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A RAPID, LIGHT-INDUCED TRANSIENT IN ELECTRON PARAMAGNETIC RESONANCE SIGNAL II ACTIVATED UPON INHIBITION OF PHOTOSYNTHETIC OXYGEN EVOLUTION

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SUMMARY

A rapid, light-induced reversible component in Signal II is observed upon inhibition of oxygen evolution in broken spinach chloroplasts. The inhibitory treatments used include Tris washing, heat, treatment with chaotropic agents, and aging. This new Signal II component is in a 1 : 1 ratio with Signal I (P700). Its formation corresponds to a light-induced oxidation which occurs in less than 500 μs. The subsequent decay of the radical results from a reduction which occurs more rapidly as the reduction potential of the chloroplast suspension is decreased. The formation of this free radical component is complete following a single 10-μs flash, and it occurs with a quantum efficiency similar to that observed for Signal I formation. Red light is more effective than far-red light in the generation of this species, and, in preilluminated chloroplasts, 3-(3,4-dichlorophenyl)-1,1-dimethylurea blocks its formation. Inhibition studies show that the decline in oxygen evolution parallels the activation of this Signal II component.

These results are interpreted in terms of a model in which two pathways, one involving water, the other involving the rapid Signal II component, compete for oxidizing equivalents generated by Photosystem II. In broken chloroplasts this Signal II pathway is deactivated and water is the principal electron donor. However, upon inhibition of oxygen evolution, the Signal II pathway is activated.

INTRODUCTION

We have recently shown that the light-induced formation of the Signal II species involves oxidation of its precursor, F, by the States S_2 and S_3 on the water side of Photosystem II [1, 2]. While this reaction occurs initially with high quantum efficiency, the kinetics of both the formation ($t_{\frac{1}{2}} = 1$ s) and decay ($t_{\frac{1}{2}} = 1$ h) are

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

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sufficiently slow to preclude an integral role for Signal II in the transport of electrons from water to Photosystem I. These results were interpreted in terms of a model in which the two processes, Signal II formation and water oxidation, compete for oxidizing equivalents generated by Photosystem II.

A number of treatments which inhibit electron flow at a point between the site of water oxidation and the Photosystem II reaction center have been developed recently. Included in this classification are Tris washing, aging, heat treatment, incubation with chaotropic agents and hydroxylamine treatment [3–7]. Chloroplasts which have been subjected to these treatments show lower chlorophyll *a* fluorescence in the light, diminished oxygen evolution capability and much higher concentrations of EPR-detectable Mn^{2+} [3, 5, 8]. However, treated chloroplasts will oxidize exogenously supplied ascorbate or Mn^{2+} via Photosystem II, a reaction which does not occur in untreated chloroplasts [9].

We have examined the effects of these treatments on the behavior of Signal II. In treated chloroplasts the number of Signal II spins which can be detected in saturating light is twice that detectable in untreated chloroplasts. Following a flash the light-induced increase in this new Signal II component proceeds with high quantum efficiency and is complete within 500 μs. The lifetime of this species is several hundred ms in the absence of exogenous reductants and decreases as the redox poise of the chloroplast suspension is lowered. These experiments are interpreted in terms of a model in which electron flow through a component of Signal II to the Photosystem II reaction center is activated upon inhibition of oxygen evolution by the treatments described above.

MATERIALS AND METHODS

1. Chloroplasts and reagents

Chloroplasts were isolated from growth-chamber spinach as described previously [1] except that the Tricine buffer used has been replaced by HEPES buffer. These chloroplasts are referred to in the text as untreated chloroplasts. Tris-washed chloroplasts were prepared as described by Yamashita and Butler [3] as modified by Blankenship and Sauer [8]. Treatment with chaotropic agents was carried out as described by Lozier et al. [5]. Heat treatment was performed by incubating 0.5-ml aliquots of untreated chloroplasts (2 mg chlorophyll/ml) at 50 °C ($\pm$1 °C) in the dark for the indicated time. Chloroplasts stored in the dark at 0 °C for 36 h are referred to as aged chloroplasts. EDTA (10^{-4} M) was added to all samples 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; in oxygen evolution experiments the chlorophyll concentration was 40 μg/ml.

Spinach ferredoxin and NADP were obtained from Sigma; 3-(3,4-dichlorophenyl)-1,1-dimethylurea (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.

2. Light sources, oxygen measurements and EPR measurements

10-μs white-light flashes, continuous white light and continuous monochromatic

light were obtained from sources as described previously [1]. Oxygen evolution in continuous light (intensity = 45 mW/cm^2) was measured as described by Blankenship and Sauer [8] using a reaction mixture which contained 0.05 M HEPES, pH 7.6, 0.02 M NaCl, 0.01 M NH_4Cl, 0.005 M $MgCl_2$, 0.001 M $K_4Fe(CN)_6$, 0.001 M $K_3Fe(CN)_6$.

