Volume 253, number 1,2, 257-263 FEB 07496 August 1989

Identification of photosystem I components from the cyanobacterium, *Synechococcus vulcanus* by N-terminal sequencing

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Received 13 June 1989

The photosystem I core complex isolated from a thermophilic cyanobacterium, *Synechococcus vulcanus*, is composed of eight low-molecular-mass proteins of 18, 14, 12, 9.5, 9, 6.5, 5 and 4.1 kDa in addition to the PS I chlorophyll protein. N-terminal amino acid sequences of all these components were determined and compared with those of higher plants. Clearly, the 9.5 kDa component corresponds to the protein which carries the non-heme iron-sulfur centers A and B. This protein is so poorly visualized by staining that it has probably been overlooked in gel electrophoresis analyses. The 18, 14, 12 and 9 kDa components show appreciable homology with respective subunits of higher plant PS I. In contrast, the 6.5, 5 and 4.1 kDa components do not correspond to any known proteins except that the sequence of the 4.1 kDa component matches an unidentified open reading frame (ORF) 42 (liverwort) or ORF44 (tobacco) of chloroplast DNA.

Cyanobacterium; Gene, *psaC*; Photosystem I; (*Synechococcus vulcanus*)

1. INTRODUCTION

PS I is a chlorophyll-carrying multisubunit complex, which mediates light-driven electron transfer from plastocyanin or cytochrome *c*-553 to ferredoxin [1]. In higher plants, the PS I complex consists of three groups of protein components: (i) two CP I apoproteins (*psa*A and *psa*B gene products) bearing P700, chlorophylls and a non-heme iron-sulfur center X; (ii) several components of 8 to 22 kDa which belong to the PS I core complex; (iii) several LHC I components of 20–25 kDa [1,2]. Similarly, cyanobacterial PS I complex has been reported to contain four components of 8 to 20 kDa and the two CP I apoproteins [3–6]. Since the cyanobacterial complex does not carry any LHC I-type components, these low-molecular-mass components might have structural or functional roles similar to the core components of higher plants.

Recently, some of the PS I components from higher plants and cyanobacteria have been characterized by sequencing proteins and/or corresponding genes. Sequences of CP I apoproteins, encoded by chloroplast genes, *psa*A and *psa*B, are highly conserved not only among higher plants but also between higher plants and cyanobacteria [7,8]. The protein carrying the iron-sulfur centers A and B, which is encoded by a chloroplast gene, *psa*C, is also highly conserved among higher plants [9–11]. In cyanobacteria, however, neither a protein nor a gene corresponding to the iron-sulfur center-carrying component has been identified yet, although there are many functional and molecular biological indications of its presence [12–14]. The other core components of higher plants so far sequenced are all encoded by nuclear genome and their sequences vary considerably among plant

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Abbreviations: CP I, photosystem I chlorophyll-protein; EPR, electron paramagnetic resonance; LHC I, light-harvesting chlorophyll-protein complex associated with photosystem I; ORF, open reading frame; PS I and II, photosystem I and II, respectively; PVDF, polyvinylidene difluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

species [15–20]. Some such core components from cyanobacteria have been also sequenced, but only one component has been proven to correspond to a higher plant homologue (subunit II or *psa*D product) [4,21,22]. Our present knowledge about the correspondence of the other low-molecular-mass core components remains very limited.

Recently, we developed a modified SDS-PAGE method with a high resolution in the low-molecular-mass region below 10 kDa [23]. This method enabled discrete detection of many low-molecular-mass components in the PS II complex [23–25], which could not be resolved by conventional SDS-PAGE. The resolved proteins were electroblotted to PVDF membrane, and directly subjected to amino acid sequencing. By use of this method, the N-terminal partial amino acid sequences of many new PS II components have been determined [24–26].

In this communication, we register eight low-molecular-mass components found in the PS I complex from *Synechococcus vulcanus*, and report their N-terminal sequences. These results provide direct identification of the cyanobacterial protein carrying the iron-sulfur centers A and B and the presence or absence of correspondence between the low-molecular-mass components of cyanobacteria and higher plants.

