Biochimica et Biophysica Acta, 682 (1982) 297–306
Elsevier Biomedical Press

BBA 41178

OPTICAL AND EPR CHARACTERIZATIONS OF OXYGEN-EVOLVING PHOTOSYSTEM II SUBCHLOROPLAST FRAGMENTS ISOLATED FROM THE THERMOPHILIC BLUE-GREEN ALGA *PHORMIDIUM LAMINOSUM* *

BACON KE [a,**], HIROSHI INOUÉ [a,***], GERALD T. BABCOCK [b], ZHOU-XI FANG [a,+] and EDWARD DOLAN [a]

[a] *Charles F. Kettering Research Laboratory, Yellow Springs, OH 45387 and* [b] *Chemistry Department, Michigan State University, East Lansing, MI 48824 (U.S.A.)*

(Received May 10th, 1982)

Key words: Pheophytin; Photosystem II; ESR; Oxygen evolution; Fluorescence yield; (Blue-green alga, Phormidium laminosum)

An oxygen-evolving, Photosystem II particle was isolated from the thermophilic, blue-green alga, *Phormidium laminosum*, according to the procedure of Stewart and Bendall (Stewart, A.C. and Bendall, D. (1979) FEBS Lett. 107, 308–312). Our particle has an oxygen-evolution activity of 1500–1600 μmol O_2/mg chlorophyll per h. The oxygen-evolution activity has a pH optimum at 5–6, and is abolished at pH 9. Maximum oxygen evolution occurs at approx. 47°C in whole cells, but at 29°C in the particles. The activity decreases to 50% when the cells are heated for 30 min at 55°C; with the particles, 50% inactivation occurred at 47°C for the same heating time of 30 min. Flash excitation of the particle at 100 K produced absorbance changes whose difference spectrum in the ultraviolet-to-near infrared region shows photochemical charge separation and recombination of P-680$^+$ and Q^- in the dark with $t_{1/2}$ of 1.75 ms. An EPR spectrum for the P-680$^+$ free radical, with g 2.0027 and $\Delta H_{pp} = 8$ G, was constructed from flash-induced EPR changes under conditions identical to those used for obtaining P-680 absorbance changes. The actinic light-induced variable fluorescence yield is 5-fold that induced by the weak probing beam alone. Addition of dithionite to the particle brings the fluorescence to the same maximum level. Under the reducing condition, strong actinic light caused the fluorescence to decrease. This observation is consistent with the notion that variable fluorescence yield in Photosystem II originates, as in green-plant chloroplasts, from recombination luminescence, the attenuation of which corresponds to photoaccumulation of reduced pheophytin under these conditions. Broad segments (300 nm) of the difference spectrum for pheophytin photoreduction were recorded by an intensified photodiode array in conjunction with a phosphoroscopic photometer. Kinetic spectrophotometric assays together with chemical analysis showed a rather clean and simple stoichiometry in these particles, namely, 1 P-680:1 Ph:1 Q:4 Mn:44 Chl. Initial investigation failed to reveal the doublet EPR spectrum previously observed for $Ph^- \cdot Q^-$ Fe in spinach subchloroplast particles (Klimov, V.V., Dolan, E. and Ke, B. (1980). FEBS Lett. 118, 97–100). A hyperfine EPR spectrum consisting of 16–20 lines and presumably associated with the manganese clusters in the oxygen-evolving protein has been confirmed in these particles. Tris washing but not washing with EDTA eliminates this signal. Active oxygen-evolving particles also yield the II_{vf} signal with a $t_{1/2}$ of approx. 800 μs. Upon Tris washing, the II_f signal appears which decays in 23.5 ms.

* Contribution No. 762 from the Charles F. Kettering Research Laboratory.

** To whom reprint requests and correspondence should be addressed.

*** Permanent address: Biology Department, Toyama University, Toyama, Japan.

\+ Permanent address: Biology Department, University of Beijing, Beijing, People's Republic of China.

Abbreviations: PS, photosystem; Ph, pheophytin; Chl, chlorophyll; Hepes, *N*-α-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

0005-2728/82/0000–0000/$02.75

Introduction

Over the past two decades, tne study of green-plant and algal photosynthesis has been greatly facilitated by the availability of subchloroplast particles enriched in one or the other of the two photosystems. In the case of Photosystem II (PS II) particles, although highly enriched and photochemically active particles have been prepared from spinach for some time [1–4], and more recently from blue-green algae [5–7], none of these preparations retained the capacity for oxygen evolution in the light, until Stewart and Bendall [8,9] recently succeeded in isolating an oxygen-evolving PS II particle from a thermophilic blue-green alga, *Phormidium laminosum*. Various chemical, optical and EPR characterizations have already been reported on this particle [8–13]. Very recently, more reports have appeared documenting the preparation of oxygen-evolving particles from blue-green algae [14] and also from spinach [15,16]. Such preparations will undoubtedly become valuable for future studies in the area of PS II, particularly the mechanism of oxygen evolution.

We have isolated the oxygen-evolving particles from *P. laminosum* essentially according to the method of Stewart and Bendall [9] and wish to report here some additional chemical and photochemical properties of this oxygen-evolving PS II particle.

Experimental Procedure

Cultures of *P. laminosum* (OH-1-p, Clone 1) were kindly furnished by Dr. Richard W. Castenholz of the University of Oregon. Cells were grown in medium D [17] in 5-gallon jars under cool-white fluorescent lamps at 47°C with air containing 1% CO_2 bubbling through the medium. The culture was harvested during the late exponential growth phase by centrifugation and used immediately or stored at −80°C. *Anabaena variabilis* (ATCC 29413) was grown at 30°C [18].

