Rapid electron transfer reactions associated with oxygen evolution in photosystem II preparations from spinach and *Phormidium laminosum*

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We have measured the nanosecond kinetics of Chl-a^{+}_{II} reduction in oxygen-evolving detergent preparations of PS II from the cyanobacterium *Phormidium laminosum* and from higher plants (spinach) at 824 and 680 nm. Compared to earlier studies at 680 nm with higher plant material, we obtained an improved signal: noise ratio for measurements on a ns to ms time scale. The kinetics of Chl-a^{+}_{II} reduction in the ns range are consistent in the two preparations and are comparable to other studies of higher plant and cyanobacterial material. The ns kinetics are tightly connected to the ability for O_2 evolution. Analysis of the μs kinetics indicates three phases: (a) the slow phase ($t_{1/2} \sim 150$ μs in spinach and ~ 500 μs in *Phormidium*) reflects the back reaction between Chl-a^{+}_{II} and Q^{-}; (b) the phase with $t_{1/2}$ 5–10 μs is probably due to a donor which is not connected to an intact water oxidation system; (c) the intermediate μs component ($t_{1/2}$ 30–40 μs) may be related to water oxidation.

Electron transfer *Oxygen evolution* *Photosystem II* *Spinach* Phormidium laminosum

1. INTRODUCTION

The capture of light energy by PS II in oxygenic organisms results in the oxidation of a specialized chlorophyll molecule termed Chl-a_{II} or P680 [1,2]. Re-reduction of Chl-a^{+}_{II} (either by the immediate donor or by a back reaction from the reduced acceptor, Q^{-}) has been studied by monitoring absorption changes around 680 and 820 nm. At 680 nm, a relatively large bleaching occurs which is due to the Chl-a_{II}–Chl-a^{+}_{II} transition [1,2]. The Chl-a_{II} cation gives rise to a much weaker, broad absorption band centred around 820 nm [3]. An EPR radical signal with a g-value of about 2.002 has also been associated with the Chl-a^{+}_{II} species [4].

In [3] it was reported that Chl-a^{+}_{II} is reduced monophasically after the first flash in dark-adapted samples by an electron donation with a half-life time of about 30 ns. More recent data [5,6] with an improved signal:noise ratio has shown multiple phases in the reduction kinetics under repetitive excitation. Using single flashes, it was shown in [7] that the Chl-a^{+}_{II} reduction kinetics on a ns time scale are related to the four different oxidation states, S_0–S_3 [8], of the oxygen evolving system. A phase of half-life time 20–30 ns was associated with states S_0 and S_1, respectively. Slower biphasic kinetics ($t_{1/2}$ 50 ns and 300 ns) were linked with states S_2 and S_3. The accumulation of a positive charge in S_2 and S_3 was proposed in order to explain the slower ns kinetics. The multi-phasic kinetics under repetitive excitation

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenol indophenol; DPC, 1,5-diphenylcarbazide; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine

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can be explained quantitatively by the superposition of the different kinetics related to the four states.

Interpretation of the Chl-a_{II}^{+} reduction kinetics on a μs to ms time scale has proven more difficult. Kinetics for electron donation with half-life times ranging from 2 to 500 μs have been reported [5,9–11]. In the past, these phases were assumed to be associated with water oxidation [10,12], although other reports have suggested that these kinetics may arise in PS II units with damaged or modified donor systems [3,5,13,14].

During the last few years, preparations of PS II particles have proven to be very useful for studies of PS II, especially the mechanism of water oxidation. Here, we have measured the kinetics of electron transfer to Chl-a_{II}^{+} both before and after inhibition of oxygen evolution in PS II preparations from spinach [15,16] and *Phormidium laminosum* [17,18]. These preparations have been widely used in studies on O_2 evolution. In both preparations we were able to measure the reduction kinetics of the photooxidized primary donor of PS II, Chl-a_{II}^{+}, with ns time resolution.

2. MATERIALS AND METHODS

Spinach (*Spinacea oleracea*) was purchased from a local market. Spinach PS II fractions were prepared by a detergent treatment described in [16], a procedure which is based on the original method described in [15]. Very high rates of oxygen evolution were maintained by these fractions (900–1000 μmol O_2/mg Chl per h) at saturating light intensities.

