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Low-molecular-mass proteins in cyanobacterial photosystem II: identification of *psb*H and *psb*K gene products by N-terminal sequencing

Hiroyuki Koike, Kaoru Mamada, Masahiko Ikeuchi and Yorinao Inoue

Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

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The O_2-evolving photosystem II core complex was isolated from a thermophilic cyanobacterium, *Synechococcus vulcanus* Copeland. Analysis by SDS-polyacrylamide gel electrophoresis revealed that the complex contained at least seven low-molecular-mass proteins in addition to the well characterized CP47 apoprotein, CP43 apoprotein, 33 kDa extrinsic protein, D1 protein, D2 protein and large subunit of cytochrome *b*-559. The separation profiles of these low-molecular-mass proteins were very similar between cyanobacterial and higher plant PS II. N-terminal sequences of the 6.5 kDa and 3.9 kDa proteins of cyanobacterial core complex were determined after blotting to a polyvinylidene difluoride membrane. The sequence of the 6.5 kDa protein showed high homology with an internal sequence of plant *psb*H gene product, so-called 10 kDa phosphoprotein, but did not conserve the Thr residue which is specifically phosphorylated in plants. The sequence of the 3.9 kDa protein corresponded to the K protein of higher plants (mature form of *psb*K gene product). These results indicate that the products of both *psb*H and *psb*K genes are present in cyanobacterial PS II as well as being associated with the O_2-evolving core complex.

Cyanobacterium; Oxygen-evolving core complex; Photosystem II; Gene, *psb*H; Gene, *psb*K; (*Synechococcus vulcanus*)

1. INTRODUCTION

The O_2-evolving PS II core complex, which is devoid of light-harvesting complexes and other peripheral components, has been hypothesized as the structural minimum for PS II functioning including water oxidizing capacity [1–4]. The complex isolated from both cyanobacteria and higher plants commonly consists of CP47 apoprotein, CP43 apoprotein, 33 kDa extrinsinc protein, D1 protein, D2 protein and two subunits of cytochrome *b*-559. Besides these, several low-molecular-mass proteins were recently found to be associated with the complex of higher plants [5,6]. In PS II membrane fragments retaining light-harvesting complexes, some other low-molecular-mass proteins were additionally found. Thus, for a more comprehensive understanding of the structural minimum for functional PS II, it is important to comparatively determine the common PS II components between cyanobacteria and higher plants. However, there are so far no reports describing the low-molecular-mass components of the cyanobacterial complex in comparison with those in higher plants.

In this communication we report several low-molecular-mass components found in the O_2-evolving core complex isolated from a thermophilic cyanobacterium, *Synechococcus vulcanus*. N-terminal sequencing of two of the components clearly demonstrated that they correspond to the products of *psb*H and *psb*K genes of higher plants.

Correspondence address: H. Koike, Solar Energy Research Group, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

Abbreviations: Chl, chlorophyll; PS II photosystem II; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

Thylakoids were prepared from a thermophilic cyanobacterium, *Synechococcus vulcanus* Copeland as described previously [7]. The O_2-evolving PS II particle was prepared by solubilizing the thylakoids with lauryldimethylamine *N*-oxide as described in [8]. This PS II particle still retained a significant amount of allophycocyanin, linker and anchor proteins. For further purification, the PS II particle was suspended in 100 mM NaCl, 40 mM Mes-NaOH (pH 6.0) at 0.25 mg Chl/ml, then Triton X-100 was added to a final concentration of 2.5% (w/v). The suspension was incubated for 5 min on ice with gentle stirring and placed onto a DEAE-Toyopearl 650M column (Toso, Japan) equilibrated with 100 mM NaCl, 40 mM Mes-NaOH (pH 6.0) and 0.05% (w/v) Triton X-100. Most of allophycocyanin, linker and anchor proteins and contaminating PS I particles were not adsorbed on the column. After washing out these proteins, a core complex was eluted with 180 mM NaCl containing buffer. The core complex thus obtained evolved oxygen at a rate of 1000–1400 μmol O_2/mg Chl per h with ferricyanide as electron acceptor at 40°C. The O_2-evolving PS II membrane fragments and core complex of higher plants were prepared from spinach as described in [4].

