Biochemical and Biophysical Research Communications **260,** 245–250 (1999)
Article ID bbrc.1999.0763, available online at http://www.idealibrary.com on IDEAL®

Cloning, Expression of the *psb*U Gene, and Functional Studies of the Recombinant 12-kDa Protein of Photosystem II from a Red Alga *Cyanidium caldarium*[1]

Hisataka Ohta,* Akinori Okumura,* Satoshi Okuyama,* Ai Akiyama,* Masako Iwai,* Shizue Yoshihara,* Jian-Ren Shen,† Masaharu Kamo‡ and Isao Enami*,[2]

**Department of Biology, Faculty of Science, Science University of Tokyo, Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan; †RIKEN Harima Institute, Sayo-gun, Mikazuki-cho, Hyogo 679-5143, Japan; and §Research Institute for Bioscience, Science University of Tokyo, Yamazaki, Noda, Chiba 278-8510, Japan*

Received April 15, 1999

The encoding extrinsic 12-kDa protein of oxygen-evolving PS II complex from a red alga, *Cyanidium caldarium,* was cloned and sequenced by means of PCR and a rapid amplification of cDNA ends (RACE) procedure. The gene encodes a putative polypeptide of 154 amino acids with a calculated molecular mass of 16,714 Da. The full sequence of the protein includes two characteristic transit peptides, one for transfer across the chloroplast envelope and another for targeting into the thylakoid lumen. This indicates that the protein is encoded in the nuclear genome. The mature protein consists of 93 amino acids with a calculated molecular mass of 10,513 Da. The cloned gene was successfully expressed in *Escherichia coli* and the resulting protein was purified, reconstituted to $CaCl_2$-washed PS II complex together with the other extrinsic proteins of 33 and 20 kDa and cyt *c*-550. The recombinant 12-kDa protein bound completely with the PSII complex, which resulted in a restoration of oxygen evolution equal to the level achieved by binding of the native 12-kDa protein.

Key Words: **extrinsic 12-kDa protein; oxygen evolution; *psb*U gene; photosystem II; *Cyanidium*.**

Photosynthetic oxygen evolution is catalyzed by the photosystem II (PSII) complex which contains a number of intrinsic membrane protein components commonly found from prokaryotic cyanobacteria to eukaryotic higher plants (for reviews, see 1, 2). In addition, there are three extrinsic proteins of 33, 12 kDa and cytochrome (cyt) *c*-550 present in prokaryotic cyanobacteria and some eukaryotic algae, which play important roles in maintaining the stability and ion environment of the oxygen-evolving complex (3–6). In green algal and higher plant PSII, two of the three extrinsic proteins, the 12 kDa protein and cyt *c*-550, disappeared and some of their functions have been replaced by two newly appeared extrinsic proteins, the 17 and 23 kDa proteins (4, 6–10).

Red algae are evolutionary one of the most primitive eukaryotic algae. The oxygen-evolving PSII complex of red algae in particular is closely related to its ancestor, prokaryotic cyanobacteria: Both cyanobacterial and red algal PSII use phycobilisome as light-harvesting antenna, and they have three common extrinsic proteins of 33, 12 kDa and cyt *c*-550 in the oxygen-evolving PSII complex (5, 7). The PSII complex from a red alga, *Cyanidium caldarium,* has been shown to contain the forth extrinsic protein, a 20 kDa protein, which is not found in the cyanobacterial PSII (5, 7). Among the four extrinsic proteins of the red algal PSII, the 12 kDa protein and cyt *c*-550 have a strong interaction with each other and play a role in minimizing the calcium and chloride requirement of the oxygen-evolving complex, whereas the unique 20 kDa protein functions in maintaining the proper binding of cyt *c*-550 and the 12 kDa protein to PSII (7). The 33 kDa and 20 kDa proteins have direct contact with PSII intrinsic components, whereas cyt *c*-550 and the 12 kDa protein associate with PSII indirectly through their interactions with the 33 and 20 kDa proteins (7).

The 33 kDa, 12 kDa proteins and cyt *c*-550 are encoded by genes *psb*O (6), *psb*U (11) and *psb*V (12), all of

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under Accession No. AB023805.

