Biochimica et Biophysica Acta, 723 (1983) 276–286
Elsevier

BBA 41302

ELECTRON DONATION TO PHOTOSYSTEM II IN REACTION CENTER PREPARATIONS

GERALD T. BABCOCK [a], DEMETRIOS F. GHANOTAKIS [a], BACON KE [b] and BRUCE A. DINER [c]

[a] *Department of Chemistry, Michigan State University, East Lansing, MI 48824-1322,* [b] *Charles F. Kettering Research Laboratory, Yellow Springs, OH 45387 (U.S.A.) and* [c] *Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris (France)*

(Received March 2nd, 1983)

Key words: Photosystem II; ESR; P-680; Reaction center; Photosynthesis; (Spinach, Pokeweed, C. reinhardii)

The room-temperature EPR characteristics of Photosystem II reaction center preparations from spinach, pokeweed and *Chlamydomonas reinhardii* have been investigated. In all preparations a light-induced increase in EPR Signal II, which arises from the oxidized form of a donor to P-680 $^+$, is observed. Spin quantitation, with potassium nitrosodisulfonate as a spin standard, demonstrates that the Signal II species, Z $^+$, is present in approx. 60% of the reaction centers. In response to a flash, the increase in Signal II spin concentration is complete within the 98 μs response time of our instrument. The decay of Z $^+$ is dependent on the composition of the particle suspension medium and is accelerated by addition of either reducing agents or lipophilic anions in a process which is first order in these reagents. Comparison of these results with optical data reported previously (Diner, B.A. and Bowes, J.M. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 3, pp. 875–883, Balaban, Philadelphia), supports the identification of Z with the P-680 $^+$ donor, D_1. From the polypeptide composition of the particles used in this study, we conclude that Z is an integral component of the reaction center and use this conclusion to construct a model for the organization of Photosystem II.

Introduction

Significant progress has been made in the biochemical resolution of PS II. Techniques for the isolation of membrane fractions which are devoid of PS I but which retain high rates of oxygen evolution have been reported recently [1–5]. These preparations complement the smaller, nonoxygen-evolving, but more highly resolved Photosystem II particles (e.g., see Refs. 6–8) which have been available for a somewhat longer time. With the availability of these preparations, it becomes possible to identify the various PS II electron-transfer cofactors, which have been characterized primarily by biophysical techniques, with specific polypeptides in the membrane complex. This approach, in combination with mutant studies [9,10], has been used to specify the polypeptides which bind the reaction center components of the photochemical core of PS II. It has also been possible to link the secondary acceptor, B, on the reducing side of PS II to its polypeptide-binding site [11]. A review of recent developments in this area is available [12].

In the experiments reported here, we have extended this approach to the oxidizing side of PS II by quantifying and characterizing EPR Signal II species which occur in small PS II reaction center

Abbreviations: ADRY, accelerator of the deactivation reactions of the water-splitting system Y; ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PS, photosystem; Chl, chlorophyll.

particles. This free radical arises from the oxidized form of a species, apparently a quinone [13], which serves to mediate electron transfer between the PS II reaction center chlorophyll, P-680, and the oxygen-evolving complex [14,15]. Recent kinetic results [16] indicate that Z can be identified with D_1, the immediate electron donor to $P\text{-}680^+$, in a variety of chloroplast preparations [17]. Optical studies of P-680 absorbance changes in reaction center preparations indicate the occurrence of D_1, and by inference Z, in these particles [18]. It thus becomes possible to test the conclusions reached by kinetic techniques by investigating the EPR properties of the reaction center preparations. Moreover, because the protein composition of one of the preparations we have used is well characterized, it is possible to identify likely Z-binding polypeptides. Our results confirm the identification of D_1 with Z and show that this species is an integral component of the reaction center complex.

Materials and Methods

Spinach chloroplasts were isolated and Tris-washed as described in [19]; oxygen-evolving Photosystem II fragments were isolated from spinach thylakoids following the procedures given in [2]. Triton-fractionated subchloroplast fragments (TSF-IIa particles) enriched in the reaction center components of PS II were isolated either from spinach or from pokeweed [6,20]. PS II particles were isolated from mutant F54-14 of *Chlamydomonas reinhardii* by using the digitonin and Triton X-100 solubilization procedure outlined in Ref. 8; these are referred to in the text as '*Chlamydomonas* particles'. Further fractionation of these particles to yield '*Chlamydomonas* DEAE particles' is also described in Ref. 8. All four types of PS II particles, the O_2-evolving and the three reaction center preparations, were essentially devoid of P-700 contamination as judged by the absence of any Signal I ($P\text{-}700^+$) formation upon illumination (see Fig. 1). The buffer system in which the preparations were suspended contained 0.4 M sucrose, 0.05 M Hepes, 0.01 M NaCl and 0.1 mM EDTA which was adjusted to pH 7.6. An acceptor system consisting of 5 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$ and 25 mM $MgCl_2$ was added to maintain electron flow through the PS II reaction center. The DEAE particles also contained 80 mM NaCl and 30 mM imidazole (final concentrations) as a result of the elution buffer used in their preparation. Other additions are noted in the figure captions. Chlorophyll concentrations were determined as described by Sun and Sauer [21]. Reagents were obtained from standard commercial sources and were used without further purification.