2. MATERIALS AND METHODS

The cyanobacterial PS I complex was extracted with 0.8% lauryldimethylamine-*N*-oxide from thylakoid membranes of a thermophilic cyanobacterium, *Synechococcus vulcanus* as described previously [27]. The PS I containing extract was diluted 3-fold with 25% (v/v) glycerol, 20 mM Hepes-NaOH (pH 7.0) and 10 mM $MgCl_2$, and centrifuged at 180000 × *g* for 1 h to precipitate the crude PS I complex. The pelleted crude PS I complex was suspended in 20 mm Hepes-NaOH (pH 7.0) and 10 mM $MgCl_2$ at 0.6 mg Chl/ml, treated further with 0.2% lauryldimethylamine-*N*-oxide for 10 min on ice, and then centrifuged at 10000 × *g* for 10 min to collect the PS I core complex. Most of the contaminated PS II and allophycocyanin were removed by this procedure. The obtained PS I core complex contained one P700 per 120–150 chlorophylls based on light-induced absorbance changes at 700 nm, and showed light-induced reduction of center A and B, based on EPR measurement at 19 K when illuminated during freezing. *n*-Butanol treatment of the PS I particles was done according to Oh-oka et al. [9].

SDS-PAGE with 7.5 M urea and a 16–22% (w/v) acrylamide gradient was done according to [23]. Proteins in a gel were transferred onto a PVDF membrane (Immobilon, Millipore) following [24]. Transferred proteins were stained with Amido Black 10B (Bio-Rad), cut and subjected to amino acid sequencing with a protein sequencer (model 477A, Applied Biosystems).

3. RESULTS

SDS-PAGE analysis revealed that the *Synechococcus* PS I complex consists of eight low-molecular-mass components of 18, 14, 12, 9.5, 9, 6.5, 5 and 4.1 kDa in addition to the intense CP I and its apoproteins (fig.1). Tentative subunit stoichiometry deduced from the relative staining intensity per apparent molecular mass is about 0.7:1.0:0.5:0.1:0.9:0.4:0.8:0.4 for 18, 14, 12, 9.5, 9, 6.5, 5 and 4.1 kDa, respectively. Note that the 9.5 kDa band is very weak and diffuse, in agreement with the known properties of higher plant iron-sulfur proteins [28]. Since the 6.5, 5 and 4.1 kDa proteins are not resolved by conventional SDS-PAGE (not shown), this separation profile would be basically the same as those reported for other cyanobacterial PS I complexes [3–6]. For example, *Synechococcus* PCC 6301 PS I complex has been reported to contain 18, 17.7, 16 and 10 kDa with a molar ratio of 0.7:1.0:0.5:1.6 [3], which seem to correspond to our 18, 14, 12 and 9 kDa components with a compatible ratio.

When the PS I complex was treated with *n*-butanol to extract hydrophilic proteins, most of the 18, 14, 12, 9.5 and 9 kDa proteins were extracted, in agreement with the observation for higher plants [9,29]. However, the 6.5, 5 and 4.1 kDa proteins were not extracted, suggestive of their hydrophobic nature.

The sequence of the 9.5 kDa protein (fig.2D) is highly homologous to that of subunit VII or the *psa*C gene product product of higher plants which carries the non-heme iron-sulfur centers A and B [9–11,29]. Since Cys is the only one residue which cannot be determined by our current sequencing system, it is very likely that the four undetermined residues in the middle of the analyzed sequence are Cys, which are assumed to coordinate the iron-sulfur centers. Two variations among the first three amino acid residues in N-terminal part were found. An amino acid replacement specific for *Synechococcus* is found at residue 28 (Val instead of Ile), but it is conservative. Thus, the sequence is highly conserved between cyanobacteria (*Synechococcus vulcanus*) and higher plants. This is in sharp contrast to the relatively low conserva-

