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Studies on the multiphasic charge recombination between chlorophyll a_{II}^{+} (P-680^{+}) and plastoquinone Q_A^{-} in Photosystem II complexes. Ultraviolet difference spectrum of Chl-a_{II}^{+}/Chl-a_{II}

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(1) The time-course of the recombination reaction occurring after charge separation between Chl-a_{II} (P-680) and Q_A in the presence of Z^{+} was analyzed by optical spectroscopy in the wavelength region between 250 nm and 570 nm. It could be modelled by the sum of three exponential decay phases with half-lifetimes of 170 μs, 800 μs and 6 ms, where the relative amplitudes of the three phases were very similar at all wavelengths. On the basis of the absorption difference spectra it is proposed that, despite the complex time-course, only the two components Chl-a_{II} and Q_A are involved. Thereby it is supposed that the multiphasic kinetics are due to a distribution of different structural states of the reaction center proteins. (2) A difference spectrum of Chl-a_{II}^{+}/Chl-a_{II} is evaluated by subtraction of contributions due to Q_A^{-}/Q_A from the combined spectrum Chl-$a_{II}^{+}Q_A^{-}$/Chl-$a_{II}Q_A$. The latter was derived from the amplitudes of the kinetic component dominating the recombination reaction as well as from the extrapolated initial amplitudes of absorption changes measured in oxygen-evolving and in Tris-treated PS II complexes. (3) Measurements of PS II complexes incubated with acetate according to Saygin et al. (Saygin, Ö., Gerken, S., Meyer, B. and Witt, H.T. (1986) Photosynth. Res. 9, 71–78) indicated that Z is the donor which supplies electrons to Chl-a_{II}^{+} in this system with a half-lifetime of about 160 μs.

Introduction

Electron transfer in PS II is started by the light-induced photooxidation of a special Chl-*a* molecule, Chl-a_{II} (P-680) [1,2]. The electron is very quickly (within a nanosecond [3,4]) transferred to a strongly bound plastoquinone molecule called Q_A (X-320) [5,6], which is thereby reduced to the (unprotonated) semiquinone anion [7]. In oxygen evolving PS II, Chl-a_{II}^{+} is reduced primarily in the nanosecond time range [8,9] by a donor called Z [10,11], the half-lifetime being dependent on the oxidation state (S_0–S_3) of the oxygen-evolving complex [12]. Z is supposedly a tyrosine residue of the D_1 polypeptide [11,13,13a].

After various treatments leading to an irreversible inhibition of O_2 evolution such as Tris-washing or incubation with millimolar concentrations of hydroxylamine, Chl-a_{II}^{+} reduction is retarded where the kinetics depend on the pH [14,15]. Under these conditions, Z reduces Chl-a_{II}^{+} in about 200 ns (at pH 9) to 40 μs (at pH 4). Z^{+} is re-reduced by artificial donor reagents such as hydroquinone or diphenylcarbacide. If a charge separation between Chl-a_{II} and Q_A occurs and Z is still oxidized, Chl-a_{II}^{+} is reduced by the charge recombination with Q_A^{-}, which is characterized by a dominating phase with a pH-independent half-lifetime of (150 ± 50) μs [14,16–19]. The reduction kinetics of Chl-a_{II}^{+} contain still slower components in the order of several hundreds of microseconds to milliseconds, which have tentatively been attributed to the back-reaction of the electron from the acceptor side to Chl-a_{II}^{+} [15,20–24]. Ford and Evans proposed that the slow phases arise from reaction centers in which Q_B is oxidized and Z is functionally disconnected from Chl-a_{II} [21]. Treatment with 600 mM acetate results in a reversible inhibition of oxygen

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Abbreviations: ANT-2p, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Fecy, $K_3(Fe(CN)_6)$; FWHM, full width at half maximum; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; P-680 (Chl-a_{II}), primary electron donor in PS II; Q_A,Q_B, quinone acceptors in PS II; RC, reaction centre; Z, intermediate electron carrier in PS II (very likely a tyrosoine).

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evolution [24]. Under these conditions forward electron transfer from an unidentified donor to Chl-a_{II}^{+} with $t_{1/2} = 160$ μs was observed to occur after the first and second flash. Under repetitive excitation, a 500 μs phase was observed, which has been explained by a retarded recombination reaction between Chl-a_{II}^{+} and Q_A^-.

In this paper we present a spectral analysis of the electron transfer reactions in Tris-treated as well as in acetate-treated PS II complexes from *Synechococcus* in order to characterize unambiguously the components that are involved.

In Tris-treated samples with oxidized Z, a complex time-course of Chl-a_{II}^{+} reduction with half-lifetimes between 170 μs and 6 ms was observed, which can be attributed exclusively to the charge recombination between Q_A^- and Chl-a_{II}^{+}.

In acetate-treated complexes, a 120–160 μs phase is caused by forward electron transfer from Z to Chl-a_{II}^{+}, while a 500–600 μs phase is due to a competition between the recombination with Q_A^- and forward electron transfer by an alternative, not yet identified donor in PS II.

A detailed difference spectrum of Chl-a_{II}^{+}/Chl-a_{II} in the wavelength region between 250 nm and 570 nm is presented. This spectrum was used in Ref. 11 for evaluation of the spectrum of the immediate donor to Chl-a_{II}^{+} in oxygen-evolving systems (i.e., of tyrosine).

Materials and Methods

Oxygen-evolving PS II complexes were prepared from the thermophilic cyanobacterium *Synechococcus* sp. according to Schatz and Witt [25] and further purified by sucrose density centrifugation [26,27]. They were characterized by a steady-state O_2 flash yield of about $2.5 \cdot 10^{-3}$ O_2 per Chl and flash corresponding to 100 Chl/PS II active in O_2 evolution. Tris treatment was performed with 0.8 M Tris-HCl (pH 8.3) as described before [27]. After the purification, 5 mM EDTA were added. The complexes treated in this way were characterized by about 67 Chl per photoreducible Q_A [28].