PS II particles from the cyanobacterium *Phormidium laminosum* were obtained by the detergent treatment in [17], followed by precipitation by polyethylene glycol [18]. The polyethylene glycol treatment removes most of the pycobilin pigments which normally contaminate these preparations. Rates of oxygen evolution between 1200 and 1500 μmol O_2/mg Chl per h were measured for the PS II fractions used here.

Samples were stored at 77 K prior to and during transportation, and then at 193 K for about 10 days. Spinach PS II fractions have been found to lose some oxygen evolution activity (about 10%) after freezing and thawing while the activity in cyanobacterial preparations seemed essentially unaffected. Spinach PS II samples were only frozen and thawed once prior to being assayed. Oxygen evolution was measured at 20°C in a Clark-type oxygen electrode. 2,6-Dimethylbenzoquinone (1 mM) and potassium ferricyanide (1 mM) were used as electron acceptors for oxygen evolution measurements. The reduction of DCIP (0.1 mM) was measured by monitoring absorption changes at 590 nm with an Aminco DW-2 dual-beam spectrophotometer. The assay medium consisted of 40 mM Mes, 5 mM $MgCl_2$, 10% glycerol (pH 6.0); 1,5-diphenylcarbazide (1 mM) was used as an artificial electron donor where stated in the text.

Measurements of absorption changes due to Chl-a_{II}^{+} were performed with two purpose-built spectrophotometers. Samples were excited at 532 nm with a frequency-doubled Nd/YAG laser (Quantel YG441) with a pulse width of approx. 3 ns. Average pulse energies are given in the figure legends.

The instrument used for 824 nm measurements (on a ns time scale) was that described in [6] except that a 5-cm pathlength cuvette was used. No fluorescence artefact could be distinguished from background noise in either PS II preparation. Measurements at 680 nm involved a different apparatus, described in detail in [6,19]. The fluorescence artefact (30% of the signal amplitude) was subtracted from the measured signal.

The measuring light intensity was approx. 200 $\mu W/cm^2$. The fraction of reaction centres in the closed state due to excitation by the measuring light was negligible because of the very fast turnover time of PS II in this material [16].

Measurements on a time scale from 1.5 μs to 50 ms involved a different detector (FND100 purchased from EG&G with a 5 kΩ load resistor), amplifier (AM 502 from Tektronix) and digitizer (Nicolet 1170 with plug-in 174).

Best fit of the data was performed as in [7]; the first 10 ns were not taken into account for the fit.

The standard assay medium consisted of 20 mM Mes, 10 mM $MgCl_2$, 2 mM KH_2PO_4, 0.5 M mannitol, pH 6.5. For measurements at pH 8.5, 20 mM Tricine replaced the Mes in the assay medium. Potassium ferricyanide (1 mM) and phenyl-*p*-benzoquinone (0.1 mM) were used as electron acceptors unless otherwise stated in the text.

3. RESULTS AND DISCUSSION

Measurements involving the PS II preparation from *Phormidium laminosum* proved relatively straightforward, because of lower light-scattering per PS II reaction centre compared with unfractionated preparations. Fig.1 shows the absorption changes detected at 824 nm on a time scale from about 5 ns to 15 ms. We decomposed the kinetics into exponential phases. The following parameters were found to provide the best fit to the data shown in fig.1c: $t_{1/2}$ = 25 ns (35–40%), $t_{1/2}$ = 220 ns (20–25%) and $t_{1/2} > 1\ \mu s$ (40–45%). The phases of half-life times greater than 1 μs are shown in fig.1a,b. A good adaptation of the decay in the μs time range requires phases with half-life times of 5–10 μs, 30–40 μs and 500 μs. A minor component (3–4%) of half-life time greater than 10 ms may be related to Chl-a_I^+ in PS I centres present in the preparation.

From the amplitude of the absorption change shown in fig.1c, we calculated a Chl antennae: Chl-a_{II}^+ ratio of about 90, using an extinction coefficient of 7000 $M^{-1} \cdot cm^{-1}$ for Chl-a_{II}^+ [20]. Taking into account that the excitation energy was not completely saturating (90%), the photosynthetic unit size of PS II in the *P. laminosum* preparation was about 80 antennae Chl per PS II.