[2] To whom correspondence should be addressed. Fax: +81-471-24-2150. E-mail: enami@rs.noda.sut.ac.jp.

Abbreviations used: PSII, photosystem II; cyt, cytochrome; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; chl, chlorophyll.

0006-291X/99 $30.00

which have been cloned from cyanobacteria and were found to contain leader sequences in their N-termini, consistent with their thylakoid lumenal location. The *psb*O gene has been cloned in some eukaryotic algae and higher plants and was found to be present in the nuclear genome, with a bipartite transit peptide in its N-terminus which is required for its transport across chloroplast envelope and thylakoid membrane (6). On the other hand, the *psb*V gene coding for cyt *c*-550 was found to be present in the plastid genome of three eukaryotic algae, *Cyanophora paradoxa* (13), *Porphyra purpurea* (14) and *Odontella sinensis* (15), whose complete plastid genome sequences have been determined. The *psb*U gene coding for the 12 kDa protein was not found in the plastid genome of the three eukaryotic algae, suggesting that it has been transferred to the nuclear genome in the eukaryotic organisms. This gene, however, has not been cloned in any eukaryotic organisms so far.

In this study, we cloned the *psb*U gene encoding the 12 kDa protein from the eukaryotic red alga, *Cyanidium caldarium*. The sequence determined included a bipartite transit peptide, confirming that the gene is indeed encoded by the nuclear genome and the gene product is transferred through chloroplast envelope and thylakoid membrane after its synthesis. The cloned *psb*U gene was expressed in *E. coli*, and the obtained recombinant protein was compared with the native 12 kDa protein in terms of the binding and functional properties by reconstitution studies.

MATERIALS AND METHODS

Cloning and sequence analysis of the psbU gene. Based on the N-terminal sequence of the 12 kDa protein from a red alga *Cyanidium caldarium* (5), two amino acid sequences, Ile2-Ile7 (IDYEGI) and Tyr46-Asp51 (YGTPDD), were chosen to synthesize degenerate oligonucleotide primers (512-fold degeneracy each). These primers were used to amplify a cDNA fragment with the double stranded cDNA as template which was constructed according to (16), by the polymerase chain reaction (PCR). To prepare cDNA fragments of the 5′- and 3′-flanking regions of the *psb*U gene, rapid amplification of cDNA ends (RACE) PCR was performed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto CA). DNA sequence was determined by Dye Deoxy Terminator Cycle Sequencing (Applied Biosystems) with a DNA Sequencing System (model 310) after the PCR fragment was inserted into the plasmid pCR II (TA Cloning Kit, Invitrogen, San Diego, CA).

Expression and purification of recombinant 12-kDa protein. The *psb*U gene was cloned into LIC site of plasmid pET-32×a/LIC (Novagen, WI) which has the advantage of carrying the thioredoxin and 6 × His tag sequence at the N-terminal end of the 12 kDa protein for increasing protein solubility and simple purification by His-Bind resin. The plasmid was transformed to the host cell BL21(DE3) (Novagen, WI). After the *E. coli* was grown at 37°C with vigorous aeration to $O.D._{600\,nm}$ = 1.0 in a 100 ml Luria-Bertani medium containing 100 μg/ml ampicillin, 1 mM isopropyl-β-D-thiogalactopyranoside was added for induction of the protein expression and the culture was grown for an additional 4 hr. The cell pellet collected by centrifugation was suspended in 20 mM Tris-HCl (pH 7.9)/500 mM NaCl/5 mM imidazole containing 0.1 mg/ml lysozyme and the cells were lysed by incubation at 30°C for 15 min and then by sonication. The lysate was centrifuged at 5000 × g for 20 min and the supernatant was applied to a 1 ml column packed with His-Bind resin. The fusion protein was eluted with 1 M imidazole, dialyzed against 20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and then treated with Factor Xa followed by purification with the His-Binding column in order to remove thioredoxin and 6 × His tag. All the purification steps of the recombinant protein were carried out on ice or at 4°C.