Tris-treated PS II complexes at pH 5.5 were obtained as in Ref. 11 and incubation with 600 mM acetate was performed as in Ref. 24. Absorption changes in the time domain between 15 μs and 10 ms were measured with an apparatus consisting of a DC Xenon arc lamp (XBO 150 W/1 from Osram) or a 250 W tungsten lamp (Osram) as measuring light sources and a photomultiplier (EMI 9558BQ) connected to a transient recorder (Biomation 4500 from Gould) as detection system. A monochromator (6 nm bandwidth except in the wavelength regions 410–450 nm and 540–570 nm, where the bandwidth was set to 3 nm) was placed between the measuring light source and the sample. The multiplier was protected by a combination of interference filters (Schott) and colored glasses (Hoya, Corning). The sample was excited by a saturating xenon flash of 15 μs duration (FWHM) filtered by 1 mm GG435 (Schott) and 3 mm CS 4-96 (Corning) for measurements between 250 and 370 nm or 2 mm RG665 (Schott) between 370 nm and 570 nm. The electrical bandwidth was d.c.–50 kHz. The same arrangement was used for measurements on a time-scale of up to 2 s, except that a deuterium lamp (C70-3V-H-G3 from Cathodeon) was used as measuring light source below 290 nm and the electrical bandwidth was d.c.–160 kHz. Measurements with a time resolution of 7 ns were performed with a spectrometer described in Ref. 4. The apparatus used in the 2–100 μs time domain is described in Ref. 11.

For the evaluation of the initial amplitude of absorption changes measured in the nanosecond and 100 μs time domain, the measured data were fitted to the analytical functions described in detail in Ref. 11 by means of an iterative least-squares procedure [29]. All measurements were performed at room temperature.

Results

Recombination in Tris-EDTA-treated PS II complexes

We studied flash-induced kinetics of absorption changes in the 15 μs to 10 ms time range under conditions where Z was oxidized prior to the flash. The PS II complexes were deprived of their physiological source of electrons by treatment with Tris, and no artificial electron donors or acceptors were added. Furthermore, the samples were supplied with 5 mM EDTA and excited with saturating flashes at a rate of 5 Hz in order to avoid re-reduction of Z^+ by Mn^{2+} in the dark time between the flashes. An analysis of the Chl-a_{II}^{+} reduction kinetics under these conditions yielded that kinetic components due to the forward electron transfer from Z to Chl-a_{II}^{+} were nearly absent (rel. amplitude at 824 nm approx. 5%, not shown). Thus, Z is in the oxidized state in about 95% of the reaction centers prior to the flash. (Note that Z is not functionally disconnected from Chl-a_{II}, as Chl-a_{II}^{+} reduction was found to occur with $t_{1/2} = 1.3$ μs after the first flash in dark-adapted samples.)

Fig. 1A–E shows the absorption changes in the system Tris/EDTA at different wavelengths. The time-courses at wavelengths indicative of Chl-*a* oxidation (824 nm (B), 434 nm (E)) appear to be very similar to those indicative for the reduction of a quinone to the semiquinone anion form (320 nm (A), 265 nm (D)). Also, C550 (C), a bandshift of Pheo-*a* centered at 545 nm upon reduction of Q_A [7,30] shows a time-dependence similar to that of signals in Fig. 1A, B, D and E.

Obviously, the decay of the absorption changes is not monophasic. Assuming that the time-course is adequately described by a sum of exponentials, we had to take into account at least three exponentials plus a constant in order to obtain a satisfactory fit. The best

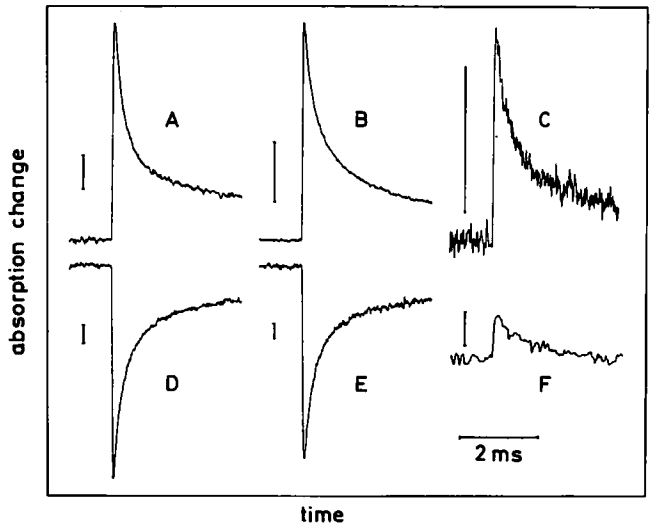


Fig. 1. Time-courses of absorption changes measured in Tris-treated PS II complexes at pH 8.3 in the presence of 5 mM EDTA and in the absence of artificial electron donors and acceptors at different wavelengths. (A) 320 nm, (B) 824 nm, (C) Difference 540–550 nm ('C550'), (D) 265 nm, (E) 434 nm, (F) 320 nm in the presence of 10 μM DCMU; 63 μM Chl, optical path = 0.12 cm; excitation flashes were spaced at 200 ms. The bar next to each time course represents $\Delta A = 2 \cdot 10^{-4}$. Average of 256 measurements.

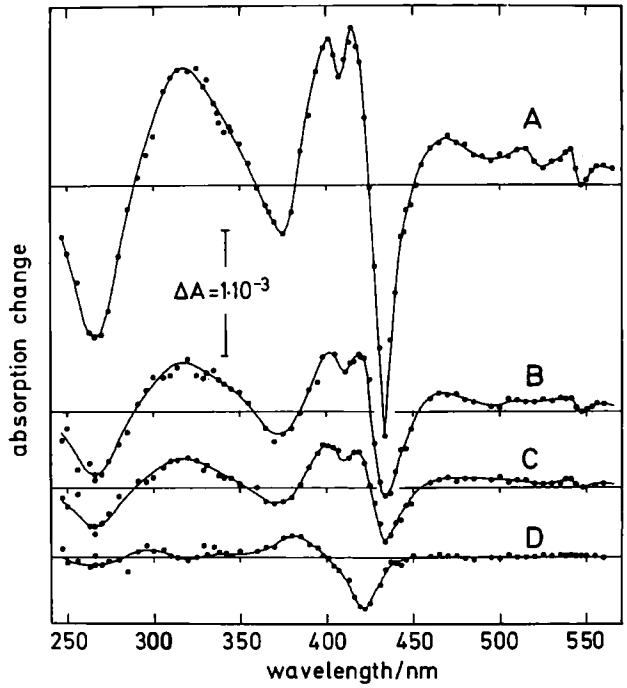


Fig. 2. Spectra of the amplitudes of kinetic phases with half-lifetimes of (A) 170 μs, (B) 800 μs, (C) 6 ms and (D) a constant. Absorption changes were measured as described for Fig. 1 and fitted to a function $\Delta A(t) = A \times \exp(-\ln 2 \times t/170\ \mu s) + B \times \exp(-\ln 2 \times t/800\ \mu s) + C \times \exp(-\ln 2 \times t/6\ ms) + D$. Note that the signals were measured (and fitted) on a total sweep of 10 ms, which is not completely shown in Fig. 1.

