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Absorbance difference spectra upon charge transfer to secondary donors and acceptors in Photosystem II *

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Charge-transfer reactions to secondary electron donors (Z, M) and acceptors (Q_A, Q_B) in Photosystem II particles isolated from a thermophilic cyanobacterium *Synechococcus* sp. (Schatz, G.H. and Witt H.T. (1984) Photobiochem. Photobiophys. 7, 1–14) were analyzed by measurements of fluorescence yield and absorbance changes in the millisecond time domain induced by repetitive flashes. (1) The electron-transfer reaction $Q_A^- Q_B \rightarrow Q_A Q_B^-$ was found to occur with kinetic phases of 0.2 ± 0.1 ms and 1.5 ± 0.5 ms half-time. At 10 ms after flashes an equilibrium distribution of $Q_A^- Q_B / Q_A Q_B^-$ of about 15/85 in oxygen-evolving and of about 25/75 in Tris-treated PS II particles was reached. (2) The absorbance difference spectra were determined for $(Q_A^- - Q_A)$, $(Q_B^- - Q_B)$, $(Z^+ - Z)$ and for $(S_4 - S_0)$, the transition associated with oxygen evolution. In the ultraviolet region they show that these electron-acceptors and -donors are the same as in spinach PS II. In the visible region all the difference spectra contain major contributions by electrochromic bandshifts due to electrostatic interaction of the reduced acceptors or oxidized donors with nearby reaction center pigments. Upon electron transfer from Q_A^- to Q_B electrochromic bandshifts due to interaction with pheophytin *a* disappeared almost completely. Bandshifts observed in the $(Z^+ - Z)$ and $(S_4 - S_0)$ spectra were attributed to chlorophyll *a*.

Introduction

In Photosystem II (PS II), the oxygen-evolving system of photosynthesis, electron transport involves at least the following steps:

$$H_2O \rightarrow M \rightarrow Z \rightarrow P\text{-}680 \rightarrow I \rightarrow Q_A \rightarrow Q_B \rightarrow Q_{pool}$$

The sequence of electron transfers (reviewed recently in Ref. 1) is initiated by P-680, the reaction center Chl *a*, which after excitation gives an electron via the intermediary pheophytin *a* molecule I [2] to the permanently bound plastoquinone molecule Q_A [3,4]. This completes the charge translocation across the photosynthetic membrane [5]. P^+-680 is reduced, perhaps via an as yet unidentified intermediate [6], by Z, which is thought to be a permanently bound plastoquinol molecule [7,8]. The resulting state $Z^+ P\text{-}680 Q_A^-$ is characterized by a several-fold increased Chl fluorescence yield. The two intrinsic semiquinone molecules Q_A^- and Z^+ are not protonated or deprotonated, and, therefore, remain at their widely different oxidation-reduction potentials.

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4'-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid; PS II, Photosystem II; RC, reaction center.

Electron transfer from Q_A^- to the pool of mobile plastoquinone molecules in the membrane requires two successive photoreactions of PS II [10]: after the first the electron is transferred from Q_A^- to Q_B, forming the plastosemiquinone anion Q_B^- [11], which remains tightly bound to the reaction center. The next photoreduction of Q_A leads to a further reduction of Q_B^-, its protonation and subsequent release as plastoquinol [12]. Electron transfer from water to Z^+ requires four successive photoreactions of PS II [13]: the first three positive charges are stored in the oxygen-evolving complex M, probably on manganese [14], inducing transitions between the so-called S-states S_0, S_1, S_2 and S_3 [15]. When Z is oxidized for the fourth time (S_4) the complex is reduced (to S_0) and O_2 released in about a millisecond. This is accompanied by the release of two protons; the other two are released after the first ($S_0 \rightarrow S_1$) and third ($S_2 \rightarrow S_3$) oxidation step [16].

To obtain information on the arrangement of these intermediates in the PS II reaction center we have studied spectral shifts which are found in the absorbance difference spectra of $(Z^+ - Z)$, $(S_4 - S_0)$, $(Q_A^- - Q_A)$ and $(Q_B^- - Q_B)$. Several such bandshifts have been reported and are believed to arise from electrochromism of reaction center pigments, perhaps P-680 and I. The first one was 'C550' [17], a blue shift of a 10 nm wide absorption band at 545 nm observed upon reduction of Q_A. When a bandshift at 685 nm was observed as well, both shifts were attributed to pheophytin *a* [3]. Around 425 nm a shift of the Soret band of pheophytin *a* may be superimposed on the absorbance increase due to Q_A reduction itself [18,19,8]. Amplitudes of the reported bandshifts were rather dependent on the sample used. A decrease of this electrochromism upon electron transfer from Q_A^- to Q_B was expected from the absence of binary oscillations of C550 [20] and recently supported by differences between absorbances of Q_A^- and Q_B^- in the blue region [21], but it has not been quantitatively determined. In the blue region, but not around 545 nm, a redshift has been observed upon the oxidation of Z and tentatively ascribed to P-680 [8]. A similar redshift was observed upon oxidation of M [8]. A redshift centered at 678 nm has been observed upon Z oxidation [22], while no data are available on absorbance changes in this region for oxidation of M.

The measurements reported here were carried out to obtain a more complete and precise set of data on the bandshifts induced by secondary electron transport, so that a quantitative comparison of the shifts induced by different electron transfer steps could be made. We present absorbance difference spectra of $(Z^+ - Z)$, $(S_4 - S_0)$, $(Q_A^- - Q_A)$ and $(Q_B^- - Q_B)$ in the spectral range from 240–560 nm and 650–700 nm as obtained with an earlier described PS II particle from the cyanobacterium *Synechococcus* sp. [23,24]. In the millisecond time scale we compared oxygen-evolving with Tris-treated PS II particles in order to separate donor side reactions from acceptor side reactions. Secondary electron transport in oxygen-evolving PS II particles will proceed from a generalized state $(MZ)^+Q_A^-Q_B$ shortly after a single turnover flash towards $(MZ)Q_AQ_B^-$ within few milliseconds. After Tris treatment which is known to release manganese from M [25], one expects within a similar time scale the transition from $Z^+Q_A^-Q_B$ towards $Z^+Q_AQ_B^-$. Hence, when the relative concentrations of these four states are known, they can be used to calculate the mentioned absorbance difference spectra by linear combinations.