Fig.2 shows some results from a more detailed investigation of the 824 nm absorption changes occurring on a μs time scale. There was a clear dependence of the Chl-a_{II}^+ reduction kinetics on both the laser-flash frequency and the acceptor regime used. With increasing flash frequency, a larger proportion of the slow component of half-life time, 500 μs, was observed at the expense of the faster 5–10 μs component. The intermediate component with $t_{1/2}$ 30–40 μs (~10%) is independent of the frequency. A small increase in the total amplitude of the absorption change with greater flash frequency was probably a result of an increase in the average pulse energy of the laser. A flash-frequency effect similar to the one in fig.2 was reported in [21] but the fast μs phases were not resolved, probably because of the slower response time of the instrument and a much larger fluorescence artefact.

The observed flash-frequency effect is characteristic of PS II reaction centres where water oxidation is inhibited. The 5–10 μs phase very likely reflects the reduction of Chl-a_{II}^+ by a donor, $\widetilde{D}$, which is either the normal immediate donor in a

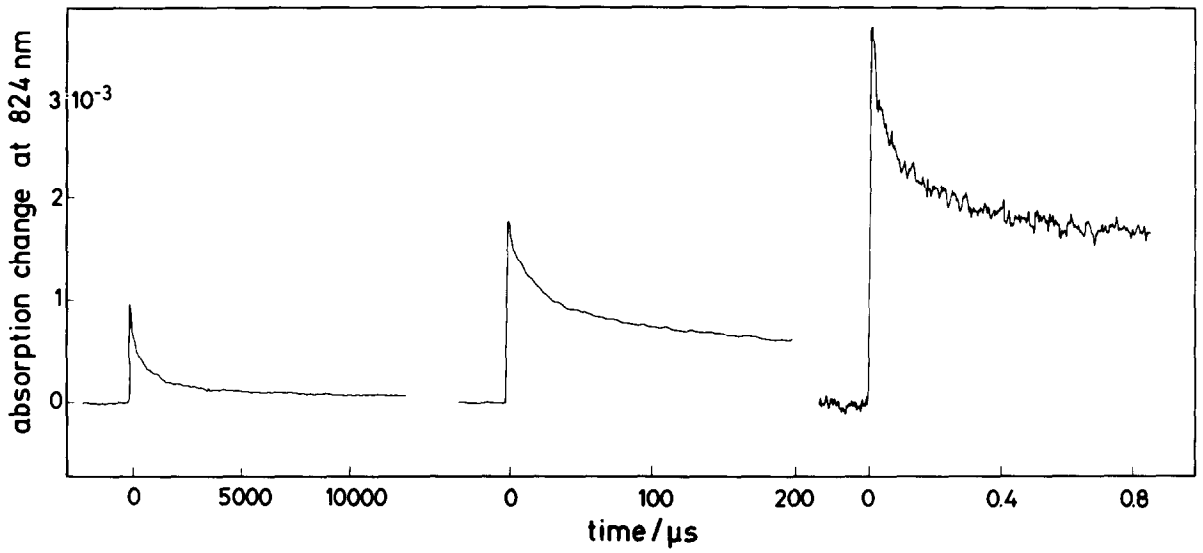

Fig.1. 824 nm absorption changes in a *Phormidium laminosum* PS II fraction suspended in 40 mM Mes/NaOH (pH 6.0), 5 mM $MgCl_2$, 10% (v/v) glycerol, with the acceptors given in the text. Chl concentration was 10 μM, path length for the cuvette, 5 cm. Excitation pulses were provided at 5 Hz with an average energy of about 1 mJ $\cdot$ cm^{-2}. Left and centre traces, 128 averages; right-hand trace, 512 averages.

