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Highly Purified Thermo-Stable Oxygen-Evolving Photosystem II Core Complex from the Thermophilic Cyanobacterium *Synechococcus elongatus* Having His-Tagged CP43

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The carboxyl terminus of the CP43 subunit of photosystem II (PSII) in the thermophilic cyanobacterium, *Synechococcus elongatus*, was genetically tagged with six consecutive histidine residues to create a metal binding site on the PSII supramolecular complex. The histidine-tagging enabled rapid isolation of an intact cyanobacterial PSII core complex from dodecyl maltoside-solubilized thylakoids by a simple one-step Ni^{2+}-affinity column chromatography. The isolated core complex was in a dimeric form with a molecular mass of about 580 kDa, consisting of five major intrinsic membrane proteins (CP47, CP43, D1, D2 and cytochrome *b-559*), three extrinsic proteins (33 kDa, 12 kDa, and cytochrome *c-550*), and a few low molecular mass membrane proteins, and evolved oxygen at a rate as high as 3,400 μmol (mg Chl)$^{-1}$ h^{-1} at 45°C with ferricyanide as an electron acceptor. The core complex emitted thermoluminescence B_2-, B_1- and Q-bands arising from $S_2Q_B^-$, $S_3Q_B^-$ and $S_2Q_A^-$ charge recombinations at respective emission temperatures of 45, 38 and 20°C, all of which were higher by about 15°C as compared with those in mesophilic spinach BBY membranes. These results indicated that the isolated core complex well retained the intact properties of thermoluminescence of thermophilic cyanobacterial cells, the deeper stabilization of PSII charge pairs. The isolated complex was extremely stable in terms of both protein composition and function, exhibiting no release of extrinsic proteins, no proteolytic degradation in any of its subunits, accompanied by only a slight (less than 10%) loss in oxygen evolution, after dark-incubation at 20°C for 8 d. These properties of the thermophilic PSII core complex are highly useful for various types of studies on PSII.

Key words: CP43 — His-tag — Oxygen evolution — PSII core complex — *Synechococcus elongatus* — Thermostability.

Photosystem II (PSII) undergoes light-driven electron transfer from water to plastoquinone in thylakoids of oxygen-evolving algae and higher plants. Its minimum structural unit capable of oxygen-evolution is called PSII core complex and consists of about ten subunits of membrane proteins and three extrinsic proteins. The membrane proteins include a pair of reaction center proteins (D1 and D2), a pair of Chl-binding inner antenna proteins (CP47 and CP43), a hemoprotein (cytochrome *b-559*) and several low molecular mass membrane proteins, whereas the extrinsic proteins include the 33 kDa Mn-stabilizing protein, the 17 kDa cytochrome *c-550*, and the 12 kDa protein (see Debus 1992 for review). The five major membrane proteins are largely homologous between cyanobacteria and higher plants, while only the 33 kDa protein has homology with its higher plant counterpart among the three extrinsic proteins of cyanobacterial PSII.

Several types of oxygen-evolving PSII core complex have so far been prepared from higher plants (Ikeuchi and Inoue 1986, Haag et al. 1990, van Leeuwen et al. 1991), green algae (Bumann et al. 1994) and cyanobacteria (Tang and Diner 1994, Nilsson et al. 1992, Bowes et al. 1983, Shen and Inoue 1993) by solubilization of their thylakoids with various detergents followed by ultracentrifugation and anion exchange and/or gel filtration chromatography. In these core complex preparations, the structure and photochemical capabilities of the PSII reaction center including the primary electron acceptor pheophytin were usually well preserved, but the structure and electron transfer capabilities on the donor side of PSII, in particular, the oxygen-evolving activity differed from one preparation to another, presumably due to different extents of damage in the water-oxidation apparatus including the tetranuclear Mn-cluster (see Debus 1992 for review). Under these circumstances, the BBY membranes have usually been employed for detailed spectroscopic studies on the water-oxidation system, e.g. X-ray absorption spectroscopy (Yachandra et al. 1993, Ono et al. 1992, George et al. 1989, Penner-Hahn et al. 1990, MacLachlan et al. 1994, Schiller et al. 1998), FTIR spectroscopy (Noguchi et al. 1992, 1995a, b) and photoactivation of the Mn-cluster

Abbreviations: DCBQ, dichlorobenzoquinone; DM, dodecyl maltoside; EPR, electron paramagnetic resonance; LHC, light-harvesting chlorophyll protein complex; P700, reaction center of PSI; PBQ, phenyl-*p*-benzoquinone; Q_A and Q_B, primary and secondary acceptor quinones of PSII; Y_D, tyrosine 160 of the D2 protein, the auxiliary second electron donor of PSII.

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(Miyao and Inoue 1991, Ananyev and Dismukes 1996) etc., because a simple preparation like BBY has been believed to retain a damage-free, exact uniformity in the nature of the water-oxidation apparatus, in spite of the difficulties arising from the low content in Mn and the heterogeneity in protein compositions.

More recently, two new-types of PSII core preparations have been reported, in which the His-tag methodology was successfully employed. The PSII core complexes thus prepared from *Chlamydomonas* (Sugiura et al. 1998, 1999) or *Synechocystis* (Bricker et al. 1998) retained good homogeneity in protein compositions and exhibited high rates of oxygen evolution, presumably due to the shorter contact period with detergents. These PSII core complexes are much improved in terms of their purity and oxygen-evolving activity as compared with those prepared by usual methods, but they are not always ideal experimental materials for studies of the water-oxidation system because of their instability of oxygen-evolving activity. In the present study, we tried to prepare a more stable PSII core complex by applying the His-tag methodology to a thermophilic cyanobacterium, *Synechococcus elongatus*. The obtained thermophilic PSII core complex was extremely stable in both structural and functional terms, exhibiting practically no changes in its protein composition and oxygen-evolving activity during incubation at 20°C for 8 d or longer. The thermophilic cyanobacterial PSII core complex we report here will be highly useful for various types of biochemical and spectroscopic studies on PSII including crystallography.

Materials and Methods

Strain and culturing conditions—Purified colony of the thermophilic cyanobacterium, *Synechococcus elongatus*, was a generous gift from Dr. U. Mühlenhoff, Biologisches Institut II, Universität Freiburg. The transformed *S. elongatus* 43-H cells were grown at 50°C under continuous illumination from white fluorescence lamps (10 to 35 mW cm^{-2}) in a 16-L DTN medium (Mülenhoff and Chauvat 1996) bubbled continuously with 3% CO_2-enriched air. For screening and maintenance of transformants, cells were grown at 45°C in the same medium supplemented with kanamycin at a concentration of 40 $\mu g\ ml^{-1}$ unless otherwise noted.