Materials and Methods

Photosystem II particles highly active in O_2 evolution were obtained from membranes of a thermophilic cyanobacterium *Synechococcus* sp. as described in Ref. 23: the membranes were suspended in a mixture of 80% (v/v) buffer 1 (20 mM Mes/NaOH (pH 6)/10 mM $MgCl_2$/2 mM KH_2PO_4/0.5 M mannitol) with 20% (v/v) glycerol to give a Chl concentration of $1.1 \cdot 10^{-3}$ M. Then, they were treated for 20 min in the dark with the detergent *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (sulfobetaine 12, SB 12) in amounts optimized as in Ref. 23, i.e., usually about 0.4% (w/w). Subsequent centrifugation at $140\,000 \times g$, 5°C for 40 min gave a supernatant (PS II suspension) which contained the PS II particles in a concentration equivalent to $1.2 \cdot 10^{-4}$ M Chl in 80% (v/v) buffer 1/20% (v/v) glycerol/0.4% (w/w) SB 12. It was stored at −20°C until use over several months. The PS II

particles showed a unit size of (80 ± 10) Chl/PS II as resulting from the amplitude of light-induced absorbance changes at 325 nm in the presence of 0.8 mM $K_3Fe(CN)_6$/50 μM DCMU/0.8 M Tris-HCl (pH 8.3), using a molar extinction coefficient for Q_A-reduction of 13000 $M^{-1} \cdot cm^{-1}$ [3]. Similar values were typically obtained from averaged O_2 yields measured with single-turnover flashes at pH 6.5 [24,25]. The PS II-to-PS I ratio of more than 20 : 1 was determined from absorbance changes at 700 nm as in Ref. 23.

Sample conditions. The two following conditions refer to measurements with (A) Tris-treated and (B) oxygen-evolving PS II particles.

(A) 0.8 M Tris-HCl (pH 8.3)/0.8 mM $K_3Fe(CN)_6$/0.2 mM $K_4Fe(CN)_6$ and further compounds from the PS II suspension (specified below). A fresh sample was first exposed to 15–20 preflashes and then measured with a flash repetition rate of 0.1 Hz. This condition was established in order to prevent accumulation of $(Q_AQ_B)^-$ or of Z^+ (see also Results).

(B) 40 mM Mes/NaOH (pH 6.5), 0.8 mM $K_3Fe(CN)_6$ and further compounds from the PS II suspension (see below). Transients were recorded after four preflashes given to the sample; the flash repetition rate was 0.2 Hz. Under these conditions no plastosemiquinone accumulated (see Results).

In both cases further compounds from the diluted PS II suspension were 1 mM KH_2PO_4/5 mM $MgCl_2$/250 mM mannitol/0.2% (w/w) SB 12/10% (v/v) glycerol; Chl *a* concentration was $6 \cdot 10^{-5}$ M.

Apparatus conditions. The measurements were performed in the flash photometer used in Ref. 8 with a cuvette of 1.2 mm optical path length. The single-beam measuring light was controlled by a shutter, opened 50 ms before and closed 30 ms after the flash. Its intensity was adjusted to induce only a negligible actinic effect at all wavelengths, and was in most cases less than 200 $\mu W/cm^2$. It was provided either by a tungsten halogen lamp (larger than 285 nm), by a deuterium lamp (less than 285 nm) or a mercury lamp. For absorbance changes actinic flashes of saturating intensity and 10 μs half-width were provided by an Xe-lamp. Electronic bandwidth for detection was 30 kHz; optical bandwidth for absorbance difference spectra was 5 nm. For measurements in the red region of the spectrum of solid angle between the sample and the photocathode was reduced until artefacts due to fluorescence and luminescence were suppressed below the noise level at 150 μs after the flash. Experimentally observed absorbance changes are given on the basis of 80 Chl facilitating the comparison with the pure difference spectra calculated on the basis of one reaction centre. *Chlorophyll fluorescence* was excited at 430 nm or 395 nm (see legends) and monitored at 690 nm (interference filter); it was generally detected by a gateable photomultiplier (Philips XP 1002) which was turned off during and until 150 μs after the actinic flash. Fast-extinguishing actinic flashes (half-width 2 μs) for fast fluorescence measurements were provided by a special Xe-lamp (Suntron 8 C, Xenon corporation, Wilmington, MA) with nearly saturating (approx. 90%) intensity. In the latter cases Chl concentration was reduced to $2 \cdot 10^{-5}$ M. All other conditions were identical to those described above and used for absorbance measurements.

Results

In order to establish appropriate conditions for measurements under repetitive flash illumination, the artificial electron donor/acceptor couple ferro-/ferricyanide was used, and the turnover of Photosystem II in its presence was studied. Fig. 1 shows that the flash-induced absorbance increase at 325 nm (the absorption maximum both of Q_A^- and of Q_B^- [11]) in Tris-treated PS II particles was found to decay largely (75–80%) by a slow reaction with ferricyanide. The second-order rate constant was $1.7 \cdot 10^3$ $M^{-1} \cdot s^{-1}$ under the conditions used (pH 8.3, 25°C, ionic strength 0.83 M, 0.2% SB 12). As standard concentration for all subsequent experiments we used 0.8 mM ferricyanide, implying an 0.5 s half-time of the semiquinone anion. Thus, with a flash repetition rate of maximally 1 per 5 s the reoxidation of $(Q_AQ_B)^-$ between flashes was essentially complete. Also the reduction of Z^+ was essentially complete in that time, as was checked by measurements of the luminescence caused by $P\text{-}680^+Q_A^-$ recombination ($t_{1/2} \approx 0.2$ ms) as a function of the dark time between flashes. The half-time of Z^+ estimated