The spin standard, potassium nitrosodisulfonate ($K_2(SO_3)_2NO$), was obtained from Aldrich Cehmical Co. and dissolved, without further purification, in 0.05 M K_2CO_3 aqueous buffer previously saturated with gaseous nitrogen. The concentration of the standard solution was determined optically before and after EPR measurements by using an extinction coefficient of 20.8 $M^{-1} \cdot cm^{-1}$ at 545 nm [22]. Solutions prepared in this way were fairly stable, the concentration of $K_2(SO_3)_2NO$ decreasing to one-half its original value in about 3 weeks when the stock solution was stored in a stoppered flask in the dark. Spin quantitation experiments were also carried out in which the $K_2(SO_3)_2NO$ standard was dissolved in deaerated 0.4 M sucrose, 0.05 M Hepes, 0.01 M NaCl, pH 7.4. Although the much more rapid decay of the free radical at this lower pH ($t_{1/2} \approx$ 1.5 h) introduced greater uncertainty, values for the EPR signal intensities at given spin concentrations were similar to those obtained in the carbonate buffer system.

EPR spectroscopy was carried out on a Bruker ER 200D spectrometer operated at X-band. The instrument modifications necessary to permit efficient kinetic operation are described in Ref. 23 as are the xenon flashlamp circuitry and the protocol followed in signal-averaged, flashing-light kinetic experiments. Continuous illumination was provided by a Bausch and Lomb microscopic illuminator. Signal averaging in kinetic experiments and integration in spin concentration determinations were carried out in the Nicolet 1180 computer which is interfaced to the spectrometer.

Light-induced carotenoid optical absorbance changes were measured by using a flash-detection spectrophotometer similar to that described by Joliot et al. [24]. The actinic flashes (xenon flashlamp, 1 μs width at half-height) were saturating and filtered by a Schott RG2 filter. The detection flashes were centered at 490 mm (band pass

2–4 nm at half-height) and were also approx. 1 μs at half-height. The measurements of $\Delta I/I$ were made at 500 μs after the actinic flash.

Results

Obsrevation and quantitation of Signal II

Fig. 1 shows EPR spectra of Signal II in Tris-washed chloroplasts (a), in pokeweed TSF-IIa particles (b), and in normal and DEAE *Chlamydomonas* particles (c and d). Qualitatively, all four samples show the same behavior. In the dark, there is a stable free radical signal which increases by a factor of about two upon illumination. The absence of PS I in the particles is evident in that the strong P-700$^+$ free radical signal which dominates the central region of the spectrum in the Tris-washed chloroplast sample is not observed in the particle preparations. The slight shift in g value observed in the *Chlamydomonas* samples upon illumination probably arises from the generation of a small amount of oxidized chlorophyll or

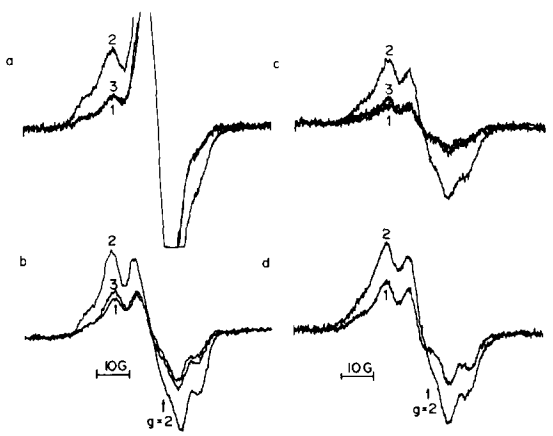

Fig. 1. Dark and light-induced, room-temperature EPR spectra in several membrane preparations: (a) Tris-washed spinach chloroplasts (2.76 mg Chl/ml), instrument gain = $1 \cdot 10^6$; (b) pokeweed TSF-IIa particles (0.69 mg Chl/ml), instrument gain = $0.8 \cdot 10^6$; (c) *Chlamydomonas* PS II particles (0.53 mg Chl/ml), instrument gain = $1.25 \cdot 10^6$; (d) *Chlamydomonas* PS II DEAE particles (0.44 mg Chl/ml), instrument gain = $1 \cdot 10^6$. A modulation amplitude of 4 G and a time constant of 0.2 s were used in recording all spectra. For a–d, the spectrum labeled 1 was recorded in the dark, spectrum 2 was recorded during continuous illumation of the sample and spectrum 3 (in a–c) was recorded immediately following continuous illumination.

possibly oxidized carotenoid [25] (less than 1% of the concentration of Signal II) in the light. For the particle samples, we found that maximal generation of the light-induced signal required the $K_3Fe(CN)_6$, $K_4Fe(CN)_6$ and $MgCl_2$ acceptor system described above. This observation can be rationalized by the results of Diner and Bowes [18] who showed that the addition of these reagents was required for efficient reoxidation of Q. Thus, in the presence of this acceptor system we expect and observe maximal formation of the oxidized form of the donor, Z, which gives rise to Signal II.

The observation of Signal II in the particle preparations, particularly in the biochemically well characterized *Chlamydomonas* preparations, suggests that the donor Z is an integral component of the PS II reaction center complex. This possibility is supported by the observation of a Signal II species at low temperature in the *Chlamydomonas* particles [26] and at room temperature in a spinach PS II particle preparation [27] and, further, by optical work indicating the presence of D_1 (which has been identified recently with Z [16]) in both *Chlamydomonas* [18] and spinach [28] particles. If this conclusion is correct, then we expect that the concentration of Z in the particles will be approximately stoichiometric with the P-680 concentration. We have explored this by carrying out spin concentration determination for Signal II in a variety of membrane preparations.