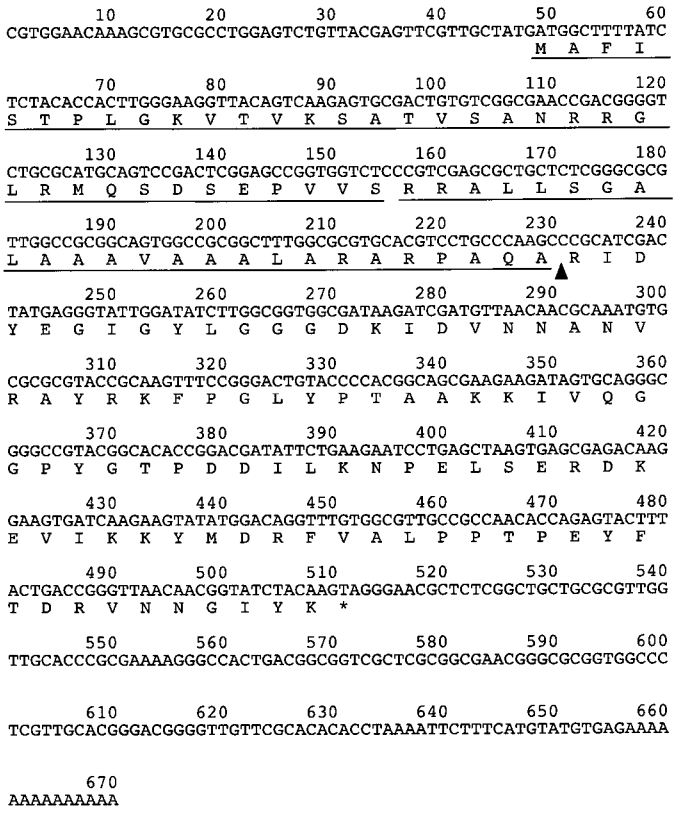

FIG. 1. Nucleotide sequence of the *psb*U gene encoding the 12-kDa extrinsic PSII protein from the red alga, *Cyanidium caldarium*. The deduced amino acid sequence is shown below the nucleotide sequence in the single-letter code. The putative chloroplast envelope transit domain and thylakoid transfer domain are underlined separately. Arrowhead indicates the cleavage site generating the mature 12-kDa protein, and the asterisk indicates the stop codon.

Reconstitution and electrophoresis. The oxygen-evolving PSII was purified from a red alga, *C. caldarium*, according to (5). The four extrinsic proteins were prepared from $CaCl_2$-extracts of the pure PSII by the method reported previously (7). For reconstitution experiments, $CaCl_2$-washed PSII, from which all the four extrinsic proteins had been removed, was incubated with the purified 33 kDa, 20 kDa proteins, cyt *c*-550 and the native or recombinant 12 kDa protein in 50 mM Mes (pH 6.5), 25% glycerol (Buffer A) for 30 min at 0°C in the dark at a chlorophyll (chl) concentration of 0.1 mg/ml. The amount of each protein added to the incubation mixture was 6.6 μM, which is approximately 3 times that of the reaction center. Following incubation, an aliquot of 50% polyethylene glycol 6000 was added to a final concentration of 10%, and then the PSII particles were collected by centrifugation at 100,000 × g for 30 min, washed once with and resuspended in buffer A (for details see Ref. 7).