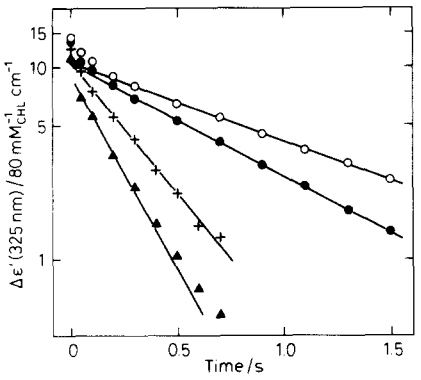

Fig. 1. Semilogarithmic plot of decays of semiquinone-absorbance changes at 325 nm in Tris-treated PS II particles with different ferricyanide concentrations: (○) 0.5 mM; (●) 0.8 mM; (+) 1.8 mM; (▲) 3 mM.

from such measurements was about 1 s, presumably due to reduction of Z^+ by ferrocyanide. It appeared that the backreaction of $Z^+(Q_AQ_B)^-$ was insignificant. To verify that point, 50 μM DCMU was added – which should promote the backreaction by keeping the electron on Q_A^- – and the decay of the 325 nm absorbance change was measured. No decay (i.e., less than 5%) was observed within 10 ms. Thus we concluded that measurements up to 10 ms after the flash were not affected significantly by any back reactions and that a flash spacing of 10 s was enough for complete recovery of Z between the flashes. Under such conditions in Tris-treated particles only one

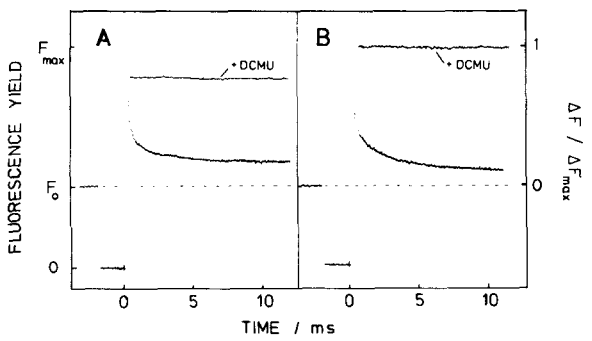

Fig. 2. Changes of chlorophyll fluorescence yield (excited at 430 nm) in (A) Tris-treated and (B) oxygen-evolving PS II particles in absence and presence of 50 μM DCMU. Average of 16 transients. Conditions as described in Materials and Methods for (A) and (B), but chlorophyll concentration 20 μM.

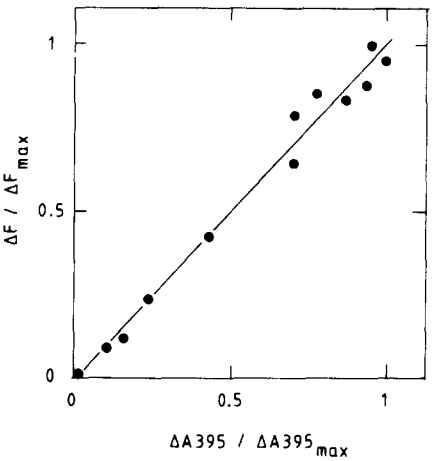

Fig. 3. Relative changes of variable chlorophyll fluorescence yield (excited at 395 nm) and of absorbance changes at 395 nm measured simultaneously in oxygen-evolving PS II particles. Sample condition (B) as described in Materials and Methods plus 50 μM DCMU. Observed time range, 2 s; electronic bandwidth 100 Hz. Dark-adapted samples were excited by single flashes (10 μs half-width) of different intensities adjusted with neutral density filters.

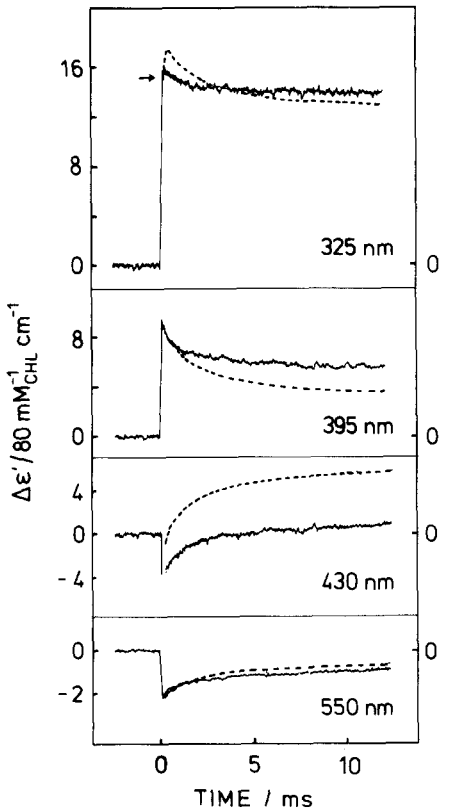

Fig. 4. Flash-induced absorbance changes in Tris-treated (solid traces, condition (A)) and oxygen-evolving (dashed curves, condition (B)) PS II particles. Averages of 24 or 48 transients.

process is expected: electron transfer from Q_A^- to Q_B.

The latter is most easily detected by measuring the Chl fluorescence yield, which depends primarily on the redox state of Q_A [27]. Fig. 2 shows the fluorescence changes observed during the first 10 ms after repetitive flashes in (A) Tris-treated and (B) oxygen-evolving PS II particles at the same Chl concentration. In both preparations the flash-induced fluorescence increase decayed in three phases with half-times of 0.2 ($\pm$0.1) ms, 1.5 ($\pm$0.5) ms and several hundred ms (not shown). Control experiments on dark-adapted samples with 50 μM DCMU (upper traces) confirmed that all of these decay phases were blocked by DCMU and therefore were not due to back reaction. The slowest phase probably corresponds to the decay of the absorbance change at 325 nm as mentioned above (Fig. 1). The millisecond decay, however, is not associated with a substantial decrease in the semiquinone anion absorbance at 325 nm and may thus be attributed to electron transfer from Q_A^- to Q_B. The ratio F_{max}/F_0 in Fig. 2 was relatively low due to a constant contribution to fluorescence yield from the auxiliary pigment allophycocyanin [28,29] which is still bound to the PS II particle from *Synechococcus* [23]. When fluorescence was excited at longer wavelengths (more than 500 nm) favouring even more the excitation of allophycocyanin the F_{max}-to-F_0 ratio became even lower.