fit was obtained at all wavelengths with half-lifetimes of about 150–190 μs, 700–900 μs and 2–10 ms. In a second approach, we used a fitting procedure with fixed half-lifetimes of 170 μs, 800 μs and 6 ms plus a constant. The signals at all wavelengths could be fitted satisfactorily with this set of time constants, and as a result we found that the relative amplitudes of the decay phases were very similar, i.e., about 60% 170 μs, 25% 800 μs and 15% 6 ms. It should be mentioned that these values were found in measurements at room temperature, while at lower temperatures ($T = 250$ K) higher relative amplitudes of the slower phases were observed (not shown). The spectra of the three phases in the wavelength region between 250 nm and 570 nm are shown in Fig. 2A–C. From the similarity of these spectra we conclude that all three phases arise from the charge recombination between Chl-a_{II}^+ and Q_A^-, which thus occurs in nearly all reaction centers (see below). The spectra in Fig. 2A–C are therefore attributed to the absorption difference of Chl-$a_{II}^+Q_A^-$/Chl-$a_{II}Q_A$.

When the electron transfer from Q_A to Q_B was inhibited by DCMU, only small signals were observed on a time-scale of milliseconds under repetitive excitation (see Fig. 1E). As the state $Z^+Q_A^-$ formed by the first flash decays only slowly (see below), subsequent flashes can give only Chl-a_{II}^+Pheo$^-$, which recombines in 11 ns [31].

In order to prove that the difference spectrum shown in Fig. 3, circles (redrawn from Fig. 2A), represents the electron-transfer reaction Chl-$a_{II}^+Q_A^- \rightarrow$ Chl-$a_{II}Q_A$, we measured absorption changes in oxygen-evolving PS II complexes with a time resolution of 7 ns. The extrapolated initial amplitudes should represent the absorption difference due to the formation of the state Chl-$a_{II}^+Q_A^-$ and the disappearance of Chl-$a_{II}Q_A$, since with this time resolution signals due to the electron transfer from Pheo$^-$ to Q_A escape detection [3,32,4], while the reduction of Chl-a_{II}^+ by Z [8,9,11] can be recorded and extrapolated to its beginning. Q_A^- is stable on a time-scale of microseconds [33].

In Fig. 3, triangles, a partial spectrum of the extrapolated initial amplitudes measured in oxygen-evolving PS II complexes is shown. This spectrum seems to be identical to the ultraviolet part of the spectrum obtained from the analysis of the back-reaction. We also determined the spectrum of the extrapolated initial amplitudes of flash-induced absorption changes in Tris-treated PS II complexes at pH 5.5 (see Fig. 3, squares). A time resolution of 2 μs was used in this case, which assured that the fastest kinetic component in this system, i.e., the re-reduction of Chl-a_{II}^+ by Z ($t_{1/2} = 15$ μs) could be extrapolated to time zero (see Materials and Methods). Also, this spectrum is very similar to the spectrum obtained from the analysis of the back reaction (Fig. 3, circles).

While the bleaching around 265 nm and the absorption increase around 320 nm are due to the formation of

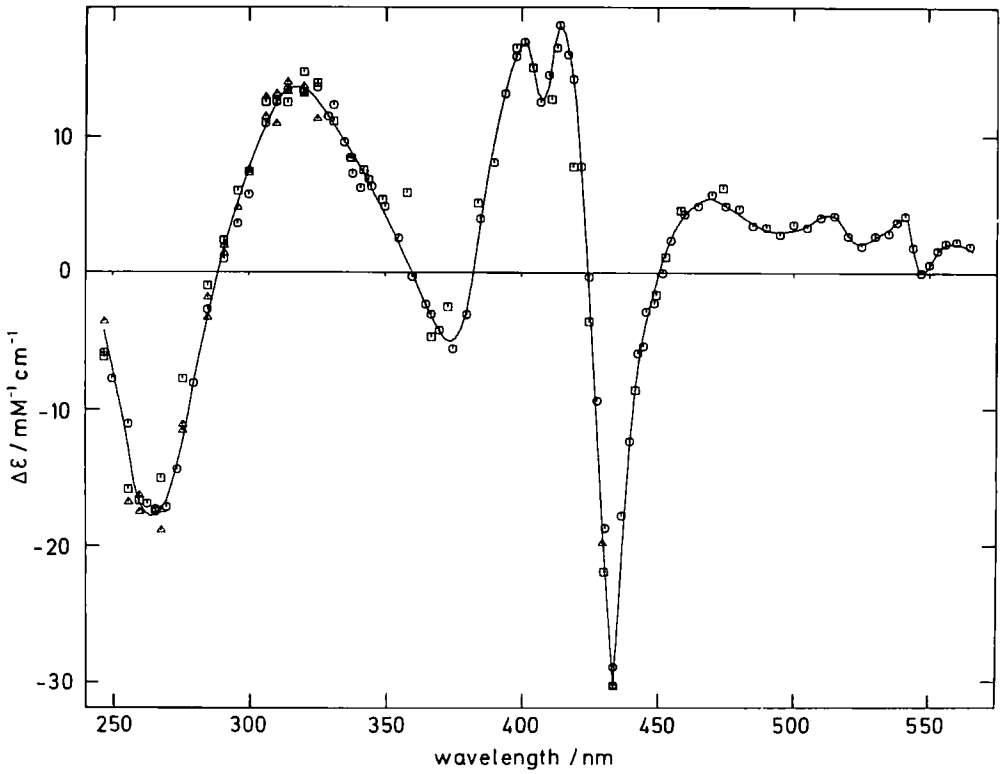


Fig. 3. Circles: Spectrum of the amplitudes of the 170 μs kinetics reproduced from Fig. 2(A). The Δε scale was calculated on the basis 67 Chl/RC and the observations that the back-reaction occurred in 95% of the reaction centers and that the 170 μs kinetics represent 65% of these centes (see text). Triangles: Spectrum of the extrapolated initial amplitudes of absorption changes measured in oxygen-evolving PS II complexes with a time resolution of 7 ns. 60 μM Chl, 1 mM Fecy, 20 mM Mes/NaOH (pH 6.5), 20 mM $CaCl_2$, 10 mM $MgCl_2$, 0.05% (w/w) 8-dodecyl D-maltoside, optical path 0.21 cm, 80% saturation. Squares: Spectrum of the extrapolated initial amplitude of absorption changes measured in Tris-treated PS II complexes with a time resolution of 2 μs. 50 μM Chl, 1 mM Fecy and 1 mM ferrocyanide, 20 mM succinic acid/NaOH (pH 5.2), 20 mM $CaCl_2$, 10 mM $MgCl_2$, 0.05% (w/w) 8-dodecyl D-maltoside, optical path 0.11 cm, 80% saturation.

