PHOTOSYSTEM II COMPLEX \textit{IN VIVO} IS A MONOMER.
Takeshi Takahashi\textsuperscript{1*}, Natsuko Inoue-Kashino\textsuperscript{1,2*}, Shin-ichiro Ozawa\textsuperscript{2}, Yuichiro Takahashi\textsuperscript{2}, Yasuhiro Kashino\textsuperscript{1} and Kazuhiko Satoh\textsuperscript{1}
\textsuperscript{1}Graduate School of Life Science, University of Hyogo, 3-2-1 Kohto, Kamigohri, Ako-gun, Hyogo 678-1297, Japan and \textsuperscript{2}Graduate School of Natural Science and Technology, Okayama University, 3-1-1 Tsushima-Naka, Okayama 700-8530, Japan
*These two authors contributed equally to this work.
Address correspondence to: Yasuhiro Kashino, Graduate School of Life Science, University of Hyogo, 3-2-1 Kohto, Kamigohri, Ako-gun, Hyogo 678-1297, Japan; Phone & Fax: +81 791 58 0185; E-mail: kashino@sci.u-hyogo.ac.jp

Photosystem II (PS II) complexes are membrane protein complexes that are composed of more than 20 distinct subunit proteins. Similar to many other membrane protein complexes, two PS II complexes are believed to form a homo-dimer whose molecular mass is ~650 kDa. Contrary to this well-known concept, we propose that the functional form of PS II \textit{in vivo} is a monomer, based on the following observations. Deprivation of lipids caused the conversion of PS II from a monomeric form to a dimeric form. Only a monomeric PS II was detected in solubilized cyanobacterial and red algal thylakoids using blue-native polyacrylamide gel electrophoresis. Furthermore, energy transfer between PS II units, which was observed in the purified dimeric PS II, was not detected \textit{in vivo}. Our proposal will lead to a re-evaluation of many crystallographic models of membrane protein complexes in terms of their oligomerization status.

Photosystem II (PS II) complexes convert solar energy to biological redox energy. Through this reaction process, water molecules are oxidized and molecular oxygen is released as a byproduct (reviewed in ref. (1)), which is the only source of molecular oxygen upon which all aerobic organisms on earth rely. PS II core complexes are membrane protein complexes that are composed of more than 20 distinct subunit proteins and many functional cofactors, including chlorophylls (Chls), carotenoids, plastoquinone and metal ions (2-5). Similar to many other membrane protein complexes (6-10), two PS II core complexes are believed to associate together to form a homo-dimer with a molecular mass of ~650 kDa, as shown by crystallographic models (2,3,5).

The PS II complex turns over dynamically, although it is quite an integrated complex; our current understanding is that the PS II complex that is damaged by high light is disintegrated into a monomeric form and is further dissociated to replace a degraded D1 protein with a \textit{de novo} synthesized D1 (reviewed in refs. 11,12). After the replacement, the PS II complex is integrated into a functional form as a dimer. It is supposed that PS II subunit proteins such as PsbI (13) or PsbTc (14) participate in the formation of the PS II dimer.

Crystallographic models of PS II have enabled the determination of the accurate molecular architecture of PS II complexes, all of which are in a dimeric form. The most recent crystallographic model of the PS II dimer at 3.0Å resolution revealed the presence of six detergent molecules located at the interface of the two monomers (5). Small structural fluctuations during the purification process might allow the invasion of those detergent molecules. However, it is also probable that the PS II complexes exist in the form of a monomer \textit{in vivo} and the two distinct monomers become a dimer during the purification step incorporating detergents between their interfaces. This idea led us to investigate the actual form of PS II \textit{in vivo}.

Contrary to the above well-known dimeric model of a functional PS II core complex, here we show that the PS II core complex functions and exists in a monomeric form \textit{in vivo}.

\textbf{Experimental Procedures}

\textit{Culture of strains}
A thermo- and acidophilic red alga,
Cyanidioschyzon merolae (15), was cultivated in medium (16) at 40°C for ~one week. The HT-3 strain (17) of a mesophilic cyanobacterium, Synechocystis sp. PCC 6803 (Synechocystis 6803, hereafter), with a hexahistidyl tag at the C-terminal end of the CP47 protein (provided by Prof. T. M. Bricker, Louisiana State University) was grown as described in (4) in a BG11 medium (18) at 30°C. A thermophilic cyanobacterium, Thermosynechococcus vulcanus, was grown as in (19) in a medium reported in (20) at 56°C. A marine centric diatom, Thalassiosira pseudonana (UTEX LB FD2) (21), was grown in an F/2 medium (22) supplemented with artificial sea water (Sigma, St. Louis, MO) at 20°C.

