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Isolation of a photosystem II reaction center consisting of D-1 and D-2 polypeptides and cytochrome *b*-559

(oxygenic photosynthesis/charge separation/pheophytin acceptor/pigment–protein complex)

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ABSTRACT **A photosystem II reaction center complex consisting of D-1 and D-2 polypeptides and cytochrome *b*-559 was isolated from spinach grana thylakoids, treated with 4% (wt/vol) Triton X-100, by ion-exchange chromatography using DEAE-Toyopearl 650S. The isolated complex appears to contain five chlorophyll a, two pheophytin a, one β-carotene, and one or two cytochrome *b*-559 heme(s) (molar ratio) and exhibits a reversible absorbance change attributable to the photochemical accumulation of reduced pheophytin typical for the intermediary electron acceptor of photosystem II reaction center. These results strongly suggest that the site of primary charge separation in photosystem II is located on the heterodimer composed of D-1 and D-2 subunits.**

It has been well established that the photosystem II reaction center of oxygenic photosynthetic organisms is contained in a chlorophyll–protein complex consisting of the following six polypeptide subunits: 47- and 43-kDa polypeptides, D-1 and D-2 polypeptides of ≈30 kDa, and two polypeptides of cytochrome *b*-559 (1, 2). The 47- and 43-kDa components can easily be isolated as chlorophyll-carrying polypeptides (3). On the other hand, the other subunits have never been isolated in combination with chlorophylls (1). The D-1 polypeptide has been identified as a herbicide-binding polypeptide and is thus ascribable to the site of secondary quinone acceptor (Q_B) binding (4). The function of D-2 polypeptide as well as of cytochrome *b*-559 in the photosystem II reaction center has not yet been clarified.

There is a controversy concerning the localization of the site of primary charge separation in the photosystem II reaction center complex (5–10). Some evidence supports the proposal that the 47-kDa subunit is the site of primary photochemistry (5–8). However, the amino acid sequence homology between the D-1 and D-2 subunits and the L and M subunits of reaction center from purple photosynthetic bacteria has led to a proposal (9, 10) that the D-1 and D-2 polypeptides play a role in the photosystem II reaction center similar to that of L and M subunits in the bacterial reaction center whose structure has been determined by x-ray crystallographic analysis (11).

In this paper we provide experimental evidence supporting the latter proposition. We have succeeded in isolating a pigment–protein complex consisting of D-1 and D-2 polypeptides and cytochrome *b*-559 that is capable of reversible photochemical accumulation of reduced pheophytin. A preliminary account of this work has been presented (12).

MATERIALS AND METHODS

The membrane preparation of grana thylakoids (Triton/photosystem II particles) was prepared from spinach as described by Kuwabara and Murata (13).

Pigments and quinones were extracted with 80% (vol/vol) acetone and quantitatively determined by HPLC using a reverse-phase column (ZORBAX-ODS). Following the procedure described (14), methanol/water, 49:1 (vol/vol) and methanol/isopropanol, 3:1 (vol/vol) were used for chromatographic development, and the elution of components was monitored by the absorption either at 440 nm (chlorophylls and carotenoids) or at 255 nm (pheophytin a and plastoquinone-9). The spectrophotometry of the components was based on the absorption coefficients described by Eskins *et al.* (14) for chlorophylls and carotenoids, by Vernon (15) for pheophytins, and by Barr and Crane (16) for plastoquinones. The amounts of cytochrome *b*-559 with different midpotentials were determined by the method of Hind and Nakatani (17) using a difference millimolar absorption coefficient (559–570 nm) of 15 (18).

$NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as described (19) with the modifications that 6 M urea was included in the gels to increase the focusing of D-1 and D-2 bands (4) and a 10–20% (wt/vol) acrylamide gradient was used.

Rabbit polyclonal antibodies against spinach 47- and 43-kDa subunits were a kind gift from K. Aoki (20). Anti-cytochrome *b*-559 was generated in rabbit from the purified spinach cytochrome with two polypeptide subunits. Proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane again by electrophoresis. The blot was incubated sequentially with antiserum and peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad). The peroxidase-containing bands were developed by incubating the strips with hydrogen peroxide and 4-chloro-1-naphthol.