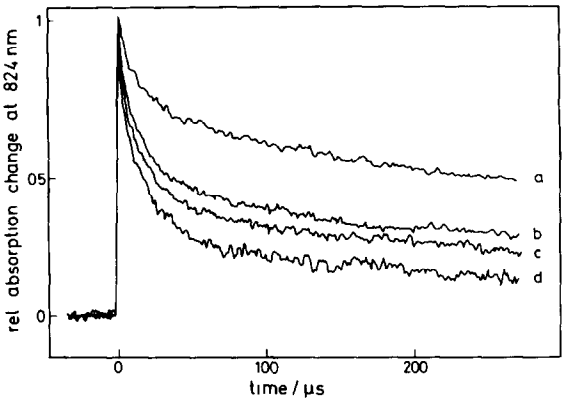

Fig.2. Microsecond absorption changes at 824 nm in the *Phormidium laminosum* PS II fraction under various conditions: (a) 64 averages at 10 Hz in the presence of 10 mM potassium ferricyanide; (b) 64 averages at 5 Hz with 1 mM potassium ferricyanide; (c) 64 averages at 1 Hz with 1 mM potassium ferricyanide; (d) 32 averages at 1 Hz with 0.2 mM phenyl-*p*-benzoquinone. Maximum relative absorption changes correspond to: (a) 1.8×10^{-3}; (b) 1.6×10^{-3}; (c) 1.4×10^{-3}; (d) 1.4×10^{-3}. Other conditions as described in fig.1.

modified state or a side donor in PS II [11]. The 500 μs phase is probably due to the back reaction between Chl-a_{II}^{+} and Q^{-} which has been reported earlier for *P. laminosum* PS II particles [14]. Under repetitive excitation, the proportion of both phases is determined by the rate of re-reduction of $\tilde{D}^{+}$ and the repetition frequency. At higher flash frequencies less $\tilde{D}^{+}$ is re-reduced between flashes so that a larger proportion of the 500 μs back reaction is observed. The dependence on the acceptor regime used (see fig.2) might be explained by an indirect influence on the rate of $\tilde{D}^{+}$ reduction.

The intermediate μs phase with $t_{1/2}$ 30–40 μs (~10%) was found to be roughly independent of flash frequency and acceptor regime used. This may indicate that this phase can be attributed to PS II centres which are active in O_2 evolution. This assumption is supported by the analysis of the μs decay of Chl-a_{II}^{+} measured in a series of flashes given to dark-adapted samples (unpublished).

With the spinach PS II preparation we performed comparative measurements of the Chl-a_{II}^{+} reduction kinetics at 824 nm (fig.3) and 681 nm (fig.4) on a ns and μs time scale. Incubation at pH 8.5 inhibits oxygen evolution in this preparation [15,16] and was found to slow down the ns components into the μs range (for discussion, see below). The kinetics of the ns decay at pH 6.5 are very similar at 681 and 824 nm. The best fit analysis led, at both wavelengths, to the following results: $t_{1/2}$ = 25–30 ns (35–45%), $t_{1/2} \simeq$

