ELSEVIER

Biochimica et Biophysica Acta 1232 (1995) 208–216

BBA Biochimica et Biophysica Acta

Isolation and characterization of a Photosystem II complex from the red alga *Cyanidium caldarium*: association of cytochrome *c*-550 and a 12 kDa protein with the complex

Isao Enami [a,*], Hiroshi Murayama [a], Hisataka Ohta [a], Masaharu Kamo [b], Katsuyoshi Nakazato [c,d], Jian-Ren Shen [d]

[a] *Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo 162, Japan*
[b] *Research Institute for Biosciences, Science University of Tokyo, Yamazaki, Noda, Chiba 278, Japan*
[c] *PRESTO, JRDC, RIKEN, Wako, Saitama 351-01, Japan*
[d] *Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan*

Received 8 June 1995; revised 7 August 1995; accepted 22 August 1995

Abstract

A Photosystem II (PS II) complex was purified from an acidophilic as well as a thermophilic red alga, *Cyanidium caldarium*. The purified PS II complex was essentially devoid of phycobiliproteins and other contaminating components, and showed a high oxygen-evolving activity of 2375 μmol O_2/mg Chl per h using phenyl-*p*-benzoquinone as the electron acceptor. The expression of this high activity did not require addition of exogenous Ca^{2+}, although EDTA reduced the activity by 40%. This effect of EDTA can be reversed not only by Ca^{2+} but also by Mg^{2+}; a similar Mg^{2+} effect has been observed in purified cyanobacterial PS II but not in higher plant PS II. Immunoblotting analysis indicated the presence of major intrinsic polypeptides commonly found in PS II from cyanobacteria and higher plants as well as the extrinsic 33 kDa protein. Antibodies against the extrinsic 23 and 17 kDa proteins of higher plant PS II, however, did not crossreact with any polypeptides in the purified PS II, indicating the absence of these proteins in the red alga. In contrast, two other extrinsic proteins of 17 and 12 kDa were present in the red algal PS II; they were released by 1 M Tris or Urea/NaCl treatment but not by 1 M NaCl. The 17 kDa polypeptide was identified to be cytochrome *c*-550 from heme-staining, immunoblot analysis and N-terminal amino acid sequencing, and the 12 kDa protein was found to be homologous to the 12 kDa extrinsic protein of cyanobacterial PS II from its N-terminal sequence. These results indicate that PS II from the red alga is closely related to PS II from cyanobacteria rather than to that from higher plants, and that the replacement of PS II extrinsic cytochrome *c*-550 and the 12 kDa protein by the extrinsic 23 and 17 kDa proteins occurred during evolution from red algae to green algae and higher plants.

Keywords: Oxygen evolution; Photosystem II; Extrinsic protein; Cytochrome *c*-550; Protein, 12 kDa ; (Red algae)

1. Introduction

Photosynthetic oxygen evolution takes place in the PS II complex that is commonly found from cyanobacteria to higher plants. The essential components of the PS II complex are almost identical between cyanobacteria and higher plants; they include the D1, D2 reaction center proteins, the intrinsic Chl-binding proteins CP47 and CP43, the α and β subunits of cyt *b*-559 (9 kDa and 4.5 kDa, respectively), the *psb*I gene product, the extrinsic 33 kDa protein and perhaps some other low-molecular-mass polypeptides functioning mostly in maintaining a functional structure of PS II (for reviews, see [1–3]). In contrast to these similarities, however, there are two extrinsic proteins that are significantly different between PS II complexes from higher plants and cyanobacteria. In higher plant PS II, the extrinsic 23 and 17 kDa proteins serve in maintain-

---

Abbreviations: CBB, Coomassie brilliant blue R-250; Chl, chlorophyll; Cyt, cytochrome; DM, *N*-dodecyl β-D-maltoside; EDTA, ethylenediaminetetraacetate; HTG, *N*-heptyl β-D-thioglucoside; Mes, 4-morpholineethanesulfonic acid; Q_B, the secondary quinone acceptor of Photosystem II; PS I and PS II, Photosystem I and Photosystem II; TMBZ, 3,3′,5,5′-tetramethylbenzidine.

[*] Corresponding author. Fax: +81 471 242150.

0005-2728/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0005-2728(95)00122-0

ing maximum oxygen-evolving activity and protecting the Mn cluster from attack by exogenous reducing agents; without these two extrinsic proteins, non-physiologically high concentrations of Ca^{2+} and Cl^- are required in order to maintain oxygen evolution and functional binding of the Mn cluster (for reviews, see [4–6]). In cyanobacterial PS II, on the other hand, these two extrinsic proteins are absent, but a low-potential cyt *c*-550 and a 12 kDa protein have been found as the alternative extrinsic components in maintaining oxygen evolution and stability of PS II in vivo [7–12]. Although these two extrinsic components have no homology in their primary sequences with the 23 and 17 kDa proteins of higher plant PS II [11,13], it has been shown that they have some binding properties in cyanobacterial PS II similar to the properties of the 23 and 17 kDa proteins in higher plant PS II [9–12,14]. This suggested a replacement of cyt *c*-550 and the 12 kDa protein by the 23 and 17 kDa proteins during evolution from cyanobacteria to higher plants. There do, however, exist some functional differences between the two sets of extrinsic proteins which may be responsible for the different requirement of Ca^{2+} and Cl^- between cyanobacterial and higher plant PS II; for example, the enhancing effect of Ca^{2+} on oxygen evolution in cyanobacterial PS II can be partially replaced by some other cations that can never be effective in higher plant PS II [5,7,10,15,16].

