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Purification and properties of an oxygen-evolving Photosystem II reaction-center complex from spinach

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A chlorophyll-protein complex, capable of photochemical water oxidation and consisting of only one extrinsic protein of 33 kDa in addition to six intrinsic proteins of the Photosystem II reaction center, has been isolated from spinach thylakoids by digitonin extraction, performed at pH 6.0, followed by chromatographic separations using DEAE-Toyopearl 650S as described briefly (Tang, X.S. and Satoh, K. (1985) FEBS Lett. 179, 60–64). The protein complex contained approx. 3–4 manganese atoms, 2 mol plastoquinone-9 and 2 mol low-potential forms of cytochrome *b*-559 heme per mol of the photoactive primary acceptor, Q_A. The oxygen evolution of the complex was highly stimulated by the presence of $CaCl_2$ and stabilized by glycerol; the typical rate of 400–500 μmol O_2 per mg Chl per h was attained with 2,5-dichlorobenzoquinone and potassium ferricyanide as electron acceptors in the presence of 50 mM $CaCl_2$. The protein complex exhibited a dark-stable EPR Signal II; the microwave power saturation profile of the signal was almost identical with that of oxygen-evolving membrane preparations. The multiline EPR signal ascribable to Kok's S_2-state was elicited in this protein complex by illumination at 200 K, as in membrane preparations. These results indicate that the basic machinery of photosynthetic water oxidation is preserved in an almost intact state in the isolated chlorophyll-protein complex.

Introduction

In the previous preliminary report [1], we have described a chlorophyll-protein complex capable of oxygen evolution and consisting of only one extrinsic polypeptide of 33 kDa in addition to those of the PS II reaction center complex, i.e., 47-, 43-, 32- and 30-kDa components [2] and those of cytochrome *b*-559 [3]. This was the first demonstration that machinery of photosynthetic oxygen-evolution is solely associated with a single protein complex rather than with membranes. Similar preparations were subsequently isolated from *Synechococcus* sp. [4] and Triton-PS II particles of spinach [5,6] by using octylglucoside treatment. These preparations are expected to facilitate largely the analysis of oxygen-evolving mechanism in PS II, since the polypeptide and chemical composition is extremely simple as compared with that of membrane preparations and the particle size is fairly small (nearly 450 kDa in case of the sample in Ref. 1) and thus suitable for spectroscopic analysis of the components involved in the oxygen-evolving system.

Abbreviations: PS II, Photosystem II; DCIP, 2,6-dichlorophenolindophenol; Mes, 4-morpholinethanesulfonic acid; HPLC, high-performance liquid chromatography.

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This paper will present a detailed description of the isolation procedure and the biochemical, photochemical and EPR spectroscopic characterization of the oxygen-evolving chlorophyll-protein complex previously communicated [1].

Materials and Methods

Thylakoid and PS II preparations. NaBr-washed thylakoid membranes treated with 0.25% digitonin for 15 min and the PS II reaction center core complex derived from the thylakoids were prepared from spinach as previously described [7]. The oxygen-evolving PS II membranes were isolated from spinach, following the procedure given in Ref. 8.

Measurement of photochemical activities. The rate of DCIP photoreduction, either with or without added electron donor, 1,5-diphenylcarbazide, was measured photometrically (A_{580}, 12.9 $mM^{-1} \cdot cm^{-1}$) at 25°C in a medium containing 40 μM DCIP/50 mM $CaCl_2$/30 mM Mes-NaOH at pH 6.5 and PS II preparations. The concentration of 1,5-diphenylcarbazide was 500 μM and that of PS II preparations was equivalent to 10 μg chlorophyll per ml. Saturating actinic light was provided by an incandescent lamp combined with heat-absorbing filters (HA-50, Hoya, Tokyo, Japan) and a red cut-off filter (V-R 65, Toshiba, Tokyo, Japan). The rate of oxygen-evolution was measured at 20°C with a Clark-type oxygen electrode (model 53, Yellow Springs Instruments, U.S.A.) in a medium containing 400 mM sucrose/50 mM $CaCl_2$/50 mM Mes-NaOH at pH 6.5, PS II preparations and an approximate oxidant in the concentration shown in the text. The concentration of pS II preparations was equivalent to 10 μg chlorophyll per ml. White actinic light was provided by an incandescent lamp combined with a heat-absorbing filter. The light intensity used was sufficient for saturating the oxygen evolution.

