FEBS Letters 426 (1998) 140–144 FEBS 20095

Rapid and discrete isolation of oxygen-evolving His-tagged photosystem II core complex from *Chlamydomonas reinhardtii* by Ni^{2+} affinity column chromatography

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Received 17 February 1998

Abstract We have developed a simple and rapid procedure to isolate an oxygen-evolving photosystem II (PS II) core complex from *Chlamydomonas reinhardtii*. A His-tag made of six consecutive histidine residues was genetically attached at the carboxy terminus of D2 protein to create a metal binding site on the PS II supramolecular complex. The recombinant cells producing the His-tagged variant of D2 protein grew photoautotrophically as well as the wild-type cells. Characterization of the oxygen evolution and the thermoluminescence properties revealed that the His-tagging did not affect the functional integrity of the PS II reaction center. A PS II core complex was isolated from the detergent-solubilized thylakoids of the recombinant cells in 4 h by a single one-step Ni^{2+} affinity column chromatography. This preparation consists of D1, D2, CP43, CP47, 33 kDa, and a few low molecular weight proteins, and retains a high rate of oxygen-evolving activity (= 1000 μmol/mg Chl/h).

Key words: Photosystem II; *psbD*; *Chlamydomonas reinhardtii*; His-tag; Ni^{2+}-chelate chromatography

1. Introduction

Photosynthetic oxygen evolution takes place in a supramolecular complex denoted as photosystem II (PS II) composed of more than ten subunit proteins and several cofactors [1,2]. To elucidate the functional structure of PS II with the aim of understanding the mechanism of oxygen evolution, which has been of biologists' and biophysicists' interests, a multidisciplinary approach has been taken, including physical, molecular genetical, and crystallographical studies [2–4]. For these studies, isolation of a pure oxygen-evolving PS II core complex is a crucial prerequisite among others.

Several types of oxygen-evolving PS II core complexes have been purified from thylakoids of higher plants [5,6], green alga [7,8], and cyanobacteria [9–11], mostly by detergent solubilization followed by ultracentrifugation, gel filtration, or anion exchange chromatography. The oxygen-evolving activities retained in these preparations differed variously, due probably to denaturation during the time-consuming purification steps required for eliminating light-harvesting complex (LHC) and PS I complex, so that development of a rapid and simple protocol for isolating the oxygen-evolving PS II core complex has been desired. Tagging of a target gene with an affinity tag consisting of consecutive histidine residues has been demonstrated to be effective for discriminating separation of contaminating proteins [12]. Several membrane protein complexes have been successfully purified by this method, e.g. cytochrome oxidases from *Rb. sphaeroides* [13] and from *E. coli* [14]. Recently, the photosynthetic reaction center was also purified by His-tagging the M subunit from *Rb. sphaeroids* [15], which suggested to us this approach for the supramolecular complex, the PS II complex.

The transgenic green alga *Chlamydomonas reinhardtii* undergoes oxygenic photosynthesis similar to higher plants and cyanobacteria, and has been widely used as a model organism for studying various aspects of photosynthesis. In the present study, we applied the His-tag strategy to purify the PS II complex from this organism. Histidine residues were genetically attached to D1 or D2 proteins to confer a high affinity metal binding site on the supramolecular PS II core complex. From detergent-solubilized thylakoids of the recombinant cells producing the D2 protein fused with a His-tag at the carboxy terminus, we were able to prepare the oxygen-evolving PS II core complex in 4 h.

2. Materials and methods

2.1. Materials

Restriction endonucleases and other modifying enzymes were purchased from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA). Expand High Fidelity PCR System was obtained from Boehringer Mannheim (Amsterdam, The Netherlands). All chemicals used were of reagent grade.