The absorbance change induced by continuous, saturating light from a xenon lamp (500 W) were measured at 5°C in a 10-mm cuvette with a Hitachi-356 spectrophotometer equipped with a cross-illumination system. Appropriate filter combinations were used for protecting photomultiplier from strong actinic illumination as follows: below 600 nm, two Corning 4-96 filters for the measuring beam, a red cutoff filter (VR-67, Toshiba, Tokyo), and heat- and UV-absorbing filters for the actinic beam; above 600 nm, a red cutoff filter (VR-61, Toshiba), an appropriate interference filter for the measuring beam, two Corning 4-96 filters, and heat- and UV-absorbing filters for the actinic beam. The sample in 60 mM Tris·HCl, pH 8.5, containing 1 μM methyl viologen and 1–5 mg of sodium dithionite per ml was incubated for a few minutes before each measurement.

DEAE-Toyopearl 650S was purchased from Toyo Soda (Tokyo).

RESULTS

The photosystem II particles (1 mg of chlorophyll per ml) were further treated with 4% (wt/vol) Triton X-100 in 50 mM

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Tris·HCl (pH 7.2) for 1 hr at 4°C with stirring. The resultant mixture was centrifuged at 100,000 × *g* for 1 hr at 4°C, and the supernatant solution was adsorbed onto a DEAE-Toyopearl 650S column equilibrated with 50 mM Tris·HCl, pH 7.2/0.05% Triton X-100/30 mM NaCl. The column was then washed with the same buffer solution until the eluent became colorless. This procedure eluted the major portion of chlorophyll. The eluents were enriched in the polypeptides of 47- and 43-kDa components of photosystem II reaction center, light-harvesting chlorophyll a/b–proteins, and 18-, 24-, and 33-kDa components of the oxygen-evolving system, but not with those of D-1 and D-2 subunits. The column was then subjected to the gradient elution of NaCl (30–200 mM) in the presence of 50 mM Tris·HCl, pH 7.2/0.05% Triton X-100. Two chlorophyll-containing fractions were obtained by this procedure as shown in Fig. 1. The major fraction eluted at about 95 mM NaCl was enriched in both chlorophyll a and pheophytin a (see later), and the absorption spectrum of the minor fraction, which eluted at about 130 mM NaCl, was similar to that of the photosystem II reaction center complex prepared from digitonin extracts (4).

The $NaDodSO_4$/polyacrylamide gel electrophoretic profile obtained by Coomassie brilliant blue staining of the major fraction shown in Fig. 2 has polypeptides with the same mobilities as the D-1 and D-2 subunits of the photosystem II reaction center and as the cytochrome *b*-559 polypeptides (9- and 4.5-kDa components). On the other hand, practically no polypeptides corresponding to the 47- and 43-kDa subunits are observed. In addition to the four polypeptide bands mentioned above, variable amounts of a small but clearly distinct protein band(s) of about 60 kDa was usually observed in the profile. This band(s) was not a stable component in the profile of the starting material (Triton photosystem II particles) but usually is observed in the purified preparations of photosystem II reaction center (4) and thus identified as the aggregated form(s) of lower molecular weight subunits of this hydrophobic complex. This higher molecular weight band(s) was reactive with none of the following rabbit polyclonal antibodies prepared against spinach polypeptides; anti-47-kDa subunit, anti-43-kDa subunit, or anti-cytochrome *b*-559, as shown in Fig. 3. Since all of the five (including the higher molecular weight band) polypeptide bands mentioned above comigrate together with pigments as a sharp band upon electrophoresis in digitonin under nondenaturating conditions (data not shown), it seems obvious that they are present as a single protein–pigment complex. The ratio of the amounts of polypeptides of D-1 and D-2 subunits and 9-kDa component of cytochrome *b*-559 in this preparation was estimated to be roughly identical with that in the purified photosystem II reaction center complex (Fig. 2, lane B).