For resolution of low-molecular-mass proteins, SDS-PAGE with 7.5 M urea and a 16 to 22% (w/v) polyacrylamide gradient was performed as described in [5]. Separated proteins were stained with Coomassie brilliant blue R-250 (Bio-Rad) or transblotted to a polyvinylidene difluoride membrane (Millipore) using a semidry-type electroblotter as described in [9]. Transferred proteins were stained with Amido black 10B (Bio-Rad) and subjected to a protein sequencer (model 477A, Applied Biosystems).

3. RESULTS

Polypeptide compositions of thylakoids, PS II particles and the O_2-evolving core complex are shown in fig.1. Bands of CP47 apoprotein, CP43 apoprotein, 33 kDa extrinsic protein, D1 protein, D2 protein and the large subunit of cytochrome *b*-559 were identified in PS II particles by monospecific antibodies raised against respective spinach proteins (Koike, H., Ikeuchi, M. and Inoue, Y., unpublished). All of these proteins were nearly quantitatively recovered in the core complex, while lipids, allophycocyanins, linker and anchor proteins were mostly removed by DEAE-Toyopearl column chromatography. In the low-molecular-mass region at least seven bands were separately detected in both PS II particles and the core complex in addition to the band of the large subunit of cytochrome *b*-559. The separation profiles were almost identical between the core complex and PS II particles and similar to those of O_2-evolving PS II membrane fragments and the core complex from spinach. It is thus inferred that these low-molecular-mass proteins are intrinsic components of the cyanobacterial PS II as well, but are not related with the light-harvesting complex. Although accompanied by several comigrating dense bands, all these low-molecular-mass proteins could be detected as faint bands in thylakoid membranes.

Of the low-molecular-mass proteins found in the cyanobacterial PS II core complex, 6.5 kDa and 3.9 kDa bands were well separated from other bands in SDS-PAGE profile. After transblotting to a polyvinylidene difluoride membrane, these bands were cut out and subjected to protein sequencing. N-terminal 20 amino acid signals and 31 amino acid signals were successfully resolved for 6.5 kDa and 3.9 kDa proteins, respectively (figs 2 and 3).

Computer-assisted homology search revealed that the determined sequence of the 6.5 kDa protein was homologous to an internal sequence of the *psb*H gene product, so-called 10 kDa phosphoprotein, of *Chlamydomonas* [10] and higher plants [11–14] (fig.2). Alignment of these sequences indicates that the *Synechococcus* sequence is most homologous to the tobacco sequence but is least homologous to the *Chlamydomonas* sequence, and that an 11 amino acid insert found in *Chlamydomonas* is missing in *Synechococcus* as well as in higher plants. Notably, the N-terminal segment including consensus Ala-Thr-(Gln)-(Thr)- of *Chlamydomonas* and higher plants is missing in the *Synechococcus* protein. This is not due to proteolysis during preparation, since the same 6.5 kDa polypeptide can be detected in PS II particles and thylakoid (fig.1). In contrast to this big difference in N-terminal sequence, the following sequence showed high homology with the corresponding sequences of plants. Based on the reported sequence of the *psb*H gene [11–14], the wheat, tobacco and spinach proteins are predicted to consist of 72 amino acid residues while the liverwort protein is of 73 amino acid residues (note that the first Met residue is lost after translation [9,11]). All of them show very high homology especially in C-terminal side including the single membrane-spanning segment (not shown in fig.2). If we assume that this C-terminal side is conserved in cyanobacteria, the *Synechococcus* protein is predicted to consist of 60 or 61 amino acid residues, and its molecular mass is calculated to be

