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Properties of *Chlamydomonas* Photosystem II Core Complex with a His-Tag at the C-Terminus of the D2 Protein

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A His-tagged PSII core complex was purified from recombinant *Chlamydomonas reinhardtii* D2-H thylakoids by single-step Ni^{2+}-affinity column chromatography and its properties were partially characterized in terms of their PSII functions and chemical compositions. The PSII core complex that has a His-tag extension at the C-terminus of the D2 protein evolved oxygen at a high rate of 2,400 μmol (mg Chl)$^{-1}$ h^{-1} at the optimum pH of 6.5 with ferricyanide and 2,6-dichlorobenzoquinone as electron acceptors in the presence of Ca^{2+} as an essential cofactor, and approximately 90% of the activity was blocked by 10 μM DCMU. The core complex exhibited the thermoluminescence Q-band but not the B-band regardless of the presence or absence of DCMU, although both bands were observed in the His-tagged thylakoids. The core complex was free from PSI and contained one Y_D, Tyr 160 of the D2 protein, four Mn atoms, two cytochrome *b-559*, about 46 Chl *a* molecules, and probably one Q_A, the primary acceptor quinone of PSII. It was inferred from these results that His-tagging at the C-terminus of the D2 protein does not affect the functional and structural integrity of the PSII core complex, and that the 'His-tag strategy' is highly useful for biochemical, physicochemical, and structural studies of *Chlamydomonas* PSII.

Key words: *Chlamydomonas reinhardtii* — D2 protein — His-tag — Oxygen evolution — PSII core complex — Purification.

PSII performs light-driven electron transfer from water to plastoquinone in thylakoids of oxygen-evolving organisms such as higher plants, cyanobacteria and algae. The PSII core complex is the structural minimum required for evolution of molecular oxygen from water and consists of more than 10 subunit proteins including the heterodimer of the D1 and D2 proteins, Chl-binding inner antenna proteins of CP43 and CP47, a hemoprotein of cytochrome *b-559*, and several low-molecular-weight membrane proteins (see Debus 1992 for review). To elucidate the mechanism of oxygen evolution, numerous investigations have been done on a variety of PSII preparations obtained from various organisms, using multidisciplinary approaches including biochemical, biophysical, molecular-genetical, or crystallographical studies (Nixon et al. 1992, Britt 1996). The genetical approach has been considered to be promising among others, and a number of amino acids in cyanobacterial PSII reaction center proteins have been replaced to those with opposite charges or without chemical bonding side chains (see Vermaas 1993 for review). In many of these studies, however, phenotypes of the resulting mutant cells have been examined without isolating the PSII core complex, although use of purified PSII core complexes is more desirable to analyze the details of functional or structural changes brought about by the mutation.

A green alga, *Chlamydomonas reinhardtii*, undergoes oxygenic photosynthesis and its PSII resembles that of higher plants with respect to the light-harvesting system (Glazer 1989). Chloroplast gene of *C. reinhardtii* can be transformed like in cyanobacteria by means of homologous recombination, and the resulting recombinant cells can be grown heterotrophically in acetate-containing culture media, even if their photosynthetic functions are impaired by the mutagenesis. We can make better use of these advantages if a *C. reinhardtii* PSII core complex can be prepared more easily. An Oxygen-evolving PSII core complex of *C. reinhardtii* was first purified by Bumann and Oesterhelt (1994) from DM-solubilized thylakoids of wild-type cells by use of ultracentrifugation and ion-exchange chromatography followed by size-exclusion chromatography. Although their preparation has been reported to evolve oxygen at a high rate of 1,000 μmol (mg Chl)$^{-1}$ h^{-1}, the preparation does not always seem to be widely used, presumably because of the complicated isolation procedures. Stimulated by the successful isolation of *Rhodobacter spheroides* reaction center by use of 'His-tag strategy' (Goldsmith and Boxer 1996), we have constructed a transformant *C. reinhardtii* D2-H cell which carries an affinity tag consisting of consecutive six histidine

Abbreviations: BQ, *p*-benzoquinone; DCBQ, dichlorobenzoquinone; DM, *n*-dodecyl β-D-maltoside; EPR, electron paramagnetic resonance; LHC, light-harvesting complex; P700, reaction center of PSI; Q_A, primary acceptor quinone of PSII; Q_B, secondary acceptor quinone of PSII; Y_D, tyrosine 160 of the D2 protein.