In unfractionated thylakoid membranes, Babcock and Sauer [29] used P-700$^+$ as a spin standard and showed that Signal II was approximately (±50%) stoichiometric. A better approach is to use an external standard that allows for absolute spin quantitation. Fremy's salt ($K_2(SO_3)_2NO$) is a useful choice of a standard in that it can be quantified optically and is water soluble [30] (see above). Because spin quantitation by EPR is somewhat prone to error, we have developed a fairly detailed protocol for our quantitation procedure. First, we determined that both Fremy's salt and the Signal II species in the various preparations show EPR signals which are linear in the square root of applied microwave power to at least 5 mW. Consequently, we choose this power for our quantitation studies. Second, the linear relationship between H_1 modulation ampli-

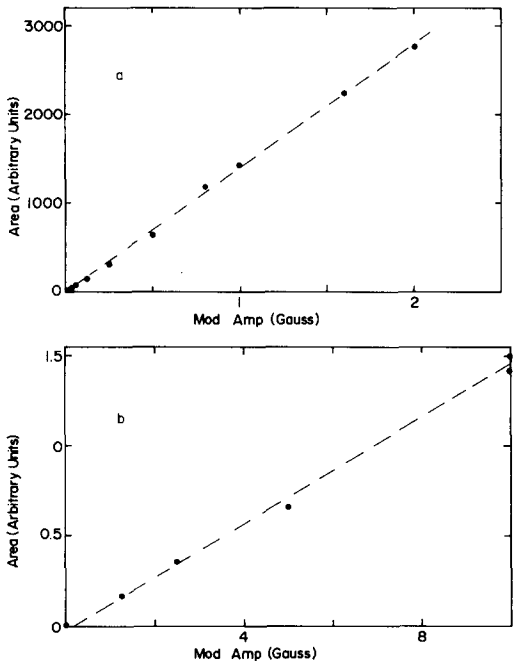

Fig. 2. Area under EPR absorption curves for: (a) $K_2(SO_3)_2NO$ and (b) Signal II (dark) in spinach chloroplasts as a function of modulation amplitude (Mod Amp). For $K_2(SO_3)_2NO$, the concentration was 3.00 mM, the sweep range was 10 G and only the central hyperfine line of the three line spectrum was integrated. For Signal II, the chlorophyll concentration was 4.11 mM, the sweep range was 100 G and the entire spectrum was integrated. The units of area for a and b are the same; the lines fitted to the data points are least-squares lines. For details of the quantitation procedure, see Ref. 30.

tude and the area under the EPR absorption curve [30], even under conditions of mild overmodulation, provides a convenient means by which to increase the accuracy of the determinations. Thus, we carried out double integration of the first-derivative EPR signal of Fremy's salt and of Signal II under a range of modulation amplitudes. By plotting the area vs. the modulation amplitude we are able to extract a least-squares value for area/gauss of modulation amplitude for the known and unknown species. Typical data for Fremy's salt and for Signal II in unfractionated membranes are shown in Fig. 2. Finally, from this value and the known concentration of $K_2(SO_3)_2NO$, we can determine the Signal II spin concentration in the membrane preparations (see legend to Fig. 2). These are given in terms of chlorophylls per spin in Table I for several different preparations. We have also expressed the ratio of Signal II spins per P-680 by using the estimated antenna sizes for the various particle preparations [8,20]. Two trends are clear from these data: (a) the reaction center particles are enriched in Signal II concentration compared to either the unfractionated membranes or to the O_2-evolving PS II preparation and (b) the Signal II_f spin concentration (i.e., that fraction of Signal II which is observed upon illumination) is approximately stoichiometric with P-680 con-

TABLE I

SIGNAL II (SII) SPIN QUANTITATION

Preparation	Chl [a]/ SII (dark)	Chl [b]/ SII (light)	SII (dark) [c]/ P-680	SII (light)/ P-680	SII (total)/ P-680
O_2-evolving chloroplasts	388 ± 28	d	1.03	d	1.03
Tris-washed chloroplasts	404	390	0.99	1.02	2.01
O_2-evolving spinach PS II particles	270	d	0.93	d	0.93
Pokeweed TSF-IIa	90	72	0.5	0.63	1.13
Chlamydomonas particle preparation 1	115	94	0.39	0.48	0.87
Chlamydomonas particle preparation 2	150	73	0.3	0.62	0.92
Chlamydomonas DEAE particles	52	66	0.86	0.68	1.54

[a] Chl per Signal II spin following 3 min dark period.

[b] Chl per Signal II spin induced by continuous illumination.

[c] Estimates of dark, light and total Signal II spins per P-680 in the various preparations were made by assuming 400 Chl per P-680 in unfractionated chloroplasts [19], 250 Chl per P-680 in the O_2-evolving PS II particles [2], 45 Chl per P-680 in the *Chlamydomonas* PS II reaction center preparations [8] and 45 Chl per P-680 in the TSF-IIa preparation (Ke, B., unpublished data).