Preparation of thylakoid membranes and purification of photosystem complexes

Cells of C. merolae were collected at 3,000 x g for 15 min and resuspended in medium A containing 10 mM MgCl₂, 5 mM CaCl₂, 50 mM MES-NaOH (pH6.0), 5% glycerol and 0.8 M betaine. The collected cells were broken at 1 mg Chl a/mL with the same volume of glass beads at 4°C by ten cycles of 5 sec breaking and 2 min cooling in the presence of DNase I (0.5 µg/mL) (Sigma) and a protease inhibitor cocktail (250 µL/100 mL cell suspension) (Sigma). Thylakoid membranes (~150 mg Chl a) were harvested by centrifuging the broken cells at 35,000 x g for 20 min and then resuspended in medium A supplemented with 175 mM NaCl, unless indicated otherwise. To purify PS II, after solubilization by 1.0% n-dodecyl-β-D-maltoside (DDM) (Anatrace, Maumee, OH) for 20 min on ice, solubilized membranes were separated from the insoluble fraction by centrifugation (35,000 x g for 20 min at 4°C) and subjected to anion-exchange chromatography (Q-Sepharose HP; GE Healthcare, Buckinghamshire, England). The resin was packed in a XK26/40 column (GE Healthcare) and was pre-equilibrated with medium A supplemented with 175 mM NaCl and 0.04% DDM. After washing-out the unbound proteins with four column volumes of equilibration medium, monomeric and dimeric PS II complexes were eluted by increasing the NaCl concentration to 150 mM at a flow rate of 1.5 mL/min and precipitated by 15% PEG 2,000, followed by resuspension in medium B (supplemented with 0.04% DDM). Size exclusion chromatography using HiPrep 16/60 Sephacryl S-300 HR (GE Healthcare) with medium B containing 0.04% DDM at a flow rate of 0.5 mL/min was performed to further purify PS I complexes. The elution was monitored at 280 nm.

Thylakoid membranes and PS II complexes from Synechocystis 6803 were prepared as described in (4). Thylakoid membranes from T. vulcanus were isolated as described in (23). To prepare thylakoid membranes from T. pseudonana, cells were collected by centrifugation at 3,000 x g for 15 min, resuspended in medium A at 1 mg Chl a/mL, and were broken with glass beads as described above. After removal of debris (1,000 x g, 10 min), thylakoid membranes were precipitated at 35,000 x g for 15 min at 4°C and resuspended in the same medium. Thylakoid membranes and PS II enriched membranes from spinach obtained in a local market were prepared as in (24).

DEAE chromatography to assess oligomeric status of photosystem II

Thylakoid membranes from C. merolae suspended in medium A at 1 mg Chl/mL were solubilized by 0.8% DDM. After removal of insoluble materials by centrifugation (35,000 x g for 20 min at 4°C), an aliquot of solubilized membranes equivalent to 0.5 mg Chl in the initial thylakoid membranes was applied to DEAE-Toyopearlpack650S (Tosoh,
Tokyo, Japan) pre-equilibrated with medium A (supplemented with 0.04% DDM) and washed with various volumes of medium A (supplemented with 0.04% DDM) at a flow rate of 1.0 mL/min. Elution was performed by increasing the concentration of NaCl linearly at a ratio of 200 mM/h at the same flow rate. The elution pattern was monitored at 280 nm.

**Electrophoretic analysis**

Blue-native polyacrylamide gel electrophoresis (BN-PAGE) was performed according to the method described in (25), with slight modifications, using a slab gel containing 5 - 10% gradient acrylamide as a resolving gel and 1.5% acrylamide as a stacking gel, at 60 V at 4°C for ~20 h. Electrophoresis was run only with slightly-blue cathode buffer without the deep-blue cathode buffer. This modification does not alter the results of our experiments, as assessed by preliminary experiments. Second-dimensional electrophoresis after BN-PAGE and the denaturing electrophoresis were performed by the method described in (26,27) with a slab gel containing 18 - 24% gradient acrylamide and 6 M urea at 11 mA (180 mA·h). Molecular mass standards were purchased from Invitrogen (Carlsbad, CA) for BN-PAGE and BioRad (Hercules, CA) for SDS-PAGE.

