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FURTHER CHARACTERIZATION OF A PHOTOSYSTEM-II PARTICLE ISOLATED FROM SPINACH CHLOROPLASTS BY TRITON TREATMENT: THE REACTION-CENTER COMPONENTS*

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SUMMARY

Re-examination of the chlorophyll *a*/*b* ratio in the Photosystem-II reaction-center particle isolated from spinach chloroplasts by Triton treatment yielded a value of 28 ± 2. The Photosystem-II particle (as well as the Photosystem-I particle, TSF-1) is enriched in manganese (chlorophyll/Mn, 10 and 10–20, respectively). Heat treatment does not release the manganese from the Photosystem-II reaction-center particle as readily as from native chloroplasts.

The fluorescence-induction phenomena of the Photosystem-II particles have been correlated with their photochemical properties in the presence of secondary electron acceptors and (or) donors which further confirm the Photosystem-II character of the particle.

C550 and P680, which are currently considered to be the primary electron acceptor and donor, respectively, of Photosystem II, have been detected in the Photosystem-II reaction-center particle by light-induced difference spectra at 77 °K. The presence of P680 has further been corroborated by a free-radical signal revealed by low-temperature EPR spectroscopy. The Photosystem-II particle contains no detectable P700 or bound iron–sulfur proteins. The photobleaching of both C550 and P680 is practically irreversible at 77 °K. The Photosystem-II reaction-center particle is enriched in P680, C550 as well as cytochrome b_{559} approximately 10-fold relative to the unfractionated chloroplasts.

INTRODUCTION

The concept of two photosystems in green-plant photosynthesis has been amply documented [1, 2] and is now generally accepted. Some of the most convincing supporting evidence comes from the fractionation of chloroplasts into two

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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types of particles whose properties are consistent with those expected of the two photosystems [1–3]. Although highly purified (or enriched) Photosystem-I subchloroplast fragments have been obtained by a wide variety of fractionation techniques, comparable Photosystem-II subchloroplasts have only been prepared recently.

A small particle approximately 10 nm in diameter (designated as TSF-2a in ref. 4) with a high chlorophyll *a*/*b* ratio and Photosystem-II activity was prepared in this laboratory in 1970 and reported first by Vernon et al. [4]. It was subsequently found that this particle is highly enriched in cytochrome b_{559}, and that the cytochrome can undergo a light-induced photoreduction at room temperature [5]. Furthermore, the particle can recombine with the Photosystem-I particle (TSF-I), with good reconstitution activity [6]. This particle has a relatively simple fluorescence emission spectrum, with a single peak at 680 nm at room temperature and 685 nm at 77 °K [5].

This paper reports a re-evaluation of the chlorophyll *a*/*b* ratio by a different assay method as well as a determination of the manganese content in the Photosystem-II particles. Certain properties relative to the primary electron acceptor and primary electron donor of Photosystem II have been examined. The spectral species, C550, which has been postulated as the primary electron acceptor of Photosystem II [7], has been detected by light-induced absorption changes. Fluorescence phenomena exhibited by the reaction-center particles are typical of those expected of Photosystem II. Light-minus-dark difference spectra measured with the Photosystem-II particles at 77 °K are representative of the spectral species, P680, which has been postulated as the reaction-center chlorophyll of Photosystem II [8–10]. The presence of P680 is further corroborated by a free-radical signal revealed by low-temperature EPR spectroscopy.

EXPERIMENTAL

Detailed procedures for preparing the small Photosystem-II particles can be found in refs 5 and 11. 2,6-Dichlorophenolindophenol (DCIP) reduction activity was assayed by measuring the absorption decrease at 590 nm with 1,5-diphenylcarbazide as the donor (actual activity of particles used in the present work was typically about 1000 μmoles/mg chlorophyll per h).

Light-induced absorption changes due to C550 reduction were measured with a Phoenix dual-wavelength spectrophotometer or a scanning instrument constructed in this laboratory. For the measurement of P680 absorption changes, it is essential that the interference by fluorescence is accounted for, or, more preferably, eliminated from the measured signal. For this purpose, we have modified (details will be reported elsewhere) the dual-wavelength spectrophotometer built in this laboratory by providing an isolated dark period between the sample- and reference-beam periods, so that a pure fluorescence signal can be extracted during this dark period and subtracted from the final light-induced transmission-change signal. With this device, a fluorescence interference with an intensity equivalent to twice the transmission could be tolerated. The fluorescence intensity was attenuated further by moving the photomultiplier tube away from the sample cuvette. Under these experimental conditions, typical fluorescence intensity encountered with the Photosystem-II reaction-center particles was about 50-60% of the transmission level.