EPR experiments were carried out using the Varian E-3 (X-band, 9.5 GHz) EPR spectrometer described previously [1]. The microwave power in all experiments was 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 time constant and scan rate are noted in figure legends. Signal averaging was performed using a 1024 channel Northern Scientific NS544 Digital Memory Oscilloscope. The unfiltered output of the E-3 was fed into a preamplifier where d.c. background levels were biased off and time constants as noted in the text were imposed. This signal was then stored in the averager. Appropriate timing circuits (Tektronics 160, 161 and 162) synchronized the initiation of the averager sweep and the flash-lamp discharge. All experiments were carried out at room temperature.

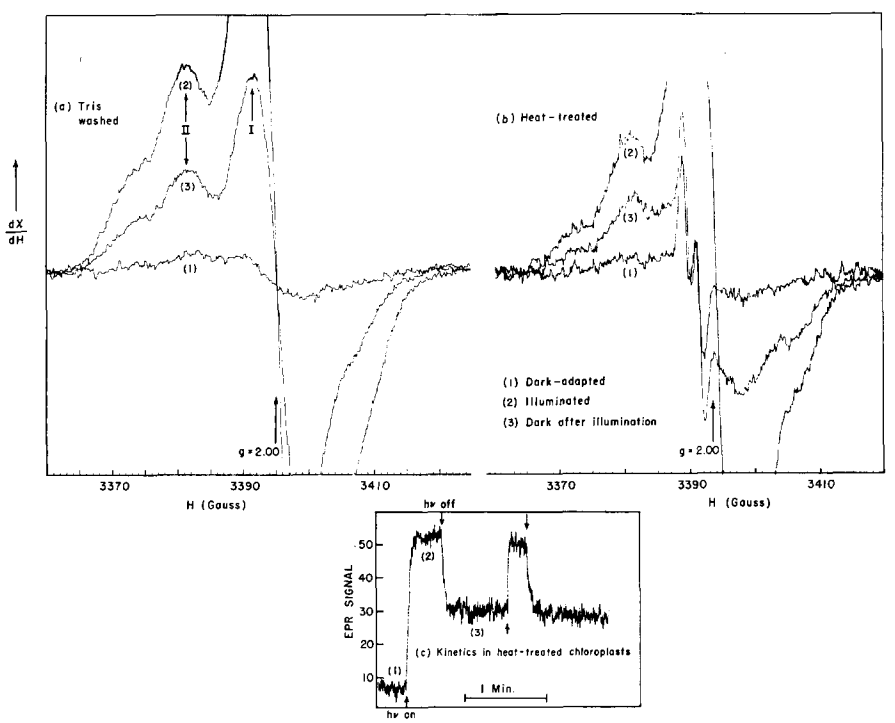

Fig. 1. Signal II in Tris-washed (a) and heat-treated chloroplasts (b, c) under various illumination conditions. In (a) and (b) EPR spectra for the sample in the dark prior to illumination, during illumination, and in the dark following illumination are labeled (1), (2) and (3), respectively. The instrument time constant was 0.3 s with a scan rate of 25 G/min. In (c) the kinetics of these light-induced changes of Signal II were followed at the low-field maximum labeled II in (a). The regions in this trace that match the conditions under which the spectra in (a) and (b) were recorded are correspondingly labeled.

RESULTS

Effect of Tris washing and heat treatment on Signal II: spin concentration and illumination kinetics

Fig. 1 shows EPR spectra of Tris-washed (1a) and heat-treated (1b) chloroplasts under various illumination conditions. In the dark the level of Signal II is low (Spectra 1) in both types of chloroplast samples, since these inhibitory treatments tend to destabilize the usual free-radical state of Signal II [10]. Illumination increases the level of both Signal II and Signal I (Spectra 2), while in the dark following illumination (Spectra 3) Signal II decays to a level about half that observed in the light.

