Isolation of a photosystem 2 preparation from higher plants with highly enriched oxygen evolution activity

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Detergent-treatment of higher plant thylakoids with Triton X-100 at pH 6.3 has been used to purify a PS2 fraction with very high rates of oxygen evolution (>1000 μmol.mg chl^{-1}.h^{-1}). A photosynthetic unit size of about 300 chlorophyll (chl) molecules has been determined by optical methods, suggesting an average turnover time for PS2 of about 2 ms. The donor system for P680$^+$ is particularly well preserved in the preparation, as judged by P680$^+$ reduction kinetics, the detection by EPR of Signal II$_{LT}$ and the presence of the high potential form of cytochrome *b*-559 (at a ratio of 1:1 with the reaction centre).

Photosystem 2 *Oxygen evolution* *P680$^+$* *Cytochrome* b-*559* *Signal II$_{LT}$* *Chloroplast*

1. INTRODUCTION

Progress towards the resolution of the donor system of photosystem 2 and the water oxidation mechanism in higher plant chloroplasts has often been difficult because of the presence of components and signals from photosystem 1 and the cytochrome b_6–f complex which can confuse the analysis. For instance, optical studies of P680$^+$ reduction have been hampered by uncertainty over the extent of the contribution of P700$^+$ to the signals [1–4]. Similarly, studies of cytochrome *b*-559 in chloroplasts are hindered by the presence of two other cytochromes with absorption peaks at 563 nm and 554 nm [5]. Attempts to solve this problem have involved the purification of photosystem 2 by phase-partition methods [6], and detergent treatments [7–9], but until recently this has yielded preparations with little [6–8], or no oxygen evolution activity [9], suggesting that significant damage to the donor system had occurred. Some of the preparations were also contaminated by the cytochrome b_6–f complex [10].

Recently, two major advances have been made towards the purification of a PS2 fraction which is highly active in water oxidation.

Detergent preparations of PS2 from the cyanobacterium, *Phormidium laminosum*, have been obtained which can maintain rates of oxygen evolution of up to 2000 μmol O_2.mg chl^{-1}.h^{-1}. The particles obtained have a small photosynthetic unit size of about 70 chl molecules [11,12].

The cumulative problems of culturing large volumes of the cyanobacterium, the long preparation time, low yield and the practical difficulties involved in the isolation procedure may explain why relatively few groups have used this PS2 preparation.

A detergent preparation of PS2 from spinach which retained about 50% of the oxygen evolution capacity was described in [13,14]. We have now modified their procedure, as detailed below, so that all of the water oxidation activity is retained. The major advantages of this preparation over the cyanobacterial PS2 fraction are the speed, ease and

Abbreviations: chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; MES, 2-(*N*-morpholino) ethanesulfonic acid; PS2, photosystem 2; PS1, photosystem 1; Tris, Tris-(hydroxymethyl)aminomethane; DMBQ, 2,6-dimethyl-benzoquinone

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high yield of the procedure. Only 1.5 h work is required for making both chloroplasts and PS2 fraction, with a further 1.5 h free time in the middle. A further advantage is that only one cytochrome (*b*-559) can be detected in this preparation. The PS2 particles from *P. laminosum* appear to have at least two cytochromes present [11]: *b*-559 and *b*-549. The PS2 preparation described below is thus ideally suited to optical and EPR studies of the donor side of P680, and some initial experiments are presented.

2. MATERIALS AND METHODS

Intact chloroplasts were obtained with high rates of oxygen evolution (400–550 μmol O_2.mg $chl^{-1}.h^{-1}$) by the method in [15] except that L-ascorbate (sodium salt), 5 mM, was added to the grinding medium. The chloroplasts' envelope membranes were removed by incubation in a hypotonic medium (5 mM $MgCl_2$), for 30 s, followed by the addition of an equal volume of 0.66 M sorbitol, 5 mM $MgCl_2$, 40 mM MES (pH 6.5). The suspension was then centrifuged at 3000 $\times$ *g* for 2 min. The final chloroplast pellet was resuspended in ice-cold buffer containing 2 mM MES, 5 mM $MgCl_2$, 15 mM NaCl (pH 6.3 at room temperature). The chloroplasts were then left on ice under subdued light for about 1.5 h to allow a more complete stacking and separation of PS2 from PS1. After 1.5 h the chloroplasts were added to 20% Triton X-100 so that the final conc. was 50 mg detergent/ml and 2 mg chl/ml. Following mixing by inversion once or twice, the suspension was left at 4°C in the dark for 25 min, followed by centrifugation at 40000 $\times$ *g* at 4°C for 30 min. The pellet was resuspended in the same buffer, and for storage was frozen with 20% glycerol at 77 K.

