Molecular Identification of a Novel Protein That Regulates Biogenesis of Photosystem I, a Membrane Protein Complex*

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Photosystem I (PSI) is a multisubunit pigment-protein complex in the thylakoid membranes of cyanobacteria and chloroplasts. BP26, a random photosynthesis-deficient mutant strain of the cyanobacterium Synechocystis 6803 has a severely reduced PSI content, whereas its photosystem II is present in normal amount. The BP26 mutant is complemented by a novel gene, btpA, that encodes a 30-kDa protein. In this strain, a missense mutation in the btpA gene, resulting in the replacement of a valine by a glycine residue, significantly affects the accumulation of the PSI reaction center proteins, PsaA and PsaB. Northern blot analysis revealed that the steady-state levels of the transcripts from the psaAB operon, encoding these proteins, remain unaltered in the mutant strain. Hence the BtpA protein regulates a post-transcriptional process during the life cycle of the PSI protein complex such as 1) translation of the psaAB mRNA, 2) assembly of the PsaA/PsaB polypeptides and their associated cofactors into a functional complex, or 3) degradation of the protein complex. Close relatives of the BtpA protein have been found in nonphotosynthetic organisms, viz. the archaeabacterium Methanococcus jannaschii, the eubacterium Escherichia coli, and the nematode, Caenorhabditis elegans, suggesting that these proteins may regulate biogenesis of other protein complexes in these evolutionarily distant organisms.

Integral membrane proteins participate in a wide array of biological processes. The thylakoid membranes of oxygenic photosynthetic organisms contain four major multisubunit protein complexes: photosystem I (PSI),1 photosystem II (PSII), cytochrome b6/f complex and ATP synthase. During recent years, the biochemical compositions as well as functions of these protein complexes have been studied in considerable details. However, significantly less is known about the molecular mechanisms of biogenesis and assembly of these multisubunit membrane proteins.

In the thylakoid membranes of chloroplasts and cyanobacteria, the PSI complex mediates light-induced electron transport from plastocyanin or cytochrome c553 to ferredoxin (1–3). In cyanobacteria, PSI consists of eleven major polypeptides (4). Among them, a heterodimer of two integral membrane pro-
A Thylakoid Biogenesis Protein in Cyanobacteria

During recent years, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter called *Synechocystis 6803*) has been widely used for molecular analysis of the form and function of the photosynthetic apparatus (3, 14). This organism is naturally transformable with exogenous DNA (15). Under appropriate conditions, the introduced DNA molecules undergo homologous double reciprocal recombinations with chromosomal DNA, thus allowing directed gene replacement as well as phenotypic complementation of defined mutant strains (16). In addition, *Synechocystis 6803* cells can grow phototrophically, so that mutant strains impaired in photosynthesis can be propagated in glucose-enriched media.

We have recently developed a method for selection of random photosynthesis-deficient mutant strains of *Synechocystis 6803* (17). This method utilizes a strain of *Synechocystis 6803* for which glucose is toxic during prolonged incubation in the light. However, it can grow in the presence of glucose when its photosynthetic activity is inhibited (16). Using this selection scheme, we have isolated a variety of photosynthesis-deficient mutants. For example, analysis of one of these mutant strains has led to the identification of an ABC transporter protein for manganese, the first high affinity transporter system for this metal identified in any organism (17).

In the present study, we have characterized another such mutant strain, BP26. Using a complementation approach, we have cloned and sequenced a novel gene *btpA*, a mutation in which has significantly affected the form and function of the PSI complex in this mutant. Functional analysis of the BP26 mutant has indicated that the *btpA* gene is involved in the regulation of biogenesis of PSI reaction center complex at a post-transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Enzymes for recombinant DNA work were from New England Biolabs; modified T7 polymerase (Sequenase) was from U.S. Biochemical Corp.; [α-32P]dCTP (2000 Ci/mmol) for radioactive labeling of DNA fragments and α-35-S-dATP (>1000 Ci/mmol) for DNA sequencing were from Amersham Corp. All chemicals used were of reagent grade.

