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A Novel 3.5 kDa Protein Component of Cyanobacterial Photosystem I Complexes

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A novel protein component of 3.5 kDa was detected in photosystem I complexes prepared from several cyanobacteria, viz. *Synechococcus vulcanus, Synechococcus elongatus* BP-1, *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803. The complete amino acid sequence of this component was determined by direct protein sequencing. The sequences of the 3.5 kDa proteins from these four organisms were highly homologous to each other, featuring a hydrophobic domain in the middle. The cyanobacterial consensus sequence exhibits significant homology to the presumed product of ORF32 in the chloroplast DNA of liverwort (*Marchantia polymorpha*), but no homologous ORF is present in the chloroplast DNA of tobacco or rice. Since this protein appears to interact strongly with the PS I reaction center complex, it may play some role in the function and maintenance of the structure of PS I.

Key words: Cyanobacteria — Photosystem I — *psa*M — *Synechococcus elongatus* — *Synechococcus* sp. PCC 7002 — *Synechococcus vulcanus* — *Synechocystis* sp. PCC 6803.

The PS I complex in the photosynthetic apparatus consists of a number of protein subunits, the functions of most of which are unknown (Bryant 1992, Scheller and Møller 1990). We have been studying the protein composition of PS I preparations from various cyanobacteria and higher plants and examining the evolutionary conservation of various subunits. When the PS I complex isolated from the thermophilic cyanobacterium *Synechococcus vulcanus* was analyzed by high resolution SDS-urea-PAGE and protein sequencing, three proteins of 6.5 kDa, 4.8 kDa and 4.1 kDa were newly identified as PS I components (Koike et al. 1989). Of these three components, homologues of the 6.5 kDa and 4.1 kDa proteins were found in PS I complexes from higher plants and were designated PS I-K and PS I-J, respectively (Franzén et al. 1989, Hoshina et al. 1989, Ikeuchi et al. 1990). The other component, the 4.8 kDa protein [tentatively named PS I-N by Bryant (1992)], was also detected in the PS I complex from *Anabaena variabilis* ATCC 29413 but has not yet been found in higher plants. It appears, therefore, that PS I-N is a cyanobacterium-specific component of PS I (Koike et al. 1989, Ikeuchi et al. 1991c). Besides these three components, another small protein of 3.9 to 4.5 kDa (named PS I-I) has been identified in PS I complexes of higher plants (Scheller et al. 1989, Ikeuchi et al. 1990) and also of *Anabaena* (Ikeuchi et al. 1991c). Thus, PS I-I, PS I-J and PS I-K have been shown to be conserved evolutionarily between cyanobacteria and higher plants as PS I components.

In the course of our studies on the protein composition of the PS I complex from *S. vulcanus*, we noticed that a faint band of protein (below the 4.1 kDa band of PS I-J) was sometimes visible after SDS-urea-PAGE. However, resolution of this band was not always reproducible because of interference by lipids. In the present study, we unambiguously identified this protein by protein sequencing. It turned out to be a novel PS I component that interacts strongly with the reaction center complex.

Abbreviations: CPI, chlorophyll-protein complex I of photosystem I reaction center; ORF, open reading frame; PAGE, polyacrylamide gel electrophoresis; PS I-X, protein in photosystem I encoded by the *psa*X gene.

Materials and Methods

Organisms and preparation of PS I complexes—A crude preparation of the PS I complex was obtained from thylakoids of thermophilic *Synechococcus vulcanus* by solubilization with lauryldimethylamine *N*-oxide followed by sucrose density gradient centrifugation according to Koike et al. (1989). The PS I complex of thermophilic *Synechococcus elongatus* strain BP-1 was prepared by solubilization of thylakoids with digitonin followed by digitonin-PAGE as described in Nakayama et al. (1979) and Yamagishi and Katoh (1983). These two thermophilic cyanobacteria, which had been collected from different locations in Japan, were cultured at 55°C as described by Hirano et al. (1980). PS I complexes of *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002 were prepared from thylakoids by solubilization with dodecylmaltoside and subsequent column chromatography on a MonoQ column (Pharmacia, Sweden) by the methods of Barry et al. (1988) and Rögner et al. (1990). *Synechocystis* PCC 6803 was cultured in BG-11 medium (Rippka 1988) supplemented with 20 mM TES-KOH (pH 8.0). *Synechococcus* PCC 7002 was cultured in medium A supplemented with 1 mg ml^{-1} $NaNO_3$ as described by Stevens and Porter (1980).