Red algae, in terms of the presence of nucleus, chloroplast and mitochondria within their cells, are evolutionarily closer to higher plants than to cyanobacteria. Their photosynthetic apparatus, however, is more similar to that of cyanobacteria, since it contains phycobilisome as light-harvesting antenna instead of Chl *a*/*b* binding proteins as found in higher plants, although a Chl *a*-binding antenna protein has recently been reported to be present in PS I of a red alga [17]. Consequently, thylakoid membranes are not stacked in chloroplasts of red algae. Comparative studies on the level of photosynthetically functional membrane-protein complexes, in particular the PS I and PS II complexes, from red algae with those from cyanobacteria and higher plants, however, have been rare partly due to difficulties in obtaining purified PS I and PS II complexes from red algae. Wolfe et al. [18] recently isolated PS I and PS II complexes from a red alga, *Porphyridium cruentum*, and reported that the PS I from this red alga is structurally more similar to that of higher plants rather than to that of cyanobacteria. The PS II complex they purified, however, had lost all the extrinsic components required for oxygen evolution, which prevented a direct comparison of PS II complex from the red alga with that of cyanobacteria and higher plants. In particular, cyt *c*-550 is known to be present in red algae [19,20], but its cellular location and function have not been clarified. In order to determine whether this cytochrome is associated with red algal PS II and to compare the PS II complex of red algae in detail with that of cyanobacteria and higher plants, we purified an intact PS II complex highly competent in oxygen evolution from a red alga, *Cyanidium caldarium*. This alga is acidophilic, with an optimum growth pH range of 1–3, as well as being thermophilic, growing maximally at 40–50°C [21,22]. Our results revealed that, in addition to the extrinsic 33 kDa protein, the PS II from *C. caldarium* contained cyt *c*-550 and a 12 kDa protein as extrinsic components; their N-terminal amino acid sequences were homologous to those of cyt *c*-550 and the 12 kDa protein found in cyanobacterial PS II. We also showed that Mg^{2+} could enhance oxygen evolution suppressed by EDTA in the purified PS II to an extent comparable to the enhancing effect of Ca^{2+}, a feature also found in cyanobacterial PS II but not in higher plant PS II. The present results therefore indicated that, unlike PS I, PS II of red algae is closely related to PS II of cyanobacteria rather than to that of higher plants.

2. Materials and methods

2.1. Cell culture and thylakoid membrane preparation

Cells of *Cyanidium caldarium* Geitler were grown at 40°C in a 40 L incubator in an inorganic medium described in [21], with continuous agitation by bubbling of 5% CO_2-containing air under illumination provided by white fluorescent tubes at 3000 lux. The pH of the growth medium was adjusted to 3 by H_2SO_4. 1-week-old cells of the alga were filtered through three layers of gauze, collected by centrifugation at $3000 \times g$ for 5 min, washed three times with distilled water, and then resuspended in medium A that contained 25% glycerol, 10 mM $CaCl_2$ and 50 mM Mes (pH 6.0). The cells were disrupted by agitation with glass beads of 100 μm in diameter on ice in the dark for 8 cycles; with 15 s on and 1 min off for each cycle. This gave a maximum yield of cell disruption without affecting the oxygen-evolving activity. The thylakoid membranes were collected by centrifugation at $100\,000 \times g$ for 20 min and resuspended in medium A.

2.2. Preparation of crude PS II particles

Thylakoid membranes were solubilized with 2% HTG in medium A at 0.5 mg Chl/ml for 30 min at 0°C in the dark. The HTG-treated membranes were centrifuged at $100\,000 \times g$ for 20 min to remove unsolubilized materials, and the resultant supernatant was loaded onto a layer of 1 M sucrose in medium A followed by centrifugation at $410\,000 \times g$ for 1 h. This resulted in a green band at the surface of the 1 M sucrose layer and a dark blue precipitate, with some free phycobiliproteins present between the green band and the precipitate. The precipitate showed a high oxygen-evolving activity and was referred to as crude PS II particles, whereas the green band was rich in PS I and phycobiliproteins and had only a very low oxygen-evolving activity.

2.3. Preparation of pure PS II complexes

The crude PS II particles were further solubilized with 2% DM in 25% glycerol and 50 mM Mes (pH 6.0) at 1.2 mg Chl/ml, and the solubilized particles were loaded onto a DEAE-Sepharose CL-6B column (Pharmacia Biotech) equilibrated with 0.05% DM, 25% glycerol, 0.2 M NaCl and 50 mM Mes (pH 6.0). Washing the column with the same medium first eluted a blue fraction rich in phycobiliproteins, followed by elution of a green fraction rich in PS I. The purified PS II complexes were eluted with the same medium containing 0.33 M NaCl. The eluted PS II complexes were either concentrated by membrane filtration with Centriprep-10 or collected by centrifugation at 35 000 × *g* for 15 min following incubation with 17% poly(ethylene glycol) 6000 at 0°C for 30 min in the dark.

2.4. Characterization of the purified PS II complexes

SDS-polyacrylamide gel electrophoresis was carried out as described in [23], with a gradient gel of 16–22% containing 7.5 M urea, in the buffer system of Laemmli [24]. Samples were solubilized with 2% lithium lauryl sulfate and 70 mM dithiothreitol. For Western blotting, proteins on the gel were transferred onto a nitrocellulose membrane (Schleicher & Schuell, pore size of 0.45 μm), reacted with respective antibodies, and visualized with biotinylated anti-rabbit IgG. Antibodies against D1, D2, CP47, CP43, the α subunit of cyt *b*-559 and the extrinsic 33 kDa, 23 kDa, 17 kDa proteins were raised against respective proteins from spinach; antibodies against cyt *c*-550 and the 12 kDa extrinsic protein were raised against the proteins from thermophilic *Synechococcus vulcanus* [25]. Heme-staining was carried out with TMBZ/H_2O_2 on the gel, according to the procedure described in [25–27].

For dissociation of extrinsic proteins, the purified PS II was treated with either 1 M Tris (pH 8.5) containing 10 mM $MgCl_2$ or 2.6 M urea/0.2 M NaCl or 1 M NaCl in the suspension medium at 0.5 mg Chl/ml for 30 min on ice in the dark. The treated complex was precipitated by centrifugation at 35 000 × *g* for 20 min after incubation with 10% poly(ethylene glycol) 6000 at 0°C for 30 min in the dark. For determining N-terminal amino acid sequences, the extrinsic proteins released by 1 M Tris wash were separated by electrophoresis, and the proteins on the gel were transferred onto a PVDF membrane (Clear Blot Membrane-p; ATTO, Japan), stained with 0.1% CBB and destained with 40% methanol, 10% acetic acid. Each band on the membrane was cut out, and the N-terminal sequence was determined by the Edman degradation method with an automatic amino acid sequencer (Model 477A, Applied Biosystems).

