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Stoichiometric association of extrinsic cytochrome c_{550} and 12 kDa protein with a highly purified oxygen-evolving photosystem II core complex from *Synechococcus vulcanus*

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A highly purified, native photosystem II (PS II) core complex was isolated from thylakoids of *Synechococcus vulcanus*, a thermophilic cyanobacterium by lauryldimethylamine *N*-oxide (LDAO) and dodecyl β-D-maltoside solubilization. This native PS II core complex contained, in addition to the proteins that have been well characterized in the core complex previously purified by LDAO and Triton X-100, two more extrinsic proteins with apparent molecular weights of 17 and 12 kDa. These two proteins were associated with the core complex in stoichiometric amounts and could be released by treatment with 1 M $CaCl_2$ or 1 M alkaline Tris but not by 2 M NaCl or low-glycerol treatment, indicating that they are the real components of PS II of this cyanobacterium. N-Terminal sequencing revealed that the 17 and 12 kDa proteins correspond to the apoprotein of cytochrome c_{550}, a low potential *c*-type cytochrome, and the 9 kDa extrinsic protein previously found in a partially purified PS II preparation from *Phormidium laminosum*, respectively. In spite of retention of these two extrinsic proteins, no homologues of higher plant 23 and 17 kDa extrinsic proteins could be detected in this cyanobacterial PS II core complex.

O_2 evolution; Photosystem II; Extrinsic protein; Cytochrome c_{550}; 9 kDa protein; *Synechococcus*

1. INTRODUCTION

The cyanobacterial PS II complex largely resembles the PS II complex of higher plants. Despite the big differences in antenna pigment complexes, up to 15 subunits have so far been identified in an O_2-evolving core complex prepared with LDAO and Triton X-100 (LDAO/Triton–PS II) from a thermophilic cyanobacterium, *Synechococcus vulcanus*, and most of them are homologous to the respective corresponding subunits of higher plant PS II [1]. However, there lies a substantial difference in the composition of extrinsic proteins: the two extrinsic proteins of 23 and 17 kDa involved in Ca^{2+} and Cl^--effects in higher plant O_2-evolving system are absent in cyanobacterial PS II complex, while the 33 kDa extrinsic protein is commonly present in PS II from both origins [2,3].

Exceptionally, however, two extrinsic proteins of 15 kDa (apoprotein of cyt c_{550}) and 9 kDa were reported to be present in an O_2-evolving PS II preparation obtained from *Phormidium laminosum* by successive treatment of thylakoids with LDAO and DM [3,4], and the 9 kDa protein was suggested to modulate O_2 evolution in a way apparently different from that of the 23 or 17 kDa protein in higher plant PS II [3,5,6]. In spite of these reports, it has been suggested that these two proteins are the extrinsic components particular for *P. laminosum*, because the two proteins failed to be detected in LDAO/Triton–PS II complex from *S. vulcanus* [1,7,8] or various PS II preparations from any other cyanobacteria [9,10]. In addition, there remains a general question as to how tightly the cyt c_{550} and the 9 kDa protein are associated with the PS II core complex, since the *Phormidium* PS II preparation retained a large amount of PB and other unknown components. To address these questions and ambiguities, we purified a native O_2-evolving core complex from *S. vulcanus* that is almost completely devoid of PB and other unknown components by use of LDAO and DM as solubilizing detergents. It was revealed that cyt c_{550} and a 12 kDa protein homologous to the 9 kDa protein of *Phormidium* PS II, are the real extrinsic components of cyanobacterial PS II and are stoichiometrically associated with the O_2-evolving core complex. We also confirmed that cyanobacterial PS II contains no homologues of the extrinsic 23 and 17 kDa proteins of higher plant PS II.

Abbreviations: cyt, cytochrome; DM, *n*-dodecyl β-D-maltoside; LDAO, lauryldimethylamine *N*-oxide; MES, 4-morpholine-ethanesulphonic acid; PB, phycobiliproteins; PS I and II, photosystem I and II.