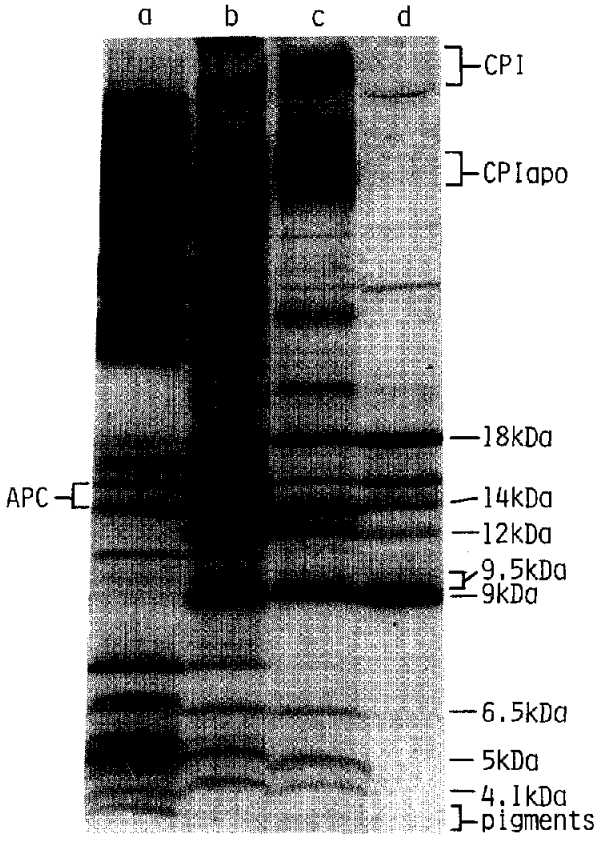

Fig.1. Polypeptide composition of the cyanobacterial (*Synechococcus vulcanus*) PS II core complex (lane a), thylakoids (lane b), the PS I complex (lane c) and *n*-butanol extract of the PS I complex (lane d). APC stands for allophycocyanin of PS II.

tion found for the other components (see below).

The 9.5 kDa protein is poorly stained in the gel by Coomassie brilliant blue as mentioned above or on the PVDF membrane by amido black. However, when analyzed by sequencing, this protein gave intense amino acid signals comparable to those from the other components. Moreover, EPR measurements indicated almost complete retention of the iron-sulfur centers A and B in the PS I preparation (not shown). Hence, it can be assumed that the iron-sulfur protein is present in an almost stoichiometric amount in our preparation.

The determined N-terminal sequence of the 18 kDa protein (fig.2A) shows high homology to those of the 17.7 kDa protein of *Synechococcus* PCC 6301 [4] and the 16 kDa protein of *Synechocystis* PCC 6803 [21]. It is also homologous to the internal sequence of subunit II (20–22 kDa) of spinach [17] and tomato [15]. Clearly, all the three cyanobacterial proteins lack the N-terminal amino acid stretch found in higher plant homologues. Only the first Met residue of *Synechocystis* protein is removed posttranslationally [21]. This will probably be the case in *S. vulcanus* as well. This subunit is believed to interact with ferredoxin based on crosslinking experiments [30]. Corresponding genes have been isolated in *Synechocystis*, spinach and tomato and designated *psa*D [15,17,21].

The sequence of the 14 kDa protein (fig.2B) shows homology with that of spinach subunit IV, whose gene has been designated *psa*F [16]. However, no corresponding sequence has so far been reported for any other cyanobacteria. Interestingly, the homology between the 14 kDa and subunit IV is high in N-terminal part while low in the internal part. In spinach, the subunit IV is suggested to be attached on the lumen side of the PS I complex, since its mature protein sequence is preceded by a presequence having an amphipathic β-sheet and a hydrophobic domain in its C-terminal part, both of which are peculiar to lumen-located proteins such as plastocyanin and PS II extrinsic proteins related to water oxidation [16]. If so, the cyanobacterial homologue might also be processed when translocated into the lumen space of thylakoids.

The sequence of the 12 kDa protein (fig.2C) shows homology with that of 17 kDa protein of *Synechococcus* PCC 7002, and very limited homology with spinach subunit V, designated *psa*G [16]. However, appreciable homology can be seen in internal sequence between the 17 kDa protein of *Synechococcus* PCC 7002 and the subunit V of spinach and pea, although the corresponding region for the *S. vulcanus* protein was not determined in this study. Based on these, we tentatively postulate that the 12 kDa protein corresponds to the subunit V of spionach (*psa*G).

