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The flash number dependence of EPR Signal II decay as a probe for charge accumulation in Photosystem II

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The decay kinetics of EPR Signal II has been used to monitor accumulation of positive charge in dark-adapted O_2-evolving and inhibited PS II preparations from spinach. In fully active samples the Signal II transients following four saturating flashes exhibit periodic oscillations in amplitude and decay time. These oscillations result because electron transfer from the O_2-evolving complex to Z^+, the species giving rise to Signal II, becomes slower in the higher S-states. A small signal is induced following the first flash because of rapid rereduction of Z^+ in the S_1 state, but transients are resolved following the second and third flash, corresponding primarily to the S_2 and S_3 states, respectively, with half-times of 0.6 and 1.3 ms. The oscillatory pattern and decay times are similar to those previously reported in whole chloroplasts (Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289). The period-four behavior is confirmed by extension of our observations to eight flashes. Upon partial inhibition of O_2-evolution by NaCl washing, which extracts the 16 and 24 kDa peptides, or upon incubation at pH 7.75, a large transient is observed following the first flash, and subsequent flashes induce signals of similar amplitude and decay time. Extraction of the 16, 24 and 33 kDa peptides by $CaCl_2$ washing also eliminates the oscillations and markedly slows the decay kinetics. The absence of period-four oscillations in these inhibited samples indicates that turnover of the O_2-evolving complex is blocked. Furthermore, the large signals induced following the first flash indicate slow rereduction of Z^+ in the lower S-states, suggesting that even partial advancement is inhibited. Addition of Ca^{2+} to NaCl-washed preparations restored the period-four oscillations. Thus, the 16 and 24 kDa peptides are not necessary for turnover of the O_2-evolving complex, but in their absence Ca^{2+} is required for electron transfer from the O_2-evolving complex to Z.

Introduction

The O_2-evolving complex of Photosystem II catalyzes the four-electron photooxidation of water to oxygen. Kok and co-workers proposed a model in which Photosystem II advances during photochemical turnover through five successive oxidation states S_i ($i = 0$ to 4), the state S_4 spontaneously decaying to produce oxygen and S_0 [1,2]. The S_2 and S_3 states decay to S_1, producing a

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; DCBQ, 2,5-dichlorobenzoquinone; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PS, Photosystem.

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distribution of 75% S_1 and 25% S_0 within minutes following dark adaptation. Manganese is an essential cofactor for O_2-evolution and is thought to be associated with the accumulation of oxidizing equivalents [3,4]. Recently, spectroscopic techniques have directly probed the chemical nature of the S-state intermediates. A low-temperature multiline EPR signal, assigned to a mixed-valence tetrameric Mn complex [5,6], is associated with the S_2 state [5,7]. EXAFS studies of S_1 are consistent with a dimeric bridged manganese structure [8]. The $S_1 \rightarrow S_2$ transition is accompanied by a shift in the manganese X-ray absorption edge to higher energy, indicative of an increase in oxidation state [9]. Flash-induced absorption changes in the ultraviolet [10] and infrared [11] have also been assigned to manganese oxidation state changes. The path of electron transfer from the O_2-evolving complex to the photochemical reaction center is not well understood, but it is thought that intermediate electron carrier(s) are involved [12]. Reduction of the reaction center, denoted P-680, proceeds with several microsecond and millisecond phases which are dependent on the S-state [13,14]. A semiplastoquinone cation species, Z^+, which gives rise to EPR signal II, is directly oxidized by P-680 [15]. The rereduction kinetics of Z^+ also varies with flash number, suggesting that it functions as an intermediate electron carrier between the O_2-evolving complex and P-680 [16]. Slower electron transfer from the higher S-states is thought to reflect accumulation of net positive charge at the O_2-evolving complex. The species D (which gives rise to EPR signal II_{slow}) and cytochrome *b*-559 are associated with PS II, but their specific functions have not yet been assigned.