Oxygen evolution was measured with a Clark-type oxygen electrode at 25°C, in a medium of 25% glycerol, 20 mM NaCl and 50 mM Mes (pH 6.0) with 0.6 mM phenyl-*p*-benzoquinone as an electron acceptor unless otherwise indicated. Chlorophyll *a* was extracted with 80% acetone and its concentration was determined according to [28].

3. Results

The procedure we used to purify PS II complex from the red alga *C. caldarium* was designed based on the methods previously described for purification of PS II from higher plants [29] and cyanobacteria [9,30–33], and is schematized in Fig. 1. First, cells of the red alga were disrupted with glass beads, a method commonly used to disrupt cyanobacterial cells [31–33]. The thylakoid membranes obtained were then solubilized with HTG, which has been used successfully to remove light-harvesting proteins from PS II membranes of higher plants [29]. The solubilized membranes were centrifuged on a layer of 1 M sucrose [30] resulting in a crude PS II particle. This crude PS II particle may still reside in the membrane fragments of the thylakoids and be present in an aggregated form, since it was found in the pellet after centrifugation. Lane 3 in Fig. 2 shows that this crude PS II particle retained a large amount of phycobiliproteins and some PS I components. To further purify the PS II complex, the crude PS II particle was treated with DM and separated with an anion-exchange column. This one-step column chromatography successfully removed the phycobiliproteins and PS I components by elution at a low concentration of NaCl, resulting in a purified PS II complex which eluted at a higher NaCl concentration (Fig. 2, lanes 4–6). It should be noted here that in the present conditions, the PS I fraction was eluted before the PS II fraction from the column, whereas in some cyanobacteria, PS II was eluted from anion-ex-

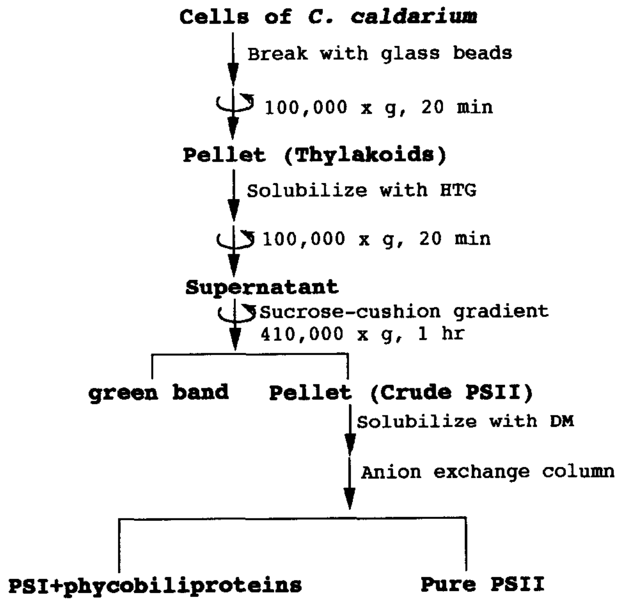

Fig. 1. Purification procedure for the PS II complex from the red alga, *Cyanidium caldarium*. See text for details.

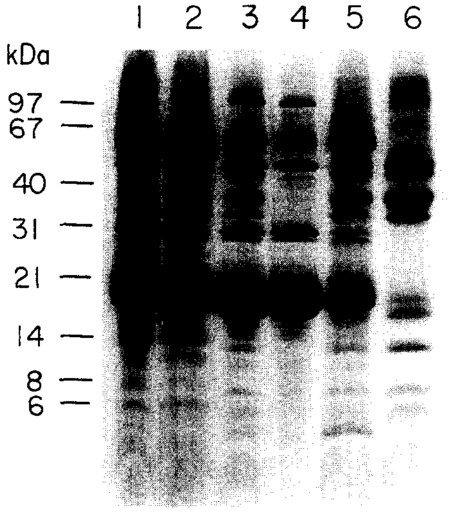

Fig. 2. SDS-polyacrylamide gel electrophoresis of each fraction obtained during purification of red algal PS II complex. Lane 1, thylakoid membranes; lane 2, green band obtained by 1 M sucrose-cushion centrifugation of HTG-solubilized thylakoids; lane 3, crude PS II; lanes 4 and 5, blue and green fractions, respectively, eluted from the column directly with equilibrium medium containing 0.2 M NaCl; lane 6, purified PS II.

change columns before a major PS I fraction [32,33]. This is mostly because the starting material of the column chromatography in the present study was a crude PS II particle which had been largely depleted of PS I; in fact, a similar elution pattern has been observed for a crude PS II from a thermophilic cyanobacterium [9]. It is possible that the major PS I fraction eluted after PS II in some cyanobacteria [32,33] is a trimeric form of PS I, which may have been removed by HTG treatment or may not occur at all in the present work, since we did not include any divalent cations in the solutions used for the column chromatography.

Table 1
Recovery of Chl and oxygen-evolving activity in each fraction obtained during purification of PS II from *Cyanidium caldarium*

	Total Chl		Oxygen evolution	
	mg Chl	%	µmol O_2 /mg Chl per h	%
Thylakoids	7.0	100 [a]	412	100 [b]
Sucrose-cushion centrifugation				
Green band	4.1	58	112	16
Crude PS II	1.5	21	843	43
Anion exchange				
Green fraction	0.48	7	726	12
Pure PS II	0.22	3	2514	18

[a] Chl yield with the amount of Chl in starting thylakoids as 100%.

[b] Yield of oxygen-evolving activity with that of thylakoids as 100%, multiplied by the Chl yield.