The 9 kDa protein (fig.2E) gave two sequences. The minor one corresponded exactly to the major one except for the absence of the first Met residue. This means that 20–30% of the 9 kDa protein loses its first Met residue posttranslationally. The sequence is homologous to that of the 10 kDa protein of *Synechococcus* PCC 6301 [4]. These two

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                                                                    ref.
(A) 18 kDa protein (psaD)
S. vulcanus                                  TTLTGQPPLYGGSTGGLL-SADT-EEKYAIT
S. PCC6301 (17.7 kDa)                        AE....KT.VF........K..E.-.......    4
S. PCC6803 (16 kDa)                          M.E.S....KF........-.KANR.......   21
Spinach (S.U.II, 22 kDa)     AAAAEGKAATPTETKEAPKGFTPPELDPNTPS.IFA.......-RKAQV..F.V..   17
Tomato  (S.U.II, 21 kDa)       AE--APAATEEKPAPAGFTPPQLDPNTPS.IF........-RKAQV..F.V..   15

(B) 14 kDa protein (psaF?)
S. vulcanus                  DVAGLVPAKDSPAFQKRAAAAVNTTAD
Spinach (S.U.IV, 18 kDa)     .I...T.C.E.KQ.A..EKQ.LKKLQA                             16

(C) 12 kDa protein (psaG?)
S. vulcanus                  AEELVKPYNGDPFVGHLSTP
S. PCC7002 (17 kDa)            MDIIQHG...Q..N.A..(INASAFIKAKI----NRLPGYKQGLKAQKIG)   22
Spinach (S.U.V, 16 kDa)             ELSPSL.IS...G(L--.L.LGRFVFFNF..ENMA..VPE-.-N./)  16

(D) 9.5 kDa protein (psaC)
S. vulcanus                  AHTVKIYDT?IG?TQ?VRA?PTDVLEMVP?D
Spinach (9 kDa)              S.S......C..C..C...C.......I.W.                    9,10,29
Tobacco (9 kDa)              MS.S......C..C..C...C.......I.W.                        33
Liverwort (9 kDa)            M..A......C..C..C...C.......I.W.                        32

(E) 9 kDa protein (psaH?)
S. vulcanus                      MVQRGSKVKILRPESYVYNEVGTVAS
S. PCC6301 (10 kDa)              AIA..D..R.......WF........                          4
Spinach (S.U.VI? 12 kDa)     KPSPIGPK.....R.M?KR?.                                   *

(F) 6.5 kDa protein
S. vulcanus                  TLPDTTWTPSVGLVVILSNLFAIALGRYAI

(G) 4.8 kDa protein
S. vulcanus                  ATKSAKPTYAFRTF?AVLLLAINFLVAAY

(H) 4.1 kDa protein
S. vulcanus                  MKHFLTYLSTAPVL
Liverwort                    .QDVK.........                                          32
Tobacco                      .RDLK....V....                                          33

*Ikeuchi, M., Hiyama, T. and Inoue, Y., unpublished data
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Fig.2. N-terminal sequence of *Synechococcus* PS I low-molecular-mass proteins and their alignments with corresponding sequences of other cyanobacteria and higher plants. Only N-terminal parts of the reported sequences are presented for alignment. Amino acid differences between *Synechococcus* and the other proteins are indicated: the same residue is shown by a dot, a hyphen denotes a deletion, and question marks indicate residues which were not determined. A Met residue at a translation initiation site but missing in the mature protein is underlined. Hydrophobic region, which may span the membrane, is indicated by a horizontal bracket. *S.* PCC6301, *S.* PCC7002 and *S.* PCC6803 stand for *Synechococcus* PCC 6803, *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803, respectively.

cyanobacterial sequences are also homologous to that of spinach 10 kDa protein, although the spinach protein has several additional residues at its N-terminus (Ikeuchi, M., Hiyama, T. and Inoue, Y., unpublished). The spinach sequence does not correspond to any sequences reported before other than the above two cyanobacterial proteins, and its molecular mass is close to that of subunit VI judging from its close migration with the iron-sulfur protein (the subunit VII). Based on these data, we postulate that this protein is probably equivalent to the subunit VI of Nelson's designation [2].

The sequence of the 6.5 kDa protein (fig.2F) is new and does not correspond to any PS I component so far reported for higher plants or

cyanobacteria. A hydrophobic segment consisting of 22 amino acid residues is bounded by a pair of charged residues (Asp at 4th and Arg at 26th), which probably spans the thylakoid membrane. This view is supported by the *n*-butanol extraction experiment.

Also the sequence of the 4.8 kDa protein (fig.2G) does not correspond to any PS I component, but is exactly the same as the 5 kDa protein found in the O_2-evolving PS II core complex from the same *S. vulcanus* [31]. Although the determined sequence is partial, there may be a hydrophobic stretch starting after the 12th Arg, as is also suggested by the *n*-butanol extraction experiment.

