Properties of oxygen-evolving Photosystem-II particles from *Phormidium lamosum*, a thermophilic blue–green alga

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1. O₂-evolving Photosystem-II particles from the thermophilic blue–green alga *Phormidium lamosum* contained 1 mol of Mn/13–17 mol of chlorophyll *a* compared with 1 mol of Mn/65–75 mol of chlorophyll *a* in unfractionated membranes. 2. At least two-thirds of the Mn in the Photosystem-II particles was removed by mild heating and by treatment with Tris or EDTA, with concomitant loss of O₂ evolution. However, irreversible inactivation was also caused by washing in buffers without MgCl₂, and this inactivation was not accompanied by a corresponding loss of Mn. 3. Bivalent cations (Mg²⁺ or Ca²⁺), Cl[−] or Br[−] ions and at least 20% (v/v) glycerol were required for maximum stability of O₂ evolution. 4. The Photosystem-II particles were enriched in high-potential cytochrome *b*-559 (1 mol of cytochrome/50–60 mol of chlorophyll *a*) and in component C-550, and had a photosynthetic-unit size of 40–70 molecules of chlorophyll *a*. 5. The absorption spectrum at 77 K showed a preponderance of shorter-wavelength forms of chlorophyll *a* in the Photosystem-II particles, and in the fluorescence emission spectrum at 77 K there were major chlorophyll fluorescence bands at 684 nm and 695 nm, with almost no fluorescence in the far-red region. 6. Analysis of the lipid and protein contents showed that the Photosystem-II particles were not chemically pure (for example, all of the membrane-bound cytochromes and cytochrome *c*-549 were present), but their high O₂-evolution activity and good optical properties make them useful for functional studies on Photosystem-II and O₂ evolution.

Recently, attempts to fractionate chemically the photosynthetic membranes of oxygenic organisms without irreversible loss of O₂ evolution have met with some success. Spector & Winget (1980) have reported the purification of an Mn-containing protein that restored O₂ evolution to cholate-extracted spinach (*Spinacia oleracea*) chloroplast membranes incorporated into liposomes. Adopting a somewhat different approach to the problem, we have used the detergent dodecyl dimethylamine oxide to extract a highly active O₂-evolving Photosystem-II particle from the thylakoids of a thermophilic blue–green alga, *Phormidium lamosum* (Stewart & Bendall, 1979). The preparation was greatly enriched in O₂ evolution, showing rates of up

to 1300 μmol of O₂/h per mg of chlorophyll *a* with the lipophilic electron acceptor 2,6-dimethyl-1,4-benzoquinone. The particles were depleted in pigment P-700, containing only 1.0–1.2 nmol of pigment P-700 per mg of chlorophyll *a*, compared with 6.2–6.9 nmol of pigment P-700 per mg of chlorophyll *a* in the unfractionated thylakoids. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis under mild conditions revealed that the particles were enriched in a chlorophyll–protein complex that is generally thought to be associated with the reaction centre of Photosystem-II (Stewart, 1980).

In the present paper we describe in more detail the properties of the active O₂-evolving Photosystem-II particles from *P. lamosum*.

Materials and methods

Membrane-fragments, Photosystem-I- and Photosystem-II-enriched particles were prepared, and

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Mes, 4-morpholine-ethanesulphonic acid; CF₁, extrinsic part of chloroplast coupling factor.

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rates of O_2 evolution were measured, as described previously (Stewart & Bendall, 1979, 1980).

Mn was assayed with a Perkin-Elmer model-380 atomic-absorption spectrophotometer in the graphite-furnace mode. Samples were diluted 10–100-fold with deionized water and compared with standards prepared by dilution of standard Mn solution (1 mg/ml; BDH Chemicals, Poole, Dorset, U.K.). Ashing of samples before analysis was found not to be necessary.

Mn was also assayed by e.p.r. spectroscopy, a Varian E-4 e.p.r. spectrometer being used. Spectra were recorded at room temperature with the following instrument settings: microwave power, 5 mW; microwave frequency, 9.49 GHz; modulation amplitude, 3.5 mT; modulation frequency, 100 kHz. Amounts of e.p.r.-detectable Mn were determined on samples of Photosystem-II particles by comparing the peak-to-trough height of the $Mn^{2+}, 6H_2O$ line at $g = 2.12$ with that for a $10 \mu M$ solution of $MnCl_2$. For the determination of total Mn, samples were first acidified with 0.1 M-HCl.

High-potential cytochromes were assayed as described by Bendall *et al.* (1971), except that particles at a concentration of $20 \mu g$ of chlorophyll *a*/ml were suspended in a buffer containing 0.5 M-sorbitol, 10 mM- $MgCl_2$ and 50 mM-Mes/NaOH buffer, pH 6.5. Low-potential *b*-type cytochromes were assayed by the method of Rich & Bendall (1980), with particles at a concentration of $20 \mu g$ of chlorophyll *a*/ml suspended in 0.5 M-sorbitol/10 mM- $MgCl_2$ /50 mM-Mes-NaOH buffer, pH 6.0. Absorption difference coefficients of $\Delta \epsilon \ 20000 M^{-1} \cdot cm^{-1}$ were assumed for all the cytochromes.

Dark-minus-light difference spectra at 77 K were recorded with a sensitive split-beam spectrophotometer (Johnson Foundation, University of Pennsylvania, Philadelphia, PA, U.S.A.), by using the method of Bendall & Sofrová (1971). Low-temperature absorption spectra were also recorded using the Johnson-Foundation spectrophotometer.