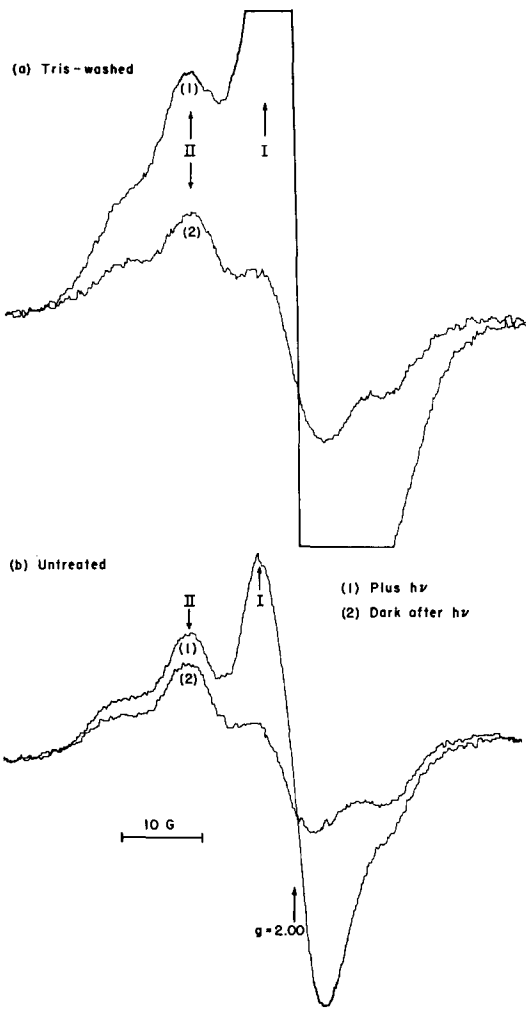

Fig. 2. EPR spectra of Signal II in Tris-washed (a) and untreated (b) chloroplasts in the light (1) and in the dark following illumination (2). The chlorophyll concentration in each sample was 3.6 mg/ml. The spectra were recorded with identical gain and modulation amplitude settings with an instrument time constant of 0.3 s and scan rate of 25 G/s.

Fig. 1c shows the kinetics of these light-induced changes in Signal II concentration. A dark-adapted sample of heated chloroplasts was monitored at the magnetic field strength labeled II in Fig. 1a. The initial low level of Signal II is rapidly increased by saturating white light and cessation of illumination results in a rapid decay to about half this value. As shown, subsequent illumination increases spin concentration to the original light-induced level and rapid decay follows again upon darkening.

Fig. 2 shows a comparison between the number of Signal II spins in Tris-washed (2a) and untreated (2b) chloroplasts. Both samples were adjusted to the same chlorophyll concentration. In the dark following illumination (Spectra 2) both samples show the same Signal II spin concentration; however, in saturating continuous light (Spectra 1) Signal II shows a 2-fold increase in the Tris-washed chloroplast sample, whereas there is only a 20 % increase in Signal II in the untreated chloroplasts. Signal I magnitude is higher in Tris-washed than in untreated chloroplasts because the normal flow of electrons from Photosystem II has been interrupted by the inhibitory treatment.

These results indicate that inhibition of oxygen evolution by Tris or heat activates a component of Signal II not normally observed in untreated chloroplasts. This newly observed free radical species exhibits rapid rise and decay kinetics in response to illumination and is present in a 1 : 1 ratio with the conventional Signal II species. The EPR spectra of these two components of Signal II are indistinguishable, as evidenced by identical field positions for the low-field peak and shoulder in the spectra shown in Figs 1a, 1b and 2a. We shall refer to the kinetically fast component of Signal II observed in treated chloroplasts as Signal IIf and to the classical component observed in both treated and untreated chloroplasts as Signal IIs. Previously

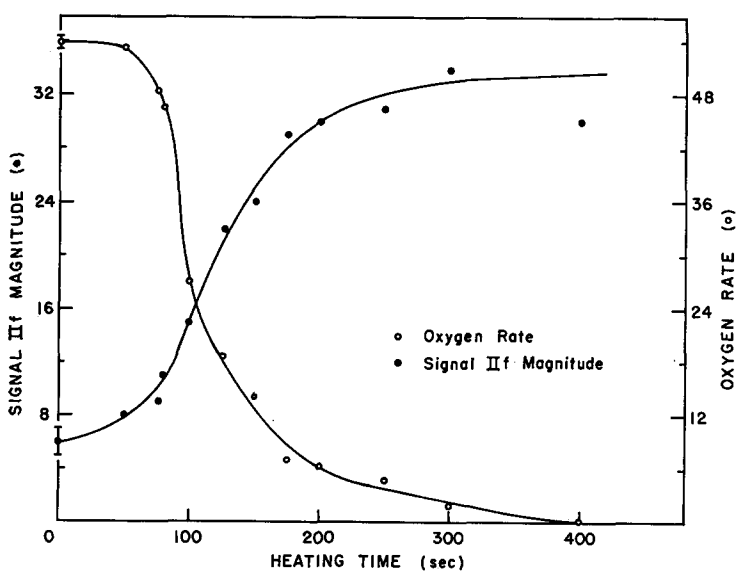

Fig. 3. Oxygen evolution rate and Signal IIf magnitude in spinach chloroplasts as a function of incubation time at 51 °C. Signal IIf magnitude was measured as the rapidly decaying component at the low-field peak of Signal II. Both Signal IIf and the oxygen rate are given in relative units. The rate of oxygen evolution for untreated chloroplasts was about 100 μm O_2 · (mg chlorophyll)$^{-1}$ · h^{-1}.

we showed that in untreated chloroplasts the ratio of Signal IIs to P700 (Signal I) was 1 : 1 [1]; therefore Signal IIf is also 1 : 1 with P700.