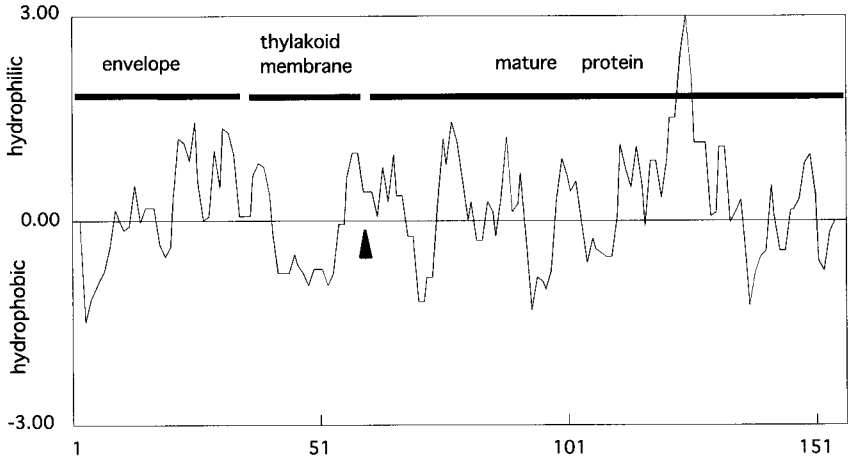

FIG. 2. Hydropathy profile of the deduced amino acid sequence of the red algal 12-kDa protein. Hydropathy analysis was performed with the program GENETYX (Software Development Co., LTD, Tokyo, Japan), with 5 residues each as the averaging number for calculation of the hydrophilicity.

Sodium dodecyl sulfate-polyacryoamide gel electrophoresis was carried out with a gradient gel of 16-22% acrylamide containing 7.5 M urea (17). Samples were solubilized with 2% lithium dodecyl sulfate and 70 mM dithiothreitol in buffer A for 30 min. Oxygen evolution was measured with a Clark-type oxygen electrode in buffer A at 25°C, with 0.4 mM phenyl-*p*-benzoquinone as the electron acceptor. Chl *a* concentration was determined according to (18).

RESULTS AND DISCUSSION

Cloning and sequence analysis of the psbU gene coding for the 12-kDa protein. The *psb*U gene from the red alga *C. caldarium* was cloned using the two-step PCR method. First, PCR was conducted to amplify the DNA fragment corresponding to the N-terminal part of the protein and a 150-bp cDNA fragment was amplified from cDNA prepared from a *C. caldarium* cDNA library. Subsequent cloning and sequencing of the amplified fragment revealed that the deduced amino acid sequence from the amplified fragment was identical to the N-terminal sequence of the protein. The second PCR step involved the RACE procedure (19) by which the DNA fragments of the 5′- and 3′-flanking regions of the 12 kDa protein cDNA were amplified using primers newly synthesized based on the 150-bp cDNA fragment, which yielded a 360-bp cDNA fragment and a 600-bp cDNA fragment from the 5′- and 3′-RACE, respectively. The nucleotide sequences of these cDNA fragments were confirmed to contain the cDNA for the 12 kDa protein. These sequences were combined with the partial sequence corresponding to the N-terminal part of the gene to yield the whole sequence of the gene, which is shown in Fig. 1. This sequence contained three in-frame ATG codons upstream of the N-terminal arginine residue of the mature polypeptide; they are located at positions 46, 49 and 127, respectively. Among them, the ATG codon at nucleotide number 49 was assigned as the start codon, because this codon is consensus to the start codon (AnnATGGC) for translation initiation in higher plants (20). According to this assignment, the gene encodes a polypeptide of 154 amino acid residues with a total molecular mass of 16714 Da. The N-terminal sequence determined for the mature 12 kDa protein corresponds to the sequence starting from residue number 62 of the gene-derived amino acid sequence. This indicates the cleavage of the first 61 residues after synthesis of the protein, which gave rise to a mature 12 kDa protein of 93 residues with a calculated molecular mass of 10513 Da. Thus, the first 61 residues served as a transit peptide for transport of the protein across membranes.

