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# Thermal Protection of the Oxygen-Evolving Machinery by PsbU, an Extrinsic Protein of Photosystem II, in *Synechococcus* species PCC 7002[1]

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**The evolution of oxygen is the reaction that is the most susceptible to heat in photosynthesis. We showed previously that, in the cyanobacterium *Synechococcus* sp. PCC 7002, some protein factors located on the thylakoid membranes are involved in the stabilization of this reaction against heat-induced inactivation, and we identified cytochrome $c_{550}$ as one such factor (Y. Nishiyama, H. Hayashi, T. Watanabe, N. Murata [1994] Plant Physiol 105: 1313–1319). In the present study we purified another protein that appears to be essential for the stabilization of the oxygen-evolving machinery. The purified protein had an apparent molecular mass of 13 kD, and the gene encoding the 13-kD protein was cloned from *Synechococcus* sp. PCC 7002 and sequenced. The deduced amino acid sequence revealed that the protein was homologous to PsbU, an extrinsic protein of the photosystem II complex, which has been found in thermophilic species of cyanobacteria. Western analysis showed that the level of PsbU in thylakoid membranes was constant, regardless of the growth temperature. Our studies indicate that PsbU, a constituent of the photosystem II complex, protects the oxygen-evolving machinery against heat-induced inactivation.**

The PSII complex is a pigment-protein complex that utilizes light energy to drive the transport of electrons and the oxidation of water to oxygen. It is widely recognized that the PSII complex is the most susceptible to heat among various components of the photosynthetic apparatus (Berry and Björkman, 1980; Mamedov et al., 1993). The inactivation of the PSII complex by heat occurs primarily at a catalytic site for the evolution of oxygen, which is composed of a cluster of four Mn atoms (Katoh and San Pietro, 1967; Yamashita and Butler, 1968; Santarius, 1975; Thompson et al., 1989; Mamedov et al., 1993). The loss of two of the four Mn atoms results in the complete loss of oxygen-evolving activity (Nash et al., 1985). Thus, the stabilization against heat of the oxygen-evolving machinery in the PSII complex seems likely to play an essential role in protecting the entire photosynthetic system from heat inactivation.

We have studied the molecular basis of the stabilization of the oxygen-evolving machinery against heat using thylakoid membranes from the cyanobacterium *Synechococcus* sp. PCC 7002. To investigate the factors that are responsible for the heat stability of the oxygen-evolving machinery, which is located on the luminal side of the thylakoid membranes, we disrupted the vesicular structure of the membranes using a low concentration of Triton X-100. This treatment resulted in a remarkable decrease in the heat stability of the oxygen-evolving machinery. Furthermore, heat stability was recovered upon reconstitution of the membranes with the components that had been extracted with Triton X-100. Using a reconstitution assay, we determined that one of the factors responsible for the heat stability of the oxygen-evolving machinery was Cyt $c_{550}$, a Cyt with a low-redox potential (Nishiyama et al., 1994). However, Cyt $c_{550}$ was insufficient to account for the entire restorative ability of the extract obtained from the thylakoid membranes with Triton X-100. This observation implies that other important factors remain to be identified in the extract.

In the present study we purified another protein factor that contributes to the heat stability of the oxygen-evolving machinery from the material that had been extracted from thylakoid membranes with Triton X-100. Nucleotide sequence analysis revealed that the protein obtained was homologous to PsbU, an extrinsic protein of the PSII complex, which has been found in thermophilic cyanobacteria.

## MATERIALS AND METHODS

### Organism and Culture Conditions

*Synechococcus* sp. PCC 7002 was obtained from the Culture Collection of the Pasteur Institute (Paris, France). The cells were grown photoautotrophically at 38°C for 3 to 5 d as described previously (Nishiyama et al., 1993).

### Preparation and Treatment of Thylakoid Membranes

Thylakoid membranes were isolated from cells, as described previously (Nishiyama et al., 1993). The isolated thylakoid membranes were suspended in medium A (50

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Abbreviation: Chl, chlorophyll.

mM Hepes-NaOH, pH 7.5, 800 mM sorbitol, 30 mM $CaCl_2$, 1.0 M glycine betaine, and 1 mM 6-amino-*n*-caproic acid) and stored at 0 to 4°C for a maximum of 1 d. To disrupt the closed vesicular structure of the thylakoid membranes, we incubated the membranes in darkness at 4°C for 5 min in medium A that contained 0.1% (w/v) Triton X-100, at a ratio of Triton X-100:Chl of 2:1 (w/w).

### Assay of Heat Stability

Reconstitution of thylakoid membranes with individual fractions after column chromatography or with concentrated solutions of protein was performed as described previously (Nishiyama et al., 1994). Reconstituted membranes were incubated at designated temperatures for 20 min in darkness. After incubation the suspension of membranes was cooled to 25°C and the oxygen-evolving activity was measured. For chromatographic assays, aliquots of thylakoid membranes that had been reconstituted with a given fraction were incubated at 25 and 38°C, and the oxygen-evolving activity after incubation at 38°C was compared with that after incubation at 25°C. The increase in the ratio of activities was regarded as the thermostabilizing activity of the fraction.