Inhibition of O_2 evolution and activation of Signal IIf by heat

Fig. 3 shows the effect of heating time at 50 °C on O_2 evolution and Signal IIf magnitude. Samples (0.5 ml) of untreated chloroplasts were heated for the indicated times, and following the heat treatment both oxygen evolution and Signal IIf magnitude were assayed for the same sample. The decline in O_2 evolution parallels an increase in Signal IIf magnitude; O_2 evolution is 50 % inhibited after 100 s of heat treatment while Signal IIf is 50 % activated at a heating time of 110 s. The magnitude of the small Signal IIf component observed in the unheated sample varies with the chloroplast preparation and may correspond to the fast transient in Signal II recently observed by Beinfeld [11] (see Discussion).

Effect of red vs far-red illumination on Signal IIf formation

The results of Fig. 3 suggest that the light-induced formation of Signal IIf is a System II reaction which is activated as oxygen evolution is inhibited. Fig. 4 shows

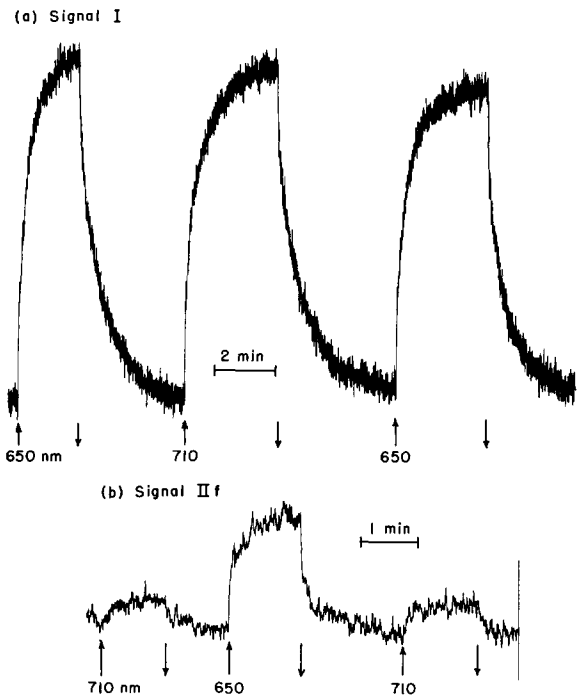

Fig. 4. Effect of red and far-red light on Signal I (a) and Signal IIf (b) formation in Tris-washed chloroplasts. The intensity for 650 nm light was 40 μW/cm^2; for 710 nm light 30 μW/cm^2. The reaction mixture contained $2 \cdot 10^{-3}$ M NADP, 60 μg ferredoxin/ml, $1 \cdot 10^{-3}$ M $K_4Fe^{II}(CN)_6$ and $1 \cdot 10^{-4}$ M DCMU for the Signal I determination. For the Signal II experiment DCMU, which inhibits Signal IIf formation in preilluminated chloroplasts, was excluded. The instrument time constant was 0.3 s. Signal IIf was monitored at 3381 G, the low-field peak of Signal IIf in Fig. 1. Signal I was monitored at 3392 G, where the Signal II derivative amplitude is zero (see Fig. 1).

the effect of red and far-red light on the formation of Signal IIf. The non-saturating intensities of 650 nm and 710 nm light were adjusted to give equal steady-state rates of P700 (Signal I) oxidation in Tris-washed chloroplasts (Fig. 4a). DCMU was added to block any residual flow of electrons from Photosystem II. The results of this experiment indicate that equal numbers of photons are being absorbed by Photosystem I for the two wavelengths. The extent of Signal IIf formation in response to these two intensities (Fig. 4b) shows that 650-nm light is 3.5 times more effective than 710-nm light and indicates that the generation of Signal IIf is a Photosystem II-mediated reaction. In these experiments the intensities of both 650- and 710-nm light were sufficiently low that both Signal I and IIf formations were linear with light intensity. Under these conditions the steady-state rate is proportional to the initial rate of formation and can be used as a measure of the initial rate. At the high chloroplast concentrations used for the EPR studies, the samples absorbed nearly all of the incident light at both wavelengths, so the steady-state yields should reflect the relative quantum efficiencies.