2.2. Construction of His-tagged *psbA* and *psbD* genes

A carboxy terminal His-tag was attached by inserting an oligonucleotide linker A (Fig. 1) at the *Eco*72I site on plasmid pBD110, which carries the *psbD* gene of *C. reinhardtii* on the 5.6-kbp *Pst*I-*Hin*dIII fragment from plasmid pH3 [16] (a kind gift from Dr. J.M. Erickson) and a bacterial *aadA* gene on tandem. An amino terminal His-tag was attached by inserting an oligonucleotide linker B (Fig. 1) at the *Nde*I site on plasmid pBA158, which was derived from pBA157 [17] by introducing several silent mutations in the *psbA* gene. The resultant plasmids, pBD110H carrying His-tagged *psbD* and pBAH158 carrying His-tagged *psbA* gene, were introduced into a *psbD* deficient mutant ΔD2-1 and a *psbA* deficient mutant ac-u-ε strain, respectively. Transformation and screening of *C. reinhardtii* cells were performed as described previously [17]. Details of the construction of the plasmids and *C. reinhardtii* host strains will be described elsewhere.

2.3. Isolation of PS II core complex

Chlamydomonas cells were grown in liquid TAP medium [18] under dim light (≈1 μE m^{-2} s^{-1}) at 25°C with continuous aeration. Thylakoids were prepared from 12 l of a mid-logarithmic culture by the method of Chua and Bennoun [19]. The thylakoids (2 mg of Chl/ml)

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Abbreviations: Chl, chlorophyll; DCBQ, dichlorobenzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DM, *n*-dodecyl β-D-maltoside; LHC, light-harvesting complex; PS I, photosystem I; PS II, photosystem II; PAGE, polyacrylamide gel electrophoresis; WT, wild type

PII S0014-5793(98)00328-7

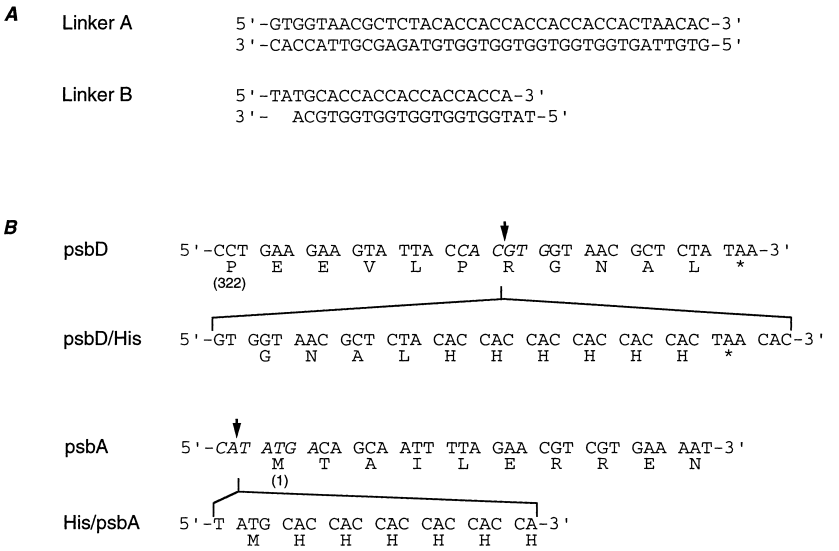

Fig. 1. Sequences of linkers A and B (*A*), and the *psbD* and *psbA* genes with the inserted sequences for His-tags (*B*). Linkers A and B were inserted in the *psbD* and *psbA* genes, respectively, at the sites indicated by the arrows. Resulting sequences are shown as psbD/His and His/psbA. The numbers in parentheses indicate the amino acid number of D2 and D1 proteins.

were solubilized with 25 mM HEPES/NaOH (pH 7.5), 100 mM NaCl, 10% glycerol, and 2% *n*-dodecyl β-D-maltoside (DM) (Sigma Chemical Company, St. Louis, MO) by stirring for 45 min on ice and then centrifuged at 12 000×*g* for 10 min. After addition of imidazole to make 15 mM, the supernatant was loaded onto a ProBond™ resin column (Invitrogen, San Diego, CA) equilibrated with 25 mM HEPES/NaOH (pH 7.5), 100 mM NaCl, 0.03% DM, and 10% glycerol. The column was washed with 10 volumes of the equilibrating buffer containing 15 mM imidazole. The PS II core complex was eluted with 40 mM MES/NaOH (pH 6.0), 100 mM NaCl, 0.03% DM, 10% glycerol, and 200 mM imidazole at a flow rate of 0.8 ml/min. The eluted PS II core complex was diluted with 10 volumes of 40 mM MES/NaOH (pH 6.5) and concentrated by using an Amicon Centriprep 50 (A Grace Company, Beverly, MA).