### Purification of Protein

Thylakoid membranes corresponding to 60 mg of Chl were treated with 0.1% Triton X-100 under the same conditions as described above, with the exception that medium A was replaced by medium B (20 mM Tris-HCl, pH 7.5). The suspension of membranes was then separated into soluble and membrane fractions by centrifugation at 200,000*g* for 2 h. The soluble fraction was applied to a column (2.6 cm i.d. × 10 cm) of DEAE-Toyopearl 650C (Tosoh, Tokyo, Japan) that had been equilibrated with medium B. The column was washed with 300 mL of medium B to remove Triton X-100 and nonadsorbed components. The fractions eluted with medium B that contained 0.5 M NaCl were collected and dialyzed against 2 L of medium B for 12 h at 4°C. The dialysate was applied to a column (1.5 cm i.d. × 5 cm) of DEAE-Toyopearl 650S that had been equilibrated with medium B. The column was washed with 50 mL of medium B and was then developed with a linear gradient of NaCl from 0 to 0.3 M in 150 mL of medium B. Active fractions were collected and dialyzed against medium B, as indicated above, and the dialysate was applied to a Mono-Q HR 5/5 column (Pharmacia) that had been equilibrated with medium B. The column was developed with a linear gradient of NaCl from 0 to 0.5 M in 20 mL of medium B. Active fractions were collected and dialyzed for 12 h at 4°C against 1 L of medium C (25 mM *N*-methylpiperazine, pH 5.5), and the dialysate was applied to a Mono-P HR 5/5 column (Pharmacia) that had been equilibrated with medium C. The column was washed with 20 mL of medium D (10% [v/v] Polybuffer 74-HCl, pH 4.0; Polybuffer 74 was purchased from Pharmacia), and then it was re-equilibrated with medium C. The column was developed with a linear gradient of NaCl from 0 to 0.5 M in 20 mL of medium C. Active fractions were recovered and dialyzed for 12 h at 4°C against 1 L of medium E (20 mM Hepes-NaOH, pH 7.5, and 10 mM NaCl). The dialysate was concentrated 20-fold using a Centricon-10 centrifugal microconcentrator (Amicon, Beverly, MA).

### Analysis of Proteins and Quantitation of Chl

SDS-PAGE was performed as described by Laemmli (1970) on a 10 to 20% gradient polyacrylamide gel. After electrophoresis the gel was stained with 0.1% (w/v) Coomassie brilliant blue R-250. For western-blotting analysis, antibodies against the purified 13-kD protein were raised in a rabbit. After SDS-PAGE the proteins on the gel were transferred electrophoretically to a PVDF membrane (Clear Blot Membrane-p, Atto, Tokyo, Japan). The immunochemical detection of the protein was performed with the antiserum according to the instructions supplied with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Protein concentrations were determined with a protein assay solution (Bio-Rad) with BSA as the standard (Bradford, 1976). Concentrations of Chl were determined as described by Arnon et al. (1974).

### Measurements of Photosynthetic Activity

The photosynthetic evolution of oxygen was measured by monitoring the concentration of oxygen with a Clark-type oxygen electrode. The activity of PSII in thylakoid membranes was measured at 25°C in the presence of 100 μM phenyl-1,4-benzoquinone. Red actinic light, at an intensity of 2 mE $m^{-2}$ $s^{-1}$, was provided by an incandescent lamp that was used in conjunction with an HA50 heat-absorbing optical filter (Hoya, Tokyo, Japan) and an R-60 red optical filter (Toshiba, Tokyo, Japan).

### Analysis of the Amino Acid Composition and Amino-Terminal Amino Acid Sequence

The purified protein was hydrolyzed in 5 N HCl at 110°C for 24 or 72 h, and the amino acid composition was determined with an automated amino acid analyzer (model 835, Hitachi, Tokyo, Japan). The amino-terminal sequence was determined by automated Edman degradation with a protein sequence analyzer (model 492, Applied Biosystems).

### Molecular Cloning

The sequence of 58 amino acids at the amino-terminal end of the purified protein was determined. A DNA fragment corresponding to the 58 amino acid residues was amplified by PCR as follows: Genomic DNA was isolated from *Synechococcus* sp. PCC 7002 cells as described by Williams (1988), and it was used as the template for PCR. Degenerate primers GA(GA)CG(CG)GT(GTC)AA(TC)-CCCGC(CT)GA(TC)AAAGT (amino acid residues 1 to 9 of the amino-terminal sequence of the protein) and GG-(GAT)AT(AG)TT(CAG)A(GA)(GAC)AC(AG)TC(TC)TC-(GAC)AC (amino acid residues 58 to 51) were used as forward and reverse primers, respectively. The product of

the PCR of 190 bp was cloned into the plasmid vector pT7Blue (Novagen, Madison, WI), and the nucleotide sequence of the cloned DNA was determined to ensure that the cloned gene encoded a portion of the purified protein.