Q_A^-, the sharp bleaching around 434 nm is caused by the oxidation of Chl-a_{II}.

Absorption difference spectra

The Chl-a_{II}^+/Chl-a_{II} spectrum alone can be evaluated by subtracting the Q_A^-/Q_A spectrum from the Chl-$a_{II}^+Q_A^-$/Chl-$a_{II}Q_A$ spectrum shown in Fig. 3.

In order to have a reliable Q_A^-/Q_A spectrum of our *Synechococcus* PS II complexes for this purpose, we performed measurements as described by Dekker et al. [28] with PS II preparations from spinach. In short, the samples are supplied with the redox couple ferri/ferrocyanide and with DCMU, which inhibits the electron transfer from Q_A^- to the secondary quinone. The latter conditions induce a cyclic electron flow around the photosystem. The re-oxidation of Q_A^- by ferricyanide is slow ($t_{1/2} = 4$ s under our conditions), while the re-reduction of Z^+ ($t_{1/2} = 165$ ms under our conditions) is due to a competition between charge recombination with Q_A^- and forward electron transfer from ferrocyanide. The time-course of the absorption change observed at 320 nm under these conditions is shown in Fig. 4, inset. The initial amplitude reflects the absorption change due to $Z^+Q_A^-/ZQ_A$; its spectrum is shown in Fig. 4B.

When the re-reduction of Z^+ has been completed, the remaining part of Q_A is still reduced in that fraction of the reaction centers where Z^+ has been reduced by ferrocyanide. Therefore, the shape of the spectrum of the signal amplitudes at this time is determined by $\Delta\epsilon(Q_A^-/Q_A) + \Delta\epsilon$(ferri/ferrocyanide). This is still true at any later point in time during the reoxidation of Q_A^- by ferricyanide. We determined the spectrum at 800 ms after the flash and, after subtraction of the difference spectrum of ferri/ferrocyanide, that of Q_A^-/Q_A is obtained (Fig. 4A). To calibrate the Δε-scale we used the condition that the absorption difference of Q_A^-/Q_A at 320 nm should be $\Delta\epsilon = 12\,500\ M^{-1} \cdot cm^{-1}$ (7, 34). There are only minor differences between this spectrum and the one reported previously for BBY-preparations from spinach [28], such as a slight blue shift of the 325 nm/265 nm absorption bands and the absence of 475 nm/505 nm bands. A similar blue shift has been observed previously by Schatz and van Gorkom [35] in PS II particles from *Synechococcus* sp. However, the Q_A^-/Q_A spectrum of the latter authors shows a larger absorption increase in the range between 320 nm and 350 nm.

Fig. 4C shows the Z^+/Z difference spectrum obtained by subtraction of the Q_A^-/Q_A spectrum (Fig. 4A)

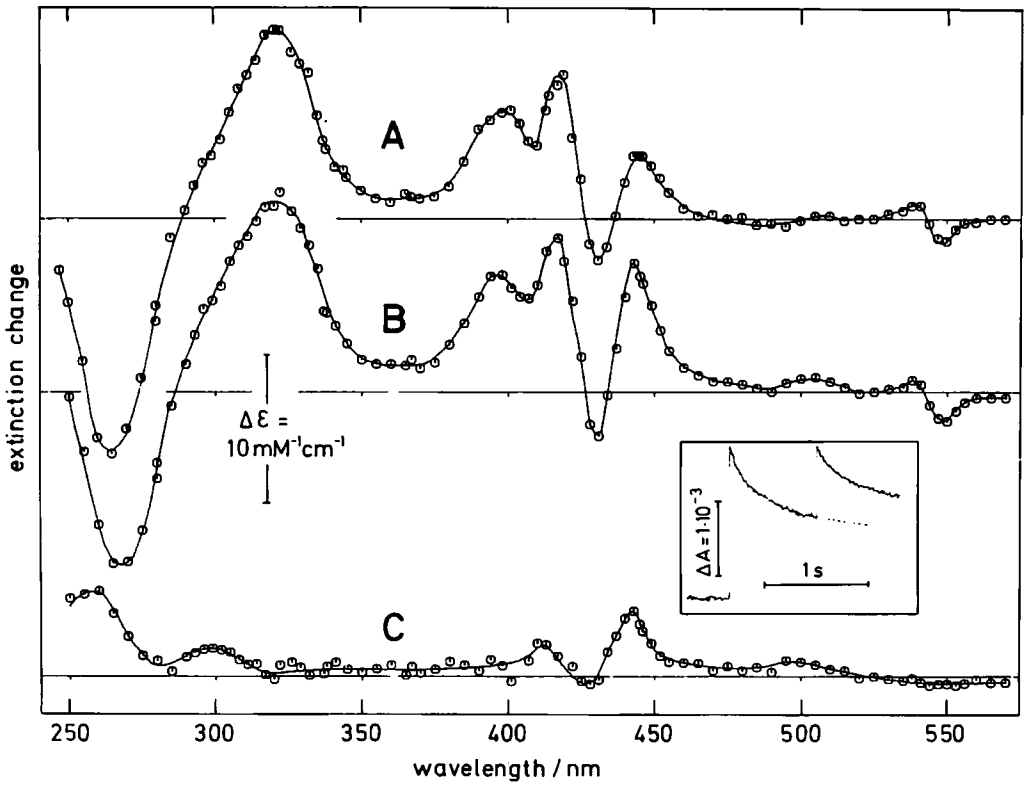


Fig. 4. Difference spectrum of (A) Q_A^-/Q_A, (B) $Z^+Q_A^-/ZQ_A$, (C) Z^+/Z measured according to the method described in Ref. 25 with the PS II complexes from *Synechococcus* sp. used in this work. 94 μM Chl, 2.5 mM Fecy, 2.5 mM ferrocyanide, 10 μM DCMU, 100 mM Tris-HCl (pH 8.3), 20 mM $CaCl_2$, 10 mM $MgCl_2$, about 1 M sucrose, optical path 0.12 cm. Excitation flashes were spaced at 10 s. For further details see text. Inset: Time-course of absorption changes measured at 320 nm in a double-flash experiment under the conditions described above. The dotted line represents the signal observed when the second flash is omitted. The second flash is given about 805 ms after the first flash.

from that of $Z^+Q_A^-/ZQ_A$ (Fig. 4B), which is very similar to previously reported Z^+/Z spectra [28,35–37].