Construction of His-tagged CP43 gene and tansformation of S. elongatus—Strategy of construction of a pUC43-H plasmid for expression of His-tagged *psbC* in *S. elongatus* is shown in Fig. 1A. The 3′ region of *psbC* coding for the C-terminus of CP43 in 43K/pBK-CMV (a kind gift from Dr. M. Hirano, Toray Research Center) was amplified by PCR using the C1899 primer (5′-CCCAGTGAATTCTAT-GGTCC-3′) and the C2423R primer (5′-TTTGGTACCAGATCAAGGCTGGGCATCGAGAGG-3′). After inserting the amplified DNA fragment of 531 bp into pUC19, the Tr/His linker

5′-CACGCGGTTCTCACCACCACCACCACCACTAGT-3′
3′-CATGGTGCGCCAAGAGTGGTGGTGGTGGTGATCAGATC-5′

was ligated between the *Eco*RI and *Xba*I sites of the plasmid. Then, a 978-bp DNA fragment corresponding to the 3′ non-coding region of *psbC*, which was prepared by digestion of the 43K/pBK-CMV at *Hin*cII and *Hin*dIII sites, was inserted into the plasmid containing the Tr/His linker and the amplified 531-bp fragment DNA to make pUCLF. The sequence of the C-terminal region of His-tagged CP43 is shown in Fig. 1B. Finally, pUC43-H was constructed by inserting into pUCLF a 1,339-bp kanamycin resistant gene cassette obtained by digestion of pRL161 at *Xba*I site (Elhai and Wolk 1988). The resulting plasmid DNA was introduced into *S. elongatus* cells by electroporation according to Mühlenhoff and Chauvat (1996). All the constructed plasmids were maintained in the *E. coli* DH5α strain using standard techniques (Sambrook et al. 1989). For PCR, an Expand High Fidelity PCR System (Boehringer Manheim) was used. Ligation of the amplified DNA with the linker fragments was confirmed by sequencing.

Preparation of thylakoids—The transformed *S. elongatus* 43-H cells were grown for 5 d to reach an OD_{730} of 3–3.5 in a 16-L DTN medium, and harvested by centrifugation at 5,000 × *g* at 25°C. After one wash in 25 mM HEPES/NaOH (pH 7.5), 15 mM $CaCl_2$ and 15 mM $MgCl_2$ (washing buffer), the cells were re-suspended in 100–200 ml of the same washing buffer supplemented with 0.2% bovine serum albumin, 1 mM benzamidine, 1 mM aminocaproic acid and 50 $\mu g\ ml^{-1}$ DNase I (bovine pancreas type IV, Sigma), and 20 $\mu g\ ml^{-1}$ RNase A at a Chl concentration of 1 mg ml^{-1}. The cells were treated with lysozyme (0.2%, w/v) at 40°C in darkness for 1 h under gentle stirring, and then broken with a French Press at 400 kgf. After removing cell walls and unbroken cells by centrifugation at 5,000 × *g* for 5 min, thylakoids were pelleted by centrifugation at 25,000 × *g* for 15 min at 4°C. Pellets were re-suspended in 40 mM MES/NaOH (pH 6.5), 15 mM $CaCl_2$, 15 mM $MgCl_2$ and 25% glycerol (suspension buffer) at a Chl concentration of 2–3 mg ml^{-1}, and stored in liquid N_2.

Isolation of PSII core complex—Thylakoids prepared from the transformed *S. elongatus* cells were solubilized with 1% DM (Sigma Chemical Company, St. Louis, U.S.A.) in 40 mM MES/NaOH (pH 6.5), 100 mM NaCl, 15 mM $CaCl_2$, 15 mM $MgCl_2$, and 10% glycerol at a Chl concentration of 1 mg ml^{-1} for 30 min at 4°C in darkness under gentle stirring. The suspension was then centrifuged at 25,000 × *g* for 10 min at 4°C, and the resulting supernatant was mixed with a half volume of ProBond™ resin (Invitrogen, San Diego, CA) that had been equilibrated with 40 mM MES/NaOH (pH 6.5), 100 mM NaCl, 15 mM $CaCl_2$, 15 mM $MgCl_2$, 0.2% DM and 10% glycerol (equilibrating buffer), and gently stirred in darkness for 60 min at 4°C. The resulting slurry was transferred to an empty column and washed with 5-bed volumes of the equilibrating buffer supplemented with 15 mM imidazole at a flow rate of 0.8–1.0 ml min^{-1}. The column was further washed with 5-bed volumes of 40 mM MES/NaOH (pH 6.5), 100 mM NaCl, 15 mM $CaCl_2$, 15 mM $MgCl_2$, 15 mM imidazole, 0.03% DM and 10% glycerol at the same flow rate. After the second wash, PSII core complex was eluted with 40 mM MES/NaOH (pH 6.5), 100 mM NaCl, 15 mM $CaCl_2$, 15 mM $MgCl_2$, 200 mM imidazole, 0.03% DM and 10% glycerol at a flow rate of 0.5 ml min^{-1}. The eluted PSII core complex was precipitated by centrifugation at 25,000 × *g* for 10 min at 4°C after addition of an equal volume of PEG-solution [40 mM MES/NaOH (pH 6.5), 15% polyethylene glycol-8000, 20 mM NaCl, 20 mM $CaCl_2$, 20 mM $MgCl_2$]. Pellets were resuspended in 40 mM MES/NaOH (pH 6.5), 10 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.03% DM and 25% glycerol at a Chl concentration of 2–3 mg ml^{-1} and stored in liquid N_2. This sample was used for characterization studies described in this paper. For size-separation, the PSII core complex was further treated with 1.5% DM at a Chl concentration of 1.5

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mg ml^{-1} for 30 min at 4°C in darkness, and then subjected to a Superdex-200 column (PC 3.2/30) chromatography (SMART™ System, Pharmacia Biotech) at a flow rate of 10 $\mu l\ min^{-1}$. Molecular mass of the PSII core complex was estimated by using a Gel Filtration Calibration Kit (Pharmacia Biotech) as standards eluted in the same solvent system under the same conditions.

Preparation of Mn-depleted PSII—The PSII core complex was incubated in 40 mM MES/NaOH (pH 6.5), 20 mM NaCl, 0.4 M sucrose, 0.5 mM EDTA 2Na, 10 mM NH_2OH at a Chl concentration of 0.5 mg ml^{-1} at 25°C for 30 min in darkness to dissociate Mn, and the Mn-depleted core complex was pelleted by centrifugation at 25,000 × *g* for 10 min. The pellets were washed for three times in 40 mM MES/NaOH (pH 6.5), 20 mM NaCl and 0.4 M sucrose, and then resuspended in the same medium. For Mn depletion from BBY membranes, the membranes were incubated with the above NH_2OH solution for 20 min at 0°C.

Stability test—BBY membranes capable of oxygen evolution at a high rate were prepared from spinach according to Berthold et al. (1981) with slight modifications by Ono and Inoue (1986). Spinach BBY, *S. elongatus* 43-H thylakoids and purified PSII core complex were suspended in 40 mM MES/NaOH (pH 6.5), 10 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.03% DM and 10% glycerol at a Chl concentration of 1 mg ml^{-1} and incubated at 20°C in darkness as long as for 21 d. An aliquot of test sample was taken out from the container every day during the beginning 8 d and at the last day, and was subjected to examinations of oxygen-evolving activity and polypeptide compositions (only for PSII core complex). For examination of polypeptide compositions, the incubated PSII core complex samples were mixed with the same volume of 40 mM MES/NaOH (pH 6.5) and centrifuged at 10,000 × *g* for 60 min. The pellets were resuspended in 40 mM MES/NaOH (pH 6.5), 10 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.03% DM, and then applied to SDS-PAGE.

SDS-PAGE and immunoblot analysis—PSII core complex and thylakoids of *S. elongatus* suspended in 40 mM MES/NaOH (pH 6.5), 10 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 0.03% DM were solubilized with 1% SDS, and then electrophoresed in an SDS-PAGE with a 16–22% gradient gel containing 7.5 M urea as described by Ikeuchi and Inoue (1988). Western blotting on a nitrocellulose membrane was conducted according to Towbin et al. (1979). Antisera against D1, D2, CP43, CP47 and extrinsic 33 kDa protein of spinach (gifts from Dr. M. Ikeuchi, University of Tokyo), and extrinsic 12 kDa protein and cytochrome *c-550* of *Synechococcus vulcanus* (gifts from Dr. J.-R. Shen, RIKEN Harima Inst.) were used to detect respective proteins on the blots. The blots were developed with a goat anti-rabbit IgG-alkaline phosphatase conjugate (Jackson Immuno Research Laboratories, West Grove, PA).

