Short Communication

Presence in Photosystem II Core Complexes of a 34-Kilodalton Polypeptide Required for Water Photolysis[1]

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ABSTRACT

Photosystem II (PSII) reaction center core complexes have been isolated and characterized from wild type (WT) *Scenedesmus obliquus* and from its LF-1 mutant. LF-1 thylakoids are blocked on the oxidizing side of PSII and have a reduced Mn content. Visible absorption and low temperature fluorescence spectra of both core complexes are identical and resemble those reported for spinach (Satoh, Butler 1978 Plant Physiol 61: 373–379). Lithium dodecyl sulfate-polycrylamide gel electrophoresis reveals that a protein alteration, originally observed in thylakoid membranes (Metz, Wong, Bishop 1980 FEBS Lett 114: 61–66), is retained in the PSII core particles. That is, a 34-kilodalton (kD) polypeptide, present in the WT core complex, is missing in the mutant, and the core complex of the mutant contains a 36-kD protein not present in the WT. The 34-kD intrinsic protein is also observed in O₂-evolving PSII preparations and PSII core complexes from spinach. It is distinct from the 33-kD extrinsic protein first reported by T. Kuwabara and N. Murata (1979 Biochim Biophys Acta 581: 228–236). We suggest that the 34-kD protein is a site of Mn binding in the PSII membrane.

Analysis of low fluorescent mutants of the green alga *Scenedesmus obliquus* has indicated that a thylakoid polypeptide with an apparent molecular mass of approximately 34-kD is involved in the water-splitting reaction of PSII (12, 14). These data also suggested a possible role for the 34-kD protein in binding Mn required for this reaction and that the protein is inserted into the membrane in a 36-kD precursor form. These conclusions arose from the observation that thylakoids of the non-O₂-evolving LF-1[3] mutant were depleted of Mn and that a 36-kD protein replaced the 34-kD protein present in WT thylakoids.

Other proteins with apparent molecular masses in the 30- to 34-kD range have also been associated with PSII. Kuwabara and Murata (9) purified a 33-kD protein which has been implicated in the water-splitting reaction (10, 21). It is an extrinsic protein which does not require detergents to remain soluble in aqueous solution. A protein which functions on the reducing side of PSII and contains the binding site for atrazine-type herbicides also has been identified. This polypeptide has an apparent molecular mass of 30- to 32-kD and has been located on polyacrylamide gels by a photoaffinity labeling technique (16). It is an intrinsic membrane protein and is part of the PSII reaction center core particle (18). It apparently does not stain well with Coomassie Brilliant Blue.

Several techniques are now available with higher plants for: obtaining highly enriched OES II preparations (see 8 for a survey; 3, 10, 21); selectively removing the extrinsic polypeptides at 17, 23, and 33 kD from the preparations (1, 10, 15, 21); and obtaining PSII core complexes from the OES II (20). We have adapted these techniques to obtain PSII core complexes from WT *Scenedesmus* and the LF-1 mutant. In this report, we show that the 34-kD/36-kD protein mobility change observed in thylakoids of these strains is retained in PSII core particles. Also, by comparison to various fractions obtained during the preparation of spinach PSII core complexes, we demonstrate the distinction between the extrinsic 33-kD protein of Kuwabara and Murata (9) and the 34-kD intrinsic membrane protein.

MATERIALS AND METHODS

Spinach thylakoid and OES II membranes were prepared as before (8, 10) except that 0.5 mM PMSF was included in the grinding solution. Extrinsic proteins were removed from the OES II preparations by washing them with a 1 M CaCl₂ solution (15). The resultant supernatant (containing the extrinsic proteins) was dialyzed overnight against 10 mM Tricine-NaOH (pH 7.8), 0.1 mM PMSF. The salt-treated PSII pellet was washed once with 0.2 M sucrose, 10 mM MgCl₂, 10 mM Mes-NaOH (pH 6.5), and suspended at 1 mg Chl/ml in the same solution. PSII core particles were prepared from this suspension using a modification of the procedure of Westhoff *et al.* (20). The suspension was brought to 1% (w/v) dodecyl-β-D-maltoside using a 20% (w/v) stock solution, incubated in the dark for 10 min at 22°C, and centrifuged for 10 min at 10,300g (4°C). One-half-ml aliquots of the supernatant were layered onto 15 to 30% sucrose gradients containing 0.05% dodecyl-β-D-maltoside in 10 mM Mes (pH 6.5). The gradients were centrifuged for 20 h at 200,000g in a Beckman SW41 rotor. The denser of the two major green bands which form contained the PSII core complex and was collected with a syringe. This sample was prepared for a second centrifuge run on identical sucrose gradients by use of a Centricon microconcentrator with a YM30 membrane (Amicon Corp., Danvers, MA). The sample was brought up to 0.5% dodecyl-β-D-maltoside prior to loading 0.2-ml aliquots on half the gradients used during the first spin. After centrifugation, the PSII-core-containing fractions were collected, concentrated, and stored at −70°C until needed.

