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STRUCTURAL AND CATALYTIC PROPERTIES OF THE OXYGEN-EVOLVING COMPLEX

CORRELATION OF POLYPEPTIDE AND MANGANESE RELEASE WITH THE BEHAVIOR OF Z^+ IN CHLOROPLASTS AND A HIGHLY RESOLVED PREPARATION OF THE PS II COMPLEX

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Treatment of intact thylakoid membranes with Triton X-100 at pH 6 produces a preparation of the PS II complex capable of high rates of O_2 evolution. The preparation contains four manganese, one cytochrome *b*-559, one Signal II_f and one Signal II_s per 250 chlorophylls. By selective manipulation of the preparation polypeptides of approximate molecular weights of 33, 23 and 17 kDa can be removed from the complex. Release of 23 and 17 kDa polypeptides does not release functional manganese. Under these conditions Z^+ is not readily and directly accessible to an added donor (benzidine) and it appears as if at least some of the S-state transitions occur. Evidence is presented which indicates that benzidine does have increased access to the oxygen-evolving complex in these polypeptide depleted preparations. Conditions which release the 33 kDa species along with Mn and the 23 and 17 kDa polypeptides generate an alteration in the structure of the oxidizing side of PS II, which becomes freely accessible to benzidine. These findings are examined in relationship to alterations of normal S-state behavior (induced by polypeptide release) and a model is proposed for the organization of functional manganese and polypeptides involved in the oxygen-evolving reaction.

Introduction

The linear four-step oxidation of water by PS II requires the coordination of redox reactions driven by light, through an intermediate Z, with a set of redox reactions involving the so-called S-states where manganese is a cofactor. Efforts to characterize components of the oxygen-evolving reaction have led to the suggestion that the Z/Z^+ redox couple may be identical with the plastohydroquinone/plastosemiquinone cation radical couple [1,2] and that the kinetic behavior of Z^+ may be modified by treatments such as exposure to Tris or addition of ammonia, which inhibit oxygen evolution [3,4]. The microwave power saturation properties of Z^+ are affected by the nature of amine inhibition (bulkier amines, such as methylamine, decrease the microwave power required to saturate Z^+); for amines which release manganese (Tris, NH_2OH), we have shown that these reagents produce different patterns of manganese extraction [5]. A model for the organization of functional manganese in the oxygen-evolving complex and its relationship to Z has

Abbreviations: PS, Photosystem; Chl, chlorophyll; BZ, benzidine; Z, primary donor to P-680$^+$; HQ, hydroquinone; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ADRY, acceleration of the deactivation reactions of water-splitting enzyme system Y.

been proposed [5]. Recently, the well-established chloride requirement for O_2 evolution [6] has been characterized and interpreted in the context of this model [7].

Concurrent with the biophysical studies cited above, biochemical techniques have been developed for the isolation of O_2-evolving PS II preparations [8–10]. The reduced polypeptide content of such preparations has made it possible to show that Tris treatment not only inactivates O_2 evolution but also releases certain polypeptides with molecular weights of 33000, 23000 and 17000 [9]. This result has been confirmed and extended by others [11,12], and it is now apparent that PS II preparations can be utilized for resolution and reconstitution experiments following procedures developed by Åkerlund et al. [13] with the so-called 'inside-out' thylakoid vesicles.

In this communication we report the properties of the PS II complex isolated by a modification of the procedure of Berthold et al. [8]. Polypeptides are released from this preparation by a variety of treatments and the effects of those treatments on the extent of manganese release and on the behavior of Z^+ are reported. These results are used to refine previous models [5] developed to describe the organization of PS II. Preliminary accounts of this research have appeared [14–16].

Materials and Methods

Thylakoid membranes were isolated according to the procedure given in Ref. 17. Isolation of the PS II complex was carried out as outlined in Refs. 11 and 18 and is described in detail below. The final pellet was resuspended in 50 mM Mes (pH 6) containing 5 mM $MgCl_2$/15 mM NaCl/1 mM ascorbate; Triton X-100 (25% stock solution in 50 mM Mes (pH 6), 5 mM $MgCl_2$, 15 mM NaCl to which 100–200 μg/100 ml of catalase had been added) was added dropwise with stirring until the suspension contained 25 mg Triton per mg Chl. We routinely adjust the conditions, so that the final Chl concentration is 2 mg/ml. After 30 min of stirring in the dark at 4°C, the suspension was centrifuged (40000 × g, 30 min) and the pellet was resuspended in the Mes/NaCl/$MgCl_2$ solution. The Chl concentration was determined and Triton X-100 was added to a final concentration of 5 mg/mg Chl. After a 5 min dark incubation period at 4°C, the suspension was again centrifuged (40000 × g, 30 min). The pellets were resuspended in 400 mM sucrose containing 50 mM Mes/5 mM $MgCl_2$/15 mM NaCl; following centrifugation, the pellets were resuspended to a final concentration of 3 mg/ml and stored at −80°C. Inactivation of thylakoid preparations by NH_2OH was carried out as follows: chloroplasts were incubated for 20 min, in the dark, in the presence of 5 mM NH_2OH at pH 7.5. Following centrifugation the pellets were washed twice with a pH 7.5 solution containing 0.4 M sucrose/50 mM Hepes/15 mM NaCl and finally were resuspended in a small volume of this solution. Tris inactivation was carried out as described in Ref. 5. Salt treatment to release polypeptides from the PS II complex was carried out by diluting a suspension of the preparation (3 mg Chl/ml in 400 mM sucrose/50 mM Mes (pH 6.0)/5 mM $MgCl_2$/15 mM NaCl) with an equal volume of 4 M NaCl (in 50 mM Mes, pH 6.0). After mixing the suspension was allowed to stand on ice in the dark for 1 h. Centrifugation (40000 × g, 30 min) pelleted the membranes, which were washed once with the original suspending medium and stored at a final concentration of 3 mg Chl/ml in 400 mM sucrose/50 mM Hepes/15 mM NaCl at pH 7.5 or 400 mM sucrose/50 mM Mes/15 mM NaCl at pH 6.0.