Fluorescence was detected with an EMI Model 9558 BQ photomultiplier used in conjunction with a PAR Model 220 lock-in amplifier. The fluorescence-rise curves were obtained with an actinic beam isolated by a 650-nm interference filter at an intensity of 1850 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} and modulated by a shutter.

Conditions for EPR spectroscopy are described in the legend of Figs 5 and 6.

RESULTS AND DISCUSSION

Re-examination of the chlorophyll a/b ratio

The chlorophyll *a/b* ratio of the small Photosystem-II particles was reported to be about 8 (refs 4, 5), as determined by the formula of Arnon [12]. As this formula is not sufficiently sensitive for an accurate determination for a low concentration of chlorophyll *b* in the presence of a high concentration of chlorophyll *a*, we subsequently re-examined the chlorophyll *a/b* ratio by the method developed by Ogawa and Shibata [13]. This method is based on a reaction of hydroxylamine with the aldehyde group of chlorophyll *b* to produce a derivative whose red absorption band is at the same position as chlorophyll *a*. The concentrations of both chlorophylls *a* and *b* are then determined from the absorbance values measured at a single wavelength (666 nm) before and after the hydroxylamine reaction. By this procedure, the chlorophyll *a/b* ratios for unfractionated chloroplasts, the digitonin-fractionated System-I (D-144) particles, the Triton-fractionated Photosystem-I (TSF-I) and Photosystem-II reaction center (or TSF-2a) particles are: 3.0, 5.6, 14 ± 1 and 28 ± 2, respectively. Note that while the values for unfractionated chloroplasts and the digitonin-fractionated Photosystem-I particles are in good agreement with literature values, the ratios obtained by this method for the two Triton-fractionated particles are substantially higher. It is of interest to note that Wessels et al. [14] recently reported the fractionation of a similar Photosystem-II reaction-center particle from spinach chloroplasts by digitonin treatment, for which they reported a chlorophyll *a/b* ratio of 25.

Manganese content; effect of heat treatment on manganese removal and photochemical activity

Since manganese is generally considered to play a role in O_2 evolution or water oxidation in Photosystem II of green plants, we have examined the manganese content in these Photosystem-II reaction-center particles. Contrary to the previously reported partition of manganese among the two photosystems after digitonin fractionation, the Triton-fractionated Photosystem-I and the small Photosystem-II reaction-center particles both showed several fold enrichment in manganese. The small Photosystem-II particles had a chlorophyll/manganese ratio of 10. These findings indicate that, presumably as a result of the fractionation procedure and/or the action on Triton, manganese had become concentrated in the reaction-center particles of both photosystems. In view of these unexpected results, it would be hazardous to attach any significance to the enrichment of manganese in the Photosystem-II particles.

Experiments on the effect of heat treatment for the removal of manganese from native chloroplasts and Photosystem-II particles are shown in Fig. 1. In contrast to the situation in native chloroplasts, which lose manganese by simple heat treatment, the manganese in Photosystem-II particles is much less readily released.

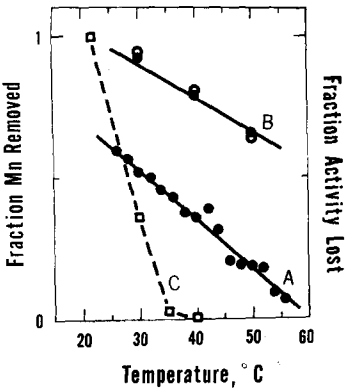

Fig. 1. Effect of heat treatment on the fraction of manganese removed from spinach chloroplasts (Curve A) and the Photosystem-II particles (Curve B) and on the DCIP-reduction activity of the Photosystem-II particles (Curve C). Samples contained in test tubes were immersed for 5 min in a water bath thermostated at the temperatures given. Mn was analyzed by atomic absorption spectroscopy.

This again suggests that the manganese protein in the reaction-center particles is no longer present in the native state. The appearance of an EPR signal of Mn^{2+} in reduced Photosystem-II particles (see Fig. 5) is also consistent with this notion.

Fig. 1 also shows the extreme sensitivity of the DCIP-reduction activity to heat treatment; about 65% of the photochemical activity was lost when the sample was immersed in a 30 °C bath for 5 min; heating at 35 °C for 5 min completely abolished the activity.