Fluorescence emission at 77 K was measured from the front surface of samples ($6 \mu g$ of chlorophyll *a*/ml, frozen in 0.35 M-sucrose/10 mM-NaCl/50 mM-Tris/HCl buffer, pH 8.0) in a 1 mm Perspex (lucite) cuvette. Fluorescence was excited at 632.8 nm by a helium-neon laser and detected after passing through a monochromator placed at 45° to the measuring beam.

Measurements of the half-time of activation of O_2 evolution were made on fresh preparations of lettuce (*Lactuca sativa*) chloroplasts (prepared as described by Rich & Bendall, 1980) and *P. laminosum* Photosystem-II particles, by using a modulated oxygen electrode as described by Joliot (1972). The modulated beam (frequency 20 Hz) was passed through a 2.6 mm heat filter (Corning 5600) and a 3 mm far-red filter (Schott RG695). Lettuce chloro-

plasts and Photosystem-II particles were dark-equilibrated for 20 and 45 min respectively before measurements were made.

Flash-induced rapid absorbance changes of pigment P-680 at 820 nm were measured by Dr. P. Mathis (Département de Biologie, Centre d'Etudes Nucléaires de Saclay, Gif-sur-Yvette, France), by using apparatus and techniques that have been described previously (Van Best & Mathis, 1978; Conjeaud *et al.*, 1979; Conjeaud & Mathis, 1980). Flash illumination was provided by a dye laser (Rhodamine B, giving broad-band illumination around 600 nm, energy ≈ 20 mJ) which was flash-pumped by a frequency-doubled YAG laser (emission 530 nm, duration $\approx 20 \mu s$). Other conditions were as described by Van Best & Mathis (1978). For these experiments, Photosystem-II particles were treated with Tris by 7-fold dilution into 0.8 M-Tris/HCl, pH 8.0, incubated for 20 min in the light and collected by centrifuging (200 000 g, 4 h). Flash-induced absorbance changes were measured in the Tris-treated particles diluted to a concentration of $20 \mu g$ of chlorophyll *a*/ml in 50 mM-Mes/NaOH buffer, pH 6.0, plus 1.7 mM- $K_3Fe(CN)_6$. A molar absorption difference coefficient (reduced minus oxidized) of $7000 M^{-1} \cdot cm^{-1}$ was assumed for pigment P-680 at 820 nm (Mathis, 1977).

Lipids were extracted from lettuce chloroplasts and *P. laminosum* Photosystem-II particles with chloroform/methanol (1:1.2, v/v) plus 0.7% (w/v) NaCl. After phase separation the lower phase was collected, evaporated to dryness under a stream of N_2 , re-dissolved in a small amount of chloroform and chromatographed on silica-H t.l.c. plates in chloroform/methanol/acetic acid/water (75:25:8:3, by vol.). Lipids were made visible by charring, or by Dittmer's spray for phospholipids (Dittmer & Lester, 1964).

Sodium dodecyl sulphate / polyacrylamide-gel electrophoresis was performed at room temperature by using the buffer system of Laemmli (1970). Samples containing $50 \mu g$ of chlorophyll *a* were treated for 5 min at room temperature or $60^\circ C$ with $100 \mu l$ of buffer containing 6.2 mM-Tris/HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol (omitted when gel was to be stained with tetramethylbenzidine) and 0.001% Bromophenol Blue. Treated samples were centrifuged to remove undissolved material, and $20 \mu l$ of the clear supernatant was applied to the gel. Molecular-weight markers were bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000), lysozyme (14 300) and mammalian cytochrome *c* (12 400). The stacking gel (1.5 cm) and resolving gel (5 cm) were 5 and 15% (w/v) in acrylamide respectively. Electrophoresis was for 3 h at 100 V. Gels were stained either with Coomassie Blue R by the method of Fairbanks *et al.*

(1971) or with tetramethylbenzidine by the method of Thomas *et al.* (1976). All chemicals used were of the higher degree of purity available from BDH.

EDTA-washed membranes were prepared by diluting a sample of membranes 50-fold with 0.7 mM-EDTA (adjusted to pH 7.7 with NaOH), collecting the membranes by centrifuging at 38 000 g for 20 min and resuspending them in 0.7 mM-EDTA. The washing procedure was repeated five times and the final pellet resuspended in a small volume of buffer containing 0.5 M-sorbitol, 10 mM-MgCl₂, 10 mM-Hepes/NaOH buffer, pH 7.5, and 5 mM-phosphate buffer (NaH₂PO₄/K₂HPO₄), pH 7.5. Membranes washed with 1 M-NaBr were prepared by a similar procedure.

Results

Manganese content of the Photosystem-II particles

The O₂-evolving Photosystem-II particles from *P. laminosum* were highly enriched in Mn, containing 1 mol of Mn/13–17 mol of chlorophyll *a*, compared with 1 mol of Mn/65–75 mol of chlorophyll *a* in unfractionated membranes and 1 mol of Mn/250–300 mol of chlorophyll *a* in the Photosystem-I fraction. The value for the amount of Mn in Photosystem-II particles determined by atomic-absorption spectrophotometry was confirmed by room-temperature e.p.r. spectrometry of acidified samples. Virtually all of the Mn in the Photosystem-II particles, representing a recovery of approx. 75–80% of the total Mn in the thylakoids, was apparently bound to protein, since only a very small free Mn (Mn²⁺, 6H₂O) signal was observable in e.p.r. spectra of concentrated preparations, even at liquid-helium temperature (results not shown).