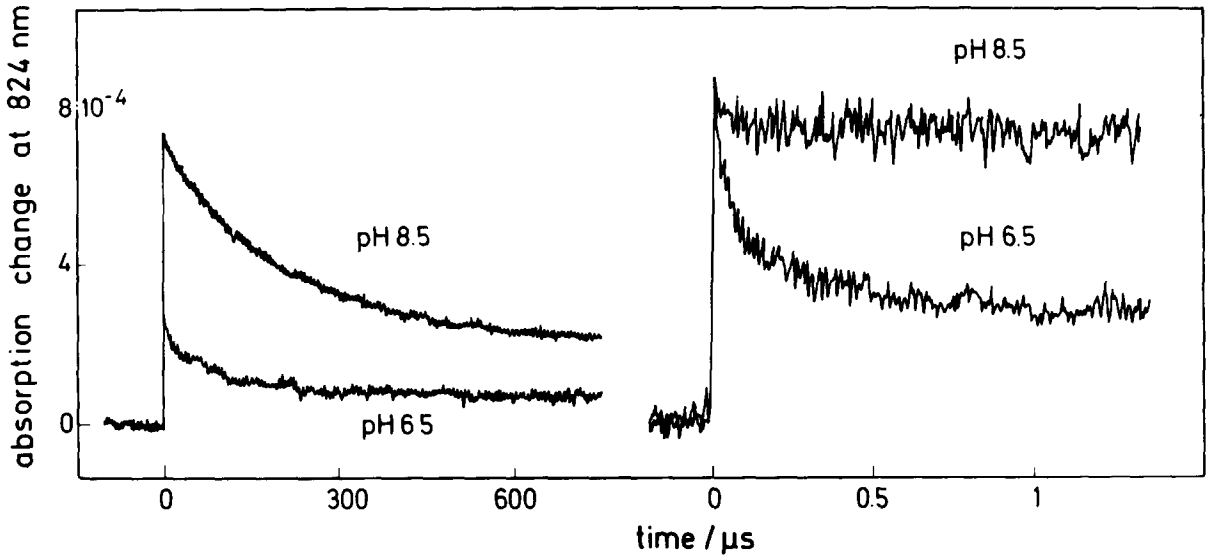

Fig.3. 824 nm absorption changes in spinach PS II fractions suspended at pH 6.5 and pH 8.5 with the conditions given in the text. Chl concentration was 8.7 μM, cuvette path length, 5 cm. Excitation pulses were provided at 5 Hz with an average energy of 0.5 mJ · cm^{-2}. The left-hand traces are the result of 128 averages, the right upper trace, 1024 averages, the right lower trace, 2048 averages (the sample was changed after 1024 flashes).

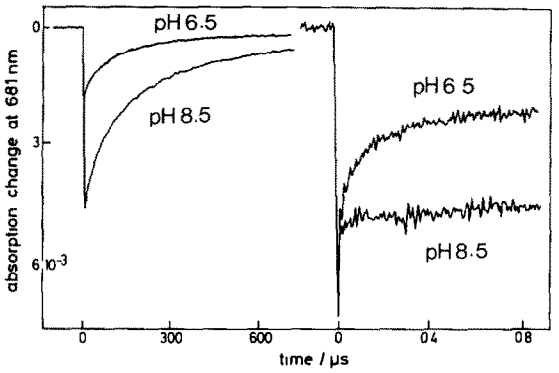

Fig.4. 681 nm absorption changes in a spinach PS II fraction suspended at pH 6.5 and pH 8.5. Excitation pulses at 5 Hz were provided with an average energy of 2 $mJ \cdot cm^{-2}$. Chl concentration was 10.6 μM, cuvette path length, 2 cm. The intensity of the measuring beam was 200 $\mu W \cdot cm^{-2}$. The left-hand traces show the average of 256 measurements; right (upper) trace, 1024 measurements; right (lower) trace, 512 measurements. A fluorescence artefact was subtracted from the right-hand traces as described in the text.

200–250 ns (20–25%) and $t_{1/2} > 1$ μs (30–40%). From the signal sizes at 680 and 824 nm we calculated a Chl : Chl-a_{II}^{+} ratio of 300, using an extinction coefficient of 7000 $M^{-1} \cdot cm^{-1}$ for Chl-a_{II}^{+} at 824 nm and taking into account the scattering signal (see below). With the 680 nm measurement and using an extinction coefficient of 65000 $M^{-1} \cdot cm^{-1}$, approximately 250 Chl per reaction centre can be calculated.

Recently, absorption changes at 688 nm in Class II chloroplasts from spinach have been adapted in the ns range by two phases with $t_{1/2} = 50$ ns (35% of the total signal) and 500 ns (20%) [22], which is somewhat slower than the kinetics reported for the spinach PS II preparation used in the present study. Perhaps this deviation reflects an effect of a more acidic internal pH in the unfractionated thylakoids due to proton 'pumping' by the measuring light [23]. However, the first 25 ns of the decay could not be taken into account for the adaptation in [22], and the signal to noise ratio was considerably worse than the one mentioned here. Hence, once might also suppose that the contribution from phases with $t_{1/2} < 50$ ns has been underestimated in [22].

The decay in the μs range is multiphasic; it can be adapted with components of $t_{1/2} = 5$–10 μs, 30–40 μs and ~150 μs. The faster half-life times are consistent with the results obtained with the *Phormidium* PS II preparation. The 150 μs phase can probably be assigned to a back reaction between Chl-a_{II}^{+} and Q^{-} in centers where water oxidation is inhibited [11]. The difference in the kinetics of the back reaction between spinach PS II particles (150 μs) and the *P. laminosum* preparation (500 μs, see above) has been reported earlier [14] and might be due to the different photosynthetic material. Another possibility would be that the inhibited PS II centre can exist in different states which give rise to different rate constants for the charge recombination between Q^{-} and Chl-a_{II}^{+}. This assumption is supported by further investigations (unpublished).