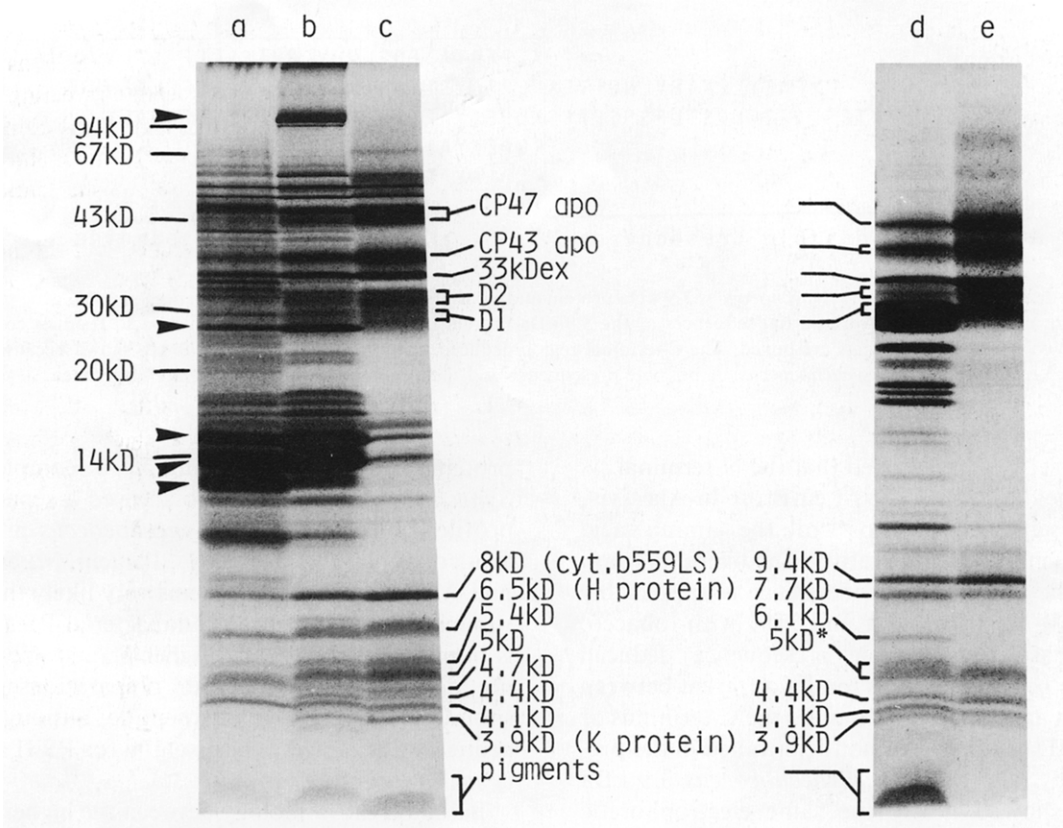

Fig.1. Polypeptide composition of thylakoids (lane a), PS II particles (b) and the core complex (c) from *S. vulcanus*, and PS II membrane fragments (d) and the core complex (e) from spinach. The diffused band of 5 kDa in spinach preparations consisting of at least three different components [9] is indicated by an asterisk. Bands of phycobiliproteins, linker and anchor proteins are indicated by arrowheads. Positions of molecular standards are also shown.

about 6.3 kDa. This estimation is in accordance with the molecular mass determined by SDS-PAGE (6.5 kDa) within error.

The determined sequence of the 3.9 kDa polypeptide was homologous to a part of the *psb*K gene product of liverwort and tobacco [13,14] and to the N-terminal sequence of the 3.9 kDa protein from spinach and wheat [9,15] (fig.3). Alignment

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                        *            ARR                  TWLGDILRPLNSEYGKV      ref.
Synechococcus
Chlamydomonas        ATGT    SKAKPSKVNSDFQEPGLVTPLGTLLRPLNSEAGKV      10
Liverwort         (M)ATQIIDDTPKTKGKK            SGIGDILKPLNSEYGKV      14
Tobacco           (M)ATQTVENSSRSGPRR            TAVGDLLKPLNSEYGKV      13
Wheat             (M)ATQTVEDSSKPRPKR            TGAGSLLKPLNSEYGKV      9,12
Spinach           (M)ATQTVESSSRSRPKP            TTVGALLKPLNSKYGKV      11
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Fig.2. Determined sequence of *Synechococcus* 6.5 kDa protein and its alignment compared with sequences of *psb*H gene products of *Chlamydomonas* and higher plants. Amino acid residues conserved between *Synechococcus* and others are boxed. C-terminal region deduced from *psb*H gene sequence is not shown because it was not determined in *Synechococcus*. The second Thr residue, the phosphorylation site, of *Chlamydomonas* and spinach is indicated by an asterisk. The Met residue for translation initiation, which is missing in the mature protein, is shown in parentheses.