Hydropathy plot of the gene-derived amino acid sequence was analyzed to characterize the transit peptide of 61 residues (Fig. 2). As shown in Fig. 1, the first 36 residues of the 12 kDa presequence were enriched in basic (K and R) and hydroxylated (S and T) residues which are characteristic features for the transit peptide of chloroplast envelope (21). In contrast, the residues from 37 to 61 of the *psb*U gene presequence have characteristic common features for thylakoid transfer peptide (21), e.g., its N-terminal part is hydrophilic and positive, its central part is hydrophobic which is predicted to constitute a α-helical structure, and its C-terminal part contains alanine residues at positions -3 and -1 upstream from N-terminus of the mature protein which is consistent with the consensus sequence A-X-A for recognition site of the thylakoidal processing peptidase of higher plants (Figs. 1 and 2). Based on these features, the residues from 37 to 61 were assigned as a thylakoid transfer signal. Thus, the gene-derived amino acid sequence contained two char-

```
                                                                    ↓
C. caldarium  MMAFISTPLGKVTVKSATVSANRRGLRMQSDSEPVVSRRALLSGALAAAVAAALARARPAQA
P. laminosum  ------------------MKRLVGVLMILGLMLTSWGLLGSPQTAIAASLSPLSFNPSPVLA
S. PCC7002    ----------------------MSRVVSALMGLVLMFGCAFFSVQPQAQALDLSNGFVSAA
S. PCC6803    -------------------------MKFISRLLVACSVLIGLMGFLGADLAQALTPNPILA

C. caldarium  ----R-IDYEGIGYLGGGDKIDVNNANVRAYRKFPGLYPTAAKKIVQGGP
P. laminosum  EQQFR-NAMDDKLATDFGKKIDLNNTNVRAFMQYPGMYPTLARMILKNAP
S. PCC7002    VLGERVNPADKVLESEYGKKIDLNNASVRLFRELRGFYPTLAKRIIENAP
S. PCC6803    ---EL-NAVDAKLTTDFGQKIDLNNSDIRDFRGLRGFYPNLASEIIKNAP
                          * *** **   *      * **  *
C. caldarium  YGTPDDILKNPELSERDKEVIKKYMDRFVALPPTPEYF--TDRVNNGIYK
P. laminosum  KESVEDVLKMPGLTDTQKEILKNNFSNFVVSPPLDALVEGGDRFNNGIYR
S. PCC7002    YDSVEDVLNIPDLSEKQLARLEENLERFTVTPPADVFIDGDQRLNTGDY-
S. PCC6803    YDTVEEVLDIPGLSETQKSRLEANLGSFTVTEPSIELTSGDDRINPGVY-
                    *  * *             *    *         *
```

	C. caldarium	*P. laminosum*	*S.* PCC7002	*S.* PCC6803
C. caldarium	———	44.6 %	40.2 %	36.6 %
P. laminosum	———	———	48.4 %	52.7 %
S. PCC7002	———	———	———	57.4 %
S. PCC6803	———	———	———	———

FIG. 3. Comparison of the 12-kDa protein sequence among the red alga and three cyanobacteria. The complete sequences of the 12-kDa protein from the red alga *C. caldarium* (this study) and three cyanobacteria *P. laminosum* (22), *Synechococcus* sp. PCC7002 (23), and *Synechocystis* sp. PCC6803 (11) were compared. The downward arrowhead indicates the cleavage site; asterisks indicate identical residues among all the sequences compared. The table below the sequence shows the ratio of identical residues out of the total residues of the mature 12-kDa protein compared.

acteristic transit peptides, one for chloroplast envelope and the other for thylakoid membrane transfer signal. This indicates that the extrinsic 12 kDa protein in the red algal PSII is encoded in the nuclear genome. This clearly shows that the *psb*U gene coding for the 12 kDa protein is transferred to the nuclear genome already in the most primitive eukaryotic algae, red algae.

Figure 3 compared the gene-derived 12 kDa protein sequence from *Cyanidium caldarium* with those from cyanobacteria, *Phormidium laminosum* (22), *Synechococcus* sp. PCC7002 (23) and *Synechocystis* sp. PCC6803 (11). The signal peptide of the red algal 12 kDa protein from *C. caldarium* was clearly longer than those from three cyanobacterial 12 kDa protein, consistent with the nuclear genome location of the *psb*U gene in the red alga. The sequence homology of the mature part of 12 kDa protein was 44.6%, 40.2% and 36.6% between *C. caldarium* and *P. laminosum*, *S.* PCC7002 or *S.* PCC6803, respectively. These numbers are slightly lower than the sequence homology among the three species of cyanobacteria which range from 48.4% to 57.4%, suggesting a close evolutionary linkage of the 12 kDa protein in the oxygen-evolving complex from prokaryotic cyanobacteria and eukaryotic red algae.