Oxygen evolution was measured in a Clark-type oxygen electrode at 20°C. The standard assay medium consisted of 10% glycerol, 5 mM $MgCl_2$, 1 mM $K_3Fe(CN)_6$, 1 mM 2,6-dimethylbenzoquinone and 20 mM MES (pH 6) or 20 mM Tris (pH 8). Ammonium chloride (3 mM) was also added for measurements involving unfractionated chloroplasts. EPR signals were recorded with a Jeol FE-1X spectrometer with 100 kHz field modulation, as detailed in [16].

Absorption changes at 820 nm were measured using a purpose-built spectrophotometer, employing a large-area photodiode (UDT, California), coupled to a low-noise amplifier (EG/G Parc Model 113). The limit of the time-resolution of the instrument was about 2 μs. Excitation light was provided by a Chromatix tunable dye laser which provided pulses at 660 nm with a full width at half maximum intensity of 600 ns. Signals were recorded using a Datalab 920 transient recorder and averaged with a Datalab 4000 B signal averager. Optical measurements of cytochromes were taken with an Aminco DW2 spectrophotometer.

3. RESULTS AND DISCUSSION

Initially, we prepared PS2 fractions by following the procedure in [13], i.e., employing two detergent treatments at pH 7.5. We obtained a good purification of PS2, but rates of oxygen evolution up to only 300 μmol O_2.mg $chl^{-1}.h^{-1}$ could be obtained, and the initial rate was not stable, being rapidly reduced during illumination. With the modified procedure described here, rates up to 1180 μmol O_2.mg $chl^{-1}.h^{-1}$ have been recorded, and the stability of the initial rate is greatly improved.

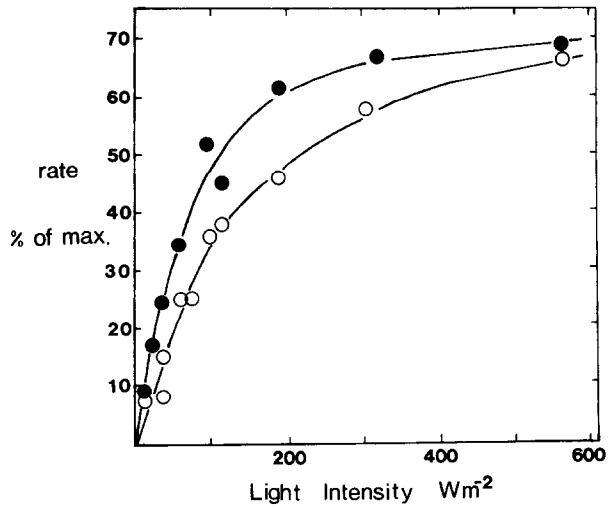

Fig.1. Light intensity-dependence of the rate of oxygen evolution in chloroplasts at pH 8 (○) and in the PS2 preparation at pH 6 (●). Maximum rates, at 1500 W.m^{-2}, were 400 μmol.mg $chl^{-1}.h^{-1}$ for chloroplasts and 800 μmol.mg $chl^{-1}.h^{-1}$ for the PS2 preparation; chl was 9 μg/ml; assay medium was as described in the text.

Fractionation at pH 6.3 appears to protect the donor system of PS2 from the effects of the detergent, although the 1.5 h period allowed for the incubation of chloroplasts in the stacking medium may also be important in this respect, and could allow increased separation of PS2 from PS1 in the thylakoid membrane [6,17]. The fractionation procedure described has been applied to pea, lettuce, and spinach chloroplasts with equal success.