EPR spectroscopy. For low temperature EPR measurements, the sample in quartz tubes (i.d., 0.4 mm) was dark-adapted for 30–60 min at 0°C before being freezed. For observing the multiline EPR signal originating from S_2-state [21], the samples were illuminated for 3 min by the light from a 400 W tungsten lamp, while the temperature of the sample was maintained with a solid CO_2-methanol mixture at about 200 K. EPR spectra were recorded with a Brucker ER-200 X-band spectrometer. The temperature of the sample was maintained with a liquid helium cryostat (ESR 900, Oxford Instrument, U.K.) as described in Ref. 9.

SDS-polyacrylamide gel electrophoresis. The polypeptide profiles were analyzed by SDS-polyacrylamide gel electrophoresis carried out as in ref. 10 with modifications; 6 M urea was included in the analyzing gels and a 10–20% acrylamide gradient was made.

Spectral analysis. Absorption spectra at room temperature were measured with a dual-beam spectrophotometer (model 557, Hitachi, Tokyo, Japan). Fluorescence emission spectra at 77 K were measured with a home-made spectrofluorometer described in ref. 11.

Chemical compositions. Plastoquinone-9 and pheophytin *a* were determined for 90% acetone extracts with HPLC using a reverse-phase column (ZORBAX-ODS, Shimadzu, Kyoto, Japan). Following the procedure in Ref. 12, methanol/water (49 : 1) and methanol/isopropanol (3 : 1) were used for the chromatographic development and the elution of the components was monitored by the absorption at 255 nm. The amounts of different potential forms of cytochrome *b*-559 were determined by the method of Hind and Nakatani [13] using a difference millimolar absorption coefficient of 15 at 559 nm [14]. The primary electron acceptor, Q_A, was determined by measuring light-induced absorption change at 325 nm, with a Hitachi 356 spectrophotometer equipped with a strong continuous side illumination passed through a blue filter (4-96, Corning, U.S.A.) to ensure the complete reduction. A difference millimolar absorption coefficient of 13 at 325 nm [15] was used. Manganese contents were determined with an inductively coupled plasma emission spectrometer (Spectra Span V Dual Source Multielement System, Beckman, U.S.A.). Chlorophyll concentration was determined in 80% acetone by the method of Arnon [16].

Chemicals. Digitonin was purchased from B.D.H. Chemicals Co. Ltd. (Broom Road, U.K.) and DEAE-Toyopearl 650S was from Toyo Soda Co. Ltd. (Tokyo, Japan).

Results

I. Purification

Digitonin extraction

In the experiment shown in Fig. 1. NaBr-washed spinach thylakoids briefly treated with a low concentration of digitonin (see Materials and Methods) were extracted by 1 h incubation at 1% digitonin in a medium containing 350 mM NaCl/50 mM pH buffer of different kinds at varied pH conditions. The presence of NaCl in the extracting medium largely affected the specific activity of oxygen evolution in the extracts as well as the extent of solubilization, and the maximum activity was attained at the concentration of about 350 mM. The specific activity of DCIP photoreduction with water of the extracts measured at pH 6.5 decreased when the extraction was carried out at neutral pH irrespective of the buffer system used: Mes-NaOH or Tris-HCl, whereas the DCIP photoreduction with an artificial electron donor, 1,5-diphenylcarbazide, was independent of the pH within the range of 5.5–8.0. In the acidic conditions below 5.5, the specific activity of DCIP photoreduction, both with or without 1,5-diphenylcarbazide, decreased with lowering the pH. In typical experiments, the specific activity of DCIP photoreduction with water of the extracts obtained at pH 6.0 was 70–100 μmol O_2 evolved per mg Chl per h and was about one-half of the same activity with 1,5-diphenylcarbazide. The photochemical activity of oxygen evolution in the extracts was relatively unstable even if the sample was kept at 0°C (half-time of about 24 h). However, the presence of glycerol at about 20% in the medium largely prevented the inactivation (see below).

