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OPTICAL CHARACTERIZATION OF PHOTOSYSTEM II ELECTRON DONORS

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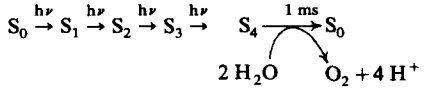
Detailed absorbance difference spectra are reported for the Photosystem II acceptor Q, the secondary donor Z, and the donor involved in photosynthetic oxygen evolution which we call M. The spectra of Z and Q could be resolved by analysis of flash-induced kinetics of prompt and delayed fluorescence, EPR signal II_f and absorbance changes in Tris-washed system II preparations in the presence of ferricyanide and 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU). The spectrum of Z oxidation consists mainly of positive bands at 260, 300 and 390–450 nm on which a chlorophyll *a* band shift around 438 nm is superimposed, and is largely pH-independent as is also the case for the spectrum of Q reduction. The re-reduction of Z^+ occurred in the millisecond time range, and could be explained by a competition between back reaction with Q^- (120 ms at pH 6.0) and reduction by ferrocyanide. When the Tris treatment is omitted the preparations evolve oxygen, and the photoreduction of Q (with DCMU present) is accompanied by the oxidation of M. The Q spectrum being known, the spectrum of the oxidation of M could be determined as well. It consists of a broad, asymmetric increase peaking near 305 nm and of a Chl *a* band shift, which is about the same as that accompanying Z in Tris-washed system II. Comparison with spectra of model compounds suggests that Z is a bound plastoquinol which is oxidized to the semiquinone cation and that the oxidation of M is an Mn(III) → Mn(IV) transition.

Introduction

Kinetically the mechanism of photosynthetic oxygen evolution has been characterized quite well [1,2]. Four successive photoreactions of system II are required in order to obtain oxygen evolution, so the system can be in five different redox states, the so-called S states:



Abbreviations: Chl, chlorophyll; DCMU, 3(3′,4′-dichlorophenyl)-1,1-dimethylurea; DPC, *sym*-diphenylcarbazide; Mes, 4-morpholineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, PS II, Photosystem II.

Attempts to identify the successive oxidation steps have until now not been very rewarding. Optical or EPR signals associated with the $S_0 \rightarrow S_1$ and the $S_2 \rightarrow S_3$ transition have not yet been observed. The $S_1 \rightarrow S_2$ transition is accompanied by an absorbance increase in the ultraviolet [3], which is reversed during the $S_4 \rightarrow S_0$ transition [4,5]. The redox component causing these changes will be designated M in this paper. The $S_1 \rightarrow S_2$ transition was also found to induce a multiline EPR signal which is ascribed to strongly magnetically interacting manganese ions [6–8].

The state S_4 is accompanied by the EPR signal 'II_{vf}' [9,10]. This signal is ascribed to the oxidized form of Z, the electron donor which normally reduces P^+-680, the photooxidized reaction center chlorophyll, within a microsecond, is an inter-

mediate in the $S_2 \rightarrow S_3$ transition [10] and may be in the $S_0 \rightarrow S_1$ and $S_1 \rightarrow S_2$ transitions as well. The evidence for this, however, is not yet conclusive and is based to a large extent on the properties of a similar, but much longer lived, EPR signal 'II_f' [11], observed after inactivation of oxygen evolution by for example, Tris treatment. In Tris-washed system II the decay kinetics of P^+-680 coincide with the rise kinetics of Z^+ [12,13]. Tris treatment appears to remove manganese from the membranes (see for a review Ref. 14) and the $S_1 \rightarrow S_2$ transition is not observed. The EPR signal of Z^+ is ascribed to a semiquinone [15,16]. In order to be sufficiently oxidizing, this would have to be the protonated, cationic form [17], which suggestion was recently supported by simulation experiments [18–20]. Optically, Z^+ was reported to be detectable around 300 nm [21].

In this paper, we describe flash-induced changes in PS II preparations, observed by optical and EPR spectroscopy, and report detailed absorbance difference spectra of the oxidation of Z, the reduction of the primary acceptor Q [22] and of the oxidation of M. The spectra support the identification of Z^+ as a plastosemiquinone cation and the oxidation of M as an Mn(III) → Mn(IV) transition.