[d] No continuous light-induced increase in Signal II spin concentration (see Ref. 14).

centration in the particles, thus providing additional support for the association of Z^+ with the reaction center complex. The decrease in spins per P-680 apparent in the particle preparations compared to unfractionated membranes is consistent with optical data on the *Chlamydomonas* particles [18] (see below).

Signal II flash kinetics in PS II reaction center preparations

By using a $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox buffer system and $MgCl_2$ to shield surface charges, it is possible to carry out repetitive-flash signal-averaged experiments on the transient kinetics of Signal II in the various particle preparations. Fig. 3 shows kinetic traces at the indicated field values relative to the Signal II spectrum in *Chlamydomonas* DEAE particles. The amplitude and sense of the signal change and the decay kinetics all indicate that the light-induced changes observed arise from Signal II. The slight, negative-going spike observed at field position C is reproducible

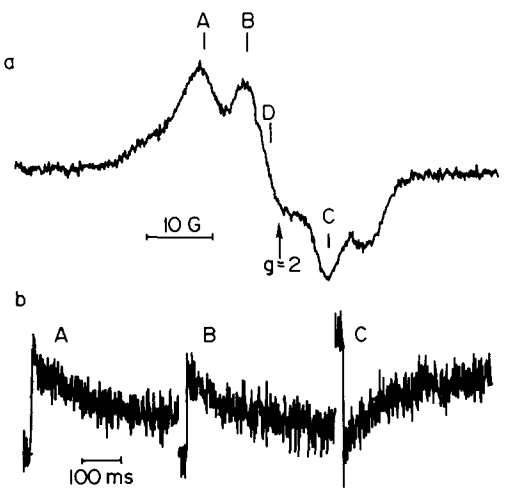

Fig. 3. Signal II kinetic transients as a function of magnetic field in *Chlamydomonas* DEAE PS II particles. The spectrum of Signal II recorded in the dark is shown in a; field positions at which the kinetic traces shown in b were recorded are indicated as points A, B and C on the spectrum. Field point D is the zero crossing of Signal II. For the spectrum, the following instrument parameters were used: 0.2 s time constant, 200 s sweep, 4 G modulation amplitude, 20 mW microwave power. For the kinetic traces, 200 flashes, given at a rate of 0.25 Hz, were averaged, the instrument time constant was 500 μs. DEAE particles containing 0.44 mg Chl/ml were used.

and apparently arises from the light-induced generation of another radical in the $g = 2$ region. Because the change is small and the spectrum of the radical is narrow (no spike is apparent at field set B), we have not pursued this in more detail. At the zero crossing of Signal II (position D in Fig. 3), we noted essentially no (approx. 10% the amplitude at A) flash-induced increase in EPR signal amplitude. Signal shape analysis of the light induced change in spinach TSF-IIa particles yielded results similar to those in Fig. 3 for the *Chlamydomonas* DEAE particles. A number of other experiments were carried out in order to determine the characteristics of Signal II transient kinetics in the reaction center preparations. The results (not shown) can be summarized as follows. (1) The rise time of the signal is instrument limited at 98 μs, consistent with the recent determination of the Signal II_f rise time in Tris-washed chloroplasts [16] and with the properties of D_1 in *Chlamydomonas* particles [18]. (2) The light-induced signal increase shows microwave power saturation in the 20 mW range. This behavior is characteristic of Signal II when the enhanced relaxation induced by a nearby paramagnet, presumably manganese [19], has been removed. Manganese determinations on intact and DEAE *Chlamydomonas* particles are consistent with this and show decreased levels of Mn per P-680 relative to those observed in untreated chloroplasts [19]: three determinations of Mn/P-680 in intact particles showed less than 2 Mn/PS II center (1.86 ± 0.4, 1.19 ± 0.1 and 0.27 ± 0.1 Mn/center); one measurement in DEAE particles gave 1.53 ± 0.14 Mn/P-680 (Diner, B., Nakatani, H. and Durosay, P., unpublished results). The relatively low power saturation observed in the particles, together with the lower manganese stoichiometry, is consistent with the reaction center nature of the preparation. (3) The flash-induced increase in Signal II saturates at incident light intensities which are comparable to those required to saturate Signal II_f in Tris-washed chloroplasts at comparable P-680 concentration. This indicates that Signal II responds with high quantum efficiency in the particle preparation. (4) The transient response of Signal II is fairly stable in the particle preparations. We noted, for example, decreases of approx. 25 and 50% of the initial flash amplitude after 600 flashes on *Chlamydomonas*

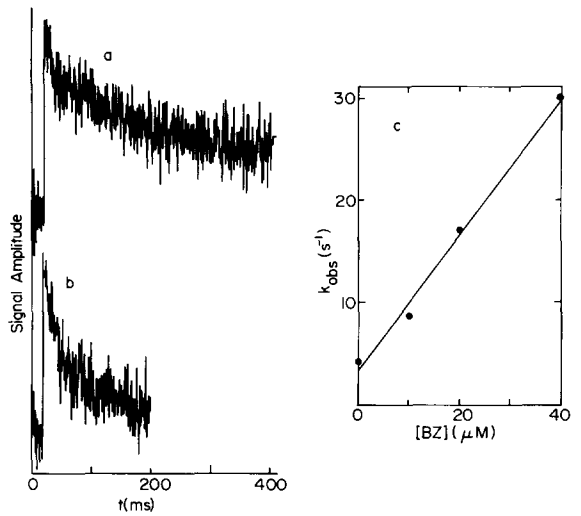

Fig. 4. Effect of donor addition on Signal II decay in *Chlamydomonas* DEAE PS II particles. The decay of Signal II in DEAE particles (0.44 mg Chl/ml) without (a) and with (b) the addition of 40 μM benzidine is shown. Each trace is the average of 200 flashes, given at a frequency of 0.25 Hz. Instrument conditions: 0.5 ms time constant, 4 G modulation amplitude, 20 mW microwave power, field set at field point A (see Fig. 3). In (c), the benzidine (BZ) concentration dependence of the observed decay rate constant is summarized.