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residues at the C-terminus of the D2 protein and succeeded in rapid isolation of an oxygen-evolving PSII core complex (Sugiura et al. 1998). In the present study, we have characterized some of the properties of our His-tagged PSII core complex. The results suggest that introduction of His-tag at the C-terminus of the D2 protein gives rise to no harmful influence on PSII functions, and the complex isolated by 'His-tag strategy' is free from PSI contaminations.

Materials and Methods

C. reinhardtii wild-type 2137 cells and transformant D2-H cells expressing *psbD* with an extension of hexa-histidine residues were grown as described (Sugiura et al. 1998). A His-tagged PSII core complex was isolated from thylakoids of the D2-H strain according to Sugiura et al. (1998) and precipitated from the affinity column eluate by centrifugation at $12,000 \times g$ for 5 min at 4°C after dilution with the same volume of ice-cold suspension buffer (40 mM MES/NaOH (pH 6.5), 10 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM NaCl and 25% glycerol) supplemented with 20% polyethylene glycol-3,000. Pellets were resuspended with the suspension buffer and stored in liquid N_2. Chl concentrations were determined according to Arnon (1949). SDS-PAGE of PSII core complex was done with a 16–22% gradient gel containing 7.5 M urea as described by Ikeuchi and Inoue (1988).

Fluorescence emission spectra were measured at 77 K using a Hitachi 850 fluorometer (Tokyo, Japan). Samples were suspended in 40 mM MES/NaOH (pH 6.5) containing 10% polyethylene glycol-4,000 and kept in darkness for 10 min before frozen in liquid N_2. Absorption spectra were recorded at room temperature using a Shimadzu UV-3100PC spectrophotometer (Kyoto, Japan) at a 2-nm resolution. Cytochrome *b-559* was assayed by means of reduced-minus-oxidized difference absorption spectra according to Fujita and Murakami (1987) using a Hitachi 557 spectrophotometer. Redox difference molar extinction coefficient of 15 $mM^{-1}\ cm^{-1}$ at 559 nm (Garewal and Wasserman 1974) was used.

EPR spectra were recorded with a JEOL JES-FE1XG EPR spectrometer (Tokyo, Japan) as described by Miller and Brudvig (1991). Samples were illuminated at 273 K for 30 s to fully induce the Y_D^+ signal, cooled to 77 K to trap Y_D^+, and then the Y_D^+ spin density was determined by use of double integrated intensity of the Cu^{2+} signal of $CuSO_4$ crystals measured separately as a standard (Alger 1968). After the EPR measurement, Chl content of the same sample was determined, and then the number of Chl molecules per Y_D^+ was calculated. This method yielded a value of 213 ± 20 Chl molecules per PSII reaction center when applied to spinach BBY membranes (Berthold et al. 1981). The intensities of Y_D^+ signal in thylakoids and PSII core complexes were estimated from the heights of the low-field-side shoulders of the Y_D^+ spectra in order to avoid overestimation due to overlapping of free radicals.

Bound Mn was determined with a Shimadzu atomic absorption spectrophotometer AA-640-13 (Kyoto, Japan) equipped with a GFA-3 graphite furnace atomizer as described by Ono and Inoue (1983). Oxygen evolution was measured with a Clark-type oxygen electrode at 25°C in a reaction medium containing 40 mM MES/NaOH (pH 6.5), 0.2 M sucrose, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM NaCl using appropriate electron acceptors.

Thermoluminescence glow curves were recorded by essentially the same method as described by Ohad et al. (1990). Thylakoids (50 μg Chl) or PSII core complexes (10 μg Chl) were suspended in 50 μl of 40 mM MES/NaOH (pH 6.5), 0.2 M sucrose, 10 mM $MgCl_2$, 10 mM NaCl, and 20% glycerol. For generation of $S_2Q_A^-$ and $S_2Q_B^-$ charge separated states, the suspensions were kept in darkness for 20 min at 5°C, and then excited at −10°C by a saturating actinic flash (~2 μs duration) from a Xenon lamp.