Materials and Methods

PS II particles were prepared from spinach chloroplasts according to the method of Berthold et al. [23], with the exception that the second Triton X-100 incubation step was omitted. We observed that it was necessary to increase the concentration of solubilized Triton X-100 during the first incubation step rather slowly to avoid complete membrane solubilization and inactivation of PS II activities. Tris washing was performed with 0.8 M Tris-HCl at pH 8.3 in moderate light for 15 min with a Chl concentration of 0.2 mg/ml. The Tris treatment removed, in addition to the oxygen-evolving capacity, most of the residual PS I activity, leaving about one P-700 to 10000 Chl molecules in the particles. The amount of PS II was 1 to 280 ± 20 Chl molecules, as measured by reduction of the primary acceptor Q with continuous light [22]. The amount of cytochrome *b*-559 was about 1 to 220 Chl molecules, as measured by the method of Bendall et al. [24]; this cytochrome appeared mainly (approx. 90%) in the low-potential form, even before Tris treatment. Neither the cytochromes *f* or b_6, nor the electrochromic band shift (P-515) could be detected.

The optical measurements were performed with a Chl concentration of 200 μg/ml in 20 mM Mes-NaOH at pH 6.0, unless stated otherwise. Since we found that with Mg^{2+} less light was needed to obtain saturation of the variable fluorescence, all experiments were done in the presence of 5 mM $MgCl_2$. The experiments were carried out in a single-beam apparatus with repetitive, saturating 10 μs xenon flashes, spaced at 10 s. The optical path length was 1.2 mm. Above 290 nm, a tungsten halogen lamp was used for the measuring light and the photomultiplier was protected from the flashes by using appropriate filter combinations. Below 290 nm, a deuterium lamp was used in combination with a solar-blind photomultiplier (EMR model 541Q-05M).

The difference spectra were corrected for particle flattening, determined according to the method of Pulles et al. [25]. The differential correction factor varied above 240 nm between 1.01 (at 550 nm) and 1.17 (at 435 nm).

The EPR experiments were performed with a Varian E9 X-band spectrometer with 100 kHz modulation. EPR signal II kinetics were measured at the negative peak at $g = 2.0039$ with 12.5 G modulation amplitude, 3 ms time-constant, and 20 mW microwave power. Chlorophyll concentrations, buffers and flash frequencies were the same as in the optical measurements. To prevent aggregation, 0.03% Triton X-100 was added to the final preparations. This addition appeared to have no effect on the kinetic properties of the particles.

Results

Kinetics

We studied the flash-induced kinetics of prompt and delayed chlorophyll fluorescence, EPR signal II_f, and absorbance changes in Tris-washed system II particles. DCMU (10 μM) was added to inhibit electron transfer beyond the primary acceptor Q, and 2.5 mM ferricyanide was added to reoxidize Q^- within the 10 s between the flashes. The luminescence (Fig. 1c) and EPR signal II_f (Fig. 1d) showed monophasic decay kinetics in the

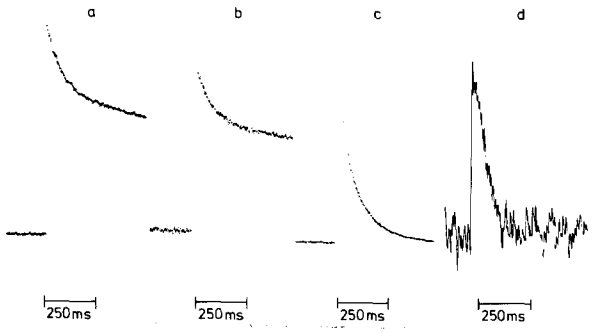


Fig. 1. Decay kinetics of the flash-induced variable fluorescence (a), the absorbance change at 325 nm (b), the luminescence (c) and EPR signal II_f (d) in Tris-washed PS II particles, suspended at a Chl concentration of 200 μg/ml in 20 mM Mes-NaOH (pH 6.0)/5 mM $MgCl_2$/2.5 mM ferricyanide/10 μM DCMU. In the case of the EPR experiments, 0.03% Triton X-100 was added. The emission measurements were carried out alternately without and with a weak continuous excitation of 490 nm, the former yielding the luminescence trace and the difference yielding the fluorescence trace. The recordings are the average of 10 (a, c), 50 (b) and 1000 experiments (d), respectively.