Our understanding of the donor side of PS II has been aided by the development of various procedures which selectively inhibit O_2-evolution, and in some cases a specific electron transfer step may be assigned as the point of inhibition. Selective release of 16 kDa and 24 kDa extrinsic polypeptides by NaCl washing partially inhibits O_2-evolution, and full activity can be restored by rebinding of the 24 kDa peptide [17,18] or addition of nonphysiological concentrations of Ca^{2+} [19,20]. Ca^{2+} is a cofactor for O_2-evolution [21], and the 24 kDa peptide functions to promote high-affinity binding of Ca^{2+} to a site on the PS II membrane [22,23]. The 16 kDa peptide is not necessary for reconstitution if at least 3 mM Cl^- is present [24].

The mechanism by which O_2-evolution is inhibited in the absence of bound Ca^{2+} and the 24 kDa peptide is unclear. Measurements of fluorescence induction [17], P^+-680 reduction [25] and P^+-680 Q^- recombination [26] all indicate that the site of inhibition is on the donor side of PS II. Åkerlund et al. [26] also observed that O_2-evolution yields under continuous and flash illumination are inhibited to the same extent and concluded that the inhibition is an all-or-none phenomenon. In contrast, Ono and Inoue [27] did not observe any inhibition under flash illumination without addition of EDTA; presumably, loosely bound Ca^{2+} was removed during sample preparation in the former but not the later study. Two groups reported that the residual O_2-evolution exhibits period-four oscillations similar to those of intact PS II preparations [23,27,28], suggesting that remaining active centers are unaffected by the NaCl treatment. In contrast, Radmer et al. [29] observed a 2-fold increase in the miss parameter, α, and changes on the acceptor side. Similar results were obtained with luminescence and ultraviolet absorption measurements [30]. These changes are not reversed upon binding of the 24 kDa peptide or addition of Ca^{2+}, and their origin is not well understood. These workers also report reversible 'disconnection' of the O_2-evolving complex from P-680 in 25% of the PS II centers following NaCl extraction [29–31]. The electron-transfer step at which O_2-evolution is blocked in the absence of the 24 kDa peptide or added Ca^{2+} also remains controversial. Several groups have reported a substantial decrease in amplitude of the S_2 multiline EPR signal [32–34]. However, De Paula et al. [35] recently reported that most of the multiline signal amplitude is retained in the absence of the 24 kDa peptide, but addition of EGTA reduces the amplitude to the level observed in the earlier studies. Thus, Ca^{2+} is required for generation of the multiline EPR signal, and the discrepancy reflects depletion of bound Ca^{2+} in the earlier studies. In the absence of bound Ca^{2+} a steady-state light-induced increase in EPR Signal II is observed [35,36], suggesting that the blockage occurs between the O_2-evolving complex

and Z. Thermoluminescence oscillations (B-band) were recently modeled on the basis of two modes of inhibition: some of the inactive centers are capable of partial S-state advancement while others are blocked prior to S_2 [27]. Luminescence measurements locate the block at the $S_3 \rightarrow S_0$ transition [28]. Under repetitive flash conditions, dramatically slower rereduction of Z^+ was reported, and addition of Ca^{2+} partially reversed this effect [30,37]. However, more recent studies report that Z^+ rereduction is largely unchanged upon NaCl washing, even in the absence of added Ca^{2+}, possibly indicating the presence of an intermediate donor prior to Z [34,36]. Addition of EGTA does not slow the decay kinetics in these preparations (Cole, J. and Sauer, K., unpublished observations).

Extraction of the 16 kDa, 24 kDa and 33 kDa peptides by $CaCl_2$ washing inhibits O_2 evolution without concomitant release of Mn [38]. Upon incubation at low ionic strength, half of the Mn is slowly released [39], but all four Mn are retained in the presence of 200 mM Cl^- [40] or 50 mM $CaCl_2$ [39]. Significant restoration of O_2-evolution activity occurs on rebinding of the 33 kDa peptide [41–43] or with 200 mM Cl^- [40,43], albeit with a greatly increased miss parameter, α, in the latter case [27]. In the absence of the 33 kDa peptide or high Cl^-, the loss of the S_2 EPR signal [32,33] and the marked retardation of Z^+ reduction [34,36] imply that electron transfer from the O_2-evolving complex to Z is blocked. In contrast, oscillations in the B-band thermoluminescence are unchanged through the second flash, suggesting that the block occurs at the $S_3 \rightarrow S_4$ transition [44].