The Mn content of unfractionated membranes of *P. laminosum* was similar to that reported for higher-plant chloroplasts (Radmer & Cheniae, 1977), and by Tel-Or & Stewart (1975) for intact filaments and spheroplasts of the blue-green algae *Phormidium luridum* and *Anabaena cylindrica*. However, Tel-Or & Stewart (1975) reported a 90% loss of Mn from *P. luridum* membranes, and a corresponding loss of 90% of the O₂-evolution capacity, when spheroplasts were ruptured by 10-fold dilution in a hypo-osmotic buffer. We reported previously that membrane fragments from *P. laminosum* lost O₂-evolution capacity when they were washed in a hypo-osmotic buffer in the absence of MgCl₂ (Stewart & Bendall, 1980). This observation has been confirmed for the Photosystem-II particles from *P. laminosum*, but there did not appear to be a general correlation between loss of O₂ evolution and loss of Mn from the particles (Table 1). For example, washing in hypo-osmotic buffer either with or without MgCl₂ resulted in the loss of less than 20% of the Mn (this amount was rather variable, and in

Table 1. Effect of glycerol, MgCl₂, EDTA and heat treatment on the Mn content and O₂-evolution activity of Photosystem-II particles from *P. laminosum*

Samples (0.1 ml) of Photosystem-II particles (1.24 mg of chlorophyll *a*/ml) were diluted to 5.6 ml with the buffers shown. 'HP' denotes a buffer containing 10 mM-Hepes/NaOH buffer, pH 7.5, and 5 mM-phosphate buffer (NaH₂PO₄/K₂HPO₄), pH 7.5. The mixtures were incubated for 45 min at 5°C, centrifuged at 200 000 g for 4 h and the particles resuspended in 0.7 ml of buffer HP plus 25% (v/v) glycerol and 10 mM-MgCl₂. Heat treatment was performed by incubating a 0.1 ml sample of Photosystem-II particles for 2 min at 60°C. The treated sample was diluted to 5.6 ml with buffer HP plus 25% (v/v) glycerol and 10 mM-MgCl₂, and the particles centrifuged and resuspended as described above. An O₂-evolution activity of 100% corresponded to 850 µmol of O₂/h per mg of chlorophyll *a*. A 100% retention of Mn corresponded to 4.43 µg of Mn/mg of chlorophyll *a*. O₂ evolution was measured at 25°C in a Hansatech oxygen electrode in a final volume of 1.0 ml of a buffer containing 0.5 M-sorbitol, 10 mM-MgCl₂, 20 mM-Hepes/NaOH, pH 7.5, plus 2 mM-K₃Fe(CN)₆, 1 mM-2,6-dimethyl-1,4-benzoquinone and Photosystem-II particles equivalent to 10 µg of chlorophyll *a*.

Treatment	Mn retained (% of control)	O ₂ -evolution activity retained (% of control)
HP	83.6	18
HP + 10 mM-MgCl ₂	80.9	57
HP + 25% (v/v) glycerol	90.3	70
HP + 25% (v/v) glycerol + 10 mM-MgCl ₂	100	100
HP + 2 mM-EDTA	31.6	0
HP + 2 mM-EDTA + 25% (v/v) glycerol	57.1	38
0.8 M-Tris/HCl, pH 8.0	12.2	0
Heat	34.5	0

one experiment only 10% of the Mn was removed by these treatments), but the loss of activity was 80–90% in the absence of MgCl₂ compared with 40–45% when MgCl₂ was present. In the presence of 25% (v/v) glycerol, very little Mn was lost during washing, but maximal activity was only retained when MgCl₂ was also present. E.p.r. spectrometry showed that, in all cases, at least 85% of the Mn remaining after washing was e.p.r.-undetectable, unless samples were acidified, thus eliminating the possibility that differing activities were due to different proportions of the retained Mn remaining bound to protein. The effect of ionic conditions on the activity and stability of the Photosystem-II particles is discussed further below.

There were indications that the pool of Mn in Photosystem II of *P. laminosum* may be more

susceptible to extraction by EDTA and high concentrations of alkaline Tris buffer than is the case with higher-plant chloroplasts. In higher-plant chloroplasts, treatment with EDTA is ineffective in removing Mn from Photosystem II or inhibiting O_2 evolution (Cheniae & Martin, 1966), and either mild heating or treatment with 0.8 M-Tris/HCl, pH 8.0, although totally inhibiting O_2 evolution, removes only about two-thirds of the Mn pool (Cheniae & Martin, 1970; Homann, 1968). We have confirmed (Table 1) that mild heating removed about two-thirds of the Mn from *P. laminosum* Photosystem-II particles, but the same proportion could be removed by washing in a hypo-osmotic buffer containing 2 mM-EDTA, whereas a single extraction with 0.8 M-Tris/HCl, pH 8.0, resulted in the loss of up to 90% of the Mn. All three treatments completely inhibited O_2 evolution. Glycerol (25%, v/v) afforded some protection against extraction of Mn by EDTA, but nevertheless there was considerable inhibition of activity.