SDS-urea-PAGE and protein sequencing—For separation of low-molecular-mass proteins from PS I, high resolution SDS-urea-PAGE with 7.5 M urea and 16–22% acrylamide was performed at 25°C according to Ikeuchi and Inoue (1988a). PS I complexes were solubilized with 2% lithium dodecylsulfate, 60 mM dithiothreitol and 60 mM Tris-HCl (pH 8.5) at a Chl concentration of 0.8 mg ml^{-1} and heated at 70°C or 100°C for 1 min. In some cases, PS I complexes were delipidated by extraction with a mixture of methanol and ether (1 : 9, v/v) according to Ikeuchi et al. (1991c). For protein sequencing, PS I equivalent to 64 μg Chl was delipidated. Proteins were resolved by SDS-urea-PAGE and electroblotted onto a polyvinylidene difluoride membrane (ProBlot™; Applied Biosystems, U.S.A.). Each band, visualized by Amido Black staining was cut out and subjected to N-terminal sequencing. Alternatively, it was treated with 0.6 M HCl for 24 h to release the N-terminal block prior to sequencing according to Ikeuchi and Inoue (1988b).

Homology search—A computer-assisted homology search was made using the TFASTA program developed by Pearson and Lipman (1988). This program compares a protein query sequence to a DNA database sequence by translating the DNA sequence into all six frames and performing a protein sequence comparison in each frame. Both EMBL and GenBank databases were searched.

Results and Discussion

When the PS I complex from *S. vulcanus* was analyzed by SDS-urea-PAGE, a faint band was just detectable below 4 kDa (Fig. 1, left). The resolution of this band was not reproducible because of interference by comigrating lipids. These lipids were selectively removed by extraction with a mixture of methanol and ether (1 : 9; v/v) without any effect on the separation profile of other components, although the CPI was converted to its constituent apoproteins. As a result, the band at 3.5 kDa was clearly resolved by SDS-urea-PAGE (Fig. 1, right). The complete amino acid sequence of the 3.5 kDa protein was successfully determined by N-terminal sequencing after electroblotting onto a polyvinylidene difluoride membrane (Fig. 2). It was revealed that this protein consisted of 31 amino acid residues with no N-terminal block. The calculated molecular mass was 3,424 Da, which agrees well with the apparent value of 3.5 kDa estimated from the electrophoretic mobility (Fig. 2). The amino acid sequence of the 3.5 kDa protein did not match any region of the PS I proteins identified to date, indicating that the protein is a novel PS I component.

A similar 3.5 kDa protein was found in the PS I complex from *S. elongatus* (Fig. 3). Neither the 3.5 kDa protein nor PS I-L was undetectable in the non-heated non-extracted sample. Upon lipid extraction, these proteins be-

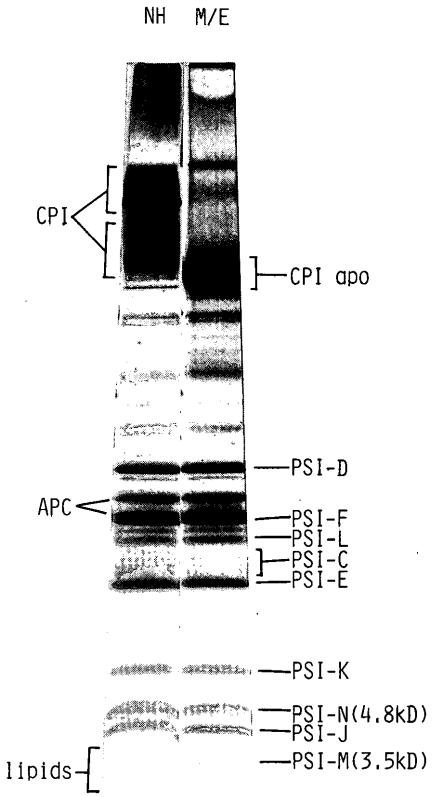

Fig. 1 SDS-urea-PAGE profile of the PS I complex from *Synechococcus vulcanus*. NH stands for the non-heated sample, and M/E stands for the lipid-extracted sample. APC, contaminating allophycocyanins.