Fig.1 shows the light-intensity dependence of oxygen evolution in chloroplasts, and in the PS2 fraction prepared from spinach. The efficiency of the reaction appears to be greater in the PS2 fraction, which may be a reflection of much lower excitation energy spillover from PS2 to PS1 [17]. Contamination of the PS2 fraction by PS1 has been estimated by optical and EPR methods, and was found to be very low, with a ratio of $P700^+$ to $P680^+$ of about 0.04 compared to about 1 in chloroplasts.

The light-induced changes in signal II observed at cryogenic temperatures have been termed signal II_{LT} [16], and we have proposed that components giving rise to this signal are early donors to the reaction centre, P680 [16,18]. Fig.2a shows the signal II_{LT} in a Triton X-100 preparation which has been fractionated at pH 6.3. The change in signal II is much smaller in our earlier preparations which were fractionated at pH 7.5 (fig.2b), and the overall shape of signal II is also different.

The high-potential form of cytochrome *b*-559 has also been reported as a low-temperature donor

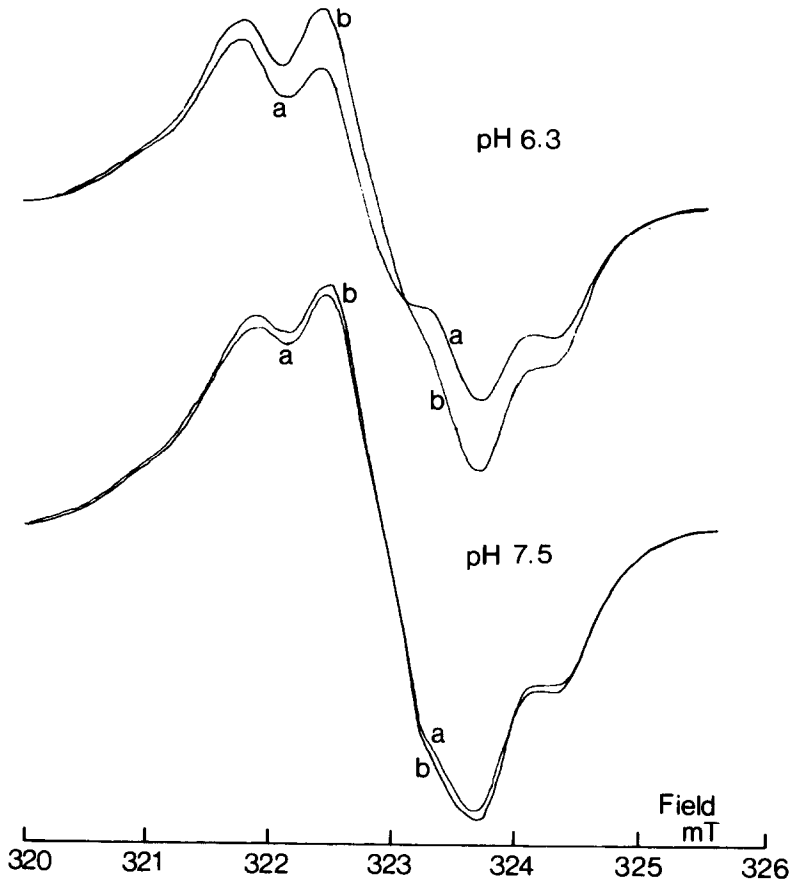

Fig.2. Light-induced changes in the EPR signal II recorded at 15 K in preparations fractionated at: pH 6.3 (5.2 mg chl/ml; instrument gain, 200); pH 7.5 (2.3 mg chl/ml; instrument gain, 500). The spectra shown are from samples frozen after 30 min dark adaptation at 4°C (a), and the same sample during 15 K illumination (b). Instrument settings were: microwave power, 100 μW; frequency, 9.1 GHz; modulation amplitude, 0.2 mT.

to $P680^+$, although its role at physiological temperatures is not clearly understood [18]. The ratio of high-potential *b*-559 present in the PS2 preparation is about 1:1 with P680, as shown in fig.3. All of the high-potential form appears to be reduced in the samples, since the addition of 5 mM hydroquinone did not increase the amplitude of the absorption difference spectrum shown in fig.2a.

