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The effect of mono- and divalent salts on the rise and decay kinetics of EPR signal II in Photosystem II preparations from spinach

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The rise and decay kinetics of EPR signal II have been used to probe the organization of the donor side of Photosystem II (PS II) before and after extraction of PS II preparations with high concentrations of salt. 800 mM NaCl or 500–800 mM NaBr substantially depletes the preparations of the 16 and 24 kDa proteins and decreases the steady-state rate of O_2-evolution by 70–80% from control rates. These treatments do not largely alter the decay kinetics of Signal II; the rise kinetics remain in the instrument limited time range (2 μs or less) during the first 8–12 flashes. Treating PS II preparations with 800 mM $CaCl_2$ removes the 16, 24 and 33 kDa proteins with at least 95% inhibition of the steady-state rates of O_2 evolution. The additional removal of the 33 kDa polypeptide decreases the rates of oxidation and rereduction of Z, the species responsible for Signal II. Preparations treated with either mono- or divalent salts show a steady-state light-induced increase in Signal II similar to that seen in Tris-washed samples. Such a steady-state increase indicates that the rate of electron transport from water to Z is greatly decreased or blocked. The data are interpreted within a model in which there is an intermediate electron carrier between the O_2 evolving complex and Z.

Introduction

Recent advances [1,2] in the preparation of O_2-evolving PS II preparations have stimulated extensive research into the mechanism of the water-splitting reactions and the organization of electron-transport components on the donor side of PS II. The ability of high concentrations of salts to inhibit the steady-state O_2-evolution activity of these preparations has been well documented [3–6]. This inhibition is attributed to the extraction of three extrinsic proteins, namely the 16 kDa [7], the 24 kDa [3,4] and the 33 kDa proteins [5,6]. Washing with 0.8–2 M NaCl depletes the preparations of the 24 and 16 kDa proteins [3,4] and produces 70–80% inhibition of O_2 evolution [3,4,6], while extraction with 0.8 to 1 M $CaCl_2$ or $MgCl_2$ causes at least 95% inhibition of O_2 evolution, and extracts the 33 kDa protein as well as the 16 and 24 kDa proteins [5,6]. Reconstitution studies [3,6–9] indicate that both the 24 and 33 kDa proteins, and probably the 16 kDa protein [7] are needed for competent water-splitting activity, although none of the three proteins appears to carry a transition metal or prosthetic group capable of electron transport [5,6,10]. More recently, the 33 kDa protein has been suggested to stabilize Mn

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; Chl, chlorophyll; PS I and II, Photosystem I and II; DCBQ, 2,5-dichlorobenzoquinone.

within its membrane binding site [11,12], while the 24 kDa protein has been implicated as a Ca^{2+}-binding protein [13]. These proteins appear to maintain a proper conformation of electron-transport components on the donor side of PS II, so that facile electron transport from the oxygen-evolving complex to the reaction center of PS II, P-680, can occur.

We have previously observed that treatment of PS II preparations with NaCl or $MgCl_2$ blocks the formation of the low-temperature multiline EPR signal attributed to Mn in the S_2 state of the O_2-evolving complex [6]. To examine further the effects of salt inhibition on the electron-transfer components of the donor side we have measured the rise and decay kinetics of EPR Signal II in untreated and salt-inhibited samples. This signal arises from a semiplastoquinone cation [14] which passes oxidizing equivalents from the PS II reaction center ($P\text{-}680^+$) to the oxygen-evolving complex [15,16]. Inhibition of O_2 evolution activity by Tris has been shown to slow both the rise and decay kinetics of Signal II [17–19]. In contrast, we find that extensive depletion of the 16 and 24 kDa proteins by NaCl or NaBr extraction causes the loss of 70–80% of the steady-state O_2-evolution activity, but leaves the rise and decay kinetics of S II substantially unaltered (within instrument resolution). However, after removal of the 33 kDa protein in addition to the 16 and 24 kDa proteins by $CaCl_2$ treatment, we find that the rise and decay kinetics are similar to those seen in Tris-treated preparations.