3.5 kDa protein (PSI-M)

	* * * * ** ** ***	amino acid	MW(Da)
Synechococcus vulcanus	M-ALTDTQVYVALVIALLPAVLAFRLSTELYK*	31	3424
Synechococcus elongatus	M-ALTDTQVYVALVIALLPAVLAFRLSTELYK*	31	3424
Synechococcus PCC 7002	M-GISDTQVLVALAIALIPGVLAFRLSTELYK*	31	3304
Synechocystis PCC 6803	M-ALSDTQILAALVVALLPAFLAFRLSTELYK/	31(?)	3380(?)
Cyanophora paradoxa	M--LADGQIFTALAVALVPGILALRLALELYKF*	31	3332
liverwort	MTSISDSQIIVILLSVFITSILALRLGKELYQ*	32	3566
	└──────18 aa──────┘		

Fig. 2 Sequence alignment of cyanobacterial 3.5 kDa proteins (PS I-M) and hypothetical gene products of ORF31 of *Cyanophora paradoxa* and ORF32 of liverwort. Asterisks at the C-terminal position of the sequences show stop codons or the absence of an amino acid signal, while a slash shows the end of sequencing. Amino acid residues that are totally conserved are indicated by asterisks on the top line. An internal hydrophobic domain is indicated by the bottom line. Sequences from *Cyanophora paradoxa* and liverwort are from D.A. Bryant and V.L. Stirewalt (unpublished results) and Umesono et al. (1988), respectively.

came detectable, while the chlorophyll-containing high-molecular-mass CPI complexes was no longer visible on the stacking gel. Heat treatment at 70°C or 100°C for 1 min also gave similar results: the 3.5 kDa protein, as well as the other proteins, was fully resolved despite the presence of lipids. Likewise, a similar 3.5 kDa protein was detected in the PS I complexes of *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803 after lipid extraction (Figs. 4, and 5). The elution profile of the 3.5 kDa protein during the col-

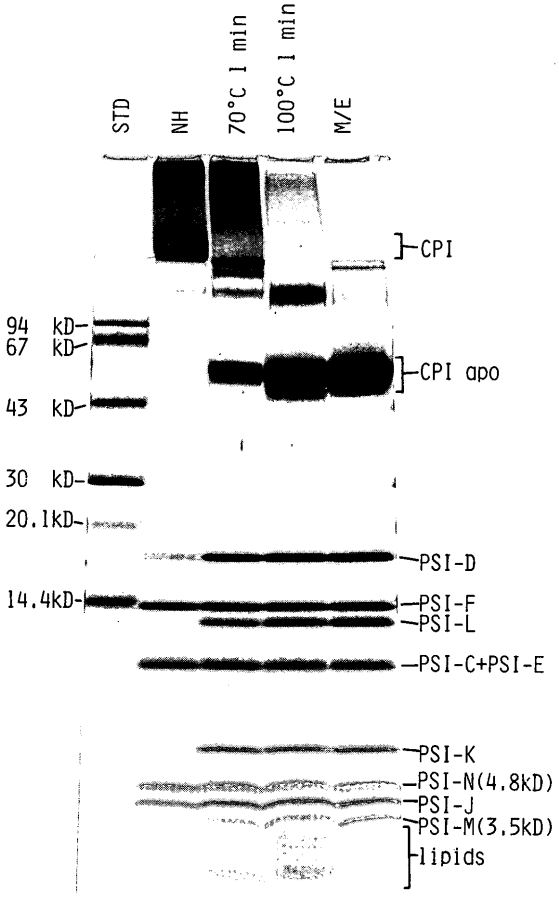

Fig. 3 SDS-urea-PAGE profile of the PS I complex from *Synechococcus elongatus*. The solubilized samples were heated where indicated. NH stands for the non-heated sample, and M/E stands for the lipid-extracted sample. Molecular standards are shown in the left lane.