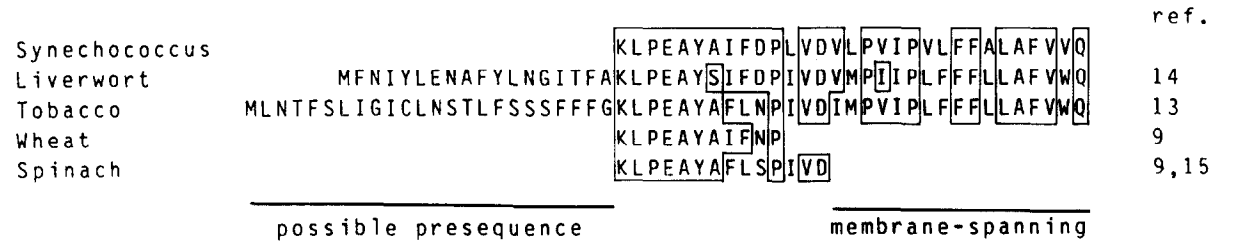

Fig.3. The determined sequence of *Synechococcus* 3.9 kDa protein and its alignment compared with sequences of *psb*K gene products of liverwort and tobacco, and with partial sequences of the 3.9 kDa protein of wheat and spinach. Amino acid residues conserved between *Synechococcus* and others are boxed. The C-terminal region deduced from *psb*K gene sequence is not shown because it was not determined in *Synechococcus*. A possible presequence and membrane-spanning segment are underlined.

of these sequences revealed that the N-terminal six amino acid residues, Lys-Leu-Pro-Glu-Ala-Tyr-, are consensus, and most of the amino acid replacements are conservative. The overall homology of the *Synechococcus* sequence is appreciably high, 77% with liverwort and 71% with tobacco. In contrast, the possible presequence deduced from the gene sequence is totally different between liverwort and tobacco. Although the C-terminus of the 3.9 kDa protein was not determined in the present study, the fact that *Synechococcus* 3.9 kDa protein showed nearly the same electrophoretic mobility as the corresponding spinach protein (fig.1) suggests that the *Synechococcus* protein also consists of nearly 38 amino acid residues like that of higher plants.

4. DISCUSSION

Satoh et al. [3] first isolated the cyanobacterial O_2-evolving PS II core complex devoid of phycobiliproteins and linker proteins from *Synechococcus* sp., although they did not analyze its low-molecular-mass components. Here we isolated a similar O_2-evolving core complex from a different strain of *Synechococcus* and registered at least seven low-molecular-mass proteins as intrinsic components of cyanobacterial PS II core. We also found that the separation profiles by SDS-PAGE of these components are similar between cyanobacteria and higher plants. N-terminal sequencing of two of those revealed the presence of comparable *psb*H and *psb*K gene products in *Synechococcus* as well as in higher plants. In view of our observation that the low-molecular-mass proteins of the O_2-evolving core complex of *Synechocystis* PCC6803 also showed a separation profile similar to that of *Synechococcus* proteins (Sotiropoulou, G., Koike, H., Ikeuchi, M. and Inoue, Y., unpublished), it seems very likely that the subunit structure of the cyanobacterial PS II core is homologous to that of higher plants. We could not detect any band in our preparations corresponding to the 9 kDa polypeptide, although this protein was claimed to be essential for PS II activity in *Phormidium* [16].