millisecond region. At pH 6.0, a 50 ms half-time was observed in both cases. The decay of the variable fluorescence (Fig. 1a) and of the absorbance change at 325 nm (Fig. 1b), however, showed biphasic kinetics. About 40% of the amplitude of both showed the 50 ms phase, while 60% showed a decay time of 3 s.

Because the relative amplitudes of both phases were the same when measured at 541 minus 551 nm ('C-550', a pheophytin band shift induced by Q^- [22,26]), we conclude that the absorbance change at 325 nm was due to Q^- only. The total absorbance change corresponded to one semiquinone anion ($\Delta\varepsilon = 13\ mM^{-1} \cdot cm^{-1}$ [22]) per 280 Chl molecules, i.e., practically 100% of the reaction centers.

The data indicate that 40% of Q^- are involved in a back reaction with Z^+, accompanied by luminescence emission. The remaining 60% of Q^- are probably reoxidized by ferricyanide, since the half-time of the slow phase was dependent on the ferricyanide concentration. The biphasic kinetics of Q^- are most easily explained by assuming that Z^+ is being reduced by some other component as well. Since no slower luminescence decay phases were detected, this reduction of Z^+ was virtually irreversible and probably due to ferrocyanide. Thus, a very simple kinetic scheme results (Fig. 2), involving only Q, Z and ferricyanide. According to this scheme the half-times of back reaction and reduction by ferrocyanide must be 120 and 80 ms, respectively, in order to explain the 50 ms decay of Z^+ and the 40% of Q^- that are involved in the back reaction.

Kinetics in the absence of DCMU and kinetics in the presence of DPC as electron donor confirmed this model. Omission of DCMU appeared to have no effect on the decay and amplitude of EPR signal II_f and the luminescence. The slow phase in the fluorescence and ΔA_{325}, however, became faster and biphasic with half-times of about 150 and 700 ms, respectively.

Addition of DPC (and DCMU) accelerated the

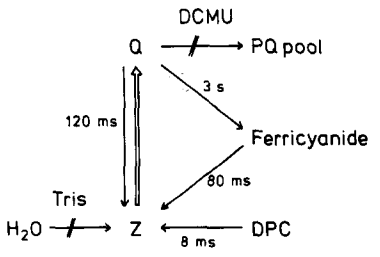


Fig. 2. Flash-induced electron transfer in the ms time range in Tris-washed PS II preparations at pH 6.0 with 2.5 mM ferricyanide, 5 mM $MgCl_2$, 10 μM DCMU and 1 mM DPC (see text). Numbers represent half-times.

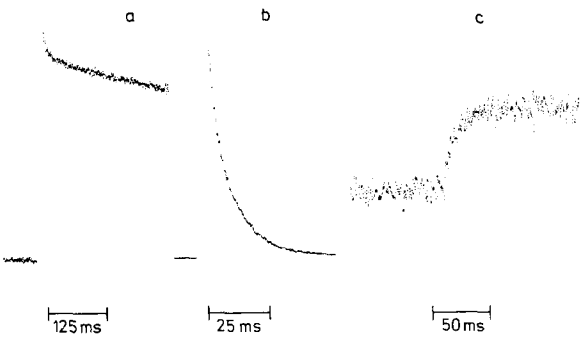


Fig. 3. Decay kinetics of the flash-induced variable fluorescence (a) and the luminescence (b), and oxidation kinetics of DPC (c) in Tris-washed PS II preparations at conditions as for Fig. 1, except that DPC (1 mM) was added. DPC oxidation was measured as an absorbance increase at 290 nm, where the changes due to the photosystem were relatively small. The absorbance change was averaged 200 times.

decay of signal II_f and the luminescence to about 8 ms (Fig. 3b), and prevented the 50 ms fluorescence decay (Fig. 3a). Because the donation to Z^+ is much faster now, only a very small contribution of the back reaction between Q^- and Z^+ may be expected. Indeed, there is a small, about 10 ms, decay phase apparent in the fluorescence decay with DPC. The amount of DPC oxidized in a flash was uncertain, since the absorbance difference spectra indicated that another form of DPC is generated than by chemical oxidation in vitro [27]. The DPC oxidation kinetics, however, could be measured at 290 nm, where the absorbance changes of Q and Z are relatively small (see below). The 8 ms rise-time of the absorbance increase at this wavelength (Fig. 3c) indicates that DPC donates directly to Z^+.