EPR spectroscopy was carried out on a Bruker ER-200D spectrometer operated at X-band and interfaced to a Nicolet 1180 computer. Instrument modifications as well as the xenon flash lamp circuitry and the protocol for signal averaged, flashing-light kinetic experiments are described in Ref. 5.

Results

Assay of O_2 evolution activity

The PS II complex requires a lipophilic mediator to sustain high rates of oxygen evolution, and as we reported elsewhere [8], the optimum pH for activity is between 5.5 and 6. We have further examined optimal conditions for assay of the complex and have observed that sucrose stimulates activity, whereas $MgCl_2$ is slightly inhibitory (Table I). The assays in Table I were carried out at

TABLE I

EFFECT OF ASSAY COMPONENTS ON O_2 EVOLUTION BY THE PS II COMPLEX

The basic reaction mixture contained 50 mM Mes (pH 6)/2.5 mM $Fe(CN)_6^{3-}$/250 μM dichlorobenzoquinone. Other additions are as shown in the table.

Addition	Activity (μmol O_2/h per mg Chl)
–	325
0.2 M sucrose	500
0.4 M sucrose	620
0.6 M sucrose	580
0.4 M sucrose + 2.5 mM $MgCl_2$	560
0.4 M sucrose + 5.0 mM $MgCl_2$	525

high light intensity ($8 \cdot 10^3$ J · m^{-2} · s^{-1}), since this is critical for optimal activity. We are limited to this intensity, so we cannot be certain that we have achieved light saturation. The data of Table I indicate that sucrose enhances activity, by a mechanism that might involve partitioning of residual Triton from the membranes into the sucrose, and that $MgCl_2$ is inhibitory to activity. Others [19] have reported similar high rates of O_2 evolution using a modification of the isolation procedure we describe here.

Redox components of the isolated PS II complex

Fig. 1 presents the results of experiments to characterize the cytochrome *b*-559 content of the PS II complex. No light-induced changes from the cytochrome are seen (data not shown) but the oxidation/reduction experiments of Fig. 1 show that about 50% of the *b*-559 is in the high potential form (hydroquinone reducible), while another 50% is reduced by ascorbate. As the figure shows, after ascorbate addition, dithionite addition produces no further reduction. We have also repeated the quantitation of Mn^{2+} in these preparations by using the EPR technique described previously [5] (data not shown). A summary of these data along with other results is presented in Table II. We would conclude from these data that the PS II complex contains one O_2-evolving unit per 240–250 Chl. Because there are approx. 400 Chls per PS II unit in our starting spinach chloroplasts [5], the PS II preparations represent an enrichment of approx. 2-fold. In contrast to the report of Lam et al. [21]

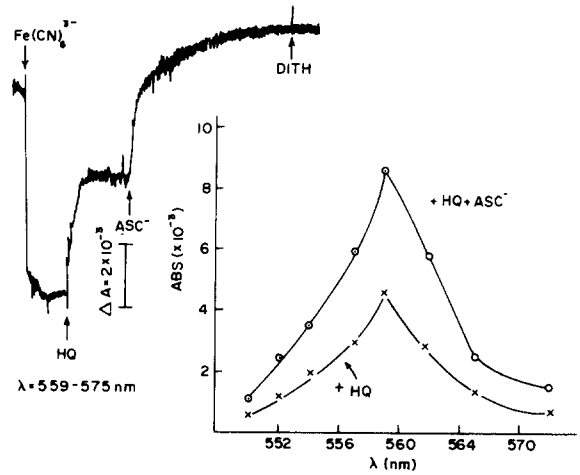

Fig. 1. Chemically induced oxidation and rereduction of cytochrome *b*-559 in the isolated PS II complex. The procedure described by Horton and Croze [20] for addition of oxidants and reductants was used. The sample compartment of the Aminco DW-2 was thermostatted at 20 °C; the sample (90 μg Chl/ml) was suspended in a medium containing 400 mM sucrose/50 mM MES (pH 6)/15 mM NaCl/5 mM $MgCl_2$.

we find only one *b*-559 per reaction center in our preparation, in agreement with Ford and Evans [19]. Likewise, Yamamoto and Nishimura [22] report only two manganese per reaction center in another PS II preparation; this may be a reflection of the low rates of O_2 evolution (and hence denatured material) in this preparation.