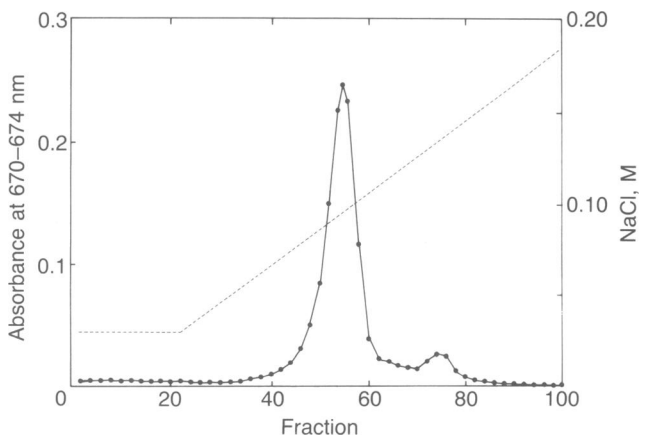

FIG. 1. Elution profile of ion-exchange column chromatography using DEAE-Toyopearl 650S. NaCl concentration was as indicated by dashed line.

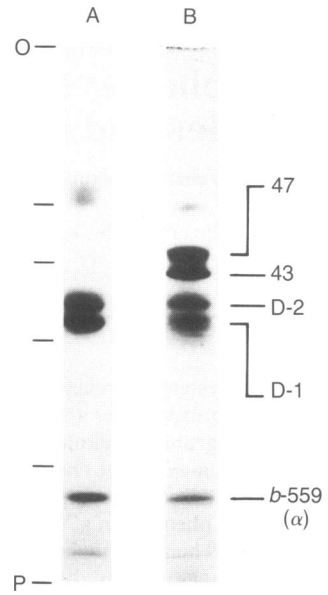

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of the major fraction eluted at 95 mM NaCl shown in Fig. 1 (lane A). For comparison, the profile for the photosystem II reaction center complex prepared from digitonin extracts (4) is also shown (lane B). The fraction (lane A) and the reaction center complex (lane B) equivalent to 1.5 and 7.5 μg of chlorophyll, respectively, were loaded onto the gel. Tick marks on the left side indicate the positions of the origin of analyzing gel (O), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome *c* (12.4 kDa), respectively, from the top, and the pigment front (P).

In the room-temperature absorption spectrum of the isolated D-1–D-2–cytochrome *b*-559 complex shown in Fig. 4, the chlorophyll a absorption peak in the red region is observed near 673 nm and practically no absorption due to chlorophyll b is evident. The presence of carotenoid is indicated by the absorption shoulders around 460–490 nm. One of the striking feature of the absorption spectrum is the presence of a strong absorption peak at about 415 nm,

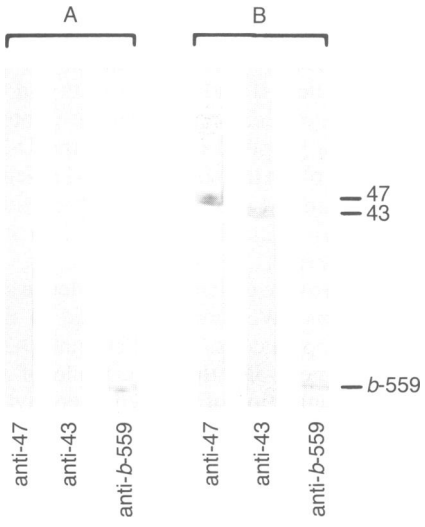

FIG. 3. Reactivity of blotted protein bands with rabbit polyclonal antibodies against 47-kDa component (anti-47), 43-kDa component (anti-43), or cytochrome *b*-559 (anti-*b*-559) of photosystem II reaction center. Lanes A, major fraction eluted at 95 mM NaCl. Lanes B, photosystem II reaction center complex from digitonin extracts. Conditions were exactly identical with those used in Fig. 2.