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2. MATERIALS AND METHODS

Synechococcus vulcanus Copeland was grown at 53–55°C as described in [11]. Thylakoid membranes were prepared by osmotic shock after lysozyme treatment of 5-day old cyanobacterial cells [11]. Crude PS II particles were prepared by solubilizing the thylakoids with

LDAO as described previously [12], suspended in 25% glycerol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid–NaOH buffer (pH 7.0), 10 mM $MgCl_2$ at 1 mg chl/ml and stored at −80°C. For further purification, the crude PS II particles were thawed, diluted to 0.5 mg chl/ml with 50 mM MES (pH 6.0) and treated with 4% DM for 5 min at 0°C. The DM-solubilized materials were passed through a 22 μm membrane filter, loaded immediately onto a Mono Q HR 10/10 anion exchange column (Parmacia) and then eluted with 50 mM MES (pH 6.0)/NaCl containing 0.05% DM at 18°C. The isolated native PS II core particles were diluted ten-fold with 40 mM MES (pH 6.0) and concentrated by ultrafiltration with an Amicon YM 100 membrane. O_2 evolution was measured in 40 mM MES/NaOH (pH 6.0) containing 10 mM $MgCl_2$ at 25°C with a Clark-type electrode, with 0.6 mM phenylbenzoquinone and 1 mM ferricyanide as electron acceptors.

For dissociation of the extrinsic proteins, the native PS II core particles were treated with 2 M NaCl or 1 M $CaCl_2$ containing 40 mM MES (pH 6.0) and 10 mM $MgCl_2$ or with 1 M Tris (pH 8.5) containing 10 mM $MgCl_2$ at a chlorophyll concentration of 0.5 mg/ml for 30 min at 0°C. Low-glycerol treatment was carried out according to [5] by incubating the PS II complex in a glycerol-free medium (40 mM MES, pH 6.0, 10 mM $MgCl_2$) at 0.5 mg chl/ml for 30 min at 0°C. After these treatments, the PS II complex was collected by centrifugation at 300,000 × g for 1 h and subjected to analysis of protein composition. SDS gel electrophoresis was carried out with a 16%–22% acrylamide gradient gel containing 7.5 M urea [13]. N-Terminal sequencing of proteins extracted by 1 M Tris was carried out as described in [8]. Absorption spectra were recorded with a Shimadzu MPS 2000 spectrophotometer at room temperature.

3. RESULTS

The conditions for anion exchange chromatography of DM-solubilized crude PS II particles were determined using a micropurification system (SMART™, Pharmacia) equipped with a mini-Mono Q column (Mono Q PC 1.6/5). Fig. 1 shows the elution profile of proteins from the mini-Mono Q column during a step-wise gradient elution with NaCl. Upon loading the DM-solubilized crude PS II particles to the column, a polypeptide of 13 kDa (the dense band below PB in lane 1

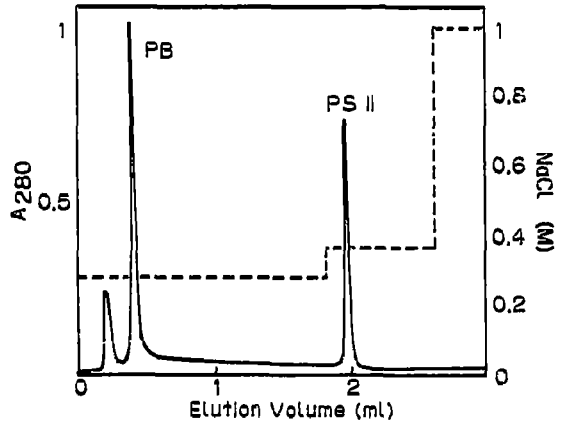

Fig. 1. Elution profile of DM-solubilized crude PS II particles of *S. vulcanus* from a Mono Q PC 1.6/5 column during elution with a step-wise gradient of NaCl. The flow rate was 0.1 ml/min at about 2.8 MPa. The eluate was photometrically monitored by recording the absorbance at 280 nm. PB, phycobiliprotein fraction; PS II, native PS II core complex fraction.

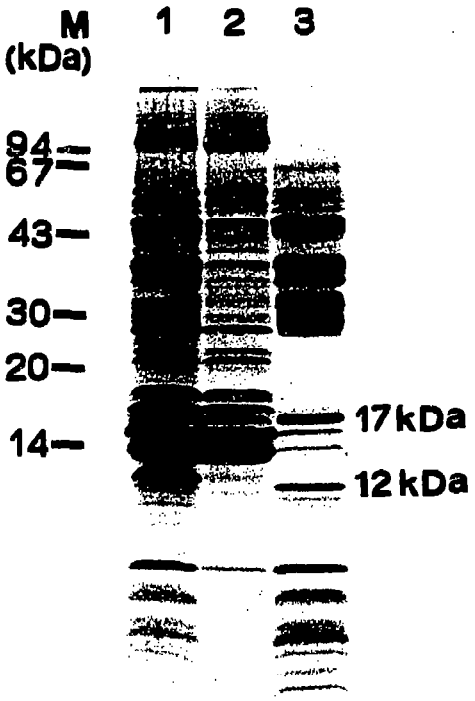

Fig. 2. SDS gel electrophoresis profile of crude PS II particles and purified native PS II core complex of *S. vulcanus*. (Lane 1), LDAO-prepared crude PS II particles; (Lanes 2 and 3), phycobiliprotein fraction and native PS II core complex fraction, respectively, separated chromatographically with the Mono Q column from DM-solubilized LDAO-prepared crude PS II particles as shown in Fig. 1. Molecular markers used are: 94 kDa, phosphorylase; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase; 20 kDa, soybean trypsin inhibitor and 14 kDa, α-lactoalbumin.