[3] Abbreviations: LF, low fluorescent; WT, wild type; OES II, oxygen-evolving PSII; PMSF, phenylmethylsulfonyl fluoride; LDS, lithium dodecyl sulfate.
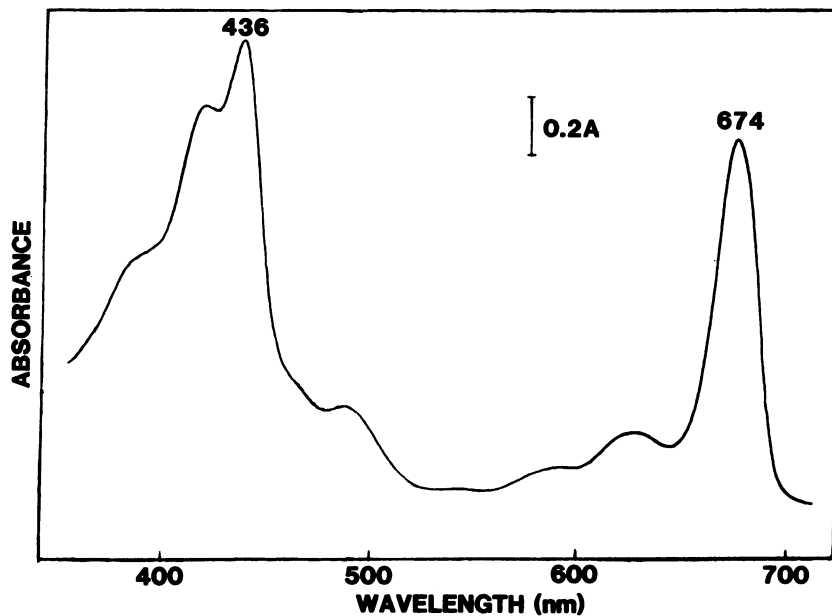
FIG. 1 Absorption spectrum of PSII core complexes isolated from the *Scenedesmus* mutant, LF-1.
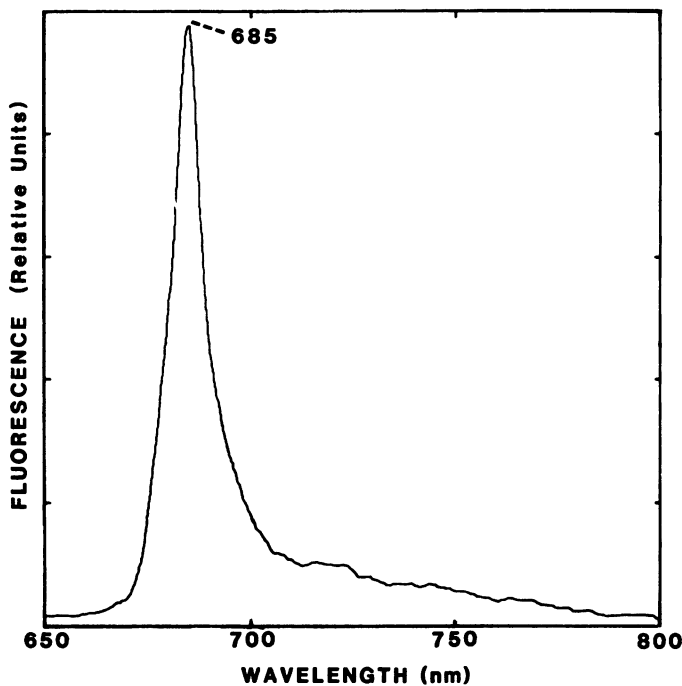

FIG. 2. Low temperature (77K) fluorescence emission spectrum of PSII core complexes isolated from WT *Scenedesmus*.