The irreversible inhibition of O_2-evolution that occurs at pH $\geqslant$ 8.0 may be assigned to the release of the three extrinsic peptides and Mn [45], while a reversible mode of inactivation between pH 7.0 and 8.0 is not mediated by release of these components [46]. The decrease of the S_2 multiline signal without concomitant effect on Signal II decay kinetics implies that the reversible alkaline inhibition blocks electron transfer at the same point as does NaCl washing [46]. In contrast, only a 20% inhibition of the amplitude of the ultraviolet absorption changes assigned to Mn is observed at pH 8.3, and S_0 becomes the predominant stable state in the dark [47]. This discrepancy may reflect different conditions for incubation at alkaline pH.

In this study, we have measured the millisecond decay kinetics of Z^+ in dark-adapted preparations subjected to a series of flashes. In chloroplasts, reduction of Z^+ was previously observed to be faster than the 100 μs instrument response for the states S_0 and S_1, but decay phases of 0.4 and 1 ms were reported for S_2 and S_3, respectively [16]. Thus, the transient response of Z^+ following a series of saturating flashes is a direct probe for charge accumulation at the O_2-evolving complex. We observe that the flash-number-dependence of Z^+ reduction in PS II preparations is quite similar to that reported in chloroplasts. In contrast, following inhibition of O_2-evolution by NaCl or $CaCl_2$ washing or incubation at pH 7.75 we observe significant millisecond decay components following the first flash, suggesting that electron transfer from the lower S-states is blocked or greatly slowed. In addition, the normal period-four flash number dependence of the decay amplitudes and half-times following subsequent flashes is abolished in the inhibited samples, indicating that accumulation of oxidizing equivalents at the O_2-evolving complex is blocked or greatly altered. Based on these observations, we conclude that these inhibited preparations are incapable of even partial S-state advancement. Restoration of period-four oscillations in the NaCl-washed samples by added Ca^{2+} implies that the 24 and 16 kDa peptides are not necessary for turnover of the O_2-evolving complex, but that Ca^{2+} is required for electron transfer between the O_2-evolving complex and Z.

Materials and Methods

O_2-evolving PS II preparations free of PS I were obtained by Triton X-100 extraction of spinach chloroplasts. Destemmed leaves were disrupted in a buffer containing 0.4 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$ and 20 mM Hepes at pH 7.5. The homogenate was strained through eight layers of cheesecloth and centrifuged at 6000 $\times g$ for 10 min. The pellets were resuspended in a washing buffer containing 150 mM NaCl, 4 mM $MgCl_2$, 5 mM EDTA and 20 mM Hepes at pH 7.5. The suspension of broken chloroplasts was centrifuged as above, and the resulting pellets

were resuspended in the extraction buffer containing 15 mM NaCl, 5 mM $MgCl_2$ and 50 mM Mes at pH 6.0. Dense debris was removed by centrifugation at $400 \times g$ for 5 s, and the chloroplasts were recovered from the supernatant by centrifugation at $6000 \times g$ for 10 min. Triton X-100 extraction and salt treatment was performed under ambient light as previously described [32]. Treatment with 0.8 M NaCl extracts about 80% of the 16 and 24 kDa peptides without release of the 33 kDa peptide, while 0.8 M $CaCl_2$ extracts at least 95% of all three peptides [36]. Control and salt-treated preparations were resuspended in assay buffer containing 0.4 M sucrose, 15 mM NaCl and 50 mM Mes at pH 6.0, centrifuged at $34000 \times g$ for 10 min and resuspended in the same buffer. Reversible, alkaline pH treatment was performed by incubating samples in a buffer containing 0.4 M sucrose, 15 mM NaCl, 50 mM Hepes at pH 7.75 for 1 h under ambient light followed by centrifugation and resuspension at pH 7.75. Steady-state O_2-evolution rates were measured in assay buffers as described previously [32]. Typically, control rates of 300–500 μmol O_2/mg Chl per h were obtained. Immediately before EPR measurements, 1 mM ferricyanide, 1 mM ferrocyanide and 0.5 mM DCBQ were added to the sample as a redox buffer and acceptor system. In all samples, the final chlorophyll concentration was adjusted to 2.5 mg/ml.