For the evaluation of the Z^+/Z spectrum in Fig. 4C we used the supposition that the extinction difference of Z^+/Z at 320 nm is negligibly small [28,35,36]. As this was doubted recently by Weiss and Renger [37], we want to present further evidence in favor of our assumption, which was derived from a double-flash experiment performed with the above-described system at 320 nm (see Fig. 4, inset). The amplitude of the absorption change caused by the second flash (applied at about 805 ms after the first flash) reaches exactly the same level as that of the first flash. When the second flash is fired, Z has been re-reduced in all reaction centers, while Q_A is re-oxidized in only about half of the centers. Therefore, the second flash can form the state $Z^+Q_A^-$ only in about half of the centers. Since $\Delta\epsilon$(ferri/ferrocyanide) at 320 nm is negligible, the double-flash experiment yields the following result:

$$\Delta\epsilon(Z^+Q_A^-/ZQ_A) = 0.5\times\Delta\epsilon(Z^+Q_A^-/ZQ_A) + 0.5\times\Delta\epsilon(Q_A^-/Q_A)$$

Therefore, it follows that the contribution of Z^+/Z must be very small at 320 nm.

Fig. 5 shows the difference spectrum of Chl-a_{II}^+/Chl-a_{II} which results from the subtraction of the above-derived Q_A^-/Q_A spectrum (Fig. 4A) from the combined spectrum Chl-$a_{II}^+Q_A^-$/Chl-$a_{II}Q_A$ (Fig. 3). The reliability of the Chl-a_{II}^+/Chl-a_{II} spectrum obtained in this way depends (especially in the ultraviolet region) quite sensitively on the appropriate calibration of the ordinate in Fig. 3 in $\Delta\epsilon$ units, which is based on measurements of the ratio of antenna chlorophylls to photoreducible Q_A ($\Delta\epsilon = 12\,500\ M^{-1}\cdot cm^{-1}$ at 320 nm). This result is consistent with findings from a different type of measurement. The secondary electron tranfer Chl-$a_{II}^+Z \rightarrow$ Chl-$a_{II}Z^+$ causes only small absorption changes in the order of $\Delta\epsilon \approx 400$-$700\ M^{-1}\cdot cm^{-1}$ at 320 nm [11,37]. With the condition that contributions due to Z^+/Z are negligible at this wavelength (see above), these changes must be due to Chl-a_{II}^+/Chl-a_{II}, which is in agreement with our data shown in Fig. 5.

The Chl-a_{II}^+/Chl-a_{II} spectrum presented in this work is dominated by a very sharp bleaching around 434 nm and further characterized by absorption increases around 305, 345, 400, 415 and above 450 nm and absorption decreases below 290 nm and around 375 nm. The absorption difference of the bleaching centered at 434 nm ($\Delta\epsilon = 28\,000\ M^{-1}\cdot cm^{-1}$) is significantly smaller than the one observed upon oxidation of Chl-*a* in vitro ($\Delta\epsilon = 50\,000\ M^{-1}\cdot cm^{-1}$, Ref. 38) (see Discussion). In order to exclude a measuring artifact, excitation of the

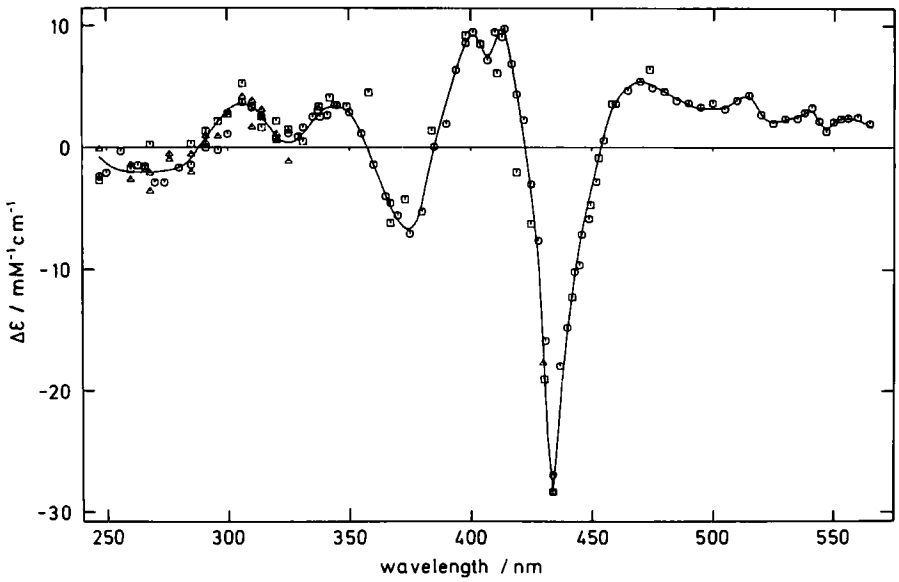


Fig. 5. Difference spectrum attributed to the oxidized minus reduced form of Chl-a_{II}, evaluated by subtraction of the spectrum of Q_A^-/Q_A (Fig. 4A) from the combined spectrum of Chl-$a_{II}^+Q_A^-$/Chl-$a_{II}Q_A$ (Fig. 3) on the same $\Delta\epsilon$-scale. The symbols are used analogous to Fig. 3. Circles: spectrum of the 170 μs phase of the recombination reaction between Chl-a_{II}^+ and Q_A^- minus Q_A^-/Q_A; triangles: spectrum of the extrapolated initial amplitude of absorption changes in oxygen-evolving PS II complexes minus Q_A^-/Q_A; squares: spectrum of the extrapolated initial amplitude of absorption changes in Tris-treated PS II complexes minus Q_A^-/Q_B; for further details, see legend of Fig. 3.

sample by the measuring light was tested to be insignificant and a spectral bandwidth of 3 nm was chosen in the blue region. Different spectral features of the Chl-a_{II}^+/Chl-a_{II} spectrum evaluated in this work compared to the Chl-a_{II}^+/Chl-a_{II} spectrum in vitro might be caused by bandshifts resulting from the positive charge on Chl-a_{II}^+ and the negative charge on Q_A^-. Bandshifts caused by a negative charge on Q_A^- and a positive charge on Chl-a_{II}^+ at the same time might not necessarily represent the sum of the effects of only one charge on either Q_A^- or Chl-a_{II}^+, resp.