**Bacterial Strains, Plasmids, and Growth Conditions**—A glucose-sensitive isolate of *Synechocystis* sp. PCC 6803 was a kind gift of Dr. C. P. Wolk, who obtained it from the American Type Culture Collection. The BP26 mutant was isolated during a previous study (17). Cyanobacterial cells were grown in BG11 medium (18) at 30 °C under 50 μE m⁻² s⁻¹ of fluorescent light without glucose (autotrophic growth), or under 10 μE m⁻² s⁻¹ of light in the presence of 5 μM glucose (heterotrophic growth). Solid medium was supplemented with 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate and 10 mM TES-KOH, pH 8.2. Liquid cultures were grown with vigorous bubbling with room air. Growth of cyanobacterial strains was monitored by the measurement of light scattering at 730 nm on a DW2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL).

**Escherichia coli** strain TG1 (supE hsdS thi Δlac-proAB F (traD86 proAB + lacIq lacZAM15)), used for the propagation of various plasmids, was grown at 37°C in Luria-Bertani medium (19). The plasmid pUC119 (20) was used as the basic cloning vector.

**Measurement of Electron Transport Rates and Spectroscopy**—Rates of photosynthetic electron transfer reactions of intact *Synechocystis 6803* cells were measured essentially as described previously (21). During these assays, the wild-type and BP26 mutant samples were adjusted to equal cell densities at which the chlorophyll concentration of the wild-type cells was 5 μg/ml. Concentrations of chlorophyll in intact cells were measured after methanol extraction (22). Absorption spectra and P700 chemical difference spectra were recorded on a DW2000 spectrophotometer (SLM-Amino Instruments, Urbana, IL). P700 was quantitated as ascorbate-reduced minus ferricyanide-oxidized chemical difference spectra of thylakoid membranes as described elsewhere (23). The samples were adjusted to 25 μg of Chl/ml (wild-type) or 8 μg of Chl/ml (BP26). Fluorescence spectra of intact cyanobacterial cells were recorded at 77 K on a home-built diode-array instrument as described previously (24).

**RESULTS**

**Isolation and Biochemical Analysis of the BP26 Mutant**—A collection of random, photosynthesis-deficient mutant strains of *Synechocystis 6803* was generated by using glucose as an agent for selection (17). A number of these spontaneous mutants were deficient in their PSI activity. One such mutant, BP26, was chosen for further study.

Under photoautotrophic growth conditions, the BP26 cells grew almost four times slower than the wild-type cells (Table I). However, in the presence of glucose (heterotrophic growth), the doubling rate of these mutant cells was comparable to that of the wild-type cells (Table I). Using artificial electron donors and acceptors, we examined the PSI- and PSI-mediated electron transport rates in intact cells that have been grown photoautotrophically. As shown in Table I, the PSI-mediated oxygen evolution rate in the BP26 cells was near normal, whereas its PSI-activity was only ~20% of that in the wild-type cells. These data suggested that the BP26 mutant strain is specifically impaired in its PSI activity.

Fig. 1A shows the absorption spectra of wild-type and mutant cells. In the BP26 strain the absorbances at 440 and 680 nm, originating from chlorophyll a molecules, were significantly reduced. In contrast, the peak at 625 nm, reflecting absorption by phycobilins, was not affected. The chlorophyll content of the mutant strain was estimated to be 15–20% of that in the wild-type cells. In addition, absorption spectra of isolated thylakoid membranes (Fig. 1B) revealed that the peak of chlorophyll a in the mutant strain was shifted to 675 nm. Similar
phenomena have been observed in other cyanobacterial PSI-deficient mutant strains, and have been attributed to a loss of PSI-associated chlorophyll and retention of PSII-associated chlorophyll molecules (31). The loss of chlorophylls and the consequent increase in the phycobilin-to-chlorophyll ratio resulted in the bluish color of the BP26 cells.

In *Synechocystis* 6803 cells, most of the chlorophyll molecules are associated with the PSI complex. Hence, a reduced cellular content of chlorophylls may be due to a decreased level of this protein complex. Using chemical difference spectroscopy, we determined that on a total chlorophyll basis, the content of P700, the reaction center chlorophylls of PSI, was nearly 5-fold less in this mutant (Table I). In addition, low temperature (77 K) fluorescence spectra of intact cells revealed that the emission band at 725 nm, originating from the PSI complex, was substantially reduced in the mutant (data not shown).