Results

In our previous study, we reported a purification strategy of oxygen-evolving PSII core complexes from *C. reinhardtii* D2-H cells, in which the C-terminus of the D2 protein was modified by attaching a His-tag (Sugiura et al. 1998). The oxygen-evolving activity of the His-tagged PSII core complex purified by a single-step Ni^{2+}-affinity column chromatography was measured with various electron acceptors. As compiled in Table 1, the highest rate of oxygen evolution was 2,400 μmol $(mg\ Chl)^{-1}\ h^{-1}$ when 2 mM ferricyanide plus 0.5 mM 2,6-DCBQ was used as the electron acceptor in the presence of 10 mM $CaCl_2$ at pH 6.5. Omission of $CaCl_2$ caused approximately 60% reduction of the activity even in the presence of the same electron acceptor. In general, oxygen-evolving PSII core complexes of spinach and cyanobacteria require $CaCl_2$ as high as 10 mM to express their maximum rates (Ghanotakis et al. 1984, Ikeuchi et al. 1985, Pauly et al. 1992) and to be resistant against photoinhibition (Boussac and Ratherford 1988). This Ca^{2+} requirement is ascribed to the absence of the extrinsic 23- and 17-kDa polypeptides (see Debus 1992 and Nugent 1996 for reviews). The His-tagged PSII core complex seemed to contain neither the 23- nor 17-kDa polypeptides, since none of these two polypeptides could be detected in the supernatant obtained by treatment of the complex with 1 M NaCl or 1 M Tris (data not shown). This is why 10 mM $CaCl_2$ was required for the His-tagged PSII core complex to express the maximum oxygen evolution. Among the electron acceptors tested, combined use of ferricyanide plus 2,6-DCBQ was most effective as compared with single use of ferricyanide, 2,6-DCBQ or BQ. Judging from its relatively high efficiency as an electron acceptor, ferricyanide appears to accept electrons from Q_A. However, it should be noted that approximately 90% of the activity was inhibited by 10 μM DCMU (Table 1). This suggests that the Q_B site of the His-tagged PSII core complex retained high affinity for DCMU as in wild-type or His-tagged thylakoids, although the site in the isolated core complex seemed to be depleted of the Q_B quinone molecule as will be discussed later. In contrast, the His-tagged thylakoids exhibited the same rate of oxygen evolution regardless of the presence or absence of $CaCl_2$.

Figure 1 shows the room temperature absorption spectra of His-tagged thylakoids and His-tagged PSII core complex obtained from D2-H cells. Both preparations showed a red absorption maximum at 675 nm, and the A_{675}/A_{650} absorbance ratio was 2.1 for thylakoids but in-

Table 1 Dependence on electron acceptors and Ca^{2+} of oxygen evolution by His-tagged PSII core complex isolated from *C. reinhardtii* D2-H cells

Electron acceptor	$CaCl_2$ (10 mM)	DCMU (10 μM)	Oxygen evolution μmol (mg Chl)$^{-1}$ h^{-1}	Rel. %
Ferricyanide (2 mM)/2,6-DCBQ (0.5 mM)	+	−	2,430	100
Ferricyanide (2 mM)/2,6-DCBQ (0.5 mM)	−	−	1,030	42
Ferricyanide (2 mM)	+	−	1,480	61
2,6-DCBQ (0.5 mM)	+	−	1,210	50
BQ (0.3 mM)	+	−	1,080	45
Ferricyanide (2 mM)/2,6-DCBQ (0.5 mM)	+	+	335	14

creased to 6.3 for the PSII core complex, indicating extensive removal of Chl *b* during the isolation procedure. In the spectrum of the PSII core complex, the A_{450}/A_{500} absorbance ratio, an index for the content of carotenoid species, was clearly decreased, and a low but significant peak was seen at around 545 nm, indicative of enrichment of pheophytin *a*. These spectral features of His-tagged PSII core complex were identical to those of so far reported PSII core complexes prepared from *C. reinhardtii* wild-type (Bumann and Oesterhelt 1994), a cyanobacterium, *Synechocystis* sp., (Rögner et al. 1990), or spinach (Tang and Satoh 1985).

The polypeptide composition of the purified His-tagged PSII core complex was analyzed by SDS-PAGE containing urea as shown in Fig. 2. The PSII core complex consisted of CP47, CP43, D2, D1, extrinsic 33 kDa protein and cytochrome *b-559* with apparent molecular masses of 47, 44, 32, 30, 28 and 10 kDa, respectively. There was no detectable contamination of PSI in the preparation as judged from the CBB-staining profile of the SDS-PAGE. The absence of PSI was further confirmed by means of fluorescence and EPR measurements. Upon excitation at 435 nm, His-tagged thylakoids gave a 77 K emission spectrum as shown in Fig. 3A. The emission band at 710 nm is attributed to PSI Chl, and the emission band at 685 nm to PSII Chl. These fluorescence features of His-tagged thylakoids were identical to those of thylakoids from wild-type cells (Shim et al. 1990). The 77 K emission spectrum of the PSII core complex measured under the same conditions exhibited only the major single emission band at 685