Absorbance difference spectra

According to the kinetic scheme in Fig. 2, the two phases (observed in the absence of DPC) can be represented as follows:

$$1.0\,Z^+ + 0.4\,Q^- + 0.6\,\text{ferro} \xrightarrow{50\,\text{ms}} 1.0\,Z + 0.4\,Q + 0.6\,\text{ferri}$$

$$0.6\,Q^- + 0.6\,\text{ferri} \xrightarrow{3\,\text{s}} 0.6\,Q + 0.6\,\text{ferro}$$

So the slow phase minus the spectrum of ferricyanide reduction yields the $Q^- - Q$ absorbance difference spectrum, and the fast phase minus 40% of the $Q^- - Q$ spectrum and minus 60% of the spectrum of ferrocyanide oxidation yields the $Z^+ - Z$ difference spectrum (Fig. 4).

The $Q^- - Q$ spectrum in the ultraviolet appears to be nearly identical to that in chloroplasts [28]. In the blue part, sharp peaks are seen at 398, 416 and 449 nm, and sharp minima at 407 and 433 nm. Part of this complicated structure is probably due to a blue shift of the intermediary acceptor pheophytin *a* [26] upon reduction of Q, as is observed also around 545 and 685 nm [22]. The small peaks at 505 and 475 nm may be due to a carotenoid.

The $Z^+ - Z$ difference spectrum is characterized by two positive bands in the ultraviolet, peaking near 260 and 300 nm, and probably by a shoulder at 275 nm. In addition a positive change is seen at 395 nm, and a sharp band shift around 438 nm,

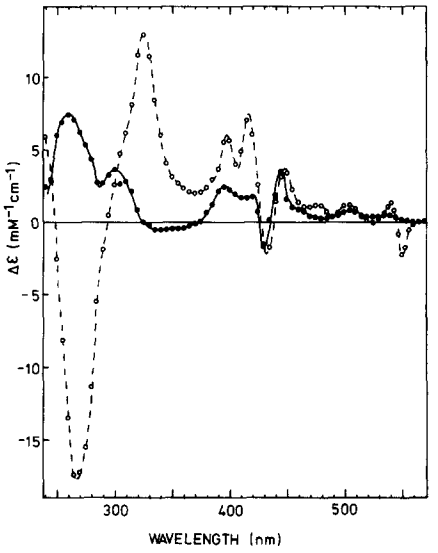


Fig. 4. Reduced-minus-oxidized absorbance difference spectrum of the primary acceptor Q (open circles) and oxidized-minus-reduced difference spectrum of the secondary donor Z (solid circles) in Tris-washed PS II preparations, obtained as described in the text. The spectra are calculated using an extinction coefficient for the reduction of Q of 13 $mM^{-1} \cdot cm^{-1}$ at 325 nm.

possibly due to a red shift of the primary donor P-680 upon oxidation of Z. Also in this spectrum, a small positive band around 505 nm can be seen.

In the region where DPC does not show absorbance changes (above 380 nm), the $Z^+ - Z$ difference spectrum could also be measured by analysis of the 8 ms decay phase. In this case, only small contributions by Q^- and ferrocyanide may be expected. The difference spectrum was found to be nearly identical to that shown in Fig. 4, with the exception that the Chl *a* band shift seemed to be smaller (not shown). This may indicate that DPC^+ remains bound to the reaction center for much longer than 8 ms and preserves some of the electrochromism of P-680.