Assay of oxygen evolution—Oxygen evolution was measured with a Clark-type oxygen electrode in the presence of appropriate electron acceptors at various temperatures. Activity of intact cells was measured in 25 mM HEPES/NaOH (pH 7.5) or 40 mM MES/NaOH (pH 6.5), both containing 10 mM NaCl. Activities of spinach BBY membranes, cyanobacterial thylakoids and the purified PSII core complex were assayed in 40 mM MES/NaOH buffer (pH 6.5) containing 0.4 M sucrose, 15 mM $MgCl_2$, 15 mM $CaCl_2$ and 10 mM NaCl.

Thermoluminescence measurements—Thermoluminescence glow curves were measured by essentially the same method as described by Inoue (1996). *S. elongatus* cells (20 μg of Chl) were suspended in 70 μl of 25 mM HEPES/NaOH (pH 7.5), 0.2 M sucrose, 10 mM NaCl, 15 mM $MgCl_2$, 15 mM $CaCl_2$, and 20% glycerol. *S. elongatus* thylakoids (20 μg of Chl), PSII core complex (15 μg of Chl) and spinach BBY membranes (15 μg of Chl) were suspended in 70 μl of 40 mM MES/NaOH (pH 6.5), 0.2 M sucrose, 10 mM NaCl, 15 mM $MgCl_2$, 15 mM $CaCl_2$, and 20% glycerol. After dark-relaxation for 20 min on ice, the samples were illuminated at 5°C with saturating actinic flashes (≈2 μs duration) given from a xenon lamp, and then rapidly cooled to 77 K. The thermoluminescence emission was measured during warming the sample at a heating rate of 40°C min^{-1}. For measurements of the A_T-band arising from $His^+Q_A^-$ charge recombination, Mn-depleted PSII core complex (15 μg of Chl) and Mn-depleted spinach BBY membranes (15 μg of Chl) were suspended in 70 μl of 40 mM MES/NaOH (pH 6.5), 0.2 M sucrose, 10 mM NaCl, 15 mM $MgCl_2$, 15 mM $CaCl_2$, and 20% glycerol, and illuminated at −25°C with continuous red light (100 mW cm^{-2}) for 20 s, and then cooled to 77 K.

EPR measurements—EPR spectra were recorded with a JEOL JES-FE1XG EPR spectrometer (Tokyo, Japan). PSII core complex and cyanobacterial thylakoids contained in quartz sample tubes were illuminated at 273 K for 30 s to fully induce the Y_D^+ signal, and then cooled to 77 K to trap Y_D^+. The signal intensity was doubly integrated to determine the Y_D^+ spin density by comparing with the Cu^{2+} signal of crystal $CuSO_4$ as a standard (Miller and Brudvig 1991). To avoid overestimation due to overlapping of Chl free radicals, only the low-field-side signal of the Y_D^+ spectrum was used for the calculation.

Chl content of the purified PSII core complex was determined on the basis of Y_D^+ by use of the same sample that had been subjected to EPR measurement, and expressed as a number of Chl molecules per PSII reaction center. This method gave a value of 210 ± 10 Chl molecules per PSII reaction center, when applied to spinach BBY membranes depleted of PSI.

Results

Purification of His-tagged PSII core complex—In previous papers (Sugiura et al. 1998, 1999) we have reported that attachment of six consecutive histidine residues (His-tag) at the C-terminus of D2 protein of the PSII reaction center of a green alga, *Chlamydomonas reinhardtii*, was extremely useful, enabling isolation of pure and highly active oxygen-evolving PSII core complex by a simple one-step Ni^{2+}-affinity column chromatography. In this study, we attempted to purify a more stable oxygen-evolving PSII core complex from transformed cells of a thermophilic cyanobacterium, *Synechococcus elongatus*, which was genetically attached with a His-tag at the C-terminus of CP43 gene (*psbC*) as illustrated in Fig. 1. The mutant *S. elongatus* 43-H cells were selected by kanamycin screening, and oxygen-evolving PSII core complex was isolated by one-step Ni^{2+}-affinity column chromatography after solubilization of the His-tagged thylakoids with DM.

As shown in Table 1, the thylakoids prepared from 43-H cells were very intact as judged from the retention of high oxygen-evolving activity almost the same as that of intact cells [300–400 μmol (mg Chl)$^{-1}$ h^{-1}] when measured at 25°C in the presence of 2,6-DCBQ plus ferricyanide as electron acceptors. A 30-min treatment of the thylakoids with DM resulted in solubilization of about 87% of chlorophyll *a*, and the recovery of His-tagged PSII core com-

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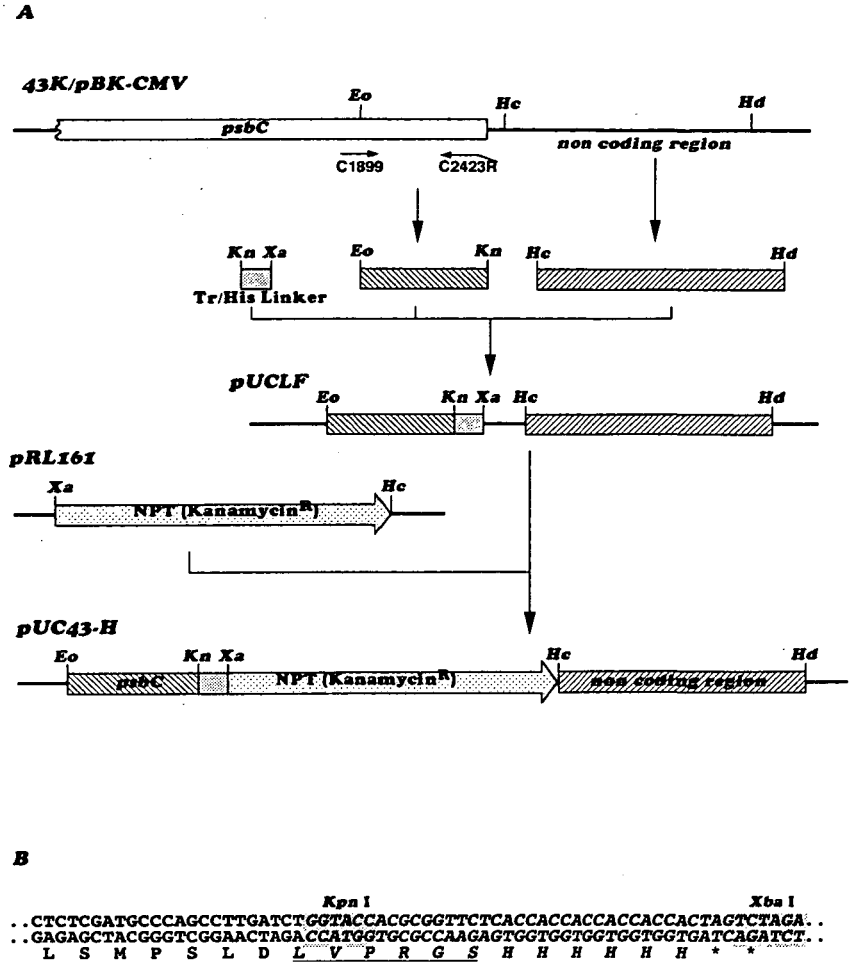

Fig. 1 Strategy of plasmid pUC43-H construction for expression of His-tagged *psbC* in *S. elongatus* (A) and the sequence of C-terminal region of His-tagged CP43. Panel A: *Eo, Eco*RI; *Hc, Hin*cII; *Hd, Hin*dIII; *Kn, Kpn*I; *Xa, Xba*I. Panel B: Additional sequence for expression of the thrombin recognition site (underlined). The His-tag is shown in italic.

plex in the affinity column eluate was 3.3% in terms of chlorophyll *a* concentration. The rate of oxygen evolution by the purified core complex was 2,200 μmol (mg Chl)$^{-1}$ h^{-1} under the same conditions as above, and was enhanced to 3,400 μmol (mg Chl)$^{-1}$ h^{-1} when measured at 45°C with ferricyanide alone as the electron acceptor.