Materials and Methods

Oxygen-evolving PS II preparations were obtained and incubated in high concentrations of salt, as previously described [6]. The extracted preparations were then centrifuged (10 min, 34000 $\times g$) and washed free of the salt by suspension in 50 mM Mes (pH 6.0)/10 mM NaCl followed by centrifugation. Steady-state O_2-evolution rates were measured polarographically, using a Yellow Springs Instrument 4004 electrode and a high-sensitivity teflon membrane. Actinic light from a 200 W projector lamp was filtered through 2.5 cm of water and a cut-off filter (Corning C.S. 3-68) and focused onto the polarographic vessel of 4.3 ml capacity. Oxygen evolution was assayed at 21 ± 2°C in 50 mM Mes (pH 6.0)/10 mM NaCl, which additionally contained 3 mM ferricyanide/3 mM ferrocyanide/500 μM 2,5-dichlorobenzo-

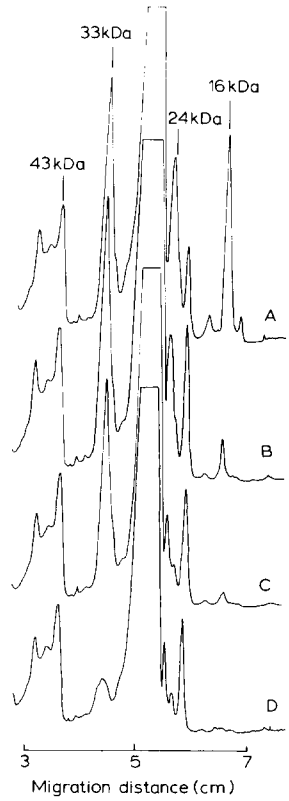


Fig. 1. Densitograms of SDS-polyacrylamide gel electrophoresis gel illustrating the effects of salt extractions on the peptide content of PS II preparations. The procedure for salt extraction is described in the text and in Ref. 6. (A) Control preparation, (B) 800 mM NaCl, (C) 800 mM NaBr, (D) 800 mM $CaCl_2$. Prior to electrophoresis 100 μl of each sample (Chl concentration, 1.5 mg per ml) was diluted into an equal volume of a solution containing 0.125 M Tris-HCl (pH 6.8)/4 M urea/4% SDS/20% glycerol/10% 2-mercaptoethanol. 25 μg of Chl was loaded in each case. Protein composition was analyzed by SDS-polyacrylamide gel electrophoresis in the buffer system of Laemmli [22], additionally containing 2 M urea. The samples were electrophoresed under constant power (25 mW). The gel was stained with Coomassie brilliant blue R-250, and the densitograms were recorded on a Hoeffer Scientific Instruments GS 300 scanning densitometer. The residual band at the 32–34 kDa position in the $CaCl_2$-treated preparations apparently is due to the atrazine binding protein [23] and/or the protein described by Metz et al. [24], because in other samples this band remained after extraction with 960 mM Tris-HCl (pH 9.3), a treatment known to release the 16, 24 and 33 kDa proteins completely [4].

quinone (DCBQ) as an electron acceptor system. Chlorophyll concentrations in the assay were about 5 μg per ml. Chlorophyll concentrations were obtained by the method of Arnon [20]. Conditions for electrophoresis are described in the legend to Fig. 1. Samples used for Signal II decay measurements were resuspended in 50 mM Mes (pH 6.0)/10 mM NaCl to a chlorophyll concentration of 3 mg/ml. Samples used for risetime measurements were resuspended in the same buffer except that the pH was set at 5.5. Before the kinetic measurement, unless otherwise noted, 2.5 mM ferricyanide/2.5 mM ferrocyanide/500 μM DCBQ were added to the sample as redox buffers and external electron carriers. Signal II kinetic measurements were performed and analyzed as previously described [19,21]. Sample excitation was achieved by pulsing the sample with a Phase-R DL-1400 pulsed dye laser using rhodamine 640 (Exciton) in methanol as the dye. Kinetic measurements in the millisecond and longer time regime were done on a Varian E-109 EPR spectrometer using 100 kHz magnetic-field modulation and no filter on the output of the receiver. Faster kinetics (down to 2 μs resolution) were obtained on the same spectrometer modified for 1 MHz field modulation. In all cases the laser repetion rate was 2 Hz, and kinetics were measured at $g = 2.010$. Instrument settings are listed in the figure and table legends.