Table 1 shows recovery of Chl and oxygen-evolving activity in each fraction obtained during the purification procedure. Upon solubilization of the thylakoid membranes with HTG and centrifugation, most of the Chl appeared in the green band which contained mainly PS I and phycobiliproteins as judged from its polypeptide composition and a low oxygen-evolving activity (lane 2 of Fig. 2, Table 1). The crude PS II particle evolved oxygen at a rate twice as high as the thylakoid membranes did. In contrast, the purified PS II complex obtained by DM solubilization and column chromatography was largely devoid of phycobiliproteins and other contaminating components (lane 6 of Fig. 2, see below also), and showed an oxygen-evolving activity more than 5-fold higher than that of the starting thylakoid membranes (Table 1). It should be pointed out, however, that the yield of purified PS II complex was only 3% on the basis of Chl of the starting thylakoid membranes, which corresponds to a total yield of oxygen evolution of 18% if we multiply the Chl yield by

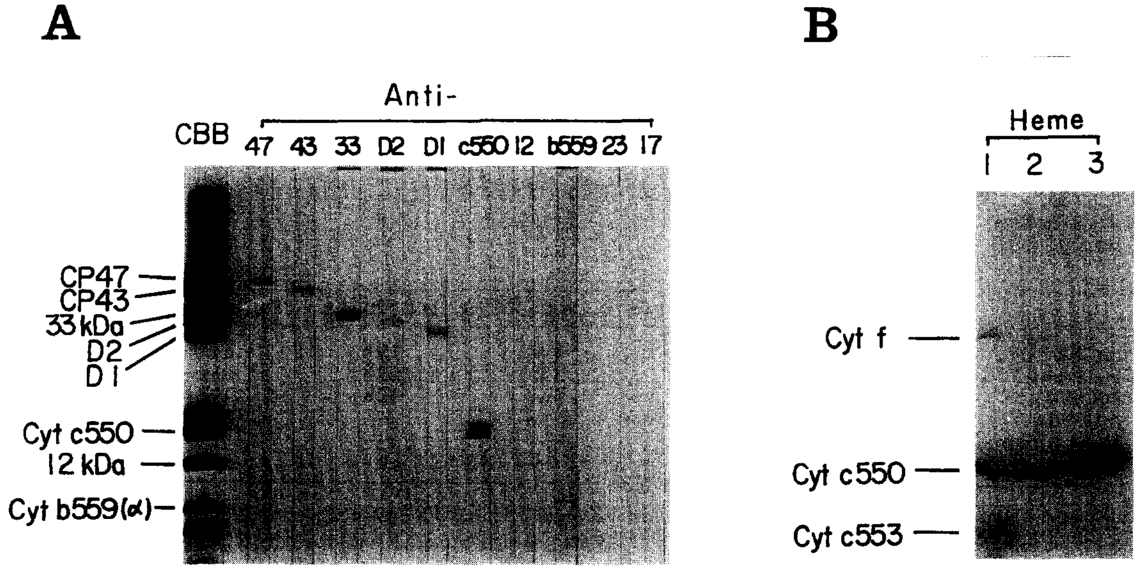

Fig. 3. (A) Identification of red algal PS II components by immunoblot analysis. Antibodies used were indicated on top of each lane. (B) Heme staining of thylakoids (lane 1); crude PS II (lane 2) and pure PS II (lane 3) from the red alga, *Cyanadium caldarium*.

the relative oxygen-evolving activity with the activity of thylakoids as 100%. Efforts to increase the Chl yield of purified PS II complex by increasing the DM concentration and solubilization time, however, resulted in a decrease in the oxygen-evolving activity. The sum of Chl recovered in each fraction of the sucrose cushion centrifugation or column chromatography shown in Table 1 was slightly less than that of the respective starting materials because of loss of a slight amount of PS I and PS II during the centrifugation or column chromatography.

In order to identify the protein components in the purified PS II complex from *C. caldarium*, immunoblotting analysis was carried out with antibodies raised against major PS II polypeptides of spinach as well as against two extrinsic proteins from a thermophilic cyanobacterium, *Synechococcus vulcanus*. As Fig. 3A shows, antibodies against spinach D1, D2, CP47, CP43, the α subunit of cyt *b*-559 and the extrinsic 33 kDa protein crossreacted with the corresponding components in the PS II complex from the red alga, confirming the presence of these homologous components in the red alga. Antibodies against spinach extrinsic 23 and 17 kDa proteins, however, did not crossreact with any polypeptides in the red algal PS II complex, suggesting that these two extrinsic components are not present in the complex. On the other hand, an antibody against *S. vulcanus* cyt *c*-550 crossreacted with a polypeptide with an apparent molecular mass of 17 kDa, suggesting that this cytochrome is present in the PS II complex from the red alga. The antibody against the 12 kDa extrinsic protein of *S. vulcanus*, however, did not crossreact with any polypeptide.

