Identification of *psb*I and *psb*L gene products in cyanobacterial photosystem II reaction center preparation

Masahiko Ikeuchi, Hiroyuki Koike and Yorinao Inoue

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

Photosystem II (PS II) reaction center complex of higher plants was revealed to contain a new low-molecular-mass component of 4.8 kDa encoded by a chloroplast gene, *psb*I [(1988) FEBS Lett. 241, 99–104], in addition to D1, D2 and two subunits of cytochrome *b*-559. Here we isolated a PS II reaction center complex from the thermophilic cyanobacterium, *Synechococcus vulcanus*, by column chromatography using Triton X-100 and urea, and attempted to identify the same gene product. SDS gel electrophoresis of the cyanobacterial reaction center complex resolved three protein bands of 8, 5.4 and 5 kDa in addition to D1, D2 and a partially remaining 47 kDa protein. N-terminal sequencing revealed that the 5 kDa band consisted of two components: one, having an open N-terminus, was homologous to the *psb*L gene product of higher plants, and the other, having a blocked N-terminus, was homologous to the *psb*I gene product of higher plants. The 8 kDa and 5.4 kDa were the large and small subunits of cytochrome *b*-559, respectively. Based on these results, we conclude that the subunit structure of cyanobacterial PS II reaction center complex is essentially the same as that of higher plants except that the cyanobacterial *psb*L product is more tightly associated with the reaction center.

Cyanobacterium; Photosystem II; Reaction center; Gene, *psb*I; Gene, *psb*L; (*Synechococcus vulcanus*)

1. INTRODUCTION

There are many protein components registered for various types of photosystem II (PS II) preparations from plants [1–3]. Among these, the D1–D2 heterodimer has been claimed to form the reaction center to mediate photochemical reactions [4], based on the sequence homology with L and M subunits and the structural analogy with the crystallized reaction center complex of photosynthetic purple bacteria [5,6]. However, no active D1–D2 heterodimer has been successfully isolated yet, and the minimum active complex so far isolated is the so-called 'D1–D2 cytochrome–*b*-559' complex, that was first reported for spinach by

Correspondence address: M. Ikeuchi, Solar Energy Research Group, The Institute of Physical and Chemical Research, Wako, Saitama 351-01, Japan

Abbreviations: PVDF, polyvinylidene difluoride; PS II, photosystem II; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

Nanba and Satoh [7]. Although this complex has a pigment composition (4 to 5 Chl and 2 pheophytin per unit) similar to that of bacterial reaction center [7], it contains three low-molecular-mass proteins (two subunits of cytochrome *b*-559 of 9.4 kDa and 4.4 kDa and the newly found protein of 4.8 kDa) in addition to D1 and D2 [8]. Likewise, crystallized bacterial reaction center complexes contain such additional components as the H subunit and the cytochrome with four hemes as reported for *Rhodopseudomonas viridis* [5]. It is not known, however, whether there are any structural or functional relationships with respect to these additional components between the higher plant PS II and purple bacterial reaction centers.

From the evolutionary point of view, cyanobacteria, oxygenic procaryotes, are the organisms of an intermediate type located between photosynthetic bacteria and green plants. Accumulating data show that cyanobacterial PS II is similar to plant PS II in many aspects such as subunit com-

position and photochemistry. For example, the structural genes of cytochrome b-559 subunits (psbE and psbF), D1 (psbA) and D2 (psbD) were identified in cyanobacterial chromosomes [9–11] and their expression was confirmed immunologically using antibodies raised against corresponding plant proteins [9,12]. Recently we also identified the psbH and psbK gene products in Synechococcus PS II [13], both of which had been found in higher plants before [14,15].

These similarities lead us to assume that the cyanobacterial PS II reaction center must also contain the 4.8 kDa component, that was recently identified in higher plants [8] to be encoded by a chloroplast gene designated psbI [16,17]. There is an attempt reported to isolate the reaction center complex from Synechocystis [18], but nothing has been described about the low-molecular-mass protein components probably because of a poor resolution in SDS-PAGE [18]. Here we isolated a cyanobacterial PS II reaction center complex from a thermophilic cyanobacterium, Synechococcus vulcanus, and report a clear identification of all the low-molecular-mass proteins by N-terminal sequencing.