The published procedure of Stewart and Bendall [9] was used with some modification for the preparation of spheroplasts, membrane fragments and purified particles. Chlorophyll content was determined by the formula of Arnon [19]. Oxygen evolution was assayed at 22°C except where noted otherwise by a home-made Clark-type oxygen electrode precalibrated by using glucose-glucose oxidase to remove oxygen from air-saturated water or monitoring the response to the oxygen evolved from a known amount of hydrogen peroxide by catalase-catalyzed decomposition [20]. P-700 was estimated by redox difference absorption spectroscopy using 64 $M^{-1} \cdot cm^{-1}$ as the millimolar differential extinction coefficient for the difference band near 700 nm [21]. P-680 was estimated from the flash-induced absorbance change at 810 and 680 nm using 7 and 65 $M^{-1} \cdot cm^{-1}$, respectively, for the ϵ_{mM}. The intermediary electron acceptor, pheophytin, was estimated by comparing the light-induced absorbance change at 685 nm accompanying pheophytin reduction [22] with the total chlorophyll absorbance.

Absorption spectra were routinely recorded on a Cary spectrophotometer. Flash-induced absorbance at room temperature and cryogenic temperatures were carried out as previously described [23]. For slower absorbance changes, a phosphoroscopic photometer was used [22] in which the chopped measuring beam was simultaneously modulated at 100 kHz and the signal extracted by a PAR 5101 lock-in amplifier. With the appropriate modification, the same photometer was also used for measuring fluorescence-yield changes, with the modulated probing beam now decreased to an intensity of 10–20 erg/cm^2 per s. The actinic beam was obtained from a 150-W xenon lamp after passing through a heat-removing filter and a cut-on filter (Corning 2-58). The phosphoroscopic device allows the probing and excitation beams to overlap in their wavelengths without signal interference. For relatively slow-decaying absorbance changes such as that of pheophytin (see Fig. 5), a 300-nm wide segment of the difference spectrum was also recorded by a photodiode-array detector (the intensified DARSS system of Tracor Northern) adapted to the phosphoroscopic photometer using unfiltered white light as the measuring beam.

A Varian model 4500 with 100-kHz field modulation or a Brucker model ER200D EPR spectrometer was used for recording EPR spectra and flash-induced EPR signal changes. An Air Products liquid-helium transfer line or an Oxford Instruments ESR9 siphon/cryostat system was used for experiments at cryogenic temperatures. EPR

experimental conditions are described in the figure legends. EPR spectroscopy was also used to assay the manganese content in the PS II particles, using a precision 1-mm quartz tube as the sample cell. Calibration curves were constructed from assays with known manganese concentrations prepared from standard AA Mn^{2+} solution.

Results and Discussion

Some general properties

In general, the PS II particles we have isolated from *P. laminosum* are very similar to those reported previously by Stewart and Bendall [10]. Absorption and fluorescence spectra of the particles at room temperature [9] and at cryogenic temperatures [10] have been reported.

We have run the SDS-polyacrylamide gel electrophoresis analysis and compared the polypeptide composition of the *P. laminosum* PS II particles with PS II particles prepared from spinach. In agreement with previous reports [11], we found the polypeptide pattern of the *Phormidium* particles (our results not shown here) to be substantially more complex than those of TSF-IIa [2] or those prepared by the methods of Satoh [24] or Mullet and Arntzen [25]. This higher level of complexity is expected for a particle that still retains a high level of oxygen-evolution activity. It is of interest to note, however, that a very high concentration of a 34 kDa band is present in the polypeptide pattern of the *Phormidium* PS II particle but absent in the patterns of TSF-IIa [2] or other PS II particles [24,25]. A 34 kDa protein has recently been identified as a possible component of the manganese-binding water-splitting complex in green algae by Bishop and co-workers [26] and in spinach thylakoid by Åkerlund and Jansson (Ref. 27; also cf. Refs. 28–30).

The PS II particle is not totally devoid of P-700 either [9]; in our particles, the P-700 content is one P-700/1083 Chl, and the P-680/P-700 ratio is about 25 (see below). As has been indicated previously [10], an attempt to reduce further the level of P-700 in the PS II particles without substantial loss of activity was not successful.

Our particles have a consistently high oxygen-evolution activity, 1500–1600 μmol O_2/mg Chl per h. To preserve the oxygen-evolution activity, 'buffer C' [9] (10 mM Hepes, 5 mM phosphate, 10 mM $MgCl_2$, 25% glycerol, pH 7.5) was used as the suspending medium.

With red light excitation (Corning filter 2-58 and 1 cm of water), the oxygen-evolution rate increased linearly below $3 \cdot 10^5$ erg/cm^2 per s and became saturated at $4 \cdot 10^5$ erg/cm^2 per s. The optimum pH for oxygen evolution was between 5 and 6, while at pH 9, the activity was nearly abolished. After the thermophilic *P. laminosum* cells had been maintained at approx. 55°C for 30 min, their oxygen-evolution activity was reduced to 50% and was virtually abolished after heating