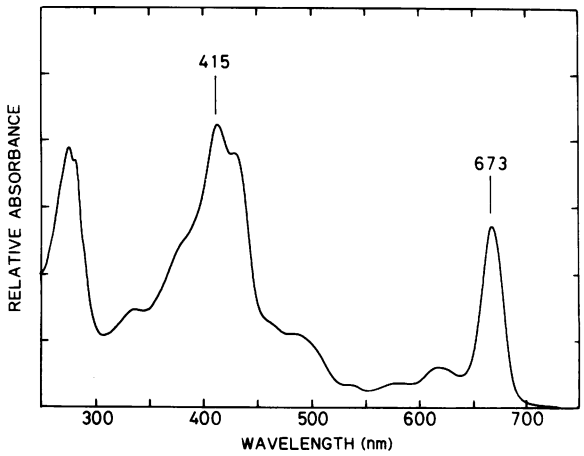

FIG. 4. Absorption spectrum of the isolated complex measured at room temperature.

suggesting the presence of higher concentration, relative to chlorophyll a, of pheophytin and/or cytochrome *b*-559 heme in this complex.

The HPLC analysis of pigment and chemical composition indicated that the complex appears to contain a molar ratio of five chlorophyll *a*, one β-carotene, and one or two cytochrome *b*-559 heme per two pheophytin a (Table 1). The complex contains virtually no plastoquinone-9 (less than 0.1 mol per 2 mol pheophytin a).

The purified complex was totally inactive in the photoreduction of 2,6-dichlorophenol indophenol by 1,5-diphenylcarbazide. However, the complex exhibits a reversible absorption change upon illumination as measured in the presence of dithionite and methyl viologen (Fig. 5). The characteristic negative peaks at 422, 515, 545, and 682 nm and a positive peak at 450 nm in the light-induced spectrum as well as their kinetic behavior coincides well with those of photoaccumulation of reduced pheophytin in the photosystem II reaction center as reported by Klimov *et al.* (21) although the negative peak position at the red region is blue-shifted 2–3 nm

Table 1. Chemical composition of the purified pigment–protein complex

Component	Molar ratio
Pheophytin a	2.0
Chlorophyll a	5.1 ± 0.5
Chlorophyll b	<0.1
β-carotene	1.0 ± 0.3
Plastoquinone-9	<0.1
Cytochrome *b*-559	1.3 ± 0.4

Molar ratio was determined on the basis of 2.0 pheophytin a molecules. Data are from five independent experiments and expressed as mean ± SEM.

in this preparation. Using millimolar difference absorption coefficient of pheophytin a–pheophytin a^- of 32 at the red maximum (22), the absorption change upon saturating actinic illumination in this study was roughly estimated to correspond to about one pheophytin a molecule photoreduced out of two chemically estimated molecules in the complex. Spin-polarized EPR triplet at low temperature has been observed in this material (25).

DISCUSSION

The pigment–protein complex isolated in this study contains the polypeptides of D-1 and D-2 subunits of photosystem II reaction center and of cytochrome *b*-559. However, in addition to these well-characterized polypeptides, Coomassie blue stained gels have a band of about 60 kDa that is hardly detectable in the $NaDodSO_4$/polyacrylamide gel electrophoretic profile of the starting material. The following two lines of evidence, however, support the interpretation that this protein band(s) originates from aggregation of D-1 and D-2 subunits of photosystem II reaction center complex. (*i*) This band(s) is reactive with none of the polyclonal antibodies against the 47-, 43-kDa component, or cytochrome *b*-559 of spinach photosystem II reaction center complex. (*ii*) Radioactive iodine labeling through the photochemical oxidation of $K^{125}I$ by photosystem II (which is very specific for both D-1