A genomic DNA library of *Synechococcus* sp. PCC 7002 was constructed in the phage vector λ*DASH*II (Stratagene), and it was screened with the radioactively labeled probe of 190 bp. Recombinant phage DNA was isolated from the four phages that gave a positive signal and was subjected to restriction mapping and Southern-blotting analysis. A DNA fragment of 2.3 kb, which contained a full-size *psbU* gene, was obtained by digestion with *Hin*dIII. It was subcloned into the *Hin*dIII site of pUC19 (Pharmacia), and the resultant plasmid was designated pUH239. The nucleotide sequence of a 649-bp fragment within the insert of pUH239 was determined with an automated DNA sequencer (model 373A, Applied Biosystems).

## RESULTS

### Purification and Identification of a Protein That Contributes to the Heat Stability of the Oxygen-Evolving Machinery

We reported previously that disruption of the vesicular structure of thylakoid membranes by treatment with 0.1% Triton X-100 resulted in a marked decrease in the heat stability of the oxygen-evolving machinery and that the heat stability could be restored when the membranes were reconstituted with the components that had been extracted by this treatment (Nishiyama et al., 1994).

The activity in the extract that was capable of restoring heat stability was recovered in fractions that were eluted in 0.5 M NaCl from the first anion-exchange column (DEAE-Toyopearl 650C). When the active fractions were subjected to a second round of anion-exchange chromatography on DEAE-Toyopearl 650S, the activity was separated into three peaks upon the elution with a linear gradient of NaCl (Fig. 1). The first peak of activity was assigned to Cyt $c_{550}$, which had been identified as one of the factors that contribute to the heat stability of the oxygen-evolving machinery (Nishiyama et al., 1994). The material in the second peak was investigated in the present study.

We purified a protein that was responsible for the second peak of activity by anion-exchange chromatography on a Mono-Q HR 5/5 column and subsequent chromatofocusing on a Mono-P HR 5/5 column. The fractions obtained from the Mono-Q column that were able to restore heat stability were eluted between 0.2 and 0.3 M NaCl. These fractions included a trace of Cyt $c_{550}$, which was, as shown previously, able to increase heat stability. During chromatography on the Mono-P column most of the proteins were eluted between pH 5.5 and 4.0. No relevant activity was detected, except for the residual Cyt $c_{550}$, which was eluted at pH 4.0. Activity was, however, recovered as a single peak when the column was subsequently developed with a gradient of NaCl after re-equilibration at pH 5.5 (Fig. 2). This elution property suggested that the functional protein might have a pI of less than 4.0.

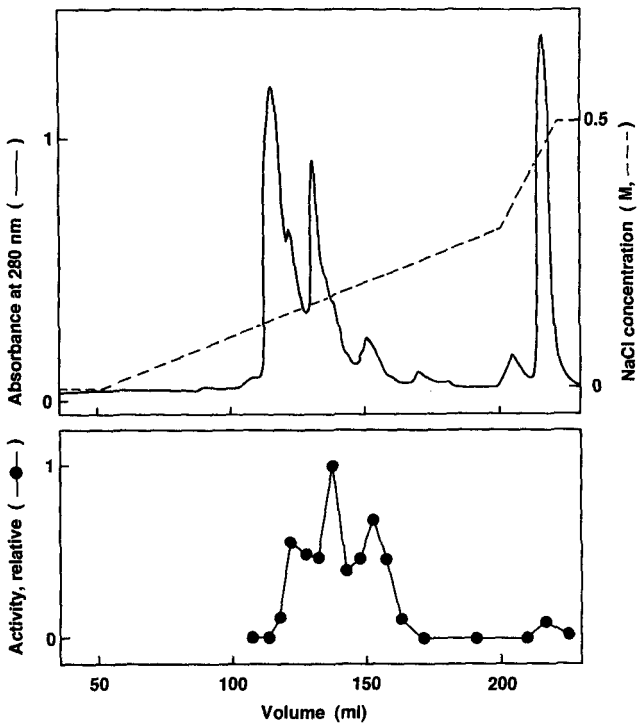


**Figure 1.** Results of the chromatography on the second anion-exchange column (DEAE-Toyopearl 650S) of the active fractions that were obtained after the anion-exchange chromatography on the first column. Activity of individual fractions was assayed as described in "Materials and Methods."

Figure 3 shows the protein profiles obtained after SDS-gel electrophoresis of the active fractions from each step in the purification. The active fraction obtained from the Mono-P column contained a single protein of 13 kD, without any contaminants.