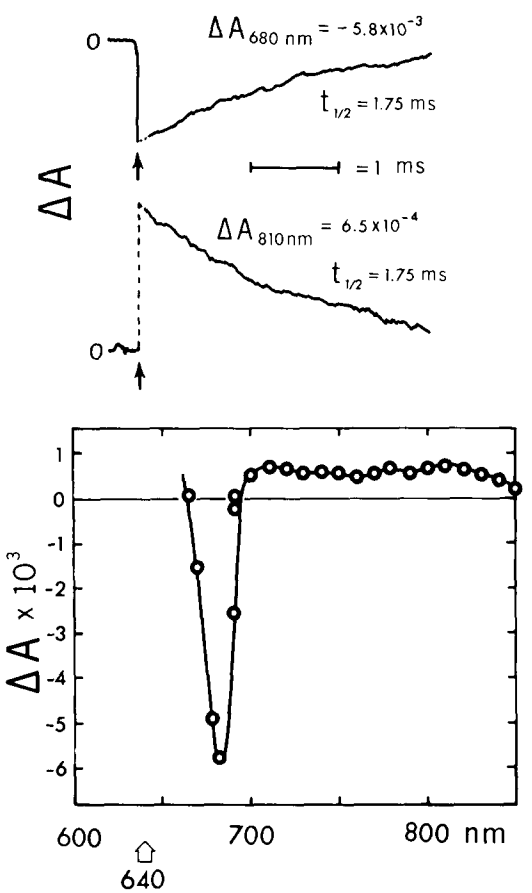

Fig. 1. Light-induced absorbance changes associated with P-680 photooxidation in *Phormidium* PS II particles at approx. 100 K. The reaction mixture was a phosphate buffer (pH 6.4) containing 60% glycerol and 74 μg Chl/ml; cuvette path length, 1 mm. Excitation light: 250-ns, 640-nm dye-laser flashes. Top: absorbance changes at 680 and 810 nm (each average of two sweeps). Bottom: the light-minus-dark difference spectrum.

above 65°C. On the other hand, the membrane fragments had a half-inactivation temperature of approx. 47°C and an upper limit of approx. 50°C, very similar so that of *A. variabilis* cells. The optimum reaction temperature for *Phormidium* cells was approx. 47°C, whereas for the membrane fragments it was 29°C.

The primary electron donor, P-680, and the stable primary electron acceptor, Q

In PS II reaction centers, when the secondary electron donor is active, P-680$^+$ is reduced in about 25 ns [31] following the first flash on dark-adapted material and in approx. 400 ns under steady-state conditions [32]. Thus, in these oxygen-evolving particles, where the donor side is expected to be intact, flash-induced absorbance changes due to P-680 photooxidation would not readily be seen without adequate time resolution in the detection system. When the PS II particles are Tris washed, however, so that the endogenous electron donor is somehow modified P-680$^+$ reduction monitored at 820 nm occurs in 3.5–20 μs (depending on pH) [13]. Thus far, only measurement at 820 nm has been reported for the *Phormidium* PS II particles [10,13].

We have chosen to use cryogenic temperature as the means for altering the electron-transport pathway after initial charge separation in PS II to facilitate the observation of P-680 photooxidation and the subsequent recombination between P-680$^+$ and the reduced primary acceptor, Q^- [33,34]. Through these measurements, a difference spectrum for the P-680 $\rightarrow$ P-680$^+$ reaction has been constructed and the involvement of P-680 can be definitively established. By also measuring the associated ultraviolet absorbance changes, it has been possible to confirm that charge recombination involved P-680$^+$ and the plastosemiquinone radical, as well as to estimate their stoichiometry.

Fig. 1 (top) shows the absorbance-change transients at approx. 100 K in *Phormidium* PS II particles induced by 250-ns, 640-nm dye-laser pulses. Both the 680- and 810-nm transients decayed in 1.75 ms, and their amplitudes are consistent with differential extinction coefficient values of 65 and 7 $mM^{-1} \cdot cm^{-1}$, respectively. The assignment of the light-minus-dark difference spectrum (Fig. 1, bottom) to P-680 is reinforced by the identical decay times found at all wavelengths examined.

The free-radical nature of P-680 was further investigated by the use of time-resolved EPR spectroscopy at cryogenic temperatures. In Fig. 2 (left), flash-induced EPR changes at several field values show that their decay times at approx. 100 K are 1.75 ± 0.25 ms, nearly the same as that of the absorbance changes attributed to P-680$^+$ decay (see Fig. 1, top). The spectrum of flash-induced EPR changes at various field values (Fig. 2, right) has a g value of 2.0027 ± 0.003 and ΔH_{pp} of 8 G, in good agreement with that reported previously for P-680$^+$ [34,35]. The EPR spectrum for the sample in the dark after flash excitation has the typical shape of the so-called 'Signal II'. This spectrum may contain some contribution from a light-induced, irreversible free-radical signal with ΔH of about 12 G [3,36].

Other aspects of the EPR characteristics of the *Phormidium* PS II particles have recently been examined by Nugent et al. [12]. They reported that steady illumination of the PS II particles at cryogenic temperatures produced an additional Signal II (Signal II_{LT}), which has been attributed to a donor species close to the PS II reaction center and may represent part of the charge-accumulation system of water oxidation.

