

The *ctpA* gene encodes the C-terminal processing protease for the D1 protein of the photosystem II reaction center complex

(photosynthesis/oxygen evolution/*Synechocystis* 6803/posttranslational modification/interphotoreceptor retinoid-binding protein)

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ABSTRACT The D1 protein of the photosystem II (PSII) complex in the thylakoid membrane of oxygenic photosynthetic organisms is synthesized as a precursor polypeptide (pD1) with a C-terminal extension. Posttranslational processing of the pD1 protein is essential to establish water oxidation activity of the PSII complex. We have recently identified a gene, *ctpA*, a mutation in which resulted in a loss of PSII activity in the cyanobacterium *Synechocystis* sp. PCC 6803. To study the function of the CtpA protein, we inactivated the *ctpA* gene by inserting a kanamycin-resistance gene into its coding sequence. The resultant mutant strain, T564, had no PSII-mediated water oxidation activity, but it had normal cytochrome *b₆f* and photosystem I activities. Measurements of thermoluminescence profiles and rates of reduction of 2,6-dichlorophenolindophenol indicated that PSII complexes in the mutant cells had functional reaction centers that were unable to accept electrons from water. Immunoblot analysis showed that D1, D2, CP47, CP43, and the α subunit of cytochrome *b₅₅₉*, five integral membrane proteins of PSII, were present in T564 cells. Interestingly, the D1 protein in the mutant cells was 2 kDa larger than that in wild-type cells, due to the presence of a C-terminal extension. We conclude that the CtpA protein is a processing enzyme that cleaves off the C-terminal extension of the D1 protein. Interestingly, the CtpA protein shows significant sequence similarity to the interphotoreceptor retinoid-binding proteins in the bovine, human, and insect eye systems.

Many proteins in prokaryotic as well as eukaryotic cells are synthesized in their precursor forms. They are converted to the mature forms after proteolytic processing and removal of short peptides at the N- or C termini of the precursor proteins. Many proteins have cleavable N-terminal signal sequences that are necessary for their translocation across specific membranes. Signal peptidases responsible for the endoproteolytic cleavage of such precursor proteins have been identified and studied extensively (1). A number of proteins are synthesized with cleavable C-terminal extensions (2, 3), the functions of most of them being largely unknown. Moreover, the processing proteases for such C-terminal extensions have not been identified in most cases.

Linear electron transport during oxygenic photosynthesis is mediated by three protein complexes—photosystem II (PSII), cytochrome *b₆f*, and photosystem I (PSI)—in the thylakoid membranes of green plants, algae, and cyanobacteria. PSII mediates light-induced electron transfer from water to plastoquinone, with concomitant evolution of molecular oxygen (see ref. 4 for a recent review). Upon excitation with light, P680, the reaction center chlorophyll(s) (Chl), releases electrons that move successively through a

pheophytin and two plastoquinone (Q_A and Q_B) molecules on the acceptor side of PSII. The oxidized P680 molecule is subsequently reduced by electrons arriving from water via a cluster of four Mn atoms and a redox-active tyrosine residue (Y_Z) on the donor side of PSII (4). The catalytic core of PSII comprises two proteins, D1 and D2. Closely associated with these polypeptides are other integral membrane proteins—namely, CP47, CP43, cytochrome *b₅₅₉*, PsbL, and a number of other small polypeptides (5).

In most oxygenic photosynthetic organisms, the D1 protein is synthesized with a C-terminal extension (6) anywhere between 8 and 16 amino acid residues long (7). The D1 protein is present in its precursor (pD1) form in LF1, a photosynthesis-deficient mutant strain of the eukaryotic alga *Scenedesmus obliquus* (7, 8). In the presence of the C-terminal extension on D1, a functional Mn cluster is not formed, as a result of which the PSII complex in the LF1 mutant is unable to oxidize water. The C-terminal processing enzyme for the D1 protein has been partially purified from pea and spinach (9, 10). However, biochemical purification of this processing enzyme to homogeneity has not yet been reported.