Signal II kinetics was measured with a Varian E109 spectrometer at $g = 2.010$ with no filter on the output (half-time of instrument rise, 0.2 ms). Saturating flashes (15 mJ/pulse) of 0.5 μs duration were provided by a Phase R DL-1400 dye laser, with Rhodamine 640 (Exciton) as the dye. The sample was dark adapted in a reservoir on ice for at least 5 min prior to data collection. Fresh sample was pumped through a light-tight flow system into the EPR flat cell prior to each train of flashes. The laser, pump, and data acquisition were synchronized by a laboratory-designed timing module. Typically, 1000–2000 flash sequences were averaged for each experiment, and two or three experiments were summed for each kinetic trace. There were no systematic changes in the kinetic traces or loss of O_2-evolution activity during the course of signal averaging. In particular, there was no evidence of the flash-induced release of loosely bound Ca^{2+} in the NaCl-washed preparations reported in Ref. 30; our preparations were presumably already in the Ca^{2+}-depleted state (vide infra).

The data were analyzed using a nonlinear least-squares fitting program. Transients were fit to the sum of a rising component (determined by the instrument time response) and one or two decaying components. The amplitudes of decay components with half-times shorter than about 1.5 ms were not uniquely determined using this procedure; presumably, the decay components become convoluted with the instrument rise. In this case, the fast decay amplitude was estimated by taking the difference between the initial signal maximum and the amplitude of the slow component.

Results

The sequential pattern of Signal II millisecond decay transients in O_2-evolving and inhibited PS II preparations following four saturating laser flashes is shown in Fig. 1. In the control (active) preparation, the overall signal amplitude and decay kinetics change as a function of flash number, with the maximal signal amplitude occurring on the third flash. Because subsaturating microwave power (5 mW) was used in this study, the changes in amplitude are not a result of differences in the relaxation properties of Z^+ in various S-states. The decay traces following each flash were fit to the sum of two components as described in Materials and Methods, and the results are summarized in Table I. Following the first flash, a small transient signal is observed. A minor component of about 0.5 ms half-time is observed, with most of the amplitude contained in a 120 ms component. A component of similar amplitude to the 120 ms phase but with about a 50 ms half-time is present following subsequent flashes. Because this component does not exhibit period four oscillations, it does not appear to be connected to the O_2-evolving complex and may originate from PS II centers damaged during sample preparation. The slight offset in the baseline prior to the second flash represents signal amplitude which does not decay in the 0.5 s between the flashes. The decay kinetics following the second flash is similar to that following the first, except that the 0.5–0.7