The sequence of the 4.1 kDa protein (fig.2H) was obtained only after 0.6 N HCl treatment for 24 h, indicative of N-terminal blockage. This sequence is not homologous to any known PS I component. However, computer-assisted homology search revealed that the determined sequence is homologous to a hypothetical product of ORF42 (liverwort) [32] and ORF44 (tobacco) [33] of chloroplast DNA (fig.2H and fig.3), although expression of these ORFs has not yet been proven. Both deduced products contain a hydrophobic segment consisting of 22 residues which may span the membrane. The corresponding segment appears to be hydrophobic in *S. vulcanus*, although the obtained sequence is partial. The N-terminal part is not conserved between *Synechococcus* and higher plants. Possibly, the C-terminal region is not conserved as well, since the termination codon is found at different positions between liverwort and tobacco. Overall homology between the two ORFs is 71.6% at the nucleotide level and 81.4% at the amino acid level. Deduced products of liverwort

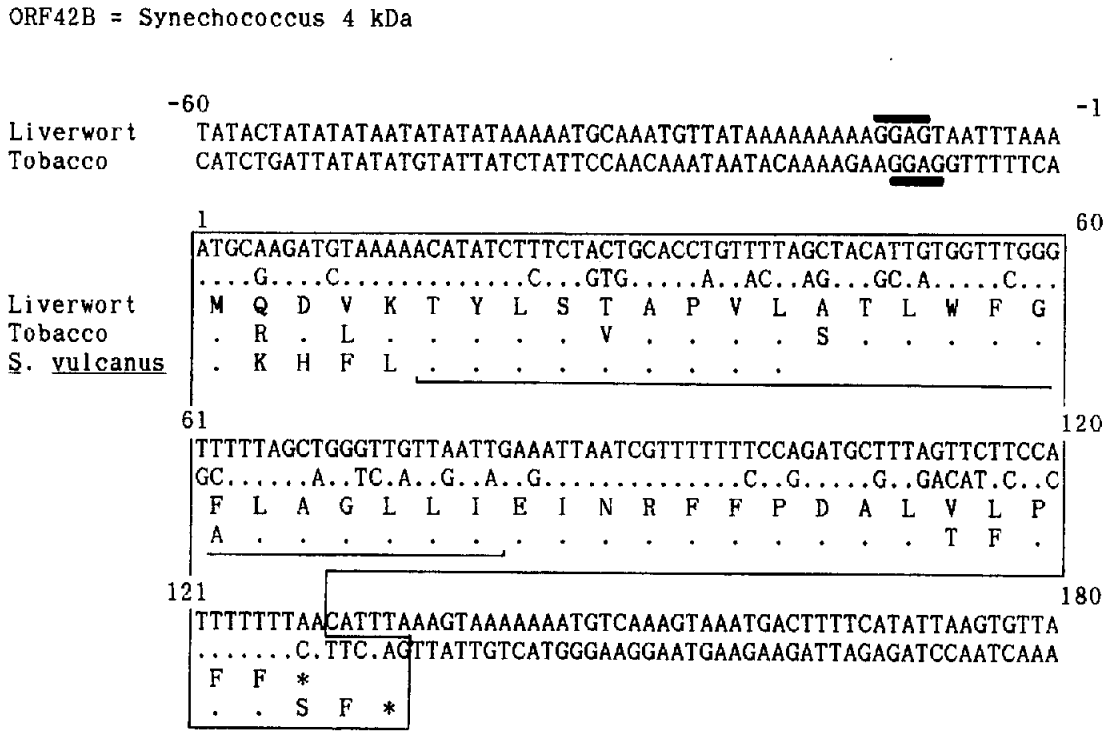

Fig.3. The nucleotide sequences of ORF42 of liverwort and ORF44 of tobacco encoding the 4.1 kDa protein and its flanking region in the chloroplast DNA from liverwort [32] and tobacco [33]. ORF42 or ORF44 is located downstream of *rpl*33 in both species. Numbering starts at the ATG codon in liverwort. Corresponding amino acids deduced from the liverwort sequence are indicated below the codon. The identified gene is boxed. Nucleotide sequences in the coding region between liverwort and tobacco and any resulting amino acid differences are shown below the liverwort sequence. A dot indicates the same residue. An asterisk denotes a stop codon. A possible ribosome-binding site is shown by bold lines above and below. The hydrophobic region, which may span the membrane, is indicated by a horizontal bracket.

and tobacco consist of 42 and 44 amino acid residues with molecular masses of 4777 and 5072 Da, respectively. These values are close to that of the cyanobacterial counterpart (4.1 kDa). These support the idea that the 4.1 kDa protein is encoded by a cyanobacterial gene homologous to the plant ORF42/ORF44.