Results

Peptide content and O_2-evolution activity of the salt-washed preparations

The effects of salt-washing on the O_2-evolution activity and peptide content of the PS II preparation are shown in Fig. 1 and Table I, and are reasonably consistent with previously published work [3,4,9]. We find that washing with 800 mM NaCl inhibits the O_2-evolution activity by 70% and depletes the preparations of only the 16 and 24 kDa proteins (Fig. 1B, Table I), while washing with 800 mM $CaCl_2$ (or $MgCl_2$) produces at least 95% inhibition of O_2-evolution activity and completely removes the 16, 24 and 33 kDa proteins (Fig. 1D, Table I). In accord with our previous work [6], Fig. 1C and Table I show that extraction with 800 mM NaBr results in greater inhibition of O_2-evolution than does washing with 800 mM NaCl, and produces nearly complete release of the 24 and 16 kDa proteins, but only a small loss of the 33 kDa protein.

TABLE I

THE EFFECT OF MONO- AND DIVALENT SALTS ON THE O_2-EVOLUTION ACTIVITY AND PEPTIDE CONTENT OF PS II PREPARATIONS

PS II preparations were salt treated as described in the text. O_2-evolution activities were obtained as described in the text and in Ref. 6. The amount of the peptides was estimated from the peak height of the stained bands in densitograms (Fig. 1), using an untreated sample as reference for no release, and a sample treated with 960 mM Tris (pH 9.3) as reference for complete release [4]. The peak heights of the 16, 24 and 33 kDa bands were normalized to the 43 kDa band to account for minor variations in the amount of Chl loaded in each lane. Decreasing the Chl load from 25 μg to 10 μg provided results which were within the indicated uncertainty.

Treatment	Inhibition of O_2 evolution activity [a] (%)	Inhibition of release of peptides [b] (%)		
		16	24	33
800 mM NaCl	69	82	68	0
500 mM NaBr	74	90	77	≦5
800 mM NaBr	82	≧95	≧95	≦10
800 mM $CaCl_2$	≧95	100	100	100

[a] Control activity: 410 μmol O_2 per mg Chl per h. Estimated uncertainty, ±5%

[b] Estimated uncertainty, ±10%.

Signal II rise kinetics

Previous work has established that untreated PS II preparations exhibit a rise-time of Signal II that is instrument-limited (to 2 μs) [21], while for Tris-inhibited samples the risetime is increased to 10 μs at pH 5.5 [19]. Fig. 2 reveals the effects of salt washing on the kinetics of Signal II appearance under comparable conditions. Preparations treated with 800 mM $CaCl_2$ exhibit a risetime similar to that seen in Tris-inhibited samples (Fig. 1). In contrast, samples extensively depleted of the 16 and 24 kDa proteins by treatment with 500 mM NaBr show a risetime that is instrument limited (Fig. 2). Thus, it appears that the loss of the 33 kDa polypeptide is correlated with the slower donation of electrons from Z to $P\text{-}680^+$ in Tris- and $CaCl_2$-inhibited preparations.