Purification by DEAE-Toyopearl column chromatography

The oxygen-evolving PS II complex extracted at pH 6.0 was further purified at the same pH by two-step chromatographic method using DEAE-Toyopearl as developed for the isolation of PS II reaction center core complex [7]. The digitonin extracts containing about 10 mg chlorophyll were diluted to about 7–10 times by 0.2% digitonin solution and then loaded onto a DEAE-Toyopearl 650S column (3 × 25 cm) equilibrated with 50 mM Mes-NaOH (pH 6.0) containing 0.2% digitonin. The column was washed with 100 ml of the same buffer and then most of the light-harvesting chlo-

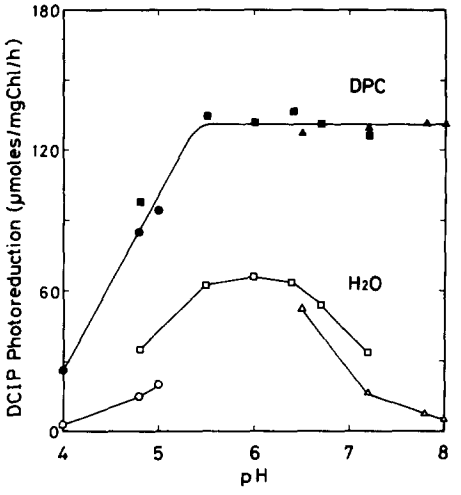

Fig. 1. Digitonin extraction of spinach thylakoids at different pH. Spinach thylakoid membranes were treated with digitonin in Tris-HCl (triangles), Mes-NaOH (circles) or potassium phthalate-NaOH (squares) buffer at 50 mM. DCIP photoreduction either with water (open symbols) or with 1,5-diphenylcarbazide (closed symbols) was measured. See text for further explanation.

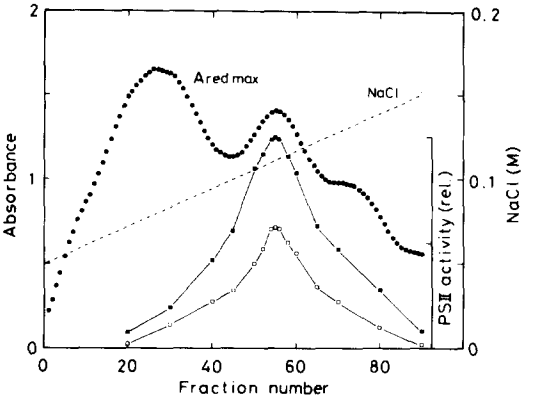

Fig. 2. Chromatographic separation of digitonin extracts using DEAE-Toyopearl 650S. NaCl concentration in the elution medium was increased as indicated by the broken line. Closed circles; absorbance measured at the red absorption maximum; closed squares; relative rate of DCIP photoreduction with 1,5-diphenylcarbazide; open squares: relative rate of DCIP photoreduction with water. See text for further explanation.

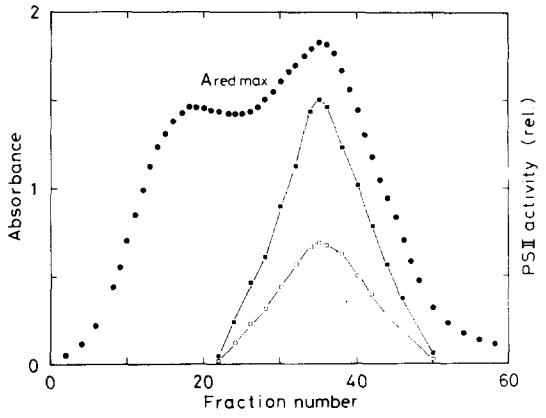

Fig. 3. Separation at the second DEAE-Toyopearl chromatography. Closed circles: absorbance measured at the red absorption maximum; closed squares: relative rate of DCIP photoreduction with 1,5-diphenylcarbazide; open square: relative rate of DCIP photoreduction with water. See text for further explanation.