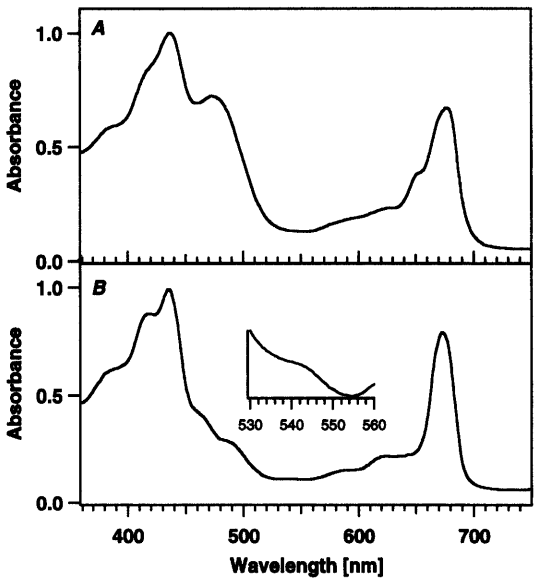

Fig. 1 Room temperature absorption spectra of His-tagged thylakoids (A) and His-tagged PSII core complex (B) isolated from *C. reinhardtii* D2-H cells. Inset in panel B: expanded spectrum in 530–560 nm region.

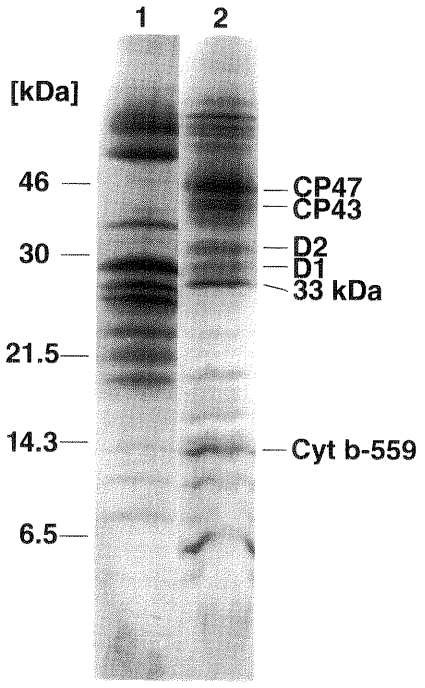

Fig. 2 SDS-PAGE profile showing the polypeptide composition of the PSII core complex fraction obtained by Ni^{2+} affinity column chromatography. Lane 1, His-tagged thylakoids (20 μg of Chl) from D2-H cells; lane 2, His-tagged PSII core complex eluted from Ni^{2+} affinity column by imidazole (8 μg of Chl).

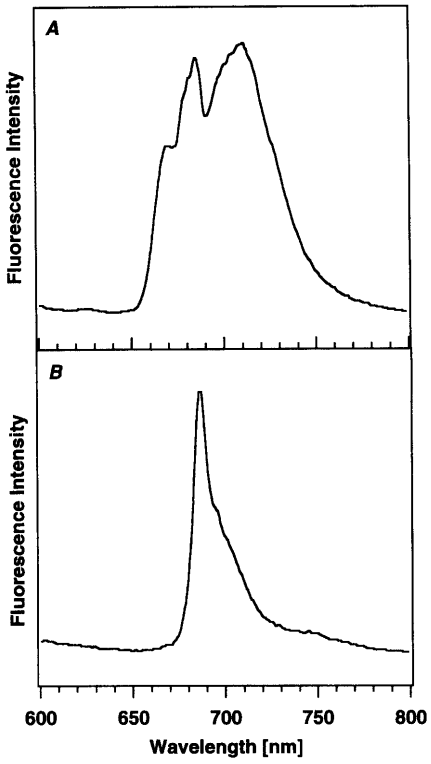

Fig. 3 77 K fluorescence emission spectra of His-tagged thylakoids (A) and His-tagged PSII core complex (B) isolated from *C. reinhardtii* D2-H cells. Chl concentrations were 0.4 μg and 0.2 μg Chl ml^{-1} for (A) and (B), respectively. Excitation wavelength was 435 nm.