The sequence of 58 amino acids at the amino-terminal end of the purified protein was determined. A homology search revealed that the protein was homologous to PsbU, which was formerly known as the 9- or 12-kD extrinsic protein of the cyanobacterial PSII complex. This protein has been found in the thermophilic cyanobacteria *Phormidium laminosum* (Stewart et al., 1985a) and *Synechococcus vulcanus* (Shen et al., 1992) and in the red alga *Cyanidium caldarium* (Enami et al., 1995). Thus, we identified the 13-kD protein as PsbU of *Synechococcus* sp. PCC 7002.

### Molecular Cloning of the *psbU* Gene

A genomic DNA library of *Synechococcus* sp. PCC 7002 was constructed in the phage vector λ*DASH*II and it was screened with the 190-bp probe, obtained by amplification of a portion of the *psbU* gene by PCR. The recombinant phage DNA was isolated from four plaques that gave a positive hybridization signal. The restriction maps of the isolated DNAs allowed us to select a 2.3-kb *Hin*dIII fragment that contained a full-size *psbU* gene. We subcloned this fragment into the *Hin*dIII site of pUC19 and sequenced a 649-bp portion of the fragment. An open-reading frame of

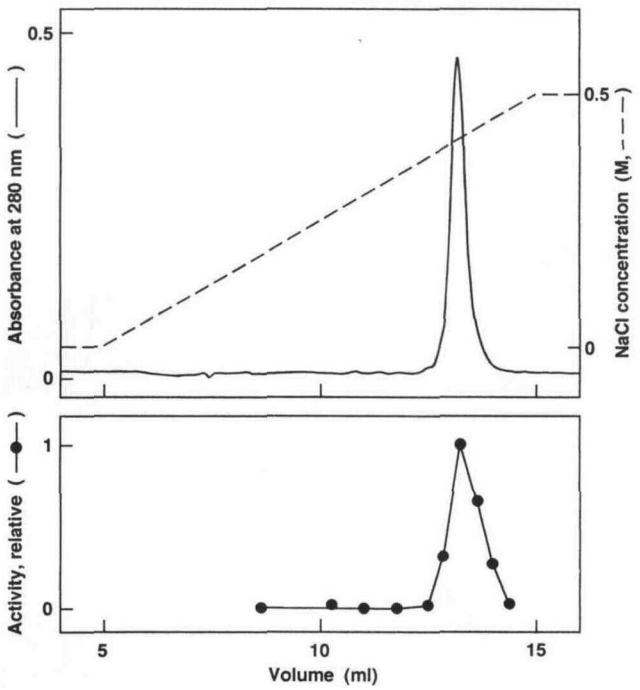


**Figure 2.** Results of the chromatographic separation on the Mono-P HR 5/5 column using the active fractions obtained after chromatography on the Mono-Q HR 5/5 column.

414 bp, which encoded PsbU, was located within the sequenced fragment (Fig. 4). The deduced amino acid sequence indicates that the product of the open-reading frame consists of a transit peptide of 42 residues and a mature protein of 96 residues. The deduced amino acid composition of the mature protein was identical to that of the purified protein, confirming the identity of the gene. The presence of a transit peptide suggests that PsbU is located on the luminal side of thylakoid membranes.

### Thermoprotective Role of PsbU

Figure 5A shows the effects of the addition of purified PsbU on the temperature profile of heat inactivation of the oxygen-evolving machinery in thylakoid membranes that had been treated with 0.1% Triton X-100. The protein was added to a suspension of thylakoid membranes with a Chl concentration of 5 $\mu$g mL$^{-1}$ to a final concentration of 5 $\mu$g mL$^{-1}$. The addition of the protein resulted in a marked increase in the heat stability of the membranes, with the temperature for 50% inactivation shifting from 37 to 41°C. In terms of the temperature for 50% inactivation, heat stability appeared to return to the initial level upon the addition of PsbU. However, at higher temperatures the restoration of heat stability was less effective. A similar profile of restoration was observed when only Cyt $c_{550}$ was added to thylakoid membranes that had been treated with Triton X-100 (Nishiyama et al., 1994), suggesting that neither PsbU nor Cyt $c_{550}$ alone is sufficient for full restoration. It is noteworthy that either the removal of PsbU by treatment with Triton X-100 or the addition of PsbU barely affected the oxygen-evolving activity itself in the absence of heating. The oxygen-evolving activities before and after treatment with Triton X-100 were 185 and 170 $\mu$mol $O_2$ mg$^{-1}$ h$^{-1}$, respectively, upon incubation at 25°C. The oxygen-evolving activity after addition of PsbU to thylakoid membranes that had been treated with Triton X-100 was 180 $\mu$mol $O_2$ mg$^{-1}$ h$^{-1}$ upon incubation at 25°C. These observations suggest that PsbU is not essential for the catalytic activity of the oxygen-evolving system.