Other treatments which activate Signal IIf

The upper trace in Fig. 5a shows the flash-induced response of Signal IIf in guanidine-washed chloroplasts, while the lower trace shows that this response can be inhibited by DCMU if the acceptor pool on the reducing side of Photosystem II has

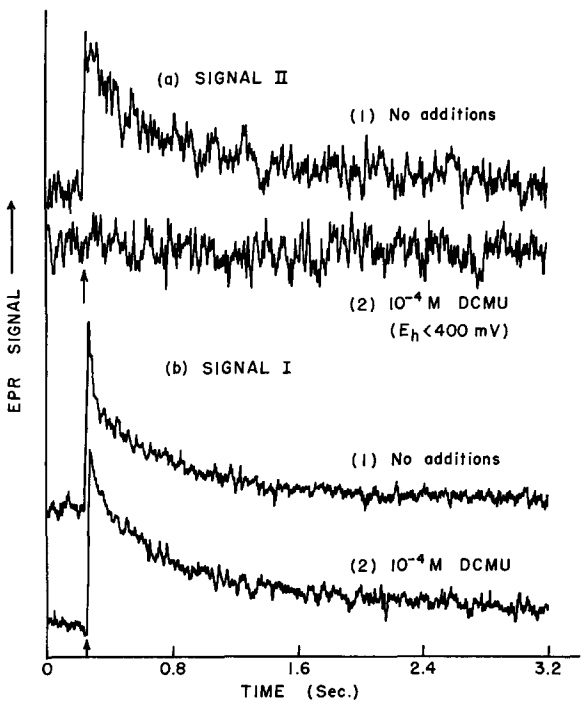

Fig. 5. Time course for the flash-induced formation of Signal IIf (a) and Signal I (b) in the absence (1) and presence (2) of $1 \cdot 10^{-4}$ M DCMU in guanidine-washed chloroplasts. The instrument time constant was 10 ms; each trace is the average of 64 scans. The arrow designates the time at which the lamp was fired in each scan. Signal IIf and Signal I monitored at field values described in Fig. 4.

been filled by preillumination prior to the light flash. Fig. 5b is a control experiment in which we monitored the flash response of Signal I in the same chloroplasts; as expected, DCMU does not inhibit Signal I formation although alterations in the decay kinetics can be observed. These experiments demonstrate that treatment with chaotropic agents, in this case guanidine, activates Signal IIf. The DCMU sensitivity shows that the light-induced transients observed in this new component are not due to Signal I. Finally we shall document in a subsequent publication that, while DCMU inhibits Signal IIf in preilluminated chloroplast suspensions of low redox poise ($E < +400$ mV), at higher potentials the inhibition by DCMU is relieved.

In addition to guanidine washing, we have also found that thiocyanate washing, aging, incubation at acid pH (pH 5 for 30 min) and hexane extraction (incubation at 0 °C for 10 min in 10 ml hexane/mg chlorophyll) also serve to activate Signal IIf to different extents. As shown previously [2], however, carbonylcyanide *m*-chlorophenylhydrazone (CCCP) treatment does not activate Signal IIf and bicarbonate depletion has also been found to be ineffectual.

Single-flash studies on the quantum efficiency of Signal IIf formation

In addition to the activation of Signal IIf reported here, Tris-washed chloroplasts have also been shown to oxidize cytochrome b_{559} [12] and carotenoids [13]. Therefore, it becomes necessary to determine whether the light-induced transients in Signal IIf proceed with high quantum efficiency or represent relatively inefficient and non-specific photoreactions mediated by Photosystem II. The results shown in Fig. 6 demonstrate that in Tris-washed chloroplasts the increase in Signal IIf spin concentration has the same magnitude whether generated by a single flash (Fig. 6a) or by saturating continuous light (Fig. 6b). Therefore, during a single 10-μsec flash full turnover in Signal IIf is observed. Fig. 7 shows a comparison of single-flash saturation curves for Signal I and Signal IIf and indicates that the intensity requirements for the

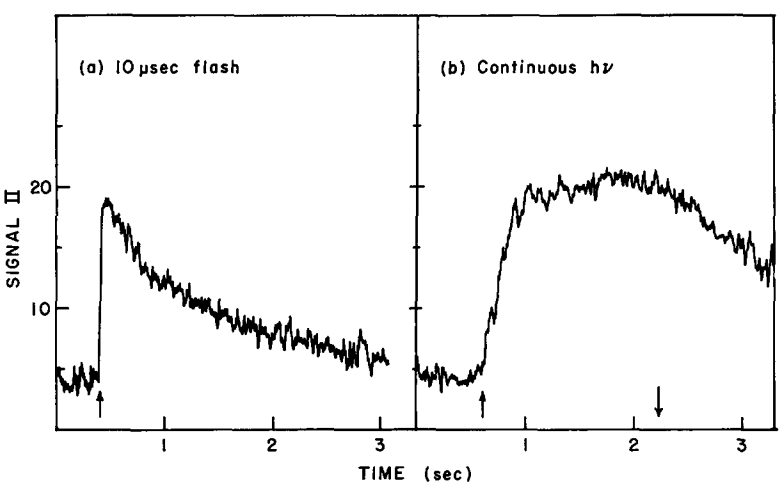

Fig. 6. Signal IIf formation in response to a single 10-μs flash (a) and saturating continuous light (b) in Tris-washed chloroplasts. The instrument time constant was 50 ms; each trace is the average of 16 scans. Signal IIf monitored at field value described in Fig. 4.