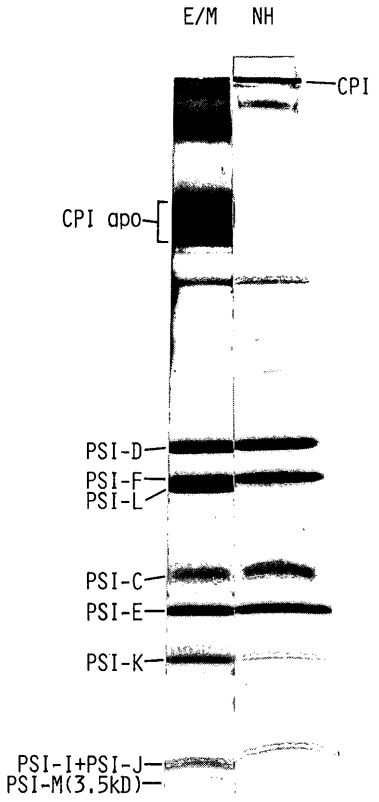

Fig. 4 SDS-urea-PAGE profile of the PS I complex from *Synechococcus* sp. PCC 7002. NH stands for the non-heated sample, and M/E stands for the lipid-extracted sample.

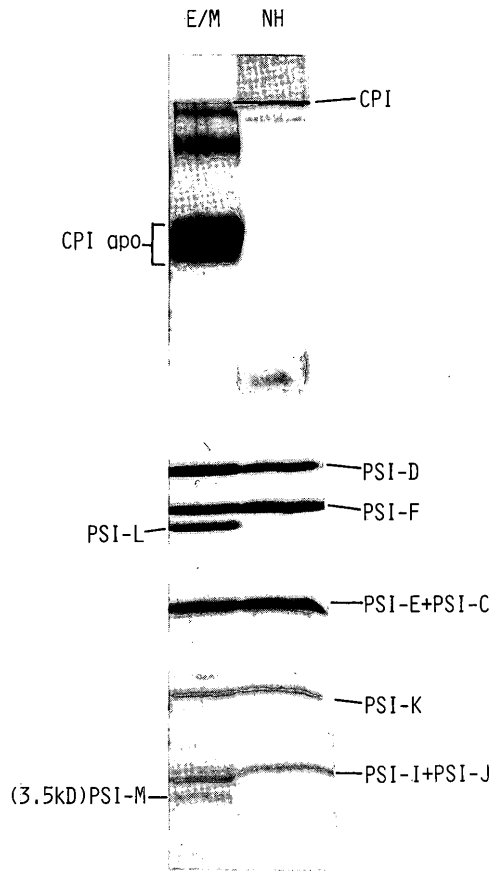

Fig. 5 SDS-urea-PAGE profile of the PS I complex from *Synechocystis* sp. PCC 6803. NH stands for the non-heated sample, and M/E stands for the lipid-extracted sample.

umn chromatography resembled that of the CPI apoproteins in the case of both *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803 (not shown), supporting the hypothesis that the 3.5 kDa protein is a true component of PS I. In agreement with the results for *S. elongatus*, neither the 3.5 kDa protein nor PS I-L could be resolved from non-heated samples of *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803, with concomitant preservation of the high-molecular-mass CPI complex. This result suggests that both the 3.5 kDa protein and PS I-L are tightly associated with the CPI complex even under such harsh conditions as SDS-PAGE in the presence of 7.5 M urea at 25°C. By contrast, these proteins were dissociated from the CPI of *S. vulcanus* under the same experimental conditions (Fig. 1), perhaps because the PS I complex of this alga was partially destabilized during preparation or storage.

Almost the entire sequence of the 3.5 kDa protein from these cyanobacterial preparations could be determined by N-terminal sequencing (Fig. 2). The N-terminus of the protein from *Synechococcus* PCC 7002 was blocked in vivo but could be deblocked by treatment with HCl prior to sequencing. By contrast, the results of sequencing the proteins from *S. vulcanus, S. elongatus* or *Synechocystis* PCC 6803 were not significantly affected by the same treatment with HCl, suggesting that their N-termini were not blocked. Since the same procedures for isolation and protein analysis were used for both *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803, it is very unlikely that the difference in terms of the N-terminal blocking of the 3.5 kDa protein was an experimental artifact. It is clear that the 3.5 kDa proteins from the four cyanobacteria are homologous to each other (Fig. 2). The amino acid sequence of the protein from *S. vulcanus* was identical to that of the protein from *S. elongatus*, suggesting a close similarity between the two thermophilic cyanobacteria. Since these sequences are not homologous to those of any known components of PS I, we can conclude that the 3.5 kDa protein is a novel PS I component that is commonly present in cyanobacteria. The only exception known to date comes from the PS I complex of *Anabaena*, in which only the PS I-I protein and not the newly identified 3.5 kDa protein was detected in a 3.5 kDa band (Ikeuchi et al. 1991c). This result might be due to the experimental difficulties encountered in attempts at sequencing a protein with a blocked N-terminus: the treatment with HCl used here for deblocking is not always very efficient. Since the band of 3.5 kDa from the PS I complex of *Anabaena* fortuitously contains PS I-I with an unblocked N-terminus, sequencing signals from a blocked protein, if any, might be masked by the strong signals from the unblocked PS I-I. It is also possible that the 3.5 kDa protein was lost from the *Anabaena* complex, since this complex was prepared by two cycles of chromatography in the presence of dodecylmaltoside and Triton X-100, in contrast with the single chromatographic fractionation used for the preparation of PS I from *Synechococcus* PCC 7002 and *Synechocystis* PCC 6803 in the present study.