Acetate

In PS II preparations treated with high concentrations (600 mM) acetate, Chl-a_{II}^+ reduction has been reported to occur with 120–160 μs after the first and second flash. When the sample was illuminated with more than two flashes, the half-lifetime of Chl-a_{II}^+ reduction was slowed down to $t_{1/2} = 500$ μs, which was interpreted to reflect mainly the recombination between Chl-a_{II}^+ and Q_A^- under these conditions [24].

Since forward electron transfer with half-lifetimes in the order of 160 μs had not been reported before, we investigated whether Z or another component donates to Chl-a_{II}^+ in the acetate system by measuring the spectrum of the 120–160 μs phase in the wavelength region of 250–320 nm. As it was necessary to add several measurements for a satisfactory signal-to-noise ratio, we tried to find a method which maintained the

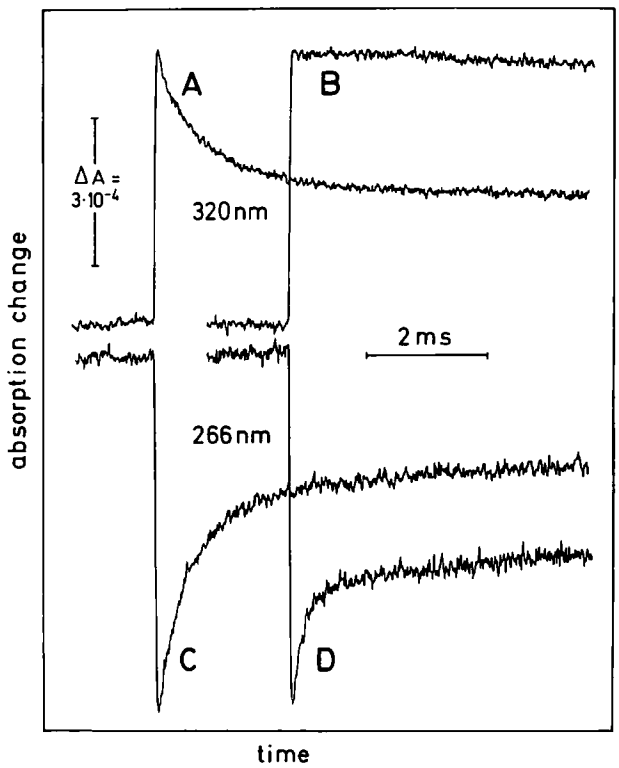


Fig. 6. Time-course of absorption changes at 320 nm and 266 nm observed after incubation of oxygen-evolving PS II complexes with 600 mM acetate. (A, C) 24 μM Chl, 100 μm dichlorobenzoquinone, 600 mM sodium acetate/NaOH (pH 5.5), 20 mM $CaCl_2$, 10 mM $MgCl_2$. (B, D) Same as in (A, C) but in the presence of 2 μM ANT-2p. Optical path, 0.12 cm. Excitation flashes were spaced at 2.5

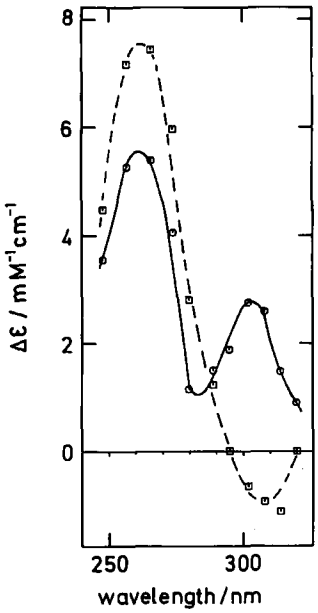


Fig. 7. Squares: spectrum of the extrapolated amplitudes of absorption changes decaying with $t_{1/2} = 120–160$ μs in acetate-treated PS II complexes in the presence of ANT-2p as described for Fig. 6(B, D); attributed to the reduction of Chl-a_{II}^+ and the oxidation of its donor. Circles: spectrum of the 120–160 μs phase (squares) minus that of Chl-a_{II}/Chl-a_{II}^+ (Fig. 5, inverted); attributed to the oxidized minus reduced form of the immediate electron donor to Chl-a_{II}^+ in the system acetate/ANT-2p.

situation of the first two flashes also in higher flash numbers. This appeared to be possible by addition of the lipophilic electron donor ANT-2p [39].

Fig. 6B, D shows that signals measured in the presence of ANT-2p can be modeled by a phase with a half-lifetime of 120–160 μs and a kinetic component decaying more slowly than $t_{1/2} \approx 20$ ms. The former phase reflects the re-reduction of Chl-a_{II}^+, since the same kinetics were observed at 824 nm (not shown). Little recombination with Q_A^- seems to occur in this case because the amplitude of the 120–160 μs kinetics is very small at 320 nm (see Fig. 6B). The slowly decaying part of the signal is attributed to reduced Q_A and the oxidized donor.

Fig. 7, dotted line, shows the amplitude of the 120–160 μs kinetics as a function of the wavelength. This spectrum is very similar to that of the electron transfer from Z to Chl-a_{II}^+ [11,37]. As can be seen in Fig. 7, solid line, the difference spectrum of the donor alone obtained after subtraction of the Chl-a_{II}/Chl-a_{II}^+ spectrum (Fig. 5, inverted) is indeed that of Z^+/Z [28,35–37]. This is compatible with EPR measurements performed with the same types of sample where the oxidized form of the donor supplying electrons to Chl-a_{II}^+ with $t_{1/2} = 170$ μs gave rise to a signal II spectrum [40].