About 40% of the cytochrome *b*-559 present is in the low-potential form. No other cytochromes can be detected in the fraction, and in both spectra shown in fig.3, the full width at half maximum remains at 11 nm. With our earlier samples, prepared by detergent fractionation at pH 7.5, 80% of the cytochrome *b*-559 is present in the low potential form. These results also suggest that the fractionation procedure we have developed preserves the donor side of PS2.

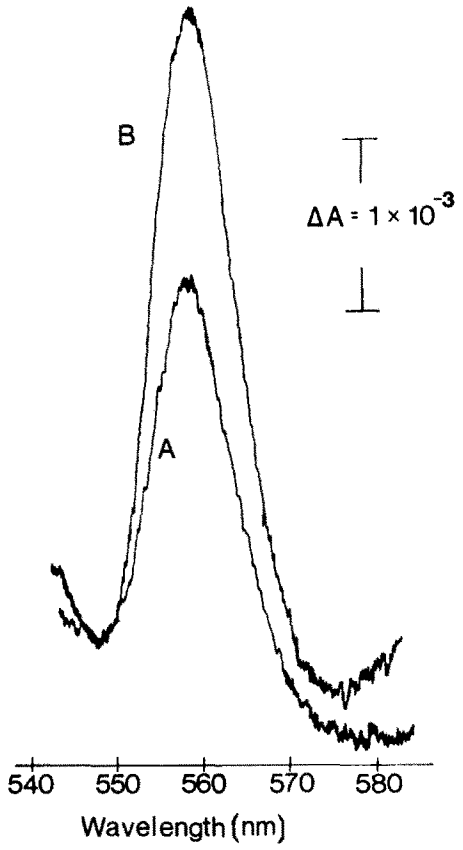

Fig.3. Absorption changes characteristic of cytochrome *b*-559 in the PS2 preparation (suspended in the standard assay medium) at pH 6: (A) obtained with reference cuvette, 1 mM $K_3Fe(CN)_6$; sample cuvette, no addition. (B) As in (A) but with excess sodium dithionite added to the sample cuvette; chl was 43 μg/ml.

In [5] no evidence was found for a close correlation between the level of high-potential cytochrome *b*-559 and oxygen evolution in chloroplasts. However, the presence of the cytochrome in this PS2 preparation, and in the highly active *P. laminosum* particles [11], suggests that there is some requirement for the cytochrome in maintaining high rates of oxygen evolution. In both preparations, the ratio of high-potential *b*-559 to P680 is about 1:1.

The amplitude and kinetics of the reduction of $P680^+$ in the Triton X-100 PS2 preparation has been measured by following rapid absorption changes at 820 nm as in [3,4]. Fig.4a shows the absorption change which occurs under conditions where oxygen is actively being evolved by the preparation. A major phase (77%) with a lifetime of 70–80 μs is detected, and a slower phase of lifetime 600 μs is also present. Not all of the $P680^+$ is detected in the experiment shown in fig.4a, since the addition of 10 mM hydroxylamine roughly doubles the maximum amplitude of the absorption change (fig.4b). Taking a millimolar extinction coefficient of 7 cm^{-1} for $P680^+$ at 820 nm [3], a ratio of 300–350 chl molecules/reaction centre has been determined for the experiment shown in fig.4b and other similar experiments. With this unit size, an average turnover time of about 2 ms can be calculated for the PS2 reaction centre during maximum oxygen evolution activity.

The effect of hydroxylamine in increasing the $P680^+$ signal detected on a microsecond timescale has been reported in [2–4], although these publications did not agree on the kinetics of $P680^+$ reduction, which was found to be much slower in the experiments in [2]. We have found that prolonged incubation of the PS2 preparation with hydroxylamine or strong illumination of the hydroxylamine-inhibited sample results in a change from the fast kinetics of $P680^+$ reduction ($\tau \simeq 17$ μs) to the much slower phase shown in fig.4c ($\tau \simeq 400$ μs). The results emphasize the complexity of the inhibitory action of hydroxylamine in photosystem 2. At least two sites for inhibition in the PS2 donor system have been reported [14]. If the primary electron acceptor of PS2 (Q), is com-