**Identification of polypeptides**

Western blotting and N-terminal sequencing were performed as described in (27) after BN-PAGE or denaturing electrophoresis. Polypeptides specific to antisera against PsbC (intrinsic Chl binding antenna protein, CP43, associated with PS II) and PsaA/B (PS I reaction center protein complex, CP1-e) (28) were detected with WestPico (Pierce, Rockford, IL) and Fuji LAS1000plus (FujiFilm, Tokyo, Japan). N-terminal sequences were determined using protein sequencer PSQ-1 (Shimadzu, Kyoto, Japan) and Fuji LAS1000plus (FujiFilm, Tokyo, Japan). N-terminal sequences were determined using protein sequencer PSQ-1 (Shimadzu, Kyoto, Japan). Heme was detected by chemiluminescence (29) using WestFemto (Pierce) and Fuji LAS1000plus after electroblotting onto a polyvinylidene fluoride (PVDF) membrane (30). Matrix-assisted laser desorption mass spectroscopy (MALDI) was performed on a Voyager DEPRO instrument (PerSeptive Biosystems Inc., Framingham, MA) after in-gel digestion by trypsin (Promega, Madison, WI) following a standard method, as described in (30). Identification of polypeptides by LC-MS/MS was performed as described in (31) using Finnigan LTQ (Thermo Fisher Scientific, Waltham, MA) connected to a multi-dimensional HPLC system Paradigm MS4 (Michrom BioResources, Auburn, CA). All solvents were HPLC grade from Sigma-Aldrich (St. Louis, MO), Wako (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan). The trypsin-digested and dried peptides were resuspended in a solution containing 0.2% trifluoroacetic acid (TFA) and 2% acetonitrile, passed through a C-Tip T-300 (AMR, Tokyo, Japan), and applied to Paradigm MS4. Data analysis and polypeptide identification after LC-MS/MS were performed by BioWorks (version 3.3; Thermo Fisher) (31) using a genomic database for C. merolae (15).

**Spectroscopic analysis**

Absorption spectra were recorded on a Uvikon 922 spectrophotometer (Kontron Instruments, Milan, Italy) at 5 µg Chl a/mL at room temperature. Steady-state fluorescence emission spectra at 77 K were measured using a H-20 UV monochromator (Jobin Yvon, Cedex, France) as described in (32). Chl concentration was determined by the method described in (33).

**Analysis of pigments and quinones**

The amount of plastoquinone and photosynthetic pigments were determined by reverse-phase HPLC with a Prodigy 5 (ODS 3, 100Å) column (150 × 4.60 mm) (Phenomenex Inc., Torrance, CA) equipped to a Shimadzu LC-10 AD system with a SCL-10A controller using a gradient of solvents, as described previously (34,35), after extraction using N,N-dimethylformamide (36). Extracted materials equivalent to 5 µg Chl a were applied to HPLC analysis to determine their relative molar ratios to pheophytine a.

**Lipid analysis**

Assessment of lipids was performed by TLC in chloroform:methanol:acetic acid:water (70:20:2:2, v/v/v/v). Monomeric and dimeric PS II, equivalent to 5 µg Chl a, were spotted directly onto TLC without extraction of lipids, to exclude any plausible loss of them. Lipids on TLC were detected fluorometrically using 2′,7′-dichlorofluorescein (Nacalai Tesque). Standard lipids from plants (digalactosyl diglyceride, monogalactosyl diglyceride, phosphatidyl glycerol and sulfoquinovosyl diglyceride) were purchased from Lipid Product (South Nutfield, England).

**Oxygen evolution assay**
Steady-state oxygen evolution was measured with a Clark-type electrode as described in (32) at 30°C using medium A as a reaction mixture. Duroquinone (Tokyo Chemical, Tokyo, Japan) or 2,5-dichloro-p-benzoquinone (DCBQ) (Sigma-Aldrich) was used as an artificial electron acceptor.

Assessment of energy transfer between reaction centers
Occurrence of energy transfer between reaction centers was assessed by measuring fluorescence induction using a pulse amplitude modulation fluorometer, PAM 101 (Walz GmbH, Effeltrich, Germany) in the presence of 10 µM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) and 1 mM sodium hydroxylamine at room temperature. A medium containing 10 mM MgCl₂, 5 mM CaCl₂, 1 M betaine and 50 mM HEPES (pH7.0) was used as a reaction mixture for PS II complexes, thylakoids, and C. merolae cells, and corresponding fresh culture media were used for Synechocystis 6803 and T. pseudonana. Chl a concentrations were 10 µg/mL for PS II complexes and cells, and 50 µg/mL for thylakoids. The fluorescence signal was recorded on a digital oscilloscope, VP-5710A (Panasonic, Osaka, Japan). The intensity of actinic light was 8 µmol photons/m²/s (intensity level 9 of measuring light on PAM 101).