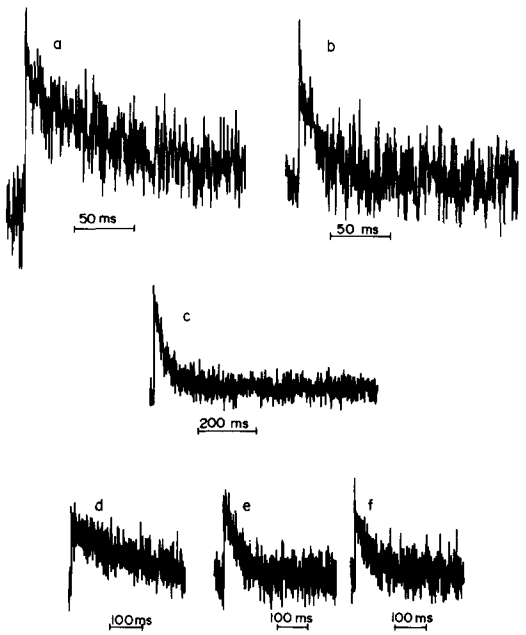

Fig. 5. Signal II kinetic transients in different PS II reaction center preparations. In a and b, *Chlamydomonas* particles (0.39 mg Chl/ml) were used. No further additions were made in a, overall decay half-time = 36 ms (see text); in b 40 μM benzidine was added as an electron donor, decay half-time = 5 ms. Each trace is the average of 200 flashes, the instrument time constant was 200 μs. In c spinach TSF-IIa particles (0.78 mg Chl/ml) were used and 40 μM benzidine was added as an electron donor. The decay half-time was 30 ms, the instrument time constant was 500 μs and 150 flashes were averaged. In d–f, pokeweed TSF-IIa particles (0.39 mg/Chl/ml) were used. In d no further additions were made, decay half-time = 170 ms; in e 20 μM benzidine was added, decay half-time = 45 ms; in f 40 μM benzidine was added, decay half-time = 22 ms. For these traces, an instrument time constant of 200 μs was used and 255 flashes were averaged. In traces a–f, a modulation amplitude of 5 G and microwave power of 20 mW were used. All kinetic traces were recorded at field setting A in Fig. 3.

DEAE particles and spinach TSF-IIa particles, respectively.

In Tris-washed chloroplasts, benzidine ($E_{m,7}$ = 0.55 V), is an effective donor to Signal II_f [31]. The data of Fig. 4 demonstrate that in PS II reaction center preparations this species is also an efficient Signal II reductant. In Fig. 4a, the decay of Signal II in *Chlamydomonas* DEAE particles is at least biphasic and shows an overall half-time of approx. 160 ms when only the $K_3Fe(CN)_6/K_4Fe(CN)_6$ buffer system is used. The kinetic trace in Fig. 4b shows that upon addition of 40 μM benzidine the decay of Signal II is markedly accelerated; the half-time is 23 ms. In Fig. 4c, we summarize the benzidine concentration dependence of the acceleration in Signal II decay. From the slope, a second-order rate constant of $7 \cdot 10^5\ M^{-1} \cdot s^{-1}$ can be obtained.

The behavior of benzidine as a reductant to Signal II is observed in the other PS II reaction center preparations. Kinetic traces showing the effect of benzidine at various concentration levels in *Chlamydomonas* particles and in spinach and pokeweed TSF-IIa particles are shown in Fig. 5; the decay half-times are given in the figure legend. The second-order rate constants which we determined from the data of Figs. 4 and 5 are collected in Table II. In general, the benzidine rate constant is similar for the various particles and close to that which we observed previously for benzidine in Tris-washed spinach chloroplasts [31]. The increased efficiency of benzidine in the *Chlamydomonas* particles was reproducible and thus appears to be a real effect. The origin of this phenomenon is unclear although it may represent

TABLE II

PS II KINETIC PARAMETERS - CHLOROPLASTS AND PARTICLES

Preparation	Reagent	k ($M^{-1} \cdot s^{-1}$)
Spinach chloroplasts Tris-washed [a]	benzidine	$1.3 \cdot 10^6$
Spinach TSF-IIa	benzidine	$6 \cdot 10^5$
Pokeweed TSF-IIa	benzidine	$8 \cdot 10^5$
Chlamydomonas particles	benzidine	$3 \cdot 10^6$
Chlamydomonas DEAE particles	benzidine	$7 \cdot 10^5$
Spinach chloroplasts Tris-washed [b]	ANT-2p	$2.7 \cdot 10^6$
Chlamydomonas particles	ANT-2p	$6 \cdot 10^6$
Chlamydomonas particles	tetraphenyl-boron	$9 \cdot 10^5$
Chlamydomonas DEAE particles	ANT-2p	$1.3 \cdot 10^6$

[a] From Ref. 31.
[b] From Ref. 23.