These spectroscopic data were supported by an analysis of thylakoid membrane proteins from these cyanobacterial cells. As shown in Fig. 2A, there is no detectable difference in the profiles of soluble proteins from the wild-type and mutant cells, while there is a significant difference in the polypeptide profiles of their thylakoids. In particular, in the mutant strain, the levels of PsaA and PsaB, the reaction centers proteins of PSI, have been significantly decreased. It is noteworthy that the levels of a number of smaller polypeptides are also decreased in the thylakoids from BP26. Some of these proteins may be subunits of the PSI complex, since in the absence of the reaction center proteins, a number of other polypeptides of PSI do not accumulate (2). Antibodies raised against both PSI reaction center proteins, PsaA and PsaB, recognized a diffused band in the thylakoid membranes from the wild-type cells. On a protein basis, the BP26 cells had only 10–15% as much of these two polypeptides. In contrast, the amounts of D1 and D2, the reaction center proteins of PSII, as well as CP43 and CP47, the chlorophyll-binding proteins of PSII, were similar in the wild-type and BP26 cells, a finding that agreed well with the PSII-mediated electron transport activities described above. Taken together, these data indicated that the spontaneous mutation in the BP26 strain had affected the cellular content of the PSI complex.

**Transcription of the psaAB Gene Cluster in the BP26 Mutant**—Based on the analysis of various targeted PSI-deficient mutant strains, it is known that the absence of other proteins of PSI does not affect the accumulation of the PsaA and PsaB proteins in cyanobacterial thylakoids (3). Hence, we reasoned that the dramatic decrease in the contents of these two polypeptides in the BP26 strain (Fig. 2) must have resulted because the mutation influences the accumulation of these reaction center proteins in the thylakoid membranes. To determine at what level this mutation effect the regulation of PsaA and PsaB biogenesis, we examined the transcript levels of the *psaAB* gene cluster in the mutant cells (Fig. 3). In *Synechocystis* 6803, two major transcripts are observed from this operon: a 5-kb transcript, that corresponds to the entire gene cluster, and a 2-kb transcript that corresponds to each of the individual genes, *psaA* and *psaB* (32). Both of these transcripts were...
present in similar amount in wild-type and BP26 cells (Fig. 3), indicating that the lesion in this mutant did not significantly affect the transcription of these genes. Thus, the defect in the BP26 mutant must effect the biogenesis of PSI at a post-transcriptional level.

**Complementation Analysis of the BP26 Mutant Strain**—We determined that the BP26 mutant strain could not be complemented by any known structural genes for PSI, indicating that the mutation in this strain maps in an unidentified genetic locus. Next, chromosomal DNA from wild-type *Synechocystis 6803* cells was digested with *Bam*HI and *Hind*III restriction enzymes and fractionated on a 0.7% low melting agarose gel. DNA fragments from individual slices of this gel were assayed for their ability to complement the BP26 cells. Fragments in the 1.5–2.5-kb range yielded the greatest number of transformants, and were cloned in the plasmid vector pUC119. One resultent recombinant plasmid could complement the BP26 mutant strain. Fig. 4A shows a restriction map of the 2-kb *Bam*HI-*Hind*III DNA fragment cloned in this plasmid. Using deletion analysis, we mapped the mutation on a 0.6-kb *Bsp120I*-BgII fragment (Fig. 4B). Complementation of the BP26 mutant cells with this DNA fragment yielded transformants that had normal pigmentation, growth rates and photosynthetic activities (data not shown). Southern blot analysis of chromosomal DNA from wild-type cells revealed that the genetic information in this fragment is present in a single copy in the genome of *Synechocystis 6803* (Fig. 4C).

**Molecular Characterization of the DNA Fragment That Complements the BP26 Mutant**—We determined the nucleotide sequence of the larger *Sal*I-*Hind*III DNA fragment shown in Fig. 4A. In this 1281-nucleotide long sequence (Fig. 5A), only one open reading frame (ORF) of significant length was identified. This ORF starts with a GU initiation codon at nucleotides 289–291 and ends with a UGA termination codon at nucleotides 1150–1152. As shown in Fig. 5A, this ORF codes for a polypeptide of 287 amino acids with a predicted molecular mass of 30 kDa. Analysis of this sequence also revealed the presence of a gene encoding an Arg-tRNA that extends between nucleotides 42 and 107, and its deduced direction of transcription is opposite to that of the ORF287.