Cytochromes and component C-550

The Photosystem-II particles were enriched, relative to chlorophyll, in all of the membrane-bound cytochromes as well as the soluble low-potential cytochrome *c*-549. [We have previously reported that exhaustive washing is necessary for the removal of this cytochrome from unfractionated thylakoids (Stewart & Bendall, 1980).] However, the degree of enrichment was greatest for cytochrome *c*-549 and for the high-potential (i.e. 1,4-benzoquinol-reducible) form of cytochrome *b*-559 (Table 2). The Photosystem-II particles were enriched 5–6-fold in high-potential cytochrome *b*-559, containing 1 mol of cytochrome *b*-559 (high-potential)/50–60 mol of chlorophyll *a*.

At 77K, approximately one-third to half of the total high-potential cytochrome *b*-559 underwent light-induced oxidation (Fig. 1a). (The proportion oxidized was determined by comparison with a

low-temperature chemical difference spectrum). A peak that was assumed to be attributable to component C-550 was also observed in the dark-minus-light difference spectrum. The peak was present in both untreated and ferricyanide-treated samples (though it was slightly diminished in size in the presence of ferricyanide; Fig. 1b), and it appeared at a shorter wavelength (543.5 nm) than has been reported for component C-550 in higher-plant chloroplasts (Knaff & Arnon, 1969; Bendall & Sofrová, 1971) or other blue-green algae (Knaff, 1973; Fujita, 1976). The Photosystem-II particles were enriched in component C-550, since the peak was virtually undetectable in dark-minus-light difference spectra of unfractionated membranes (results not shown).

Photosynthetic-unit size

We decided to determine the photosynthetic unit size in the particles so that it could be related to the

Fig. 1. Low-temperature (77K) dark-minus-light difference spectra of *P. laminosum* Photosystem-II particles. Dark-minus-light difference spectra were recorded by using the method of Bendall & Sofrová (1971), with Photosystem-II particles ($50 \mu\text{g}$ of chlorophyll *a*/ml) in a buffer containing 0.5 M-sorbitol, 10 mM- MgCl_2 and 50 mM-Mes/NaOH buffer, pH 6.5. Illumination of the negative sample was for 3 min at 580 nm. (a) Difference spectrum for untreated samples; (b) difference spectrum in the presence of 0.25 mM- $\text{K}_3\text{Fe}(\text{CN})_6$.

Table 2. Cytochrome content of Photosystem-II particles and unfractionated membranes of *P. laminosum*

Amounts of the cytochromes were measured from their reduced-minus-oxidized difference spectra, assuming absorption difference coefficients of $20000 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Cytochrome	Amount present (nmol/mg of chlorophyll <i>a</i>)	
	Photosystem-II particles	Unfractionated membranes
<i>b</i> -559 (high-potential)	22.4	3.8
<i>b</i> -559 (low-potential)	3.4	1.0
<i>f</i>	4.0	1.1
<i>b</i> -563	4.2	1.8
<i>c</i> -549	19.9	4.0

measured amounts of components such as Mn and high-potential cytochrome *b*-559. It has been shown that, under light-limited conditions after total dark equilibration, the rise time of the activation curve for O₂ evolution is related to the photosynthetic unit size (Forbush *et al.*, 1971). We previously estimated the photosynthetic unit size in the Photosystem-II particles from *P. laminosum* to be approx. 100 molecules of chlorophyll *a*, by comparison of the activation time for O₂ evolution with that in lettuce chloroplasts (Stewart & Bendall, 1979). These experiments were repeated with the following modifications: firstly, a dark-equilibration time of at least 45 min was found to be necessary for the Photosystem-II particles, and secondly, far-red (695 nm) rather than orange actinic light was used, to eliminate absorption by both chlorophyll *b* and phycocyanin. Under these conditions, the activation times for lettuce chloroplasts and *P. laminosum* Photosystem-II particles were respectively 300 and 1100 ms. The interpretation of this result depends on obtaining a value for the number of chlorophyll *a* molecules in Photosystem II of higher-plant chloroplasts. Estimates for this number vary from 130 to 190 (Thornber & Barber, 1979), giving extreme values of 35 and 50 molecules of chlorophyll *a* for the photosynthetic unit size of *P. laminosum* Photosystem-II particles.

The photosynthetic-unit size was also determined by the more direct method of measuring the concentration of the Photosystem-II reaction centre, pigment P-680, relative to total chlorophyll. Reactions of pigment P-680 are difficult to measure at 680 nm because they are complicated by absorption changes of bulk chlorophyll occurring at the same wavelength. However, Mathis (1977) has developed techniques for measuring flash-induced rapid absorbance changes of both pigment P-700 and pigment P-680 in the near infrared, at 820 nm. In untreated higher-plant chloroplasts, pigment P-680 oxidized by a laser flash is re-reduced in less than 1 μ s by the physiological donor to the reaction centre (Van Best & Mathis, 1978), but in Tris-treated chloroplasts in the presence of ferricyanide (to oxidize pigment P-700), a flash-induced absorbance change decaying with $t_1 \approx 3.5$ –20 μ s (depending on pH) has been attributed largely to the oxidation of pigment P-680, followed by its re-reduction by either a secondary donor or a form of the physiological donor that has been modified by Tris treatment (Conjeaud *et al.*, 1979; Conjeaud & Mathis, 1980).

Fig. 2 shows the fast absorbance change at 820 nm for Tris-treated Photosystem-II particles from *P. laminosum* in the presence of 1.7 mM-K₃Fe(CN)₆. Assuming that the whole of the signal was due to P-680⁺, a value of 1 mol of pigment P-680/65 mol of chlorophyll *a* can be calculated. Considered together with the value obtained by

Fig. 2. Absorbance change at 820 nm induced by a laser flash in Tris-treated Photosystem-II particles from *P. laminosum*.