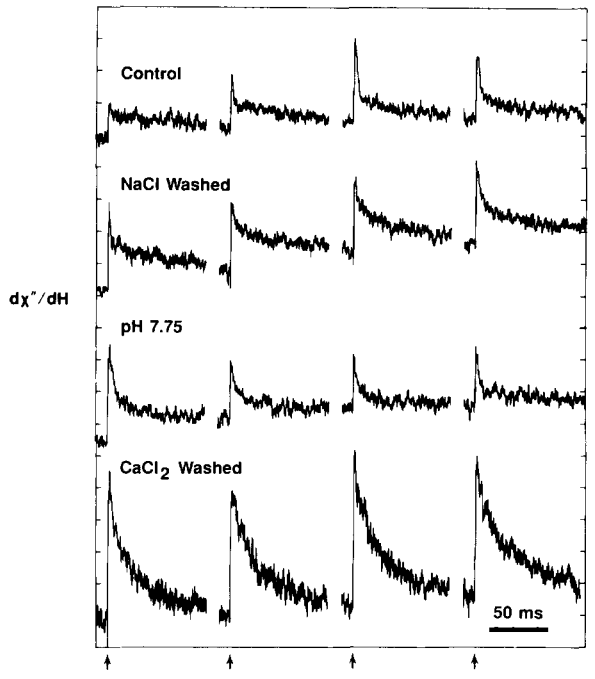

Fig. 1. Transient decay kinetics of Signal II in dark-adapted O_2-evolving and inhibited PS II preparations. Four saturating flashes were given with 0.5 s between flashes. From top to bottom: control, NaCl-washed, pH-7.75-incubated and $CaCl_2$-washed. All preparations except the pH 7.75 samples were assayed in a medium containing 0.4 M sucrose, 15 mM NaCl, 50 mM Mes, 1 mM ferricyanide, 1 mM ferrocyanide and 0.5 mM DCBQ at pH 6.0. The pH 7.75 preparations were assayed in the same medium as above, except that the buffer was 50 mM Hepes and the pH was 7.75. Instrument conditions: microwave power, 5 mW; modulation amplitude, 5 G; modulation frequency, 100 kHz. The recorder was DC-coupled, so that baseline offsets represent signal that does not decay between flashes. Each trace represents 1400–4400 events. Data were averaged from two or three separate experiments, so that each preparation was subjected to fewer than 1800 flash trains.

TABLE I

THE FLASH NUMBER DEPENDENCE OF SIGNAL II DECAY KINETICS

The transient decay kinetics of Signal II in dark-adapted O_2-evolving (control) and inhibited (NaCl-washed, pH-7.75-incubated and $CaCl_2$-washed) PS II samples. Sample preparation and experimental conditions are described in Materials and Methods and the caption to Fig. 1. Amplitudes (A) and half-times (t) were obtained by fitting the kinetic traces in Fig. 1 to the sum of a rise component and fast (f) and slow (s) decay components as described in Materials and Methods. Amplitudes are normalized such that the average total signal amplitude of the $CaCl_2$-washed sample equals 1.0. Estimated uncertainty in amplitudes and half-times, ±20%.

Sample	Flash	A_f	t_f (ms)	A_s	t_s (ms)
Control	1	< 0.10	–	0.15	120
	2	0.20 [a]	0.6	0.16	70
	3	0.40 [a]	1.3	0.14	50
	4	0.27 [a]	1.4	0.14	50
NaCl	1	0.27 [a]	0.9	0.30	100
	2	0.30	2.4	0.26	130
	3	0.27	4.3	0.22	80
	4	0.37	2.9	0.22	90
pH 7.75	1	0.51	3.9	0.21	230
	2	0.28	4.3	0.11	140
	3	0.34	3.0	0.08	110
	4	0.27 [a]	1.3	0.11	70
$CaCl_2$	1	0.54	4.2	0.49	30
	2	0.46	6.6	0.47	30
	3	0.75	10.5	0.22	80
	4	0.43	3.1	0.64	40

[a] Amplitude was estimated as described in Materials and Methods.

ms component amplitude is increased to about half of the total signal. The large transient following the third flash is dominated by a 1.3 ms component which represents about 75% of the observed decay amplitude. The amplitude of this component is decreased to about 65% of the total signal following the fourth flash. Fig. 2 demonstrates that the period-four oscillations are observable through a series of eight flashes, albeit with significant damping.

We have observed that preparations depleted of the 16 and 24 kDa proteins by NaCl washing retain 30–40% of the steady-state O_2-evolution of the control preparations when assayed at 15 mM Cl^- in the absence of Ca^{2+} [32]. Fig. 1 shows the flash-induced Signal II decay transients in NaCl-washed samples. In contrast to the intact preparation, a large signal is observed following the first flash, and subsequent flashes induce signals of similar amplitude to the first. The decay following the first flash is biphasic with 1 ms and 100 ms components contributing about equal amplitude. On all subsequent flashes, 2–4 ms and 100 ms components are observed which are again of approximately equal amplitude. We have previously observed similar millisecond kinetics in NaCl-washed samples assayed under repetitive flash

conditions [36]. The total signal intensity is about equal to that observed in the intact preparation following the third flash. It should be noted that the signal does not decay to the baseline in the 0.5 s between flashes, and the baseline shift following four flashes is about 3-times that observed in the O_2-evolving sample.