Photoreduction of C550 at 77 °K

Knaff and Arnon [7] observed in chloroplasts at 77 °K a light-induced absorption decrease near 550 nm, which they ascribed to a spectral species designated as C550 which was thought to serve as the primary electron acceptor of Photosystem II. This observation was subsequently confirmed at a number of other laboratories (refs 16–18). In chloroplasts, the net light reaction at 77 °K appears to be the reduction of C550 and oxidation of a high-potential cytochrome b_{559}. The latter was presumably oxidized by the photooxidized primary electron donor. Erixon and Butler [19] further measured the redox potential of C550 and found its behavior to be consistent with that of the fluorescence quencher. In view of the inherent Photosystem-II characteristics of C550, we thought it appropriate to look for this spectral component in the Photosystem-II particles.

As reported previously, the net steady-state light-induced reaction in the Photosystem-II particles at room temperature is a photoreduction of cytochrome b_{559} which has a redox potential of about +60 mV. If an artificial secondary electron acceptor such as DCIP is present, its reduction instead of that of cytochrome b_{559} is observed. We found that if ferricyanide is present, the absorption increase near 550 nm associated with the reduction of cytochrome b_{559} is replaced by an absorption decrease that is characteristic of C550 reduction. To further confirm that the component with this difference spectrum was C550, absorption changes were measured at 77 °K. A typical absorption-change transient at 548 nm is shown in

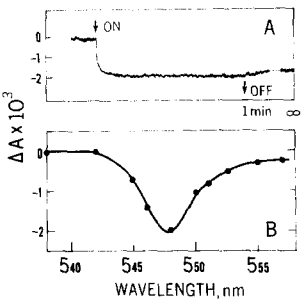

Fig. 2. (A) Light-induced absorption decrease at 548 nm due to C550 reduction at 77 °K. Chlorophyll concentration 40 μg/ml; cuvette pathlength, 1 mm. The Photosystem-II particles were suspended in 0.01 M phosphate buffer (pH 6.4) containing 50 % glycerol, 0.5 mM diphenylcarbazide and 0.1 mM ferricyanide. Excitation wavelength 650–750 nm; intensity 10^5 ergs · cm^{-2} · s^{-1}. Reference wavelength 538 nm. (B) Light-minus-dark difference spectrum for C550 constructed from data points obtained with a separate sample for each wavelength point. All conditions were the same as in A.

Fig. 2A. The onset of the reaction appeared very rapid and was limited only by the response time of the instrument (15 ms). A difference spectrum in the 540 to 555 nm range (Fig. 2B) showed a typical profile for C550 as first reported by Knaff and Arnon [7]. Note that approximately 10% of the absorption change decayed very slowly in the dark. The reason for this slow decrease is not known. The C550 concentration in these small Photosystem-II particles may be estimated relative to that in unfractionated chloroplasts. The maximum absorption decrease at 548 nm observed by us was $2 \cdot 10^{-3}$ absorption unit, almost identical to that reported in ref. 7, except that the number of chlorophyll molecules in the light path was only 10% that used by Knaff and Arnon [7]. On the assumption that the intensity of the excitation light was saturating in both cases, the results would suggest that C550 in the Photosystem-II reaction-center particles is enriched approximately 10-fold. Since the extinction

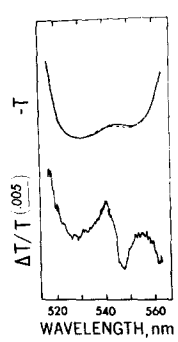

Fig. 3. Absorption spectra (recorded as 'inverted' transmission) of Photosystem-II particles before (–) and during (- - -) illumination at 77 °K (top), measured with a continuous scanning dual-wavelength spectrophotometer. Sample preparation was the same as for Fig. 2. Chlorophyll concentration 100 μg/ml. Cuvette pathlength 1 mm. Reference wavelength 538 nm. The excitation light was isolated with a 650–750-nm broad-band interference filter at an intensity of $5 \cdot 10^4$ ergs · cm^{-2} · s^{-1}. The photomultiplier was protected with a Corning 4-96 filter. The light-minus-dark difference spectrum is shown at the bottom.

coefficient of C550 is not known, the absolute concentration of C550 in either chloroplasts or the particles cannot yet be estimated.

Fig. 3 shows spectra of the Photosystem-II particles measured with the continuous scanning dual-wavelength spectrophotometer. At the top are the absorption spectra taken at 77 °K before and during illumination. The light-minus-dark difference spectrum (bottom) shows the typical spectral shift (decrease at 548 nm and increase at 542 nm) observed by others [16–18] in spectra obtained by continuous scanning. It should be noted that a light-induced difference spectrum showing active C550 reduction was observed only when ferricyanide was present in the sample prior to freezing, which suggests that C550 may be largely present in the reduced state.