To determine the molecular nature of the spontaneous mutation in the BP26 mutant strain, we cloned the corresponding 0.6-kb *Bsp120I*-BgII DNA fragment from these cells and determined its sequence. A comparison of this sequence with that from the wild-type cells revealed a single base substitution in the BP26 DNA. At the nucleotide position 440, a T to a G mutation (Fig. 5A) resulted in the substitution of valine 51 by a glycine residue in the ORF287-encoded polypeptide in the mutant strain. A directed inactivation of the ORF287 resulted in a phenotype similar to that of BP26 (data not shown). As discussed above, the ORF287 protein regulates the biogenesis of the PSI reaction center protein complex, and we have named this gene *btpA* (biogenesis of thylakoid proteins), and the encoded protein, BtpA.

**Analysis of the BtpA Protein Sequence**—An analysis of the hydrophobicity profile of the sequence of the BtpA protein (Fig. 5B) indicated that this polypeptide is largely hydrophilic. The only significant hydrophobic segment is between residues 74 and 127, with two putative transmembrane spans. The deduced sequence of the BtpA protein was used to search for similar sequences in the nondegenerate GenBank<sup>TM</sup> sequence data base (September 1996). Such analysis revealed that the *btpA* gene has not been previously identified in *Synechocystis 6803*. The BtpA protein shared a high degree of similarity (79%) with the partial sequence (derived from a random sequencing project) of an ORF (GenBank<sup>TM</sup> accession no. Z47150) in *Calothrix*, another cyanobacterium. Interestingly, a highly significant similarity (64%) was found between...
BtpA and a hypothetical 28.9-kDa protein (GenBankTM accession no. U67554) in the archaebacterium Methanococcus jannaschii (Fig. 6). The sequence of this ORF has been determined during the M.jannaschii genome sequencing project. However, the function of this protein in M.jannaschii cell is not known.

In addition, the BtpA protein shared 55% sequence similarity with a hypothetical 29.4-kDa protein (GenBankTM accession no. P39364) in the bacterium E. coli and with a hypothetical 32.1kDa protein (GenBank accession Z69383) in the nematode, Caenorhabditis elegans. These sequences of these ORFs have also been determined during the respective genome sequencing projects, and their biological functions are currently unknown. Limited homologies were also found with a number of dehydrogenases, e.g. glyceraldehyde phosphate dehydrogenase. However, the regions of such homologies did not correspond to known cofactor-binding domains in these well studied enzymes.

Finally, a significant similarity was found between BtpA and the product of the hypE gene in several bacterial species (34). Genetic analysis has indicated that the hypE gene is necessary for the formation of the hydrogenase protein complex, although the protein encoded by this gene is not a structural component of the hydrogenase enzyme.

DISCUSSION

Reduced Level of the PSI Complex in the BP26 Mutant Strain—The major objective of this communication has been the genetic and biochemical characterization of BP26, a random photosynthesis deficient mutant strain of the cyanobacterium Synechocystis 6803. Deduced amino acid sequence of the BtpA protein is shown below the corresponding nucleotide sequence. A purine-rich region which may serve as a ribosome-binding site is underlined. Boldface letters above the nucleotide and below the amino acid sequence show the mutation in the BP26 strain. The dashed underlined region corresponds to an Arg-tRNA gene. The nucleotide sequence shown here has been deposited in the GenBankTM-EMBL database under the accession no. U37695.

BtpA and a hypothetical 28.9-kDa protein (GenBankTM accession no. U67554) in the archaeabacterium Methanococcus jannaschii (Fig. 6). The sequence of this ORF has been determined during the M.jannaschii genome sequencing project. However, the function of this protein in M.jannaschii cells is not known. In addition, the BtpA protein shared 55% sequence similarity with a hypothetical 29.4-kDa protein (GenBankTM accession no. P39364) in the bacterium E. coli and with a hypothetical 32.1kDa protein (GenBank accession Z69383) in the nematode, Caenorhabditis elegans. The sequences of these ORFs have also been determined during the respective genome sequencing projects, and their biological functions are currently unknown. Limited homologies were also found with a number of dehydrogenases, e.g. glyceraldehyde phosphate dehydrogenase. However, the regions of such homologies did not correspond to known cofactor-binding domains in these well studied enzymes. Finally, a significant similarity was found between BtpA and the product of the hypE gene in several bacterial species (34). Genetic analysis has indicated that the hypE gene is necessary for the formation of the hydrogenase protein complex, although the protein encoded by this gene is not a structural component of the hydrogenase enzyme.