The trace shown is the average result for four experiments. The amplified band-width was 1 MHz. Tris-treated Photosystem-II particles (19 μ g of chlorophyll *a*/ml) were suspended in a buffer containing 50 mM-Mes/NaOH, pH 6.0, plus 1.7 mM-K₃Fe(CN)₆. Other conditions were as described in the Materials and methods section. The arrow denotes excitation by a flash-pumped Rhodamine B dye laser.

comparison of activation times for O₂ evolution, a Photosystem-II unit size within the range of 40–70 molecules of chlorophyll *a* is indicated. This suggests that only one molecule of high-potential cytochrome *b*-559 was present per Photosystem-II reaction centre, and it is noteworthy that apparently only half of those molecules became operative as donors to Photosystem II at 77 K.

Unfortunately the range for the photosynthetic unit size is too wide to give very accurate information about the number of molecules of Mn per Photosystem-II reaction centre: approx. 3–5, by taking an average value of 1 mol of Mn/15 mol of chlorophyll *a* in the Photosystem-II particles. This result is somewhat lower than the value of 5–8 Mn molecules per Photosystem-II trap reported by Cheniae & Martin (1968). However, there is still controversy over the amount of Mn functioning on the oxidizing side of Photosystem II and the proportion of that Mn that is associated with the O₂-evolving complex itself.

Low-temperature (77 K) absorption and fluorescence emission spectra

Room-temperature absorption spectra for Photosystem II, Photosystem-I particles and unfractionated membranes of *P. laminosum* have been published previously (Stewart & Bendall, 1979). The different forms of chlorophyll could be distinguished more clearly at 77 K (Fig. 3). In unfractionated membranes, chlorophyll *a* forms were present that have been designated from the peak or shoulder

Fig. 3. Absorption spectra at 77K for unfractionated membranes, Photosystem-I and Photosystem-II fractions from *P. laminosum*

The spectra shown are for unfractionated membranes (.....), Photosystem-I (—) and Photosystem-II particles (----) diluted to a concentration of 5 μg of chlorophyll *a*/ml in a buffer containing 25% (v/v) glycerol, 10 mM-MgCl₂, 10 mM-Hepes/NaOH buffer, pH 7.5, and 5 mM-phosphate buffer (NaH₂PO₄/K₂HPO₄), pH 7.5.

Fig. 4. Low-temperature (77K) fluorescence emission spectra of unfractionated membranes, Photosystem-I and Photosystem-II particles from *P. laminosum*

Unfractionated membranes (.....), Photosystem-I (—) and Photosystem-II particles (----) were suspended at a concentration of 6 μg of chlorophyll *a*/ml in a buffer containing 0.35 M-sucrose, 10 mM-NaCl and 50 mM-Tris/HCl buffer, pH 8.0. Fluorescence was excited at 632.8 nm by a helium-neon laser. The relative vertical scale expansions for the three spectra are shown.

positions as Ca668, Ca672, Ca679, Ca687, Ca697 and Ca710. In the Photosystem-II particles, Ca697 and Ca710 were almost completely absent, and there was enrichment in Ca668, Ca672 and Ca677, as well as a new shoulder in the spectrum at 684 nm. The presence of allophycocyanin was evident in a shoulder at 655 nm. In contrast, the Photosystem-I particles were relatively depleted in Ca672, but retained the longest-wavelength forms of chlorophyll. The spectra presented in Fig. 3 are very similar to those reported by Nakayama *et al.* (1979) for Photosystem-I and Photosystem-II fractions prepared from the thermophilic blue-green alga *Synechococcus* sp., and are consistent with a large body of evidence assigning the longest-wavelength forms of chlorophyll *a* to Photosystem I and shorter-wavelength forms to Photosystem II (French *et al.*, 1972).

The Photosystem-II particles showed a high fluorescence at 77 K (Fig. 4; note different vertical scales for the three spectra). Excitation at 633 nm by a helium-neon laser produced a fluorescence peak at 684 nm, with shoulders at approx. 662 nm and 695 nm, and a smaller peak in the far-red at about 720 nm. Unfractionated membranes had a lower overall fluorescence, with the major peaks now at 658 and 721 nm, and shoulders at approx. 683 and 695 nm. Compared with unfractionated thylakoids, Photosystem-I particles showed a similar

fluorescence peak at 721 nm, but the 685 nm peak was relatively larger, the 695 nm shoulder virtually absent, and fluorescence around 660 nm diminished.

There is still some uncertainty about the origins of the various peaks in low-temperature fluorescence emission spectra, but the far-red fluorescence is usually attributed to the long-wavelength Photosystem-I antenna chlorophyll and the 695 nm peak to antenna chlorophyll closely associated with the reaction centre of Photosystem II (Murata *et al.*, 1966). In the blue-green and red algae, where excitation at 633 nm excites fluorescence both of bile pigments and of chlorophyll, peaks at approx. 640 nm and 660 nm are attributed to phycocyanin and allophycocyanin respectively (Ley *et al.*, 1977; Katoh & Gantt, 1979). The emission spectra for the fractions from *P. laminosum* were consistent with the foregoing interpretation. In particular, the fact that the 695 nm band was virtually absent from Photosystem-I particles supports the assignment of 695 nm fluorescence to antenna chlorophyll closely associated with the Photosystem-II reaction centre. Nakayama *et al.* (1979) have reported a prominent 695 nm fluorescence-emission band in a Photosystem-II fraction from *Synechococcus* sp., and more recently Satoh (1980) has shown that, under

appropriate conditions, highly purified Photosystem-II particles from spinach also showed low-temperature fluorescence at 695 nm.