Fluorescence-induction phenomena

The components lying on the reducing side of Photosystem II, namely, the primary electron acceptor or the fluorescence quencher, and the pool of secondary acceptors have been extensively studied by means of fluorescence induction, i.e., the change of light-induced fluorescence yield as a function of time. According to the interpretation of Duysens and Sweers [20], fluorescence induction reflects the photoreduction of the primary electron acceptor (Q), which quenches fluorescence in its oxidized state by utilizing the absorbed photon for its own reduction to Q^-. Initially Q^- is reoxidized by the pool of secondary oxidant (A). When all A is reduced and can no longer reoxidize Q^-, the fluorescence rises to the maximum level. Thus the area above the fluorescence-rise curve represents the number of equivalents put into the total oxidant pool on the reducing side of Photosystem II [21].

We have correlated the fluorescence induction in the Photosystem-II particles with their photochemical activities as a further confirmation of their Photosystem-II character. A typical light-induced fluorescence-rise curve for the particle in the presence of the donor 1,5-diphenylcarbazide is shown in Fig. 4, Curve 1. The rapid

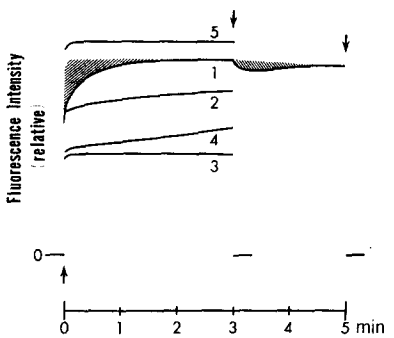

Fig. 4. Fluorescence-rise curves observed with the Photosystem-II particles. Chlorophyll concentration 2.4 μg/ml; suspension medium, 0.01 M phosphate buffer (pH 6.4). Excitation light 650 nm (isolated by interference filters); intensity 1850 ergs · cm^{-2} · s^{-1}; upward arrow, light on; downward arrow, light off (only the second downward arrow applies to Curve 1). Fluorescence detection: 680 nm fluorescence isolated with interference filters; photomultiplier, EMI 9558BQ. Curve 1, sample contained only 0.3 mM diphenylcarbazide. Curve 2, sample contained 0.3 mM diphenylcarbazide and 6 μM DCMU. Curve 3, sample contained 90 μM ferricyanide only. Curve 4, sample contained 90 μM ferricyanide and 0.3 mM diphenylcarbazide. Curve 5, 0.25 mM dithionite was added to the sample beforehand. See text for other details.

initial fluorescence rise is followed by a slower rise to the maximum level in about 3 min. If an oxidant, say, ferricyanide, is added, its reduction creates a new steady state in which Q is more oxidized, and the fluorescence decreases at first and then rises again to the maximum. In fact, if the amount of ferricyanide is known, the area represented by the temporary change in the fluorescence curve could be used as a standard to estimate the size of the total internal oxidant pool in the Photosystem-II particle. Four separate experiments defining the area shown by the second shaded region on Curve 1 in Fig. 4 yielded 1 equiv. per approx. 4 chlorophyll.

When DCMU is present, the initial rapid portion rises higher, but the induction portion rises more slowly (Curve 2). The anomaly is consistent with the fact that the Photosystem-II reaction-center particles are less sensitive to the specific inhibitor, DCMU, thus still allowing electrons to leak to the secondary acceptors such as cytochrome b_{559} [5]. The anomaly is also consistent with the suggestion that a possible cyclic reaction, i.e., reoxidation of the photoreduced primary acceptor by the oxidized primary donor may be facilitated when the linear electron transport pathway is partially blocked by DCMU.

Fluorescence induction, reflecting the redox state of the quencher, is shown in the presence of the acceptor ferricyanide with and without the donor diphenylcarbazide present. At 90 μM ferricyanide (Curve 3), Q is maintained largely in the oxidized state, and thus the fluorescence remains at a low level after the initial rapid rise; at 90 μM ferricyanide plus 0.3 mM diphenylcarbazide (Curve 4), the fluorescence rises slightly faster, indicating a faster accumulation of reduced quencher. A similar phenomenon can be observed with DCIP as the oxidant. When 0.25 mM dithionite is added, which presumably reduces all the internal oxidant pool, the fluorescence rises to the maximum level immediately upon illumination (Curve 5).