We have used a genetic approach to identify the *ctpA* gene that complements SK18, a spontaneous PSII-deficient mutant strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (11). Interestingly, the sequence of the CtpA protein exhibited a significant homology to Prc (or Tsp), a recently identified C-terminal processing protease in the bacterium *Escherichia coli* (2, 12), suggesting that the CtpA protein may also be a C-terminal processing enzyme in *Synechocystis* 6803. The data presented in this report demonstrate that CtpA is the processing protease for the D1 protein of PSII.

MATERIALS AND METHODS

Cell Culture. *Synechocystis* sp. PCC 6803 cultures were grown at 30°C as described (13). The T564 strain was grown in medium supplemented with 5 mM glucose and 25 μ g of kanamycin (Km) per ml. Cultures of wild-type and LF1 mutant strains of the green eukaryotic alga *Scenedesmus obliquus* were grown in the dark in a minimal medium supplemented with glucose as described (14).

Protein Electrophoresis and Immunodetection. To obtain total protein extracts, *Synechocystis* 6803 cells were ruptured

Abbreviations: PSI, photosystem I; PSII, photosystem II; Chl, chlorophyll; DCIP, 2,6-dichloroindophenol; DPC, diphenyl carbazide; IRBP, interphotoreceptor retinoid-binding protein; Km^r, kanamycin resistance; Y_Z , redox-active tyrosine in PSII.

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by sonication as described (15). Thylakoid membranes from cyanobacterial cells were isolated essentially as described (16). Concentrations of Chl were measured in methanol (17). Fractionation of proteins on SDS/polyacrylamide gels and immunodetection of specific proteins on Western blots were performed as described (18). Rabbit polyclonal antibodies raised against D1, D2, CP47, and CP43 proteins were kind gifts from M. Ikeuchi and Y. Inoue (The Institute of Physical and Chemical Research, Tokyo). Antibodies against the α subunit of cytochrome *b*₅₅₉ (F. Sutterwala and H.B.P., unpublished data) were raised in our laboratory. For production of antibodies against the CtpA protein, we used the pET plasmid system (19) to overexpress the *ctpA* gene in *E. coli* cells essentially as described (20).

Steady State Electron Transport Rates. A Clark-type oxygen electrode was used to measure the rates of light-mediated oxygen evolution or consumption from intact cyanobacterial cells at 30°C as described (21). Rates of PSII-mediated electron transfer from diphenyl carbazide (DPC) to 2,6-dichloroindophenol (DCIP) were measured at 25°C on a modified Hitachi model 160 spectrophotometer. The concentration of P700, the reaction center Chl of PSI, was estimated from ascorbate-reduced minus ferricyanide-oxidized chemical difference spectra of thylakoid samples as described (22).

Thermoluminescence. The intensity of thermoluminescence from intact cyanobacterial and algal cells as a function of temperature was measured as described (23).

Comparisons of Sequences. The BLAST (24) service at the National Center for Biotechnology Information was used to search for proteins with sequence similarities to the CtpA protein. For a detailed comparison of the sequences of the CtpA protein and the interphotoreceptor retinoid-binding protein (IRBP), the BESTFIT program in the Genetics Computer Group software package (25) was used.

RESULTS

Insertional Inactivation of the *ctpA* Gene. The chromosome of *Synechocystis* 6803 contains a single copy of the *ctpA* gene. Molecular cloning of this gene and determination of its nucleotide sequence (GenBank accession no. L25250) have been described elsewhere (11).

The scheme for inactivation of the *ctpA* gene is shown in Fig. 1A. In the plasmid pSL523, a 1407-bp *EcoRI* fragment of *Synechocystis* 6803 genomic DNA contains the coding region of the *ctpA* gene. A 1.1-kbp *Bam*HI fragment from the plasmid pRL442, containing a Km^r gene (26), was inserted at a unique *Bsm*I site in the coding region of *ctpA*. The resultant plasmid, pSL564, was used to transform wild-type *Synechocystis* 6803 cells. T564, one of the resultant Km^r transformants, was used for further analysis.