RESULTS

Similarities in biochemical characteristics between monomeric and dimeric photosystem II complexes.
To investigate the intact architecture of PS II in vivo, monomeric and dimeric PS II complexes were purified from a thermo- and acidophilic primitive red alga, C. merolae (Fig. 1 and Supplemental Tables S1 – S3). The oligomerization statuses of the complexes were verified by BN-PAGE (25) (data not shown, but see Fig. 5). There were only small distinctions between them in terms of biochemical properties. Their polypeptide profiles (Fig. 1, lanes 1-4), including the low molecular mass region (lanes 3 and 4) with a typical composition of PS II complexes (4), were quite similar to each other. The amounts of pigments and plastoquinone, which account for around 10% of the molecular mass of PS II, were almost the same between the PS II monomer and the dimer, with additional ~2 Chl and ~3 zeaxanthin molecules in the PS II monomer (Table 1). Several additional minor polypeptides that were found in the monomer were not tightly associated with the PS II complex because they disappeared after size exclusion chromatography (Fig. 1, lanes 5 and 6), although there remains a possibility that the additional pigments in the monomer (Table 1) are bound to some of these polypeptides. Among the additional polypeptides, the only polypeptide known to be PS II-related is Psb27 (band e in Fig. 1 and Supplemental Table S3); however, it was present in quite low amounts compared with other subunits (Fig. 1). Psb27 is known to bind during the assembly process of PS II to facilitate the Mn₄Ca₁Clₓ cluster to assemble properly, preventing the advancing association of extrinsic proteins such as PsbO, PsbQ, PsbU and PsbV (37). In our PS II monomer, these extrinsic proteins were associated in an identical amount with those of the PS II dimer (Fig. 1), indicating that the amount of such pre-mature PS II was negligible in our PS II monomer. Absorption spectra at room temperature were almost identical, although the contribution of zeaxanthin in the monomer is evident (Supplemental Fig. S1A), reflecting the difference of its content (Table 1). Their 77K fluorescence spectra were identical, showing the absence of chromophores other than PS II (Supplemental Fig. S1B). Furthermore, the oxygen-evolving activity of the PS II monomer was only slightly lower than that of the PS II dimer when 2,5-DCBQ was used at 0.5 mM as an artificial electron acceptor; 3,040 and 3,090 µmoles O₂/mg Chl/h were shown by the monomeric and dimeric PS II, respectively, demonstrating that these complexes were intact (see below, and Table 2).

Deprivation of lipids causes the conversion of PS II from a monomeric form to a dimeric form.
The most prominent feature of the PS II monomer on the electrophoresis profile is that a huge amount of lipids were present, which disturbed the precise resolution of low molecular mass polypeptides (Fig. 1, lane 1). TLC analyses of lipids supported the electrophoresis profile, in that the amount of lipids, such as sulfoquinovosyl diglyceride, was remarkably higher in the monomer than in the dimer (Fig. 1, lanes 7 and 8), which agrees with previous reports (38,39). These
results suggest that lipid deprivation leads to the conversion of a monomer to a dimer. To clarify if there are any relationships between the amount of the PS II dimer and removal of lipids, PS II complexes after solubilization of thylakoids were bound to a DEAE column and washed to remove lipids with variable amounts of medium containing 0.04% DDM. After washing, the NaCl concentration was linearly increased to elute monomeric and dimeric PS II complexes (Fig. 2). As is frequently observed (14,40), monomeric PS II was eluted at lower NaCl concentrations than dimeric PS II (Figs. 2 and 3). When the washing volume was small, the monomeric form of PS II was dominant and the relative amount of PS II monomer to dimer decreased with increasing amounts of wash volume (Figs. 2 and 4), indicating that deprivation of lipids caused the conversion of originally existing monomer to the dimer.

This experiment was performed using a DEAE column to avoid any side-effect of higher concentrations of salts, because PS II complexes were eluted at higher salt concentrations when Q-sepharose was used. When PS II complexes purified by use of Q-sepharose, were applied to the DEAE column, monomeric PS II were converted to a dimeric form by extensive washing while dimeric PS II remained to be a dimer (data not shown).