2. MATERIALS AND METHODS

PS II particles and the O_2-evolving core complex were prepared from thylakoid membranes of Synechococcus vulcanus Copeland as described in [13]. The core complex was treated with 50 mM Tris-HCl (pH 8.0) to remove the 33 kDa extrinsic protein, and then solubilized with 4% (w/v) Triton X-100 at 1 mg Chl/ml in 4 M urea, 30 mM NaCl and 50 mM Tris-HCl (pH 7.2). After 1 h of treatment at 0°C, the solubilized materials were passed through a Millipore filter (pore size 0.2 μm) and loaded onto a DEAE-Toyopearl column which had been equilibrated with 0.05% (w/v) Triton X-100, 4 M urea, 30 mM NaCl and 50 mM Tris-HCl (pH 7.2). The column was washed with the equilibration buffer for 2 h to remove unbound proteins. Then the urea concentration was reduced to 2 M and washing was continued for about 15 h until the absorption at 415 nm of the eluate reached the level comparable to Chl absorption at 435 nm. The reaction center complex was eluted by a NaCl linear gradient from 30 to 400 mM in the same buffer. Reaction center-enriched fractions around 150 mM NaCl were pooled, diluted 10 times with 50 mM Tris-HCl (pH 7.2), and then precipitated by centrifugation at 250000 × g for 1 h.

SDS-PAGE with 7.5 M urea and a 16–22% (w/v) acrylamide gradient was performed following [8]. For protein sequencing, proteins in the gel were transferred onto a PVDF membrane (Immobilon, Millipore) using a semidry-type electroblotter according to the method in [3] except for omission of Tris from the blotting solution. This modification was indispensable to transfer the 5 kDa proteins to the PVDF membrane, although the other low-molecular-mass proteins were well blotted irrespective of the modification. Transferred proteins were stained with Amido Black 10B (Bio-Rad), cut out, and then subjected to a protein sequencer (model 477A, Applied Biosystems). Each protein band on the PVDF membrane was sequenced before and after an acid treatment (0.6 N HCl for 24 h) to check N-terminal blockage.

3. RESULTS

The PS II reaction center complex isolated from Synechococcus showed enhanced absorption peaks at 415 nm and at 539 nm as compared with those in the original core complex (fig.1). The 415 nm peak may be attributed to the Soret band absorption of both pheophytin and cytochrome b-559, while the 539 nm peak is due to pheophytin, as in the higher plant PS II reaction center complex [7]. Notably, however, the absorption by Chl at 435 nm remained as a low but distinct independent peak (fig.1), while it decreased to only a shoulder superimposed on the prominent absorption peaking at 415 nm in higher plant preparations. This indicates a higher content of Chl in the cyanobacterial preparation than in higher plant preparation, and suggests an incomplete removal of antenna Chl-protein complexes. In accordance with this, a doublet band due to the 47 kDa Chl-binding protein could still be found in the reaction center preparation from cyanobacteria, while the 43 kDa Chl-binding protein and the 33 kDa extrinsic protein were completely removed (fig.2).