Effect of various treatments on Z behavior and polypeptide and manganese content

Exposure of isolated thylakoid membranes to NH_2OH is known to inactivate the oxygen-evolving reaction system by a mechanism different from that of Tris-inactivation [23]. Sharp and Yocum [24], using NMR relaxation techniques, showed that higher S-states are immune to attack by NH_2OH and that NH_2OH-induced Mn extraction occurs only from the lower S-states. Tris, on the other hand, is known to exert its inhibitory effects when the oxygen-evolving complex is in higher S-states, S_2 and S_3 [25,26]. A study of the inhibitory action of the various amines revealed that NH_2OH inactivation of the oxygen-evolving system is unique [1], and we have characterized its behavior in more detail by examining the behavior of Z^+ and polypeptide content in NH_2OH ex-

TABLE II

REDOX COMPONENTS OF THE PS II COMPLEX

Component	Concentration	Reference
Cytochrome b_{559}	1 per 235 Chl	This work
Mn^{2+}	4 per 250 Chl	11; this work
Signal II_s (dark stable signal)	1 per 260 Chl	35
Signal II_f (Z^+)	1 per 260 Chl	35

tracted chloroplasts and PS II membranes.

Since in addition to its well-known inhibitory action on the oxygen-evolving complex, hydroxylamine also disrupts physiological electron flow from Z to P-680 [18], we avoided the second inhibitory effect by a washing procedure which removes residual NH_2OH once its inhibition has gone to completion. When these precautions are taken, the behavior of Z^+ is identical to that observed in Tris-inhibited chloroplasts. Kinetic transients of Z^+ in NH_2OH extracted chloroplasts are shown in Fig. 2 and the major features can be summarized as follows: (a) Z^+ formation in the millisecond range is stoichiometric with P-680, (b) the decay is monophasic and (c) the decay of Z^+ is accelerated by addition of exogenous donors (Fig. 2b and c) in a straightforward, second-order process. As shown in Fig. 2A neutral reductants (e.g., benzidine) are much more effective donors than are negatively charge donors (e.g., ascorbate), a behavior which parallels that observed in Tris-inhibited chloroplasts [27]. In Fig. 2B we present data from which the second order rate constants for the donor activities of benzidine and hydroquinone can be determined. A plot of k_{obs} vs. the corresponding donor concentration permits extraction of the second-order rate constant which describes the decay of Z^+ in the presence of this donor. The rate constants observed, $k(BZ) = 1.1 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and $k(HQ) = 3.2 \cdot 10^5\ M^{-1} \cdot s^{-1}$, are essentially the same as those observed in Tris-

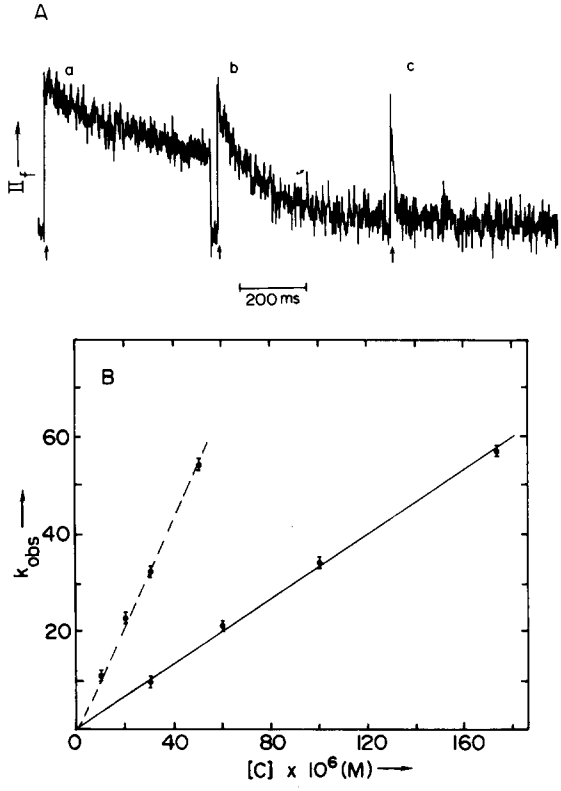

Fig. 2. (A) Kinetic transients of Z^+ at room temperature in NH_2OH-extracted chloroplasts at pH 7.5; (a) no further addition, (b) 4 mM ascorbate and (c) 50 μM benzidine and 4 mM ascorbate. Time constant 1 ms, 150 scans averaged at a rate of 0.25 Hz. (B) Graphical determination of the second order rate constants for benzidine (- - - - -) and hydroquinone (———) donation to Z^+ in NH_2OH-extracted chloroplasts.

TABLE III

RELEASE OF POLYPEPTIDES AND MANGANESE FROM THE PS II COMPLEX BY NH_2OH

PS II membranes (6 mg Chl/30–35 mg protein) were treated for 20 min in the dark at 4°C with the indicated concentrations of NH_2OH; the 2 M NaCl extraction was carried out at 4°C for 1 h. Manganese was quantitated by EPR.

Treatment	O_2 rate (μmol/h per mg Chl	Polypeptides released			Mn^{2+}/250 Chl
		33 kDa	23 kDa	17 kDa	
–	600	–	–	–	4
2 M NaCl	128	–	++	++	3.9
5 mM NH_2OH (pH 7.5)	0	+	++	++	1.6
10 mM NH_2OH (pH 6.0	0	+	++	++	2.0

inhibited chloroplasts [27]. We also used benzidine as a donor in Tris as well as NH_2OH treated PS II preparations, with ferricyanide and ferrocyanide as an acceptor system, and we found that benzidine reduces Z^+ directly with a second order rate constant similar to that observed for chloroplasts (data not shown).