As was noted previously [19,20], addition of 5 mM Ca^{2+} to NaCl-washed preparations restores steady-state O_2-evolution to 70–90% of the activity of the unextracted preparations (data not shown). Fig. 3 shows that the period-four oscillations of the Signal II decay kinetics are also restored. However, a 3–4 ms decay component is observed following the third flash, whereas a 1.3 ms phase is present in the control sample. In addition, Ca^{2+} does not eliminate the large baseline shifts observed in the NaCl-washed samples.

Incubation of samples at pH 7.75, which reversibly decreases the steady-state O_2-evolution to 30% of the rate at pH 6.0, also eliminates the periodicity of the Signal II decay kinetics (Fig. 1). The largest transient is observed following the first flash. The decay fits to a sum of components with half-times of 3.8 ms and several hundred ms, with the latter contributing about 30% of the amplitude. Subsequent flashes induce similar decay transients, except that the overall amplitude is decreased to about 50–60% of that observed following the first flash, and the half-time of the slower phase decreases with each flash. In addition, a large baseline shift is induced primarily following the first flash, with much smaller shifts occurring on subsequent flashes. The baseline shift induced by four flashes at pH 7.75 is double that at pH 6.0. To determine if the large transient signal and baseline shift occurring on the first flash exhibits period-four behavior, we examined the flash transients at pH 7.75 for a train of eight flashes (Fig. 2). There is no corresponding change after the fifth flash, suggesting that this phenomenon occurs only after dark adaptation, and is not related to the period-four oscillations observed at pH 6.0.

Extraction of the 33, 24, and 16 kDa peptides by $CaCl_2$-washing, which inactivates O_2-evolution activity, eliminates the variation of Z^+ decay transients with flash number (Fig. 1). Additionally, the average signal amplitude is increased to about twice that observed in the other preparations. While the decay transients are not obviously biphasic and could be fit to a single decay component of about 10–15 ms half-time, a significant decrease in the residuals was observed with a two-component fit. In this case, each trace was fit to the sum of 3–10 ms and 30–80 ms components, with a somewhat slower decay observed following the third flash.

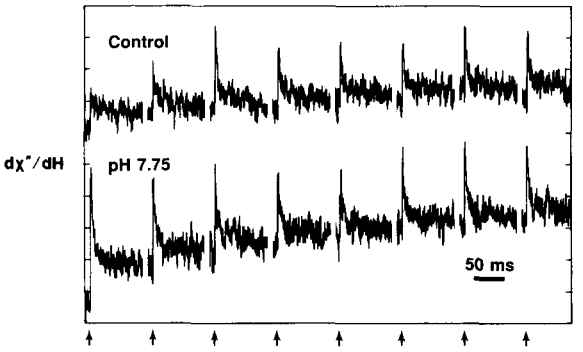

Fig. 2. Transient decay kinetics of Signal II in dark-adapted control O_2-evolving (pH 6.0) and inhibited (pH 7.75) PS II preparations. Eight saturating flashes were given at a frequency of 2 Hz. Experimental conditions were the same as in Fig. 1. Each trace represent the average of 1600 (pH 6.0) or 1300 (pH 7.75) events.

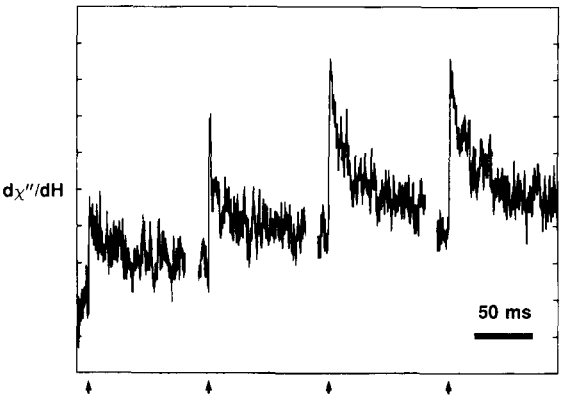

Fig. 3. Transient decay kinetics of Signal II in a dark-adapted NaCl-washed preparation in the presence of 5 mM Ca^{2+}. Experimental conditions were the same as in Fig. 1 except that the medium contained 0.4 M sucrose, 10 mM NaCl, 5 mM $CaCl_2$, 50 mM Mes, 1 mM ferricyanide, 1 mM ferrocyanide and 0.5 mM DCBQ at pH 6.0. The trace represents the average of 1700 events.