Figure 2 shows an elution profile upon further gel-filtration of the purified PSII core complex with a Super-

Table 1 Purification of oxygen-evolving PSII core complex from *S. elongatus* 43-H

Fraction	Oxygen evolution μmol (mg Chl)$^{-1}$ h^{-1}	Total Chl (mg)	Yield (%)
Cells	350[a]	210	
Thylakoids	360[a]	150	100
DM extracts	310[a]	130	87
PSII core complexes	2,200[a]	5.0	3.3
	3,400[b]		

[a] Measured at 25°C using potassium ferricyanide plus 2,6-DCBQ as electron acceptors.
[b] Measured at 45°C using potassium ferricyanide alone as an electron acceptor.

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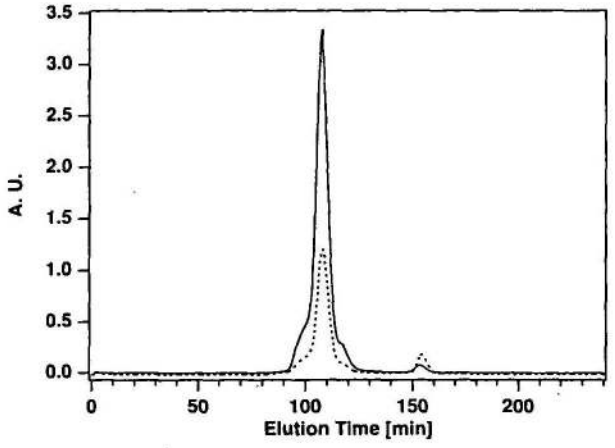

Fig. 2 Elution profile from a Superdex 200 gel-filtration column of His-tagged PSII core complex. Absorbance was recorded at 435 nm (solid line) and 280 nm (dotted line).

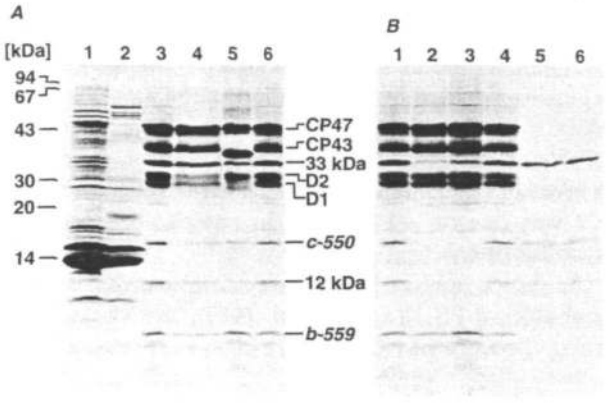

Fig. 3 SDS-PAGE profiles showing the changes in polypeptide compositions of the *S. elongatus* 43-H thylakoids before and after Ni^{2+}-affinity column chromatography (A) and effects of various treatments on the composition of extrinsic proteins (B). Panel A: lane 1, thylakoids (20 μg Chl); lane 2, flow through fraction (20 μg Chl) from Ni^{2+}-affinity column; lanes 3 and 6, His-tagged PSII core complex eluted by imidazole (10 μg Chl); lane 4, His-tagged PSII core complex (580 kDa) eluted from gel-filtration column (10 μg Chl); lane 5, PSII core complex purified from *S. vulcanus* for reference (10 μg Chl). Arrows indicate low molecular mass polypeptides of 6.5 kDa, 5.4 kDa and 5.0 kDa. Molecular size markers used were: 97 kDa, phosphorylase; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, soybean trypsin inhibitor and 14 kDa, α-lactoalbumin. Panel B: lane 1, untreated His-tagged PSII core complex (10 μg Chl); lane 2, 1 M Tris-treated pellet; lane 3, 1 M $CaCl_2$-treated pellet; lane 4, 2 M NaCl-treated pellet; lane 5, 1 M Tris-treated supernatant; lane 6, 1 M $CaCl_2$-treated supernatant.

dex-200 column. The profile exhibited a major peak at 107 min, which was preceded by an obscure shoulder at around 100 min and followed by a slightly more distinct shoulder at around 116 min. The peak was also accompanied by a clearly separated small peak at 154 min. The major component eluted at 107 min and the faint shoulder eluted at 116 min corresponded to the molecular sizes of 580 kDa and 350 kDa, respectively, and were assigned respectively to dimeric and monomeric forms of cyanobacterial PSII core complex. Notably, as opposed to the case in higher plant PSII, the abundance of the monomeric complex was less than 1%, indicating that most of the cyanobacterial PSII core complex take a dimeric form in DM-solubilized solutions and during the affinity chromatography as well. The obscure shoulder eluted at around 100 min corresponded to the molecular size of about 610 kDa, which was probably assignable to the PSII core complex in association with phycobiliproteins. The small peak at 154 min corresponded to a molecular size of 100 kDa. This component seemed to be a Chl-less protein, since its absorbance at 435 nm (due to Chl *a*) was much lower than the absorbance at 280 nm, in contrast to the other components. It is not clear at present whether this protein is a component(s) dissociated from the PSII core complex upon the second solubilization with a higher concentration of DM (1.5%) that was needed for gel-filtration column chromatography, or an unknown protein (complex) not related to the PSII-subunit proteins but happened to have an affinity for Ni^{2+}-column.

Polypeptide composition—Polypeptide composition of the purified PSII core complex was analyzed by urea-containing SDS-PAGE and immuno-blotting. Figure 3A shows the polypeptide profiles of cyanobacterial thylakoids, the flow-through fraction from the Ni^{2+}-affinity column, the PSII core complex eluted from the affinity column, and the dimeric form of PSII core complex purified by gel-filtration. Oxygen-evolving PSII core complex purified from *S. vulcanus* (gift from Dr. J.-R. Shen, RIKEN Harima Institute) was also run as a reference. Large amounts of 16- and 14-kDa phycobiliproteins present in thylakoids (lane 1) were recovered in the flow-through fraction of the affinity column chromatography (lane 2), indicative of their efficient removal by Ni^{2+}-affinity separation. The purified PSII core complex clearly exhibited the polypeptide bands of CP47, His-tagged CP43, extrinsic 33-kDa protein, D2 and D1 reaction center proteins, cytochrome *c-550*, extrinsic 12-kDa protein, and the L-subunit of cytochrome *b-559* (lanes 3, 4 and 6), but only faintly the bands due to phycobiliproteins. Notably, the migration of CP43 polypeptide in *S. elongatus* 43-H preparations (lanes 3, 4 and 6) differed clearly from that in *S. vulcanus* preparation (lane 5), indicative of the difference in its molecular mass due to the five amino acid extension for the thrombin recognition site and the six consecutive histidine residues added to the C-terminus of *S. elongatus* CP43. In low molecular mass region, there were seen at least three bands of 6.5 kDa, 5.4 kDa and 5.0

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kDa as already reported for the crude PSII core preparation from *S. vulcanus* (Koike et al. 1989). When the PSII core complex fraction eluted from the Ni^{2+}-affinity column was further purified by gel-filtration, a few faint bands including those due to phycobiliproteins disappeared (lane 4). This supports the idea that the shoulder at around 100 min preceding the main band on the gel-filtration profile of Fig. 2 was due to a PSII core in complex with residual phycobiliproteins.