The presence of cyt *c*-550 in the red algal PS II complex was confirmed also by heme-staining of the gel, which permitted detection of *c*-type cytochromes on the gel, since other types of cytochrome lost their hemes upon electrophoresis in the denatured condition [25,27]. As Fig. 3B shows, three *c*-type cytochromes, cyt *f*, cyt *c*-550 and cyt *c*-553, were detected in the red algal thylakoid membranes by the heme-staining method, which is the same with that observed in cyanobacterial thylakoid membranes [25,27,34]. Of these cytochromes, cyt *c*-550 together with a small amount of cyt *f* remained in the crude PS II particle but only cyt *c*-550 was detected in the purified PS II complex and the other two cytochromes were apparently removed during the purification procedure. This strongly suggests that cyt *c*-550 is associated with PS II in the red alga. We also determined the N-terminal amino acid sequence of this cytochrome from the red alga, which showed that it is homologous to the low-potential cyt *c*-550 found in cyanobacterial PS II (Fig. 4). The sequence similarity for the cytochrome between the red alga and cyanobacteria, however, is not very high as compared with the

```
C. caldarium               IEVAKDTNGGILNIAPEQLKRGKRLFNSHXSDXH/                     34
S. vulcanus         AELTPEVLTVPLNSEGKTITLXEKQYLEGKRLFQYAXAS/                       39
S. PCC6803          VELTESTRTIPLDEAGGTTTLTARQFTNGQKIFVDTCTQCHLQGKTKTNNNVSLGLADLA   60
S. PCC7002          TALREVDRTVNL-NETETVVLSDQQVAKGERIFINTCSTCHNSGRTKSNPNVTLSLVDLE   59
A. flos-aquae       LELDETIRTVPLNDKGGTVVLSLEQVKEGK-LFNYACAQCHAGGVTKTNQNV--GL-E--   54
M. aeruginosa       LELDEKTLTITLNDAGESVTLTSEQATEGQKLFVANCTKCHLQGKTKTNNNVSLGLGDLA   60
                      Δ     Δ  Δ        Δ   *   *   *

C. caldarium
S. vulcanus
S. PCC6803          GAEPRRDNVLALVEFLKNPKSYDGEDDYSELHPNISRPDIYPEMRNYTEDDIFDVAGYTL   120
S. PCC7002          GAEPRRDNILAMVDYLKNPTSYDVELDLSQLHPNTVRADIWSSMRNLNEEDLQNVSGYVL   119
A. flos-aquae       -PEAL--AG-ALPNRMKNPTTYDGEEEISEI-PSIKSANIF---RNLTDEDLKAIAEHIL   106
M. aeruginosa       KAEPPRDNLLALIDYLEHPTSYDGEDDLSELHPNVSRPDIYPELRNLTEDDVYNVAAYML   120

C. caldarium
S. vulcanus
S. PCC6803          IAP-KLDERWGGTIYF                                               135
S. PCC7002          VQAQVRGVAWGGGKTVN                                              136
A. flos-aquae       LEPLVVGTKWGGK/                                                 119
M. aeruginosa       VAP-RLDERWGGTIYF                                               135
```

	C. caldarium	*S. vulcanus*	*S.* PCC6803	*S.* PCC7002	*A. flos-aquae*	*M. aeruginosa*
C. caldarium	—	27%	19%	19%	34%	19%
S. vulcanus	—	—	35%	27%	51%	38%
S. PCC6803	—	—	—	48%	44%	70%
S. PCC7002	—	—	—	—	42%	47%
A. flos-aquae	—	—	—	—	—	46%
M. aeruginosa	—	—	—	—	—	—

Fig. 4. N-terminal amino acid sequence of cyt *c*-550 from *Cyanidium caldarium* aligned with known sequences of the cytochrome from *Synechococcus vulcanus; Synechocystis* sp. PCC 6803; *Synechococcus* sp. PCC 7002; *Aphanizomenon flos-aquae* and *Microcystis aeruginosa*. The symbol '*' indicates the residues identical among all the sequences compared, and 'Δ' indicates the residues conserved among N-terminal part of the cyanobacterial sequences but not in the red algal sequence. The lower table shows sequence identity for cyt *c*-550 between the red alga and cyanobacteria and among different species of cyanobacteria as obtained by dividing the number of matched residues with that of total residues compared.

sequence similarity found among different species of cyanobacteria, although our partial sequence data did not allow a detailed comparison.

The dense band with an apparent molecular mass of 12 kDa found in the CBB-stained gel of purified PS II did not crossreact with any antibodies employed. However, this component could be released upon 1 M Tris wash (vide infra). In order to identify this band, we determined its N-terminal amino acid sequence and searched for homologous polypeptides in the databases. As Fig. 5 shows, the N-terminal sequence of this polypeptide showed a moderate homology with the 12 kDa protein of PS II from *S. vulcanus* and *Phormidium laminosum*, which is also an extrinsic component associated with cyanobacterial PS II functioning in maintaining oxygen-evolving activity. Based on its apparent molecular mass, its release upon Tris or urea/NaCl wash (see below) and the moderate sequence homology, we may conclude that the 12 kDa band present in the red algal PS II is a homologous polypeptide of the 12 kDa protein found in cyanobacterial PS II. The sequence similarity of the 12 kDa protein between the red alga and cyanobacteria, however, is much lower than the similarity between the two species of cyanobacteria so far reported. This low homology may be the reason for the failure of a crossreaction of the red algal 12 kDa protein with the antibody against the corresponding protein from the thermophilic *S. vulcanus*. This appears to be compatible with the low sequence homology of the 17 kDa extrinsic protein among different organisms: the spinach 17 kDa protein has only 28% homology with the corresponding protein of *Chlamydomonas reinhardtii* [35,36].

Cyt *c*-550 and the 12 kDa protein in cyanobacterial PS II can be released upon treatment with alkaline Tris or high concentrations of divalent cations [9]. In order to check whether a similar situation occurs with cyt *c*-550 and the 12 kDa protein present in the red algal PS II, we treated the red algal PS II with 1 M Tris, Urea/NaCl or 1 M NaCl. As Fig. 6 shows, red algal cyt *c*-550 and the 12 kDa protein were released, together with the extrinsic 33 kDa protein, by 1 M Tris or Urea/NaCl but not by 1 M NaCl. This indicates that both cyt *c*-550 and the 12 kDa protein are real extrinsic components of the red algal PS II, and their release features resemble those of the homologous cyt *c*-550 and 12 kDa protein found in PS II from the thermophilic cyanobacterium, *S. vulcanus*. It must be noted here that all the salt treatments shown in Fig. 6 were carried out in the presence of 25% glycerol, which is known to be able to protect release of PS II extrinsic components [7,8,37]. Upon omission of glycerol, these two extrinsic proteins were largely released even at a lower