Absence of bound iron–sulfur proteins in the Photosystem-II reaction-center particles; low-temperature EPR detection of a free-radical signal produced by chemical oxidation and by illumination

Since separate analysis based on oxidized-minus-reduced difference spectra showed the P700 content in the Photosystem-II particles to be within the noise level (also cf. Figs 7 and 8), we were interested in knowing whether this could be confirmed by a corresponding absence of the EPR spectrum associated with the P700 free radical. We were also interested in determining whether this particle shows the EPR signal of bound iron–sulfur proteins.

As shown in Fig. 5, the Photosystem-II particles reduced by dithionite under anaerobic conditions and further illuminated in the presence of the reductant showed only a manganese signal but no EPR signal (13 °K) attributable to bound iron–sulfur proteins [22]. For comparison, the EPR spectrum of a Photosystem-I particle similarly reduced is also shown in Fig. 5. The only other green-plant chloroplast fragments known to be devoid of such signals were those derived from the No. 8 mutant of *Scenedesmus* (unpublished experiments), which also contains no P700 as determined by difference spectroscopy.

The Photosystem-II particle was examined at different oxidation states by EPR spectroscopy at 34 °K, a temperature more suitable for the observation of readily saturated signals. In Fig. 6, Spectrum A obtained from the Photosystem-II particles in the presence of 1 mM diphenylcarbazide is shown. The sample was kept in the

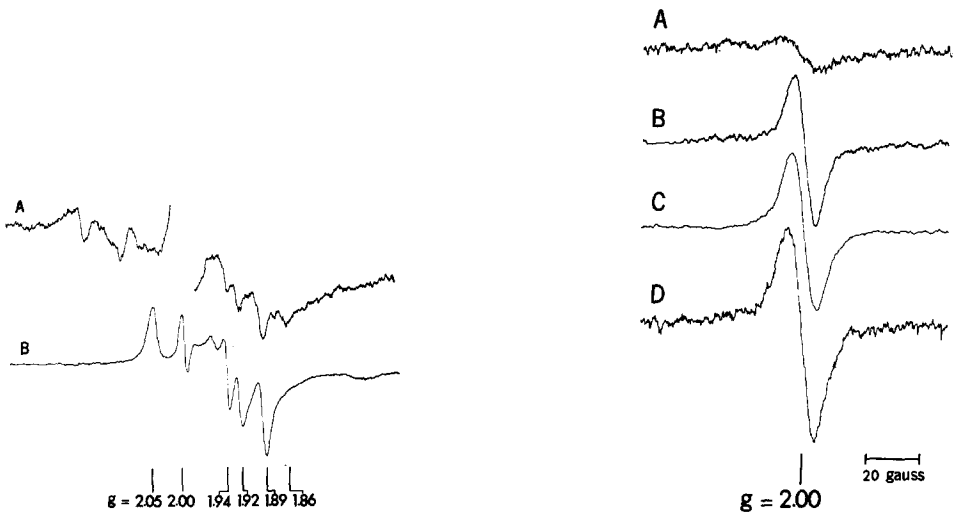

Fig. 5. EPR spectra of the Photosystem-II reaction-center particle (A) and a Photosystem-I particle (B). (A) Chlorophyll concentration $4.4 \cdot 10^{-4}$ M; in 0.05 M, phosphate buffer (pH 6.4); (B) Chlorophyll concentration $6.05 \cdot 10^{-4}$ M; P700 concentration $1.44 \cdot 10^{-5}$ M; in 0.1 M Tris buffer (pH 8) (cf. ref. 22). The EPR sample was prepared as follows: the sample was made anaerobic in a side arm attached to the EPR tube before 40 μl of 0.1 M dithionite solution were injected (see ref. 22 for details). The tube containing the reduced sample was illuminated for 1 min under a microscope lamp and then immersed into liquid N_2 while the tube was still being illuminated.

EPR spectroscopy was performed in a modified Varian spectrometer with the following conditions: microwave frequency 9.18 GHz, power 3 mW, modulation frequency 100 kHz, modulation amplitude 10 G for Spectrum A and 6.3 G for Spectrum B, temperature 13 °K, scanning rate 400 G/min for Spectrum A and 200 G/min for Spectrum B, time constant 0.5 s. The amplification of Spectrum A is 4 times that of Spectrum B.