At pH values other than 6.0, in the range 5.0–8.3, similar kinetics of luminescence, fluorescence and 325 nm absorbance were observed. In Fig. 5, it is shown that the back reaction of Z^+Q^- was clearly pH-dependent, but not sufficiently so to indicate a stoichiometric deprotonation. The difference spectra of $Q^- - Q$ and $Z^+ - Z$ were determined also at pH 5.0 and 8.3, in the same way as described above for pH 6.0. The difference

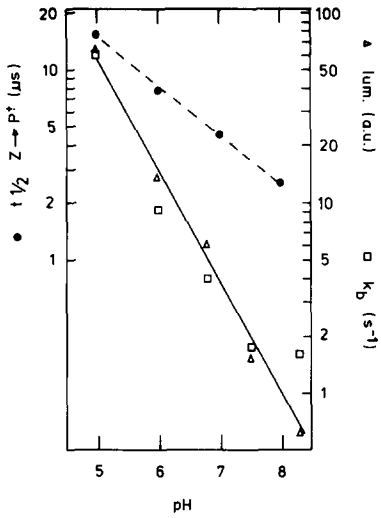


Fig. 5. Half-times of the rate constant k_b of the Z^+Q^- back reaction (open squares), and of the initial amplitude of the luminescence due to this back reaction (open triangles) as a function of pH in the Tris-washed PS II preparations. k_b was calculated from the half-time of the Z^+ decay, measured by luminescence and the fast phase in the absorbance change at 325 nm, and the amount of Q^- that was involved in back reaction with Z^+ (see text). The measurements were carried out in 30 mM sodium phosphate citrate (pH 5.0), 20 mM Mes-NaOH (pH 6.0), 30 mM sodium phosphate (pH 6.8), 20 mM Hepes-NaOH (pH 7.5), or 20 mM Tris-HCl (pH 8.3), with 5 mM $MgCl_2$, 2.5 mM ferricyanide, 2.5 mM ferrocyanide and 10 μM DCMU. The half-times of the electron transfer from Z to P^+-680, reported in Refs. 12 and 13, are also plotted (solid circles).

spectra of $Q^- - Q$ were identical at the three pH values (not shown). The most obvious pH effect in the $Z^+ - Z$ spectra was the absence of the shoulder at 275 nm at pH 8.3 (Fig. 6).

Knowing the precise shape of the difference spectrum of $Q^- - Q$ in these particles, we could also measure a precise and extended difference spectrum of the absorbance changes caused by the first S-state transition induced after dark-adaptation in an intact system II. The Tris treatment was omitted, the particles were suspended in the pH 6.0 buffer with 5 μM ferricyanide, and kept in darkness for at least 15 min, after which 10 μM DCMU was added and the first flash-induced absorbance change was measured. From the spectrum of this change, the spectrum due to Q reduction (Fig. 4) was subtracted after normalization on

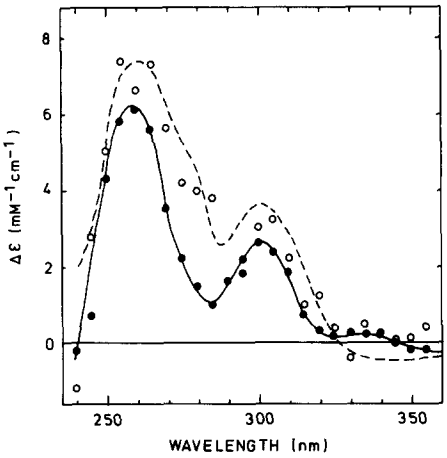


Fig. 6. The $Z^+ - Z$ absorbance difference spectrum in Tris-washed PS II preparations at pH 8.3 (solid circles, continuous line), pH 5.0 (open circles) and pH 6.0 (dashed line). The pH 6.0 spectrum was taken from Fig. 4. The pH 8.3 and pH 5.0 spectra were recorded in the buffers mentioned in Fig. 5, and corrected for particle flattening and absorbance changes of Q^- and ferrocyanide as described in the text, with the exception that the amount of Q^- that was involved in back reaction was different in these cases: 25% for pH 8.3 and 55% for pH 5.0.

the amplitude of the C550 band shift. The resulting difference spectrum is shown in Fig. 7. Since in these conditions (but without DCMU) a normal