Using the spinach PS II preparation, the measurements at 824 nm proved more difficult than those at 681 nm. This may be surprising in view of a recent report [7], where a signal to noise ratio of about 5 has been obtained at 824 nm in the ns range without averaging. One reason for the difficulty is that the signal to noise ratio around 820 nm depends strongly on light scattering of the sample; the lower scattering per PS II, the higher the usable Chl-a_{II} concentration and the higher the signal to noise ratio. In the spinach PS II preparation, scattering per PS II is considerably higher than in the subchloroplasts and the PS II preparations from *Synechococcus* used in [7]. Using highly scattering samples, another problem was encountered with the 824 nm measuring wavelength. The high intensity of the excitation pulse causes a transient scattering phenomenon [5] which gives rise to an apparent increase in transmission. This 'scattering signal' is therefore in the opposite direction to the normal 824 nm absorption changes and it could be observed when no electron acceptors were present and when DCMU (10 μM) was added. Its rise time is below 10 ns and it decays with $t_{1/2} \simeq 50$ μs. Therefore, the signal in fig.4 is somewhat distorted by the superimposed scattering signal (approx. 15% of the total amplitude).

The signal to noise ratio around 680 nm depends essentially on the absorption at 680 nm per PS II and on the turnover time of PS II. Compared to unfractionated samples, the PS II preparation from spinach has lower absorption per PS II. Hence, a higher concentration of Chl-a_{II}^{+} can be used, resulting in a better signal to noise ratio. The

short turnover time of the spinach PS II preparation (2 ms [16] compared to about 20 ms in chloroplasts [24]) allows application of a relatively high measuring light intensity around 680 nm without closing a considerable proportion of PS II reaction centres by the absorbed light. For these reasons, the absorption changes around 680 nm in the spinach PS II preparation could be measured with a higher precision than previously in class II chloroplasts from spinach [22].

Figs 3 and 4 show measurements at pH 8.5 to demonstrate that incubation of spinach PS II fractions at pH 8.5 slows down the re-reduction of Chl-a_{II}^{+}. The slow phase with $t_{1/2} \simeq 150\,\mu s$ is increased at the expense of the ns phases. Parallel to that, water oxidation is inhibited. Incubation at pH 8.5 appears to be a very mild treatment. We observed normal ns kinetics and O_2 evolution upon returning to pH 6.5, if the incubation time at 8.5 was short (<3 min) and if the sample was kept in the dark. This is in accordance with observations in chloroplasts where a temporary incubation at alkaline pH (up to pH 9.3, with and without uncoupler) in the dark does not inactivate O_2-evolution [25].

It is most likely that the inhibition of O_2-evolution by the alkaline pH requires illumination (e.g., flash excitation) because the S_2 state was suggested to be the sensitive state for alkaline inactivation [26]. Illumination at pH 8.5 causes an irreversible inhibition of water oxidation in spinach PS II fractions, which is different from chloroplasts where a decrease of the internal pH by the light-induced proton transport may occur. Since the spinach PS II fractions are uncoupled, illumination does not cause a pH gradient across the membrane. In the presence of uncouplers, illumination at alkaline pH causes inhibition also in chloroplasts [25].

Addition of the artificial electron donor, DPC, restores high electron transport rates (1500 μequivs/mg Chl per h) through PS II after the irreversible inhibition of water oxidation, although this only occurs upon returning to pH 6. In parallel with the restoration of electron transport by DPC, we also observed a major μs phase in the Chl-a_{II}^{+} reduction with a half-life time of 5 μs. To maintain an electron transport rate of 1500 μequivs/mg Chl per h, D^{+} must be re-reduced in less than 10 ms.

Although high rates of electron transport could be recovered, no ns kinetics could be observed, suggesting that the immediate donor to Chl-a_{II}^{+} was modified by the loss of the water oxidation activity.

To date, all treatments which inactivate O_2-evolution have been found to slow down the reduction rate of Chl-a_{II}^{+} [3,5,16,27]. This indicates a very close relationship between the water oxidation process and the immediate (ns) electron donor to Chl-a_{II}^{+}. The ns reduction kinetics of Chl-a_{II}^{+} in the PS II centres which are active in O_2-evolution are very similar in the two PS II preparations described, and compare closely with all the photosynthetic material studied to date [5–7]. On the basis of the kinetic evidence it would seem that on the donor side of PS II a very strong similarity exists between higher plant and cyanobacterial PS II reaction centres.

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