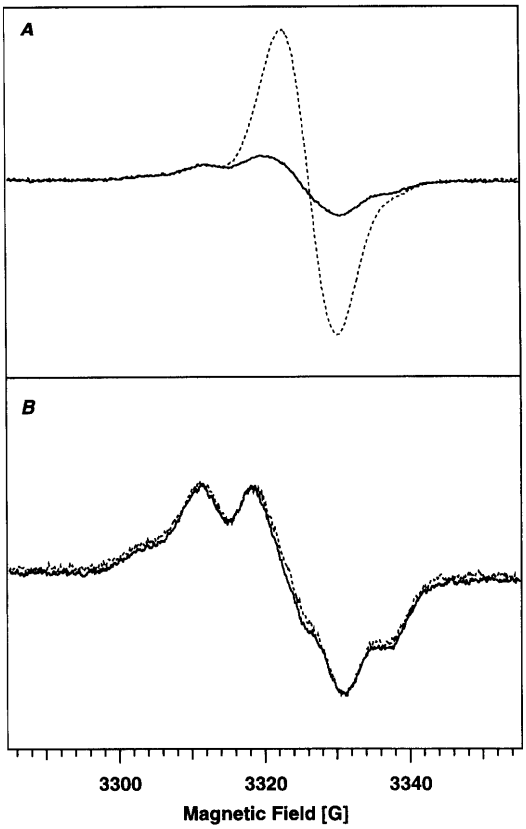

Fig. 4 EPR spectra of His-tagged thylakoids (A) and His-tagged PSII core complex (B) isolated from *C. reinhardtii* D2-H cells. The dotted- and the solid-curves indicate spectra measured in the presence and absence of 5 mM ferricyanide, respectively. Chl concentrations of thylakoids and isolated PSII core complex were 3 mg and 0.6 mg Chl ml^{-1}, respectively. Measuring conditions were as follows: microwave frequency, 9.3 GHz; microwave power, 0.05 mW; modulation frequency, 100 kHz; field modulation, 5 G; time constant, 0.3; scan time, 2 min; gain, 1×10^3.

nm, and notably, the band at 710 nm arising from PSI Chl was extremely diminished (Fig. 3B), indicative of excessive removal of PSI.

The absence of PSI in our His-tagged PSII core complex was further confirmed by low temperature EPR measurements. Solid curve in Fig. 4A shows the EPR spectrum of His-tagged thylakoids. When measured at 77 K after preillumination (30 s at 273 K) in the absence of ferricyanide followed by relaxation (30 s) of Y_Z^+ in darkness, the His-tagged thylakoids exhibited a weak but clear Y_D^+ signal. When measured in the presence of ferricyanide, however, a huge signal centering at $g \approx 2$ due to $P700^+$ was induced (dotted curve), indicative of chemical oxidation of PSI reaction center by ferricyanide.

EPR spectra of the His-tagged PSII core complex measured under the same conditions are shown in Fig. 4B. The spectrum measured in the absence of ferricyanide (solid curve) exhibited a strong double-peaked derivative band characteristic of the Y_D^+ signal arising from Tyr 160 of the D2 protein, indicative of extreme enrichment of PSII in the preparation. It is of note that the spectrum measured in the presence of ferricyanide (dotted curve) coincided exactly with the solid curve, indicating the total absence of chemically oxidizable P700 in the preparation. It is also of note that these Y_D^+ spectra coincide well with those reported in literatures (Miller and Brudvig 1991). This implies that the presence of six histidine residues at the C-terminus of D2 protein does not influence the molecular environment of Y_D^+. These results indicate clearly that our preparation strategy was successful in separating the PSII core complex from PSI.

Thermoluminescence is an outburst of light emission at characteristic temperatures when preilluminated PSII reaction centers are rapidly cooled and then rewarmed gradually in darkness (Inoue 1996). Figure 5 shows the thermoluminescence glow curves of thylakoids and the purified PSII core complex prepared in this study. When excited with a single flash, thylakoids from wild-type and D2-H cells showed similar thermoluminescence bands peaking at the same emission temperature at around 30°C