2.4. SDS-PAGE and Western blotting

Chlamydomonas cells were harvested at $OD_{750} \approx 0.6$, washed with 25 mM HEPES/NaOH (pH 7.5), 0.33 M sucrose, 1 mM $MgCl_2$, and 1.5 mM NaCl, and suspended in 25 mM HEPES/NaOH (pH 7.5), 10 mM EDTA, and 1.5 mM NaCl. The cell suspension was sonicated for 1 min at 4°C, and then centrifuged at 2500×*g* for 10 min. The supernatant was subjected to SDS-PAGE (12%) without urea according to Laemmli [20]. SDS-PAGE of PS II core complex was done with a 16–22% gradient gel containing 7.5 M urea as described by Ikeuchi and Inoue [21]. Western blotting on a nitrocellulose membrane was conducted according to Towbin [22]. Antisera against D1, D2, CP43, CP47, and extrinsic 33-kDa protein of spinach (gift from Dr. Ikeuchi) were used to detect the respective proteins on the blots. Anti-His (C-term) monoclonal antibody (Invitrogen, San Diego, CA) was used to detect proteins that contained a His-tag at the carboxy terminus.

2.5. Assay of oxygen evolution

Oxygen evolution was measured with a Clark-type oxygen electrode in the presence of 0.5 mM 2,6-DCBQ and 2 mM $K_3Fe(CN)_6$ at 25°C. Activity of the PS II core complex was measured in the reaction medium containing 40 mM MES/NaOH (pH 6.5), 0.4 M sucrose, 2 mM $MgCl_2$, and 5 mM NaCl. Activity of the cells was measured in TAP medium.

2.6. Thermoluminescence measurement

Thermoluminescence was measured essentially as described [23]. *Chlamydomonas* cells (40 μg Chl) were suspended in 80 μl of 25 mM MES/NaOH (pH 6.5), 0.2 M sucrose, 20 mM $MgCl_2$, 10 mM NaCl, and 20% glycerol. Before the measurements, the cell suspensions were kept in darkness for 20 min at 5°C. Actinic flashes were given from a Xenon lamp at 5°C to measure the B-band and at −10°C to measure the Q-band.

3. Results

3.1. Construction of amino-terminal His-tag of D1 protein and carboxy-terminal His-tag of D2 protein

In order to purify the PS II core complex rapidly, we employed 'His-tag' strategy, where the high affinity of consecutive histidine residues to Ni^{2+} ion was used [12]. We attempted to attach a His-tag to each of the heterodimer subunits, D1 and D2 proteins. The His-tag for D1 protein was designed at the amino terminus, since the carboxy terminus of D1 protein was suggested to be indispensable for forming the functional Mn-cluster [24,25]. The His-tag for D2 protein was designed at the carboxy terminus, since there had been a successful report of His-tagging at the carboxy terminus of M subunit,

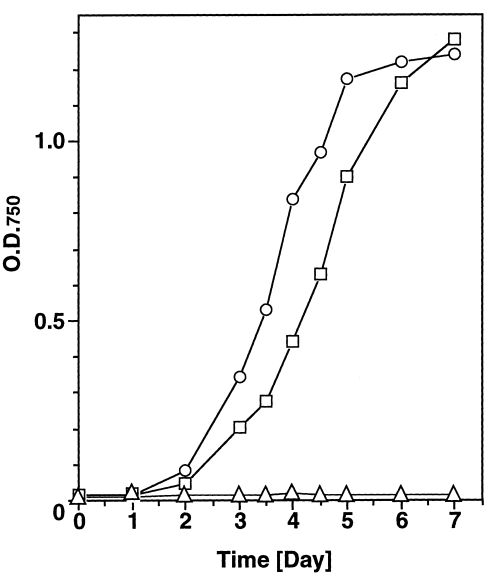

Fig. 2. Growth curves of WT, ΔD2-1 and D2-H cells of *C. reinhardtii*. Cells in mid-logarithmic phase (2.4×10^6 cells) were inoculated into 150 ml of HS minimal media [29] and cultured at 25°C with continuous aeration in the light (100 μE m^{-2} sec^{-1}). Circles, WT strain; squares, D2-H strain; triangles, ΔD2-1 strain.