rophyll *a/b*-proteins on the column was washed out by adding 50 mM NaCl to the buffer solution. The remaining materials on the column were eluted with a gradient of NaCl (50–150 mM) in the presence of 50 mM Mes-NaOH (pH 6.0) and 0.2% digitonin. This procedure separated three chlorophyll-containing fractions as shown in Fig. 2. Of the free fractions, only the second fraction was enriched in the PS II activity as measured by DCIP photoreduction either with or without 1,5-diphenylcarbazide and exhibited the absorption spectrum similar to that of the typical PS II core complex [7[. The first and the third fractions were enriched in Photosystem I complex as indicated by the SDS-polyacrylamide gel electrophoretic profile and the absorption spectrum (data not shown).

The PS II enriched fractions eluted by the NaCl gradient between 100 and 120 mM were collected and then concentrated with a small DEAE-Toyopearl column. The 3 ml of the concentrated materials containing about 2 mg chlorophyll were applied to the second DEAE-Toyopearl (3.6 × 100 cm) equilibrated with 50 mM Mes-NaOH (pH 6.0), containing 120 mM NaCl and 0.2% digitonin and developed under the same conditions. Two chlorophyll-containing fractions were separated by this procedure as shown in Fig. 3. The second fraction with higher chlorophyll *a/b* ratio (larger than 4.5) was highly active in the DCIP photoreduction with or without 1,5-diphenylcarbazide, whereas the first fraction was inactive in PS II. Based on the absorption spectrum and the polypeptide profile, this fraction was ascribed to the Photosystem I complex with light-harvesting chlorophyll *a/b*-protein of the photosystem. The second step of DEAE-Toyopearl chromatography had to be repeated twice to get pure preparations because of the poor separation in this step. Table I summarizes the purification process. The specific activity of DCIP photoreduction with water of the purified materials in this typical experiment was nearly one-half of the same activity with 1,5-diphenylcarbazide and increased 3–4 times by this purification. Nearly 10% of chlorophylls in the extracts were recovered in the final fraction.

II. Properties of the purified chlorophyll-protein complex

Photochemical activities

The oxygen-evolving activity measured either directly by oxygen-electrode or indirectly by DCIP reduction of the preparations after digitonin extraction was largely stimulated by the addition of $CaCl_2$ in the reaction mixture as repoted for the other preparations [5,6]. This stimulation was shown to be mainly due to the protection against photoinactivation (date not shown). The rate of oxygen-evolution of the purified chlorophyll-protein complex in the presence of 50 mM $CaCl_2$ was compared in Table II for various electron accep-

TABLE I

PURIFICATION OF OXYGEN-EVOLVING CHLOROPHYLL-PROTEIN COMPLEX

DPC, 1,5-diphenylcarbazide.

Preparation	DCIP photoreduction (μmol DCIP per mg Chl per h)	
	without DPC	with DPC
Digitonin extracts	72	147
After the first chromatography	142	309
After the second chromatography	238	548

TABLE II

ACCEPTOR DEPENDENCY OF OXYGEN EVOLUTION BY THE OXYGEN-EVOLVING CHLOROPHYLL-PROTEIN COMPLEX

Acceptor	Oxygen-evolving activity (μmol O_2 per mg Chl per h)
Ferricyanide (1 mM)	250
Phenylbenzoquinone (0.2 mM)	272
Dimethylbenzoquinone (1 mM)	90
Tetramethylbenzoquinone (0.5 mM)	0
2,5-Dichlorobenzoquinone (0.2 mM)	334
2,6-Dichlorobenzoquinone (1 mM)	269
Benzoquinone (1 mM)	109
DCIP (0.06 mM)	203
2,5-Dichlorobenzoquinone (0.2 mM) plus ferricyanide (1 mM)	444