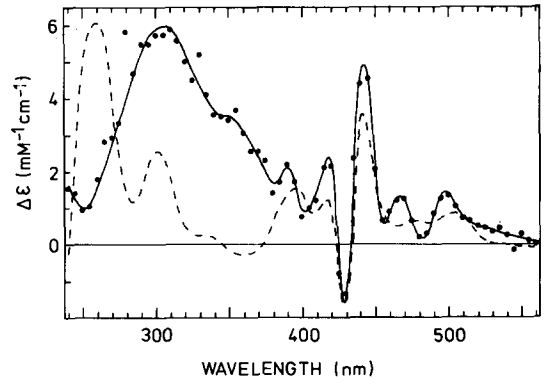


Fig. 7. Spectrum of the oxidation of M (full line), measured as the absorbance change on the first flash in dark-adapted oxygen-evolving PS II preparations from which the changes due to Q reduction (Fig. 4) are subtracted, as described in the text. The measurements were carried out at pH 6.0 with 5 μM ferricyanide, 5 mM $MgCl_2$ and 10 μM DCMU. The differential extinction coefficients were calibrated to the amount of Q reduced. For comparison, the $Z^+ - Z$ difference spectrum in Tris-washed particles at pH 8.3, measured as in Fig. 6, is shown as well (dashed line).

pattern of oxygen evolution in a flash series was observed (Wensink, J., unpublished data), we ascribe the absorbance changes to the $S_1 \rightarrow S_2$ transition (the oxidation of M). The broad maximum around 305 nm confirms earlier observations [3,4]. The shoulder near 350 nm is probably significant. The blue part of the difference spectrum bears a striking resemblance to that caused by the oxidation of Z in Tris-washed system II. Apparently, the local electrical field in the reaction center is very similar in both cases.

Discussion

In this study, two of the redox transitions at the oxidizing side of PS II have been characterized by their absorbance difference spectra in the visible and near-ultraviolet region. As was pointed out in the introduction, M is oxidized in the $S_1 \rightarrow S_2$ transition, Z is oxidized in the $S_3 \rightarrow S_4$ transition, and both are reduced during the $S_4 \rightarrow S_0$ transition. The multiline EPR signal of M^+ was reported to disappear in the $S_2 \rightarrow S_3$ transition [6], but since the ultraviolet absorbance does not change [4], this cannot be a redox change of M^+. Possibly, it is due to a magnetic interaction with the oxidant produced in the $S_2 \rightarrow S_3$ transition. The successive S states may thus be represented as in Fig. 8. The involvement of hydroxyl groups indicated between brackets (cf. Ref. 29) is merely a convenient formalism to account for the known sequence of proton release [30,31]; the oxidants produced in the $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$ transitions have so far escaped detection. The redox complexes Z/Z^+ and M/M^+ may tentatively be identified by comparison of their absorbance difference spectra to those of chemical species which might account for

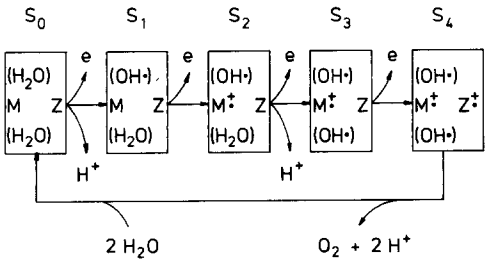


Fig. 8. Formal representation of the S states turnover (see text).

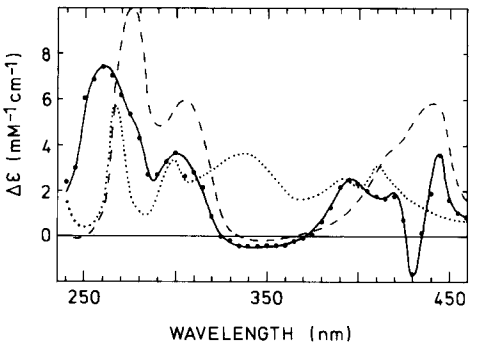


Fig. 9. Comparison of the $Z^+ - Z$ absorbance difference spectrum at pH 6.0 in Tris-washed PS II preparations (full line, from Fig. 4) with the spectrum of the neutral plastosemiquinone [32] minus plastoquinol in ethanol (dotted line) and the spectrum of the protonated cationic form of durosemiquinone minus duroquinol (dashed line). The latter spectrum was obtained by radiolysis experiments on duroquinone in 9 M H_2SO_4 and 1 M methanol (Land, E.J., personal communication).

the known EPR properties of Z^+ and M^+.