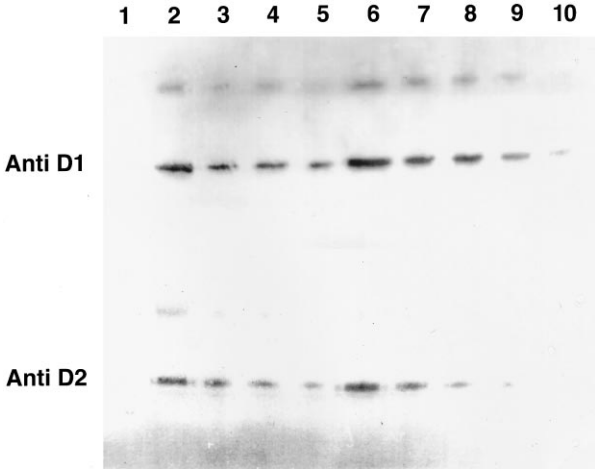

Fig. 3. Immunoblot analysis of D1 and D2 proteins in D2-H cells of *C. reinhardtii*. Lane 1, ΔD2-1 cells equivalent to 1 μg of Chl; lanes 2, 3, 4 and 5, D2-H cells equivalent to 1, 0.5, 0.4 and 0.3 μg of Chl, respectively; lanes 6, 7, 8, 9 and 10, WT cells equivalent to 1, 0.5, 0.4, 0.3 and 0.2 μg of Chl, respectively.

the counterpart of D2 protein of the bacterial reaction center in *Rb. sphaeroides* [15]. The amino-terminal His-tag for D1 protein and the carboxy-terminal His-tag for D2 protein were designed in oligonucleotide linkers and placed at the 5′-end of the intron-free *psbA* gene [19] on a plasmid vector pBA158 and at the 3′-end of the *psbD* gene on pBD110, respectively (Fig. 1). The resultant plasmids pBAH158 and pBD110H harboring His-tagged D1 and D2 proteins, respectively, were introduced into the *psbA* and *psbD* deficient mutants, ac-u-ε and ΔD2-1, respectively.

3.2. Photosynthetic activity of the cells carrying His-tagged D1 and D2 protein

We first examined the effects of the addition of His-tags to the PS II at the cell level. The transformant D2-H, which carried the His-tagged D2 protein, grew photoautotrophically as well as the wild-type (WT) control. The doubling time was 1.2 days, which was comparable to that of WT cells (Fig. 2). On the other hand, the recipient ΔD2-1 strain did not grow photoautotrophically at all. Since ΔD2-1 strain has the disrupted *psbD* gene, the photoautotrophic growth of the transformant must be due to functional expression of the His-tagged D2 protein. Oxygen-evolving activity of those cells is shown in Table 1. It was clearly indicated that the oxygen-evolving activity of the ΔD2-1 cells was rescued to 80% of the WT level by the introduction of a gene encoding the His-tagged D2 protein. In contrast to the successful tagging of D2 protein, H-D1 transformant carrying D1 protein fused with a His-tag at the amino terminus did not grow photoautotrophically (data not shown), and the recovery of oxygen-evolving activity was only 30% of the WT cells (Table 1).