tors. The affinity to electron acceptors of the purified materials was different from that of PS II membrane preparations described in Ref. 5, indicating modification on the acceptor side. The maximum rate of 400–500 μmol O_2 evolved per mg Chl per h was obtained with 1 mM ferricyanide and 0.2 mM 2,5-dichlorobenzoquinone. DCIP, however, was also a potent electron acceptor for this preparation. The oxygen-evolving activity supported by these acceptors was totally insensitive to DCMU or atrazine in the concentration range of 1–10 μM, where these reagents suppress the photochemical activity of PS II in intact thylakoids [2]. This further suggests the modification on the reducing side, and indicates that the secondary electron acceptor, Q_B, is not functioning in these conditions. This was also supported by the flash response of the chlorophyll fluorescence (data not shown).

The oxygen-evolving activity of the purified chlorophyll-protein complex was relatively unstable in a dilute solution even at 0 °C. However, the inactivation was prevented by the presence of glycerol or sucrose in the medium; the stability increased with increasing concentration of either reagent up to 20 or 40%, respectively. Thus it is possible to prepare the complex with high oxygen-evolving activity of about 800 μmol O_2 evolved per mg Chl per h by conducting the entire process in the presence of 20% glycerol, although the separation at the stages of DEAE-Toyopearl is relative poor.

Polypeptide composition

Fig. 4 shows a typical profile of SDS-polyacrylamide gel electrophoresis of the purified oxygen-evolving PS II complex together with those of PS II core complex and the 33 kDa polypeptide prepared by urea-treatment of $CaCl_2$-washed membranes [17]. The PS II core complex consists of six polypeptides; two large polypeptides of chlorophyll-binding (47 and 43 kDa subunits), two medium size polypeptides (32 and 30 kDa subunits) and two small subunits of cytochrome *b*-559, 9 kDa and 4.5 kDa, although the latter subunit is not resolved in Fig. 4. Only one additional polypeptide corresponding to the 33 kDa of Kuwabara and Murata [18] is present in the oxygen-evolving complex. The relative abundance of the 33 kDa polypeptide in the purified materials varied with preparations, and there was a tendency that when the activity was low the amounts of the polypeptide were reduced. A

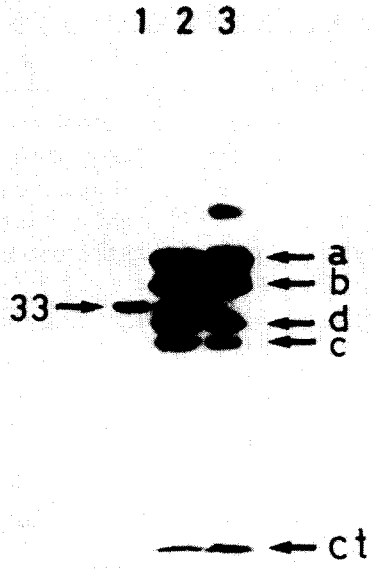

Fig. 4. SDS-polyacrylamide gel electrophoretic patterns of the purified oxygen-evolving chlorophyll-protein complex. (1) 33 kDa protein; (2) oxygen-evolving chlorophyll-protein complex; (3) PS II core complex. The symbols, a, b, c, d and Ct on the right side denote polypeptide bands of 47, 43, 30, 32 and 9 kDa, respectively. See text for further explanation.

Coomassie brilliant blue-stainable band(s) of about 60 kDa often appeared in the profile (Fig. 4). Recent analysis has shown that this band(s) originates from aggregation of 32/30 kDa components [28].

Spectral properties and chemical composition

Absorption spectrum at room temperature of the purified chlorophyll-protein complex retaining oxygen-evolving activity shown in Fig. 5 is essentially similar to that of the PS II reaction-center core complex described [7]. The chlorophyll *a* absorption peak in the red region was observed near 674 nm and practically no absorption due to chlorophyll *b* is evident. The presence of carotenoid species identified to be solely β-carotene (1 per 5 Chl *a*) by HPLC analysis was indicated by the absorption shoulders around 460–490 nm.