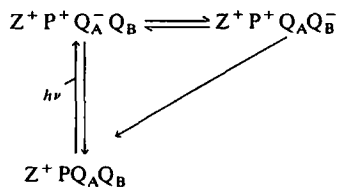
Fig. 6A, C shows signals obtained in the presence of acetate only. They are characterized by 500–600 μs kinetics and a slow (≈ 20 ms) phase. Measurements at 824 nm and 680 nm showed that Chl-a_{II}^+ decays mainly with $t_{1/2} = 500–600$ μs in most of the reaction centers, while about 10% decay with 120–160 μs (not shown). The signals observed in the ultraviolet region can be explained by a competition between the recombination of Chl-a_{II}^+ with Q_A^- and forward electron transfer from an alternative donor to Chl-a_{II}^+. From the time-course of C550 (see above) we estimated that the back-reaction occurred in about 55–65% of the reaction centers, while Chl-a_{II}^+ was reduced by the 'alternative donor' in about 35–45% of the centers (not shown). Variations of this ratio were observed for different preparations. We do not want to speculate about the nature of the 'alternative donor', as the analysis of the spectral data would require a more precise knowledge of the relative amounts of centers giving forward and back-reactions. It does not appear likely that the reduced artificial acceptor ($DCBQH_2$) donates directly to Chl-a_{II}^+, since in this case the oxidized donor would absorb at 320 nm, which is not compatible with the signal shown in Fig. 6A. Only small absorption changes were observed at 559 nm and at 980 nm, indicating that cytochrome *b*-559 [41,42] or carotenoid [43] can be excluded as candidates for the alternate donor. Since the time-courses of absorption changes observed at 320 nm and at 824 nm differ significantly, it can be concluded that the alternative donor is not a chlorophyll [44,45]. It is probably also different from Z, as EPR data obtained under the same conditions indicated that Z remained oxidized between the flashes [40]. The EPR measurements also revealed kinetics with $t_{1/2} = 500–600$ μs which were considered to originate from the cation radical form of Chl-a_{II} [40]. As the spectrum of this component was unusually broadened (1.4 mT), it appears likely that the retardation of the electron transfer at the donor side of PS II by acetate is related to a modification of Chl-a_{II}.

Discussion

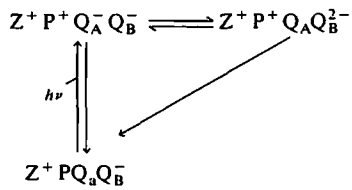
In this work, we presented a detailed analysis of microsecond electron transfer characteristics of inhibited PS II complexes from *Synechococcus*. With Tris/EDTA-treated complexes, it appeared possible to observe recombination between Chl-a_{II}^+ (in the following abbreviated by P^+) and Q_A^- in virtually all reaction centers (see below). Taking into account the two stable forms of Q_B, the system is in the state ZPQ_AQ_B or $ZPQ_AQ_B^-$ after dark adaptation. Q_B is present in our PS II preparations. After the first flash, P^+ is reduced by Z and, consequently, the state $Z^+PQ_AQ_B^-$ or $Z^+PQ_AQ_B^{2-}$, which lastly results in $Z^+PQ_AQ_B$, is formed. The second and further flashes induce the recombination between P^+ and reduced quinone (see below), since Z^+ remains oxidized between the flashes. The time-course of the recombination reaction can be described by three exponential decay phases with half-lifetimes of approx.

170 μs, 800 μs and 6 ms. The absorbance difference spectra of the three phases were found to be identical. Recombination kinetics slower than the usual 100–200 μs phases have been observed before [20–22]. It was proposed that the slow phases (600 μs–1 ms) arise from reaction centers where Z is functionally disconnected from Chl-a_{II} and Q_B is in the oxidized form [21]. Our results suggest that this interpretation is less likely, since we observed slow phases in a considerable portion (40%) of the reaction centers, although Z was well connected to Chl-a_{II} in nearly all centers (see above). Furthermore, the centers yielding the slow phases should be insensitive to DCMU according to the interpretation in Ref. 21, but this was not observed (Fig. 1F). It should be noted that the addition of EDTA was necessary to induce the back-reaction to its fullest extent, while in the absence of EDTA forward electron transfer through PS II was observed in part of the centers even in the absence of artificial donors and acceptors and at an excitation rate of 5 Hz. This means that Z^+ has been reduced in the dark time between the flashes in these centers. A likely candidate for the reductant is Mn^{2+} [46,47], which may have been liberated from the water-splitting complex by the Tris treatment and which would be removed by EDTA. Satoh and Katoh [48] studied the effect of EDTA on oxygen-evolving preparations from *Synechococcus* and concluded that it induced Chl-$a_{II}^+Q_A^-$ recombination by inhibiting electron transfer from Z to Chl-a_{II}^+. Our results do not confirm this conclusion; it has instead to be concluded that Chl-a_{II}^+ can still be reduced by Z. The question arises whether, for some reason, the recombination between only two partners, i.e., Chl-a_{II}^+ and Q_A^- occurs with differing rates in different reaction centers or whether more than two redox-active components are involved in this process.

To begin with, we discuss the case that several states of the acceptor side of PS II, i.e., $Q_A^-Q_B$, $Q_AQ_B^-$, $Q_A^-Q_B^-$ or $Q_AQ_B^{2-}$ might be involved in the reaction. The complete reaction pattern might be represented by the following schemes:



and



taking into account different redox states of Q_B prior to the flash. Each of the kinetic schemes would yield two phases.

However, from an analysis of our data we derived the conclusion that Q_B is probably not at all involved in the observed recombination reaction. If the state $Q_AQ_B^{2-}$ is involved (lower scheme), the time course of absorption changes at 320 nm should be at least partly determined by the disappearance and reappearance of semiquinones and should therefore differ significantly from that at 434 nm or 824 nm, where the concentration of Chl-a_{II}^+ is monitored. This was not observed.

The involvement of the state $Q_AQ_B^-$ (upper scheme) is also not appropriate to explain our data. According to the scheme, the state $Z^+P^+Q_A^-Q_B$ would decay essentially monophasically, were the rate for the decay is the sum of the rates for the electron transfer from $Q_A^-Q_B$ to $Q_AQ_B^-$ on the one hand and that for direct recombination between Q_A^- and Chl-a_{II}^+ on the other hand. This is so, if the equilibrium constant for $Q_A^-Q_B\,Q_AQ_B^-$ is large compared to 1. Therefore, the time-course of 'C550', the bandshift of Pheo-*a* caused mainly by the negative charge on Q_A should show a monophasic decay which was not observed (see Fig. 1C). From the presence of slow kinetic components with considerable relative amplitudes in the measured signal 'C550', we conclude that the state $Q_AQ_B^-$ is not involved in the observed reaction. According to the data shown in Fig. 5, the bandshift 'C550' can also be induced upon oxidation of Chl-a_{II}, although to a minor extent compared to that of the reduction of Q_A. However, the contribution of Chl-a_{II}^+ to 'C550' can account for only a rather small part of the slow kinetics observed in 'C550' during the recombination reaction.