Southern hybridization analysis of the chromosomal DNA demonstrated a complete segregation of the insertion-bearing chromosome in the T564 strain. As shown in Fig. 1B, the *ctpA* probe hybridized to a 1.4-kbp *EcoRI* fragment of the chromosomal DNA from wild-type cells (lane 1) as well as to an *EcoRI* fragment of similar size in the parent plasmid pSL523 (lane 3). In contrast, the same probe hybridized to a 2.5-kbp *EcoRI* fragment of the genomic DNA from the mutant T564 cells (lane 2), as expected for an interrupted *ctpA* gene (Fig. 1A and Fig. 1B, lane 4).

Absence of the CtpA Protein in T564 Cells. Antibodies raised against a CtpA fusion protein recognized a relatively diffuse band on a Western blot of a cellular extract of the wild-type strain (Fig. 2, lane 1). The mobility of this band on this SDS/urea gel system was 39 kDa. No such protein band was observed when the corresponding preimmune sera were used (data not shown). This immunostained band was absent in a cellular extract from the T564 mutant strain (lane 2), demonstrating that the insertion of the Km^r cartridge in the

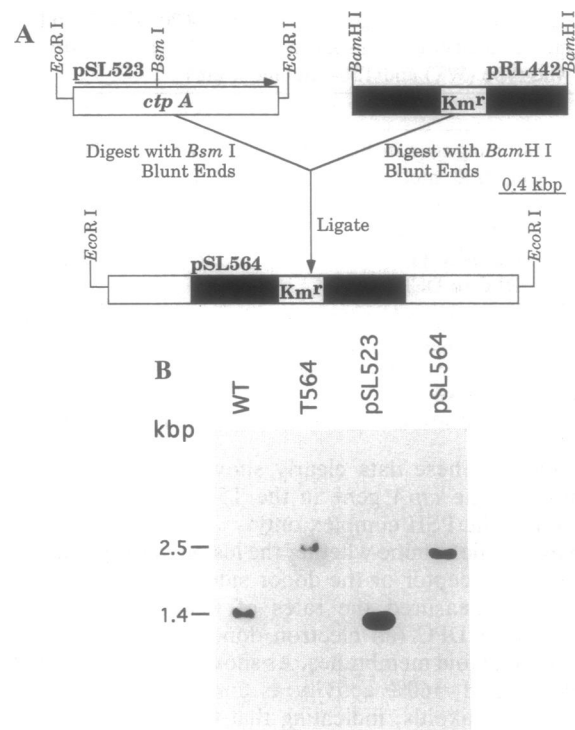


FIG. 1. (A) Construction of the pSL564 recombinant plasmid for insertional inactivation of the *ctpA* gene. (B) Southern hybridization analysis of chromosomal DNA from wild-type (WT) and T564 mutant strains of *Synechocystis* 6803 as well as the plasmids pSL523 and pSL564. Each DNA sample was digested with *EcoRI*, fractionated on an agarose gel, blotted onto nitrocellulose paper, and hybridized to a ³²P-labeled 1.4-kbp *EcoRI* fragment of pSL523.

ctpA gene has resulted in loss of the CtpA protein from these cyanobacterial cells.

Absence of Water-Oxidation Activity in the T564 Mutant Cells. Mutant strains of *Synechocystis* 6803 with no functional PSII complex cannot survive if their growth media are not supplemented with glucose. As shown in Table 1, the T564 strain was unable to grow in the BG11 medium. However, it grew well in glucose-supplemented medium. Moreover, there was no detectable PSII-mediated oxygen evolution activity in the T564 mutant cells. In contrast, the rates of light-induced electron transfer from duroquinol to methyl viologen, a reaction mediated by both cytochrome *b*_{6f} and PSI complexes as well as the soluble carrier protein(s) between these two complexes (21), were similar in the wild-type and the T564 cells. The P700 contents in the thylakoid membranes of the wild-type and mutant cells were

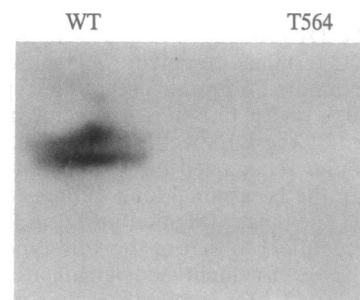


FIG. 2. Immunoblot analysis of proteins in total cellular extracts from wild-type (WT) and T564 mutant strains of *Synechocystis* 6803. Protein samples were fractionated on denaturing SDS/polyacrylamide gel, transferred to nitrocellulose filter, and immunostained with rabbit antibodies raised against a T7 gene 10-CtpA fusion protein. Samples were loaded at 100 μ g of protein per lane.