We have previously shown that several conditions (exposure to high pH, to 0.8 M Tris, or to 2 M NaCl) will release polypeptides from the PS II complex [11]. High salt exposure releases only the 23 and 17 kDa species, whereas high pH or Tris releases the 33 kDa species as well as the 23 and 17 kDa polypeptides. We have extended these investigations to NH_2OH; Table III summarizes the effects of this inhibitor on the PS II complex with respect to polypeptide release as well as with respect to the release of functional manganese. Data from an experiment with 2 M NaCl are also shown. As these data demonstrate, exposure to NH_2OH at two pH values causes a release of the 33 kDa polypeptide from the PS II complex. Fig. 3 presents the gel electrophoresis patterns of control, NH_2OH (pH 6) and 2 M NaCl treated membranes. Lane 3 shows the polypeptide banding pattern of a concentrated supernatant from the NH_2OH extraction experiment. In the NH_2OH-treated sample, the staining intensity of the 33 kDa polypeptide is decreased, and this polypeptide appears in the concentrated extract shown in lane 3. In lane 3 of the gel (Fig. 3) several higher molecular weight proteins are seen, which arise from aggregation of the 23 and 17 kDa proteins in the low ionic strength NH_2OH extract. This aggregation is not observed in concentrated extracts of the 17 and 23 kDa proteins released from PS II membranes by 2 M NaCl (data not shown). These data along with those of Fig. 2 confirm the similarities of Tris and NH_2OH action on the oxygen-evolving complex, namely manganese release, enhanced access of exogenous donors to Z^+, and, in purified PS II preparations, the release of polypeptides required for oxygen-evolution activity. This latter observation would suggest that in intact thylakoid membranes Tris and NH_2OH may not only cause release of manganese, but may also cause a partial rearrangement in the membrane of the polypeptides required for oxygen evolution.

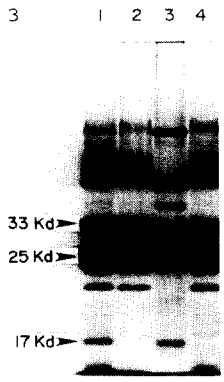

Fig. 3. Gel electrophoresis patterns of (1) the untreated PS II complex; (2) after exposure to 10 mM NH_2OH (pH 6); (3) concentrated NH_2OH extract; and (4) membranes exposed to 2 M NaCl (pH 6). Electrophoresis was carried out as described by Chua [28]. PS II samples: 30 μg Chl per lane; extract volume in lane 3 equivalent to material derived from 90–100 μg Chl.

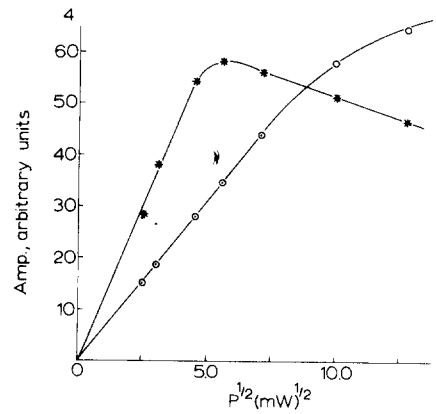

Fig. 4. Effect of microwave power (P) on the amplitude of the EPR signal of Z^+ (Amp) in Tris/EDTA (*)- and high-salt/EDTA (⊙)-extracted PS II particles. The chlorophyll concentration was 2.8 mg/ml, the instrument time constant was 100 ms and the modulation amplitude was 4 Gpp.

Mn quantitation by EPR has shown that high-salt treatment of the PS II complex does not release functional manganese [11]. Carrying out the high salt treatment in the presence of EDTA gave the same result (data not shown). Since in oxygen-evolving chloroplasts manganese apparently acts as a paramagnetic relaxing species in its interaction with Z^+ [29], we have studied the microwave power saturation and kinetic behavior of Z^+ in high-salt extracted PS II preparations. As shown in Fig. 4 the EPR signal of Z^+ in the PS II

preparation after salt extraction retains high microwave power saturation properties as opposed to the lower microwave power required to saturate the signal after Tris or NH_2OH inhibition. This observation suggests that after high-salt extraction, manganese continues to act as a relaxing species in a manner similar to that observed in untreated or ammonia inhibited preparations [1,29], and implies that manganese has not been disturbed drastically by the high salt treatment.

A kinetic study of Z^+ after extraction of the 17 and 23 kDa polypeptides was carried out under single turnover flash conditions. The results obtained by signal-averaging individual samples are shown in Fig. 5. The trace in Fig. 5a was obtained as the average of 200 flashes with 3.5 s between each flash. The signal amplitude is only 50–60% of that observed in Tris-washed chloroplasts under the same instrumental conditions and indicates that only approx. 0.6 Z^+ spins per P-680 are detected with 1 ms time resolution in salt-extracted preparations. Increasing the time resolution to 100 μs did not result in an increase in the number of Z^+ spins detected. The decay of Z^+ in Fig. 5a is strongly biphasic, with kinetic components of $t_{1/2}^{(1)} = 25$ ms and $t_{1/2}^{(2)} = 800$ ms with both phases sensitive to the presence of EDTA. As shown in Fig. 5b addition of EDTA slows down the decay dramatically (Fig. 5b).

