{vf} show temperature dependence. This transition temperature lies in the range observed for phase changes of membrane lipids [51]. Thus the transition to Arrhenius behavior may reflect conformational changes in the membrane near the Photosystem II trapping center. A similar temperature behavior for fluorescence yield has been observed by Malkin and Michaeli [52] and tentatively attributed to a conformation change decoupling Q from the A pool. However, an alteration of fluorescence yield reflecting an increase in the reduction time of $Z^+ \cdot$ on the oxidizing side of Photosystem II could account as well for the temperature behavior [53]. Hence, assignment of Signal II_{vf} to $Z^+ \cdot$ rather than Q^- implies that below −40 °C the reaction center chlorophyll, *P*-680, is unable to oxidize Z. This assertion is supported by the recent observations of Butler et al. [16], and Floyd et al. [7], that cytochrome *b*-559 serves as the electron donor to $P\text{-}680^+$ at low temperatures.

The plastoquinone antagonist, dibromothymoquinone, inhibits non-cyclic electron flow from Photosystem II to *P*-700 and at high concentrations this lipophile appears to act near the primary electron acceptor [36]. Furthermore, this agent has no effect upon the oxygen-producing complex, since the oscillation of the flash yield of O_2 is not altered [36]. In the presence of high concentrations of reduced DBMIB the A pool will be predominantly reduced during a high repetition rate flash sequence, hence the reoxidation time for Q^- should increase and be controlled by the rate-limiting step of plastohydroquinone oxidation. In contrast the kinetics for Signal II_{vf} are unaffected by 2 mM reduced DBMIB, although the transient signal amplitude is reduced.

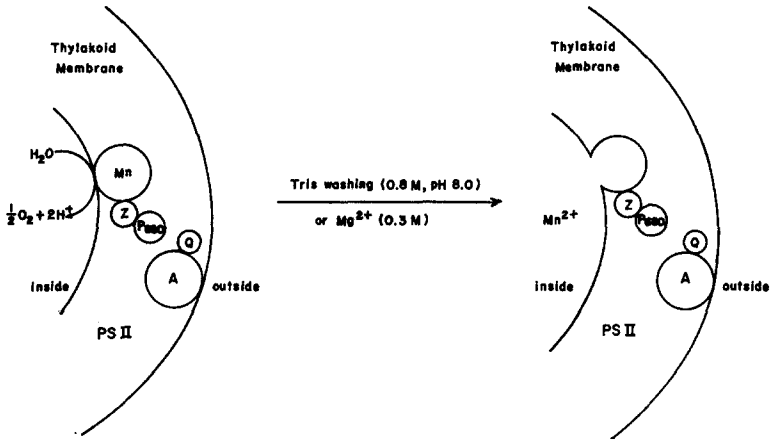

Fig. 11. Model for Photosystem II electron transport components in intact and Tris or Mg^{2+}-treated chloroplasts. Details are given in the text.

The behavior of Signal II_{vf} in the presence of inhibitors of electron transfer on the reducing side of Photosystem II, although incompatible with that predicted for the primary acceptor, is consistent with the assignment of Signal II_{vf} to $Z^+ \cdot$, since reduction in net electron flow through Photosystem II will be reflected in limited oxidation of Z by $P\text{-}680^+$. Furthermore, no alteration in the kinetics of oxidation or reduction of Z can be expected if the inhibitors act only on the reducing side of System II.

The observations presented in this paper and in earlier communications [19, 26] have been interpreted as supporting the assignment of Signal II_{vf} to the physiological donor to $P\text{-}680^+$. The relaxation and kinetic characteristics of the fast transient appear to reflect the presence of the bound manganese associated with oxygen evolution. Assuming that Signal II_{vf} arises from $Z^+ \cdot$, the paramagnetic behavior of the transient can be rationalized on the basis of the model for Photosystem II presented in Fig. 11 [56]. In untreated chloroplasts the bound manganese serves as a paramagnetic relaxer for the Z^+ radical and electron transport from water is unhindered ($t_{\frac{1}{2}}$ for reduction of $Z^+ \cdot$ approx. 400–900 μs). However, when oxygen evolution is inhibited by heating or Tris washing with release of Mn^{2+} to the inner thylakoid cavity, $Z^+ \cdot$ exhibits slower decay kinetics ($t_{\frac{1}{2}}$ approx. 1 s) and a power saturation profile similar to Signal II. This model suggests that inhibition of oxygen evolution without release of manganese would yield Signal II_{vf} with slower kinetics (e.g. Signal II_f), however, the saturation characteristics of the radical would not be altered. Experiments testing this hypothesis are currently in progress.

The chemical identity of the fast ESR transient is yet to be established. On the basis of the spectroscopic g-factor, the peak-to-peak linewidth, and spectral similarity to Signal II, Signal II_{vf} might tentatively be ascribed to a semiquinone. However, this assignment must be tempered by the following considerations: (1) a high resolution spectrum for Signal II_{vf} has not been obtained, and (2) the expected midpoint potential for Z is considerably more positive than that reported for most quinones [57]. The latter point is not prohibitive since the midpoint potentials of redox components

are markedly influenced by the local environment and the presence of proton or cation gradients [32]. A recent report by Okayama [58] suggests that plastoquinone is an essential component of the oxidizing side as well as the reducing side of Photosystem II. Preliminary experiments in our laboratory have indicated that a major portion of the chloroplast plastoquinone can be removed without loss of Signal II_f; however, total extraction of plastoquinone results in the disappearance of Signal II_f (Lucas, J. N. and Warden, J. T., unpublished results). Significantly the quantitative relationship of Signal II_{vf} to tightly bound plastoquinone remains to be ascertained.

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