Fig. 6. EPR spectra of the Photosystem-II particles [chlorophyll concentration $4.4 \cdot 10^{-4}$ M, suspended in 10 mM phosphate buffer (pH 6.4)]. (A) the sample contained 1 mM diphenylcarbazide, was dark adapted, and then frozen in the dark. (B) the sample contained 20 mM ferricyanide, was dark adapted, and then frozen in the dark. (C) the sample was the same as in B except further illuminated 5 times in a cylindrical flash illuminator at 77 °K. (D) a digitonin-fractionated subchloroplast particle (D-144) shown for purposes of comparison (Chlorophyll concentration $8.5 \cdot 10^{-4}$ M, P700/concentration = $5.62 \cdot 10^{-3}$); the sample contained 20 mM ferricyanide and was frozen in room light. Conditions for EPR spectroscopy: microwave frequency 9.18 GHz, power 30 μW, modulation amplitude 3.2 G, scanning rate 100 G/min, temperature 34 °K, time constant 0.5 s. For Spectrum C the amplification was one fourth that used for A, B, and D.

dark for several min and then frozen in the dark in liquid N_2. The observed EPR signal is relatively weak, is centered near $g = 2$ and has a peak-to-peak width of 12–15 G. This signal does not appear to correspond to the so-called Signal II. When 20 mM ferricyanide was added and the sample was kept in the dark and then frozen in the dark, a stronger EPR signal centered at g 2.00 with a line-width of 9 G was observed (Spectrum B). When the sample containing 20 mM ferricyanide was first frozen in the dark, but then illuminated while frozen in the EPR tube at 77 °K with five intense white flashes, the EPR spectrum shown by Curve C in Fig. 6 was observed. Curve D shows the EPR spectrum obtained with Photosystem-I subchloroplast (D144) particles in the presence of 20 mM ferricyanide when frozen in room light.

Since the P700 concentration of the sample used for Spectrum D was known from a separate determination and the widths and shapes of the signals shown in Curves B–D were similar, the signal height of Sample D could be used as a standard for the estimation of the spin concentration in the Photosystem-II particles produced either by the presence of ferricyanide alone in the dark (Curve B) or by a combination of ferricyanide and illumination at liquid-N_2 temperature (Curve C). EPR Spectrum B in Fig. 6 has characteristics of photooxidized $P700^+$ and one might have been tempted to attribute it to this species if it were not for the fact that the amount of $P700^+$ represented by Signal B cannot be accounted for by optical analysis for P700. On the basis of a comparison of Spectrum B with Spectrum D, the spin concentration in the sample represented by Spectrum B is 3.5 μM, corresponding to 8 spins per 1000 chlorophyll molecules.

Other alternatives would be to attribute the EPR signal to chemically-oxidized bulk chlorophyll or to a free radical originating from the Photosystem-II reaction-center chlorophyll. As shown by spectroscopic examinations to be described in the following section, the former alternative was demonstrated to be the case for Spectrum B. However, an EPR signal attributable to the reaction-center chlorophyll of Photosystem II was reported by Malkin and Bearden [23] at the time our studies were made. These workers also found that the EPR signal they observed had characteristics almost identical to those of $P700^+$. Malkin and Bearden [23] used unfractionated chloroplasts as well as digitonin-fractionated subchloroplast fragments as experimental materials. The free-radical signal produced in the chloroplasts by ferricyanide alone in the dark was ascribed to $P700^+$ and they utilized further illumination with far-red and red light to differentiate the free radicals originating from the two photosystems. They estimated the concentration of the Photosystems-II reaction-center chlorophyll in unfractionated chloroplasts to be one per 330 total chlorophyll molecules.

When the Photosystem-II particles containing ferricyanide were illuminated after the sample was frozen at 77 °K, the height of the free-radical signal increased 3-fold (Fig. 6C). Thus, the total signal and the incremental signal correspond to spin concentrations of 32 and 24 spins per 1000 total chlorophyll molecules, respectively.

Oxidation of a Photosystem-II reaction-center chlorophyll at 77 °K as detected by difference spectroscopy

Subsequently we tried to identify the free-radical signals detected by EPR spectroscopy with either oxidized bulk chlorophyll or oxidized reaction-center chlorophyll of Photosystem II. For this identification, we used low-temperature difference spectroscopy, with Photosystem-II particles of the same batch, and under comparable conditions, as those used in the EPR measurements.