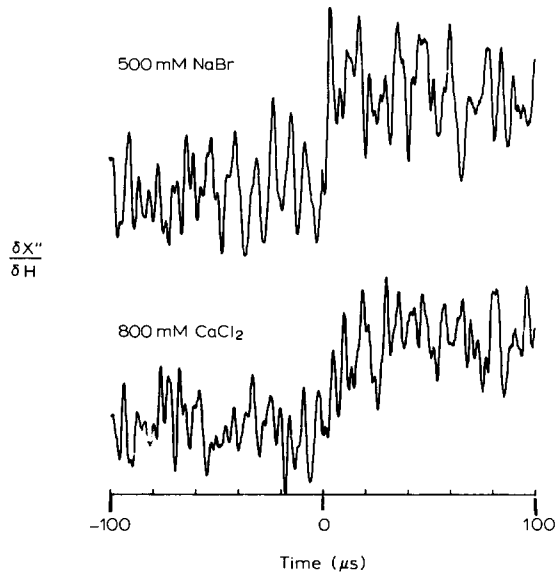


Fig. 2. Formation kinetics of Signal II under different salt deactivation conditions. Top, 500 mM NaBr deactivated; bottom, 800 mM $CaCl_2$ deactivated. EPR Signal II kinetics measured using a Varian E-109 EPR spectrometer with 1 MHz field modulation, 2 μs time resolution, 4.3 G modulation amplitude, 55 mW microwave power, kinetics measured at $g = 2.010$. Flowing sample (8–12 flashes/turnover) contained 3 mg Chl per ml/2.5 mM potassium ferricyanide/2.5 mM ferrocyanide/500 μM DCBQ/10 mM NaCl/50 mM Mes (pH 5.5). Sample temperature was 17 ± 1°C. Sample (volume, 4–8 ml) was changed after each 20000–30000 flashes. Each kinetic trace represents the sum of 50000 flashes. Gain settings were identical for each trace. Laser excitation, 5–10 mJ/pulse at the sample; flash frequency, 2 Hz.

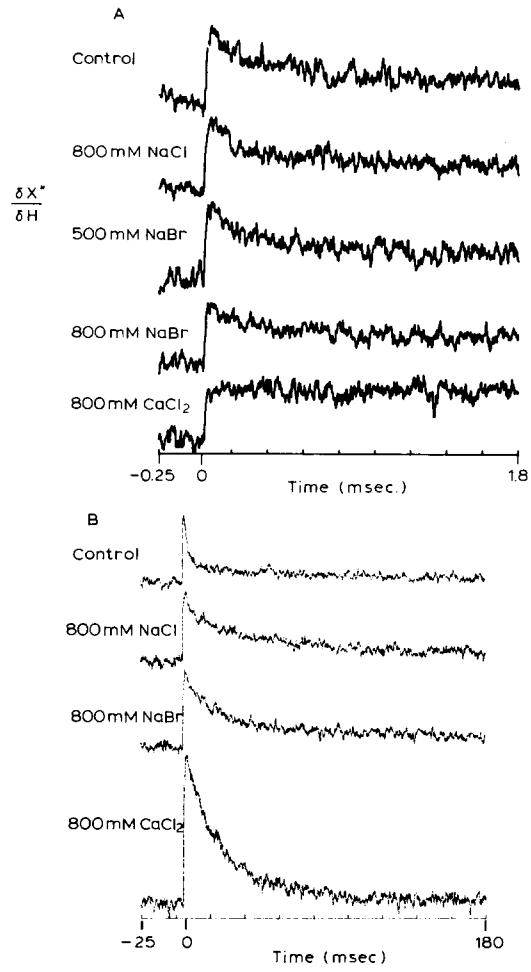


Fig. 3. The effect of salt treatments of the decay kinetics of signal II. (A). From top to bottom are: control; 800 mm NaCl-treated; 500 mM NaBr-treated; 800 mM NaBr-treated; and 800 mM $CaCl_2$-treated. Sample and instrument conditions are as in Fig. 2, except that the sample was resuspended in Mes buffer at pH 6.0. A 10 μs low pass *RC* filter was used on the output of the 1 MHz receiver. Gain settings were identical for each trace. Each trace is the sum of 50000 flashes. (B). From top to bottom: control; 800 mM NaCl-treated; 800 mM NaBr-treated; and 800 mm $CaCl_2$-treated. Signal II kinetics measured at $g = 2.010$. Varian E-109 settings: 10^4 gain, 100 kHz field modulation, 5 G modulation amplitude, 20 mW microwave power, half-time of instrument rise is 0.25 ms (time constant, out). Flowing sample contained 2.5 mM potassium ferricyanide/2.5 mM potassium ferrocyanide/500 μM DCBQ/10 mM NaCl/50 mM Mes (pH 6.0). Sample temperature was kept at 17 ± 1°C., Sample volume, 2 ml. Each trace reflects the sum of 6000 flashes. Amplitudes are normalized for differences in chlorophyll concentration. Laser excitation, 5–10 mJ/pulse at the sample; laser flash frequency, 2 Hz; 8–12 flashes per sample turnover under flow.

Signal II decay kinetics

Under the repetitive flash conditions (8–12 flashes before turnover), both untreated and salt-washed PS II preparations show multiphasic Signal II decay kinetics. Control and monovalent salt-washed samples exhibited a fast phase in the microsecond time regime (Fig. 3A) and two slower phases in the millisecond regime (Fig. 3B). In contrast, the $CaCl_2$-treated preparations showed no microsecond decay component (Fig. 3A); instead, a biphasic decay on a millisecond time-scale was observed (Fig. 3B).