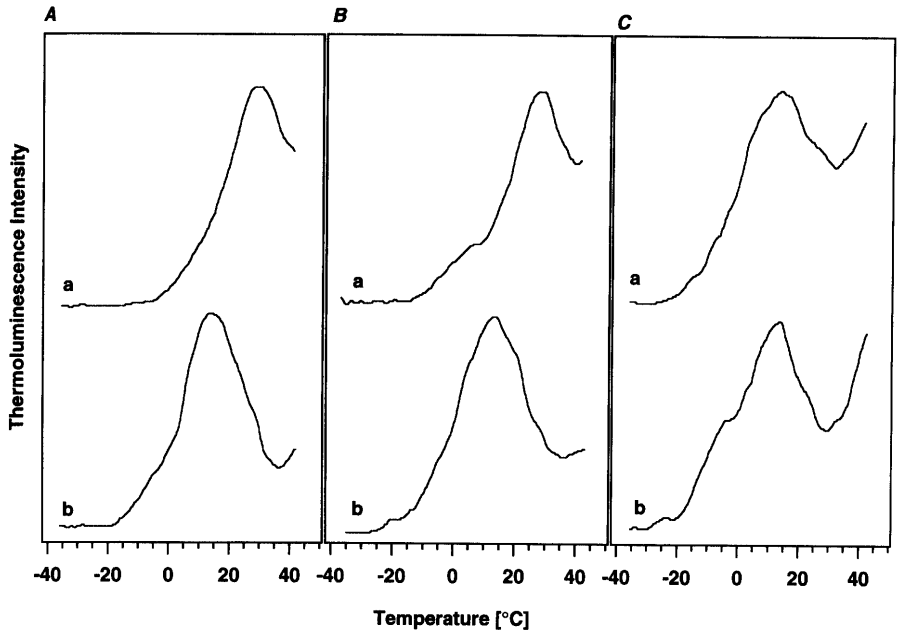

Fig. 5 Thermoluminescence glow curves of thylakoids from wild-type *C. reinhardtii* (A), His-tagged thylakoids from D2-H cells (B) and His-tagged PSII core complex (C) isolated from D2-H cells. Samples were illuminated with a single flash given at −10°C in the absence (traces a) and presence (traces b) of 10 μM DCMU, and glow curves were recorded at a heating rate of ~1°C s^{-1}.

(traces a in panels A and B), which are assigned to the B-band originating from charge recombination between S_2 and Q_B^- (Inoue 1996). When the electron transfer between Q_A and Q_B was blocked by 10 μM DCMU prior to excitation, the emission temperature downshifted to 15°C in both wild-type and His-tagged thylakoids, indicative of conversion of the B-band to the Q-band, which originated from $S_2Q_B^-$ and $S_2Q_A^-$ charge recombinations, respectively (Inoue 1996). The emission temperatures of both the B- and Q-bands were almost the same as those observed for respective intact cells (Sugiura et al. 1998). However, the His-tagged PSII core complex isolated from D2-H cells failed to exhibit the B-band: the peak temperature of thermoluminescence from the core complex was located at around 15°C (Q-band) irrespective of the presence or absence of DCMU (Fig. 5C). The failure to emit the B-band arising from $S_2Q_B^-$ recombination implies that the His-tagged PSII core complex contains no Q_B quinone. Presumably, the Q_B quinone was released out of its binding site during the isolation procedures, although the vacant Q_B pocket preserved a high affinity for DCMU.

Table 2 lists the contents of cytochrome *b-559*, Y_D^+ and Mn atom in His-tagged thylakoids and the His-tagged PSII core complexes as expressed by the number of Chl molecules relative to each component. Determination of cytochrome *b-559* based on reduced-minus-oxidized absorption difference ($\Delta\varepsilon = 15\ mM^{-1}\ cm^{-1}$, Garewal and Wasserman 1974) resulted in Chl per cytochrome *b-559* values of 405±10.5 and 23.5±1.2 for thylakoids and the PSII core complex, respectively. When the same data were processed on the basis of more recently proposed difference absorption coefficient ($\Delta\varepsilon = 17.5\ mM^{-1}\ cm^{-1}$, Cramer et al.

Table 2 Contents of cytochrome *b-559*, Y_D^+ and Mn in His-tagged thylakoids and His-tagged PSII core complex isolated from *C. reinhardtii* D2-H cells

	Chl/Cyt *b-559*[a]	Chl/Y_D^+	Chl/4 Mn
Thylakoid membranes	405 ±10.5	906 ±21.5	879 ±59
PSII core complex	23.5± 1.2	45.5± 2.5	52.4± 1.6

[a] Determined on the basis of $\Delta\varepsilon = 15\ mM^{-1}\ cm^{-1}$ by Garewal and Wasserman (1974). Data are the averages of measurements with four different preparations.

1986), the Chl per cytochrome *b-559* values of 469±12.2 and 27.3±1.4 were obtained for thylakoids and the PSII core complex, respectively. While all of cytochrome *b-559* in the thylakoids were detected as a high potential form, 75–80% of it in the PSII core complex were detected as a low potential form. Comparison of the difference spectra obtained with different reductants (dithionite-reduced minus ferricyanide-oxidized vs. hydroquinone-reduced minus ferricyanide-oxidized) confirmed the complete absence of other cytochromes, cytochromes b_6 or *f*, in our His-tagged PSII core complex (data not shown).