In order to correlate fluorescence recovery kinetics with Q_A^- oxidation kinetics we determined the dependence of ΔF on Q_A^- concentration under our conditions. The latter was monitored by absorbance changes at 395 nm, a wavelength indicative of Q_A^- [8] and efficient to probe the Chl fluorescence yield, as well. As shown in fig. 3, a linear correlation was observed between the fluorescence increase and the absorbance increase due to Q_A^- formed by a flash of various intensities

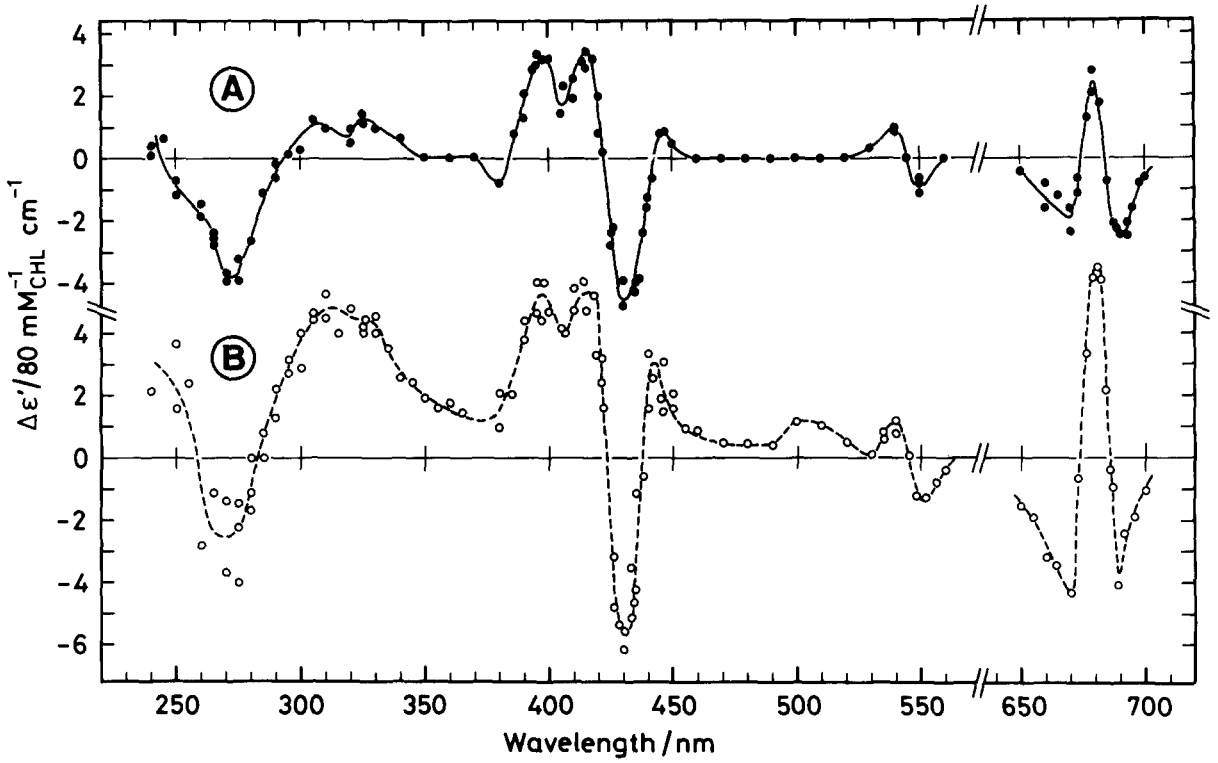

Fig. 5. Absorbance difference spectra associated with the (inverted) decay phases within 10 ms after repetitive flashes (A) Tris-treated PS II particles, attributed to $Q_A^- Q_B \rightarrow Q_A Q_B^-$ electron transfer in 75% of the reaction centers and in (B) oxygen-evolving PS II particles, attributed to the transitions $Q_A^- Q_B \rightarrow Q_A Q_B^-$ in 85% and $S_4 \rightarrow S_0$ in 25% of the reaction centers.