In order to compare our cyanobacterial preparation with the PS II reaction center preparation from higher plants, we attempted to estimate the number of Chl molecules per unit. Based on the staining intensity of the large subunit of cytochrome b-559 (8 kDa band, see below for identification), the contents of 47 kDa protein and total Chl in our reaction center preparation were estimated to be 50 and 37%, respectively, of those in the original core complex. Assuming that the core complex contains 40 to 50 Chl molecules per unit [19], the Chl content of our reaction center preparation is calculated to be 14 to 18 Chl molecules per unit. When the amount of Chl bound to the remaining 47 kDa protein was taken into account, the intrinsic number of Chl molecules per unit of cyanobacterial PS II reaction
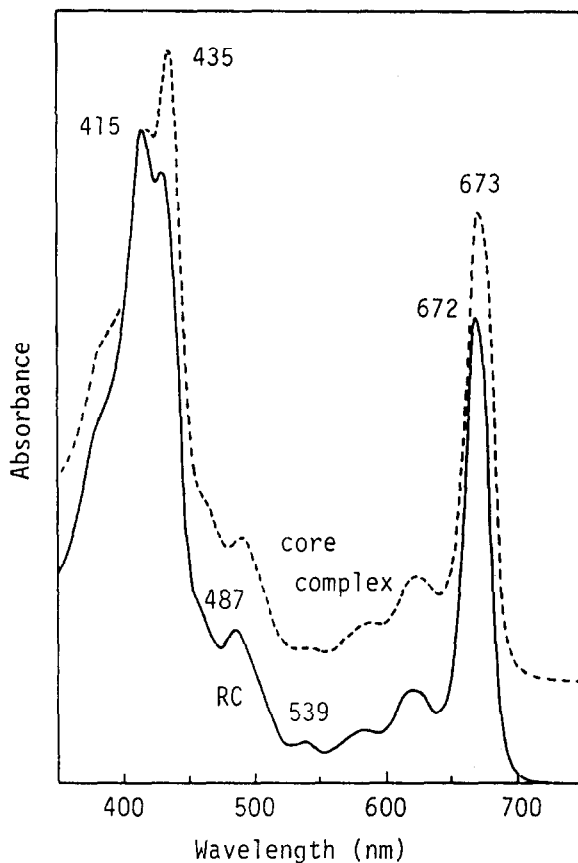
Fig.1. Absorption spectra of the reaction center (RC) complex (solid curve) and O₂-evolving core complex (dashed curve) from *Synechococcus*.

Fig.2. Polypeptide composition of PS II particles (lane a), the O₂-evolving core complex (b) and the PS II reaction center complex (c) from *Synechococcus*, and the PS II reaction center complex (d) from spinach.

center is calculated to be 4 to 8, which is close to that of higher plants. In these estimations, we used the large subunit of cytochrome *b*-559 (8 kDa band) as the most reliable standard component of PS II reaction center, since most of D1 and D2 aggregated during detergent treatment to form various aggregates recovered in high molecular mass region on the gel, resulting in an apparent poor recovery of the monomer bands.

In low-molecular-mass region, our reaction center complex exhibited three bands of about 8 kDa, 5.4 kDa and 5 kDa (fig.2). Occasionally a 4.7 kDa band could be vaguely separated from the 5 kDa band, but the core components (6.5 kDa *psb*H product, 3.9 kDa *psb*K product and the 4.1 kDa protein) could always be removed completely. The reproducibly observed three low-
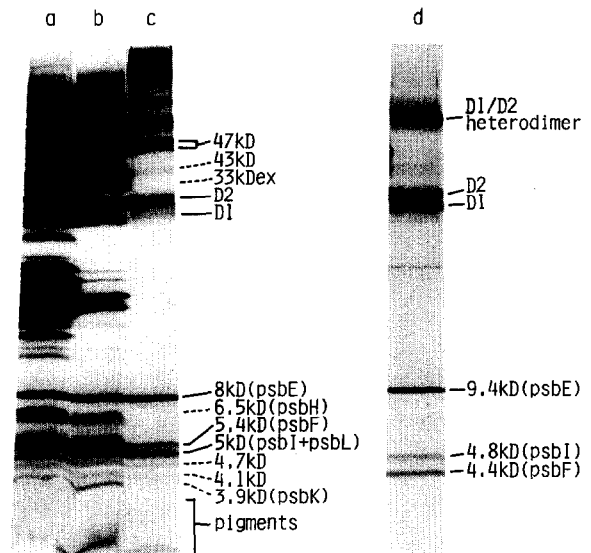
molecular-mass components were subjected to N-terminal sequencing.

The 5 kDa band of the reaction center complex gave a unique N-terminal sequence when sequenced without acid treatment (fig.3D). This sequence is homologous to the sequences deduced from the gene, which is known to be located just downstream of the *psb*E/*psb*F genes both in cyanobacteria and plants [20–22]. The product protein of this gene was recently identified in spinach and wheat PS II and the gene was designated *psb*L [3], although the same gene was once reported as *psb*I without identification of its product [22]. The sequence alignment shows that only a few residues at the N-terminus are variable but the rest is highly conserved. Note that the first Met residue is found in the *Synechococcus* product, while it is lacking in the spinach and wheat products.