Binding and functional features of the recombinant protein. The 12 kDa protein was expressed as a fusion protein with thioredoxin and 6 × His tags. The fusion protein was treated with Factor Xa to remove the thioredoxin and 6 × His tags, and then purified by column chromatography. To compare the binding and functional properties of the recombinant 12 kDa protein with those of the native 12 kDa protein, reconstitution experiments were carried out. As described previously (5, 7), four extrinsic proteins were completely released

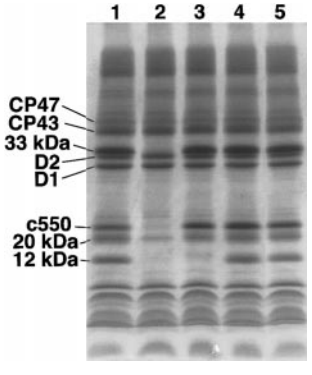

FIG. 4. Reconstitution of $CaCl_2$-treated PSII with the native 12-kDa protein or the recombinant 12-kDa protein, in combinations with the other three native extrinsic proteins of 33 kDa, 20 kDa, and cyt *c*-550. Lane 1, control PSII; lane 2, $CaCl_2$-treated PSII; lanes 3–5, $CaCl_2$-treated PSII reconstituted with the three extrinsic proteins of 33 and 20 kDa and cyt *c*-550 (lane 3), with the three extrinsic proteins *plus* the native 12-kDa protein (lane 4) and with the three extrinsic proteins *plus* the recombinant 12-kDa protein (lane 5).

TABLE 1

Oxygen Evolution of the $CaCl_2$-Treated PSII Reconstituted with the Native 12-kDa Protein or the Recombinant 12-kDa Protein Together with the Other Three Native Extrinsic Proteins

	Oxygen evolution (μmol of O_2/mg of chl/h)		
	− Ion	+ 10 mM NaCl	+ 5 mM $CaCl_2$
Untreated PSII	1586 (100)	1617 (100)	1617 (100)
$CaCl_2$-treated PSII	0 (0)	0 (0)	0 (0)
+ 33 kDa + 20 kDa + cyt *c*-550	0 (0)	418 (26)	956 (59)
+ 33 kDa + 20 kDa + cyt *c*-550 + native 12 kDa	1260 (79)	1348 (83)	1465 (91)
+ 33 kDa + 20 kDa + cyt *c*-550 + recombinant 12 kDa	1243 (78)	1270 (79)	1444 (89)

by treatment with 1 M $CaCl_2$ of the purified PSII particles from *C. caldarium* (Fig. 4, lane 2). When the 33 and 20 kDa proteins and cyt *c*-550 were reconstituted with the $CaCl_2$-washed PSII, the 33 and 20 kDa proteins were efficiently rebound, whereas cyt *c*-550 was partially rebound (Fig. 4, lane 3). Reconstitution of these three extrinsic proteins together with the native 12 kDa protein resulted in complete rebinding of the four extrinsic proteins (Fig. 4, lane 4). Reconstitution of the recombinant 12 kDa protein together with the other three extrinsic proteins also showed the complete rebinding of the four extrinsic proteins (Fig. 4, lane 5). These results indicate that the recombinant 12 kDa protein has a similar binding capability as the native 12 kDa protein.

Table 1 shows the restoration of oxygen evolution of the $CaCl_2$-washed PSII upon reconstitution with the extrinsic proteins. When the 33 and 20 kDa proteins and cyt *c*-550 were reconstituted with the $CaCl_2$-washed PSII, no oxygen-evolving activity was observed in the absence of Cl^- and Ca^{2+} but the activity recovered to a significant extent upon supplement of both Cl^- and Ca^{2+}. This is attributed to the lack of the 12 kDa protein because the 12 kDa protein and cyt *c*-550 function in maintaining requirement for Cl^- and Ca^{2+} of the red algal PSII, resembling the functions of the extrinsic 23 and 17 kDa proteins in higher plant PSII (4, 6–10). The oxygen-evolving activity in the absence of both Cl^- and Ca^{2+} was significantly restored when the native 12 kDa protein together with the other three extrinsic proteins was reconstituted with the $CaCl_2$-washed PSII. Reconstitution of the recombinant 12 kDa protein also showed a similar significant restoration of the oxygen-evolving activity as that of the native 12 kDa protein. The Cl^- and Ca^{2+} requirement of oxygen evolution disappeared similarly upon reconstitution of the recombinant and native 12 kDa protein. These results indicate that the recombinant 12 kDa protein has the same function as the native 12 kDa protein.