Scenedesmus obliquus WT and LF-1 mutant strains were grown in the dark on liquid, enriched medium (4). Cells were collected by centrifugation (2,000g, 5 min) and washed with 50 mM sodium phosphate buffer (pH 6.5). PSII-enriched algal preparations were obtained by modification of the Kuwabara and Murata (10) procedure. The washed cells (6–9 ml packed cell volume) were suspended in 50 ml of 0.1 M sucrose, 0.2 M NaCl, 50 mM Na/K phosphate buffer (pH 7.4) and 0.5 mM PMSF (buffer A). The cells were broken in the small chamber of an ice water-cooled blending apparatus (Bead Beater; BioSpec Products, Bartlesville, OK) half filled with 1.0 mm glass beads. Five 1-min blending periods were used with 1-min cooling intervals. The homogenate was separated from the beads by gravity filtration using a plastic sieve. The beads were rinsed with another 50 ml of buffer A. After centrifugation (2,000g, 5 min) to remove

unbroken cells and cell debris, the membranes were pelleted (50,000g, 10 min) and then resuspended in 0.3 M sucrose, 50 mM NaCl, 5 mM MgCl$_2$, 50 mM Na/K phosphate buffer (pH 6.9) at a Chl concentration of 1 mg/ml. The suspension was treated with Triton X-100 (25:1, detergent to Chl ratio) for 1 min, centrifuged (3,000g, 3 min), and the supernatant recentrifuged (40,000g, 10 min). The pellet was homogenized in 40 mM Na/K phosphate buffer (pH 6.9), centrifuged (2,000g, 3 min), and the supernatant recentrifuged (40,000g, 10 min) to yield a PSII-enriched fraction. The Chl a/b ratio of this preparation was about 2.7 to 1 (as compared to 2.0 to 1 for the spinach OES II), indicating there was still some PSI present. At this stage, the procedure described above for preparing PSII core particles from spinach was followed (*i.e.*, treatment with 1 M CaCl$_2$, solubilization with dodecyl-β-D-maltoside, and two sucrose density gradient centrifugations).

Visible absorption spectra were obtained with an Aminco DW2a dual wavelength spectrophotometer. Liquid nitrogen temperature fluorescence omission spectra (λ_{ex} = 435 nm) were acquired using a SPEX fluorolog (SPEX Industries, Inc., Metuchen, NJ).

LDS-PAGE using a 10 to 15% acrylamide gradient was as described by Delepelaire and Chua (7) with minor modifications (13). All samples were made up to 1% LDS, warmed to 50°C for 2 min, cooled to ice temperature, and then brought to 30 mM DTE with a 0.6 M stock solution.