Our results show that the $Z^+ - Z$ difference spectrum has positive bands near 260 nm ($\Delta\varepsilon$ = 6–7 $mM^{-1} \cdot cm^{-1}$) and 300 nm ($\Delta\varepsilon = 3$ $mM^{-1} \cdot cm^{-1}$), very small changes in the range 320–380 nm, and positive changes in the blue region to which a red shift of a Chl *a* molecule, presumably P-680, is superimposed. Fig. 9 compares the $Z^+ - Z$ difference spectrum at pH 6.0 with the in vitro difference spectra of the neutral plastosemiquinone PQH• [32] minus plastoquinol in ethanol, and that of the protonated, cationic form of durosemiquinone DQH_2^+ minus duroquinol, based on radiolysis experiments on duroquinone in 9 M H_2SO_4 and 1 M methanol by E.J. Land (personal communication). It appears that the $Z^+ - Z$ spectrum differs from that of PQH• – PQH_2 primarily by the absence of the broad band around 340 nm. In the region of 400–450 nm, however, both spectra are probably similar if the Chl *a* band shift is subtracted. The spectrum of $DQH_2^+ - DQH_2$ bears more resemblance to the $Z^+ - Z$ spectrum in the ultraviolet region, especially considering the possibility that the 275 nm peak is red-shifted by the acid environment, as is also the case for the quinone band at 260 nm. On the other hand, the large absorbance around 445 nm is missing in the $Z^+ - Z$ spectrum, but in this case such discrepancies may be expected. Z resides in a

hydrophobic [33] rather than an acidic environment, and is certainly not duroquinol. In view of these differences, the correspondence of the spectra is remarkable, indicating that Z^+ may be identified as a semiquinone cation. These results confirm the suggestions made on the basis of studies on the line shape of the EPR signal [18–20].

In Tris-washed chloroplasts [34] or inside-out vesicles [35], a retarded release of a proton is seen upon oxidation of Z and an uptake concomitant with the rereduction of Z^+. Our difference spectra indicate that this proton is not coming from Z^+ itself. Moreover, if a protonation step is involved in the rereduction of Z^+, a pH dependence of the rate of back reaction of a factor 10 per pH unit is expected. Our results indicate only a factor of about 4, and this is partially explained by the pH dependence of the electron transfer from Z to P^+ (Fig. 5: a factor of about 2 per pH unit [12,13]), so that the real pH dependence of the back reaction is only a factor 2. The reduction of Z^+, therefore, is not coupled to a stoichiometric protonation.

It is not clear which particular quinol gives the semiquinone cation radical. Kohl and Wood [36] stated that signal II had to be due to a semiquinone of plastoquinone on the basis of extraction and readdition experiments with plastoquinone and deuterated plastoquinone. However, similar effects might be caused by extraction of Q rather than extraction of Z, and the effect of deuteration on the line shape was not entirely convincing, especially in view of possible pH effects [37] in the reconstitution experiments. Nevertheless, more recent experiments tend to support the conclusion. EPR spectra of model compounds [18,19] indicate that of several compounds tested, 2-methyl-5-isopropylsemiquinone cation gives the most satisfactory fit for the signal II line shape, although the line width is still too small by a factor of 2. Thus, it appears that plastoquinol may be an acceptable candidate of Z, if the extended line width of signal II can be explained by interaction of the hydroxyl groups of the molecule with positive groups from the protein environment [19]. To our opinion, also the possibility should be considered that the molecule is covalently bound to the protein via both oxygen atoms, which might help to stabilize both the reduced and the cationic radical forms.