We further analyzed the effects of PsbU on the stabilization of the oxygen-evolving machinery by varying the amount of the protein to be added (Fig. 5B). As the amount of added protein increased, the heat stability increased sharply until the ratio of the protein:Chl was 1:10 (w/w), at which point the molar ratio of PsbU to the PSII reaction center was approximately 1:1 (w/w). Subsequently, the rate of increase became considerably lower. Cyt $c_{550}$ was shown previously to have a similar positive effect on heat stability (Nishiyama et al., 1994). To compare the thermoprotective activity of PsbU with that of Cyt $c_{550}$, we also examined the effects on heat stability of Cyt $c_{550}$ alone and in combination with PsbU. The addition of Cyt $c_{550}$ alone yielded a profile of increasing heat stability similar to that obtained with PsbU, but the restorative effect of Cyt $c_{550}$ was approximately 40% smaller than that of PsbU. The combination of PsbU and Cyt $c_{550}$ had a greater effect on heat stability than did PsbU alone. Thus, we can infer that, although PsbU and Cyt $c_{550}$ act independently to stabilize the oxygen-evolving machinery against heat, both proteins are necessary for construction of the thermally stable PSII complex.

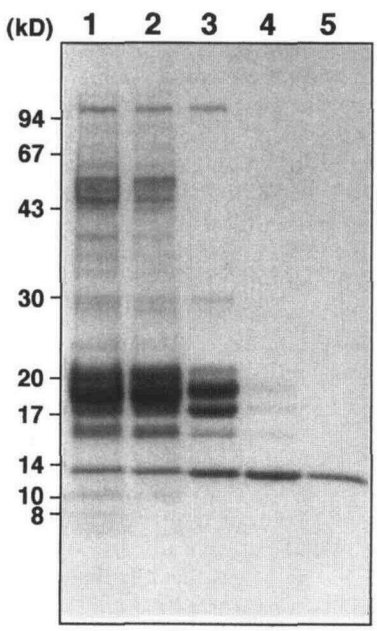


**Figure 3.** Results of SDS-PAGE of the active fractions from each step in the purification. Proteins were analyzed on a 10 to 20% gradient polyacrylamide gel. Lane 1 was loaded with the soluble fraction extracted from thylakoid membranes with Triton X-100. Lanes 2, 3, 4, and 5 were loaded with the active fractions obtained after column chromatography on DEAE-Toyopearl 650C, DEAE-Toyopearl 650S, Mono-Q, and Mono-P, respectively. Molecular masses are given in kilodaltons on the left.

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AAG CTT GGT ATT TTA CAT AAA TCT AAA AAT CAT CAA AAA AAG CTG GGA   48

AAA GCC TAG GAG GAA TCA ATC ATA ATG AGT AGA GTT GTG AGT GCA TTA   96
                                M   S   R   V   V   S   A   L     8'

ATG GGG CTC GTC CTC ATG TTC GGT TGC GCG TTC TTT AGC GTT CAG CCC  144
 M   G   L   V   L   M   F   G   C   A   F   F   S   V   Q   P    24'

CAA GCC CAA GCC CTC GAT TTA AGC AAT GGC TTT GTT TCT GCA GCC GTG  192
 Q   A   Q   A   L   D   L   S   N   G   F   V   S   A   A   V    40'

CTC GGT GAG CGT GTT AAT CCC GCT GAC AAG GTG CTT GAG TCA GAA TAC  240
 L   G   E   R   V   N   P   A   D   K   V   L   E   S   E   Y    56'

GGC AAG AAA ATC GAC CTA AAC AAC GCC AGC GTC CGT CTT TTC CGC GAG  288
 G   K   K   I   D   L   N   N   A   S   V   R   L   F   R   E    72'

CTC CGT GGA TTT TAT CCC ACA TTG GCT AAA CGC ATC ATC GAA AAT GCC  336
 L   R   G   F   Y   P   T   L   A   K   R   I   I   E   N   A    88'

CCC TAT GAC AGT GTT GAA GAT GTA TTG AAT ATC CCC GAC CTC AGC GAG  384
 P   Y   D   S   V   E   D   V   L   N   I   P   D   L   S   E   104'

AAG CAA CTG GCA CGG TTA GAG GAA AAT TTG GAG CGT TTC ACT GTA ACA  432
 K   Q   L   A   R   L   E   E   N   L   E   R   F   T   V   T   120'

CCG CCT GCT GAT GTG TTT ATT GAT GGT GAC CAA CGT CTG AAC ACC GGT  480
 P   P   A   D   V   F   I   D   G   D   Q   R   L   N   T   G   136'

GAC TAT TAA AAA TAC AAA TGA AAT CTT GCT ACG TTT AGG AAG ATG TTT  528
 D   Y                                                          138'

GTG AGT CAA ACC TAA ATT TTC AAT TGC AAT TAA CCT ATT CCT GAG GAA  576

TAG GTT TTT TTA CGA CAA TTT TTA GGA TTT GGG GCT CGG TTG GCA TAG  624

TCG ATA AAA CTG AGG TAC CCA GCC G                               649
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**Figure 4.** The nucleotide sequence of the gene for the 13-kD protein from *Synechococcus* sp. PCC 7002 (GenBank accession no. X93509). The deduced amino acid sequence is shown in the one-letter code under the nucleotide sequence. The amino-terminal sequence that was determined directly from the protein is underlined. A putative ribosome-binding site is double-underlined.