DISCUSSION

Reduced Level of the PSI Complex in the BP26 Mutant Strain—The major objective of this communication has been the genetic and biochemical characterization of BP26, a random photosynthesis deficient mutant strain of the cyanobacterium Synechocystis 6803. We have demonstrated that in this mutant, the steady state level of the PSI reaction center proteins, PsaA and PsaB, is significantly reduced, with an accompanied loss of most of the chlorophyll molecules and a 5-fold lower PSI-mediated electron transport activity. In contrast, the PSII activity of these cells is near normal, and the content of
the PSI reaction center proteins D1 and D2 as well as content of chlorophyll-binding proteins CP43 and CP47 are not decreased in this mutant strain. Hence, the lesion in the BP26 strain had specifically affected the PSI complex. It is noteworthy, that this mutant is relatively light-tolerant and can grow photautotrophically, although at a reduced rate (Table I). In contrast, a complete absence of the PSI reaction center complex in the cyanobacterium Synechocystis 6803 results in significant light-sensitivity of the cells (35, 36). The light-tolerance of the BP26 mutant was not localized in any known structural gene for the PSI complex. We have shown that a novel gene, btpA, complements this mutant, and a missense mutation in this gene has resulted in the decreased level of PSI in these cells. PSI is a well studied enzyme, and its crystal structure has been described at a 4.5 Å resolution (4). Moreover, the molecular identities of the major polypeptide components of this crystallized PSI complex are fairly well understood (2–4). Analysis of the deduced sequence of the BtpA protein clearly indicated that it is not a structural protein of PSI, suggesting that the btpA gene regulates the cellular content of the PSI complex in Synechocystis 6803.

The molecular details of the biogenesis of large multisubunit integral membrane proteins, such as PSI, remain poorly understood. A number of distinct biochemical processes are involved in the maintenance of the steady state level of such a membrane protein complex in a cell. These include transcription of the genes encoding the structural proteins, efficient translation of the mRNAs, integration of the nascent polypeptides in the thylakoid membrane, stoichiometric association of different subunit polypeptides as well as cofactors to form a functional complex, and ultimately regulated degradation of various components. Northern blot analysis of cellular RNA demonstrated that the mutation in BP 26 does not affect the accumulation of the transcripts of the psaAB gene cluster that encodes PsaA and PsaB (Fig. 3). This result strongly suggested that the BtpA protein regulates the biogenesis of the PSI reaction center proteins at a post-transcriptional level.

One of the possible roles of the BtpA protein is in the efficient translation of the psaAB messenger RNA. A number of such translational activator proteins have been identified in yeast mitochondria (37). One of them, CBS1, is an integral membrane protein (38). A second possibility is that BtpA is a membrane-bound chaperonin-like protein that stabilizes the translational intermediates of the PSI reaction center complex. Stabilization of such intermediates of the large highly hydrophobic proteins PsaA and PsaB may be an important step in the proper assembly of various polypeptides and cofactors into a functional complex. A third possibility is that the BtpA protein may regulate the biosynthesis and availability of critically important cofactors in PSI, such as P700, the reaction center chlorophylls, or Fx, an interpeptide [4Fe-4S] cluster. Finally, Collier and Grossman (39) have recently identified NhIA, a small protein, that controls the degradation of phycobilisomes, a soluble pigment-protein complex in cyanobacteria. In a similar manner, BtpA may be involved in regulating the rate of degradation of the PSI holocomplex. Further biochemical and genetic analysis of this novel protein is expected to unravel its exact function in the regulation of the steady state level of the PSI complex.

**Relatives of the BtpA Protein in Other Organisms**—A comparison of the amino acid sequence of BtpA with the GenBank™-EMBL sequence data base revealed that polypeptides homologous to BtpA are present in the archaeon *M. jannaschii*, the eubacterium *E. coli*, as well as the nematode *C. elegans*. The sequences of all of these latter proteins have an important common function in cellular metabolism. It is also noteworthy that the respective proteins in *M. jannaschii*, *E. coli*, and *C. elegans* are similar in length to BtpA, and have similar predicted secondary structures (data not shown). Given their close similarities with BtpA, we propose that these related proteins are also involved in the biogenesis or degradation of other membrane-bound multisubunit protein complexes.

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