The fluorescence emission spectrum measured at 77 K was also quite similar to that of the core complex; F_{695} emission is present and is enhanced by the addition of 1,10-phenanthroline in the presence of guanidine-HCl.

The chemical composition of the purified oxygen-evolving chlorophyll-protein complex summarized in Table III is almost identical with that of PS II core complex [7], except that the manganese content is much higher in the oxygen-evolving complex; more than three atoms of manganese are present in the oxygen-evolving complex instead of 0.6 in the PS II core complex [7] on a reaction center basis. The slightly lower manganese content in this preparation, compared to four atoms of manganese per reaction center in the membrane preparations [8], may be responsible to the relatively lower activity and is possibly related to the reduced amount of 33 kDa polypeptide in the complex. Out of the two molecules of plastoquinone in the complex, one must be ascribed to Q_A [19], the primary electron acceptor, and the function of the rest of the quinone is a matter of discussion [19,29]. The oxygen-evolving complex contains nearly two cytochrome *b*-559 per reaction center, but none in the hydroquinone-reducible form.

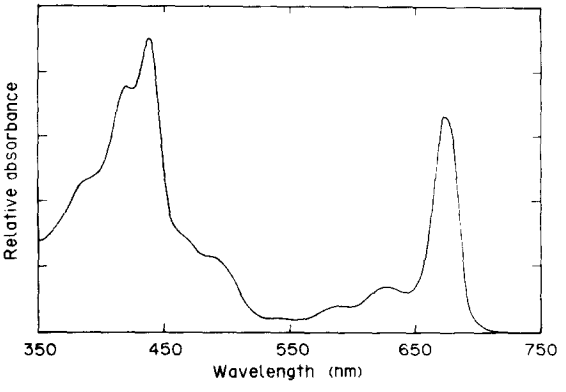

Fig. 5. Absorption spectrum of the purified oxygen-evolving chlorophyll-protein complex at room temperature.

TABLE III

CHEMICAL COMPOSITION OF THE OXYGEN-EVOLVING CHLOROPHYLL-PROTEIN COMPLEX

Component	Molar ratio
Chlorophyll *a*	55
Pheophytin *a*	2.3
Plastoquinone-9	2.2
Manganese	3.5
Cytochrome *b*-559	1.8
(Ascorbate-Hydroquinone)	0.6
(Dithionite-Ascorbate)	1.2
Primary quinone acceptor (Q_A)	1.0

EPR characterization

In the dark, the purified oxygen-evolving complex exhibits an EPR spectrum originating from a stable free radical (Signal IIs). The amplitude of the signal on a reaction center basis in this preparation was nearly identical with that in the PS II membranes or PS II core complex [20], indicating that the species responsible for the signal is almost completely preserved in the purified chlorophyll-protein complex. The oxygen-evolving chlorophyll-protein complex exhibits an almost similar power saturation behavior with that of PS II membranes, whereas slightly higher microwave power was necessary to saturate the Signal II of the PS II core complex (date not shown). When the samples were illuminated by continuous light at 200 K, the microwave power saturation profile of both the oxygen-evolving chlorophyll-protein complex and the membranes shifted to the higher microwave intensity. On the other hand, the power dependency of the PS II core complex was unaffected by the illumination (data not shown).

Fig. 6 shows the 10 K EPR difference spectra obtained by subtracting the spectrum for the dark-adapted sample from that for the sample illuminated with continuous light at 200 K for 3 min. No multiline hyperfine-structure was observable in the three preparations shown in the figure in the dark-adapted state. By the illumination at 200 K, the multiline signal ascribable to the S_2-state in the oxygen-evolving system [21] develops in the oxygen-evolving chlorophyll-protein complex as in PS II membranes, but this signal was not observed in PS II core complex. EPR signal due to Q_A^-Fe complex is also present in traces in Fig. 6 overlapped with the S_2 signal in the region of 4000 gauss ($g = 1.8$). From Fig. 6 the amplitude of the multiline signal in the oxygen-evolving complex was estimated to be about 75% of that in the PS II membrane on a reaction center basis. This value is nearly corresponded with the