4. DISCUSSION

The present study provides the first direct identification of the protein that carries the non-heme iron-sulfur centers A and B in the cyanobacterial PS I complex. The presence of this protein in the cyanobacterial PS I complex as well as in thylakoid membranes has been suggested by kinetic analysis [5,6,12–14], detection of non-heme iron-sulfur centers A and B by low temperature EPR [3,5,6] and ^{35}S labeling of Cys residues [34]. However, direct identification of the protein was difficult probably because this protein is so poorly stained on the gel: the iron-sulfur protein has been overlooked on the gel in spite of its abundance in the PS I complex. On the other hand, four low-molecular-mass proteins have been typically resolved in cyanobacteria by conventional SDS-PAGE, and their characterization and correspondence have been sporadically reported [3–6,21,22]. In this study, we resolved eight proteins and showed more clearly the correspondence between the 18, 14, 12 and 9 kDa proteins of *S. vulcanus* and the subunit II, IV, IV and VI of higher plants.

The sequence alignments we presented here suggest that there are five cyanobacterial components corresponding to respective subunits of higher plants. However, there are several more subunits in higher plants, whose counterparts are currently undetected in cyanobacteria. One is the subunit III of spinach and swisschard, which is assumed to facilitate the binding of acidic plastocyanin (p*I* = 3~4 [35,36]) to P700 [37]. However, such binding protein may not be necessary in cyanobacteria. The p*I* values of the two cyanobacterial electron donors, cytochrome *c*-553 or plastocyanin, are slightly acidic to basic (4.5–9.3) [36,38], so that no masking of the negative charge is needed for these of mobile electron carriers to have access to P700. It is also possible that the subunit III-type component is easily lost from the cyanobacterial PS I preparation, as suggested in swisschard or *Synechocystis* PCC 6803 PS I particles [21]. These possibilities must be checked by gene analysis or Western blotting analysis to detect whether any homologous gene or protein is present or not.

Two proteins of 13 and 11 kDa in the peak PS I complex [8], whose N-terminal sequences do not correspond to any of other higher plants or *S. vulcanus* are reported. Since the homologues of subunits II, IV, and V and VII were detected in the pea complex, the 13 and 11 kDa proteins may correspond to the rest, subunit III or VI, although their partial sequences show almost no homology.

The 6.5, 5 and 4.1 kDa proteins of the *Synechococcus* PS I complex do not correspond to any reported PS I proteins of higher plants. Their N-terminal sequences and *n*-butanol extraction experiments suggest that these proteins are hydrophobic. The predicted product of the plant ORF42/ORF44, which is homologous to the 4.1 kDa protein, contains a possible membrane-spanning segment. It is inferred that these three small proteins are membrane-spanning but peripheral proteins of the PS I complex. Although we do not have any evidence suggesting their function, they may not be involved in PS I reactions, since all the electron transfer components have been assigned to be located within the proteins encoded by *psa*A, *psa*B and *psa*C. The possible function of the small hydrophobic proteins might be structural as follows: (i) to support PS I assembly or (ii) to facilitate interaction of the PS I complex with other PS I or PS II complexes. The latter helps energy transfer between the complexes to attain efficient energy conservation. With regard to this it is of note that the 5 kDa protein also has an affinity for the PS II complex [31]. This may suggest that the 5 kDa protein facilitates the interaction between the PS I and PS II complexes.

Acknowledgements: We thank Dr T. Yasunaga (RIKEN) for computer-assisted homology search and Dr Y. Narahashi (RIKEN) for helpful instructions for protein sequencing. We also thank Dr T. Ono for EPR measurements and Mr K. Mamada and Miss R. Fujii for helping prepare PS I particles. This work was supported by a Grant on Solar Energy Conversion by Means of Photosynthesis awarded to The Institute of Physical and Chemical Research (RIKEN), by the Science and Technology Agency (STA) of Japan, and partly by a Grant on Frontier Research Program at RIKEN awarded by STA.

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