Absorbance changes of the PS II particles in the

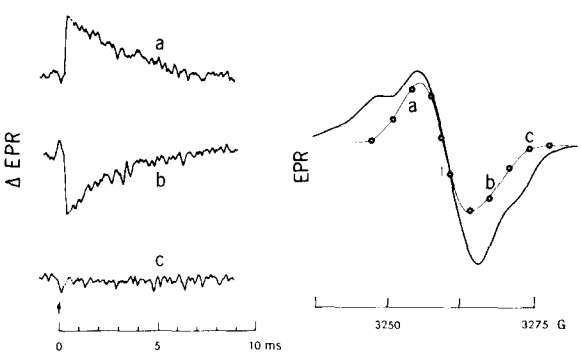

Fig. 2. Flash-induced EPR changes at different field values at approx. 100 K. The reaction mixture containing buffer C, 25% glycerol, 210 μg Chl/ml and 1 mM ferricyanide was placed in quartz tubes of 3 mm inner diameter. Microwave frequency, 9.12 GHz. Microwave power, 5 mW. Modulation amplitude, 9 G. Time constant, 100 μs. Individual field values for EPR changes (left) are indicated on the composite EPR spectrum (right). EPR changes were averaged from 32 sweeps.

ultraviolet region induced by 640-nm dye-laser flashes at approx. 100 K are shown in Fig. 3 (left), and the light-minus-dark difference spectrum plotted in Fig. 3 (right). The decay times for all wavelengths were 1.8 ± 0.2 ms, consistent with a species recombining with P-680$^+$. The shape of the difference spectrum also agrees well with that reported earlier by Van Gorkom [37] for the PS II primary acceptor, being a plastosemiquinone, and also with that for X-320 reported by Stiehl and Witt [38]. The ratio of the amplitudes of the 325- and 265-nm bands are in good agreement with the differentual extinction coefficient values of 11000 and $(-)17000\ M^{-1} \cdot cm^{-1}$ for ϵ_{325} and ϵ_{270}, respectively [37].

Light-induced fluorescence-yield changes

Light-induced fluorescence-yield changes in PS II are commonly considered to reflect the redox state of the stable primary electron acceptor, Q, of PS II. In the absence of added electron acceptors, actinic illumination increases the fluorescence yield of chlorophyll in chloroplasts. Upon turning off the actinic light, the level of fluorescence decreases to the base level. Duysens and Sweers [39] have explained this phenomenon by assuming that the primary acceptor also functions as a fluorescence quencher when it is in the oxidized state. Thus, the increase in fluorescence yield upon illumination would reflect Q being photoreduced, and the decline in fluorescence in the dark would then signal the reoxidation of reduced Q.

Fig. 4 shows light-induced fluorescence-yield changes in *Phormidium* PS II particles with no

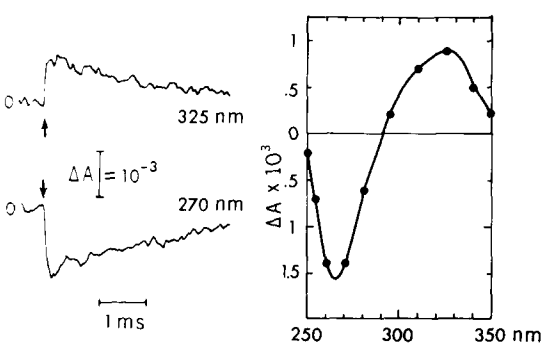

Fig. 3. Flash-induced ultraviolet absorbance changes in *Phormidium* PS II particles at approx. 100 K. Sample and excitation conditions same as in Fig. 1.

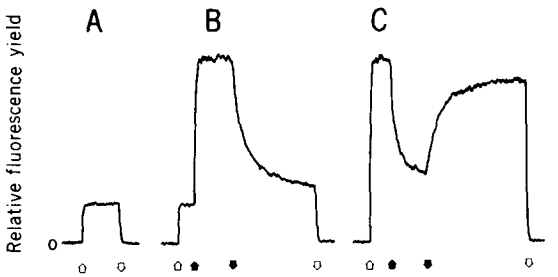

Fig. 4. Light- and chemically induced fluorescence-yield changes in *Phormidium* PS II particles. The reaction mixture contained 0.2 M Tricine buffer, pH 8, and particles at 9 μg Chl/ml. Empty arrows (upward on, downward off) represent the weak probing beam (20 erg/cm^2 per s) at 483 nm. The solid arrows represent the strong actinic light ($4 \cdot 10^5$ erg/cm^2 per s) at 680 nm (illumination time, 100 s). The probing beam was modulated at 100 kHz by an elasto-optic modulator; the sample chamber is a rotating-cup phosphoroscope [22] with a chopping frequency of approx. 100 Hz. (A) Application of the probing beam; (B) effect of actinic beam; (C) fluorescence yield of sample reduced by dithionite and the effect of subsequent actinic illumination.

acceptors added. Illumination with strong actinic light increased the fluorescence yield induced by the weak, modulated probing beam about 5-fold (Fig. 4A and B), indicating that the quencher Q is being reduced. Upon cessation of illumination, the fluorescence yield declined (as Q is reoxidized). When dithionite was added to sample to bring the redox potential to approx -500 mV, and maintain Q in the reduced state prior to excitation, the probing beam alone produced a fluorescence-yield level equal to the induced previously by the strong actinic light without dithionite (Fig. 4C). However, under this reducing condition, the application of actinic light now caused the fluorescence yield to decrease. Upon cessation of actinic illumination, the fluorescence yield increased again, albeit relatively slowly. This unusual phenomenon was first observed by Klimov and co-workers (Ref. 40; cf. Ref. 42), and they proposed a different explanation for this actinic light-induced fluorescence decrease as well as the normally observed fluorescence increase caused by actinic illumination under oxidizing conditions. In their model, an earlier electron acceptor before Q, probably pheophytin (Ph), is operative in PS II. They further proposed that the variable fluorescence may actually be luminescence arising from charge recombination

between the oxidized P-680$^+$ and reduced Ph$^-$. Thus, the recombination luminescence would appear upon reduction of Q by strong light or dithionite, but then disappears when Ph$^-$ is phototrapped. Subnanosecond and picosecond fluorometry revealed a luminescence with a decay time of approx. 4 ns [41,43] when Q is reduced. Separate absorbance-change measurements under the same conditions indicate that P-680$^+$ and Ph$^-$ recombine with a half-time of 4 ns also [43].