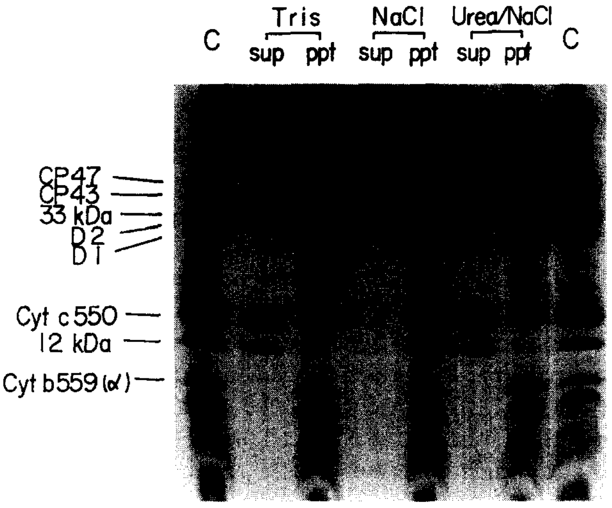

Fig. 6. Dissociation of extrinsic proteins from the purified PS II of *Cyanidium caldarium* by alkaline Tris or high concentrations of salts. The treatments were carried out as described in Materials and Methods. 'C', 'sup' and 'ppt' stand for 'control', 'supernatant' and 'pellet', respectively.

```
C. caldarium               XIDYEGIGYLGGGDKIDVDNANVRAYRKIPGLYPTAAKKIVQGGPYGTPDD   51
S.vulcanus        ATASTEEELVNVVDEKLGTAYGEKIDLNNTNIAAFIQYRGLYPTLAKLIVKNAPYEXVED   60
P.laminosum            EQQFRNAMDDKLATDFGKKIDLNNTNVRAFMQYPGMYPTLARMILKNAPFESVED   55
                                       * ***  * *  *     * *** *  *    *     *

C. caldarium      ILKNPNLdsvDKXvI/                                              66
S.vulcanus        XLNIP/                                                        65
P.laminosum       VLKMPGLTDTQKEILKNNFSNFVVSPPLDALVEGGDRFNNGIYR                  99
                   *  *
```

	C. caldarium	S.vulcanus	P.laminosum
C. caldarium	—	39%	38%
S.vulcanus	—	—	62%
P.laminosum	—	—	—

Fig. 5. N-terminal amino acid sequence of the 12 kDa protein from *Cyanidium caldarium* aligned with known sequences of the protein from *Synechococcus vulcanus* and *Phormidium laminosum*. '*' indicates identical residues. The lower table shows sequence identity for the 12 kDa protein between the red alga and cyanobacteria and between the two species of cyanobacteria, obtained by dividing the number of matched residues with that of total residues compared.

concentration (0.5 M) of NaCl (data not shown); in this condition, no release of the extrinsic components were observed in the thermophilic cyanobacterial PS II [9]. Thus, these two extrinsic components seem to associate with the red algal PS II more weakly than they do with the thermophilic cyanobacterial PS II. In fact, these two extrinsic components are also more loosely associated with PS II from a mesophilic cyanobacterium, *Synechocystis* sp. PCC 6803 (Shen et al., unpublished observations). Perhaps, their association with the thermophilic cyanobacterium is exceptionally tight as compared with that in PS II from red algae or mesophilic cyanobacteria.

On the CBB-stained gel of the extract of the Tris or urea/NaCl wash, we also noticed the presence of a band at 20 kDa which appeared slightly above the band of cyt *c*-550. This band is more weakly stained by CBB as compared with other extrinsic components or intrinsic polypeptides in the purified PS II complex; its release upon Tris or urea/NaCl wash but not by NaCl wash implied that it is also an extrinsic component of the red algal PS II. N-terminal amino acid sequencing indicated that this 20 kDa polypeptide has no significant homology with any known PS II polypeptides (data not shown), suggesting that it may be a new extrinsic component of PS II from the red alga.

In addition to the components described above, there are two bands in the purified PS II complex that were not identified; one is a band of about 100 kDa and another comigrates with cyt *c*-550 but could not be released by a Tris or urea/NaCl wash. The band of about 100 kDa may be an aggregate of some PS II components, whereas the identity of the band comigrated with cyt *c*-550 is not known at present, although it is apparently not a cytochrome since it cannot be stained by the heme-staining method (data not shown).

Table 2 shows the oxygen-evolving activity of the purified red algal PS II measured with different electron acceptors in the presence or absence of divalent cations. The PS II showed a high activity of 2375 μmol O_2 per mg Chl per h when phenyl-*p*-benzoquinone was used as an acceptor; this activity is comparable with that of PS II particles purified from cyanobacteria but higher than those from higher plants. The activity was almost completely inhibited by 3-(3,4-dichlorophenyl)-1,1-dimethylurea, and also decreased significantly when potassium ferricyanide was used as the acceptor, indicative of the retention of an intact Q_B site in the purified PS II complex. These activities were not enhanced by the addition of either Cl^- or Ca^{2+}, indicating that the purified PS II complex is intact also at the donor side. The presence of 2 mM EDTA in the assay medium, however, decreased the activity to 63% of the control. This can be due either to a chelation of residual Ca^{2+} present in the assay medium, or removal of loosely bound Ca^{2+} from the PS II complex. At present, we cannot determine which is the case, but, notably, this effect can be reversed by addition of an excess Ca^{2+}. Significantly, exogenous Mg^{2+} also largely restored the lost activity. A similar enhancing effect of Mg^{2+} has been observed in cyanobacterial PS II but not in higher plant PS II, again suggesting that the red algal PS II is more closely related to cyanobacterial PS II than to higher plant PS II.

Table 2
Effects of electron acceptors, divalent cations and chloride on oxygen evolution of the PS II complexes purified from *Cyanidium caldarium*

	Oxygen evolution [a]
Acceptors	
0.6 mM PBQ [b]	2375 (100)
0.6 mM DCBQ	1831 (77)
2.0 mM Fecy	742 (31)
0.6 mM PBQ + 2 mM Fecy	2523 (106)
0.6 mM PBQ + 10 μM DCMU	74 (3)
Divalent cations [c]	
Control	2375 (100)
+ 10 mM $CaCl_2$	2424 (102)
+ 2 mM EDTA	1485 (63)
+ 10 mM $CaCl_2$	2573 (108)
+ 10 mM $MgCl_2$	2226 (94)
Chloride [c]	
Control (– NaCl)	2429 (100)
+ 20 mM NaCl	2336 (96)
+ 100 mM NaCl	2382 (98)

[a] Oxygen evolution was measured in 50 mM Mes (pH 6.5), 25% glycerol and 20 mM NaCl, and the activity was expressed as μmol O_2/mg Chl per h.