Discussion

The O_2-evolving PS II preparation utilized in this study exhibits a flash-number dependence of the half-times and amplitudes of Z^+ decay transients similar to that previously observed in chloroplasts. Low signal amplitude is observed following the first flash because reduction of Z in S_0 and S_1 is faster than the instrument response time. The half-times for the fast decay components following the second and third flash (0.6 ms and 1.3 ms, respectively) agree well with those reported previously in chloroplasts (0.4 ms and 1 ms) [16], indicating that electron transfer from the O_2-evolving complex to Z^+ is unaltered by the detergent extraction. In contrast, the amplitude of the fourth transient relative to the third is greater in the PS II preparation than in the chloroplast study. Since the signal amplitude due to the fourth flash arises from centers residing in S_2 or S_3, this indicates a greater extent of damping. Assuming a dark distribution of 75% S_1, 25% S_0, and no double hits, the present data are consistent with about 15–20% miss parameter for each flash. About a 10% miss parameter has been observed in flash O_2 yield [28] and ultraviolet absorption [30] measurements with similar PS II preparations. The greater dephasing observed in the present study is probably related to the experimental difficulties associated with generating saturating flashes in the samples at the high optical absorbance used for EPR measurements.

The period-four oscillation in the observed amplitudes and half-times of Z^+ reduction reflects successively slower electron transfer from the higher S-states, presumably due to accumulation of net positive charge at the O_2-evolving complex. The half-times of the fast decay phase following the second and third flash agree well with turnover times for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_0$ transitions, respectively, as determined by ultraviolet absorption changes [48]. However, these data do not necessarily support a model in which the O_2-evolving complex donates directly to Z^+. In the absence of significant back-reaction, such a model predicts monophasic decay kinetics of Z^+ which match the rate of electron donation from the O_2-evolving complex. However, the largest Z^+ transient, which is observed following the third flash, is only 50% of the amplitude observed following $CaCl_2$ washing, possibly indicating the presence of additional sub-millisecond decay components not resolved with the present detection system. Under repetitive flash conditions, which result in a scrambled S-state population, a 140 μs decay component, corresponding to about half of the total amplitude, is observed in intact samples [36]. $CaCl_2$ washing eliminates this fast component, resulting in a corresponding 2-fold increase in the amplitude of the millisecond phases. Multiphasic Z^+ decay kinetics which exhibit period-four oscillations are predicted by a model in which we proposed that an intermediate donor Y is present between the O_2-evolving complex and Z [36]. This donor equilibrates with Z^+ with an apparent equilibrium constant close to unity, resulting in biphasic Z^+ decay kinetics following each flash. Below are schematic representations of the direct donation model (1) and the model which includes an intermediate donor Y (2):

$$S_{0-3} \rightarrow Z \rightarrow \text{P-680} \quad (1)$$

$$S_{0-3} \rightarrow Y \rightleftharpoons Z \rightarrow \text{P-680} \quad (2)$$

Investigation of the flash-number dependence of Signal II reduction with microsecond time resolution will allow quantitative evaluation of these models.

The loss of the period-four oscillations following NaCl extraction or incubation at pH 7.75 indicates that turnover of the O_2-evolving complex is blocked. Furthermore, there is no evidence for partial advancement of the lower S-states, since the first flash induces about the same transient Signal II amplitude as subsequent flashes. While for NaCl-washed samples this first-flash decay is faster than on subsequent flashes, it is still much slower than expected for donation from the lower S-states. In addition, at pH 7.75 the decay transient observed following the first flash is larger than on subsequent flashes. The origin of these first flash effects is not known, but they apparently result from relatively slow changes in the state of PS II which occur during dark adaptation. Since no systematic change in the decay traces occurs during the course of signal averaging, the loss of the periodic oscillations is not caused by light-induced changes in the preparation. An alter-