Table 1
Oxygen-evolving activities and Chl *a* to *b* ratios of the two His-tagged *C. reinhardtii* cells and their respective recipient cells

Strain	Oxygen evolution (μmol/mg Chl/h)	Chl *a*/*b*
WT	230 ± 27	2.6
ΔD2-1	< 20	2.2
D2-H	194 ± 12	2.6
ac-u-ε	< 20	3.3
H-D1	65.6 ± 5	3.3

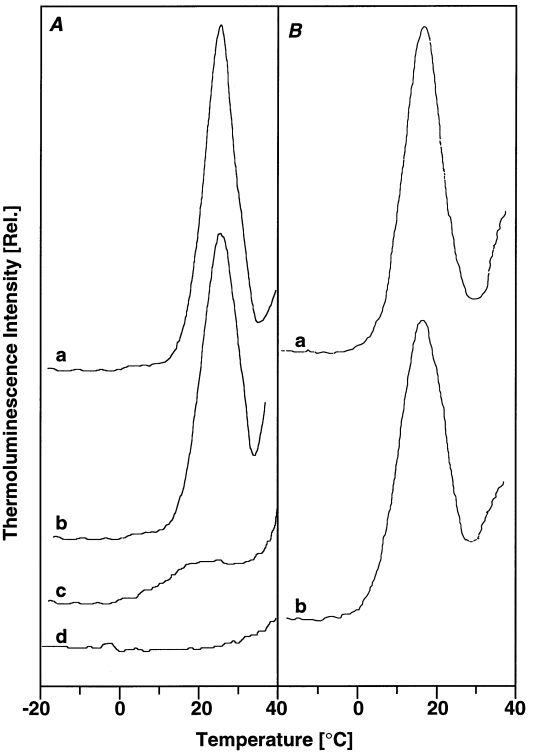

Fig. 4. Thermoluminescence glow curves after a single turnover flash illumination. Panel A shows the B-bands of WT cells (a); D2-H cells (b); H-D1 cells (c) and ΔD2-1 cells (d). Panel B shows the Q-bands of WT cells (a) and D2-H cells (b).

In D2-H cells, Chl *a* to *b* ratio was increased from 2.2 to 2.6 in parallel with the restoration of oxygen-evolving activity, while in H-D1 cells the ratio remained the same as that of D1-less mutant ac-u-ε in spite of the introduction of the *psbA* gene fused with the His-tag sequence (Table 1). This result suggested that His-tagging at the amino terminus of D1 protein even affected functional assembly of the PS II complex.

3.3. Properties of the PS II reaction center in D2-H cells

To characterize properties of the PS II reaction center in D2-H cells, expression levels of D1 and D2 proteins in the cells were determined by immunoblotting using the respective antibodies (Fig. 3). No proteins were detected in ΔD2-1 cells that crossreacted with either of the antibodies against D1 or D2 proteins (lane 1), confirming the absence of PS II reaction center in this *psbD* deficient strain. From relative intensities of the bands, the amounts of D1 and D2 proteins in D2-H cells were estimated to be 75–80% of the WT cells on Chl basis, which was in accordance with the 80% activity of oxygen evolution in D2-H cells (Table 1).

Fig. 4 shows the thermoluminescence glow curves from WT, D2-H, and H-D1 cells. Intensity and emission temperature of the B-band that originates from the charge recombination between S_2 and Q_B^-, are the measures for functional integrity of the PS II reaction center complex [26]. The B-bands from D2-H and WT cells were almost identical in terms of emission intensity and peak temperature (28°C), while the band from H-D1 strain was severely suppressed accompanied

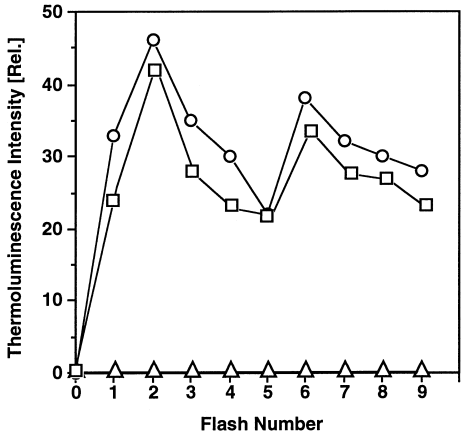

Fig. 5. Oscillation of thermoluminescence B-band intensity. Cells were illuminated with single turnover flashes: WT cells (circles), D2-H cells (squares) and ΔD2-1 cells (triangles).