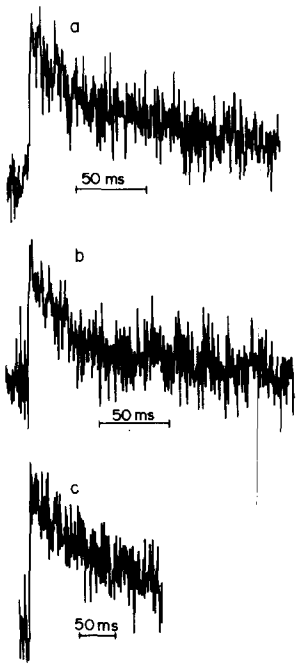

Fig. 6. The effect of lipophilic anions on the decay of Signal II in *Chlamydomonas* PS II particles and DEAE PS II particles. In a and b *Chlamydomonas* particles (0.44 mg Chl/ml) were used. In a 20 μM tetraphenylboron was added (decay half-time = 37 ms) and in b 5 μM ANT-2p was added (decay half-time = 23 ms). Instrument conditions; 200 μs time constant, 200 scans averaged. In c DEAE particles (0.40 mg Chl/ml), to which 5 μM ANT-2p was added, were used. The decay half-time is 95 ms. Instrument conditions: 500 μs time constant, 200 scans averaged. Other settings as in Fig. 5.

an increased concentration of the lipophilic benzidine species in the vicinity of Z in this preparation relative to the others rather than some more subtle kinetic effect.

An interesting aspect of the data of Figs. 4 and 5 is the decay half-times of the transient Signal II increase in the presence of only the $K_3Fe(CN)_6/K_4Fe(CN)_6$ redox buffer system. In the DEAE particles (Fig. 4a) this half-time is approx. 160 ms whereas in the parent normal *Chlamydomonas* particle (Fig. 5a) the decay is more markedly biphasic and shows an initial fast decrease which accounts for approx. one-third of the total decay (separate, higher resolution experiments show that this initial rapid decrease occurs with a half-time of approx. 1.5 ms) followed by a slower phase with a half-time of 65 ms. Diner and Bowes [18] deduced the same type of behavior for the reduction of D_1 in these two types of *Chlamydomonas* particles from their double-flash optical experiments on P-680$^+$ decay kinetics, i.e., that the rereduction of D_1 in the normal particles is significantly more rapid than is its rereduction in the DEAE-treated material. This observation provides additional support for the identification of D_1^+ with the Signal II species, Z^+.

Lipophilic anions, or ADRY reagents, have attracted renewed attention recently owing to the observation that upon addition of these species to chloroplast or subchloroplast preparations an oxidation of carotenoid, mediated by PS II, is observed [25,32]. They also appear to act as reductants to Z^+ in that the decay rate of Signal II_f in Tris-washed chloroplasts is increased by ADRY reagents in a manner which is first order in the ADRY reagent [23]. These observations have been difficult to reconcile because the two ADRY-induced phenomena, i.e., carotenoid oxidation and accelerated Z^+ reduction, do not appear to have the proper time courses necessary for a sequential kind of mechanism. In order to explore this further we have investigated the effects of ADRY reagent addition on Signal II behavior in *Chlamydomonas* particles where the carotenoid oxidation

reaction has been documented [25]. Fig. 6 shows the effect of 20 μM tetraphenylboron (a) or 5 μM ANT-2p (b) on Signal II amplitude and decay kinetics in normal *Chlamydomonas* particles and of 5 μM ANT-2p on Signal II in the DEAE-treated material (c). The signal amplitude in all three cases is 85–95% of that observed in the absence of the ADRY reagent. In the normal particles, the biphasic Signal II decay observed in the absence of the ADRY (e.g., Fig. 5a) is converted to what appears to be a monophasic decrease in signal amplitude. For tetraphenylboron we did not carry out a concentration dependence experiment but from Fig. 6a we estimate a second-order rate constant of $8 \cdot 10^5\ M^{-1} \cdot s^{-1}$ for the decay of Signal II in its presence. This and the second-order rate constants for ANT-2p in the *Chlamydomonas* particles are summarized in Table II.

In both the normal and DEAE-treated *Chlamydomonas* particles, we searched the $g = 2$ region unsuccessfully for an indication of the oxidized carotenoid free radical EPR signal. Our failure to observe this species may indicate that carotenoid oxidation in the presence of ADRY reagents is not a sustained reaction so that it becomes unobservable under our repetitive flash, signal-averaging conditions, or possibly that the species is relaxed by a nearby paramagnet so that it is not observed by room-temperature EPR. We have investigated these possibilities by carrying out experiments in which the amplitude of ADRY-induced carotenoid oxidation was determined optically as a function of flash number. The results, shown in Fig. 7, indicate that the amount of carotenoid oxidized declines with flash number in both intact and DEAE *Chlamydomonas* particles. For the intact particles the extent of carotenoid oxidation declines to 10% of that observed initially after 200 flashes; for the DEAE particles the absorbance change drops to 10% during the first 100 flashes. Thus, the carotenoid absorbance change stimulated by ADRY reagents appears to be more labile than the redox reactions of Z under similar conditions. Nonetheless, the ADRY-induced effects continue to be somewhat puzzling and we are in the process of exploring them in more detail.