As shown in Fig. 3B, the three extrinsic components of cyanobacterial PSII (Koike et al. 1989), the 33-kDa polypeptide, cytochrome *c-550* and 12 kDa, were released from the core complex upon treating the preparation with 1 M $CaCl_2$ or alkaline Tris (lanes 2, 3, 5 and 6). However, a wash with 2 M NaCl did not release these proteins at all from the PSII core complex (lane 4). These responses to salt-washing treatments of the extrinsic proteins of *S. elongatus* PSII were the same as those in *S. vulcanus* (Shen et al. 1992), but were different from those in *Phormidium* (Stewart et al. 1985) or in mesophilic cyanobacteria (Tang and Diner 1994), in which cytochrome *c-550* and the 12 kDa protein were released by high concentration salt washing, or had been lost during the preparation procedures. Probably, cytochrome *c-550* and the extrinsic 12-kDa protein in such thermophilic cyanobacteria as *S. vulcanus* and *S. elongatus* are bound to the PSII core as tightly as the 33 kDa extrinsic protein.

Absorption and EPR spectra—Figure 4 shows a room temperature absorption spectrum of the purified PSII core complex from *S. elongatus* 43-H strain. The absorption in visible wavelength region was mostly contributed by chlorophyll *a* peaking at 674.5 nm and some carotenoid species absorbing between 450 and 500 nm, with apparently no contribution by phycobilins. A small but significant peak at 545 nm indicates a substantial enrichment of pheophytin *a*,

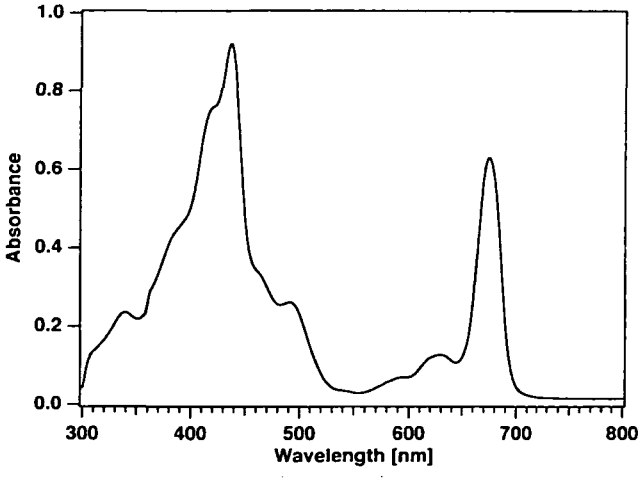

Fig. 4 Room temperature absorption spectrum of purified PSII core complex from His-tagged *S. elongatus* 43-H cells.

the primary electron acceptor of PSII. These spectral features were typical of several PSII core complexes from various sources so far reported: a green alga *Chlamydomonas reinhardtii* (Sugiura et al. 1998), a cyanobacterium *Synechocystis* sp. (Tang and Diner 1994) and spinach (Haag et al. 1990).

The number of chlorophyll *a* molecules bound to the purified PSII core complex was calculated to be 45.5±2.5 on the basis of Y_D^+ spin density. This value was comparable to the so far reported results: 40–50 Chl per reducible Q_A determined for the PSII core complex isolated from a *Chlamydomonas reinhardtii* mutant lacking PSI and ATPase (De Vitry et al. 1991), about 48 Chl per 2 pheophytin *a* molecules for spinach PSII core complex (Yamada et al. 1987), and 45±5 Chl per reduced Q_A for cyanobacterial PSII core complex (Rögner et al. 1990), whereas the value was slightly higher than that reported for the mesophilic cyanobacterial PSII core complex (37.5±2 per 2 pheophytin molecules) determined by Tang and Diner (1994).

Figure 5 shows the low temperature EPR spectra of

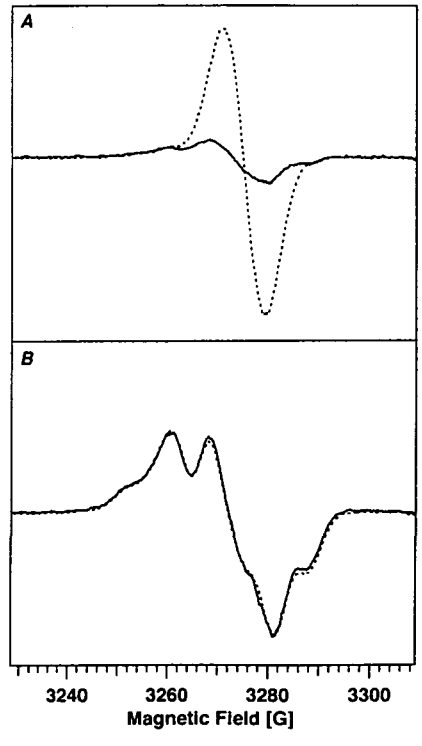

Fig. 5 EPR spectra of thylakoids (A) and isolated PSII core complex (B) from *S. elongatus* 43-H cells. Dotted- and solid-curves indicate spectra measured in the presence and absence of 5 mM potassium ferricyanide, respectively. Chlorophyll concentrations of thylakoids and PSII core complex were 3 mg 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.

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S. elongatus thylakoids and PSII core complex recorded in the presence and absence of ferricyanide. The spectrum recorded at 77 K after a 30-s preillumination at 273 K in the absence of ferricyanide followed by a 30-s dark-relaxation of Y_Z^+, exhibited a weak but clear signal assignable to Y_D^+, oxidized form of the auxiliary second electron donor of PSII reaction center (Fig. 5A, solid curve). The spectrum recorded in the presence of ferricyanide, however, exhibited an intense signal centering at $g \approx 2$ due to $P700^+$ (dotted curve), indicative of chemical oxidation of the PSI reaction center chlorophyll by ferricyanide. The EPR spectra of the purified PSII core complex recorded under the same conditions are shown in Fig. 5B. When measured in the absence of ferricyanide (solid curve), the spectrum exhibited a strong double-peaked derivative band characteristic of the Y_D^+ signal arising from the oxidized Tyr-160 of D2 protein. It should be noted that the spectrum recorded in the presence of ferricyanide (dotted curve) coincided exactly with the solid curve, indicative of complete absence of chemically oxidizable P700 in the PSII core preparation. These Y_D^+ spectra also coincided well with those reported in literatures (Miller and Brudvig 1991), suggesting that the extension of six histidine residues at the C-terminus of CP43 does not influence the molecular environment of Y_D^+. These results are the same as those observed in *Chlamydomonas* PSII core complex having a similar His-tag at the C-terminus of D2 (Sugiura et al. 1999). It is clearly indicated from these that the oxygen-evolving PSII core complex prepared from the thermophilic cyanobacterium by the His-tag methodology is completely free from PSI contamination.