Low-temperature fluorescence at 685 nm is more difficult to interpret, since it probably contains a number of components. Most reports have attributed it to chlorophyll associated with Photosystem II, and Photosystem-II fractions from both higher plants and blue-green algae have been found to be enriched in 685 nm fluorescence (Sato & Butler, 1978; Newman & Sherman, 1978; Nakayama *et al.*, 1979). However both Photosystem-II and Photosystem-I particles from *P. lamosum* showed enhanced fluorescence at this wavelength compared with unfractionated membranes, which may indicate that residual allophycocyanins in these preparations [possibly mainly allophycocyanin-B in the Photosystem-II particles (Ley *et al.*, 1977)] were still able to transfer energy to chlorophyll *a*, whereas the major proportion of allophycocyanin associated with the unfractionated membranes was functionally detached from the membrane and thus highly fluorescent at 660 nm. Free chlorophyll can also contribute to fluorescence around 680 nm in detergent-treated preparations (Sato & Butler, 1978), but the low-temperature absorption spectra (Fig. 3) suggest that the amount of free chlorophyll in the *P. lamosum* fractions was relatively small.

Stability of O_2 evolution in *P. lamosum* Photosystem-II particles: effects of glycerol and ionic conditions

As in untreated thylakoids (Stewart & Bendall, 1980), the preservation of maximum O_2 -evolution capacity in Photosystem-II particles from *P. lamosum* depended on the maintenance of optimal ionic conditions. Both chloride ions and bivalent cations were required (Table 3); 10 mM-MgCl₂ or 10 mM-CaCl₂ (but not MnCl₂) preserved the most activity, and MgBr₂ could substitute for MgCl₂. However, neither Mg²⁺ ions alone [Mg(NO₃)₂ or magnesium acetate] nor Cl⁻ ions alone (supplied as 3.2 mM-Tris/20 mM-HCl) were effective. NaCl (10 mM or 100 mM) could partially substitute for MgCl₂, but O_2 -evolution rates were less stable, decaying rapidly over 2–3 min in the light in the oxygen-electrode chamber. K⁺ ions, on the other hand, were inhibitory at concentrations above 10 mM, complete inactivation occurring in the presence of 100 mM-KCl.

We have previously reported (Stewart & Bendall, 1979, 1980) that the presence of 25% (v/v) glycerol increased the stability of O_2 evolution in *P. lamosum*. The present study has confirmed this observation (Table 4). A minimum of 20% glycerol gave the best results. 20% (v/v) ethylene glycol and 20% (w/v) sucrose also enhanced stability to some

Table 3. Effect of ionic conditions on the stability of O_2 evolution in *P. lamosum* Photosystem-II particles. Samples (0.05 ml) of Photosystem-II particles were diluted to 5.2 ml with 20 mM-Hepes/NaOH, pH 7.5, plus the additions shown. After incubation for 1 h at 5°C the particles were collected by centrifuging and resuspended in the same buffers used for washing, plus 25% (v/v) glycerol. Initial rates of O_2 evolution (measured as described in the legend to Table 1) are expressed relative to the rate (410 μ mol of O_2 /h per mg of chlorophyll *a*) for particles washed with 20 mM-Hepes/NaOH, pH 7.5, plus 10 mM-MgCl₂.

Additions to 20 mM-Hepes/NaOH, pH 7.5	Initial rate of O_2 evolution (% of control)
None	36.8*
10 mM-MgCl ₂	100
20 mM-MgCl ₂	104
50 mM-MgCl ₂	83.7
10 mM-CaCl ₂	94.9
10 mM-MnCl ₂	31.3*
10 mM-Mg(NO ₃) ₂	40.0*
10 mM-Magnesium acetate	34.5*
3.2 mM-Tris/20 mM-HCl	47.4*
10 mM-MgBr ₂	95.2
10 mM-NaCl	50.4*
100 mM-NaCl	63.2*
10 mM-KCl	37.6*
100 mM-KCl	0

* Denotes rates decaying with time in the light in the oxygen electrode.

Table 4. Effect of washing in different media on the stability of O_2 evolution in *P. lamosum* Photosystem-II particles

Samples (0.05 ml) of Photosystem-II particles were diluted to 5.2 ml with 10 mM-MgCl₂/10 mM-Hepes/NaOH, pH 7.5, plus 5 mM-phosphate buffer (NaH₂PO₄/K₂HPO₄), pH 7.5 ('buffer B'), plus the additions shown. After incubation for 1 h at 5°C the particles were collected by centrifuging (200 000g, 4 h) and resuspended in the same buffers used for washing. Activities were measured immediately in the oxygen electrode, as described in the legend for Table 1. The control activity was 790 μ mol of O_2 /h per mg of chlorophyll *a*.

Additions to buffer B	Rate of O_2 evolution (% of control)
None	57.8
10% (v/v) Glycerol	83.3
20% (v/v) Glycerol	100
30% (v/v) Glycerol	95.7
20% (w/v) Sucrose	73.7
20% (v/v) Ethylene glycol	82.1
20% (w/v) Ficoll 400	52.8

extent, but 20% (w/v) Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) did not give greater protection than washing with hypo-osmotic buffer.