As opposed to the above results, the number of Chl molecules calculated on the basis of Y_D^+ spin density exhibited higher values: 906±21.5 and 45.5±2.5 for His-tagged thylakoids and the His-tagged PSII core complex, respectively. These values were about twice as large as those determined on the basis of cytochrome *b-559*. Table 2 also lists the abundance of Mn; The numbers of Chl molecules per four Mn atoms amounted to 879±59 and 52.4±1.6 for His-tagged thylakoids and the His-tagged PSII core complex, respectively. These values are in rough agreement with those calculated on the basis of Y_D^+ spin density, and nearly twice as large as those calculated on the cytochrome *b-559* basis. These results suggest that approximately two cytochrome *b-559* molecules are associated with one unit of *C. reinhardtii* PSII. This cytochrome *b-559* content agrees with those reported for spinach core complex (Murata et al. 1984) and cyanobacterial core complex (MacDonald et al. 1994).

Summarizing the above results we conclude that one unit of the His-tagged PSII core complex from *C. reinhardtii* contains one Y_D, four Mn atoms, about 46 Chl *a* molecules, and approximately two cytochrome *b-559*. We also note that the extremely large value (800–900) of Chl per one PSII unit in *Chlamydomonas* thylakoids would be due to the high content of PSI and LHC in the thylakoids of this alga.

Discussion

The photosynthetic system of *C. reinhardtii* is closer in several aspects to that of higher plants rather than to that of cyanobacteria (Harris 1989). Moreover, chloroplast genes of *C. reinhardtii* can be transformed like in cyanobacteria via homologous recombination by means of particle bombardment (Boynton et al. 1988). In our previous study, we constructed a *C. reinhardtii* D2-H strain genetically attached with a consecutive six histidine residues at the C-terminus of the D2 protein of PSII. The resulting transformant cells exhibited almost the same oxygen-evolving activity as wild-type cells, suggesting that no harmful influence was brought about by introduction of the His-tag (Sugiura et al. 1998). A highly active oxygen-evolving PSII core complex was purified from DM-solubilized thylakoids of the cells by single-step Ni^{2+}-affinity column chromatography within several hours (Sugiura et al. 1998).

The purified His-tagged PSII core complex retained a high rate of oxygen-evolving activity of 2,400 μmol (mg Chl)$^{-1}$ h^{-1} in the presence of $CaCl_2$. This rate is comparable to those reported for several PSII core complexes from other organisms: 2,400–3,200 and 1,300–2,400 μmol (mg Chl)$^{-1}$ h^{-1} for mesophilic (Tang and Diner 1994, Nilsson et al. 1992) and thermophilic (Bowes et al. 1983, Shen et al. 1992) cyanobacterial core complexes, respectively, 1,400–1,700 μmol (mg Chl)$^{-1}$ h^{-1} for wheat core complexes (Ikeuchi and Inoue 1986), and 1,200–1,700 μmol (mg Chl)$^{-1}$ h^{-1} for spinach core complexes (Haag et al. 1990, van Leeuwen et al. 1992). The oxygen-evolving activity of our His-tagged PSII core complex was higher than 1,000 μmol (mg Chl)$^{-1}$ h^{-1}, which was the highest activity so far reported for *C. reinhardtii* core complexes prepared from wild-type cells by density-gradient centrifugation followed by ion-exchange and size-exclusion column chromatographies (Bumann and Oesterhelt 1994). In addition to the high capacity of oxygen evolution, our His-tagged PSII core complex retained a high sensitivity to DCMU: oxygen evolution by the core complex prepared previously by conventional methods was inhibited only by 40% with 10 μM DCMU (Bumann and Oesterhelt 1994), whereas approximately 90% of the activity of our His-tagged core complex was blocked by the same concentration of DCMU. The higher sensitivity to DCMU implies that the structure of the Q_B-binding site in our core complex was preserved more intact than those prepared by conventional methods, although the same detergent was employed to solubilize thylakoids.

Complete removal of LHCs and PSI is essential for obtaining a highly purified PSII core complex. In this respect, however, *C. reinhardtii* is not always a suitable material because of its high contents of LHCs and PSI: the ratio of Chl to PSII in *C. reinhardtii* thylakoids is about two times larger than that in higher plants (Table 2), and this has made purification of oxygen-evolving PSII core complex from *C. reinhardtii* difficult. This problem was managed by employing the 'His-tag strategy', and we could isolate a PSII core complex with no contamination of LHCs as judged from the extreme elimination of Chl *b* band in the absorption spectrum (Fig. 1), and also from the absence of apoprotein bands characteristic of LHCs on SDS-PAGE profile (Fig. 2). The higher Chl *a*/*b* ratio (>14) of isolated PSII core complex also supported this notion (Sugiura et al. 1998). In the present study, our PSII core complex was further confirmed to be free from PSI contamination by the extremely low PSI emission in 77 K fluorescence spectrum (Fig. 3), and also by the total absence of EPR signal due to chemically-oxidized $P700^+$ radical (Fig. 4).