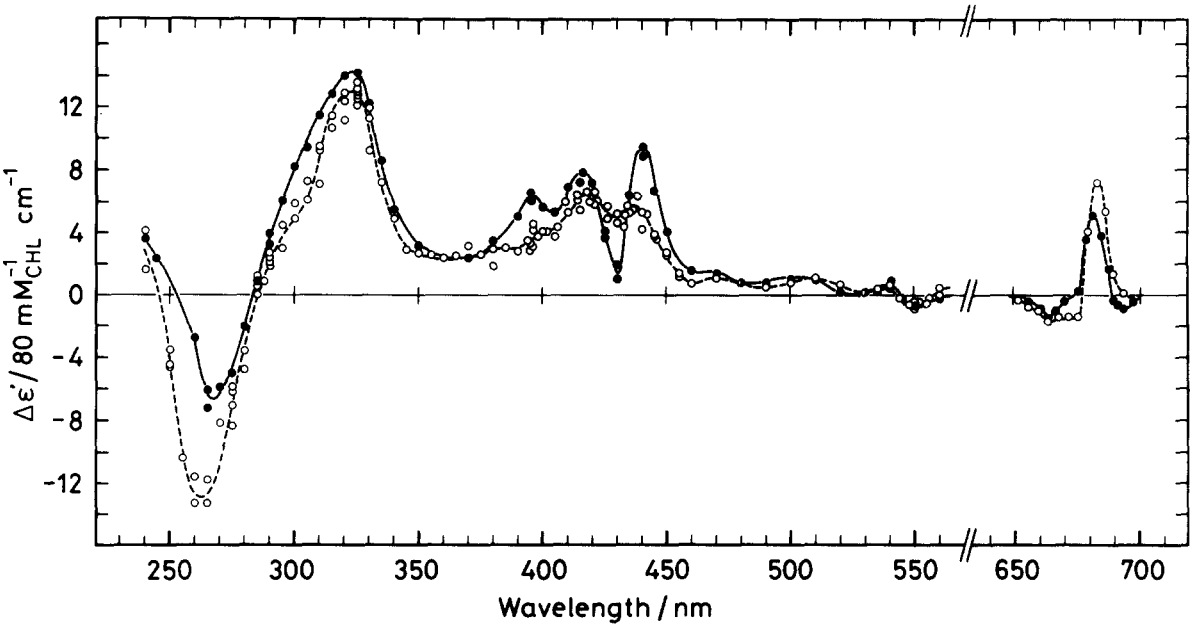

Fig. 6. Spectra of absorbance changes remaining at 10 ms after repetitive flashes in Tris-treated (closed circles, solid curve) and oxygen-evolving (open circles, dashed curve) PS II particles.

on a dark-adapted sample in the presence of DCMU. The changes were half-saturated at about 7% of the flash intensity used under standard conditions. The proportionality between variable fluorescence yield and Q_A^- indicates that PS II exists in separate units in these preparations. This is consistent with our observations of purely exponential fluorescence induction kinetics in continuous light (not shown) and a uniform 10.5 nm particle diameter (Mörschel, E. and Schatz, G.H., unpublished results). Using this proportionality and the explicit assumption that Q_AQ_B and $Q_AQ_B^-$ show the same Chl fluorescence yield, the averaged results of several measurements like those in Fig. 2 indicated that in Tris-treated about 25(±5)% and in oxygen-evolving particles about 15(±5)% of Q_A^- was still present at 10 ms after the flash.

In Fig. 4, flash-induced absorbance changes observed at different wavelength in Tris-treated particles are seen to contain several kinetic phases: a slow phase (resolved in Fig. 1) plus fast phases similar to those in the fluorescence measurements of Fig. 2. This similarity suggests quite different absorbances of $Q_A^-Q_B$ and $Q_AQ_B^-$ at various wavelengths (e.g., 395, 430 and 550 nm). Addition of DCMU resulted in step-like, irreversible (within 10 ms) absorbance changes of similar initial amplitude (averaged with 0.03 Hz flash repetition rate, not shown), confirming the assignment of the millisecond kinetics to electron transfer from Q_A^- to Q_B. At 325 nm, the similar absorbance of Q_A^- and of Q_B^- resulted in only small changes also in the absence of DCMU. Fig. 5A shows the spectrum of the millisecond decay phase of absorbance changes in Tris-treated particles as determined by the difference $A(0.1\ ms) - A(10\ ms)$. It is associated with the $Q_A^-Q_B \rightarrow Q_AQ_B^-$ transition in 75% of all reaction centers, and appears to consist predominantly of bandshifts rather than of any net increase or decrease in absorbance. Its features clearly indicate a blue shift of absorbance bands centered at 422 nm and 545 nm, a more complex bandshift in the red region and a bleaching in the ultraviolet region. Some of these changes are expected to result from a decreasing electrochromism of pheophytin *a* and perhaps other reaction center pigments upon moving the negative charge from Q_A^- to the presumably more distant Q_B.

The dashed curves in Fig. 4 show decay kinetics in oxygen-evolving particles. Their initial amplitudes are similar to those in Tris-treated particles. Additional contributions developed in the millisec-

TABLE I

STATES INDUCED BY REPETITIVE FLASHES IN PS II PARTICLES FROM *SYNECHOCOCCUS* AS WELL AS STATE TRANSITIONS OBSERVED IN FIG. 5 (SECOND ROW MINUS THIRD ROW) AND IN FIG. 6 (THIRD ROW MINUS FIRST ROW).

PS II condition	(A) Tris-treated	(B) O_2-evolving
Before flash	–	$0.25(S_0+S_1+S_2+S_3)$
0.1 ms after flash	$Z^+Q_A^-$	$Q_A^-+0.25(S_1+S_2+S_3+S_4)$
10 ms after flash	$0.25(Z^+Q_A^-)+0.75(Z^+Q_B^-)$	$0.15\,Q_A^-+0.85\,Q_B^-+0.25(S_0+S_1+S_2+S_3)$
Fig. 5	$0.75(Q_A^- - Q_A)-0.75(Q_B^- - Q_B)$	$0.85(Q_A^- - Q_A)-0.85(Q_B^- - Q_B)+0.25(S_4-S_0)$
Fig. 6	$(Z^+ - Z)+0.25(Q_A^- - Q_A)-0.75(Q_B^- - Q_B)$	$0.15(Q_A^- - Q_A)-0.85(Q_B^- - Q_B)$

ond time range. This is expected because the oxygen releasing reaction has a half-time of about a millisecond [13] and in our repetitive experiments about 25% of the centers should cause a net absorbance change due to the $S_4 \rightarrow S_0$ transition. All the other S-state transitions take place in less than a millisecond [30,31] and the rapid rise observed at 325 nm in Fig. 4 (see arrow) suggests their (partial) resolution at that wavelength. At the other wavelengths shown their contribution is expected to be much smaller [14]. If we neglect this initial transient and extrapolate the millisecond phase back to the moment of the flash we obtain its full amplitude with the spectrum given in Fig. 5B. It contains contributions from both transitions $Q_A^-Q_B \rightarrow Q_AQ_B^-$ and $S_4 \rightarrow S_0$ in 85% and 25% of the reaction centers, respectively. At 10 ms after repetitive flashes net absorbance changes due to S-state transitions are no longer expected. The remaining absorbance changes in oxygen-evolving particles are shown in Fig. 6 (open circles) and indicate (by the typical features in the ultraviolet region) that the electron is still kept on a plastosemiquinone-anion, mostly (85%) attributed to Q_B^-.