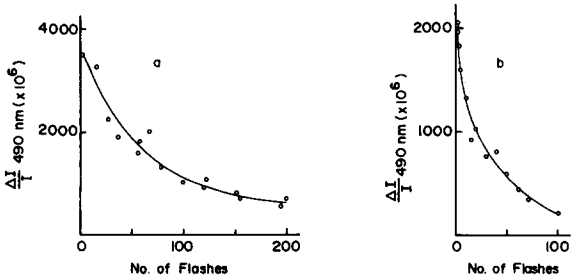

Fig. 7. Extent of carotenoid absorbance change at 490 nm as a function of flash number in intact (a) and DEAE (b) *Chlamydomonas* particles. Both samples contained 8 μg Chl/ml and were suspended in a pH 7.6 buffer containing 0.03% digitonin, 0.003% Triton X-100, 5 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 25 mM $MgCl_2$, 0.1 mM EDTA, 50 mM Hepes and 5 μM ANT-2p. Actinic flashes were given once every three seconds; $\Delta I/I$ (490 nm) was measured 500 μs after the indicated flash (data points) during a flash series. In the absence of ANT-2p, the signal at 490 nm was less than 3% of that obtained initially in the presence of the ADRY and was of the opposite sign.

Discussion

The experiments presented above provide two independent lines of evidence, kinetics and spin quantitation, which support the identification of the Signal II precursor, Z [33], with the P-680$^+$ donor, D_1 [17]. They complement well recent experiments which have determined that the oxidation time course of Z mirrors the reduction time course of P-680$^+$ [16].

The kinetic studies we have performed have been concerned with the reduction kinetics of Z. Our results can be compared with previous P-680$^+$ optical work from which some of the characteristics of D_1^+ reduction have been inferred. For example, Diner and Bowes [18] have concluded that D_1^+ is rereduced more rapidly in their normal *Chamydomonas* particles than it is in DEAE particles [18]. The reduction kinetics of Z^+ which we measure in these two types of particles are in agreement with their results (Figs. 4a and 5a, see above). A corollary conclusion which can be drawn from a comparison of the optical work on the particles [16] and our results is that the kinetic characteristics of Signal II and of the reduced acceptor Q^- are quite dissimilar. From the second-order rate constant for Q^- reoxidation by ferricyanide determined in Ref. 18, we would predict that Q^- is reoxidized in the *Chlamydomonas* particles under the conditions we have typically

used with a half-time of less than 1 ms. Signal II, on the other hand, decays with a half-time approximately two orders of magnitude longer, indicating that in the *Chlamydomonas* particle Q^- does not give rise to a Signal II-like spectrum. This point is important to establish in that if the iron associated with Q were removed during particle purification, we might expect a free radical-type spectrum [20,34]. Our data indicate that this is not the case and are in agreement with recent PS II acceptor side EPR studies in the *Chlamydomonas* particles [35].

Two other pieces of kinetic information support the identification of Z and D_1. Renger and Reuter [36] have recently shown that D_1^+ reduction is accelerated by reducing agents and by ADRY reagents. Previous results in Tris-washed chloroplasts [23,31] show that Z^+ responds similarly to these two classes of compounds and the results presented above show that faster rereduction of Z^+ in the presence of these species is also observed in PS II particles.

Our quantitation data show that light-induced increases in Signal II spin concentration occur in approx. 60% of the reaction centers. We conclude from this that Z is a major component of the PS II complex. The decrease in Signal II spin/P-680 ratio which we observe upon proceeding from unfractionated thylakoid membranes to the reaction center preparation (Table I) is similar to behavior which Mathis and Paillotin [17] and Diner and Bowes [18] have observed for D_1. In the *Chlamydomonas* particles, for example, the reduction of P-680$^+$ shows heterogeneous kinetics which indicates that D_1 is present in only two-thirds of the reaction centers [18]. Thus, like the Signal II/P680 ratio, the D_1/680 ratio apparently declines upon purification. An interesting aspect of the data of Table I, which is also implied by the optical work on D_1 (cf. Figs. 7 and 8 in Ref. 18), is the increase in Signal II spins/680 upon further purification of normal *Chlamydomonas* particles by DEAE fractionation. Whether this is a real effect or simply an example of preparation to preparation variation in the extent of Z extraction during isolation of the particles is unclear at present. However, we suspect that the former is the case which raises interesting questions regarding the activation and deactivation of Z as a P-680$^+$ donor. We are currently exploring this question in more detail.