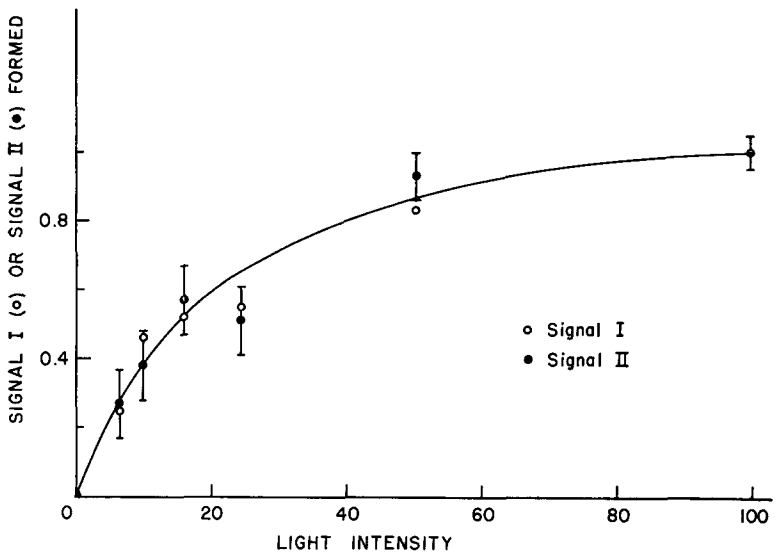

Fig. 7. Single flash saturation curves for Signal I (○) and Signal IIf (●) formation in Tris-washed chloroplasts. The chloroplast suspension contained $2 \cdot 10^{-3}$ M NADP, 60 μg ferredoxin/ml and $2 \cdot 10^{-3}$ M ascorbate. The instrument time constant was 1 ms for the Signal I determination and 10 ms for the Signal IIf determination; each experimental point was the average of 64 scans. Flash intensity was adjusted with calibrated neutral density filters. The results for both Signal I and Signal IIf have been normalized by dividing the extent of signal formation at each intensity by that formed at 100 % intensity.

generation of these two free radicals are similar in white light. Since the quantum efficiency for P700 oxidation is high [14], these two experiments allow us to conclude that, during a single flash, full turnover of Signal IIf occurs and proceeds with high quantum efficiency.

Rise and decay kinetics of Signal IIf in response to a flash

Fig. 8 shows a comparison of the rise kinetics for Signal I (Fig. 8a) in untreated chloroplasts and Signal IIf (Fig. 8b) in Tris-washed chloroplasts in response to a single 10-μs flash. The negative flash artifact spike is shown in Fig. 8c. The instrument time constant in these two experiments was 500 μs. Signal I has been shown by Warden and Bolton to be generated in less than 200 μs [15] so that in this experiment its rise is instrument limited. The rise of Signal IIf is similarly instrument limited, and we conclude that following a flash Signal IIf is fully generated within 500 μs.

As shown in Fig. 5 for guanidine-washed chloroplasts and Fig. 6 for Tris-washed chloroplasts, the decay of Signal IIf following a flash occurs in several hundred ms. Fig. 9a shows a similar experiment with heated chloroplasts; the semilogarithmic plot of these data in Fig. 9b demonstrates that the decay is first order with a halftime of 140 ms. This decay time varies both with the condition of the spinach used in the chloroplast preparation and with the specific treatment used to activate Signal IIf (Table I). Washed chloroplasts (e.g. with Tris or guanidine) have longer decay times