After acid treatment, the same 5 kDa band of the reaction center complex gave another unique sequence with comparable intensities in amino acid signals (fig.3C). This sequence is homologous to the partial sequence data obtained for the 4.8 kDa reaction center component of spinach which was recently assigned to the product of *psb*I gene

(A) 8 kDa protein (psbE)

```
Synechococcus   AG-TTGERPFSDIITSVRYWVIHSITIPALFIAG
Synechocystis   MS.-..........V..I...........M.....
Cyanophora      MS.G..........V..I......TV...FFIV..
Liverwort       MS.-.......A.....I............S.....
Tobacco         MS.-S....S.A.....I..........S.....
```

(B) 5.4 kDa protein (psbF)

```
Synechococcus   TSTNPNQEPVSYPIFTVR?VA
Synechocystis   MATQ....-..P.......WL.
Cyanophora      MN--....-.........WL.
Liverwort       M.IDRT------......WL.
Tobacco         M.IDRT------......WL.
```

(C) 5 kDa protein with blocked N-terminus (psbI)

```
Synechococcus   METLKITVYIVVTFFVLLFVFGFLSGDPA
Liverwort       .L...LF..T..I...S........N..G
Tobacco         .L...LF..T..I...S..I.....N..G
```

(D) 5 kDa protein with open N-terminus (psbL)

```
Synechococcus   ME--PNPNRQPVELNRT?L
Synechocystis   .DRNS...........S.
Cyanophora      .VSQ-.....K......S.
Liverwort       .TQP-...K.S......S.
Tobacco         .TQS-...E.N......S.
```

Fig.3. Determined amino acid sequences of *Synechococcus* proteins and their alignment with those deduced from respective gene sequences of other organisms (liverwort [20], tobacco [21], *Synechocystis* PCC 6803 and *Cyanophora paradoxa* [9,22]). Only the N-terminal regions of the deduced sequences were aligned with the *Synechococcus* data. Amino acid differences between *Synechococcus* and the others were indicated, but the same residue was shown by a dot. A hyphen denotes a deletion and a question mark means a residue which was not determined. Underlining stands for the putative membrane spanning segment or its part. Note that all the reference sequences are based on gene sequence data but not on protein sequencing. The protein sequence data for spinach and wheat [3,16] are not presented here, since they are almost the same as those deduced from tobacco genes. It is noteworthy, however, that in higher plant PS II the protein sequence data showed deletion of the first Met in *psbE*, *psbF* and *psbL* products but not in *psbI* product ([3,16,23,24]; also Ikeuchi, M. and Inoue, Y., unpublished).

reported for liverwort and tobacco chloroplast DNA [16]. Without acid treatment, this sequence could not be obtained which is indicative of N-terminal blockage. The sequence alignment shows a high homology throughout the determined part. Notably, the 5th Lys and 27th Asp residues are conserved among the three organisms showing the same putative membrane-spanning segment composed of 21 hydrophobic residues between the two residues.

The N-terminal sequences of the 8 kDa and

5.4 kDa bands were obtained without acid treatment and found to be homologous to *psbE* and *psbF* sequences, respectively, indicative of the large and small subunits of cytochrome *b*-559 with open N-termini (fig.3A,B). In higher plants, the N-terminus of the small subunit was blocked, while that of the large subunit was not [3,23,24]. The first Met residues of both subunits in *Synechococcus* were missing like those in higher plants. Alignment of the *psbF* sequences revealed that the *Synechococcus* sequence has a higher homology with *Synechocystis* and *Cyanophora* than with higher plants. The difference in apparent molecular mass of the small subunits observed by SDS-PAGE (5.4 kDa for *Synechococcus* and 4.4 kDa for higher plants) can be explained by the insertion of an additional 6 amino acid residues in the N-terminal region of the *Synechococcus* protein, since the sequence in C-terminal region (not sequenced here) is highly conserved among all organisms [22]. In contrast, the N-terminal part of the large subunit was highly conserved between *Synechococcus* and the others, and the differences were mostly found in the C-terminal end (not sequenced here).