Photoaccumulation of the intermediary electron acceptor, Ph$^-$

On the basis of recombination luminescence, the light-induced decline of fluorescence should be a consequence of photoaccumulation of reduced Ph$^-$, and can thus be monitored directly by the absorbance changes. Fig. 5 (left) shows such a correlation; both fluorescence-yield changes and absorbance changes were measured with the same sample containing dithionite in the same phosphoroscopic photometer, using a 100-kHz modulated weak probing or measuring beam and a high-intensity lamp as the actinic source.

Because of the relatively slow decay kinetics of reoxidation of phototrapped Ph$^-$, we have utilized a photodiode-array detector in conjunction with the phosphoroscopic photometer to measure its light-minus-dark difference spectrum directly with single sweeps. The absorption spectrum in the dark was measured by the diode-array detector first (Fig. 5; right, top); it is in good agreement with that reported previously [9] for the same PS II particles. The difference spectrum (Fig. 5; right, bottom) shows major and minor bleachings at 685, 670, 541, 512, 416 and 405 nm, all characteristic of pheophytin. Positive absorbance changes occur at 455, 650 and 675 nm. Note that this difference spectrum, the acquisition of which takes only a matter of seconds, is in excellent agreement with that obtained by point-by-point measurements on TSF-IIa particles from spinach chloroplasts in a similar phosphoroscopic photometer but using a conventional multiplier phototube as the detector [22].

Reduced Ph$^-$ (photoaccumulated near ambient temperature) is accompanied in TSF-IIa particles by the appearance of an EPR spectrum with g 2.0035 and $\Delta H_{pp} = 12.5$ G [22], similar to that of

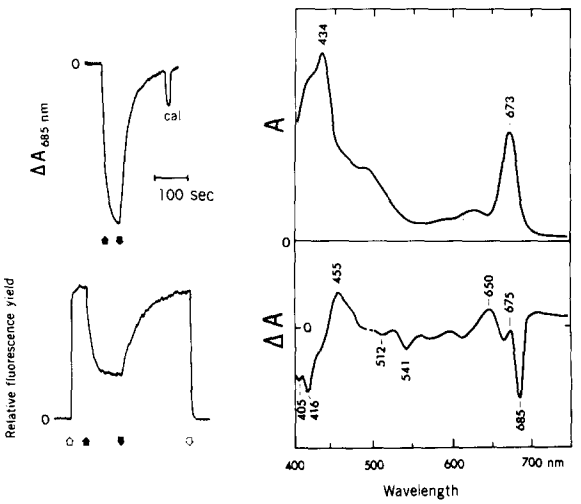

Fig. 5. Photoaccumulation of reduced pheophytin in *Phormidium* PS II particles prereduced by dithionite. Left, top: light-induced absorbance change at 685 nm (cal signal represents 1% change in transmission). Left, bottom: actinic light-induced fluorescence-yield change in the same sample as above (see Fig. 4). Right: absorption spectrum (top) and the light-minus-dark difference spectrum (bottom). Both spectra spectra were acquired by a photodiode-array detector (the intensified DARSS system of Tracor Northern) in conjunction with a phosphoroscopic photometer. 300-nm segments were measured at one time. 1536 sweeps were used to obtain the spectrum of the sample under light. See text for the amplitudes of A and ΔA.

the anion radical of pheophytin in vitro [44]. At 6 K, this signal can be seen at low microwave power, but becomes saturated at 50 mW and unobservable [22,45]. However, when pheophytin is trapped at a lower temperature (e.g., 220 K), the EPR spectrum measured at 6 K shows, in addition to the narrow EPR signal of Ph$^-$, a doublet signal centered at g 2.00 with a splitting of approx. 52 G. This split EPR spectrum, in contrast to the singlet EPR spectrum, is highly temperature sensitive, and not detectable above 15 K [22,45]. It has been attributed to Ph$^-$ perturbed by endogenous Fe associated with Q [45].

In an attempt to obtain a similar doublet EPR spectrum for the *Phormidium* PS II particles, we have not yet succeeded in observing such a split spectrum, Preliminary experiments have been carried out over a wide range of trapping temperatures ranging from 210 K (as dictated by the fluidity of the suspending medium containing 50%

glycerol) to ambient temperature. In some cases, photoaccumulations were carried out with the *Phormidium* particles and the spinach TSF-IIa particles side by side under otherwise nearly identical conditions. The reason for the absence of the split EPR spectrum in the *Phormidium* particles is not yet clear.

Composition and stoichiometry of the reaction-center components in the phormidium PS II particles

With the experiments performed thus far as described above, quantitative data are available to allow us to estimate the amount of the various major electron-transport components involved in the PS II photochemistry in the *Phormidium* particles, and a brief account of these estimations is given below.

The P-680 concentration was estimated from the light-induced absorbance change in a *Phormidium* particle containing 74 μg Chl/ml in a cuvette with 1 mm pathlength. Application of the shrinkage factor for the sample at 100 K would increase the chlorophyll concentration to 88 μM. The light-induced absorbance change in the sample at 810 nm was $1.4 \cdot 10^{-3}$. Assuming the differential millimolar extinction coefficient of P-680 at 810 nm to be 7 $mM^{-1} \cdot cm^{-1}$, the amount of P-680 undergoing photooxidation was 2 μM, or one P-680 per 44 total Chl molecules. To complete the analysis, we have also used the same sample for manganese analysis.