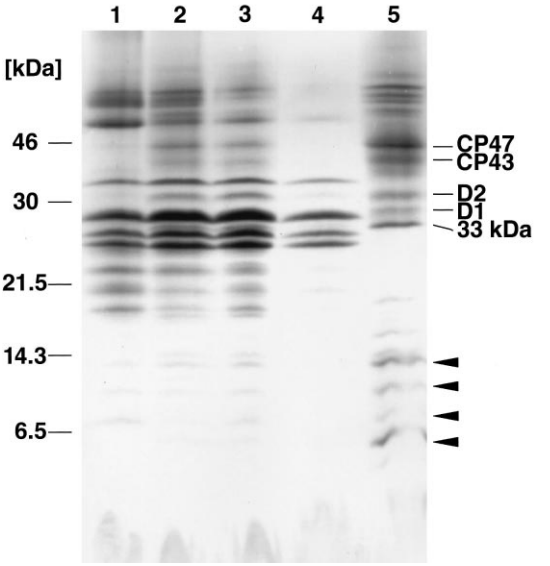

Fig. 6. SDS-PAGE profile showing the polypeptide compositions of the fractions obtained by Ni^{2+} affinity column chromatography. Lane 1, thylakoids (20 μg of Chl) from ΔD2-1 cells; lane 2, thylakoids (20 μg of Chl) from WT cells; lane 3, thylakoids (20 μg of Chl) from D2-H cells; lane 4, the flow through fraction (15 μg of Chl) from Ni^{2+} affinity column; lane 5, PS II core complex eluted by imidazole (8 μg of Chl). Arrows show gene products of *psbE* (cytochrome *b*-559 α subunit), *psbM*, *psbK* and nuclear-encoded 6.1-kDa polypeptide.

by a downshift in peak temperature (22°C) as compared with those of the other photosynthetically competent cells. The ΔD2-1 recipient strain emitted no thermoluminescence at all over the temperature range scanned. When electron transfer between Q_A and Q_B was blocked by 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) prior to the excitation, both the WT and D2-H cells emitted the Q-band at 15°C that arose from the charge recombination between S_2 and Q_A^- [26]. Turnover of the S-states was characterized to examine the effect of the His-tagging on the donor side. Upon illumination with single turnover flashes (Fig. 5), the flash yield of the B-band from D2-H cells oscillated with a periodicity of four with the maximum emission after the 2nd and 6th flashes, the same oscillation pattern as that of the WT. These results indicate clearly that the His-tagged variant of D2 protein is assembled in PS II reaction center with essentially no disturbance in the functional geometry of the donor-acceptor pairs.

3.4. Isolation of oxygen-evolving PS II core complex by one-step metal affinity column chromatography

Detergent-solubilized thylakoids from D2-H cells were loaded onto an Ni^{2+} affinity column and the column was washed until no Chl was detected in the column-through fractions. The PS II core complex which remained adsorbed on the column bed was then eluted with an imidazole-containing buffer. The Chl *a* to *b* ratio of the eluted fraction was higher than 14 and the recovery yield was 2.5 to 3% on Chl basis, and the oxygen-evolving activity was 1030 μmol/mg Chl/h in the presence of 2,6-DCBQ and $K_3Fe(CN)_6$ as electron acceptors (Table 2) with the optimum pH at 6.5 (data not shown). This oxygen-evolving activity was as high as that of the *C. reinhardtii* PS II core complex purified by sucrose density gradient centrifugation followed by anion exchange column chromatography [7].

SDS-PAGE profile of the fraction revealed a high enrichment of the PS II core complex in the preparation (Fig. 6). Polypeptide bands with apparent molecular masses of 30, 32, 44, 47 and 28 kDa were identified as D1, D2, CP43, CP47 and extrinsic 33-kDa protein by immunostaining using the respective antibodies raised against the spinach counterparts. There are at least four additional bands in the lower molecular mass region, possibly corresponding to the products of *psbE* (cytochrome *b*-559 α subunit), *psbM* and *psbK*, and the nuclear-encoded 6.1-kDa polypeptide [27]. However, these were not identified in this study due to the unavailability of antibodies for these polypeptides. This is a typical polypeptide composition of the oxygen-evolving PS II core complex that has been isolated by several other means such as sucrose density gradient centrifugation and anion exchange column chromatography [7]. The band at 60 kDa was assigned to the heterodimeric aggregate of D1 and D2 proteins based on its cross-reactivity with anti-D1 and anti-D2 antibodies (data not shown). D2 protein was also identified by immunostaining with anti-His (C-term) antibody (data not shown). LHC and PS I were essentially absent in the preparation. Notably, the time needed for the affinity chromatography was as short as