An enigmatic aspect of Signal II quantitation and behavior in unfractionated chloroplast preparations involves the occurrence of two spins per P-680 (Ref. 37, and see above). Although identical in EPR line shapes [2], these two spins display heterogeneous kinetic and microwave power saturation characteristics. Half the spins saturate at low power and are stable kinetically [19,29]; this subpopulation has been designated Signal II_s. The second half of the Signal II spin population, which also occurs with a stoichiometry of one spin per P-680, arises from Z^+ and shows variable microwave power saturation characteristics [19] and rapid kinetics [37]. In O_2-evolving preparations this subpopulation has been designated as Signal II_f; in thylakoids inhibited at the oxygen-evolving complex, Z^+ is referred to as Signal II_f. The heterogeneity in the Signal II spin population appears to be preserved in the PS II reaction center preparations. The data of Fig. 1 and Table I show the occurrence of a Signal II spin population which is relatively stable in the dark in addition to the Signal II fraction which responds rapidly to illumination. While the latter reacts with added reductants (benzidine) with a second-order rate of approx. 10^6 $M^{-1} \cdot s^{-1}$, the dark signal is much more stable in the presence of the donor. In the particles, however, the strict 1 : 1 stoichiometry we observe between Signal II_s and Signal II_f in Tris-washed chloroplasts appears to be relaxed to an extent and a departure from the equal distribution of Signal II spins is observed. While the identification of the kinetically labile fraction of Signal II spins with the donor to P-680 is justified, the role of the kinetically stable fraction remains problematic.

Of the three types of PS II reaction center preparations we have used, the *Chlamydomonas* DEAE particles are the simplest in terms of protein composition [8,18]. They appear to be composed of four major polypeptides as follows: the 47 and 50 kDa subunits which apparently form the PS II reaction center core complex, a 32 kDa polypeptide, and a low molecular mass polypeptide (approx. 8 kDa) which is apparently a cytochrome *b*-559 species. The Signal II kinetic and quantitation results presented above indicate

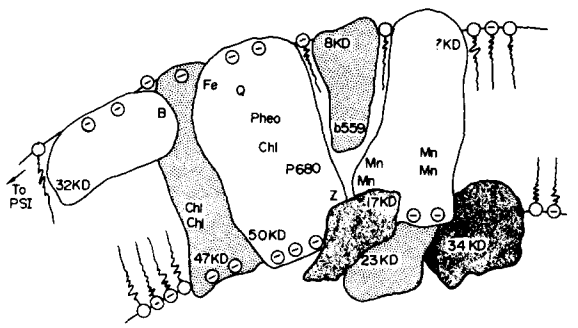

Fig. 8. Model for polypeptide composition and location of redox components in PS II; see text for details. Pheo, pheophytin; b559, cytochrome *b*-559; KD, kDa.

that the donor species, Z, is an integral component of this complex. Moreover, the experiment carried out by Satoh and Mathis [28] in which they showed that the reduction of P-680$^+$ by D_1 (Z) proceeded with the same rate constant even after they had dissociated the subunits of their reaction center preparation suggests that Z and P-680 are bound to the same subunit. These observations, together with recent work done on polypeptides associated with the water-splitting function of PS II [3,38–42], lead to the model of Fig. 8 for the organization of PS II. The reaction center components, including Q, the pheophytin intermediate, P-680 and Z occur in the PS II core composed of the 47 and 50 kDa subunits. At present, the distribution of these species over the two polypeptides is uncertain and the model of Fig. 8 is intended to leave this question open *. The polypeptide to reaction center stoichiometry is also currently unknown and we have adopted a 1 : 1 ratio for the polypeptides in Fig. 8 only for simplicity. The 32 kDa, B-binding polypeptide, which serves to interface PS II with the intersystem electron-transport chain, appears to be localized toward the outer surface of the thylakoid membrane [12]. A similar orientation is shown for cytochrome *b*-559 in accord with evidence presented previously by Horton and Cramer [43]. Water oxidation is mediated by the transfer of oxidizing equivalents generated at P-680 through Z to an as yet unidentified manganese-binding protein. Polypeptides with molecular masses in the 34, 23 and 16 kDa range have been implicated in this process but it remains uncertain at present as to whether any of these polypeptides bind manganese. (The manganese we find associated with the reaction center preparations from *Chlamydomonas* (see above) probably reflects nonfunctional adventitious binding of the cation. This conclusion is supported by the variable binding stoichiometry we observe and by data [19,45] which indicates a considerable binding capacity for manganese, nonfunctional in oxygen evolution, in chloroplast preparations.) From the procedures used in extracting the 34, 23 and 16 kDa polypeptides from the membrane, it appears as if they are extrinsic proteins held in the vicinity of PS II by electrostatic forces [10,42]; their association with the inner surface has been suggested by studies with inverted thylakoid vesicles [42]. The localization of Z toward the inside of the thylakoid membrane surface, its reasonably close proximity to the functional manganese, and the clustering of the four Mn per P-680 into two dissimilar pairs have been included in the model as the result of recent kinetic and inhibition studies of the water-oxidation process [19,44].

* Recent data on PS II particles from higher plants, which contain 43 and 47 kDa polypeptides as the PS II core, indicate that the heavier polypeptide apparently contains the reaction center chlorophyll (Nakatani, H.Y., Li, K., Kuang, T.Y. and Arntzen, C.J., presented at Midwest Photosynthesis Conference, Argonne, IL, U.S.A., October 1982).

Acknowledgements

We thank Professor C.F. Yocum for several helpful discussions. This research was supported by the National Science Foundation through grant number PCM 7900831 (B.K.), by the Délégation Général à la Recherche Scientifique et Technique (Contract No. 80.7.0154) and the Commissariat a l'Energie Solaire (Contract No. 80.75.091) (B.A.D.), and Science and Education Administration of the U.S. Department of Agriculture under Grant No. 59-2261-1-631-0 from the Competitive Research Grants Office (G.T.B.).

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