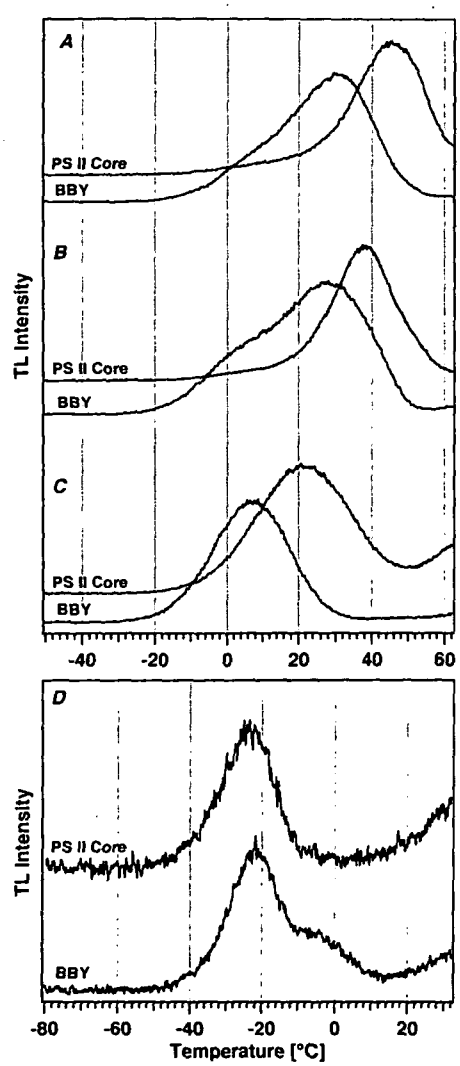

Fig. 6 Thermoluminescence glow curves of PSII core complex purified from His-tagged *S. elongatus* 43-H cells and BBY-type membranes of spinach. The glow curves were recorded at a heating rate of 40°C min^{-1} after 1 flash illumination at 5°C (A), after 2 flashes at 5°C (B), and after 1 flash at 5°C in the presence of 10 μM DCMU (C). Mn-depleted samples were illuminated with red light for 20 s at −25°C (D).

Thermoluminescence—Thermoluminescence serves as a convenient probe of both the donor and acceptor chemistry in PSII (see Inoue 1996 for review), since many of the emission bands have been assigned to result from respective recombinations between the electrons trapped on specific acceptors and the positive equivalents trapped on specific donors of PSII. Figure 6 shows the thermoluminescence glow curves of the purified PSII core complex from *S. elongatus* in comparison to those from spinach BBY. Upon excitation with a single flash, the PSII core complex exhibited a thermoluminescence band peaking at an emission temperature at around 45°C (Fig. 6A), which was assigned to the B_2-band originating from the charge recombination between S_2 and Q_B^- (Inoue 1996). As shown in Fig. 6B, excitation with two flashes resulted in emission of the B_1-band at around 38°C that has been known to arise from the charge recombination between S_3 and Q_B^-. When the electron transfer between Q_A and Q_B was blocked by 10 μM DCMU prior to the excitation, the emission temperature of the B_2-band downshifted to around 20°C (Fig. 6C), indicative of conversion of the B_2-band to the Q-band originating from $S_2Q_A^-$ charge recombination. The emission temperatures of B_2- and Q-bands were identical to those found in thylakoids and intact cells of *S. elongatus* 43-H mutant, and wild type cells as well. In spinach BBY membranes, however, the emission temperatures of the bands corresponding to B_2-, B_1- and Q-bands lower by approximately 15°C than the emission temperatures of respective bands found in *S. elongatus* PSII core complex.

Generally speaking, there are two alternative causes for a deeper stabilization of charge pair in a thermophilic PSII (Inoue 1996): either the redox potential(s) of an S-state(s) is downshifted, or the redox potential(s) of Q_A^- and/or Q_B^- is upshifted as compared with those in meso-

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philic organisms like spinach. In order to find information for this problem, the emission temperature of the A_T-band that is known to arise from charge recombination between His^+ (His_{195} or His_{190} of D1 protein, Kramer et al. 1994) and Q_A^- in Mn-depleted PSII (Ono and Inoue 1991), was examined with the PSII of *S. elongatus*. As shown in Fig. 6D, the *S. elongatus* PSII core complex depleted of Mn emitted the A_T-band at −24°C, practically the same temperature as observed for Mn-depleted spinach BBY membranes (−22°C). The fact that the emission temperature of $S_2Q_A^-$ charge recombination was upshifted by 15°C while that of $His^+Q_A^-$ was not upshifted, suggests that in PSII of a thermophile, the redox potential of S_2 was downshifted although that of Q_A^- remained unchanged.

The glow curves from BBY membranes shown in Fig. 6A and B exhibited a weak satellite emission band, superimposed on the lower temperature side of the main peak. This arises probably from the $S_2Q_A^-$ recombination in Q_B-depleted centers. The glow curves from the thermophilic PSII core complex, on the other hand, did not exhibit such satellite bands. This implies that all of the PSII reaction centers in *S. elongatus* core complex are associated with functional Q_B quinones, whereas some of those in BBY membranes are not functionally connected with Q_B quinones.

Electron acceptor- and temperature-dependencies of oxygen evolution—Figure 7 shows the temperature dependence of oxygen evolution by the purified PSII core complex measured with different species of electron acceptors. The optimal acceptor species for maximal activity differed substantially depending on the reaction temperature. When ferricyanide alone was the electron acceptor, the activity at 25°C of about 2,200 μmol O_2 (mg Chl)$^{-1}$ h^{-1} exhibited a sharp increase with increasing the temperature to reach the maximum rate of 3,400 μmol O_2 (mg Chl)$^{-1}$ h^{-1} at 45°C. When 2,6-DCBQ alone was the electron acceptor, the activity at 25°C was more or less the same as that measured with ferricyanide, but the activity

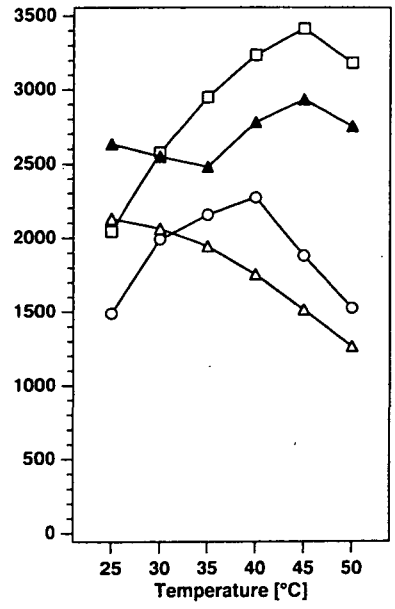

Fig. 7 Temperature dependence of oxygen evolution by purified PSII core complex of *S. elongatus* 43-H measured with different electron acceptors: 2 mM potassium ferricyanide (squares), 0.5 mM 2,6-DCBQ (open triangles), 0.6 mM PBQ (open circles), and 2 mM potassium ferricyanide plus 0.5 mM 2,6-DCBQ (solid triangles).

exhibited a gradual decrease with increasing the temperature. On combined use of the above two electron acceptors, the activity at 25°C was increased by 25% to 2,600 μmol O_2 (mg Chl)$^{-1}$ h^{-1}, which was then slightly decreased up to 35°C and then increased sharply above 35°C to exhibit the maximum rate of 2,900 μmol O_2 (mg Chl)$^{-1}$ h^{-1} at 45°C. This temperature dependence appears to consist of two parts: it followed the dependence of the 2,6-DCBQ curve below 35°C but that of the ferricyanide curve above 35°C. Probably, the electron acceptor site switches its affinity from 2,6-DCBQ to ferricyanide at around 35°C. PBQ was

Table 2 Acceptor species dependence and DCMU sensitivity of oxygen evolution by PSII core complex from *S. elongatus* 43-H

Acceptor	Oxygen evolution		Inhibition (%)
	−DCMU	+DCMU[a]	
	μmol (mg Chl)$^{-1}$ h^{-1}		
Ferricyanide (2 mM)	3,410	1,240	64
2,6-DCBQ (0.5 mM)	2,130	170	92
PBQ (0.6 mM)	2,270	190	93.5
Ferricyanide (2 mM) +2,6-DCBQ (0.5 mM)	2,930	n.d.[b]	

[a] 20 μM.
[b] not determined.