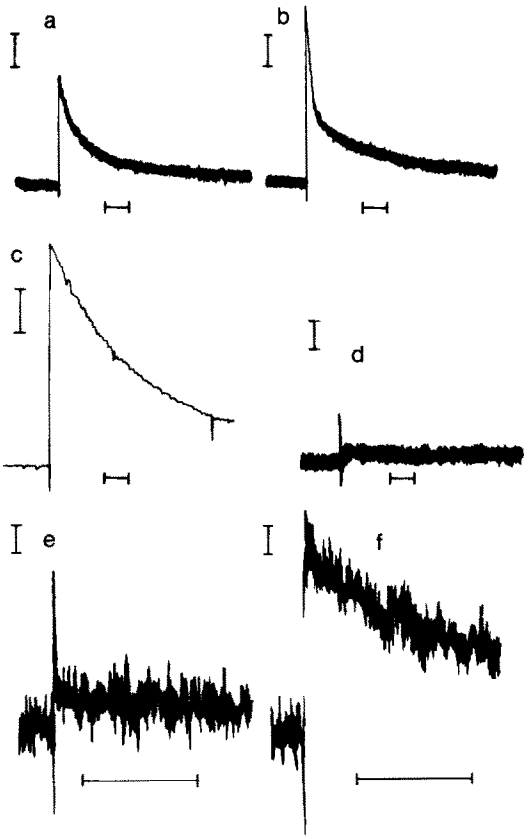

Fig.4. Absorption changes at 820 nm in the PS2 preparation: (a) suspended in the standard assay medium at pH 6 with DMBQ (1 mM) and $K_3Fe(CN)_6$ (1 mM), 16 averages; (b) as in (a), but with the addition of hydroxylamine (10 mM); (c) as in (b) but after 30 s illumination with white light, 128 averages; (d) as in (b), but with the addition of DCMU (20 μM) and sodium ascorbate (20 mM); (e) first flash, pH 8; 5 min dark adaptation with DMBQ (1 mM) and $K_3Fe(CN)_6$ (1 mM), average of 4 expt; (f) as in (e) but with >8 pre-recording flashes; horizontal bars, 100 μs; vertical bars, an absorption change of 10^{-4}; chl was 26 μg/ml; optical path length, 1 cm.

pletely reduced by the addition of DCMU and ascorbate, then only a very small absorption change can be detected in the PS2 preparation, as shown in fig.4d. Under these conditions any $P700^+$ present should be manifested by a positive absorption change decaying within about 30 ms [19].

It has been shown here that detergent fractionation at pH 7.5 causes some damage to the PS2 donor system, and, as reported in [13], virtually no oxygen evolution occurs at pH 8 in the Triton preparation. If an active preparation is suspended at pH 8, all of the $P680^+$ can be detected by the instrument, and reduction occurs relatively slowly (fig.4f), with a lifetime that is similar to the hydroxylamine-inhibited sample. However, after a few minutes of dark-adaptation at pH 8, the first flash gives rise to a very small absorption change, as shown in fig.4e. There is some indication of a fast phase of reduction in about 1 μs or less, but this is not clearly resolved with our instrument. A second flash given a few seconds after the first was found to give rise to an absorption change almost as large as in the light-adapted sample, suggesting that the immediate donor to $P680^+$ is only slowly re-reduced after treatment at pH 8.

A very fast (30 ns) reduction of $P680^+$ in dark-adapted chloroplasts which they associated with the oxygen evolving system was detected in [3]: it was shown that hydroxylamine abolished the very fast kinetics and allowed the detection of $P680^+$ in the ms time-range. From our experiments with the PS2 preparation at pH 6, we estimate that only 40–50% of the total $P680^+$ is reduced with sub-microsecond kinetics under repetitive flash conditions where oxygen evolution is occurring. The origin of the slower phases in $P680^+$ reduction are under investigation at present. The slower kinetics may be due to a fast back-reaction from Q [3,4], or possibly an alternative pathway for the donation of electrons to $P680^+$.

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