of Fig. 1) and solubilized carotenoids were not adsorbed but immediately passed through the column. Following wash of the column with 0.24 M NaCl a fraction containing mainly PB and linker polypeptides was obtained. Finally, the PS II core complex (denoted as LDAO/DM–PS II complex) was eluted with 0.35 M NaCl. Fig. 2 shows the polypeptide compositions of the PB and PS II complex fractions, in comparison with the starting material, crude PS II particles. Clearly, the PS II complex fraction is almost completely free from PB and other unknown proteins that are recovered in the PB fraction. The isolated LDAO/DM–PS II complex evolved O_2 at a rate of 1,300 μmol O_2/mg chl h when phenyl-benzoquinone and ferricyanide were supplemented as electron acceptors.

The LDAO/DM–PS II complex thus obtained contained the polypeptides of 47 and 43 kDa, the extrinsic 33 kDa protein, D2, D1, the two subunits of cyt b_{559} (8 and 4 kDa), and some recently identified low molecular mass polypeptides. This protein composition largely resembles that of LDAO/Triton–PS II complex previously isolated from *S. vulcanus* [7,8]. In the present LDAO/DM–PS II complex, however, two more bands with apparent molecular weights of 17 and 12 kDa were

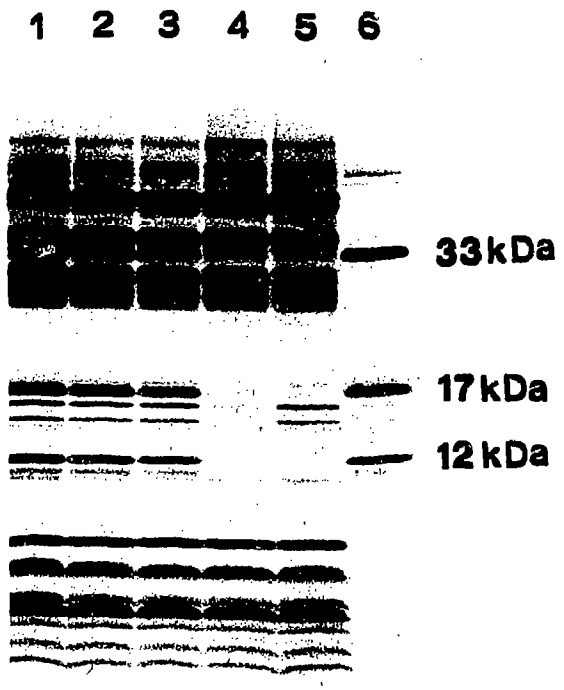

Fig. 3. Effects of various treatments on the composition of extrinsic proteins of *S. vulcanus* native PS II core complex. (Lane 1) untreated native PS II core complex; (lane 2) low-glycerol-treated; (lane 3) 2 M NaCl-treated; (lane 4) 1 M $CaCl_2$-treated; (lane 5) 1 M Tris-treated; (lane 6) 1 M Tris-extract.

additionally present. These two proteins were clearly co-purified with the PS II core complex and were almost absent in the PB fraction. Photometric scanning of the Coomassie-stained gel revealed that these two components are present in the LDAO/DM–PS II complex in stoichiometric amounts relative to the intrinsic 47, 43 or extrinsic 33 kDa proteins. Upon treatment with $CaCl_2$ or alkaline Tris, the two polypeptides were completely released, together with the extrinsic 33 kDa protein (Fig. 3, lanes 4 and 5), indicating that they are associated with PS II through electrostatic interaction. However, a wash with NaCl at a high concentration of 2 M or a low-glycerol treatment, which was reported to be effective for selective extraction of extrinsic proteins from *Phormidium* PS II preparation, did not release these two proteins at all from the LDAO/DM–PS II complex of *S. vulcanus* (Fig. 3, lanes 2 and 3). This suggests that their binding affinities for PS II core complex are as tight as that of the 33 kDa extrinsic protein, and that these two proteins are the real components of cyanobacterial native PS II.