Simultaneously with our preliminary report on the $Z^+ - Z$ difference spectrum [38], a spectrum with similar characteristics was presented by Diner and De Vitry [39]. These authors obtained the $Z^+ - Z$ spectrum by analysis of an absorbance transient observed in system II particles from a *Chlamydomonas reinhardii* mutant, in the presence of the electron donor benzidin. It should be noted that our difference spectrum of Z^+ resembles more that of the oxidation of duroquinol than of the naphthoquinone derivative measured by Diner and De Vitry.

The absence of a significant absorbance change of Z at 325 nm has a practical advantage: the concentration of Q can directly be measured by the light-induced absorbance change at this wavelength in Tris-washed system II after addition of DCMU.

In untreated oxygen-evolving system II preparations, the absorbance changes at the oxidizing side are not negligible (Fig. 7): a differential extinction coefficient of 17.5 $mM^{-1} \cdot cm^{-1}$ must then be used for the total change at 325 nm in dark-adapted material. This correction, plus that for the flattening effect (1.07 at 325 nm), converts the higher reaction-center concentration reported by Lam et al. [40] to the same value reported here: one per 280 chlorophylls.

In dark-adapted chloroplasts, the ultraviolet absorbance changes due to the oxidizing side of system II are probably the same as shown in Fig. 7: the spectrum reported by Velthuys [4] is similar, except that it peaks at 320 instead of 305 nm. This difference is probably due to a small contribution from the acceptor side, which may still be present in Velthuys' measurements. He subtracted absorbance changes measured in the presence of hydroxylamine, which is known to disconnect Z from P-680 at higher concentration [41]. An only slightly too small subtraction would already account for the apparent shift of the M^+ peak. This effect of hydroxylamine occurs at unusually low concentrations in the system II preparations (Ref. 42, and Dekker, J.P., unpublished data) and leads to a rapid charge recombination of P^+-680 Q^- in a significant fraction of the centers. Therefore, neither the method of Velthuys to determine the absorbance changes of the oxidizing side, nor the method of Thielen and Van Gorkom to de-

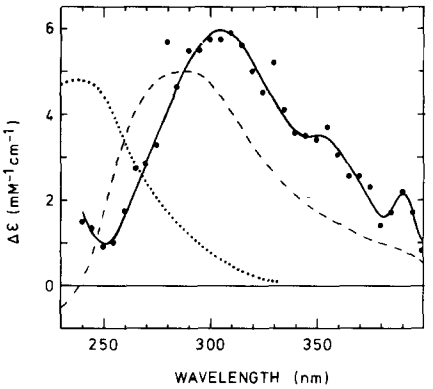


Fig. 10. Comparison of the absorbance difference spectrum due to the $S_1 \rightarrow S_2$ transition at pH 6.0 in oxygen evolving PS II preparations (full line, from Fig. 7) with the in vitro spectrum of Mn(IV) gluconate minus Mn(III) gluconate (dashed line), and with the spectrum of Mn(III) gluconate minus Mn(II) gluconate (dotted line), calculated from Ref. 45.

termine the Q concentration [43] can be used with these system II preparations.

According to our interpretation, the spectrum of Fig. 7 is due to the $S_1 \rightarrow S_2$ transition, i.e., the oxidation of M. The extinction coefficients may actually be higher than indicated in Fig. 7, because a minor fraction of the reaction centers is probably in state S_0 in the dark. Measurements of the yield of oxygen evolution as a function of flash number (Wensink, J., unpublished data) suggest that this fraction is probably not significantly different from that normally observed in dark-adapted chloroplasts. If so, the extinction coefficient should be about 30% higher. The shape of the spectrum, however, may not be affected, since absorbance changes due to the $S_0 \rightarrow S_1$ transition have not been observed (cf. Ref. 4).

In view of the evidence that manganese is involved in the $S_1 \rightarrow S_2$ transition, we compare in Fig. 10 the difference spectrum with those of the binuclear manganese-gluconate complexes described by Bodini et al. (Ref. 44, see also Ref. 45). The shape of our difference spectrum clearly indicates that an Mn(III) → Mn(IV) change is a likely candidate for the redox center M involved in the $S_1 \rightarrow S_2$ transition, and that an Mn(II) → Mn(III) change can be excluded.

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