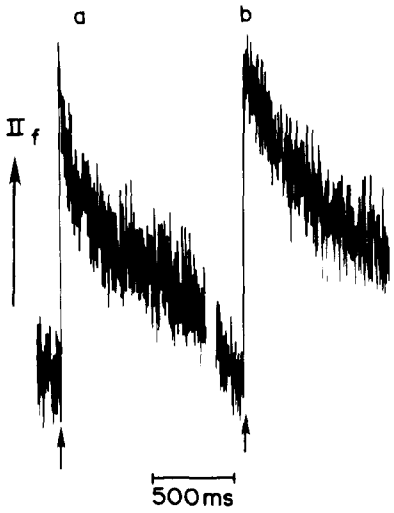

Fig. 5. Kinetic transients for Z^+ at room temperature in a high-salt-treated PS II preparation at pH 7.5. An equimolar mixture of ferricyanide and ferrocyanide was used as an acceptor system. (a) No further addition, (b) 1 mM EDTA. Each kinetic trace is the average of 200 flashes. Time constant, 1 ms; dark time between flashes t_d, 3.5 s.

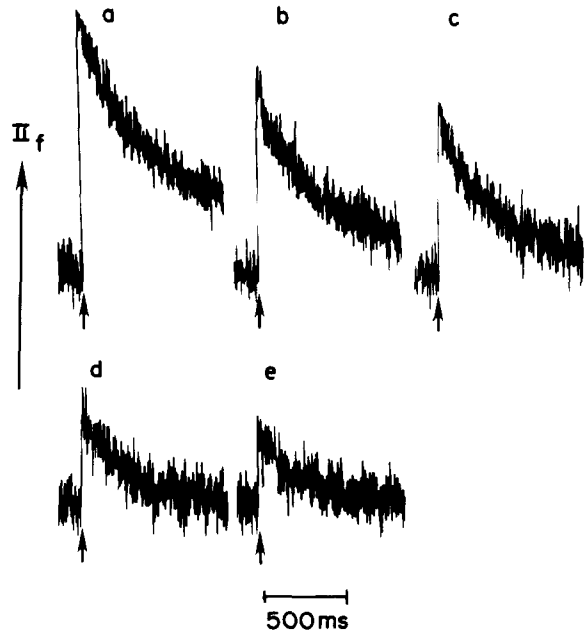

Fig. 6. The effect of benzidine on Signal II_f in a high-salt/EDTA-treated PS II preparation. No benzidine was added (a); 15 μM (b), 30 μM (c), 60 μM (d) and 120 μM benzidine (e) was added. A time constant of 1 ms was used in all five experimental traces which are each the average of 200 flashes given at a repetition rate of 0.25 Hz. An equimolar mixture of ferricyanide and ferrocyanide was used as an acceptor system.

When the exogenous donor benzidine is added (Fig. 6), the signal amplitude declines markedly. We have explored this effect of benzidine, which is radically different from the direct, second-order kinetics it exhibits in its reaction with Z^+ in Tris or NH_2OH – extracted chloroplasts, in more detail. The benzidine concentration titration is shown in Fig. 6 and demonstrates that the Z^+ signal amplitude declines progressively as benzidine concentration increases (titration of the signal in the absence of EDTA gave similar results). Fig. 7 shows that benzidine was more effective in decreasing the amplitude and the decay time when longer times between flashes were used. These results suggest that benzidine is able to reduce a component in the dark time between flashes which is then able to rereduce Z^+ following its generation in a time fast

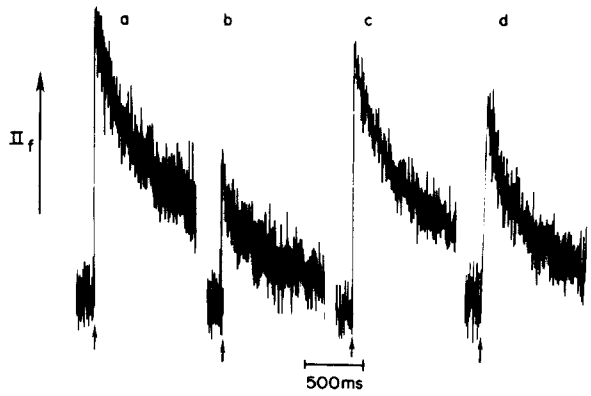

Fig. 7. Kinetic transients of Z^+ in high-salt/EDTA-treated PS II preparation. (a) No further addition, $t_d = 9$ s, (b) 30 μM BZ, $t_d = 9$ s, (c) no further addition, $t_d = 3.5$ s and (d) 30 μM BZ, t-3.5 s. Each kinetic trace is the average of 200 flashes. Time constant, 1 ms. An equimolar mixture of ferricyanide and ferrocyanide was used as an acceptor system.

compared to the detection time of our instrument, i.e.:

$$BZ_{red} + D_{ox} \overset{slow}{\rightarrow} BZ_{ox} + D_{red} \quad (1)$$

$$D_{red} + Z^+ \overset{fast}{\rightarrow} D_{ox} + Z \quad (2)$$

where D is the proposed intermediate reductant. Since manganese continues to interact closely with Z^+ in salt extracted PS II preparations, as shown above by the power saturation properties of the EPR signal of Z^+, it could very well serve as the donor, D_{red}, to Z^+.