Based on the homology between the higher plant and cyanobacterial PS II core, we may expect the presence in cyanobacteria of the other low-molecular-mass components which had been already identified in higher plants. For example, the small subunit of cytochrome *b*-559 should be identified since the large subunit has been detected immunologically, and the genes corresponding to *psb*E and *psb*F have already been sequenced in *Synechocystis* PCC6803 [17]. Another possible component is the *psb*I gene product of about 4.8 kDa which has recently been revealed to be associated with the PS II reaction center complex of higher plants, the so-called D1-D2-cytochrome *b*-559 complex [18]. The *psb*I gene has been found to be co-transcribed with *psb*K in tobacco chloroplast (Sugiura, M., personal communication). Since the operon gene organizations in chloroplast DNA are often conserved in cyanobacterial DNA [19], we may also expect that the *psb*I gene is located close to *psb*K locus in cyanobacteria.

An open reading frame of 38 amino acids was first reported just downstream of *psb*E/F genes in *Cyanophora* [20] and a corresponding product of

about 5 kDa was detected in O_2-evolving core complex in higher plants [9]. Since this gene sequence is present in *Synechocystis* PCC6803 [21], we can also expect its product in the cyanobacterial PS II core. As shown in fig.1, there are several bands in about the 5 kDa region in the PS II core complex of *Synechococcus*. Some of these will probably correspond to those identified in higher plants.

The *psb*H product of *Chlamydomonas* and higher plants is well known as the 10 kDa phosphoprotein [10,11]. Phosphorylation of the protein is dependent on light illumination, although its physiological function is still uncertain. It is established that the phosphorylation site is the second Thr residue and not the other Thr residues in *Chlamydomonas* and spinach [10,22]. However, this Thr residue and the consensus flanking sequence of *Chlamydomonas* and higher plants were missing in the *Synechococcus* protein. In addition, analysis of protein phosphorylation in *Synechococcus* PCC6301 revealed that no proteins less than 10 kDa were phosphorylated whereas the 18.5 kDa linker protein and the 15 kDa PS II chlorophyll-binding protein were phosphorylated [23]. These results strongly suggest that the *psb*H product is not phosphorylated in *Synechococcus* possibly because of the absence of the second Thr residue. Presumably, it plays some role in PS II other than phosphorylation. Considering this, we propose 'H protein' instead of '10 kDa phosphoprotein' as a general name for the *psb*H gene product. Since the H protein is associated with the O_2-evolving core complex in *Synechococcus* (fig.1) as well as in higher plants [5,24], we may assume that the H protein is essential for functional PS II.

In chloroplast DNA of higher plants, the *psb*H gene is located in an operon in which *psb*B (CP47 apoprotein), *psb*H, *pet*B and *pet*D (*pet* genes encode components of the cytochrome b_6/f complex) are aligned from 5′ to 3′ [25]. Messenger RNA transcribed from this operon is subject to complex processing [26]. However, little is known about the locations of the *psb*H, *pet*B and *pet*D genes in the cyanobacterial genome. Although the *psb*B gene and its flanking region of *Synechocystis* PCC6803 were sequenced by Vermaas et al. [27], the *psb*H sequence was not found. Since our results indicate the presence of the *psb*H gene in cyanobacteria, it would be interesting to study whether the *psb*H gene is co-transcribed with *pst*B, *pet*B and *pet*D genes or not.

In higher plants, the mature form of the *psb*K gene product, designated K protein, is not associated with the O_2-evolving core complex but with PS II membrane fragments retaining light-harvesting complexes [9,15]. This was interpreted as indicating that the K protein is not essential for the functioning of PS II. However, our finding that it was retained in the O_2-evolving core complex from *Synechococcus* suggests that it is also a common component of PS II. It is unlikely, however, to assume that the K protein functions in connecting light-harvesting complexes with the PS II core, since the light-harvesting complexes are quite different between higher plant and cyanobacterial PS II. A possible role of the K protein, if any, may be to structurally support the PS II assembly.

The K protein of both *Synechococcus* and higher plants contains a single membrane-spanning region on the C-terminal side and several charged residues on the N-terminal side. The presequence deduced from the *psb*K sequence of tobacco and liverwort DNA contains a relatively hydrophobic stretch and an Ala or Gly residue at the C-terminal end. The nature of these presequences resembles those of cytochrome *f* and plastocyanin which are translocated across the thylakoid membrane [28]. This probably indicates that the N-terminal hydrophilic region of K protein protrudes into the lumen space during maturation as a result of processing.

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