The decay curves were fit by a non-linear least-squares fitting routine to obtain the half-times (t) and amplitudes (A) of the various decay components. The slowest component in the millisecond time range (Fig. 3B) was fit using the pre-recorded

baseline as the level at t_∞. Subsequently, the other decay component in the millisecond range was fit after fixing the slower component's time (t_s) and amplitude (A_s). The amount of the fastest decay component which is visible in Fig. 3A could be determined from the data in the millisecond time range (Fig. 3B) by measuring the difference between the $CaCl_2$ treated sample's amplitude and the amplitude of any other decay trace. These differences correlated well with the contribution of the fast phase to the total amplitude seen in Fig. 3A despite the difference in microwave power used to collect the two sets of data (55 mW in Fig. 3A, and 20 mW in Fig. 3B).

The results of the analysis are presented in Table II. For the untreated preparations, a rapid decay phase of about 140 μs (t_f) contributes 55% to the observed signal amplitude, while two slower phases of about 3 ms (t_m) and 100 ms (t_s) contribute 35% and 10%, respectively. After $CaCl_2$ extraction of the 16, 24 and 33 kDa proteins the 140 μs decay disappears, leaving two decay components of 9 and 100 ms. In contrast, PS II preparations which were extensively depleted of the 16 and 24 kDa proteins by NaCl or NaBr extraction exhibited little or no loss of the 140 μs component, but they did show a 2–3 fold increase in t_m and an increase in the percentage of the slower millisecond phase, A_s (Table II).

In the above experiments, DCBQ was added so that there was a good electron acceptor for the samples. For the inhibited samples, the hydroquinone form of the molecule may act as an electron donor to Signal II. However, in the absence of quinone, we observed no increase in amplitude of the millisecond phases of the NaCl- or NaBr-inhibited samples, which indicates that the 140 μs component cannot be due to the rapid rereduction of Z^+ by the exogenous quinone (data not shown).

To investigate whether the 140 μs decay resulted from a forward donation reaction, as opposed to a back-reaction with the primary or secondary quinone acceptors, kinetic traces were collected on the control and salt-washed samples in the absence of added acceptor. The amplitude of the signal produced in control and 800 mM NaCl treated samples is only one-third that produced in $CaCl_2$ treated preparations (data not shown). Under multiple (8–12) flash conditions and in the absence of acceptor, little signal is expected if the rate of forward donation to Z^+ is much greater than the rate of back reaction. The multiple flashes result in an accumulation of reducing equivalents on the acceptor side of PS II and in the reduction of Q_A, after which stable charge separation cannot occur. The diminution of Signal II amplitude in the control and monovalent salt-washed samples implies that the fast 140 μs decay results from a forward donation, while the kinetics of Signal II reduction observed in the $CaCl_2$-treated samples appears to arise to a greater extent from a back reaction with the acceptor side. The conclusion that the 140 μs decay is due to a forward donation reaction is further supported by our observation that increasing the ferricyanide (acceptor) concentration from 2.5 mM to 10 mM did not increase the proportion of the millisecond phases in control and monovalent salt-washed samples.

TABLE II

THE EFFECT OF MONO- AND DIVALENT SALTS ON THE DECAY COMPONENTS OF SIGNAL II

Sample	A_f	t_f (μs)	A_m	t_m (ms)	A_s	t_s (ms)
Control	55±10	140±50	35±5	3.5±2	10±5	100±35
800 mM NaCl	55±10	140±50	25±5	9 ±2	20±5	100±35
500 mM NaBr	55±10	140±50	25±5	9 ±2	20±5	100±35
800 mM NaBr	50±10	140±50	35±5	9 ±2	15±5	100±35
800 mM $CaCl_2$	0	–	63±5	9 ±2	38±5	100±35

Half-times (t) and relative amplitudes (A) were obtained from kinetic traces (Fig. 3A and B) as described in Results under Signal II decay kinetics. Samples used were PS II subchloroplast preparations with 2.5 mM ferricyanide/2.5 mM ferrocyanide/500 μM DCBQ added. Instrument conditions are listed in Fig. 3A and B.