**Form of photosystem II assessed by blue-native gel electrophoresis.**

Thylakoid membranes solubilized by DDM were then applied directly to BN-PAGE to investigate the oligomerization status of PS II (Fig. 5 and Supplemental Figs. S2A and S3). When thylakoids from C. merolae were solubilized by 1.2% DDM, a sharp green band corresponding to the PS II monomer was distinguished (Fig. 5A and Supplemental Fig. S2A, lane 3, asterisk), although the molecular mass (450 kDa) was slightly larger than the purified PS II monomer (380 kDa, lane 1 in Fig. 5A and Supplemental Fig. S2A) but smaller than the purified PS II dimer (550 kDa, lane 2 in Fig. 5A and Supplemental Fig. S2A). By reducing the DDM concentration, the migration length of PS II monomer decreased, and the shape of the bands became obscure, which could be ascribed to an increase in bound lipids corresponding to the decrease in the detergent concentration. However, the migration length was clearly longer than the purified PS II dimer. Western blotting showed that the green bands with higher molecular weights were PS I complexes (open circle in Fig. 5), and the PS II dimer was below the detection level (Supplemental Figs. S2A and S3).

All crystallographic models of PS II have been obtained from dimeric forms of cyanobacterial PS II complexes, although monomeric cyanobacterial PS II complexes can be obtained (2,3,5,40,41). Therefore, the thylakoids from a mesophilic cyanobacterium, *Synechocystis* 6803, and a thermophilic cyanobacterium, *T. vulcanus* (3), were also examined. Only monomeric forms of PS II complexes (asterisks) were detected when solubilized thylakoids were directly applied to BN-PAGE (Fig. 5B and Supplemental Fig. S2B).

**Form of photosystem II assessed by photochemical reaction.**

The biochemical analyses described above strongly suggest that PS II complexes exist as a monomer in thylakoids. To confirm these results, photochemical reactions in PS II in the absence of detergent were also measured, which will exclude the possibility of plausible artifact(s) in the above results derived from the presence of detergents. In green plants, PS II core complexes are associated with 6-12 copies of light-harvesting Chl-binding proteins (LHCII) (42) and are located in the grana region of the thylakoid (43). PS II reaction centers are connected directly or via surrounding LHCII (44). In such a system, light energy captured by a PS II core complex with a closed PS II reaction center has been shown to be transferred to the neighboring connected open reaction centers (44), irrespective of the two conflicting reports on the oligomerization status of PS II based on image analysis of two-dimensional crystals (45,46). This phenomenon can be assessed by the induction kinetics of fluorescence emitted from PS II upon illumination in the presence of DCMU (44,47,48), an inhibitor of PS II. If there was no energy transfer between reaction center units, the fluorescence would increase exponentially, and if there was transfer of light energy between reaction center units, the fluorescence kinetics would show a sigmoidal increase (44,47,48). This sigmoidicity has been used as a simple assessment for the exiton migration between PS II units (49,50).
Fluorescence from spinach PS II retaining LHCII (51) and spinach thylakoids showed sigmoidal increases (Fig. 6), as has been previously reported (44,47). The proportions of sigmoidal phase (so-called PS IIα) assessed by semilogarithmic plots of the kinetics of the integrated fluorescence deficit (48) were 69% (±1.0, n=3) and 64% (±0.65, n=3), respectively, which are consistent with the reported PS IIα ratio (e.g., (48)). The purified PS II dimer from C. merolae showed only a sigmoidal component upon illumination (n=3) (Fig. 6), confirming the occurrence of energy transfer between the reaction centers. In contrast, the purified PS II monomer from C. merolae showed only a monophasic component (n=3) (Fig. 6), verifying no energy transfer between the reaction center units. Accordingly, this measurement gives us a way to address the oligomerization status of PS II in thylakoids and cells as was used in (49). Both thylakoids and cells showed only a monophasic fluorescence component (n=3), indicating that excitation energy transfer between PS II units does not occur (Fig. 6).

The same measurements were also subjected to cyanobacteria (Synechocystis 6803 and T. vulcanus) and a diatom (a centric diatom, T. pseudonana) (Fig. 6). Again, only monophasic increases of fluorescence were observed in these materials, although the increasing rates and the fluorescence Fo levels were variable, which is consistent with the previously published similar measurements in red algae and cyanobacteria (32,52-54).

**Activity of photosystem II depends on the hydrophobicity of electron acceptors.**

The value of Vmax obtained for 2,5-DCBQ was smaller in the monomer than in the dimer (3,870 vs. 4,380 µ moles O2/mg Chl/h, Table 2). The values of Km were comparable between the monomer and dimer (49.5 vs. 50.3 µM, Table 2). When duroquinone was used as an electron acceptor, Km was also comparable between the monomer and dimer (85.5 vs. 82.3 µM, Table 2). However, Vmax was larger in the monomer than in the dimer (1,830 vs. 1,570 µmoles O2/mg Chl/h, Table 2) showing an inverse trend to 2,5-DCBQ.