### Expression of PsbU

To examine whether the synthesis of PsbU is induced by high temperature, we compared the levels of expression of PsbU in cells grown at different temperatures by western analysis. The amounts of PsbU from cells grown at 25 and 38°C were the same in total cell extract, as well as in thylakoid membranes (Fig. 6). These observations suggest that PsbU was not induced by high temperature. We also examined whether posttranslational modification might occur in response to high temperature. PsbU proteins were purified from cells grown at 25 and 38°C, and they were compared in terms of molecular size, amino acid composition, and pI. All of the examined properties of both proteins were the same (data not shown). Thus, we concluded that PsbU is expressed constitutively and is localized in thylakoid membranes without any modification when growth temperature is increased.

## DISCUSSION

In the present study we identified a protein that is involved in the stabilization against heat of the oxygen-evolving machinery in *Synechococcus* sp. PCC 7002. We had assumed initially that factors that contribute to the heat stability of the oxygen-evolving system would be located near the side of evolution of oxygen, namely on the luminal side of thylakoid membranes. Disruption of the vesicular structure of thylakoid membranes with a low concentration of Triton X-100 resulted in a marked decrease in the heat stability of the oxygen-evolving machinery with a concomitant release of protein components from the membranes. The released components were capable of restoring heat stability when returned to the preparation of thylakoid membranes, an indication of their involvement in heat stability (Nishiyama et al., 1994). In the present study, using a reconstitution assay, we purified a 13-kD protein that was able to restore heat stability. The amino acid sequence deduced from the nucleotide sequence indicated that the 13-kD protein was homologous to PsbU, an extrin-

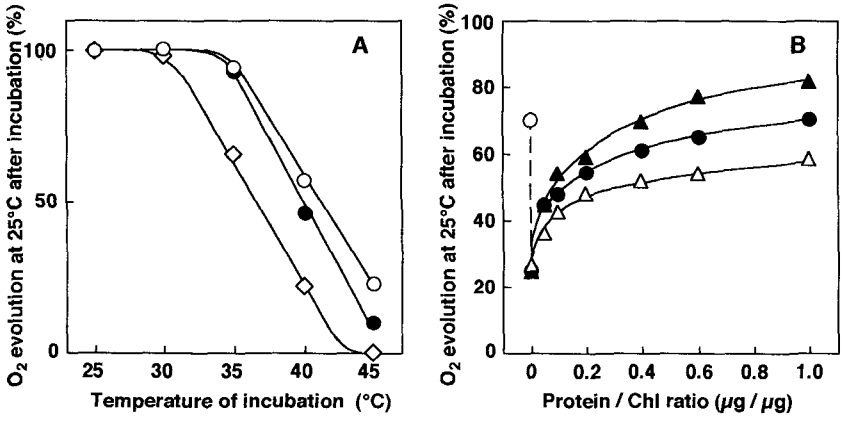


**Figure 5.** The effect of PsbU on the heat stability of the oxygen-evolving machinery of PSII. A, Purified PsbU was added to thylakoid membranes that had been treated with 0.1% Triton X-100 at a final Chl:protein ratio of 1:1 (w/w). The membranes were then incubated at the designated temperature for 20 min in darkness. Oxygen evolution was measured at 25°C in the presence of phenyl-1,4-benzoquinone. ○, No treatment with Triton X-100; ◇, after treatment with Triton X-100; ●, after treatment with Triton X-100 and addition of PsbU. The oxygen-evolving activities taken as 100% were 185, 170, and 180 μmol $O_2$ $mg^{-1}$ $h^{-1}$, respectively. B, Various amounts of PsbU and Cyt $c_{550}$ were added to thylakoid membranes that had been treated with 0.1% Triton X-100. Heat stability is presented as the oxygen-evolving activity after incubation at 38°C relative to that after incubation at 25°C for 20 min. ○, No treatment with Triton X-100; △, treatment with Triton X-100 and addition of Cyt $c_{550}$; ●, treatment with Triton X-100 and addition of PsbU; and ▲, treatment with Triton X-100 and addition of PsbU plus Cyt $c_{550}$.