Steady-state spectra

When samples competent in oxygen evolution are given saturating, continuous illumination, there is very little build-up of light-induced Signal II due to rapid rereduction of the donor side of PS II by the intact oxygen-evolving complex. In our control samples this increase consists of about 10% of the dark level of Signal II. At pH 6.0, the Tris-treated, NaCl-treated, NaBr-treated, and $CaCl_2$-treated preparations all show increases in the steady-state level of Signal II in the range of 45–65% of the dark level (Table III). Monovalent salt-washed samples display a light-induced increase which is slightly less than that seen in divalent salt- or Tris-treated cases. This level of increase in Signal II during illumination of the samples indicates that the loss of either the 16 and 24 kDa proteins or additionally the 33 kDa protein substantially decreases the rate of electron transfer from the oxygen-evolving complex to Z. However, the differences in the transient kinetics observed for the monovalent and $CaCl_2$ treated preparations indicate that the direct donor to Z is not disturbed by the removal of the 16 and 24 kDa proteins, but this donor is affected by the removal of the 33 kDa protein.

TABLE III

THE EFFECT OF MONO- AND DIVALENT SALTS ON THE STEADY-STATE LIGHT-INDUCED INCREASE IN SIGNAL II

Amount of O_2 evolution and corresponding peptide release of these samples are described in Table I. PS II preparations were salt-treated as described in the text. 1 mM ferricyanide and 1 mM ferrocyanide were added to each sample which were at a chlorophyll concentration of 3 mg/ml and buffered at pH 6.0. Spectra were collected using a Varian E-109 EPR spectrometer with 100 kHz magnetic field modulation, 6.3 gauss modulation ampliltude, 0.128 second time constant, 20 mW microwave power, gain of 50000 and a scan rate of 25 $G \cdot min^{-1}$. Signal II amplitudes were taken as the height from the low field maximum ($g = 2.010$) to the high field minimum ($g = 1.999$) in the derivative spectrum. The dark level was measured after illumination.

Sample	$\left(\frac{\text{Amplitude of light-induced Signal II}}{\text{Amplitude of Signal II in the dark}}\right)^a$
Control	0.10
800 mM NaCl	0.46
800 mM $CaCl_2$	0.64

[a] Estimated uncertainty, ±0.05.

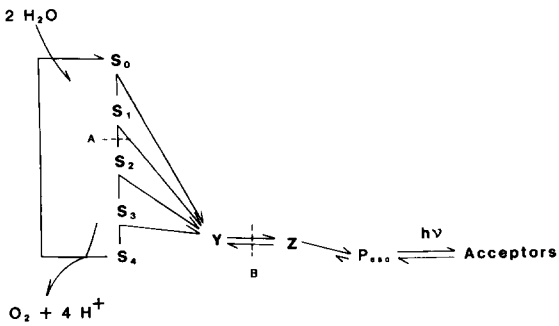
Discussion

The results presented here can be interpreted within the framework of the mechanism presented in Scheme I. In this model, there is an intermediate donor Y between the oxygen evolving complex and Z. This donor equilibrates with Z^+ during photochemical turnover with an apparent equilibrium constant of approx. 1–1.8. In control samples the 140 μs phase of Z^+ reduction then corresponds to the rapid equilibrium of Y with Z^+, while the predominant millisecond phase (3 ms) results from rate-limiting steps of the reduction of Y^+ by the oxygen-evolving complex. Upon release of the 16 and 24 kDa proteins, electron donation from the oxygen-evolving complex to Y^+ is blocked or greatly diminished at the S_1-to-S_2 transition [6]. At the chloride levels in the final suspension buffers and used in this study (10 mM), we find that, within experiental error, the loss of the multiline EPR signal is proportional to the loss of O_2-evolution activity. The multiline signal amplitude observed in samples depleted almost completely of the 16 and 24 kDa proteins (800 mM NaBr-treated samples, Table I) may be due to the chloride effect described by Toyoshima et al. [29]. The 140 μs phase of Signal II decay arise from the rapid equilibration of Z^+ with Y, while the millisecond phases of Signal II decay arise from the slow, rate-limiting reduction of Y by an exogenous or endogenous donor, and/or a back reaction with the acceptor side. This accounts for the increase in the half-time of the middle phase of Signal II, and the increase in the relative amplitude of the slow milisecond phase after monovalent salt washing. The model requires that Y be reduced during the dark cycle time (500 ms) in samples treated with monovalent salts. This condition appears to be met at pH 6.0, since we did not observe a significant difference between the amplitudes for the microsecond phase in the control and monovalent salt-washed samples under conditions in which they received 8–12 flashes before turnover. Within this model the additional release of the 33 kDa protein by $CaCl_2$ blocks or greatly decreases the rate of electron transfer be-