The manganese concentration was estimated from the peak-to-trough amplitude of the third hyperfine line (low-field side of g 2.0) in the six-line spectrum. The *Phormidium* particles appear to contain no adventitious manganese unrelated to oxygen evolution, as treatment with 50 mM Ca^{2+} yielded no detectable manganese signal. Total manganese was estimated from samples treated with 50 mM $CaCl_2$ and 0.5 M HCl. Manganese and chlorophyll determinations for this sample yielded a chlorophyll-to-manganese ratio of 12, and thus approx. 4 manganese per P-680 molecule. Three other determinations yielded a manganese-to-P-680 ratio of 4 ± 0.5.

The concentration of Q observed at low temperature may also be estimated from the light-induced absorbance changes. As described earlier, the amplitudes of the difference bands in the ultraviolet region are consistent with the molar extinction coefficients of 11–13 and 17 $mM^{-1} \cdot cm^{-1}$ at 325 and 270 nm, respectively. The extinction coefficient values together with the actual amplitudes of the absorbance changes would yield a P-680-to-Q ratio of approximately unity.

The concentration of pheophytin was estimated simply by dividing the light-induced absorbance change at 685 nm ($2.75 \cdot 10^{-2}$) by the maximum absorbance of the sample at 673 nm (1.2), yielding the [Chl]/[Ph] ratio of 44.

Taking all the estimations together, it appears the *Phormidium* PS II particles we have prepared have a rather consistent value of composition for the various electron-transport components, i.e., 1 P-680: 1 Ph: 1 Q: 4 Mn: 44 Chl *a*.

A hyperfine EPR spectrum possibly associated with the oxygen-evolving manganese protein

Manganese has been widely accepted to play a central role in oxygen evolution [46]. Lozier et al. [47] and Blankenship and Sauer [48] showed that inhibition of chloroplast oxygen evolution by washing them with Tris or a chaotropic agent resulted in the appearance of a six line EPR spectrum of Mn^{2+} (however, cf. Refs. 49 and 50). Most recently, Dismukes and Siderer [51] and Hansson and Andreasson [52] reported the observation of a multiline EPR spectrum that could be attributed to a bi- or tetranuclear manganese center in spinach chloroplasts. The 16–20-line spectrum has an average line separation of 75–90 G and a weakly anisotropic g factor of 1.96 ± 0.02. The signal peaks in intensity on the first flash and again on the fifth flash [51]. Tris washing which removes manganese also abolishes the flash-induced multiline signal.

Preliminary low-temperature EPR experiments with the *Phormidium* PS II particles confirmed the multiline EPR spectrum with characteristics very similar to those reported earlier [51] for spinach chloroplasts. Fig. 6 (left) shows the spectra in samples frozen after dark adaptation, and those for dark-adapted sample and frozen after one or two flashes. The multiline EPR signal intensity after one flash is about twice that observed after two flashes [51], which indicates that the light pulses are close to saturating and short enough to provide only a single PS II turnover per flash. A quantitation of the number of paramagnetic centers created

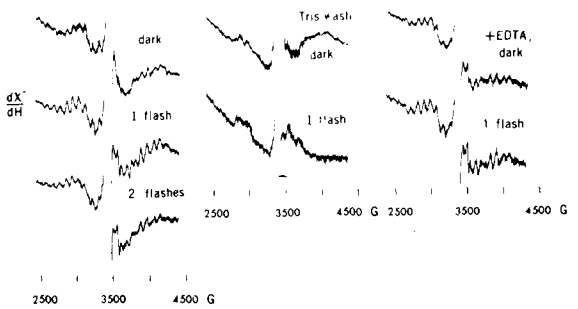

Fig. 6. EPR spectra of *Phormidium* PS II particles recorded at 158 mW microwave power at 9.6 K and 9.46 GHz microwave frequency: dark adapted and then flashed (left); Tris washed and then once flashed (center); and EDTA treated and then once flashed (right). All samples contained 0.5 mg Chl/ml in buffer C. Time constant, 0.5 s; modulation amplitude, 32 G. Illumination of the sample was with an intense, 14-μs, white-light flash at room temperature and then the sample was quickly frozen by immersing into liquid nitrogen.

by the first flash is difficult owing to the extreme instrumental conditions required to observe the signal and has not been carried out. Results similar to those shown in Fig. 6A were obtained on several occasions, which indicates that the phenomenon is reproducible. Fig. 6 (center) shows that in Tris-washed *Phormidium* particles, the multiline EPR spectrum was absent in either dark-adapted sample or sample after one flash. On the other hand, EDTA-washed *Phormidium* particles showed multiline EPR spectra with a pattern similar to that of the untreated particles. The dark-adapted, EDTA-treated sample after one flash also had a higher signal intensity than the dark-adapted unflashed sample (Fig. 6, right). Separate measurement of oxygen-evolution activity of the EDTA-treated sample showed that the activity was unaffected by EDTA treatment (cf. Ref. 10).