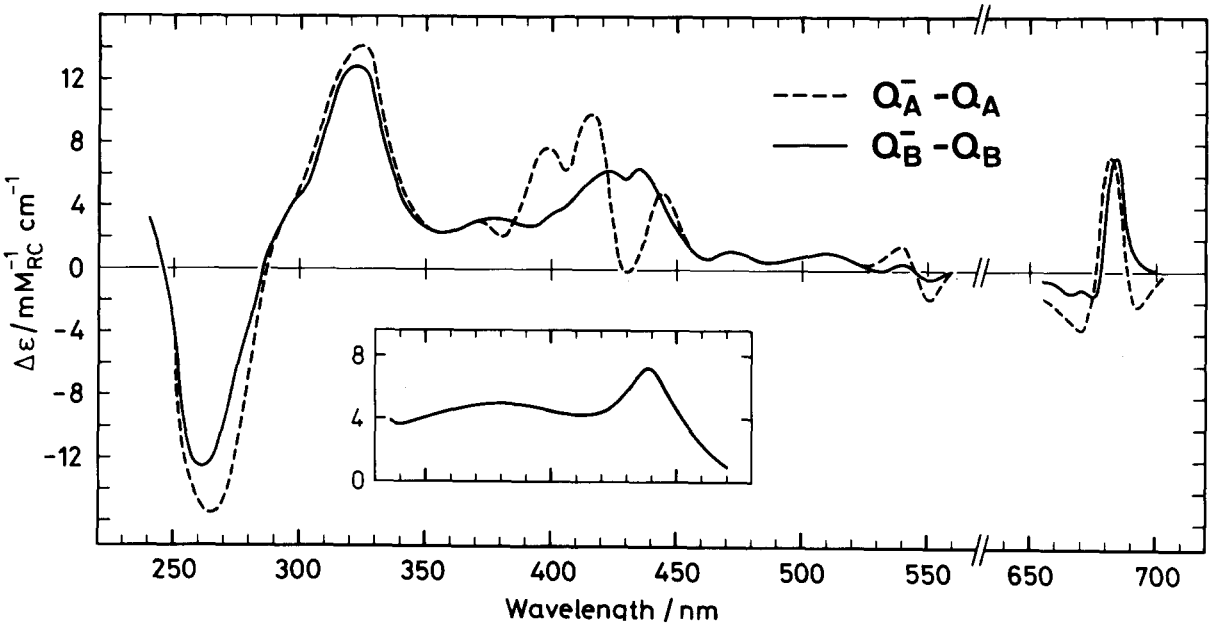

Fig. 7. Absorbance difference spectra for 100% Q_A-reduction (dashed) and 100% Q_B-reduction (solid) in PS II particles. Calculated by correcting the spectrum of oxygen-evolving particles in Fig. 6 by plus 1.133 times fig. 5A for $(Q_A^- - Q_A)$ and by minus 0.2 times fig 5A for $(Q_B^- - Q_B)$, respectively. Inset: partial absorbance difference spectrum for reduction of plastoquinone in vitro taken from Ref. 32.

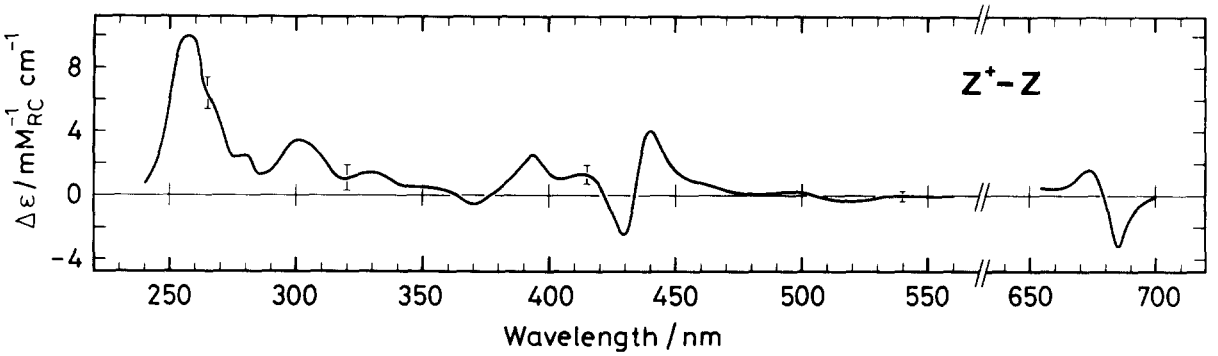

Fig. 8. Absorbance difference spectrum for 100% ($Z^+ - Z$). Calculated from the difference of the spectra in Fig. 6 (Tris-treated minus oxygen-evolving) minus 0.133 times fig. 5A.

In Tris-treated particles the corresponding changes show a quite different spectrum (closed circles in Fig. 6) which mainly reflects the additional contribution by Z^+ formation.

The states of PS II monitored by the difference spectra in Fig. 5 and Fig. 6 can be characterized (using the quantitative information from Chl fluorescence) as given in Table I. Thus, the pure absorbance difference spectra for reduction of Q_A and Q_B, for oxidation of Z and for the $S_4 \rightarrow S_0$ transition can be calculated by linear combinations of the observed difference spectra. This implies the assumption that the difference spectrum of each component remains the same under all conditions, including the different pH-conditions for (A) and (B). With this assumption we obtain the pure difference spectra for 100% Q_A- or 100% Q_B-reduction as shown in Fig. 7 by using the difference spectrum of Fig. 5A to correct the spectrum observed at 10 ms in oxygen-evolving particles (see legend of Fig. 7). The resulting ($Q_A^- - Q_A$) spectrum shows the typical features of plastoquinone reduction in vitro [32] plus the contributions by bandshifts centered at 422, 545 and 676 nm due to special RC pigments, probably pheophytin *a*. It is quite similar to that reported for PS II particles from spinach [8] up to 560 nm. The pure ($Q_B^- - Q_B$) spectrum shows in the ultraviolet region a shape like that of ($Q_A^- - Q_A$) in accordance with earlier findings in Ref. 11. The absolute $\Delta\epsilon$ values seem to be somewhat (10–20%) smaller for Q_B than for Q_A for a reason not yet understood. In the blue region the bandshift at 422 nm has almost disappeared so that the shape of the (reduced-oxidized) spectrum for Q_B is almost identical to that for plastoquinone in vitro, shown by the inset [32]. It appears that in this spectral region peaks of the in vivo spectrum are at slightly shorter wavelengths than those of the in vitro spectrum. At longer wavelengths the ($Q_B^- - Q_B$) spectrum is characterized by a small contribution of the bandshift at 545 nm and a remarkably