Therefore we have to conclude that the recombination occurs only between Chl-a_{II}^+ and Q_A^- in the states $Z^+P^+Q_A^-Q_B$ or $Z^+P^+Q_B^-Q_B^-$. This implies that the electron transfer from $Q_A^-Q_B$ to $Q_AQ_B^-$ and $Q_A^-Q_B^-$ to $Q_AQ_B^{2-}$, respectively, becomes very improbable, at least after the second and further flashes. Under these conditions, the electron transfer to Q_B might be retarded due to the presence of positive charges on Chl-a_{II} and Z. There is no contradiction between this explanation and previous reports, which state that the electron transfer from Q_A to Q_B occurs with half-lifetimes in the order of 200 to 500 μs, as the latter data were measured in the presence of reduced Chl-a_{II} and at least partly reduced Z [33,35,49]. The two states $Z^+P^+Q_A^-Q_B$ and $Z^+P^+Q_A^-Q_B^-$ can still give two phases, if the recombination occurs with different rates in either system. However, the measured data cannot be explained by the sum of two exponentials.

Therefore, we rather want to propose the following mechanism in order to explain the observed complex time-course: The recombination takes place only between Chl-a_{II}^+ and Q_A^-, whereby the multiphasic kinetics may be caused by a distribution of different struct-

ural states of the reaction center proteins. A non-exponential behavior of a reaction due to a heterogeneity of structural states has first been observed by Austin et al. [50] in studies of heme proteins. The authors explained the time-course in terms of a model in which different activation enthalpies for the reaction in question were attributed to the different structural states. The same model was used by Kleinfeld et al. [51] to describe the non-exponential time-course of the charge recombination between the oxidized primary donor and Q_A^- in bacterial reaction centers at low temperature.

The time-course of the recombination reaction between Chl-a_{II}^+ and Q_A^- measured in this work (see Fig. 1) can also be described by the analytic function used by Austin et al. [50] and Kleinfeld et al. [51]. The three exponentials used above should therefore not be attributed to three different processes, but rather be considered as a result of the familiar approach to describe the kinetics.

The protein can fluctuate between its different structural states. A non-exponential time-course is only observed when the transitions between the states occur in a time which is slow compared to the time of the measurement. Therefore, it has to be concluded that typical times for the transitions of the reaction center protein between its structural states are in the order of milliseconds or longer at room temperature. On the other hand, these times should be considerably shorter than 100 ms, since Kleinfeld et al. [51] found that the recombination reaction in bacterial reaction centers (see above) was monophasic with $\tau = 132$ ms at room temperature. Reagents such as acetate [24] or potassium iodide [23] might also cause structural changes in the protein environment of Chl-a_{II} or Q_A, which would explain the observed retardation of the forward electron transfer from Z to Chl-a_{II}^+ and the recombination reaction.

It appeared possible to determine a highly detailed spectrum of Chl-a_{II} oxidation in the ultraviolet and blue spectral region (Fig. 5) by subtraction of the Q_A^-/Q_A spectrum (Fig. 4A) from the spectrum of Chl-$a_{II}^+Q_A^-$/Chl-$a_{II}Q_A$ (Fig. 3). The Chl-a_{II}^+/Chl-a_{II} spectrum shows absorption increases around 305, 345, 400, 415 and above 450 nm and absorption decreases below 290 nm and around 375 nm and 434 nm. The latter bleaching is extremely sharp and significantly smaller than observed for Chl-*a* oxidation in vitro [38]. In the blue, the spectrum resembles very much the Chl-a_{II}^+/Chl-a_{II} spectrum measured by Döring et al. [1,2]. There is also good agreement in the blue spectral region with the results of Diner and Bowes [52]. The difference spectra reported by Van Gorkom and co-workers [53,54] appear to be characterized by contributions from both Chl-a_{II} and a quinone acceptor. Differences between our spectrum and that of Weiss and Renger [37] are probably caused by a different scaling of the Q_A^-/Q_A spectrum.

The precise knowledge of the absorbance difference spectrum of Chl-a_{II} oxidation has been of importance for the evaluation of the immediate donor to Chl-a_{II}^+ in oxygen-evolving systems [11]. It also may become important in view of recent suggestions regarding a possible involvement of a monomeric Chl-*a* molecule at the donor side of PS II [45,55]. Such a chlorophyll (characterized by a larger EPR-line width than Chl-a_{II}^+) has been shown to be a donor to Chl-a_{II} at cryogenic temperatures. Van Gorkom [53,56] investigated Chl-a_{II}^+/Chl-a_{II} spectra both optically and by EPR and found that under certain conditions the EPR spectrum broadened at the same time as the red absorbance maximum blue-shifted and the absorbance difference spectrum in the blue changed in such a way that the decrease at 434 nm became bigger and significantly broader, thereby resembling more the spectrum of the oxidation of monomeric Chl-*a* in vitro [38]. Both our difference spectra from the recombination (Fig.s 2 and 3, circles) as well as of the extrapolated initial amplitudes in Tris-treated complexes in the presence of acceptor (Fig. 3, squares) display the spectral characteristics of Chl-a_{II} coupled with the small (8 G wide) EPR bandwidth. Therefore, we conclude that the primary donor Chl-a_{II} is monitored in the spectra of Fig. 3 and that the transient involvement of monomeric chlorophyll can be excluded in the Tris-treated PS II complexes from *Synechococcus*.

A peculiar aspect of the optical difference spectrum of the primary donor, Chl-a_{II}^+/Chl-a_{II}, is the apparent absence of significant bandshifts in the whole wavelength region. Especially in the red wavelength region, the spectrum of Chl-a_{II}^+/Chl-a_{II} can be explained well by the bleaching of the ground state absorption of a chlorophyll around 680 nm [9]. The more complicated shape in the range from 250 to 600 nm might be essentially explained by the fact that both Chl-a_{II} and Chl-a_{II}^+ absorb significantly and only slightly differently in this region [38]. Differently from Chl-a_{II}, the optical difference spectrum of the primary donor, $(BChl)_2^+/(BChl)_2$, in purple bacteria [57,58] gives rise to very pronounced bandshifts. In the case of $(BChl)_2$ oxidation, the absorption band of the accessory bacteriochlorophyll at 800 nm is shifted. The absence of such a bandshift in PS II may suggest that an accessory chlorophyll is either located at a much bigger distance from the primary donor than in purple bacteria or does not exist in PS II. The absence of histidine residues in the D1/D2 polypeptides [59] homologous to the histidines in the bacterial reaction center proteins L and M that bind the accessory bacteriochlorophylls is consistent with this suggestion.

With regard to the measurement of the spectrum of

the donor to Chl-a_{II}^{+} in the presence of acetate (reaction time 160 μs), it was shown that is is the same as that of the donor in Tris-treated [28,35–37] and O_2-evolving systems [11], respectively. This indicates that one and the same component acts in this systems despite the very different kinetics (20 ns vs. 160 μs).

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