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a poorer electron acceptor than 2,6-DCBQ when measured at 25°C, but its capability as an electron acceptor increased sharply with increasing the temperature to exceed that of 2,6-DCBQ above 30°C, showing the maximum rate of 2,300 μmol O_2 (mg Chl)$^{-1}$ h^{-1} at 40°C. Table 2 summarizes these temperature dependencies with respective electron acceptors, together with the sensitivity to DCMU. It was clearly shown that oxygen evolution supported by 2,6-DCBQ or PBQ was strongly inhibited over 90% by 20 μM DCMU irrespective of the reaction temperature. The high sensitivity to DCMU implies that the structure of the Q_B-binding site in the purified PSII core complex was preserved intact. In contrast, the activity supported by ferricyanide exhibited a lower sensitivity to DCMU, being blocked by only 64% at 45°C, suggesting that ferricyanide accepts electrons not only from Q_B but also from Q_A.

Stability of the PSII core complex—In order to test the stability of the purified thermophilic PSII core complex, the courses of degradation of its subunit proteins and inactivation of oxygen evolution were examined during incubation at room temperature. Figure 8 shows the SDS-PAGE profiles of the PSII core complex sampled intermittently during dark-incubation at 20°C for indicated days (1–21 d). Samples were centrifuged to remove dissociated proteins and the precipitates were run on the gel. There was practically no change in quantity and molecular sizes of the constituent proteins at least during the beginning incubation of 8 d (lanes 1–7). Even after incubation for 21 d, these situations did not change except for a slight deformation of the spot corresponding to CP47: formation of a doublet-like spot. It is not clear at present whether the doublet formation was due to digestion by a protease. Conformational change due to dephosphorylation of initially phosphorylated CP47 is unlikely because there have been no paper reporting the phosphorylation of this pigment protein. Since the PSII core complex did not suffer any degradation of its subunit proteins even after such a long period of incubation, we may assume that our purified PSII core complex is free from proteolytic enzymes. Lane-8 profile also indicates that all the three extrinsic proteins of 33 kDa and 12 kDa and cytochrome *c-550* were not released but remained firmly associated, probably at their respective binding sites, even after 21-day dark-incubation at room temperature.

Figure 9 compares the course of changes in oxygen evolving-activity during dark-incubation at room temperature between *S. elongatus* PSII core complex and spinach BBY membranes as a reference. The *S. elongatus* PSII core complex retained more than 90% of the original activity after the beginning incubation of 8 d, whereas spinach BBY lost 35% of the original activity. After 21 d of incubation, the PSII core complex still retained approximately 80% of the original activity, while the activity of BBY membranes dropped to less than 30%. Thylakoids from *S. elongatus* retained the activity as high as that of the PSII core complex during the first 8 d, but the activity decreased gradually to approximately 60% after 21 d. The lower stability of thylakoids as compared with that of purified PSII core complex would probably be due to the presence of some protease(s) in thylakoids. The extremely high stability of oxygen evolution of the purified PSII core complex from the transformed thermophilic cyanobactrium, *S. elongatus* 43-H, together with the simple procedures

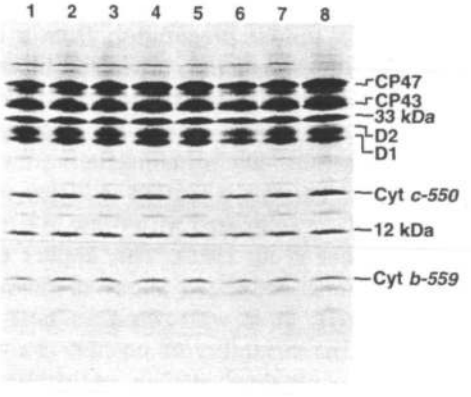

Fig. 8 SDS-PAGE profile of purified PSII core complex from *S. elongatus* 43-H after the incubation in darkness at 20°C for 0 d (lane 1), 1 d (lane 2), 2 d (lane 3), 3 d (lane 4), 4 d (lane 5), 6 d (lane 6), 8 d (lane 7), and 21 d (lane 8).

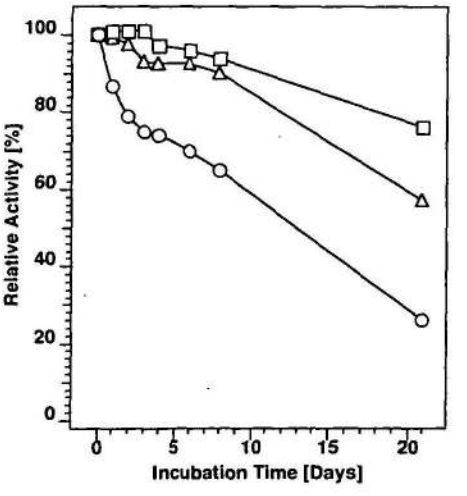

Fig. 9 Oxygen-evolving activity of purified *S. elongatus* PSII core complex, *S. elongatus* thylakoids and spinach BBY-type membranes after the incubation in darkness at 20°C. Squares, purified PSII core complex of *S. elongatus*; triangles, thylakoids of *S. elongatus*; circles, BBY membranes of spinach. Activities were measured at 25°C with 0.5 mM 2,6-DCBQ plus 2 mM ferricyanide for *S. elongatus* samples and with 0.6 mM PBQ for spinach BBY as electron acceptors, and expressed in relative activity with the 100% activities of 2,200, 360 and 560 μmol (mg Chl)$^{-1}$ h^{-1} for *S. elongatus* PSII core complex, *S. elongatus* thylakoids and spinach BBY, respectively.

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for its preparation, will be highly useful for variety of studies on PSII reaction center and the oxygen-evolving apparatus.

Discussion

The present study has demonstrated that a highly functional and highly stable PSII core complex can be isolated by a simple one-step metal-affinity chromatographic separation of detergent-solubilized thylakoids of a thermophilic cyanobacterium *S. elongatus* mutant having an artificial His-tag extension at the C-terminus of CP43. Furthermore, the purified core complex was extremely stable, showing practically no change in its protein subunit compositions during incubation at room temperature as long as 21 d, indicative of tight binding of the three extrinsic proteins and complete absence of proteolytic enzymes (Fig. 8).

Molecular size analysis by gel-filtration chromatography revealed that most of the PSII core complex particles isolated from *S. elongatus* in the present study were in dimeric form (Fig. 2). This result contrasts with those reported from other laboratories which have suggested the presence of both dimeric and monomeric forms of PSII in cyanobacterial thylakoids (Dekker et al. 1988) or in higher plant thylakoids (Hankamer et al. 1997), with a higher rate of oxygen evolution by dimeric PSII than monomeric PSII. It has also been reported that the dimeric form was converted to the monomeric form upon removal of the extrinsic 33 kDa protein (Hasler et al. 1997). If we assume that the isolation procedure employed in the present study was much milder with respect to the contact period with a detergent owing to the His-tag methodology, we may conclude that the PSII complex exists in dimeric form in vivo, at least in *S. elongatus*, and the detection of two forms of PSII is a sort of artifact arising from longer exposure to detergents. This idea corroborates the electron microscopic results that have visualized the presence of dimeric PSII particles in complex with light-harvesting chlorophyll proteins in higher plant thylakoids (Boekema et al. 1995, Lyon 1998).