RESULTS AND DISCUSSION

Algal core samples as prepared by the described procedure are highly enriched for PSII core complex and contain minimal amounts of PSI and light-harvesting Chl a/b complex. Figure 1 shows a visible absorption spectrum of the PSII core complexes isolated from the mutant, LF-1. The spectral characteristics, including peaks at 436 and 674 nm and the lack of a Chl b shoulder at 650 nm, are very similar to those published for spinach PSII core complexes (17, 18). The spectrum of PSII core complexes isolated from WT *Scenedesmus* is indistinguishable from that of the mutant. Figure 2 shows a low temperature fluorescence emission spectrum of the WT core complex. It has a single peak at 685 nm. Again, this is quite similar to the spectrum reported for spinach (18), and the spectrum of the LF-1 core complex is identical to that of the WT. These data provide additional evidence showing that the Chl organization of PSII
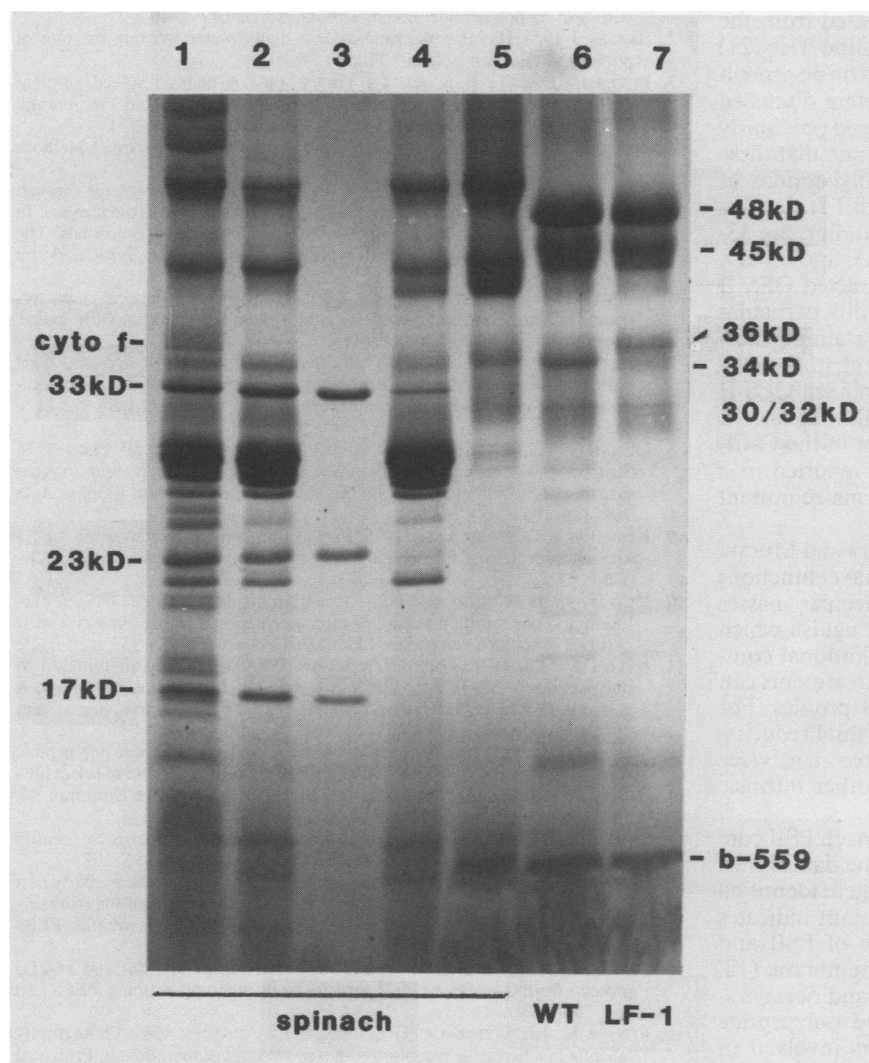
FIG. 3. Polypeptides revealed by LDS-PAGE and staining with Coomassie Brilliant Blue. Samples from spinach are: thylakoids (lane 1), OES II (lane 2), 1 M CaCl₂ extract of OES II (lane 3), calcium-extracted OESII (lane 4), and PSII core complexes (lane 5). *Scenedesmus* samples are: PSII core complexes of WT (lane 6), and of LF-1 (lane 7). Apparent molecular masses were estimated using standard proteins obtained from Bio-Rad Laboratories (not shown). The locations of Cyt *f* (cyto f) in spinach thylakoids and Cyt *b*-559 (b-559) in the core complexes are also indicated. The 45-kD reaction center band often runs as a doublet under these gel conditions.

has not been altered by the mutation (14). There is no evidence of PSI or light-harvesting Chl *a/b* complex contamination in this algal sample because expected emission peaks at 720 and 681 nm, respectively, are missing. However, PSI present on the sucrose density gradients forms a broad band just below and often extending to the PSII core band. Thus, special care must be taken when recovering PSII core complexes from algal OES II that is not necessary with spinach samples (OESII samples from spinach contain very small amounts of PSI [8]).

LDS-PAGE polypeptide analysis of spinach thylakoids (lane 1), OESII membranes (lane 2), and PSII core complexes (lane 5) as well as core complexes from *Scenedesmus* WT (lane 6) and LF-1 (lane 7) are presented in Figure 3. Both the algal core samples show four major easily observable protein bands. Minor bands present in these samples are probably due to PSI contamination. The major bands include the apoproteins of the Chl *a*-protein complexes associated with the PSII reaction center (7) at apparent molecular masses of 48 and 45 kD. The major polypeptide of Cyt *b*-559 is also present in both samples at ~8 kD. The region just below this band is obscured due to the presence of dodecyl-β-D-maltoside in the samples. Of particular interest in this report is the 30- to 36-kD region of the gel. A 34-kD protein, present in the WT core sample (lane 6), is missing in the LF-1 sample (lane 7). Instead, the mutant complex contains a 36-kD protein absent in the WT. This is identical to the protein alteration reported in thylakoid membranes from WT and LF-1

Scenedesmus (12, 14). The work of Bricker *et al.* (6), as well as the presence of the 34-kD protein in PSII core particles, indicates that it is an intrinsic membrane protein.