In order to identify the two extrinsic proteins, their N-terminal sequences were determined and the resulting sequences were compared with the proteins of known sequences in database by computer-assisted homology search. As Fig. 4A shows, the 17 kDa polypeptide is homologous to the apoprotein of cyt c_{550} found in two other cyanobacteria [14]. Absorption spectrum of the Tris extract showed a peak at 407.5 nm, and additional absorbtion peaks appeared at 549.1 and 522.3 nm when reduced by dithionite, typical of this cyt in its reduced state (Fig. 5) [15,16]. These maxima disappeared again on exposure to atmospheric oxygen (not shown), consistent with previous observations that cyt c_{550} has a low redox potential (–260 mM) and can be easily oxidized by oxygen [15,16]. Among the 37 residues determined for the *S. vulcanus* protein, 12 residues are commonly conserved in cyt c_{550} of the other two cyanobacteria, *Microcystis* and *Aphanizomenon* [14].

Fig. 4B lists the N-terminal sequence of the 12 kDa polypeptide of *S. vulcanus*. A homology search showed

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A: cytochrome c550

                 **     *  **  *    *   *  **  **
Synechococcus AELTPEVLTVPLNSEGKTITL?EKQYLEGKRLFQYA?as/
Microcystis   LELDEKTLTITLNDAGESVTLTSEQATEGQKLFVANCTKCHLQGKTKTNNNVSLGLGDLAKAEPPRDNLLALIDYLEHPTSYDGEDDLS
Aphanizomenon LELDETIRTVPLNDKGGTVVLSLEQVKEG-KLFNYACAQCHAGGVTKTNQNVGLEPEALAGALPNRMKNPTTYDGEEEISEIPSIKSAN


Microcystis   ELHPNVSRPDIYPELRNLTEDDVYNVAAYMLVAPRLDERWGGTIYF   135aa MW15039   (ref. [14])
Aphanizomenon IFRNLTDEDLKAIAEHILLEPLVVGTKWGGK/                (119aa MW12887) (ref. [14])


B: 12 kDa

                    *    *  * ** *  * ********  ** ** * *****  * **** * ***
Synechococcus  ATASTEEELVNVVDEKLGTAYGEKIDLNNTNIAAFIQYRGLYPTLAKLIVKNAPYE?VED?
Phormidium           EQQFRNAMDDKLATDFGKKIDLNNTNVRAFMQYPGMYPTLARMILKNAPFESVEDV

                   * *
Synechococcus  LNIP/
Phormidium     LKMPGLTDTQKEILKNNFSNFVVSPPLDALVEGGDRFNNGIYR        99aa MW11263 (ref. [17])
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Fig. 4. N-Terminal sequences of cyt c_{550} (A) and the 12 kDa protein (B) of *S. vulcanus* aligned with known sequences. *Indicates homologous residues.

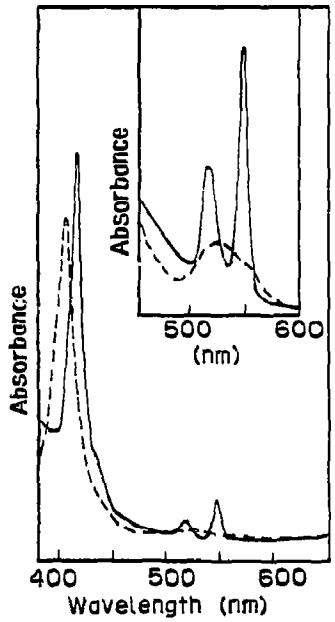

Fig. 5. Room temperature absorption spectra of Tris-extract from the native PS II core complex of *S. vulcanus* (---) no addition; (–) reduced with dithionite. The spectra between 450 nm and 600 nm were magnified in the inset.

that this sequence is highly homologous to that of the mature 9 kDa extrinsic polypeptide found in *Phormidium* PS II preparations [17]. The polypeptide is rich in hydrophilic residues, and 37 out of the 63 residues determined are conserved between *Synechococcus* and *Phormidium*. In particular, a cluster of 8 residues from Lys-24 to Asn-31 of *Synechococcus* is totally conserved in *Phormidium*, suggesting that this region may be involved in regulation of cyanobacterial PS II electron transport.