Table 1. Comparison of growth rates, Chl/P700 ratios, and light-mediated electron transport activities of *Synechocystis* 6803 wild-type (WT) and T564 mutant strains

	WT	T564
Doubling time in BG11, hr	9.5	NG
Chl/P700, mol/mol	122	119
Rates of electron transport		
H ₂ O to DCBQ*	410	<10
DQH ₂ to MV*	-305	-322
DPC to DCIP†	260	150

NG, no growth; DCBQ, 2,6-dichloro-*p*-benzoquinone; DQH₂, duroquinol; MV, methyl viologen.

*Expressed as μmol of O₂ per mg of Chl per hr. Negative numbers indicate O₂ uptake.

†Expressed as microequivalent electrons per mg of Chl per hr.

also similar. These data clearly showed that the insertion mutation in the *ctpA* gene in the T564 strain affected the function of the PSII complex only.

To further determine whether the lesion in the mutant cells was on the acceptor or the donor side of the PSII reaction center, we measured the rates of light-induced electron transfer from DPC (an electron donor to Y_Z) to DCIP in isolated thylakoid membranes. As shown in Table 1, the T564 thylakoids had $\approx 60\%$ activity as compared to that of the wild-type thylakoids, indicating that the mutation has specifically affected electron transfer from water to Y_Z. These data also implied that the PSII protein complex was present at a significant level in the thylakoid membranes of these mutant cells.

Immunoblot Analysis of PSII Proteins. Western blot analysis showed that D2, CP43, CP47, and the α subunit of cytochrome *b*₅₅₉—four integral membrane protein components of the PSII complex—were present in the T564 mutant cells. Moreover, the steady state levels of these polypeptides in the T564 cells were between 50% and 65% of their wild-type levels.

As shown in Fig. 3B, the D1 protein of PSII was also present at a reduced but significant level in the thylakoid membranes of T564 cells (lane 2). Interestingly, the mobility of D1 in the mutant cells was significantly slower (34 kDa) than that (32 kDa) in the wild-type cells (lane 1). A similar increase in the molecular mass of D1 has previously been observed in the LF1 mutant strain of the eukaryotic alga *Scenedesmus* (8) in which the pD1 protein accumulates (7). To examine whether the D1 protein in T564 cells was in its precursor form, we digested membranes isolated from wild-type and T564 cells with Lys-C, a lysine-specific endoprotease, and examined the D1 protein in these digested samples (lanes 3 and 4). The D1 protein in *Synechocystis* 6803 has a single lysine residue (at position 238) exposed to the stroma (27, 28). Digestion at this site is expected to produce two fragments of the D1 protein—a longer N-terminal fragment and a shorter C-terminal fragment. As shown in lane 3, digestion of the mature D1 protein in the wild-type thylakoids resulted in a 20.5- and a 13.5-kDa fragment, both of which were recognized by the D1-specific antibodies. (Interestingly, these antibodies recognized the C-terminal shorter fragment better than the N-terminal longer fragment of D1.) As shown in lane 4, the longer fragment of D1 in the T564 cells had a mobility identical to that of the wild-type D1 protein, whereas the shorter fragment had an apparent molecular mass of 15.5 kDa, 2 kDa larger than that of the shorter fragment of the wild-type D1 protein. Based on these data, we concluded that the D1 protein in the T564 mutant cells had a C-terminal extension. We also inferred that the CtpA protein is the processing protease that cleaves this C-terminal extension in the wild-type cells.