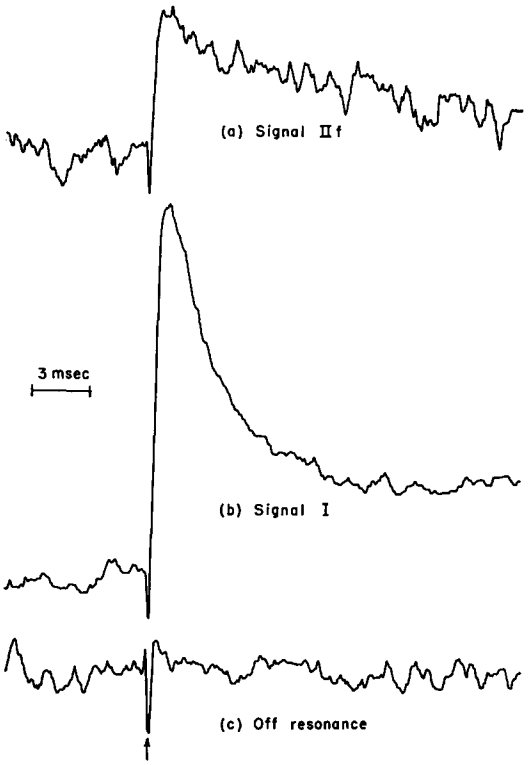

Fig. 8. Rise kinetics in response to a single 10-μs flash for Signal IIf (a) and Signal I (b) in Tris-washed chloroplasts. The instrument time constant was 500 μs. Data shown for Signal IIf are the average of 336 scans; for Signal I the average of 192 scans. Trace (c) shows the off-resonance ($H =$ 3100 G) artifact resulting from the 1 amp pulse. Reaction mixture contained $2 \cdot 10^{-3}$ M NADP, 60 μg ferredoxin/ml, $2.5 \cdot 10^{-3}$ M ascorbate and $1 \cdot 10^{-4}$ M phenylenediamine. Signal IIf and Signal I monitored at field values described in Fig. 4.

than unwashed chloroplasts (e.g. heat treated). However, the decay time for heated chloroplasts can be lengthened by washing the chloroplasts with the isolation buffer either before or after heat treatment. These data indicate that a soluble endogenous factor facilitates the decay of Signal IIf following illumination.

As shown in Table II, the addition of the oxidant, $K_3Fe^{III}(CN)_6$, slows the decay of Signal IIf in both heat-treated and Tris-washed chloroplasts while the reductants, ascorbate, phenylenediamine/ascorbate and hydroquinone/ascorbate, accelerate the disappearance of the free radical. These results indicate that the light-induced transient in Signal IIf corresponds to a Photosystem II-mediated oxidation of its precursor followed by dark re-reduction of the radical species. The acceleration of the re-reduction process by phenylenediamine/ascorbate and hydroquinone/ascorbate, which restore Photosystem II mediated electron flow to NADP in treated chloroplasts [3, 4], suggests that Signal IIf is involved in the transfer of electrons from these exogenous reductants to the Photosystem II reaction center. A detailed study of this process will be presented in a subsequent publication.

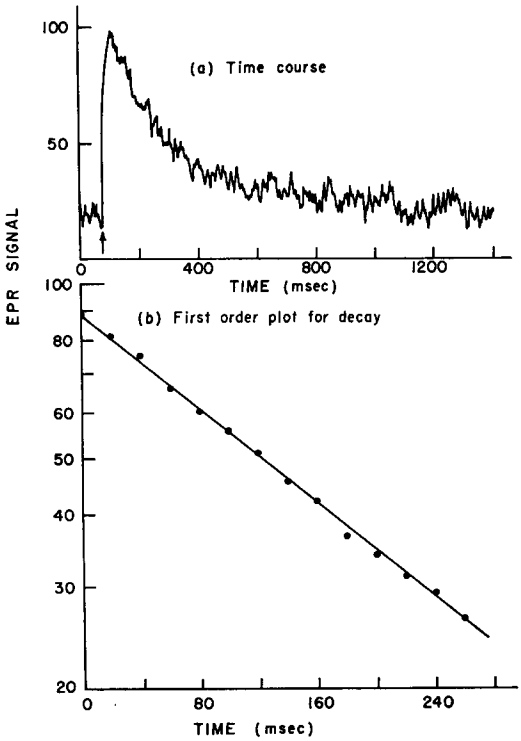

Fig. 9. (a) Time course for flash-induced transient in Signal IIf in heated (51 °C, 150 s) chloroplasts. The instrument time constant was 10 ms; data shown are the average of 48 scans. (b) First-order plot for the decay of this transient.

TABLE I

SIGNAL IIf DECAY TIME

The time course for the flash-induced transient in Signal IIf was monitored at the magnetic field point labeled II in Fig. 1a for the chloroplast samples below. The instrument time constant was 10 ms, and for each determination 30 to 100 scans were averaged. Each sample contained $2 \cdot 10^{-3}$ M $NADP^+$ and 60 μg ferredoxin/ml. The time observed for the ESR signal to decay to 1/2 its flash-induced maximal is tabulated as $t_{\frac{1}{2}}$ decay.

Chloroplast sample	$t_{\frac{1}{2}}$ decay (ms)
Guanidine-washed	400
Tris-washed	490
Heated (Prepn 1)	140
Heated (Prepn 2)	360
Isolation buffer-washed, heated (Prepn 2)	610
Heated (Prepn 3)	250
Isolation buffer-washed, heated (Prepn 3)	480
Heated, isolation buffer-washed (Prepn 3)	700

TABLE II

EFFECTS OF REDOX AGENTS ON SIGNAL IIf DECAY TIME

The time course for decay of the flash induced transient in Signal IIf was monitored as described in Table I. Chloroplast samples contained $2 \cdot 10^{-3}$ M $NADP^+$ and 60 μg ferredoxin/ml plus redox agents in the concentrations indicated. The instrument time constant was 10 ms in experiments in which $t_{\frac{1}{2}}$ was greater than 100 ms and 5 ms for $t_{\frac{1}{2}}$ less than 100 ms.