4. DISCUSSION

We showed here that two extrinsic proteins, an apoprotein of cyt c_{550} and a 12 kDa protein, are associated stoichiometrically with oxygen-evolving LDAO/DM–PS II core complex of *S. vulcanus* prepared by DM solubilization of LDAO-prepared crude PS II particles. These two proteins had not been detected previously in LDAO/Triton–PS II complex prepared from this cyanobacterium by Triton solubilization of the same crude PS II particles [1,7,8]. This is probably because they were largely released and lost during treatment with Triton. It appears that DM gives a milder solubilization of the membranes and is effective in isolating PS II core complex in such a native state as to retain the two extrinsic proteins. It is also of note that no homologues of higher plant 23 and 17 kDa extrinsic proteins could be detected in the present LDAO/DM–PS II complex, even though the complex retained the three extrinsic proteins and was capable of evolving O_2 at a significant rate. This agrees with the failure to detect the homologous 23 and 17 kDa protein in cyanobacteria by a specific extraction procedure [2] or Western blotting [3], and implies that these two other proteins are specific for the O_2-evolving system of higher plants and do not exist in cyanobacterial O_2-evolving system.

The association of cyt c_{550} with PS II has been implicated by haem-staining of partially purified PS II complex from *Phormidium*, although its amount could not be determined due to co-migration of PB [4]. The present results provide conclusive evidence for the association of cyt c_{550} with PS II and further demonstrate that the amount of cyt c_{550} present in the cyanobacterial PS II core complex is stoichiometric to other components of PS II. The same cyt c_{550} was found in thylakoid membranes of a red alga, *Porphyridium cruentum* [18], but is missing in higher plants. A questrion may arise as to the function of this cyt in PS II electron transfer or related reactions. Judging from its unusually low redox potential (−260 mV), it would be unwise to assume that the cyt is reduced by PS II photoreaction. Several reports have suggested that it may mediate cyclic electron transfer around PS I, since the cyt can be reduced by ferredoxin [19,20]. The cyclic electron transfer around PS I involves cyt b_6/f complex which resides on the acceptor side of PS II and accepts electrons from the plastoquinone pool. Due to its low redox potential, cyt c_{550} might reduce Q_B or plastoquinone upon accepting electrons from ferredoxin, and thereby modulate the balance between the cyclic electron transfer around PS I and the non-cyclic electron transport from PS II to PS I. In this respect, it is worth noting that the content of cyt c_{550} is variable depending on light conditions during growth [20] as well as the PS II/PS I ratio [21].

The *Synechococcus* 12 kDa protein is homologous to the extrinsic 9 kDa protein found in *Phormidium* PS II preparations. The *Phormidium* protein can be released from PS II by treatments with high concentrations of NaCl, $CaCl_2$, alkaline Tris or low glycerol [3], although neither of these treatments released the extrinsic 33 kDa protein from *Phormidium* PS II. In *S. vulcanus*, on the other hand, NaCl or low-glycerol treatment failed to release the 12 kDa protein from the PS II complex, although $CaCl_2$ or Tris-wash could completely release the protein together with the extrinsic 33 kDa protein. Apparently, the binding affinities of extrinsic 12 (9) and 33 kDa proteins are somewhat different between *Synechococcus* and *Phormidium* PS II, while the binding behavior of the 33 kDa protein in *Synechococcus* PS II is very similar to that in higher plant PS II.

Functional reconstitution of O_2 evolution by the 9 kDa protein has been shown in low-glycerol-treated PS II from *Phormidium*. It was suggested that removal of the 9 kDa protein slows down a dark step of S-state transition [6]. However, abiguities still remain as to its role, since removal of the protein by low-glycerol treatment did not completely depress O_2 evolution by the

Phormidium PS II preparation. This could have arisen from two possibilities, i.e. either the low-glycerol treatment does not completely dissociate the 9 kDa protein from *Phormidium* PS II or the 9 kDa protein is not absolutely required for O_2 evolution. The observation that a prolonged treatment with low-glycerol resulted in an irreversible inactivation of O_2 evolution in *Phormidium* PS II suggests that glycerol is required not only for association of the 9 kDa protein with *Phormidium* PS II but also for the maximum rate of O_2 evolution. Thus, it is difficult to identify whether the inactivation induced by low-glycerol treatment is due to the release of the 9 kDa protein or the absence of glycerol. In *Synechococcus* PS II, on the other hand, O_2 evolution does not require glycerol, and the low-glycerol treatment does not release the 12 kDa protein from *Synechococcus* PS II. Furthermore, *Synechococcus* LDAO/Triton–PS II largely depleted of the homologous 12 kDa protein showed an O_2-evolving activity of 1,000–1,400 μmol O_2/mg chl/h [7]. Thus, whether the 12 kDa protein has a role in O_2 evolution in *S. vulcanus* PS II remains to be re-examined, i.e. by sequential reconstitution of the extrinsic 33 and 12 kDa proteins of $CaCl_2$-washed LDAO/ DM–PS II core complex reported here.

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