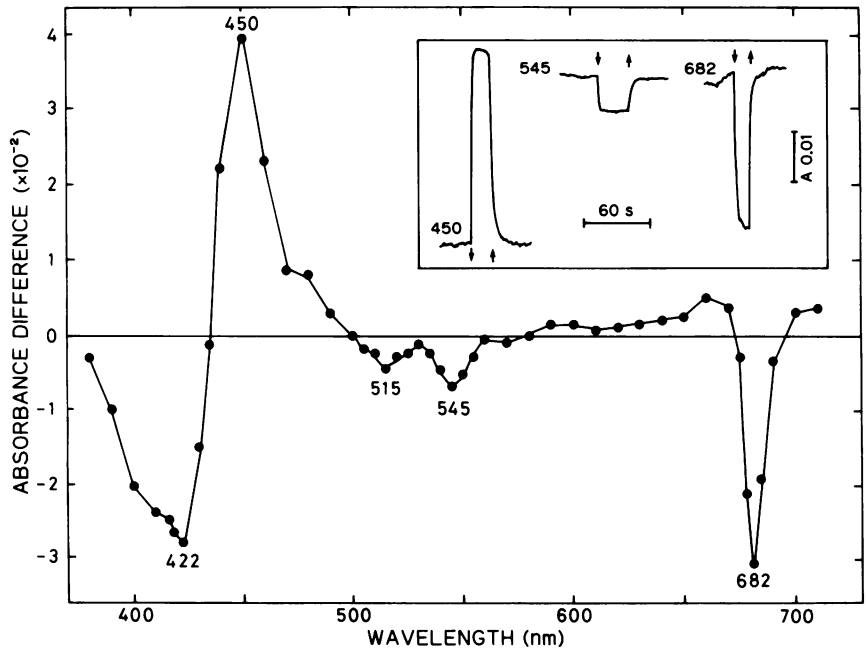

FIG. 5. Light-induced absorbance changes measured at 5°C in the presence of 1 μM methyl viologen/1–5 mg per ml of sodium dithionite/60 mM Tris·HCl, pH 8.5/20% (vol/vol) glycerol/pigment–proteins equivalent to 4.8 μM chlorophyll a. (*Insert*) Kinetic response of the absorbance changes at three different wavelengths. Arrows downward and upward indicate the time that the light was switched on and the time it was switched off, respectively.

and D-2 subunits) also labels this higher molecular weight band(s) (23). Thus it is concluded that the complex isolated here is composed entirely of D-1 and D-2 subunits of photosystem II reaction center complex and cytochrome *b*-559.

The data presented here provide strong evidence that the D-1 and D-2 subunits in the photosystem II reaction center complex are present in association with chlorophyll and pheophytin, since the polypeptides of cytochrome *b*-559 have not been isolated in combination with these pigments. It is interesting to note that there is a striking similarity in the pigment composition between this complex and the purple bacterial reaction center. The former has a molar ratio of five chlorophyll a, two pheophytin a, and one β-carotene, whereas the latter contains four bacteriochlorophyll, two bacteriopheophytin, and one carotene (24). One excess chlorophyll a molecule in the former preparation may possibly originate from contaminating trace amounts of 47- and/or 43-kDa components.

The isolated D-1–D-2–cytochrome *b*-559 complex is totally inactive in the 2,6-dichlorophenol indophenol photoreduction possibly because of the absence of both the primary quinone acceptor (Q_A) and the secondary donor (Z) as expected by the absence of chemically estimated plastoquinone-9. However, this complex is highly active in the photoreversible accumulation of reduced pheophytin a exhibiting the characteristic kinetics and spectrum of the photosystem II reaction center (21). This result strongly suggests that the complex composed of D-1 and D-2 subunits is the site of primary charge separation in photosystem II as proposed on the basis of nucleotide and amino acid sequence homology between these two subunits of photosystem II and those of bacterial L and M subunits (9, 10). On the other side, substantial experimental evidence has accumulated in favor of the proposal that the 47-kDa subunit is the site of charge separation in photosystem II (5–8). However, in most cases the presence of D-1 and D-2 subunits in the samples has not been taken into account (5, 7, 8), and none of them are conclusive (1).

It has been suggested that cytochrome *b*-559 is present in the vicinity of the photosystem II reaction center as indicated by the photooxidation at cryogenic temperatures. The result presented here is consistent with this view. The functional role of this cytochrome in the photosystem II reaction center, however, is still a matter of speculation.

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