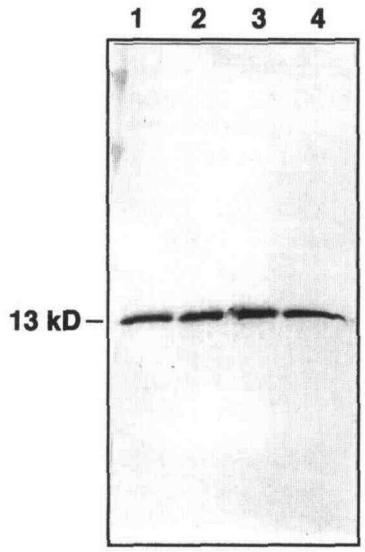


**Figure 6.** Effect of growth temperature on the expression of PsbU in *Synechococcus* sp. PCC 7002. Total cell proteins and thylakoid proteins were prepared from cells grown at 25 and 38°C, and 20 μg of proteins was loaded onto a gel. The levels of PsbU were analyzed by western blotting with the antiserum raised against the purified PsbU of *Synechococcus* sp. PCC 7002. Lane 1, Total proteins from cells grown at 25°C; lane 2, total proteins from cells grown at 38°C; lane 3, thylakoid proteins from cells grown at 25°C; and lane 4, thylakoid proteins from cells grown at 38°C.

sic protein of PSII, which has been found in thermophilic species of cyanobacteria (Wallace et al., 1989). The nucleotide sequence revealed the presence of a transit peptide, suggesting the localization of the 13-kD protein on the luminal side of the thylakoid membranes, in accordance with our assumption that the thermostabilizing factors should be located in the lumen.

In the nucleotide sequence of the *psbU* gene, three in-frame ATG codons were found 126, 102, and 87 nucleotides upstream of the codon for the amino terminus of the mature protein (Fig. 4). We postulated that the codon for initiation of translation was likely to be the codon starting at nucleotide 126, since a typical ribosome-binding site, GAGG, was found 15 nucleotides upstream of this codon. Three ATG codons were also found in the *psbU* gene from another cyanobacterium, *Phormidium laminosum*, and only the first ATG codon was accompanied by a possible ribosome-binding site (Wallace et al., 1989). The amino-terminal amino acid sequence that was determined from the purified protein may suggest that the processing site for removal of the transit peptide is located between Gly-42 and Glu-43. However, the authentic processing site might be different from this position, since the amino acid immediately before the processing site is conserved as Ala in proteins that are targeted to the lumen of thylakoid membranes (Konishi et al., 1993). We cannot exclude the possibility that the precursor to PsbU is processed between Ala-38 and Ala-39 and that the resultant mature protein of 100 amino acid residues undergoes proteolysis to produce a truncated protein of 96 amino acid residues.

PsbU was first identified as a 9-kD protein that was associated with the PSII complex from the thermophilic cyanobacterium *P. laminosum* (Stewart et al., 1985a). It was subsequently proposed that the 9-kD protein was bound to the donor side of PSII and was essential for the oxygen-evolving activity, in view of the observation that the release of the 9-kD protein from the PSII complex of *P. laminosum* was closely correlated with a decrease in oxygen-evolving activity (Stewart et al., 1985b; Stamatakis and Papageorgiou, 1993). This conclusion was supported by the results of experiments that involved the dissociation from and readdition to the PSII complex of the 9-kD protein of *P. laminosum* (Rolf and Bendall, 1989). In addition, the proposed localization of the 9-kD protein that had been deduced from the above biochemical evidence was reinforced by the finding of a leader sequence in the gene for this protein, which included a domain typical of proteins that are targeted to the thylakoid lumen (Wallace et al., 1989).

A homolog of the 9-kD protein was found in another thermophilic cyanobacterium, *Synechococcus vulcanus*, as a 12-kD protein that was associated stoichiometrically with the PSII complex (Shen et al., 1992). The 12-kD protein was located exclusively in the PSII complex (Shen and Inoue, 1993a), and it appeared to regulate the oxygen-evolving activity in cooperation with Cyt $c_{550}$, another extrinsic protein of the PSII complex in *S. vulcanus* (Shen and Inoue, 1993b). These PsbU proteins, as well as Cyt $c_{550}$, are not found in higher plants. In turn, the 18- and 23-kD extrinsic proteins of the PSII complex of higher plants are not found in cyanobacteria (Stewart et al., 1985a; Shen and Inoue, 1993a). The similarities in terms of binding and functional properties of PsbU and Cyt $c_{550}$ to the 18- and 23-kD proteins of higher plants may imply an evolutionary connection among the extrinsic proteins of the PSII complex (Shen et al., 1993b). It is of interest that the red alga *Cyanidium caldarium* has both PsbU and Cyt $c_{550}$ (Enami et al., 1995).

We compared the amino acid sequence of PsbU from *Synechococcus* sp. PCC 7002 with sequences of PsbU from other cyanobacteria (Fig. 7). The sequence of PsbU from *Synechococcus* sp. PCC 7002 is 42% homologous to the sequence of PsbU from *P. laminosum* (Wallace et al., 1989) and 48% homologous to the sequence of PsbU from *Synechocystis* sp. PCC 6803 (Kaneko et al., 1996).