Lipid analysis

Analysis of the acyl-lipid content of the Photosystem-II particles, unfractionated membranes and lettuce chloroplasts revealed no significant differences among the preparations. Virtually no phospholipid was present, the main components being mono- and di-galactosyl diacylglycerols, possibly a sulpholipid and one other minor component (results not shown). Thus it appears that significant amounts of the original membrane lipids were still present in the Photosystem-II particles, despite the detergent used in their preparation.

Polypeptide content

Despite the obvious complexity of the protein content of the Photosystem-II particles (Fig. 5), there were some clear differences in polypeptide profile between the Photosystem-II particles, Photosystem-I particles and unfractionated membranes. The pattern of bands on sodium dodecyl sulphate/polyacrylamide gels depended markedly on the solubilization temperature used for the samples. For samples solubilized at 60°C, the main polypeptide bands in the Photosystem-II particles (Fig. 5, track d) had apparent mol.wts. of 87 100, 83 200, 68 000, 57 300, 48 200, 46 400, 42 500, 40 100, 38 200, 33 900, 31 600, 30 200, 27 500, 18 000, 16 700 and 15 600. The two highest-molecular-weight bands (which in some experiments retained some chlorophyll, but did not correspond to the chlorophyll-containing bands of Photosystem-I par-

ticles or unfractionated membranes) were fainter in samples solubilized at room temperature, and there was a corresponding increase in staining intensity in the 40 000–45 000-mol.wt. region (track i). The latter region corresponded in molecular weight to the major chlorophyll–protein complex isolated from the Photosystem-II particles by non-denaturing sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Stewart, 1980), and it is possible that the bands corresponding to mol.wts. 87 100 and 83 200 may be heat-aggregated components of this complex.

Attempts were made to identify some of the other major bands in the Photosystem-II particles. Tetramethylbenzidine stained bands corresponding to 13 000–16 000, 20 000, 30 000 mol.wt. (in room-temperature-solubilized samples) and a number of fainter bands in the 40 000–50 000-mol.wt. region (Fig. 6, track f). None of the bands appeared to be attributable to *b*-type cytochromes, since neither acid-acetone extraction nor urea treatment [both of which remove the haem from *b*-type cytochromes (Bendall *et al.*, 1971)] affected the pattern (results not shown). The bands at 13 000–16 000 and 30 000 mol.wt. in the tetramethylbenzidine-stained gel were attributed to cytochrome *c*-549 and cytochrome *f* respectively, by comparison with published molecular weights for these cytochromes (Ho *et al.*, 1979). Cytochrome *f* appeared to aggregate in samples solubilized with sodium dodecyl sulphate at 60°C, since the 30 000-mol.wt. band became fainter in both tetramethylbenzidine- and Coomassie Blue-stained gels. The identities of

Fig. 5. Polypeptides of unfractionated membranes, Photosystem-II and Photosystem-I particles from *P. laminosum* separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained with Coomassie Blue R

Samples (equivalent to 10 µg of chlorophyll *a* per track) were prepared and electrophoresis performed as described in the Materials and methods section. The gel was stained with Coomassie Blue R by the method of Fairbanks *et al.* (1971). The samples in each track and the temperatures at which they were treated with sodium dodecyl sulphate were as follows: a and h, unfractionated membranes, 60°C; b and g, EDTA-washed membranes, 60°C; c, Photosystem-I particles, 60°C; d, Photosystem-II particles, 60°C; e and f, 1 µg each of bovine serum albumin (68 000 mol.wt.), ovalbumin (43 000), carbonic anhydrase (30 000) and lysozyme (14 300), 60°C; i, Photosystem-II particles, 20°C; j, Photosystem-I particles, 20°C; k, EDTA-washed membranes, 20°C; l, unfractionated membranes, 20°C.

the bands at 20 000 mol.wt. and the 40 000–50 000 mol.wt. region of the tetramethylbenzidine-stained gel are unknown. The band at 12 600 mol.wt. in unfractionated membranes may be the soluble high-potential cytochrome *c*-553.

It was thought that some of the bands in the Photosystem-II particles might be components of the coupling factor CF_1 , which was found by Satoh (1979) to co-purify with the Photosystem-II reaction centre from spinach chloroplasts. However, extensive washing with 0.7 mM-EDTA, which has been reported to remove CF_1 from chloroplasts (Høyer-Hansen *et al.*, 1979), caused only minor changes in the polypeptide pattern for unfractionated membranes (Fig. 5, tracks b, g, k), except in the low-molecular-weight (<20 000) region, where much of the difference was probably due to removal of the low-molecular-weight soluble cytochromes (as shown in the tetramethylbenzidine-stained gel; Fig. 6, tracks d and e) and biliprotein subunits. Washing with 1 M-NaBr, which has also been reported to remove CF_1 (Nelson, 1980) gave similar results. Only three higher-molecular-weight (79 000, 40 000 and 32 000) polypeptides appeared to be removed by EDTA or NaBr, and none of these corresponded in

molecular weight to published values for the five subunits of CF_1 (Høyer-Hansen *et al.*, 1979). We conclude that either more exhaustive treatment is needed to remove coupling factor from *P. laminosum* or that membrane fragments as prepared by our methods (Stewart & Bendall, 1980) were already substantially depleted in CF_1 .