To test the hypothesis outlined above further, we perturbed the manganese by using NH_3 (see Ref. 1). As shown in Fig. 8c and d, even high concentrations of benzidine have no effect on the decay time of Z^+ in the presence of 200 mM NH_3. The fact that amines only bind to the higher S-states [25] provides a further insight into the nature of the D-species in Eqs. 1 and 2 above. If D_{ox} corresponds to the manganese complex in the higher oxidation states, then the role of benzidine would be to reduce these states to the lower ones. Ammonia binding would then inhibit this process by stabilizing the higher oxidation states and thus prevent the action of the reductant. The corollary to this hypothesis is that the lower oxidation states of the complex reduce the Z^+ species in salt-extracted preparations in a time faster than the available instrument time resolution, analogous to the situation in O_2-evolving chloroplasts [30]. This suggestion is consistent with the fact that we observe only approx. 0.6 Z^+ spins per P-680 under steady-state conditions (see above) in these salt-extracted membranes. To explore this possibility we studied the behavior of Z^+ in dark-adapted samples subjected to a series of four saturating flashes. The results of such an experiment show that no signal, other than a small increase in Signal II_s, is observed on the first flash but that Z^+ does develop on subsequent flashes (data not shown). From these results the following model can be constructed for the behavior of Z^+ and manganese involved in the water-splitting complex following the release of the 17 and 23 kDa polypeptides:

$$(Mn)_x^{low} + Z^+ \rightarrow (Mn)_x^{high} + Z \qquad t_{1/2} < 100\ \mu s \quad (3)$$

$$(Mn)_x^{high} + BZ_{red} \rightarrow (Mn)_x^{low} + BZ_{ox} \qquad \text{slow} \quad (4)$$

The role of benzidine in this model is to convert the higher oxidation states of the manganese complex to the lower ones which leads to elimination of the ability to detect Z^+ on the subsequent flash. The NH_3 effect (presented in Fig. 8) is explained in this model as preventing the above conversion. It is possible that NH_3 binding blocks electron transfer from benzidine to manganese either by changing the redox properties of the manganese complex so that benzidine becomes an ineffective donor or by altering the electron transfer properties of the manganese complex itself. The overall effect of polypeptide extraction then is manifested on the higher oxidation states of the oxygen-evolving complex with minimal perturbation occurring to the lower ones. The interpretation is consistent with our earlier suggestions [15] as well as those made by Wensink and Van Gorkom [31] and by Åkerlund [32]. An important alternative to reaction 3 above has been pointed out by Bouges-Bocquet [33] who suggested that the transitions $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ proceed through an alternative intermediate which reacts in a pathway parallel to the Z-pathway for the higher S-states. Within the context of the results presented here, such a model predicts identical results.

The data of Fig. 6 can be used to estimate the

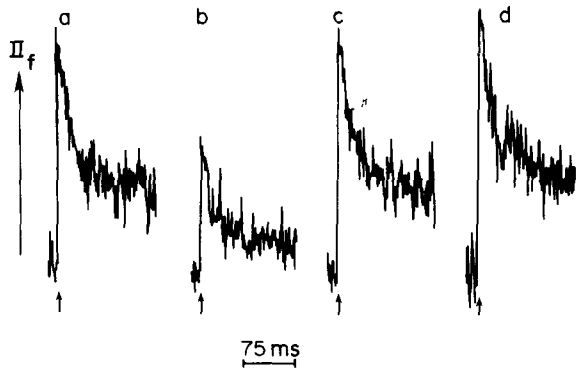

Fig. 8. Kinetic transients for Z^+ at room temperature in high-salt (2 M NaCl, pH 6.0)-treated PS II preparation at pH 7.5. An equimolar mixture of ferricyanide and ferrocyanide (3 mM each) was used as an acceptor system. (a) No further addition, (b) 50 μM benzidine, (c) 200 mM NH_3 and (d) 200 mM NH_3 and 100 μM benzidine. Each kinetic trace is the average of 200 flashes. Time constant, 0.5 ms and the dark time between flashes t_d, 5 s.

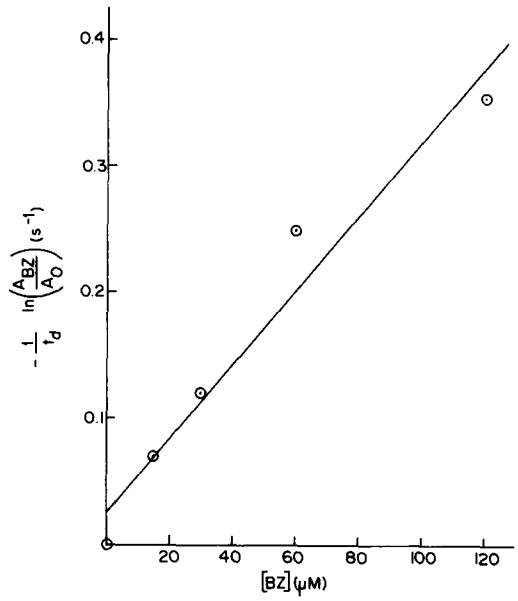

Fig. 9. Graphical determination of the rate constant of $(Mn)_x^{high}$ reduction by benzidine. The line is a fit of a least square analysis; see text for details.

rate constant for the reaction between BZ and $(Mn)_x^{high}$ in Eqn. 4. If we assume that the reaction proceeds with pseudo-first-order conditions, which is reasonable given the benzidine and oxygen-evolving complex concentrations in these experiments, then the rate of disappearance of $(Mn)_x^{high}$ is given by:

$$-\frac{d\left[(Mn)_x^{high}\right]}{dt} = k[BZ]\left[(Mn)_x^{high}\right] = k'\left[(Mn)_x^{high}\right] \quad (5)$$