Compared with the PsbU proteins from the thermophilic cyanobacteria, PsbU from *Synechococcus* sp. PCC 7002, a mesophilic cyanobacterium, exhibited distinct differences in terms of binding and functional properties. First, the release of PsbU from the PSII complex of *P. laminosum* required treatment with 1 M chloride salts, such as NaCl, $CaCl_2$ or $MgCl_2$, 0.8 M alkaline Tris, or a reduction of concentration of glycerol in the medium (Stewart et al., 1985a), whereas 1 M $CaCl_2$, but neither NaCl nor a low-glycerol medium, released PsbU from the PSII complex in *S. vulcanus* (Shen et al., 1992). By contrast, PsbU of *Synechococcus* sp. PCC 7002 was released from the PSII complex upon disruption of the vesicular structure of thylakoid membranes with a low concentration of Triton X-100 or by ultrasonic oscillation (Y. Nishiyama, D.A. Los, and N. Murata, unpublished data). Western analysis revealed that

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S. 7002          MSRVVSALMGLVLMFGC-AFF-SVQPQAQALDLSNGFVSAAVLGER--VNPADKVLESEY   56
S. 6803               MKFISR*LVA*-SLL-IGLMGFLGA**AQALTPNPI*A*---L*AV*AK*TTDF   49
P. laminosum     *K*L*GV**I*G**LTSWGLLG*P*TAIA*SLSPLS*NPSP**A*QQFR*AM*DK*ATDF   60

S. 7002          GKKIDLNNASVRLFRELRGFYPTLAKRIIENAPYDSVEDVLNIPDLSEKQLARLEENLER  116
S. 6803          *Q******SDI*D**G******N**SE**K*****T**E**D**G***T*KS***A**GS  109
P. laminosum     ********TN*PA*MQYP*M*****RM*LK***FE******KM*G*TDT*KEI*KN*FSN   20

S. 7002          FTVTPPADVFIDGDQRLNTGDY                                        138
S. 6803          ****E*SIELTS**D*I*P*V*                                       131
P. laminosum     *V*S**L*ALVE*GD*F*N*I*R                                      143
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**Figure 7.** Alignment of amino acid sequences deduced from the *psbU* genes of different cyanobacteria. Identical amino acids are indicated by asterisks. Gaps introduced to optimize alignment are indicated by dashes.

PsbU was also present in a soluble fraction that had been separated from the membranes after breakage of cells with glass beads, suggesting that a portion of the protein might be released when thylakoid vesicles are disrupted mechanically (Y. Nishiyama, D.A. Los, and N. Murata, unpublished data). Therefore, PsbU of *Synechococcus* sp. PCC 7002 seems likely to be more loosely associated with the PSII complex than is the case in the thermophilic species.

Second, in the thermophilic species PsbU is essential for oxygen-evolving activity (Rolf and Bendall, 1989; Shen and Inoue, 1993b). By contrast, in *Synechococcus* sp. PCC 7002, neither the dissociation of PsbU from thylakoid membranes with Triton X-100 nor the reconstitution of membranes with PsbU affected the oxygen-evolving activity to any significant extent. This observation indicates that PsbU is not essential for the oxygen-evolving activity in *Synechococcus* sp. PCC 7002. A similar difference in terms of the binding and functional characteristics of Cyt $c_{550}$ was observed when the thermophilic species and *Synechococcus* sp. PCC 7002 were compared. Thus, it appears that PsbU and Cyt $c_{550}$ play a protective role, without which the PSII complex in *Synechococcus* sp. PCC 7002 is susceptible to inactivation by heat. We can speculate that the thermophilic species evolved a more stable oxygen-evolving machinery, to withstand the extremely high temperatures of their natural habitats, by strengthening the association of these extrinsic proteins with the PSII complex, where these proteins participate in the catalytic activity required for the evolution of oxygen.

When the cells of *Synechococcus* sp. PCC 7002 acclimate to high temperature, the heat stability of their photosynthetic machinery is enhanced (Nishiyama et al., 1993). This acclimation response has been observed in a number of higher plants and cyanobacteria (Berry and Björkman, 1980; Lehel et al., 1993), although the molecular mechanism underlying such acclimation remains to be clarified. We have found that the oxygen-evolving machinery, which is the most sensitive to heat of all components of the photosynthetic system, is stabilized upon acclimation to high temperature (Nishiyama et al., 1993). Therefore, we should now investigate whether any factor that contributes to the stabilization of the oxygen-evolving machinery is involved in this acclimation response.

Our findings related to the mode of expression of PsbU demonstrate that this protein is expressed constitutively and is localized in the thylakoid membranes, without any modifications, during acclimation to high temperature. These observations suggest that PsbU is one of the macromolecules that constitutively stabilize the oxygen-evolving machinery and that it is not a factor that directly modifies the heat stability of the oxygen-evolving machinery during the acclimation to high temperature. However, the dramatic effect of PsbU, as well as of Cyt $c_{550}$, on heat stability suggests that these extrinsic proteins are indispensable for the construction of a thermally stable PSII complex. It remains to be determined whether these extrinsic proteins are involved in the mechanism that underlies the enhancement of the heat stability of the oxygen-evolving machinery, which occurs during acclimation to high temperature.

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