Fig. 7 (left) shows the absorption spectra of the Photosystem-II particles in the presence of diphenylcarbazide or ferricyanide taken at 77 °K, and the particles containing ferricyanide subsequently illuminated at 77 °K. The difference spectra are shown in Fig. 7 (right). The first difference spectrum indicates that the presence of 0.5 mM ferricyanide brought about a chemical bleaching (in the dark) of a chlorophyll with an absorption maximum at 683 nm at 77 °K. Illumination of the particles at 77 °K in the presence of ferricyanide resulted in an additional photobleaching. The absorption maximum of the photobleached chlorophyll lies at a longer wave-

Fig. 7. Absorption spectra (left) of the Photosystem-II particles at 77 °K before (—) and during or after (- - -) illumination. The difference spectra (right) are: 1, (sample+ferricyanide, dark) minus (sample+diphenylcarbazide, dark); 2, (sample+ferricyanide, light) minus (sample+diphenylcarbazide, dark); 3, (sample+ferricyanide, light) minus (sample+ferricyanide, dark). Sample medium was the same as that used in Fig. 2. Blue excitation light was isolated with a broad-band (400–460 nm) interference filter plus a Corning 4-96 filter at an intensity of $5 \cdot 10^4$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}. Reference wavelength 725 nm. Chlorophyll concentration 72 mg/ml. Cuvette pathlength 1 mm.

Fig. 8. Difference spectra of the Photosystem-II particles at 77 °K. 1, (sample+ferricyanide+diphenylcarbazide, dark) minus (sample+diphenylcarbazide, dark); 2, (sample+ferricyanide+diphenylcarbazide, light) minus (sample+diphenylcarbazide, dark); 3, (sample+ferricyanide+diphenylcarbazide, light) minus (sample+ferricyanide+diphenylcarbazide, dark). All other conditions were the same as those used in Fig. 7.

length than that of the chemically-bleached chlorophyll. This is more clearly seen from the third difference spectrum taken for the sample containing ferricyanide during and before illumination. The absorption maximum of the photobleached chlorophyll is at 689 nm.

Chemical bleaching of chlorophyll in the Photosystem-II particles in the dark by ferricyanide can apparently be prevented when diphenylcarbazide is also present, as shown by the first difference spectrum in Fig. 8, taken between the particle containing ferricyanide plus diphenylcarbazide and that containing diphenylcarbazide only. The difference spectrum representing the photobleached chlorophyll can be obtained by measuring either between the illuminated sample containing ferricyanide plus diphenylcarbazide and that containing diphenylcarbazide alone in the dark (second difference spectrum in Fig. 8) or between the Photosystem-II particles containing ferricyanide plus diphenylcarbazide during and before illumination (third difference spectrum in Fig. 8). The photobleaching of chlorophyll absorbing at 689 nm was irreversible at 77 °K, as the difference spectrum taken after and before illumination is practically identical with that taken during and before illumination.

From the different locations of the absorption maxima between the chemically-bleached and photobleached chlorophylls, we interpret these results by assigning the shorter-wavelength-absorbing band to the oxidized bulk chlorophyll and the longer-wavelength-absorbing band to the reaction-center chlorophyll of Photosystem II. Consistent with this assumption, the free-radical EPR signal seen in the Photosystem-II particles containing ferricyanide only but not illuminated would most likely be due to oxidized bulk chlorophyll and the incremental free-radical signal

brought about by illumination at 77 °K would then correspond to photooxidized reaction-center chlorophyll.

Assuming the extinction of the reaction-center chlorophyll is the same as that of the bulk chlorophyll, we may estimate the approximate concentration of the reaction-center chlorophyll in the Photosystem-II particle from the absorption and difference spectra. Estimations made from several separate sets of measurements as those shown in Fig. 8 yielded about 20 reaction-center chlorophylls per 1000 total chlorophyll molecules, which is in good agreement with the spin concentration estimated from the incremental EPR signal height in Fig. 6.

CONCLUDING REMARKS

Initial work on the fractionation of spinach chloroplasts by Triton treatment yielded two types of particles: a small Photosystem-I particle (TSF-1) with a chlorophyll *a*/*b* ratio of about 6, enriched in P700, and a high activity of $NADP^+$ reduction, all representative of properties of Photosystem I; the other, larger particle (TSF-2) has a chlorophyll *a*/*b* ratio near 2, negligible P700, and a low $NADP^+$-reduction activity. The Photosystem-II characteristics of this particle were further confirmed when a suitable electron donor was found and the DCIP-reduction activity could be demonstrated [24]. Subsequently, a small particle was derived from the larger TSF-2 particle by further fractionation; this particle has a chlorophyll *a*/*b* ratio of about 28, as determined in the present work, a very high activity in catalyzing DCIP reduction by diphenylcarbazide, is devoid of P700, and enriched in cytochrome b_{559}. All these characteristics are consistent with the previous suggestion that the particle may represent the Photosystem-II reaction-center complex [4].