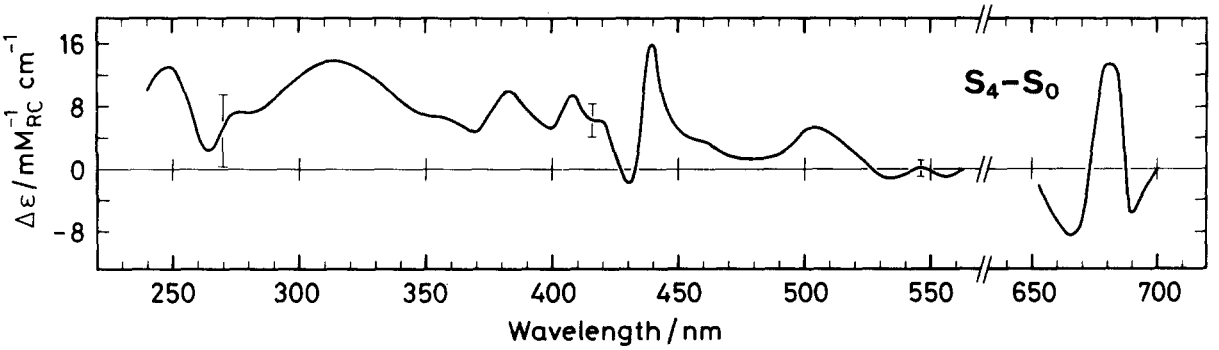

Fig. 9. Absorbance difference spectrum for 100% ($S_4 - S_0$). Calculated as the difference of Fig. 5B minus 1.133 times fig. 5A, multiplied by a factor of 4.

strong, asymmetric redshift of a band at 678 nm.

Fig. 8 shows the pure $(Z^+ - Z)$ spectrum which is calculated (see legend) from the difference between Tris-treated and oxygen-evolving PS II particles at 10 ms after the flash (Fig. 6) corrected for contributions of the acceptor side (Fig. 5A). In most features it resembles those obtained with PS II particles from spinach [8] and from a green alga [33], which was attributed in Ref. 8 to plastosemiquinol-cation formation. In our $(Z^+ - Z)$ spectrum we note two electrochromic bandshifts: a redshift centered at 435 nm and a blueshift at 680 nm, most likely of Chl *a* bands. The latter finding is at variance with Ref. 22, where for a band at 678 nm a redshift was observed (see discussion).

Fig. 9 shows the absorbance difference spectrum associated with the $S_4 \rightarrow S_0$ transition as calculated (see legend) from the millisecond transient in oxygen-evolving particles (Fig. 5B) and acceptor side contribution (Fig. 5A). Due to our repetitive flash protocol it is associated with a much larger experimental error (see error bars) than the other spectra. The $(S_4 - S_0)$ spectrum shows only positive net absorbance changes (at about 310, 380, 410 and 505 nm) plus predominant contributions by a red shift of a band at 435 nm and a more complex shift pattern in the red region. Between 270 and 460 nm it is similar to the corresponding spectrum observed in spinach PS II particles [14].

Discussion

Making use of PS II particles of the cyanobacterium *Synechococcus* sp., secondary electron transfer reactions in PS II were studied under repetitive flash illumination. From Ref. 24 it was known that in this preparation the electron acceptor side of PS II is accessible to ferricyanide. Here, conditions were chosen at which the oxidation of Q_B^- by ferricyanide was complete between flashes, but slow enough to be negligible at 10 ms after the flash. The dependences of ferricyanide reduction on pH and temperature [24] account for the different rates found in this work and in Ref. [24]. In Tris-treated particles, where oxygen evolution is inactivated, the terminal electron donor is Z. It was found that the back reaction of Z^+ and Q_B^- was negligible at 10 ms after the flash and that Z^+ was reduced with a half-time of about 1 s, presumably by ferrocyanide added at a concentration of 0.2 mM. This long lifetime of Z^+, much longer than reported in Ref. [34] for another cyanobacterial PS II preparation, was quite convenient for our purpose: no back reaction had to be taken into account.

Comparison of absorbance changes in Tris-treated and in oxygen-evolving particles (Fig. 4) showed that approximately the same amount of Q_A was reduced by a flash. The only complicating difference was in the equilibrium constant (K_{eq}) for the electron partition between Q_A and Q_B. K_{eq} could be determined from Chl fluorescence yield measurements, assuming that only the redox state of Q_A and not that of Q_B was responsible for the observed fluorescence changes. Thus, we found that the amount of Q_A^- still present at 10 ms after the flash was about 15% in oxygen-evolving and about 25% in Tris-treated PS II particles, corresponding to K_{eq} values of 5–6 and of about 3, respectively. The value for oxygen-evolving particles corresponds well with that recently determined in chloroplasts ($K_{eq} = 4.9$ [35]). Tris treatment in our case lowered the value of K_{eq}, not as strongly as reported in Ref. 11, but at variance with Ref. 36 where no effect was reported.

The states of PS II monitored by the absorbance difference spectra (Figs. 5 and 6) could be characterized according to Table I. From these data the difference spectrum upon oxidation/reduction of each of the intermediates was calculated, assuming that it is the same in all conditions. This comprises all the redox states of the other intermediates and the pH conditions which, we note, were different in oxygen-evolving (pH 6.0) and in Tris-treated (pH 8.3) samples. In spinach PS II particles the $(Q_A^- - Q_A)$ spectrum was found to be the same at these pH values while the $(Z^+ - Z)$ spectrum showed differences only near 275 nm when measured at pH 8.3 and pH 6.0 [8]. Thus, at least for the acceptor quinone pH-effects on the spectrum seem to be negligible.