EPR signals II_{vf} and II_f

At room temperature, EPR spectroscopy can be used to detect the oxidized form of a donor to P-680$^+$ [53]. This species is usually designated as Z or D, most likely is a plastohydroquinone derivative and, when oxidized, gives rise to EPR Signal II_{vf} in oxygen-evolving preparation and to EPR Signal II_f upon Tris inhibition of oxygen evolution [54]. We have investigated the behavior of the *Phormidium* particles by room-temperature EPR; the results are shown in Fig. 7. Spectra recorded in the g 2.0 region in the dark (Fig. 7A, trace a), in the light (Fig. 7A, trace b) and in the dark after illumination (Fig. 7A, trace c) for oxygen-evolving particles indicate slight P-700$^+$ contamination and also a small contribution from Signal II_f. In general, however, these EPR characteristics indicate that the preparation is fairly pure and that the oxygen-evolving apparatus has largely maintained its integrity during isolation. By setting the magnetic field at the low-field peak of Signal II, it is possible to monitor the kinetics of Signal II_{vf} in response to flash excitation. The results of this experiment are shown in Fig. 7B. The rise of the signal is limited by the 100-μs time response of the instrument; the decay is strongly biphasic. The rapidly decaying in higher-plant preparations [53], while the slower decaying component probably arises from Signal II_f in oxygen-evolving centers which have been inactivated either during the preparation of the particles or during the course of

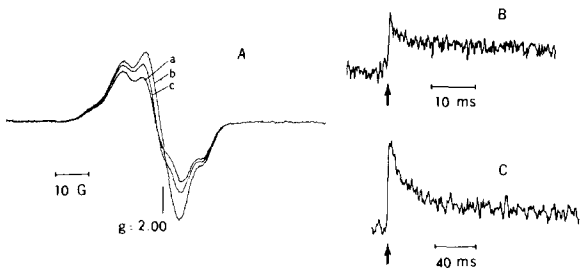

Fig. 7. (A) EPR spectra at room temperature of *Phormidium* PS II particles in the dark (a), in the light (b) and in the dark after illumination (c). Buffer C was the suspending medium to which no further additions were made. EPR conditions: modulation amplitude, 4 G; microwave power, 20 mW; instrument time constant, 100 ms. Illumination was provided by a Bausch and Lomb microscope illuminator. (B) The kinetics of Signal II in oxygen-evolving *Phormidium* particles. The magnetic field was set at the low-field peak of Signal II and a 14 μs saturating flash was fired as indicated by the arrow. Buffer C was the suspending medium to which 3 mM potassium ferricyanide was added as the acceptor. EPR conditions: 4 G modulation amplitude; 20 mW microwave power; 100 μs time constant; 200 flashes averaged. (C) The kinetics of Signal II in Tris-washed *Phormidium* particles. Tris washing was carried out by making particles 0.8 M in Tris-HCl buffer (pH 8.0) and incubating in room light for 20 min. Ferricyanide to a final concentration of 3 mM was added following the Tris treatment. EPR conditions as in B except that the instrument time constant was 1 ms and the data shown are the average of 100 scans. The chlorophyll concentration in all samples was approx. 800 μg/ml.

these signal-averaged experiments. We have also found this type of Signal II_{vf} behavior in oxygen-evolving particles prepared from higher-plant chloroplasts (Ref. 16; and Ghanotakis and Babcock, unpublished observations). In Fig. 7C, the effect of Tris washing on the kinetic behavior of Signal II is shown. The decay of Z^+ ($t_{1/2} = 23.5$ ms) is considerably slower than the 800-μs fast phase in Fig. 7B, indicating that Signal II_{vf} in the oxygen-evolving particles has been converted to Signal II_f by Tris treatment. An interesting aspect of the data in Fig. 7C is that the decay of Signal II_f in these particles is faster than Signal II_f decay in thylakoids under similar conditions.

Concluding Remarks

Results of our optical and EPR characterizations presented here show that the PS II particles isolated from the thermophilic, blue-green alga, *P. laminosum*, have a well defined composition relative to, and a high degree of enrichment in, the early electron carriers and the oxygen-evolving apparatus. The particle not only retains all the primary photochemistry of PS II but also a high oxygen-evolution activity as shown by the various physical characterizations and direct oxygen-evolution measurements. Undoubtedly this particle will serve as a very useful experimental system for further studies of PS II and, in particular, the mechanism of oxygen evolution. Recent reports [14–16] indicate a growing interest in isolating oxygen-evolving subchloroplast particles from other sources as well, which undoubtedly will add more impact to the inquiry into PS II chemistry.

Acknowledgements

The authors thank Professor R.W. Castenholz, University of Oregon, for providing the *P. laminosum* culture and culturing information. We also wish to acknowledge with thanks the assistance provided by Drs. C.T. Yerkes and C.F. Yocum in the room-temperature EPR measurements, and the SDS-polyacrylamide gel electrophoresis analysis on the *Phormidium* particles and other PS II preparations provided by Drs. C.J. Arntzen and P. Haworth. This work was supported in part by grant PCM-7900831 from the National Science Foundation (B.K.) and by the Service and Education Administration of the U.S. Department of Agriculture under grant No. 59-2261-1-1-631-0 from the Competitive Research Grants Office (G.T.B.).