**DISCUSSION**

In this work, we showed that PS II functions mostly in a monomeric form in vivo, contrary to the widely accepted concept that the functional form of PS II complexes is the dimer. This conventional concept is consistent with and stands on the crystallographic models of PS II reported so far (2,3,5,40,41). However, it is natural that the crystallographic models of PS II are dimers because all of the PS II crystals were made using purified PS II dimers. One prerequisite for obtaining crystals of membrane protein complexes is to remove lipids (26). As we have shown in this work (Figs. 2 and 4), the extensive removal of lipids, on purpose or unintentionally, during the purification steps for the crystallization work could result in the high yield of PS II dimer. The side surface opposing the interface between the two monomers in dimeric PS II might be specific to the formation of dimer, because the loss of PsbTc that locates at such an interface results in a decrease in the ratio of dimeric PS II (14).

To our knowledge, no direct evidence on the in vivo form of PS II complexes has been reported. The model of the PS II dimer is primarily based on freeze-fracture electron microscopic studies and crystallographic models. The freeze-fracture studies revealed the presence of pairs of 10 nm particles associated with phycobilisomes, which were suggested to be the dimeric forms of PS II (55-57). PS II monomers could associate together to form a dimer in some physiological statuses. Actually, Wollman reported that 10 nm particles observed on the exoplasmic fracture (EF) face are attributable to PS II and are apparently able to aggregate once the phycobilisomes are detached in Cyanidium caldarium (58). Freeze-fracture is very useful to address the configuration of biological membranes. However, the definite identification of 10 nm particles remains to be determined because of the limited resolution. In higher plants, two conflicting results on the oligomerization status of PS II are reported using image analyses of two-dimensional crystals (45,46), which were also obtained by use of detergents. There are also many reports that show the PS II dimer on BN-PAGE (e.g., refs. (11,59,60)). In some reports, higher or comparable amounts of monomer relative to dimer were found, although the authors concluded that the monomer is a transient status of PS II biogenesis (11,60). The use of detergents to solubilize membrane...
protein complexes could affect the appearance of the dimer on BN-PAGE, because the concentration of DDM seemed to affect the amount of lipids associated with PS II complexes (Fig. 5). Solubilization at a lower concentration of DDM resulted in diffuse bands, both of PS II and PS I, and some parts of them did not migrate into the resolving gel although there was no appearance of dimeric PS II (Fig. 5 and Supplemental Fig. 3). More heterogeneous amount of lipids associated with individual PS II complexes solubilized with lower concentrations of DDM could result in the defused band shape. Therefore, aiming to obtain distinct bands of membrane protein complexes, higher concentrations of detergents are used in many reports for the analyses on BN-PAGE.

To exclude the effects of detergents described above when assessing the form of PS II complexes in vivo, we measured photochemical reactions in the absence of detergents using thylakoids and cells of not only of primitive red alga, but also from cyanobacteria and a diatom. Differing from purified PS II complexes, PS I complexes are present and phycobilisomes are attached to PS II complexes in cells and thylakoids in red algae and cyanobacteria. In general, the fluorescence yield of PS I is quite low at room temperature (61), and the variable fluorescence can be attributed to PS II only. Presence of phycobilisomes can increase the antenna size of PS II, which increases the rate of QA reduction, but cannot change the fluorescence induction pattern unless the PS II core complexes are connected through the large linker protein of the phycobilisome. The presence of phycobilisomes also contributes to the increase of Fo level. In our measurements, energy transfer between PS II units, which can be determined by the sigmoidal fluorescence increase as observed in dimeric PS II, was barely detected in vivo (Fig. 6). This result strongly indicates that most of the population of PS II functions in a monomeric form in vivo. Differing from the materials above, energy transfer between the PS II units was observed in spinach thylakoids and PS II preparations (Fig. 6). This is consistent with the previous reports (44,47,62). In this sense, the relationship between individual PS II reaction center units in non-green plants is quite different from that in green plants, whose PS II complexes are assembled in the grana region and are connected directly or via surrounding LHCII.