On SDS gels, the dye-staining intensity of the 5 kDa band (*psbI* plus *psbL* products) was reproducibly higher by a factor of about 1.5 than that of the 5.4 kDa band (*psbF* product). When the apparent molecular masses of these proteins are taken into account, the molar abundance of the two 5 kDa proteins relative to the 5.4 kDa protein will be even higher. The intensity of amino acid signals in protein sequencing suggested comparable amounts of *psbI* and *psbL* products in the 5 kDa band. Based on these, it can be inferred that both *psbI* and *psbL* products are present at almost stoichiometric amounts with regard to the *psbF* product, although the exact stoichiometry could not be determined in this study because of various limitations.

4. DISCUSSION

The present study not only provided the first evidence for the presence of *psbI* gene in cyanobacteria, but also demonstrated that the *Synechococcus* PS II reaction center complex contains the product of this gene (*psbI*) as well as the

products of psbL, psbE and psbF genes. The expression of psbL gene in cyanobacteria was also confirmed. In higher plants, the products of psbI, psbE and psbF genes are identified to be the components of the PS II reaction center complex, while the product of psbL gene is lost during purification from the core complex to the reaction center [3,16]. This difference in association pattern between higher plants and cyanobacteria might be interpreted by an incomplete dissection of the reaction center from the core components in Synechococcus, as also shown by the partial retention of the 47 kDa protein. Our present results may indicate that the psbL product is more strongly associated with the reaction center than the other core components which are removed during column chromatography. To settle this, it is necessary to isolate a cyanobacterial PS II reaction center complex completely free from the 47 kDa protein. However, we can at least conclude that the PS II reaction center complex of both higher plants and cyanobacteria commonly consists of D1, D2, cytochrome b-559 and the psbI gene product. We would also like to note in this context, that the PS II reaction center preparation from the green alga, Scenedesmus obliquus, also contains a 5 kDa protein in addition to D1, D2 and the two subunits of cytochrome b-559 (Hiyama, T., Ikeuchi, M., Seibert, M. and Inoue, Y., unpublished).

It is still not known what roles the gene products of psbI and psbL play in PS II. We only know that psbL is co-transcribed with psbE and psbF [25]. The present study revealed that the psbL product is co-purified with the two subunits of cytochrome b-559 (products of psbE and psbF) up to the reaction center complex in Synechococcus (this study) and up to the core complex in higher plants [3]. This suggests that the psbL product is a peripheral component of cytochrome b-559. The answer to this question might be derived by checking the presence of psbL product in isolated, active cytochrome b-559 complex.

In chloroplast DNA, the psbI gene is located near the psbK gene but far from the psbE/psbF/psbL operon [20,21]. This may suggest that the psbI gene product has a unique function completely unrelated with cytochrome b-559. In cyanobacteria, the presence of psbI gene is now established by protein sequencing, and is going to be cloned from the cyanobacterial chromosome. It might be reasonable to assume its location near the psbK gene like in chloroplast genome, since the presence and expression of the psbK gene in cyanobacteria were also confirmed by protein sequencing [13].

In the reaction center of Rhodopseudomonas viridis, the H subunit and a cytochrome are present in addition to the L and M subunits [5]. The H subunit has a single membrane-spanning segment in its N-terminal region, while the cytochrome is a peripheral protein and does not span the membrane. Thus, it may be possible to hypothesize that the psbI product has a role similar to the H subunit. In fact, we can point out a slight homology in the membrane-spanning segment between the two proteins, although the H subunit has a huge hydrophilic domain in contrast to the tiny hydrophilic chains on both N- and C-terminal regions of the psbI product. A plausible way to study the role of the psbI product in PS II reaction center would be to use genetically engineered mutants.