Integration followed by equating the $(Mn)_x^{high}$ concentration of time zero to the amplitude of Z^+ detected in the absence of benzidine and the $(Mn)_x^{high}$ concentration of time t_d (in Fig. 6, $t_d = 4$ s) at the various benzidine concentrations to the Z^+ amplitude detected, yields the following expression:

$$-\frac{1}{t_d}\ln\frac{A_{BZ}}{A_0} = k' = k[BZ] \quad (6)$$

where A_{BZ} is the initial Z^+ amplitude in the presence of a given benzidine concentration, A_0 is the initial Z^+ amplitude detected in the absence of benzidine, t_d is the dark time between flashes and k is the second order rate constant for Eqn. 4. In Fig. 9 we have plotted the data of Fig. 6 according to Eqn. 5. From the slope we estimate a value for k of $2.9 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. A similar analysis applied to the data of Fig. 7 yields estimates of k of $(2.5–3.0) \cdot 10^3$ $M^{-1} \cdot s^{-1}$, which is in good agreement. An important point in these experiments is that the rate constant is more than two orders of magnitude slower than we observe for the reaction of benzidine with Z^+ directly in Tris-washed or hydroxylamine treated preparations (see above). Thus, although it appears according to this analysis, that salt extraction 'opens' the oxidizing side of PS II to the action of exogenous reductants, the reactions are considerably more sluggish than when one also removes the 33 kDa polypeptide.

Since ADRY reagents are known to accelerate the deactivation of the higher oxidation states of the oxygen-evolving complex [34], we also studied the behavior of high salt treated PS II preparations in the presence of CCCP, which is an effective ADRY reagent. Addition of low concentrations of CCCP (5–10 μM) results in a decrease in the amplitude of the EPR signal of Z^+ in the millisecond range (data not shown). This observation supports further the model proposed above for the effect of benzidine in high-salt-treated PS II preparations.

Discussion

Isolation of the PS II complex from thylakoid membranes provides a powerful means for examining the catalytic and structural properties of the oxygen-evolving reaction in closer detail. When viewed as a straightforward biochemical procedure, the isolation protocol we have developed for the PS II complex yields a 2-fold purification of oxygen evolution activity on a chlorophyll basis. This doubling of specific activity also yields an enrichment of Mn^{2+} and Signal II although on the same basis, we find only one cytochrome *b*-559. The extent to which quantitation of these components by various techniques agree suggests to us that a functional oxygen-evolving complex requires four Mn atoms, and that one cytochrome *b*-559 and one Signal II_s species are also closely associated with the complex. The Mn^{2+} stoichiometry observed in the PS II complex is consistent with our earlier results (four Mn^{2+} per 400 Chl) obtained with intact thylakoid membranes [5], and is at variance with the lower stoichiometries reported for other preparations possessing reduced oxygen-evolving activity [22].

Our examination of the requirements for optimal rates of O_2 evolution by the isolated PS II complex have shown that this reaction requires a lowered pH in the assay medium as well as the presence of a class III acceptor (a *p*-benzoquinone). The latter observation indicates that the reducing side of PS II in the complex is not sufficiently accessible to ferricyanide to permit high steady-state rates of O_2 evolution. The observation that maximum O_2 evolution activity in the isolated complex occurs near pH 6 may reflect the true optimum for PS II electron transport.

Our gel electrophoresis experiments combined with Mn quantitation by EPR indicate that polypeptides with molecular weights of 17000, 23000 and 33000 are important for O_2 evolution and in agreement with other results [13], we have shown that extraction of the 17 and 23 kDa polypeptides from the PS II complex can be achieved without loss of Mn^{2+}. Treatments which remove functional manganese (Tris and NH_2OH extractions) also result in partial extraction of the 33 kDa polypeptide. Since the isolated 33 kDa polypeptide contains no manganese (data not shown) we have tried to probe further the organization of manganese and the three polypeptides mentioned above by studying the kinetic behavior of Z^+. The various treatments which have been used to inactivate O_2 evolution capacity can be separated into three classes according to their effect on manganese content, Z^+ behavior and polypeptide compositions, as described below.

Class A

(1) Extraction with NH_2OH in the dark, (2) extraction with a high concentration of Tris-buffer in the light and (3) high pH. These treatments perturb the pool of functional manganese and also extract the 17, 23 and 33 kDa polypeptides from the PS II complex. After these treatments we observe full Z^+ magnitude and the radical saturates at low (approx. 25 mW) microwave power levels. The lifetime of the radical, Z^+ is extended into a time-range of hundreds of milliseconds and is decreased upon addition of exogenous reductants by a direct, second order reaction [34].

Class B

Treatment with various amines. This treatment inhibits oxygen evolution but releases neither manganese nor the 17, 23 and 33 kDa polypeptides. Only partial development of Z^+ is observed by EPR. The radical shows high (approx. 100 mW) microwave power saturation properties and is inaccessible to exogenous donors [1].