The remaining problem opposing our conclusion is that the oxygen-evolving activity in the PS II monomer is generally lower than in the dimer (e.g., ref. (39), and Table 2). However, based on our present investigation, this can be explained by higher amounts of lipids in the monomer, which affects the accessibility of artificial electron acceptors to the secondary electron acceptor Qb site (23). The artificial electron acceptor, 2,5-DCBQ, is known to support a high rate of oxygen evolution by accepting electrons via both the first and the second intrinsic electron acceptor, QA and Qb (23), and is commonly used at around 0.5 mM to assess oxygen evolving activity. At lower concentrations, it accepts electrons via Qb plastoquinone, but at higher concentrations, it replaces Qb plastoquinone and accepts electrons directly from QA, resulting in a lower activity (23). Another artificial electron acceptor, duroquinone, was shown to accept electrons only via the secondary intrinsic electron acceptor Qb not directly from QA (23). The hydrophobicity of quinones can be evaluated by the values of CLogP, the calculated logarithm of the partition coefficient; the more hydrophobic a compound is, the greater its CLogP. The CLogP values of 2,5-DCBQ and duroquinone are 1.25 and 2.63, respectively (63). The difference of Vmax can be interpreted as the difference of the abundance of lipids resulting in the different turnover rates of the artificial electron acceptors at the Qb sites. Abundant lipids in the PS II monomer might become a physical barrier for the less hydrophobic 2,5-DCBQ to access to the QB site resulting in a lower Vmax. Inversely, the abundant lipids might assist the accessibility to the Qb site for more hydrophobic duroquinone that accept electrons through Qb. Therefore, the rate of oxygen evolution can be determined by the accessibility and mobility of 2,5-DCBQ and duroquinone to the Qb site in the lipids around the Qb site.

PS II complexes are embedded in the lipid layer (thylakoid membrane), therefore our results indicate that most of the population of PS II functions and exists in a monomeric form in vivo. The stoichiometry of ~1 between isolated PS II and the bound light-harvesting phycobilisome suggested in T. elongatus (64) (formerly
Synechococcus sp.) agrees with the monomeric existence of PS II in vivo, although other stoichiometry is also reported in cyanobacterial and red algal cells/thylakoids (65-68). Our conclusion is important, because it will call for a novel hypotheses for the function of some PS II subunit proteins, the association of antenna pigment-protein complexes, and the processes of assembly and repair of PS II, due to the fact that the current hypotheses stand on the premise that the functional PS II in vivo is a dimer. Furthermore, the oligomerization status of many crystallographic models of membrane protein complexes may need to be re-evaluated because the protein complexes were purified using processes similar to the purification of PS II complexes.

REFERENCES


**FOOTNOTES**

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1The abbreviations used are: BN-PAGE, blue-native polyacrylamide gel electrophoresis; Chl, chlorophyll; ClogP, the calculated logarithm of the partition coefficient; 2,5-DCBQ, 2,5-dichloro-p-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DDM, n-dodecyl-ß-D-maltoside; LHCII, light-harvesting Chl-binding proteins; MALDI, Matrix-assisted laser desorption mass spectroscopy; PEG, polyethylene glycol; PS I and PS II, photosystem I and II; TFA, trifluoroacetic acid.

**FIGURE LEGENDS**

*Fig. 1.* Polypeptide and lipid profiles of purified monomeric and dimeric photosystem II. Polypeptides were separated on a gel and stained with Coomassie. The lipids were separated on TLC. Lane M, molecular mass standards; lanes 1, 3, 5 and 7, monomeric PS II complex; lanes 2, 4, 6 and 8, dimeric PS II complex; lanes 1 and 2, PS II complexes without lipid removal; lanes 3 and 4, PS II complexes after lipid removal; lanes 5 and 6, PS II complexes after size-exclusion chromatography. Lanes 7 and 8, lipids separated on TLC. PS II complexes equivalent to 5 µg Chl were used in each lane. Identity on the right side of lane 4 was determined in Supplemental Tables S1 and S2. The polypeptide bands indicated by dots (a–g) are identified in Supplemental Table S3. The sizes of molecular mass standards are given on the left. A, sulfoquinovosyl diglyceride; B, phosphatidyl glycerol; C, monogalactosyl diglyceride; D, Chl.

*Fig. 2.* Chromatogram of the elution of monomeric and dimeric PS II complexes after variable volumes of washing. The volumes used for washing before the gradient increase of NaCl concentration are shown on
each panel. Volume zero is set to the time when the increase of NaCl concentration was started. The gradient of NaCl concentration is given on the top chromatogram. D, dimer; M, monomer. The oligomerization statuses of these fractions were verified by BN-PAGE in Figure 3.

**Fig. 3.** Separation patterns of two fractions obtained in Supplemental Figure S2 on BN-PAGE. Washing volumes corresponding to those in Figure 2 are given on the top. Lane A, first fraction in Figure 2 representing monomeric form of PS II; lane B, second fraction in Figure 2 representing dimeric form of PS II. Top panel, picture before Coomassie-staining; bottom panel, picture after Coomassie-staining. The positions of molecular mass standards are shown on the left.

**Fig. 4.** Inter-conversion from monomeric form (open circle) to dimeric form (closed circle) of photosystem II complexes. The relative ratios were calculated based on the areas of monomer and dimer fractions eluted after washing with various volumes of buffer shown in Figure 2.