Recently Gounaris et al. [18] reported a PS II reaction center preparation from Synechocystis PCC 6803 obtained by use of lauryl maltoside and Triton X-100. The absorption spectrum of their preparation is more or less the same as that of ours, indicative of a high Chl to pheophytin ratio, although the absorption peak at 435 nm due to Chl is appreciably higher in their preparation than in ours. However, their interpretation is totally different from ours. We attributed the high content of Chl to the partial retention of the 47 kDa Chl-binding protein and inferred that the cyanobacterial PS II reaction center per se has a low Chl to pheophytin ratio like that in higher plants. In contrast, Gounaris et al. [18] did not detect any of the 47 kDa or 43 kDa proteins with their specific antibodies raised against corresponding proteins of higher plants, and concluded that the cyanobacterial PS II reaction center has a much higher Chl to pheophytin ratio. These different interpretations might be ascribed to the ambiguities we often experience in immunological detection owing to a weak cross-reactivity of an antibody. A definite conclusion must await a preparation of cyanobacterial PS II reaction center complex completely free from the 47 kDa protein.

REFERENCES

[1] Satoh, K. (1985) Photochem. Photobiol. 42, 845–853.

[2] Murata, N. and Miyao, M. (1987) in: Progress in Photosynthesis Research (Biggins, J. ed.) vol.I, pp.453–462, Martinus Nijhoff, Dordrecht.

[3] Ikeuchi, M., Takio, K. and Inoue, Y. (1989) FEBS Lett. 242, 263–269.

[4] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.

[5] Deisenhofer, J., Epp, O., Miki, K., Huber, R. and Michel, H. (1985) Nature 318, 618–624.

[6] Michel, H., Weyer, K.A., Gruenberg, H., Dunger, I., Oesterhelt, D. and Lottspeich, F. (1986) EMBO J. 5, 1149–1158.

[7] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.

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

[9] Pakrasi, H.B., Williams, J.G.K. and Arntzen, C.J. (1988) EMBO J. 7, 325–332.

[10] Curtis, S.E. and Haselkorn, R. (1984) Plant Mol. Biol. 3, 249–258.

[11] Williams, J.G.K. and Chisholm, D.A. (1987) in: Progress in Photosynthesis Research (Biggins, J. ed.) vol.IV, pp.813–816, Martinus Nijhoff, Dordrecht.

[12] Vermaas, W.F.J., Ikeuchi, M. and Inoue, Y. (1988) Photosynth. Res. 17, 97–113.

[13] Koike, H., Mamada, K., Ikeuchi, M. and Inoue, Y. (1989) FEBS Lett. 244, 391–396.

[14] Farchaus, J. and Dilley, R.A. (1986) Arch. Biochem. Biophys. 244, 94–101.

[15] Murata, N., Miyao, M., Hayashida, N., Hidaka, T. and Sugiura, M. (1988) FEBS Lett. 235, 283–288.

[16] Ikeuchi, M. and Inoue, Y. (1988) FEBS Lett. 241, 99–104.

[17] Webber, A.N., Packman, L., Chapman, D.J., Barber, J. and Gray, J.C. (1989) FEBS Lett. 242, 259–262.

[18] Gounaris, K., Chapman, D.J. and Barber, J. (1989) Biochim. Biophys. Acta 973, 261–301.

[19] Satoh, K., Ohno, T. and Katoh, S. (1985) FEBS Lett. 180, 326–330.

[20] Ohyama, K., Fukuzawa, H., Kohchi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. (1986) Nature 322, 572–574.

[21] Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubaybashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. and Sugiura, M. (1986) EMBO J. 5, 2043–2049.

[22] Cantrel, A. and Bryant, D.A. (1988) Photosynth. Res. 16, 65–81.

[23] Widger, W.R., Cramer, W.A., Hermodson, M., Meyer, D. and Guillifor, M. (1984) J. Biol. Chem. 259, 3870–3876.

[24] Widger, W.R., Cramer, W.A., Hermodson, M. and Herrmann, R.G. (1985) FEBS Lett. 191, 186–190.

[25] Cushman, J.C., Christopher, D.A., Little, M.C., Hallick, R.B. and Price, C.A. (1988) Curr. Genet. 13, 173–180.