We quantified the size of antenna Chl in one unit of PSII core complex on the bases of cytochrome *b-559*, Y_D^+, and Mn atoms. Assuming two cytochrome *b-559* molecules and one Y_D^+ spin per one unit of PSII, the antenna size of our His-tagged PSII core complex was calculated to be approximately 46 Chl *a* molecules. This value is comparable to 40–50 Chl per reducible Q_A determined for the PSII core complex isolated from a *C. reinhardtii* mutant lacking PSI and ATPase (De Vitry et al. 1991), about 48 Chl per 2 pheophytin molecules for a spinach PSII core complex (Yamada et al. 1987), and 45 ± 5 Chl per reduced Q_A for a cyanobacterial PSII core complex (Rögner et al. 1990), whereas the value is slightly larger than that for the cyanobacterial PSII core complex (37.5 ± 2 per 2 pheophytin) isolated by Tang and Diner (1994). It is thus inferred that the positively charged His-tag at the C-terminus of the D2 protein probably does not induce conformational changes in D1, D2, CP43 and CP47 which may influence the association of Chl molecules to these proteins.

The antenna size of the His-tagged PSII core complex was also calculated on the basis of Mn content. The size determined on four Mn atom basis coincided roughly with the value determined by assuming one Y_D^+ and two cytochrome *b-559* per PSII. This is consistent with the recently accepted view that an active oxygen-evolving PSII core complex contains four Mn ions per P680, reaction center Chl of PSII (see Debus 1992 for review). Since the Mn abundance per PSII did not decrease during the purification, we may assume that the tetranuclear Mn-cluster in the His-tagged PSII core complex is properly retained in its normal binding site. In other words, the six histidine residues extending at the C-terminus of the D2 protein, probably protruding towards the lumenal side, do not influence the binding of Mn atoms. This contrasts sharply with the fact that the carboxylate group at the C-terminus of the D1 protein is essential to Mn-binding (see Debus 1992 for review). The normal functioning of the Mn-cluster in the His-tagged PSII was also evidenced by the high rate of oxygen evolution and also by normal thermoluminescence and its period-four oscillations (Sugiura et al. 1998). It is of note in this context that introduction of His-tag at the N-terminus of the D1 protein resulted in the loss of PSII complexes in the recombinant cells (Sugiura et al. 1998).

Thermoluminescence measurements in the present study revealed that the His-tagged PSII core complex does not emit the B-band arising from $S_2Q_B^-$ charge recombination. One might ascribe this to a modification of the S_2-state of the Mn-cluster by the positively charged six histidine residues on the C-terminus of the D2 protein. However, judging from the fact that the same His-tagged PSII in thylakoids emitted a normal B-band (Sugiura et al. 1998), this cannot be the case. It should rather be ascribed to the absence of Q_B quinone in the Q_B-binding site.

Regarding the topology of the His-tag attached to the C-terminus of the D2 protein, we deduce that its six histidine residues are largely exposed, with considerable freedom in motion, at the lumenal surface of thylakoids. It is also deduced that the extrinsic 33-kDa protein does not cover the His-tag, since this extrinsic component was copurified with the His-tagged PSII core complex (Sugiura et al. 1998). If the His-tag site is covered with the extrinsic polypeptide(s) or folded into the molecule of the D2 protein, our purification strategy utilizing the metal-chelate affinity would not work. Judging from the substantially high recovery of PSII core complex of about 75%, coverage of the six histidine residues by the extrinsic polypeptide(s) is unlikely. It may be of note, however, the present results do not preclude the possibility that the other two extrinsic proteins (23 kDa and 17 kDa) may cover the His-tag, since these two proteins are lost from the core complex by DM-treatment.

In conclusion, His-tagging of D2 protein at the C-terminus gives rise to no inconvenient effects on PSII, neither on its structure nor on its assembly, but enables us to isolate a pure PSII core complex by single-step Ni^{2+}-affinity column chromatography within several hours. Due to the short contact time with the detergent, the isolated complex retains a high rate of oxygen evolution and high affinities for DCMU and artificial benzoquinones as well. This 'His-tag strategy' can be widely applied for biochemical, biophysical, and crystallographical studies of *Chlamydomonas* PSII.

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