**Fig. 5.** Polymerization status of photosystem II in thylakoid membrane assessed by blue-native polyacrylamide gel electrophoresis. Chl-binding protein complexes in thylakoid membranes from a red alga, *C. merolae* (panel A), and two cyanobacteria (panel B), are separated on BN-PAGE. In panel A, thylakoids were solubilized by adding 1.2 (lane 3), 1.0 (lane 4), 0.8 (lane 5) and 0.6% (w/v) (lane 6) DDM. Lanes 1 and 2, purified monomeric and dimeric PS II complexes, respectively. In panel B, thylakoid membranes from *Synechocystis* 6803 (lanes 4 - 7) and *T. vulcanus* (lanes 8 - 11) were solubilized by adding 1.2 (lanes 3 and 8), 1.0 (lanes 4 and 9), 0.8 (lanes 5 and 10) and 0.6% (w/v) (lanes 6 and 11) DDM. Lanes 1 and 2, purified monomeric and dimeric PS II complexes from *C. merolae*, respectively; lane 3 in panel B, PS II complexes purified from HT3 strain of *Synechocystis* 6803; lane M, molecular mass marker. The sizes of molecular mass markers are 1,048, 720, 480, 242, 146 and 66 kDa from the top to bottom. Gels were stained by Coomassie. The bands that were green in color before Coomassie-staining (Supplemental Fig. S4) are indicated by asterisks and open circles correspond to the PS II monomer and the PS I complex, respectively.

**Fig. 6.** Assessment of energy transfer between photosystem II reaction centers. Fluorescence induction kinetics were recorded using PS II complexes, thylakoid membranes (thy), cells (cell) of spinach, *C. merolae* (*Cm*), *Synechocystis* 6803, *T. vulcanus* (*Tv*) and *T. pseudonana* in the presence of 50 µM DCMU. The scales of abscissa are adjusted depending on the rising rate of fluorescence. Fluorescence was normalized to the averaged intensity between 8 and 9 s after onset of actinic light. Actinic light was turned-on at time 1 s.
Table 1. Contents of pigments and plastoquinone in purified monomeric and dimeric photosystem II complexes. The amounts of pigments and plastoquinone were determined by HPLC. The values are expressed based on 2 pheophytine (Phe) \( a \). (\( n = 5 \))

<table>
<thead>
<tr>
<th></th>
<th>Chl ( a ) (mol/mol)</th>
<th>Zeaxanthin (mol/mol)</th>
<th>( \beta )-Carotene (mol/mol)</th>
<th>Plastoquinone (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>37.0 (±0.5)</td>
<td>4.8 (±0.3)</td>
<td>9.4 (±0.1)</td>
<td>2.2 (±0.1)</td>
</tr>
<tr>
<td>Dimer</td>
<td>35.2 (±0.2)</td>
<td>2.1 (±0.1)</td>
<td>9.4 (±0.3)</td>
<td>2.2 (±0.1)</td>
</tr>
</tbody>
</table>

Table 2. Dependency of oxygen evolving activity on the artificial electron acceptor quinones. The rates of oxygen evolution were measured at 30°C with various concentrations of 2,5-DCBQ and duroquinone at 2 µg Chl/mL. The plots were fitted using data points with quinone concentrations below the occurrence of inhibition the rectangular hyperbola using a Lavenberg Marquardt regression algorithm by KaleidaGraph ver. 4.0 for Macintosh (Synergy Software, Reading, Pennsylvania, USA), and Vmax and Km were obtained from the resulting regression coefficients.

<table>
<thead>
<tr>
<th></th>
<th>Duroquinone</th>
<th>2,5-DCBQ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (µM)</td>
<td>Vmax (µmol O(_2)/mg Chl/h)</td>
</tr>
<tr>
<td>Monomer</td>
<td>85.5 (±6.6)</td>
<td>1,830 (±54.7)</td>
</tr>
<tr>
<td>Dimer</td>
<td>82.3 (±4.7)</td>
<td>1,570 (±35.8)</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3
Figure 6

Fluorescence (RU)

Time (sec)

Spinach PS II
Spinach thy
Cm PS II dimer
Cm PS II monomer

Cm thy
Cm cell

Synechocystis thy
Synechocystis cell

Tv thy
Tv cell
Thalassiosira thy
Thalassiosira cell
Photosystem II complex in vivo is a monomer
Takeshi Takahashi, Natsuko Inoue-Kashino, Shin-ichiro Ozawa, Yuichiro Takahashi, Yasuhiro Kashino and Kazuhiko Satoh

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