A computer-assisted homology search of DNA databases revealed that the sequences of the 3.5 kDa proteins of the four cyanobacteria are significantly homologous to the deduced sequence encoded by liverwort ORF32 (Fig. 2), which is located from position 22516 to 22614 in the chloroplast DNA (Ohyama et al. 1986). Although the sequence from liverwort is about 35% identical to those from cyanobacteria, the sequence alignment shows reasonably good conservation of the charged residues and hydrophobicity of the internal domain (Fig. 2). Since the cyanobacterial 3.5 kDa protein could be extracted from the PS I complex by chloroform and methanol (not shown), it seems likely that it interacts with a hydrophobic region of the reaction center proteins, although the hydrophobic domain (18 amino acid residues) does not seem to be long enough to span the membrane. It is, thus, suggested that liverwort ORF32 encodes a PS I component that is homologous to the cyanobacterial 3.5 kDa protein, although no PS I com-

plex has yet been purified from liverwort. Therefore, we support the designation of *psa*M for the liverwort ORF32 and the hypothetical cyanobacterial gene that encodes the 3.5 kDa protein, as proposed by Bryant (1992), on the basis of our preliminary results. It is noteworthy that a homologous gene has also been found in the cyanelle DNA of the red alga *Cyanophora paradoxa* (Bryant and Stirewalt, personal communication).

The hypothetical protein deduced from the ORF32 of liverwort is not registered in the protein database of NBRF-PIR or Swiss-Prot, probably because the hypothetical protein is so small when compared with typical cellular proteins. However, on the basis of the preferential codon usage in liverwort, ORF32 has been inferred to be a possible protein-coding gene (Umesono et al. 1988). A typical Shine-Dargano sequence and possible prokaryotic promoter −10 and −35 sequences are found upstream from this ORF, and an inverted repeat is present downstream. These suggest that ORF32 is expressed as a monocistronic message in liverwort. It is notable, however, that no homologous ORF was found in the chloroplast DNA of tobacco, although many of the genes that surround ORF32 in the chloroplast genome of liverwort are well conserved in tobacco (Shinozaki et al. 1986). No homologous ORF was detected in chloroplast DNA from rice, in which there is a rather different gene arrangement around the region that corresponds to liverwort ORF32 (Hiratsuka et al. 1989). In addition, no homologous protein has yet been detected in PS I complexes from spinach or pea (Ikeuchi et al. 1990). These observations suggest that a homologue of the 3.5 kDa protein may not be present in higher plants. If this is the case, a comparison of the properties of PS I complexes between liverwort and higher plants might provide clues to the functional role of the 3.5 kDa protein. Alternatively, we might assume that the nuclear genome in higher plants includes *psa*M, the product of which was lost from PS I complexes during preparation or was undetectable as a result of technical difficulties. Further screening for the low-molecular-mass protein or the *psa*M gene in higher plants is needed to clarify this issue.

Independently of Bryant (1992), Iwasaki et al. (1991) proposed the name *psa*M for a hypothetical gene that encodes a PS I protein of 17.5 kDa in cucumber. They concluded that its N-terminal sequence was not homologous to that of any known PS I protein. However, more careful comparison suggests that it is characteristically similar to the N-terminal region of the presumed mature form (Okkels et al. 1991) of barley PS I-L. The apparent molecular mass of the cucumber protein (17.5 kDa) is close to that of PS I-L from barley and spinach (14–18 kDa) (Okkels et al. 1991, Ikeuchi and Inoue 1991b). In addition, they did not detect PS I-L in the PS I complex from cucumber, even though the presence of many other known components of PS I was confirmed. Presumably, the 17.5 kDa protein in cucumber is not a new PS I component but is most probably PS I-L. Therefore, we would like to retain the designation *psa*M for the gene for the cyanobacterial 3.5 kDa protein.