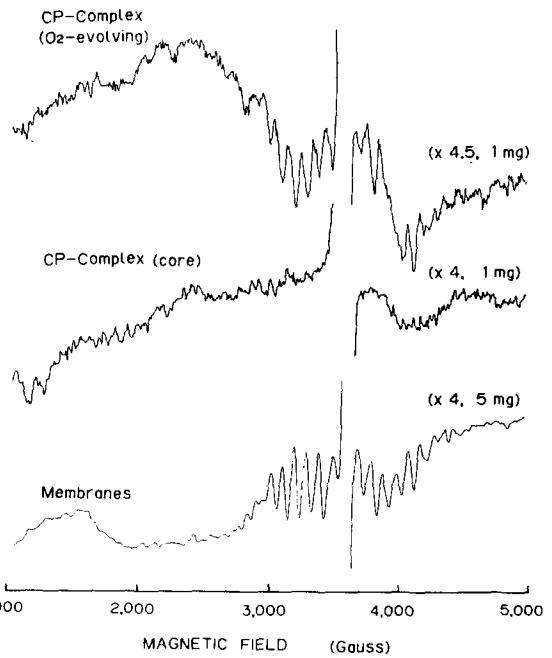

Fig. 6. Light-minus-dark EPR difference spectra recorded at 10 K in oxygen-evolving chlorophyll-protein complex (top), PS II reaction-center core complex (middle) and PS II membrane fragments (bottom). The numbers in parentheses represent the expanding scales and the concentrations of sample in mg Chl per ml. Spectrometer conditions: microwave power, 100 mW; microwave frequency, 9.66 GHz; field modulation, 40 G; modulation frequency, 100 kHz; time constant, 80 ms. Average of eight scans.

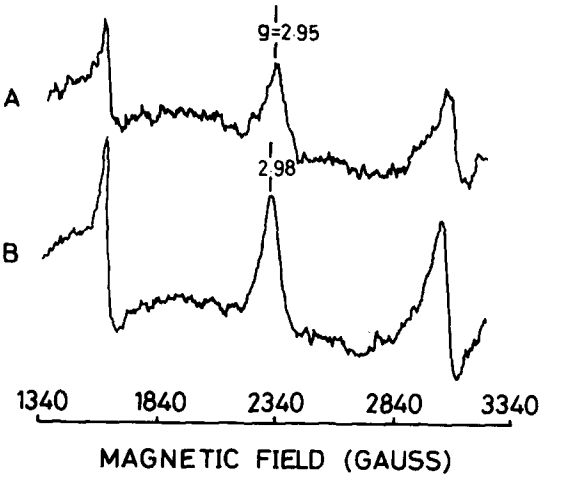

Fig. 7. EPR spectra at 10 K of dark-adapted oxygen-evolving chlorophyll-protein complex (A) and PS II reaction center core complex (B). The concentration of the oxygen-evolving complex and the core complex were 1.0 and 1.4 mg Chl per ml, respectively. Spectrometer conditions as in Fig. 6, except that the microwave power was 10 mW.

amount of manganese atoms remaining in this purified protein preparation. The power saturation profile of the signal was almost identical with that of PS II membranes.

In Fig. 7, EPR spectra of cytochrome *b*-559 recorded at 10 K for both oxygen-evolving (A) and core (B) complexes, are compared for the dark-adapted samples. In both preparations, the illumination at 200 K did not increase the signal indicating that the cytochrome is present as the oxidized form in the dark-adapted samples. The EPR signal of the cytochrome is observed with a peak at $g = 2.95$ in the oxygen-evolving complex and 2.85 in the core complex. The microwave power saturation profiles of this signal for both the oxygen-evolving complex and the core complex were practically identical, indicating that the cytochrome is present in a similar – although slightly different as shown by the *g*-values – molecular environment in both chlorophyll-protein complexes.

Discussion

In this paper we have presented a detailed description of the isolation procedure and the properties of an oxygen-evolving chlorophyll-pro-

tein complex briefly communicated previously [1]. An improvement has been made in the purification to increase the specific activity of water oxidation by including NaCl in the extracting medium. It has also been suggested that if the entire process of purification is conducted in the presence of glycerol, no appreciable part of the oxygen-evolving capacity would be inactivated.