[b] Abbreviations used in the table are: PBQ, phenyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; Fecy, potassium ferricyanide; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

[c] Measured with 0.6 mM phenyl-*p*-benzoquinone as the acceptor.

4. Discussion

4.1. Comparison of PS II from red algae with that from higher plants and cyanobacteria

We have presented here a first report on purification of an intact, highly active oxygen-evolving PS II complex from a red alga, *C. caldarium*. The activity of the purified PS II complex is comparable with that of cyanobacterial PS II but much higher than that of higher plant PS II. The following lines of evidence obtained indicate that the red algal PS II is more closely related with cyanobacterial PS II rather than with higher plant PS II. (i) While the extrinsic 33 kDa protein as well as the major membrane-spanning polypeptides of PS II were identified in the red algal PS II, the extrinsic 23 and 17 kDa proteins found in higher plant PS II but not in cyanobacterial PS II were apparently not present in the red algal PS II. Although a possibility exists that these two extrinsic proteins are present in cells of the red alga but lost during purification of the PS II complex, since we did not perform immunobolt analysis on cells or thylakoid membranes, we consider that this is rather unlikely, since the purified PS II showed a very high oxygen-evolving activity which was not enhanced by addition of divalent cations or chloride. (ii) Instead, two other extrinsic proteins, one being cyt *c*-550 and another the 12 kDa protein, were associated with the red algal PS II. These two proteins are apparently homologous to the corresponding two extrinsic proteins found in cyanobacterial PS II since they had a similar molecular mass, are all released by Tris or urea/NaCl treatment, and their N-terminal amino acid sequences are moderately homologous to the sequences of the corresponding cyanobacterial proteins. The two cyanobacterial proteins have been shown to be required for optimum oxygen-evolving activity and stability of cyanobacterial PS II in vivo [10–12], which suggests a similar function for cyt *c*-550 and the 12 kDa protein in the red algal PS II. (iii) In the presence of all the three extrinsic proteins, the oxygen-evolving activity of the red algal PS II did not require exogenous Ca^{2+}, but addition of EDTA appreciably suppressed the activity. Furthermore, the suppressed activity can be effectively restored not only by Ca^{2+} but also by Mg^{2+}. These features of the Ca^{2+} effect are similar to those observed in cyanobacterial PS II, but significantly different from those in higher plant PS II. Based on these results, we conclude that the red algal PS II is evolutionarily more closely related to cyanobacterial PS II than to higher plant PS II.

The similarity in the photosynthetic apparatus between red algae and cyanobacteria has been reported at the level of thylakoid membranes [38,39], in spite of the big difference in their cell structure, as red algae are eukaryotes but cyanobacteria are prokaryotes. Yurina et al. [40] analyzed the total polypeptide composition of thylakoids from red algae and concluded that the thylakoid polypeptides of red algae as a whole are more similar with those of cyanobacteria than those of higher plants. In particular, thylakoid membranes of red algae contain phycobiliproteins but do not contain Chl *b* and hence do not contain plant-type light-harvesting proteins. This, however, is no longer true since Wolfe et al. [17,18] have recently found a Chl-*a*-binding light-harvesting protein associated with PS I from a red alga, *Porphyridium cruentum*. They also showed that the red algal PS I is more similar to that of higher plants than that of cyanobacteria in terms of their total polypeptide composition. The PS II purified from *C. caldarium*, however, did not contain plant-type light-harvesting proteins, and its polypeptide composition was similar to that of cyanobacterial PS II. Thus, it appears that PS I and PS II of red algae are evolutionarily in different positions, with PS I closer to that of higher plants whereas PS II is more similar to that of cyanobacteria.

4.2. Cellular location of cyt c-550 and its possible function in red algae

Cyt *c*-550 has long been known to be one of the major *c*-type cytochromes present in cyanobacteria [41–43] and some eukaryotic algae [44,45] including red algae [19,20]. Shen et al. recently reported that this cytochrome is an extrinsic component stoichiometrically associated with PS II from a thermophilic cyanobacterium and is required for maintaining PS II oxygen-evolving activity and the effective binding of the 12 kDa extrinsic protein to PS II [9,10]. They also showed that the gene for this cytochrome has a leader sequence and genetic deletion of this cytochrome resulted in a reduction not only in the PS II oxygen-evolving activity but also in the PS II stability in a mesophilic cyanobacterium, *Synechocystis* sp. PCC 6803 [11,12]. These results led them to conclude that cyt *c*-550 is associated with PS II at the lumenal side of thylakoid membranes and functions in maintaining oxygen evolution and stability of PS II in cyanobacterial cells [10–12]. The present findings that PS II purified from a red alga also contains cyt *c*-550 as an extrinsic component are compatible with the results observed in cyanobacteria and thus suggest that, in red algae, cyt *c*-550 is also located in the thylakoid lumen and functions in maintaining PS II oxygen evolution. A thylakoid lumenal location of red algal cyt *c*-550 is in line with the fact that the gene for this cytochrome in red algae is located in the plastid genome [20] and has a leader sequence (Reith, M., personal communication) that is required for its transport into the thylakoid lumen. Thus, the cellular location and function of cyt *c*-550 appear to have been conserved during evolution from cyanobacteria to red algae. In the future, it remains to be clarified as to how and where the extrinsic cyt *c*-550 and 12 kDa protein are replaced by the 23 and 17 kDa proteins during the evolution of PS II.

Acknowledgements

We would like to thank Dr. Y. Inoue of The Institute of Physical and Chemical Research (RIKEN) for his critical reading of the manuscript. We also thank Drs. T. Horio and T. Kakuno of Osaka Univesity for providing the antibodies against spinach CP47, CP43, 33, 23 and 17 kDa proteins, Dr. M. Ikeuchi of University of Tokyo for providing the antibodies against spinach D1, D2 and the α subunit of cyt *b*-559. This work was supported in part by a grant-in-aid for Co-operative Research (No. 05304006) from the Ministry of Education, Science and Culture of Japan.