We also determined the N-terminal sequences of proteins in other bands from our PS I complexes. Most of them provided simple confirmation of the previous identifications. One point to be mentioned, however, is that a 4 kDa band from *Synechocystis* PCC 6803 and *Synechococcus* PCC 7002 contained both PS I-I and PS I-J. Namely, when the 4 kDa band was subjected to N-terminal sequencing after treatment with HCl, two different amino acid signals were obtained at many cycles, indicative of the superposition of two proteins in this band. From their intensities, the signals were interpreted as being derived from PS I-I and PS I-J, although it was difficult to assign the two sequences unambiguously (Fig. 6). In a homology search using the TFASTA program, we recently found a *psa*J-like frame 106 bp downstream from *psa*F in *Synechocystis* PCC 6803, the DNA sequence of which was reported by Chitnis et al. (1991). However, they did not mention the presence of this *psa*J-like frame in their original report probably because the initiation codon was absent. However, if we incorporate frame shifts at two positions in their DNA sequence, the resultant open reading frame corresponds closely to our amino acid sequence (Fig. 6). This implies that the *psa*J-like frame is a pseudogene or that the frame shift is due to an artifact in DNA sequencing. The linkage of *psa*J to *psa*F is not surprising since it has been found in both *Synechococcus* sp. (Muehlenhoff et al. 1992) and in the cyanelle genome of *Cyanophora paradoxa* (Bryant 1992).

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4 kDa band [ PSI-I    MdgSYAASYLPWILiPMVGwLFPAVTMGLLFIyIE?e/
           [ PSI-J    MDGLKSFLSTAPVMIMALLtF??GILIEFN??ypd??/
psaJ-like frame       MD▒LKS▒LSTAPVMIMALLTFTAGILIEFNAFYPDLLFHP*
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Fig. 6 N-terminal sequences obtained from the 4 kDa band from *Synechocystis* PCC 6803 and the *psa*J-like frame reported by Chitnis et al. (1991). Amino acid signals from the 4 kDa band were attributed to PS I-I and PS I-J. Residues with small letters were not conclusively determined. A slash shows the end of sequencing. The frame shift (one base insertion in each case) is assumed at ▒ in the *psa*J-like frame for the best match with the amino acid sequencing data. An asterisk at the C-terminal position of the *psa*J-like frame indicates a stop codon. Question marks indicate the absence of significant data.

We have now analyzed the protein composition of five cyanobacterial PS I complexes by high resolution SDS-urea-PAGE and protein sequencing: those of *Synechococcus vulcanus, Synechococcus elongatus* BP-1, *Synechococcus* sp. PCC 7002, *Synechocystis* sp. PCC 6803 (Koike et al. 1989 and this work) and *Anabaena variabilis* ATCC 29413 (Ikeuchi et al. 1991c, Nyhus et al. 1992). It is almost definitely established that PS I-C, PS I-D, PS I-E, PS I-F, PS I-I, PS I-J, PS I-K, PS I-L, PS I-M and PS I-N are common PS I components of PS I in cyanobacteria. From the SDS-PAGE profiles (Fig. 1, 3–5), it seems very unlikely that we shall detect any more PS I components in our cyanobacterial preparations. It seems likely that PS I-G, PS I-H and a 9 kDa protein (tentatively designated PS I-O), found in higher plants, are absent from cyanobacterial PS I. Recently Li et al. (1991) obtained the same results for the PS I complex of *Synechococcus* sp. PCC 6301, with the exception of the low-molecular-mass proteins. However, ferredoxin-$NADP^+$ oxidoreductase has been found in a PS I preparation isolated from spinach by a mild detergent treatment (Sakihama et al. 1990). It may, thus, be possible to find more proteins that interact with the PS I complex, if a still closer to native PS I complex can be isolated by use of milder conditions for solubilization or fractionation.

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Note Added in Proof

A DNA sequence of *Synechococcus* sp., which corresponds to our 3.5 kDa protein, is now registered as *psa*M in EMBL and GenBank under accession number of X59760. by Haehnel, W.H., Nelson, N. and Witt, I. Their deduced protein sequence 100% is identical to our sequences from thermophilic *Synechococcus vulcanus* and *S. elongatus* BP-1.

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