Class C

High-salt (2 M NaCl) incubation at pH 6. This treatment selectively removes the 17 and 23 kDa polypeptides but does not release functional manganese or the 33 kDa polypeptide. Only partial development of Z^+ is observed and the species exhibits high microwave power saturation (Fig. 4). The kinetic study of Z^+ under these conditions suggests that the manganese complex can still function as a donor system to Z^+. The absence of the Z^+ signal on the first flash, in a four flash experiment, could be the result of a very fast reduction of Z^+ by the manganese complex (see Ref. 30), whereas the effect of benzidine could be attributed to the reduction of the manganese complex to lower oxidation states. Such a model would also explain the NH_3 results of Fig. 8 and the fact

that the benzidine effect depends on the time between flashes.

The controlled disintegration of the oxygen-evolving complex with concurrent analyses of Z^+ rereduction kinetics which is described in this communication permits us to identify more precisely the interrelationships between manganese, water-soluble polypeptides and Z that constitute the structural-catalytic assembly responsible for oxygen evolution activity. The 17 and 23 kDa polypeptides appear to have a structural rather than a catalytic role in O_2 evolution activity. Removal of these polypeptides results in a condition whereby exogenous donors, such as benzidine, have ready access to components involved in the water-splitting process (either the manganese complex or Z itself). In contrast, exposure to Tris or NH_2OH liberates manganese as well as water soluble polypeptides (including some of the 33 kDa species), destroys the magnetic interaction between manganese and Z^+, and accelerates Z^+ reduction by added exogenous donors. This result, added to the findings of other investigators, would support the assignment to the 33 kDa polypeptide of a role in structural organization of the oxidizing side of PS II which promotes the productive binding of manganese to sites in close proximity to Z, which in turn makes possible the linear four-step oxidation of water to molecular oxygen.

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References

1 Ghanotakis, D.F., O'Malley, P.J., Babcock, G.T. and Yocum, C.F (1983) in The Oxygen-Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 87–98, Academic Press, Tokyo

2 O'Malley, P.J. and Babcock, G.T. (1984) Biochim. Biophys. Acta 765, 370–379

3 Babcock, G.T. and Sauer, K. (1975) Biochim. Biophys. Acta 376, 315–328

4 Yocum, C.F. and Babcock, G.T. (1981) FEBS Lett. 130, 99–102

5 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7507–7511

6 Kelly, P.M. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210

7 Sandusky, P.O. and Yocum, C.F. (1983) FEBS Lett. 162, 339–343

8 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234

9 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268

10 Kuwabara, T. and Murata, N. (1982) Plant Cell Physiol. 23, 533–539

11 Sandusky, P.O., Selvius DeRoo, C.L., Hicks, D.B., Yocum, C.F., Ghanotakis, D.F. and Babcock, G.T. (1983) in The Oxygen-Evolving System of Photosynthese (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 183–194, Academic Press, Tokyo

12 Kuwabara, T. and Murata, N. (1983) in The Oxygen-Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 217–222, Academic Press, Tokyo

13 Åkerlund, H.E., Jansson, C. and Andersson, B. (1982) Biochim. Biophys. Acta 681, 1–10

14 Babcock, G.T., Buttner, W.J., Ghanotakis, D.F., O'Malley, P.J., Yerkes, C.T. and Yocum, C.F. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 243–252, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague

15 Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 279–282, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague

16 Yocum, C.F., Sandusky, P.O., Ghanotakis, D.F. and Babcock, G.T. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 341–344, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague

17 Robinson, H.H., Sharp, R.R. and Yocum, C.F. (1981) Arch. Biochem. Biophys. 207, 1–8

18 Ghanotakis, D.F. and Babcock, G.T. (1983) FEBS Lett. 153, 231–234

19 Ford, R.C. and Evans, M.C.W. (1983) FEBS Lett. 160, 159–164

20 Horon, P. and Croze, E. (1977) Biochim. Biophys. Acta 462, 86–101

21 Lam, E., Baltimore, B., Ortiz, W., Chollar, S., Melis, A. and Malkin, R. (1983) Biochim. Biophys. Acta 724, 201–211

22 Yamamoto, Y. and Nishimura, M. (1983) Biochim. Biophys. Acta 724, 294–297

23 Cheniae, G.M. and Martin, I.F. (1971) Plant Physiol. 47, 567–575

24 Sharp, R.R. and Yocum, C.F. (1981) Biochim. Biophys. Acta 635, 90–104

25 Velthuys, B.R. (1975) Biochim. Biophys. Acta 396, 392–401

26 Frasch, W. and Cheniae, G.M. (1980) Plant Physiol. 65, 735–745

27 Yerkes, C.T. and Babcock, G.T. (1980) Biochim. Biophys. Acta 590, 360–372
28 Chua, N.H. (1980) Methods Enzymol. 69, 434–446
29 Warden, J.T., Blankenship, R.E. and Sauer, K. (1976) Biochim. Biophys. Acta 423, 462–478
30 Babcock, G.T., Blankenship, R.E. and Sauer, K. (1976) FEBS Lett. 61, 286–289
31 Wensink, J. and Van Gorkom, H.J. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 299–302, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
32 Åkerlund, H.E. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. I, pp. 391–394, Martinus Nijhoff/Dr. W. Junk Publishers, The Hague
33 Bouges-Bocquet, B. (1980) Biochim. Biophys. Acta 594, 85–102
34 Ghanotakis, D.F., Yerkes, C.T. and Babcock, G.T. (1982) Biochim. Biophys. Acta 682, 21–31