References

[1] Erickson, J.M. and Rochaix, J.-R. (1992) in Topics in Photosynthesis (Barber, J., ed.), Vol. 11, pp. 101–177, Elsevier, Amsterdam.
[2] Ikeuchi, M. (1992) Bot. Mag. Tokyo 105, 327–373.
[3] Vermaas, W. (1993) Annu. Rev. Plant Physiol. Plant Mol. Biol. 44, 457–481.
[4] Ghanotakis, D.F. and Yocum, C.F. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 255–276.

[5] Debus, R.J. (1992) Biochim. Biophys. Acta 1102, 269–352.

[6] Rutherford, A.W., Zimmerman, J.-L. and Boussac, A. (1992) in Topics in Photosynthesis (Barber, J., ed.), Vol. 11, pp. 179–229, Elsevier, Amsterdam.

[7] Stewart, A.C.,Siczkowski, M. and Ljungberg, U. (1985) FEBS Lett. 193, 175–179.

[8] Stewart, A.C., Ljungberg, U., Åkerlund, H.-E. and Anderson, B. (1985) Biochim. Biophys. Acta 808, 353–362.

[9] Shen, J.-R., Ikeuchi, M. and Inoue, Y. (1992) FEBS Lett. 301, 145–149.

[10] Shen, J.-R. and Inoue, Y. (1993) Biochemistry 32, 1825–1832.

[11] Shen, J.-R., Vermaas, W. and Inoue, Y. (1995) J. Biol. Chem. 270, 6901–6907.

[12] Shen, J.-R., Burnap, R.L. and Inoue, Y. (1995) Biochemistry 34, 12661–12668.

[13] Wallace, T.P., Stewart, A.C., Pappin, D. and Howe, C.J. (1989) Mol. Gen. Genet. 216, 334–339.

[14] Miyao, M. and Murata, N. (1987) in Topics in Photosynthesis (Kyle, D.J., Osmond, C.B. and Arntzen, C.J., eds.), Vol. 9, pp. 289–307, Elsevier, Amsterdam.

[15] Rolfe, S.A. and Bendell, D.S. (1989) Biochim. Biophys. Acta 973, 220–226.

[16] Pauly, S., Schlodder, E. and Witt, H.T. (1992) Biochim. Biophys. Acta 1099, 203–210.

[17] Wolfe, G., Cunningham, F.X., Durnford, D., Green, B. and Gantt, E. (1994) Nature 367, 566–568.

[18] Wolfe G.R., Cunningham, F.X. Jr., Grabowski, B. and Gantt, E. (1994) Biochim. Biophys. Acta 1188, 357–366.

[19] Evans, P.K. and Krogmann, D.W. (1983) Arch. Biochem. Biophys. 227, 494–510.

[20] Reith, M. and Munholland, J. (1993) The Plant Cell 5, 465–475.

[21] Enami, I. and Fukuda, I. (1975) Plant Cell Physiol. 16, 211–220.

[22] Enami, I., Nagashima, H. and Fukuda, I. (1975) Plant Cell Physiol. 16, 221–231.

[23] Ikeuchi, M. and Inoue, Y. (1988) Plant Cell Physiol. 29, 1233–1239.

[24] Laemmli, U.K. (1970) Nature 227, 680–685.

[25] Shen, J.-R. and Inoue, Y. (1993) J. Biol. Chem. 268, 20408–20413.

[26] Thomas, P.E., Ryan, D. and Levin, W. (1976) Anal. Biochem. 75, 168–176.

[27] Guikema, J.A. and Sherman, L.A. (1981) Biochim. Biophys. Acta 637, 189–201.

[28] Porra, R.J., Thompson, W.A. and Kriedemann, P.E. (1989) Biochim. Biophys. Acta 975, 384–394.

[29] Enami, I., Kamino, K., Shen, J.-R., Satoh, Ka and Katoh, S. (1989) Biochim. Biophys. Acta 977, 33–39.

[30] Ohno, T., Satoh, Ka. and Katoh, S. (1986) Biochim. Biophys. Acta 852, 1–8.

[31] Burnap, R.L., Koike, H., Sotiropoulou, G., Sherman, L.A. and Inoue, Y. (1989) Photosynth. Res. 22, 123–130.

[32] Boerner, R.J., Nguyen, A.P., Barry, B.A. and Debus, R.J. (1992) Biochemistry 31, 6660–6672.

[33] Tang, X.-S. and Diner, B.A. (1994) Biochemistry 33, 4594–4603.

[34] Omata, T. and Murata, N. (1984) Biochim. Biophys. Acta 766, 395–402.

[35] Jansen, T., Rother, C., Steppuhn, J., Reinke, H., Beyreuther, K., Jansson, C., Andersson, B. and Herrmann, R.C. (1987) FEBS Lett. 216, 234–240.

[36] Mayfield, S.P., Schirmer-Rahire, M., Frank, G., Zuber, H. and Rochaix, J.-D. (1989) Plant Mol. Biol. 12, 683–693.

[37] Stamatakis, C. and Papageorgiou, G.C. (1993) Biochim. Biophys. Acta 1183, 333–338.

[38] Raven, P.H. (1970) Science 169, 641–646.

[39] Cavalier-Smith, T. (1982) Biol. J. Linn. Soc. 17, 289–306.

[40] Yurina, N.P., Karakashev, G.V., Karapetyan, N.V. and Odintsova, M.S. (1991) Photosyn. Res. 30, 15–23.

[41] Holton, R.W. and Myers, J. (1963) Science 142, 234–235.

[42] Ho, K.K., Ulrich, E.L., Krogmann, D.W. and Gomez-Lojero, C. (1979) Biochim. Biophys. Acta 545, 236–248.

[43] Alam, J., Sprinkle, J., Hermodson, M.A. and Krogmann, D.W. (1984) Biochim. Biophys. Acta 766, 317–321.

[44] Yamanaka, T., DeKerk, H. and Kamen, M.D. (1967) Biochim. Biophys. Acta 143, 416–424.

[45] Kamimura, Y., Yamasaki, T. and Matsuzaki, E. (1977) Plant Cell Physiol. 18, 317–324.