In contrast to the similar preparations which utilize Triton-X 100 and/or octylglucoside [4–6], the procedure reported here uses only digitonin and thus distortion in the pigment system is expected to be small and suitable for the spectroscopic analysis. On the other hand, these preparations, including the one from a cyanobacterium [4], contain irrespective of the method of purification only one extrinsic polypeptide of 33 kDa (35 kDa in case of the cyanobacterium) in addition to 6 polypeptides of the PS II reaction center. These preparations retain the manganese at 3.5–3.7 atoms per reaction center and they exhibit relatively high rates of oxygen evolution (300–1000 μmol O_2 evolved per mg Chl per h). However, the sensitivity of the electron transport to DCMU or atrazine and the spectrum of the efficiency of electron acceptors are largely different in different preparations, indicating the different degree of modifications on the reducing side [1,4–6]. The lower content of 33 kDa polypeptides in some preparations was consistent with the lower abundance of manganese and the lower rate of oxygen evolution. The presence of 33 kDa polypeptide, however, has shown not to be indispensable for the manifestation of oxygen evolution and is rather necessary for the stabilization of manganese or the machinery of oxygen-evolving system as shown in PS II membranes [17,30] and in the protein complex [24]. The presence of an additional polypeptide of 18–22 kDa in the purified preparations was often noted [1,4,6], but its occurrence was less reproducible and unnecessary for the oxygen-evolution. From these results, now it is firmly established that the PS II core complex accompanied with a peripheral protein of 33 kDa and preserving manganese, probably four atoms per reaction center in its native state, is a structural minimum for the catalytical oxygen evolution.

Although the oxygen-evolving chlorophyll-protein complex retaining most of the manganese (more than three atoms per reaction center) exhibits nearly the same amplitude of dark-stable EPR Signal II as PS II core complex depleted in most of the manganese (nearly one atom per reaction center, Ref. 7), the microwave power saturation profile of the signal was slightly different from that of the core complex; instead it was quite similar to that of membrane preparations. On the other hand, in membrane preparations the power saturation of the signal in the sample, inactivated by Tris-treatment and lacking manganese on the active site, occurs at lower microwave intensity than that of untreated samples (Isogal, Y. and Itoh, S., unpublished data), due to slower spin relaxing rates [25]. These facts suggest that the microenvironment surrounding this signal species in the isolated oxygen-evolving complex is preserved as in oxygen-evolving PS II membranes, but it is largely different in the core complex as compared with that of Tris-treated membranes, although both preparations are inactive in the oxygen evolution.

The measurement of the multiline EPR signal which is attributable to Kok's S_2-state also indicated that the catalytic site for water oxidation is preserved in an almost intact state in the isolated chlorophyll-protein complex as in oxygen-evolving PS II membranes. However, there was a discrepancy between the extent of the multiline signal and the rate of oxygen-evolution between membrane preparations and the isolated protein complex. This may indicate that the rate-limiting step in the oxygen evolution in the isolated complex is different from that in intact membrane preparations as exemplified by the different affinity to the electron acceptors and the relatively low rate of oxygen-evolution in the isolated protein complex.

It has been reported that the oxidized high-potential form of cytochrome *b*-559 has an EPR signal at $g = 3.08$ and the oxidized low-potential form at 2.95 [9,22,23] and 2.98 [9] in the presence and absence of manganese, respectively, in the oxygen-evolving membranes. Thus it is concluded that the purified chlorophyll-protein complexes either oxygen-evolving or inactive in the oxygen evolution (core complex) contain the cytochrome only as low-potential forms and suggested that the difference in the *g*-value in both complexes is due

to differences in the manganese contents. The absence of high-potential form of cytochrome *b*-559 in the complex was also shown by spectroscopic measurement. Thus it is quite clear that the presence of high-potential form of this cytochrome is not essential for the manifestation of oxygen evolution, as already indicated in membrane preparations [31].

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