Table 2
Isolation of PS II core complex from a 12-l culture of D2-H cells by Ni^{2+}-affinity chromatography

	Oxygen evolution (μmol/mg Chl/h)	Chl *a*/*b*	Yield of Chl	
			mg	%
D2-H cells	194	2.6		
Thylakoids	375	2.5	20	100
PS II core complex	1030	>14	0.5–0.6	2.5–3.0

4 h, and the quantity of the purified PS II core complex amounted to 0.6 mg of Chl starting from a 12-l culture of *C. reinhardtii* cells.

4. Discussion

The amino acid sequences of D1 and D2 proteins, the major reaction center subunits of PS II, are homologous to each other [28], both having five membrane-spanning α-helices with long hydrophilic protrusions on both the lumenal and stromal sides. Imidazole ring and carboxylate groups of His, Glu and Asp residues present in the lumenal protrusions of D1 (and D2) protein(s) are considered to serve ligands for binding the tetranuclear manganese cluster, the catalyst for water oxidation. In the present study, we demonstrated that addition of six histidine residues to the carboxy terminus of D2 protein did not give rise to serious effects on the stability of the manganese cluster, as corroborated by the normal growth, high oxygen-evolving activity and normal thermoluminescence properties of the D2-H cells. In contrast, introduction of the same His-tag to D1 protein at its amino terminus, which is presumably of the stromal topology, resulted in a drastic lowering of oxygen evolution accompanied by severe modification of thermoluminescence properties.

From the D2-H *Chlamydomonas* cells that expressed and assembled a His-tagged D2 protein in the PS II reaction center, the PS II core complex was isolated by a simple procedure, one-step Ni^{2+} affinity column chromatography of detergent-solubilized thylakoids. The isolated PS II core complex exhibited the highest rate of oxygen evolution exceeding 1000 μmol/mg Chl/h among similar preparations of *Chlamydomonas* PS II complexes that have ever been reported [7,8]. The yield of the His-tagged PS II complex from detergent-solubilized thylakoids of D2-H cells was as low as 3% on chlorophyll basis. However, assuming that one PS II core complex binds 40 to 50 Chls [27], and that one photosynthetic unit (in WT thylakoids) binds about 1000 Chls, the net yield of the His-tagged PS II core complex from D2-H thylakoids is calculated to be as high as 75%. If we further take into account the immunoblotting data (Fig. 3) suggesting that the expression of D1 and D2 proteins is slightly suppressed in D2-H cells, the practical net yield would be even higher. The yield of PS II core complex, 3% on Chl basis starting from the thylakoids prepared from a 12-l culture of *C. reinhardtii* cells at mid-logarithmic phase is reproducible and seems to be maximally optimized as far as under the conditions employed in the present protocol. However, the oxygen-evolving activity of *C. reinhardtii* cells does not change much throughout the logarithmic phase of the growth, so that we will be able to easily increase the quantity by harvesting more cells at the late-logarithmic phase.

In this study, we demonstrated that introduction of a His-tag to only one of the ten subunits, the D2 protein, enabled us to isolate the PS II complex by a simple, high yield, one-step chromatography without affecting its activity of oxygen evolution, the most delicate activity among others. The method can be easily extended for large scale preparation of intact PS II core complex, which will no doubt help our structural studies as well as biochemical and biophysical studies on the mystery of oxygen evolution.

Acknowledgements: We are grateful to Dr. M. Ikeuchi for antibodies. We thank Drs. J. Erickson, N. Fischer, and J.D. Rochaix for making the plasmids available. We thank Drs. K.-C. Han and J.-R. Shen for helpful advice. This work was supported in part by a grant for Photosynthesis Research provided to RIKEN by the Science and Technology Agency (STA) of Japan.

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