In this connection, it is worth recalling the recent report by Wessels et al. [14] on the fractionation and characterization of a similar Photosystem-II reaction-center particle by digitonin fractionation of spinach chloroplasts. These particles resemble the Triton-fractionated small Photosystem-II (TSF-2A) particles in chlorophyll *a*/*b* ratio, cytochrome b_{559} content, and the absorption and fluorescence spectra. However, the two particles differ in the photochemical activity of cytochrome b_{559}, even though this cytochrome is present largely in the oxidized form in both types of particles. In the digitonin-fractionated F_{II} particles of Wessels et al. [14], the portion of cytochrome b_{559} which is reducible by ascorbate can undergo photooxidation at 77 °K, whereas, in TSF-2a, cytochrome b_{559} undergoes photoreduction at room temperature only but not at low temperatures. The cause for the different behavior of the cytochrome in the two particles is not clear.

Experimental results reported here on characteristic Photosystem-II components further support the suggestion that these small particles represent the reaction-center complex of Photosystem II. The fluorescence-induction phenomena shown in Fig. 4 are consistent with the interpretation of Duysens and Sweers [20] concerning the effect of exogenous electron donors and acceptors on the redox state of the presumed primary electron acceptor, and thus on its ability to quench fluorescence in Photosystem II. The anomaly in the fluorescence-rise curve in the presence of DCMU and the unusually large number of equivalents estimated by ferricyanide titration are both consistent with the notion of a small cyclic reaction taking place in this particle.

In spite of these fluorescence data, our studies do not yield information which

bears directly on the involvement of C550 as the primary electron acceptor. A direct correlation of the absorbance and fluorescence changes under comparable experimental conditions (cf. ref. 25) would be required to answer this question.

Results from optical and EPR spectroscopic investigations present a picture which is consistent with that of a reaction-center chlorophyll of Photosystem II. Such a reaction-center chlorophyll was proposed earlier by Döring et al. [8, 9], based on reaction kinetics [8] and on spectral characterization in the red and blue regions [9]; the spectral species was designated as chlorophyll a_{II}. Döring et al. [9] attributed the absorption changes of chlorophyll a_{II} to a sensitizer reaction, and that chlorophyll a_{II} is not engaged directly in the electron transfer [9]. The question of a possible effect of fluorescence-yield changes on these absorption changes was raised by Butler [26], but was responded by Döring and Witt [27], who demonstrated that fluorescence artifact was not involved in these measurements.

In a study of photooxidation of cytochrome b_{559} in chloroplasts at 77 °K, Floyd et al. [10] observed an absorption decrease at 680 nm, and noticed that its decay corresponded kinetically with the onset of cytochrome b_{559} oxidation. Floyd et al. [10] interpreted these absorption changes at 680 nm as due to a Photosystem-II reaction-center chlorophyll designated as P680, and implicated it as the primary photooxidant responsible for cytochrome b_{559} oxidation.

The combined optical and EPR experiments reported in this work appear to be consistent with the expected behavior of the Photosystem-II reaction-center chlorophyll, P680. However, because of controversies often encountered in connection with P680, several points regarding the spectral characteristics of and the measuring technique for P680 should be reiterated briefly. First, in order to rule out any possible fluorescence artifact in the absorption-change measurements in the red region, we have modified a dual-wavelength spectrophotometer so that the fluorescence was accounted for and eliminated from the final absorption-change signal. The major supportive evidence, of course, lies in the fact that an expected photobleaching of the spectral species occurs at 77 °K, which would be required for a primary reactant. Furthermore, the irreversibility of the absorption change due to both P680 and C550 is also internally consistent since they are expected to behave in parallel in their presumed roles. The risetime for P680 photobleaching (not shown) is similar to that shown for C550 in Fig. 2 (top). However, the functional role of the spectral species, P680, in Photosystem II remains to be clarified.

The presumed Photosystem-II reaction-center chlorophyll, P680, revealed by our spectroscopic measurements has an absorption maximum at 689 nm and a half bandwidth of about 7 nm. This maximum is thus at a much longer wavelength and the bandwidth is narrowed from the values reported for chlorophyll a_{II} at room temperature by Döring et al. [9] (682 nm and 18 nm, respectively). These differences may be partially attributable to the lower temperature used in this work. Note that the wavelength of the absorption maximum is also different from that of the major absorption band of the Photosystem-II particle (670–672 nm), and its fluorescence maximum (685 nm), both measured at 77 °K [5].

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