Chloroplasts	Additions	$t_{\frac{1}{2}}$ decay (ms)
Heated	—	360
Heated	14 mM $K_3Fe^{III}(CN)_6$	610
Heated	0.043 mM phenylenediamine, 1.2 mM ascorbate	30
Tris-washed	—	800
Tris-washed	20 mM $K_3Fe^{III}(CN)_6$	1150
Tris-washed	10 mM ascorbate	250
Tris-washed	0.04 mM phenylenediamine, 1.2 mM ascorbate	40
Tris-washed	0.04 mM hydroquinone, 1.2 mM ascorbate	60

DISCUSSION

The results presented above demonstrate that dramatic alterations in the properties of Signal II result from a number of treatments which have been commonly used to inhibit oxygen evolution. Fig. 10 shows the model which we propose to explain these results. P680 is the reaction center chlorophyll for Photosystem II [16], Z is a donor to P680 which serves as the branch point between water oxidation and Signal IIf generation. In untreated, broken chloroplasts, as normally isolated, the Signal IIf pathway is deactivated; and the major source of electrons to oxidized P680 is from water. Under the treatments described above, however, Signal IIf is activated and electron flow through this component to $P680^+$ is observed. This switching mechanism is best demonstrated by the results in Fig. 3 which show the parallel activation of Signal IIf and deactivation of oxygen evolution with heating time. We have also performed experiments with Tris-washed chloroplasts reactivated according to Yamashita et al. [17]. In these preparations oxygen evolution is restored, and a corresponding decline in the level of Signal IIf is observed [18].

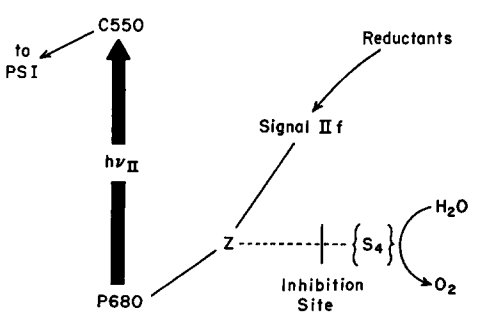

Fig. 10. Model for Signal IIf generation in chloroplasts inhibited on the water side of Photosystem II. Details described in text.

As we have shown, a variety of treatments activate Signal IIf. Of these, Tris washing and heat treatment have been the best characterized. They are similar in that both result in chloroplasts which show low fluorescence in the light, have decreased rates of oxygen evolution, and show much higher concentrations of EPR-detectable Mn^{2+} than do untreated chloroplasts [3–5]. In the presence of an exogenous electron donor, DCMU-sensitive NADP reduction is partially restored (up to 60 %), fluorescence increases are observed upon illumination, and phosphorylation associated with both coupling sites is observed [3, 19–21]. The results shown in Table II indicate that Signal IIf is integral to this process, since it is on the pathway between the site of exogenous electron-donor oxidation and P680.

It appears that the deactivated state of Signal IIf in untreated broken chloroplasts may result from the chloroplast-preparation procedure. Recently Warden and Bolton [22], using intact chloroplasts prepared as described by Jensen and Bassham [23], have described a Signal II component similar to the Signal IIf that we observe in treated broken chloroplasts. The rise time of this component in intact chloroplasts is less than 1 ms, with a decay on the order of 5–10 s. The signal is roughly stoichiometric with Signal I and disappears upon breakage of the intact chloroplasts. These results suggest that a soluble component activates Signal IIf in intact chloroplasts and that this factor is lost upon rupture, resulting in deactivation. In this model the fast transient in Signal II in broken chloroplasts recently reported by Beinfeld [11] and the slight Signal IIf component observed in Fig. 3 in the unheated sample would correspond to a fraction of the Signal IIf species which survives the chloroplast preparation procedure in the activated state. The treatments we have described above indicate that there are alternative mechanisms by which Signal IIf may be activated. These possibilities are currently being explored in our laboratory.

ADDENDUM

After this manuscript was submitted, a description of a similar correlation between loss of oxygen evolution and increase of Signal II was reported [24] for chloroplasts depleted of Mn^{2+} by exchange with Mg^{2+}. On the basis of the amplitude, spectrum and kinetics of the signals, we believe that their observations and ours probably correspond to the same entity.

ACKNOWLEDGEMENTS

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