Two of the most strongly staining bands in the electrophoretogram of the Photosystem-II particles, which were missing in that of the Photosystem-I particles (Fig. 5, track c), were the bands at 33 900 and 27 500 mol.wt. These bands remain unidentified, but it is noteworthy that Satoh (1979) found a 27 000-mol.wt. component of the Photosystem-II reaction centre in highly purified preparations from spinach chloroplasts, and a recent report (Metz *et al.*, 1980) has suggested that a polypeptide of 34 000 mol.wt. may be involved in electron transport on the oxidizing side of Photosystem-II. Spector & Winget (1980) reported a mol.wt. of 65 000 for their Mn-containing protein from spinach chloroplasts. The Photosystem-II particles did contain a polypeptide of approximately that molecular weight, but it did not appear to be a major protein component of the preparation.

Discussion

The present analysis has shown that, although highly active in O_2 evolution and enriched in Photosystem-II components (pigment P-680, Mn, component C-550 and high-potential cytochrome *b*-559), the Photosystem-II particles were not chemically pure, containing a considerable proportion of the protein and lipid components of the original membranes. So far, attempts to purify the preparation further without substantial loss of activity have not been successful. A major difficulty has been the fact that the O_2 -evolving system in the particles, although remarkably resistant to detergent, was in other respects extremely sensitive to the composition of the external medium. There have been a number of reports of similar sensitivity of Photosystem II and the O_2 -evolving system in cell-free preparations of other blue-green algae (e.g. McSwain *et al.*, 1976; Binder *et al.*, 1976; Tel-Or & Stewart, 1975), but so far there has been no convincing explanation for these effects. Inactivation under unfavourable conditions, which was irreversible, seemed to involve some sort of conformational change rather than merely dissociation, since it was not directly correlated (except in the cases of heat, Tris or EDTA treatment) with release of bound Mn. The role played by ionic conditions in blue-green-algal preparations may be rather complex. For example, particular ionic conditions may be required to maintain a component or components of the Photosystem-II- O_2 -evolving com-

Fig. 6. Haem-containing polypeptides of unfractionated membranes, Photosystem-II and Photosystem-I particles from *P. laminosum* separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and stained with tetramethylbenzidine

Samples ($10\mu\text{g}$ of chlorophyll *a* per track) were prepared and electrophoresis performed as described in the Materials and methods section. The gel was stained with tetramethylbenzidine by the method of Thomas *et al.* (1976). Samples in each track and solubilization temperatures were as follows: a, $1\mu\text{g}$ of mammalian cytochrome *c* (12 400 mol.wt.), 60°C ; b, unfractionated membranes, 20°C ; c, unfractionated membranes, 60°C ; d, EDTA-washed membranes, 20°C ; e, EDTA-washed membranes, 60°C ; f, Photosystem-II particles, 20°C ; g, Photosystem-II particles, 60°C . The arrows show the positions of stained bands that were green in the unstained gel.

plex in an active conformation, as well as perhaps to facilitate the correct conformation change in the light. This might explain why NaCl could partially preserve the capacity for O₂ evolution in *P. laminosum* Photosystem-II particles stored in the dark, so that initial rates of O₂ evolution in the light were reasonably high, but progressively more severe inhibition was observed with time in the light. The fact that K⁺ ions were inhibitory whereas Na⁺ ions were not suggests that the ionic requirements for O₂ evolution require a chemical explanation and were not due to membrane-surface-charge effects.

Compounds such as glycerol may stabilize hydrophobic interactions by decreasing the concentration of free water. Glycerol has been found to be effective in stabilizing some other intrinsic membrane proteins after their extraction from the membrane (Ichikawa & Yamano, 1967; Lu & Coon, 1968), and in the case of *P. laminosum* Photosystem-II particles, 25% (v/v) glycerol (and possibly higher concentrations of ethylene glycol and sucrose) may help to preserve the correct hydrophobic environment around the O₂-evolving complex, particularly in the presence of detergents. As pointed out by Spector & Winget (1980), the apparent requirement, noted in some reports, for the presence of membrane vesicles to preserve O₂ evolution (Stillwell & Tien, 1978) can also be explained by the idea that the O₂-evolving complex is maintained in a particular native conformation by the hydrophobic membrane environment. In the case of higher plants, part of this environment may be provided *in vivo* by the large intrinsic chlorophyll *a/b*-protein complex. The lack of this complex in blue-green algae may be partly responsible for their greater sensitivity to 'water concentration' in cell-free preparations.

However, there were indications that in some respects the O₂-evolving enzyme itself may also be inherently more fragile in the blue-green algae. Since EDTA readily removed a large proportion of the Mn from the Photosystem-II particles, the Mn-affinity of the binding sites in the complex must be lower than the affinity of Mn for EDTA. In higher-plant chloroplasts the reverse has been found to be the case (Radmer & Cheniae, 1977).

The Photosystem-II particles from *P. laminosum* showed the highest activities, expressed on a chlorophyll basis, that have been reported for any O₂-evolving preparations. They were considerably depleted in Photosystem-I components and had a relatively small Photosystem-II antenna size. These properties, together with their good optical qualities (suspensions of the particles at concentrations up to 100 µg of chlorophyll *a*/ml were only very weakly light-scattering, and could be clarified completely with minimal loss of activity by addition of a small amount of detergent) make them ideal for studies of absorbance and fluorescence changes and e.p.r.

signals associated with Photosystem-II and the O₂-evolving system.

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