The PSII core complex isolated in this study retained a high activity of oxygen evolution, which exhibited the maximum rate as high as 3,400 μmol O_2 $(mg\ Chl)^{-1}\ h^{-1}$ when measured at 45°C with ferricyanide as an electron acceptor. Notably, the activity was extremely stable, showing a less than 10% loss during the first 8 d, and a less than 20% loss after 21-day dark-incubation at room temperature (Fig. 9). This extremely high stability of oxygen-evolving activity parallels with the highly stable protein composition of this complex that is accompanied by no release of Mn-stabilizing extrinsic proteins and practically no degradation of any subunits of the complex (Fig. 8). Interestingly, different types of temperature dependence were observed depending on the species of electron acceptor: with ferricyanide or PBQ as an alone acceptor the activity was positively proportional with temperature, while with 2,6-DCBQ alone it was inversely proportional with temperature, and with a combination of ferricyanide plus 2,6-DCBQ both types of dependencies were mixed (Fig. 7). The complex also showed an efficient electron transfer to ferricyanide even at a lower temperature of 25°C with a high sensitivity to DCMU. This is another unusual aspect of the acceptor species dependence of this complex: in usual PSII core complexes, non-charged hydrophobic electron acceptors like 2,6-DCBQ are much more efficient than charged hydrophilic electron acceptors like ferricyanide, especially when the Q_B site is kept intact. As we have no data at present measured with non-His-tagged control PSII prepared from the same organism, it is difficult to argue about the cause for these unusual features of temperature- and acceptor-dependencies in relation to the presence of His-tag moiety that is expected to be located at the surface of the core complex. However, it may be interesting to hypothesize that they have resulted from some influence due to the strong positive charge of histidine residues protruding probably on the stromal side, the electron acceptor side, attracting the negatively charged ferricyanide. The assumption of His-tag protruding at stromal side may be rational as deduced from the hydropathy plot of amino acid sequence of CP43 (Bricker 1990, Sayre and Wrobel-Boerner 1994).

Thermoluminescence analyses confirmed the presence of functional Q_B quinone in the isolated PSII core complex by showing the thermoluminescence emission arising from $S_2Q_B^-$ and $S_3Q_B^-$ charge recombinations (Fig. 6). The presence of functional Q_B quinone contrasts with the His-tagged PSII core complex isolated from *Chlamydomonas*, in which the emission due to $S_2Q_B^-$ recombination could not be detected, although the Q_B-binding site retained high sensitivity to DCMU (Sugiura et al. 1999). The presence of functional Q_B quinone in isolated PSII core complex has been reported for a similar preparation from a thermophilic cyanobacterium, *S. vulcanus*, (Shen and Inoue 1993), but its thermoluminescence properties are slightly different from those of *S. elongatus* as will be briefly discussed later.

In cells of thermophilic cyanobacteria, the major thermoluminescence bands are emitted at higher temperatures by about 20°C as compared with those in mesophilic organisms (Govindjee et al. 1985). This implies a deeper stabilization of charge separated states in thermophilic PSII reaction centers. It is worthwhile to note that in thermophilic PSII the capability of positive charge accumulation must be maintained at high temperatures, presumably because it is needed for normal S-state turnovers by some reasons. In contrast to the clear upshifts in emission temperature of Q- and B-bands found for the present thermophilic PSII core complex, however, the A_T-band

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from Mn-depleted complex did not show such clear difference in emission temperature (Fig. 6D). The former two bands involved oxidized S-state whereas the latter band involved a redox-active histidine residue as the carrier of positive equivalent (Ono and Inoue 1991). If we assume that the redox potentials of Q_A and Q_B quinones are not much different between mesophilic spinach and thermophilic *S. elongatus*, the results are interpreted that the upshifted Q- and B-bands found in thermophilic cyanobacteria are due to lowered redox potentials of the oxidized S-states, the Mn-cluster. It should be noted in this context that a similar thermophilic PSII core complex prepared from another thermophilic cyanobacterium, *S. vulcanus*, did not retain this capability of deeper stabilization but emitted their thermoluminescence bands at temperatures more or less the same as those of mesophilic PSII (Shen and Inoue 1993). The reason for this difference is not clear, but there is a possibility that longer contact with detergent gave rise to a faint change in the redox potential of the thermophilic Mn-cluster. Seemingly, complete retention of the functions of Q_B and Mn-cluster, including their redox potentials, in purified PSII core complex is possible only by applying His-tag methodology to thermophilic cyanobacterial thylakoids.

There are three PSII core complex preparations so far reported from different organisms with different sites of His-tag insertion. The first one used a *Chlamydomonas* mutant having a His-tag extension at the C-terminus of D2 (Sugiura et al. 1998, 1999), the second one a *Synechocystis* mutant with a His-tag at the C-terminus of CP47 (Bricker et al. 1998) and the present work a thermophilic *Synechococcus elongatus* mutant with a His-tag at the C-terminus of CP43. In these three cases, His-tag insertion at the C-terminus was commonly successful for isolation of highly active oxygen-evolving core complex, but the topology of the hexa-histidine tags as deduced from the hydropathy plots of the target proteins is not same: the C-terminus of D2 will protrude to the lumenal side whereas those of CP47 and CP43 to the stromal side. For all cases we may assume that the tag was exposed at the surface of the core complex with some freedom in motion, as evidenced by the successful isolation of the complex by metal affinity chromatography. Since all the three PSII core complexes exhibited high rates of oxygen evolution comparable to those of respective wild type cells, we may consider that the consecutive hexa-histidine extension does not give rise to any unfavorable influence on the functioning of PSII. We presume that hexa-histidine extension at the C-terminus of CP43 does not affect the structural integrity of PSII core complex. Absorption peaks, oxygen evolution activity, and Chl/reaction center ratio were all identical to those determined for the PSII core complexes from mesophilic cyanobacteria (Nilsson et al. 1992, Tang and Diner 1994). This idea may be supported by the observation that the thermoluminescence B_2- and Q-bands of *S. elongatus* 43-H mutant cells and the thylakoids appeared at the same temperatures and those in the wild type cells and thylakoids.

In contrast to these successful cases, when the same His-tag was attached to the N-terminus of D1 of *Chlamydomonas*, which presumably protruded at the stromal side, there was no assembly of active PSII in the mutant thylakoids, indicative of strongly unfavorable effects on the assembly of PSII (Sugiura et al. 1998). Although the cause for this unfavorable effect is not clear, as far as no information was available as to the properties of the complex because of the absence of PSII assembly in the mutant thylakoids, selection of the insertion site and its effects on the functional properties of the resulting complex are matters of serious importance in future studies.

In conclusion, the present study demonstrated that application of the His-tag methodology to a thermophilic cyanobacterium *S. elongatus* was successful for rapid isolation of an extremely stable PSII core complex capable of oxygen evolution at a high rate. The obtained core complex is advantageous not only for crystallization of PSII but also for varieties of studies on the structure and function of the tetranuclear Mn-cluster, the catalyst of water oxidation for photosynthetic oxygen evolution.

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