References

1 Vernon, L.P. and Shaw, E.R. (1971) Methods Enzymol. 23, 277–288
2 Ke, B., Vernon, L.P. and Chaney, T. (1972) Biochim. Biophys. Acta 256, 345–357
3 Ke, B., Sahu, S., Shaw, E.R. and Beinert, H. (1974) Biochim. Biophys. Acta 347, 36–48
4 Wessels, J.S.C., Van Alphen-Van Wavern, O. and Voorn, G. (1973) Biochim. Biophys. Acta 292, 741–752
5 Ogawa, T., Vernon, L.P. and Mollenhauer, H.H. (1969) Biochim. Biophys. Acta 172, 216–229
6 Newman, P.J. and Sherman, L.A. (1978) Biochim. Biophys. Acta 503, 343–361
7 Nakayama, K., Yamaoka, T. and Katoh, S. (1979) Plant Cell Physiol. 20, 1565–1576
8 Stewart, A.C. and Bendall, D. (1969) Biochem. Soc. Trans. 6, 1040–1041
9 Stewart, A.C. and Bendall, D. (1979) FEBS Lett. 107, 308–312
10 Stewart, A.C. Bendall, D. (1981) Biochem. J. 194, 877–887
11 Stewart, A.C. (1980) FEBS Lett. 114, 67–72
12 Nugent, J.H.A., Stewart, A.C. and Evans, M.C.W. (1981) Biochim. Biophys. Acta 635, 488–497
13 Reinman, S., Mathis, P., Conjeaud, H. and Stewart, A.C. (1981) Biochim. Biophys. Acta 635, 429–433
14 England, R.R. and Evans, E.H. (1981) FEBS Lett. 134, 175–177
15 Yamamoto, Y., Doi, M., Tamura, N. and Nishimura, M. (1981) FEBS Lett. 133, 265–268
16 Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234
17 Castenholz, R.W. (1970) Schweiz, Z. Hydrol. 32, 537–551
18 Peterson, R.B., Dolan, E., Calvert, H.E. and Ke, B. (1981) Biochim. Biophys. Acta 634, 237–248
19 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
20 Wingo, W.J. and Emerson, G.M. (1975) Anal. Chem. 47, 351–352
21 Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171
22 Klimov, V.V., Dolan, E. and Ke, B. (1980) FEBS Lett. 112, 97–100
23 Ke, B. (1972) Methods Enzymol. 24, 25–53
24 Satoh, K. (1979) Biochim. Biophys. Acta 546, 84–92
25 Mullet, J.E. and Arntzen, C.J. (1981) Biochim. Biophys. Acta 635, 236–248
26 Bishop, N.I., Metz, J.G., and Wang, J. (1981) in Proceedings of the 5th International Congress on Photosynthesis (Akoyunoglou, G., ed.), Vol. 3, pp. 211–225, Balaban International Science Services, Philadelphia

27 Åkerlund, H.-E. and Jansson, C. (1981) FEBS Lett. 124, 229–232
28 Novak-Hofer, I. and Siengenthaler, P.-A. (1977) Biochim. Biophys. Acta 468, 461–471
29 Kuwabara, T. and Murata, N. (1979) Biochim. Biophys. Acta 581, 228–236
30 Leto, K.J. and Miles, D. (1980) Plant Physiol. 66, 18–24
31 Van Best, J.A. and Mathis, P. (1978) Biochim. Biophys. Acta 503, 178–188
32 Sonnelveld, A. Rademaker, H., and Duysens, L.N.M. (1979) Biochim. Biophys. Acta 548, 536–551
33 Ke, B. and Dolan, E. (1980) Biochim. Biophys. Acta 590, 401–406
34 Van Gorkum, H.J., Taminga, J.J., Haveman, J. and Van der Linden, I.K. (1974) Biochim. Biophys. Acta 347, 417–438
35 Bearden, A.J. and Malkin, R. (1973) Biochim. Biophys. Acta 325, 266–274
36 Malkin, R. and Bearden, A.J. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 294–297
37 Van Gorkom, H.J. (1974) Biochim. Biophys. Acta 347, 439–442
38 Stiehl, H.H. and Witt, H.T. (1969) Z. Naturforsch. 24B, 1588–1598
39 Duysens, L.N.M. and Sweers, H.E. (1963) Studies on Microalgae and Photosynthetic Bacteria (Miyachi, S., ed.), pp. 357–372, University of Tokyo Press, Tokyo
40 Klimov, V.V., Klevanik, V.A., Shuvalov, V.A. and Krasnovsky, A.A. (1977) FEBS Lett. 82, 183–186
41 Klimov, V.V. Allakherdiev, S.I. and Paschenko, V.A. (1978) Dokl. Akad. Nauk. S.S.S.R. 242, 1204–1207
42 Haehnel, W., Nair, J.A., Reisberg, P. and Sauer, K. (1982) Biochim. Biophys. Acta 680, 161–173
43 Shuvalov, V.A., Klimov, V.V., Dolan, E., Parson, W.W. and Ke, B. (1980) FEBS Lett. 118, 279–282
44 Fujita, I., Davis, M.S. and Fajer, J. (1978) J. Am. Chem. Soc. 100, 6280–6282
45 Klimov. V.V., Dolan, E., Shaw, E.R. and Ke, B. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7227–7231
46 Radmer, R. and Cheniae, G. (1977) Topics in Photosynthesis (Barber, J., ed.), Vol. 1, pp. 301–348. Elsevier, Amsterdam
47 Lozier, R., Baginski, M. and Butler, W.L. (1971) Photochem. Photobiol. 14, 323–328
48 Blankenship, R.E. and Sauer, K. (1974) Biochim. Biophys. Acta 357, 252–266
49 Siderer, Y., Malkin, S., Poupko, R. and Luz, Z. (1977) Arch. Biochem. Biophys. 179, 174–182
50 Gribova, Z.P. and Tikhonov, A.N. (1977) Biofizika 22, 651–655
51 Dismukes, G.C. and Siderer, Y. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 274–278
52 Hansson, Ö. and Andreasson, L.-E. (1982) Biochim. Biophys. Acta 679, 261–268
53 Blankenship, R.E., Babcock, G.T., Warden, J.T. and Sauer, K. (1975) Biochim. Biophys. Acta 51, 287–293
54 Yocum, C.F., Yerkes, C.T., Blankenship, R.E., Sharp, R.R. and Babcock, G.T. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 7507–7511