In conclusion, we cloned and sequenced the *psb*U gene encoding the 12 kDa extrinsic protein of PSII from the eukaryotic red alga, *Cyanidium caldarium*. The gene contained a bipartite transit peptide, indicating that it is located in the nuclear genome and transferred through chloroplast envelope and thylakoid membrane after its synthesis. The 12 kDa protein was successfully expressed in *E. coli* as a fusion protein, and then the mature protein was cut off and purified to homogeneity. Reconstitution studies showed that the recombinant protein retained the binding and reactivating capabilities as the native 12 kDa protein. This allows us to further study the structure and function of the 12 kDa protein in the red algal PSII.

REFERENCES

1. Ikeuch, M. (1992) *Bot. Mag. Tokyo* **105,** 327–373.
2. Bricker, T. M., and Ghanotakis, D. F. (1996) *in* Oxygenic Photosynthesis: The Light Reactions (Ort, D. F., and Yocum, C. F., Eds.), pp. 113–136, Kluwer Academic Publishers, The Netherlands.
3. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1992) *FEBS Lett.* **301,** 145–149.
4. Shen, J.-R., and Inoue, Y. (1993) *Biochemistry* **32,** 1825–1832.
5. Enami, I., Murayama, H., Ohta, H., Kamo, M., Nakazato, K., and Shen, J.-R. (1995) *Biochim. Biophys. Acta* **1232,** 208–216.
6. Seidler, A. (1996) *Biochim. Biophys. Acta* **1277,** 35–60.
7. Enami, I., Kikuchi, S., Fukuda, T., Ohta, H., and Shen, J.-R. (1998) *Biochemistry* **37,** 2787–2793.
8. Ghanotakis, D. F., Topper, J. N., Babcock, G. T., and Yocum, C. F. (1984) *FEBS Lett.* **170,** 169–173.
9. Andersson, B., Critchley, C., Ryrie, I. J., Jansson, C., Larsson, C., and Anderson, J. M. (1984) *FEBS Lett.* **168,** 113–117.
10. Miyao, M., and Murata, N. (1986) *Photosynth. Res.* **10,** 489–496.
11. Shen, J.-R., Ikeuchi, M., and Inoue, Y. (1997) *J. Biol. Chem.* **272,** 17821–17826.
12. Shen, J.-R., Vermaas, W., and Inoue, Y. (1995) *J. Biol. Chem.* **270,** 6901–6907.
13. Stirewalt, V. L., Michalowski, C. B., Luffelhardt, W., Bohnert, H. J., and Bryant, D. A. (1995) GenBank/EMBL/DDBJ/data bases, Accession No. U30821.
14. Reith, M. E., and Munholland, J. (1996) GenBank/EMBL/DDBJ/data bases, Accession No. U38804.
15. Kowallik, K. V., Stoebe, B., Schaffran, I., Kroth-Pancic, P., and Freier, U. (1996) GenBank/EMBL/DDBJ/data bases, Accession No. Z67753.

16. Ohta, H., Shirakawa, H., Uchida, K., Yoshida, M., Matuo, Y., and Enami, I. (1997) *Biochim Biophys Acta* **1319,** 9–13.
17. Ikeuchi, M., and Inoue, Y. (1988) *Plant Cell Physiol.* **29,** 1233–1239.
18. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) *Biochim. Biophys. Acta* **975,** 384–394.
19. Frohman, M. A., Dash, M. K., and Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8998–9002.
20. Lütcke, H. A., Chow, K. C., Mickel, F. S., Moss, K. A., Kern, H. F., and Scheele, G. A. (1987) *EMBO J.* **6,** 43–48.
21. Keegstra, K., Olsen, L. J., and Theg, S. M. (1989) *Ann. Rev. Physiol. Plant. Mol. Biol.* **40,** 471–503.
22. Wallace, T. P., Stewart, A. C., Pappin, D., and Howe, C. J. (1989) *Mol. Gen. Genet.* **216,** 334–339.
23. Nishiyama, Y. (1995) GenBank/EMBL/DDBJ/data bases, Accession No. D84098.