Thermoluminescence Analysis. Fig. 4 shows the profiles of

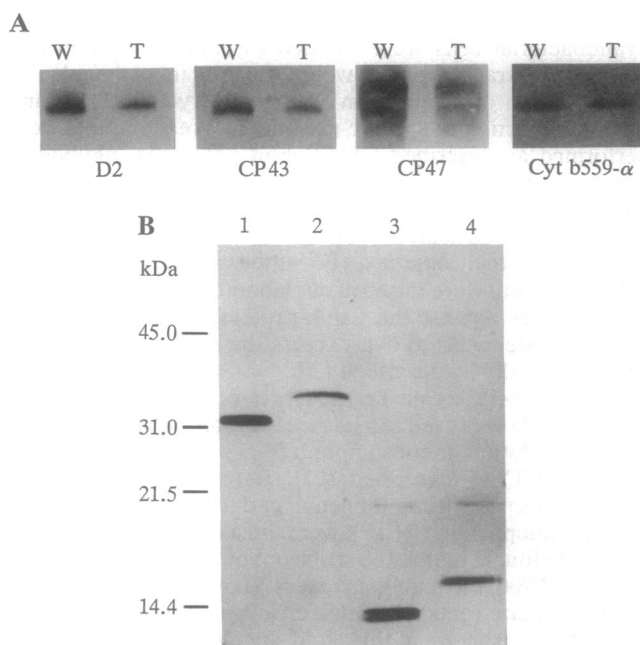


FIG. 3. (A) Immunoblot analysis of PSII polypeptides in membrane preparations. Thylakoid proteins from *Synechocystis* 6803 wild-type (W) and T564 mutant (T) cells were fractionated on denaturing SDS/polyacrylamide gel, transferred to nitrocellulose filter, and immunostained with rabbit antibodies raised against D2, CP43, CP47, and the α subunit of cytochrome *b*₅₅₉ (cyt *b*₅₅₉- α), respectively. (B) Immunoblot analysis of membrane proteins from wild-type and T564 cells of *Synechocystis* 6803. Blot was probed with antibodies raised against the D1 protein. Otherwise, the experimental conditions were as described in A. Samples in lanes 1 and 2 were untreated thylakoid proteins, whereas those in lanes 3 and 4 were similar thylakoid samples digested with a Lys-C lysine-specific endoprotease. Lanes: 1 and 3, wild-type samples; 2 and 4, T564 samples. Positions of molecular mass markers (kDa) are shown on the left.

temperature-dependent emission of luminescence from intact cells of *Synechocystis* 6803 and *Scenedesmus*. The glow peaks at 23.5°C in the wild-type *Synechocystis* cells and at 16°C in the wild-type *Scenedesmus* cells originated from recombination between S_{2,3}/Q_B whereas the shoulder at 7°C in the former sample originated from S_{2,3}/Q_A recombination (23). These peaks were missing from the T564 as well as the LF1 mutant cells. In addition, both of these mutant strains gave rise to peaks centered around -35°C. A similar peak (A_T

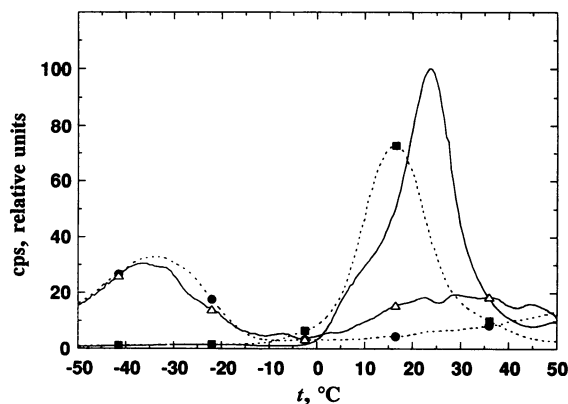


FIG. 4. Thermoluminescence intensity as a function of temperature in intact cells of wild-type (solid line) and T564 mutant (triangles) strains of *Synechocystis* 6803 as well as in cells of wild-type (squares) and LF1 mutant (circles) strains of *Scenedesmus*. cps, Counts per second.

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