native explanation for the absence of period-four changes in the Z^+ transients in these inhibited samples is that the O_2-evolving complex still cycles through the various oxidation states but with altered kinetics, such that the 3–4-fold retardation of electron transfer kinetics from successively higher S-states no longer occurs. Such a scheme predicts that flash O_2 yields would be unaltered in these inhibited preparations, which is not true for the NaCl-washed preparations [26,27]. A study of the flash-number-dependence of Signal II decay in NaCl-washed preparations by Ghanotakis et al. [37] found no Signal II induced by the first flash but substantial signal on subsequent flashes, which would indicate that the lower S-states are capable of electron donation. The origin of the discrepancy with the present results is unclear. Because we observe much slower Z^+ reduction following the first flash in the inhibited samples than in intact samples, our study suggests that the loss of the light-induced S_2 multiline EPR signal in the absence of bound Ca^{2+} [32–35] or upon alkaline pH incubation [46] occurs because formation of the S_2 state is blocked, not because of an alteration of the Mn environment which eliminates the EPR signal but does not block advancement. However, the identity of the reductant to Z^+ remains unclear. Because under repetitive flash conditions the 140 μs decay component is unchanged upon monovalent salt washing, we suggested that the intermediate donor Y is present and is rereduced by an exogenous or endogenous donor in the 500 ms between flashes. In the present study, the amplitude of the millisecond decay phases represents only about half that observed following $CaCl_2$ extraction, possibly indicating a sub-millisecond decay component corresponding to the 140 μs phase under repetitive flash conditions.

Restoration of the period four oscillations in NaCl-washed preparations by Ca^{2+} provides evidence that the 24 and 16 kDa peptides are not necessary for turnover of the O_2-evolving complex. We propose that Ca^{2+} facilitates electron transfer from the O_2-evolving complex to Z, as has been previously suggested [30,31,35]. However, the $S_3 \rightarrow S_0$ kinetics, as measured by the Z^+ decay following the third flash or by ultraviolet absorption changes [30], is irreversibly slowed to 3–5 ms by NaCl washing. The origin of this effect is unknown.

In addition to the loss of the period-four oscillations in Z^+ reduction kinetics, simultaneous extraction of the 33, 24, and 16 kDa peptides by $CaCl_2$ greatly slows the reduction of Z^+, as manifested by a 2-fold increase in the amplitude of the millisecond decay phases. Thus, the physiological donor to Z is not present upon removal of the 33 kDa peptide, and the reduction of Z^+ may proceed via back reaction with the reduced acceptor or donation by an exogenous species. The thermoluminescence results [44] may be accommodated within this model only if it is assumed that the two positive charges stored on the oxidizing side of PS II do not reside on the Mn O_2-evolving complex but are stored on other donors.

The large flash-induced baseline shifts observed in the NaCl washed and alkaline pH preparations represent formation of an EPR signal with significant intensity at $g = 2.010$ which does not decay in the 0.5 s between flashes, but which relaxes on extensive dark adaptation. The origin of this signal and its relation to other PS II components remains unclear. We are currently attempting to assign this signal by characterizing the lineshape and kinetics of its formation and decay.

The results presented here demonstrate that the flash-number dependence of the reduction kinetics of Z^+ is a sensitive and direct probe of charge accumulation associated with turnover of the O_2-evolving complex. The decay transients observed with the intact preparation exhibit period-four oscillations due to slower reduction of Z^+ in the higher S-states. In contrast, the Z^+ kinetics observed with the inhibited preparations is relatively insensitive to flash number, indicating that the blockage of electron transfer occurs prior to the O_2-evolving complex.

Recently, we have obtained X-ray absorption spectra at the Mn K-edge in NaCl and $CaCl_2$ washed PS II preparations (Cole, J. et al., unpublished data). These data suggest that the structure of the Mn-containing O_2-evolving complex is not altered by extraction of the extrinsic proteins. However, oxidation of this Mn at the $S_1 \rightarrow S_2$ transition is inhibited, supporting the model in which electron transfer from the O_2-evolving complex is blocked.

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