The differences between the $(Q_A^- - Q_A)$ and the $(Q_B^- - Q_B)$ spectrum emerge directly from the absorbance changes measured between 0.1 ms and 10 ms after each flash in Tris-treated PS II particles (Fig. 5A). These changes are expected to arise primarily from the electrochromic response of

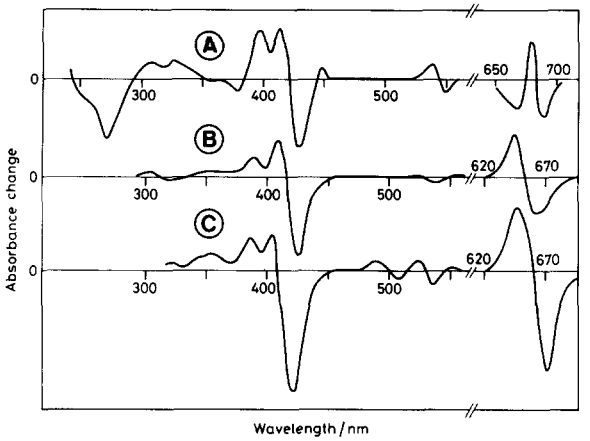

Fig. 10. (A) Absorbance difference spectrum of $Q_A^- Q_B \rightarrow Q_A Q_B^-$ transfer redrawn from Fig. 5A compared with first derivatives ($\delta\epsilon/\delta\bar{\nu} = \lambda^2 \delta\epsilon/\delta\lambda$) of absorbance spectra of pheophytin in (B) 98% (v/v) acetone/water and (C) 99.5% (v/v) ethanol/water. Note that wavelength scales of in vitro spectra (B) and (C) are shifted with respect to in vivo spectrum (A).

pheophytin *a* [3,37] (and perhaps of other RC pigments) to the presence of the electron on the acceptor quinone complex $Q_A Q_B$. If this pheophytin *a* is located closer to Q_A than to Q_B, the electrochromism is expected to decrease when the electron moves from Q_A^- to Q_B. In first approximation, the spectrum of an electrochromic band shift is proportional to the first derivative of the affected absorbance band on an energy scale [38]. In fact, such derivative spectra for pheophytin *a* in two different solvents (Fig. 10B and C) show remarkable similarities to the in vivo spectrum (Fig. 10A, redrawn from Fig. 5A), at least in the range 350–560 nm.

The amplitude of the absorbance changes in Fig. 5A was calibrated on basis of the Chl fluorescence yield measurements (Fig. 2) and used to calculate the difference spectra for 100% reduction of Q_A and Q_B, respectively. Comparison of the results (Fig. 7) clearly shows the extent of the decrease of electrochromism upon electron transfer from Q_A^- to Q_B. The shift of the pheophytin *a* Soret band, around 422 nm, is decreased by a factor of about 10, that of the Q_x band, around 545 nm, by about 4. In the region of the Q_y band, around 680 nm, a pronounced bandshift is present in the difference spectrum of Q_B as well, at slightly longer wavelength than in the Q_A difference spectrum. The PS II reaction center contains two pheophytin *a* molecules [39]. By analogy to observations in purple bacteria [40,41], it may be proposed that the charges on Q_A^- and on Q_B^- affect two different pheophytin molecules. In fact, if the arrangement of Q_A, Q_B and the two pheophytins in PS II were as symmetrical as crystallized *Rhodospeudomonas viridis* reaction centers seem to suggest [42], the similarity of the spectra in Fig. 7 in the Q_y region would be less surprising than the very different pheophytin contributions in the Soret region.

Our $(Z^+ - Z)$ spectrum shows, in agreement with an expected point charge effect [43], a red shift of a Soret band 435 nm and a blue shift of a band at 680 nm, attributed to a Chl *a*, most likely P-680. In the ultraviolet and blue region our results are consistent with earlier reports in Ref. 8 and 33. In the red region there is a blue shift in cyanobacterial, but a red shift in spinach PS II [22] (however, the possibility of a pH dependence of this spectrum has not yet been excluded). The difference spectrum of the millisecond transient in oxygen-evolving particles after subtraction of contributions by the acceptor side was attributed to the reduction of S_4 to S_0 in about 25% of the RCs. The accurate percentage may be slightly lower due to misses not compensated by double hits and/or decay of S_3 in the dark. However, both losses are estimated to be small compared to the experimental error. S_3 was found to be quite stable after extraction of PS II from the thylakoid membrane [44]. The resulting $(S_4 - S_0)$ spectrum appears to be consistent, within the large error, with that reported for spinach PS II in Ref. 14. The band at 505 nm probably corresponds to the absorbance change found to oscillate with a periodicity of 4 [45] in the same material when studied in dark-adapted samples. These changes were reported [45] to monitor the net charge of the oxygen-evolving complex, taking deprotonation sequence [16] into account. This view is consistent with the close correlation of deprotonation kinetics reported in Ref. 9 with our decay kinetics of the absorbance changes. A time-resolved analysis of the oscillation pattern at various wavelengths may provide the necessary information to establish this point unambiguously.

Conclusion

The absorbance difference spectra of ($Q_A^- - Q_A$), ($Q_B^- - Q_B$), ($Z^+ - Z$) and ($S_4 - S_0$) in the cyanobacterium *Synechococcus* sp. are similar to those in spinach. In the visible region of the spectrum they all contain pronounced features due to electrochromic bandshifts of pheophytin *a* and/or chlorophyll *a*, which can be used to discriminate between the various secondary electron-transfer reactions in PS II. In the blue region (Soret bands), the negative charge on Q_A^- causes a blue-shift and the positive charges on Z^+ and S_4 cause red-shifts. In the red region (Q_y bands) composite but predominantly red-shifts are observed; a small blue-shift, however, was observed in the ($Z^+ - Z$) spectrum. Further studies, e.g., by polarization spectroscopy on oriented samples, may help to disclose further this potentially rich source of information.

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