tween Y and Z^+. The fact that the rise of Signal II also is slower indicates that there is a major alteration of the electron-transport chain on the donor side of PS II after extraction of the 33 kDa protein. The postulated electron transfer component (Y) may be identical to that giving rise to a $g = 4.1$ EPR signal observed at 10 K after low temperature illumination [25]. Experiments to test this possibility are currently in progress.

The model presented in Scheme I is also consistent with the flash-number dependence of Signal II decay reported by Babcock et al. [26], if it is assumed that the 140 μs phase of equilibration between Y and Z^+ was not resolved in those experiments. Thus, on the first flash, which reflects primarily the $S_1 \rightarrow S_2$ transition, no signal was observed, owing to the rapid reduction of Y^+ by the oxygen evolving complex. On the two subsequent flashes, representing the $S_2 \rightarrow S_3$ and $S_3 \rightarrow S_4$ transitions, the observed amplitudes and half-times of S II decay reflects the rate-limiting steps of the reduction of Y by the oxygen-evolving complex. On the fourth flash ($S_0 \rightarrow S_1$ transition), again little signal would have been observed owing to the rapid reduction of Y.

Although they did not resolve the 140 μs phase of Signal II decay, the recent results of Ghanotakis et al. [27] on the effects of NaCl washing on the kinetics of Signal II decay are reasonably consistent with ours. However, they concluded that the unresolved rapid phase of Signal II decay was the result of rapid donation from Mn in the lower S-states of the oxygen-evolving complex. We have



Scheme I. (A) Extraction of 16 and 24 kDa proteins; (B) extraction of 33 kDa protein.

previously shown that release of the 16 and 24 kDa proteins by salt treatment inhibits the formation of the low-temperature multiline EPR signal attributed to Mn in the S_2 state of the O_2-evolving site [6]. Assuming the model of Ghanotakis et al. [27], this result would indicate either that release of the 16 and 24 kDa proteins blocks or greatly decreases the rate of electron donation from the Mn complex to Z decreasing the yield of S_2, or that the Mn complex donates to Z, but, in the absence of these proteins, can no longer give rise to the multiline signal. Although we cannot exclude the latter possibility, such a perturbation of the oxygen-evolving complex should also alter the rate of electron transfer from S_1 to Z^+. However, we have observed no significant difference between the halftimes for the microsecond phase in the control and monovalent salt-washed samples (Fig. 3, Table II). Moreover, the observation by Ghanotakis, et al. that the addition of benzidine to NaCl-washed samples decreases the amplitude of the millisecond phases of Signal II reduction through conversion of higher oxidation states of Mn to lower ones which rapidly donate to Z^+ can be equally well explained as due to benzidine donation to Y in our model (Scheme I).

Recently, Åkerlund et al. [28] found that NaCl washing of inside-out thylakoids decreases the amplitude of the nanosecond phases of Chl a_{II}^+ reduction and increases the microsecond phases, indicating that removal of the 24 kDa protein decreases the rate of electron transfer from Z to $P\text{-}680^+$ (Scheme I). This result is difficult to reconcile with our observation that the risetime of Signal II (Z in Scheme I) remains 2 μs or less after extensive depletion of the 16 and 24 kDa proteins. We are presently attempting to measure by EPR the decay kinetics of $P\text{-}680^+$ in NaBr- and $CaCl_2$-treated preparations to investigate this discrepancy.

Although a more complete understanding of the organization of electron-transport components on the donor side of PS II awaits the microsecond time resolution of the flash number dependence of the Signal II decay kinetics, the results presented here in combination with those of Casey and Sauer [25], and Blough and Sauer [6] support the idea that there is at least one donor between the oxygen-evolving complex and Z.

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