There is also evidence for a fifth core protein in the 30- to 32-kD region. This can be seen in all three PSII core samples (lanes 5-7), although there is no distinct band visible. The protein believed to contain the binding site for atrazine and to function on the reducing side of PSII has been reported to migrate in this region (11, 16, 18). Since whole cells and thylakoids of both WT and LF-1 show unimpaired electron transport on the reducing side of PSII and both are sensitive to PSII inhibitors, this protein should be present in this poorly resolved area of the gel. Note that in our LDS-PAGE system, which contains no urea, spinach PSII core complexes display two bands in the 40- to 50-kD region and at least two bands in the 30-to 35-kD region. The results of Satoh *et al.* (Ref. 18; Fig. 3) using SDS in the absence of urea show single bands in these two regions, and they indicate urea is required to separate the bands in the respective regions. Since protein band locations are sensitive to the electrophoretic conditions employed, the identification of the band at 30- to 32-kD in our gels as the herbicide binding protein must be considered preliminary.

Another PSII protein, not found in core particles, but which migrates in the 30- to 34-kD region, is the extrinsic 33-kD polypeptide first described by Kuwabara and Murata (9). It is present in OES II of higher plants and may participate in the

water-splitting reactions. This protein can be released from the OES II by a variety of treatments including alkaline Tris (21) and 1 M $CaCl_2$ (15). In the literature, this 33-kD extrinsic protein has often been equated to the 34-kD intrinsic protein discussed above (e.g. 1, 2, 5, 10, 11, 15). This has been discussed previously in Bricker et al. (6). The data in Figure 3 demonstrate that these two proteins are distinct. Lane 2 shows the polypeptides of spinach OES II. Treatment of this preparation with 1 M $CaCl_2$ is known to release three extrinsic polypeptides including the 33-kD extrinsic protein (15). These proteins, at 17, 23, and 33 kD, can be identified in the extract (lane 3). The extracted OES II (lane 4) retains a trace of the 33-kD protein in this particular experiment. A protein which shows mobility and staining characteristics similar to the 34-kD polypeptide present in the WT algal PSII core sample also can be observed in spinach OES II membranes (lane 2), in $CaCl_2$-extracted spinach OES II (lane 4), and in spinach PSII core particles (lane 5), but not in the $CaCl_2$ extract (lane 3). A 34-kD protein has also been reported in a maize OES II (6) and is missing in thylakoids of a maize mutant which lacks the entire PSII complex (13).

The 34-kD intrinsic protein, the 33-kD Kuwabara and Murata protein, and the herbicide-binding protein all have functions associated with PSII. The similarity in their molecular masses has, of course, sometimes made it difficult to distinguish which protein is being discussed in the literature. An additional complication arises from the observation that certain treatments can result in the loss of the 34-kD protein from gel profiles. For example, heating of the sample in the presence of thiol reducing agents decreases the amount of 34-kD protein visible on gels (see 14). This phenomenon has been observed with other intrinsic membrane proteins (e.g. 19).

Satoh et al. (18) reported a 34-kD protein in spinach PSII core particles but was unable to assign it a function. The data shown here suggest that it is homologous to the 34-kD protein identified in Scenedesmus. Previous work with the LF-1 mutant indicates that this protein functions on the oxidizing side of PSII and influences the binding of Mn on the thylakoid membrane (12, 14). The presence of the protein in both spinach and Scenedesmus PSII core particles, which have very limited polypeptide compositions, further defines its role. The factors involved in binding Mn, which is functional in oxygen evolution, are likely to be quite complex and more than one protein may be required. However, since some treatments can remove the extrinsic proteins from spinach OES II without releasing Mn required for water-splitting activity (15), we suggest that the intrinsic 34-kD protein is a binding site for functional Mn on the PSII membrane. It may also bind one or more of the extrinsic proteins.

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