

Amino Acid Residues That Are Critical for *in Vivo* Catalytic Activity of CtpA, the Carboxyl-terminal Processing Protease for the D1 Protein of Photosystem II*

Received for publication, March 22, 2001, and in revised form, May 26, 2001
Published, JBC Papers in Press, June 14, 2001, DOI 10.1074/jbc.M102600200

Noritoshi Inagaki^{‡§¶}, Radhashree Maitra[‡], Kimiyuki Satoh^{§¶}, and Himadri B. Pakrasi^{‡**}

From the [‡]Department of Biology, Washington University, St. Louis, Missouri 63130-4899, the [§]National Institute for Basic Biology, Okazaki 444-8585, Japan, and the [¶]Department of Biology, Okayama University, Okayama 700-8530, Japan

CtpA, a carboxyl-terminal processing protease, is a member of a novel family of endoproteases that includes a tail-specific protease from *Escherichia coli*. In oxygenic photosynthetic organisms, CtpA catalyzes C-terminal processing of the D1 protein of photosystem II, an essential event for the assembly of a manganese cluster and consequent light-mediated water oxidation. We introduced site-specific mutations at 14 conserved residues of CtpA in the cyanobacterium *Synechocystis* sp. PCC 6803 to examine their functional roles. Analysis of the photoautotrophic growth capabilities of these mutants, their ability to process precursor D1 protein and hence evolve oxygen, along with an estimation of the protease content in the mutants revealed that five of these residues are critical for *in vivo* activity of CtpA. Recent x-ray crystal structure analysis of CtpA from the eukaryotic alga *Scenedesmus obliquus* (Liao, D.-I., Qian, J., Chisholm, D. A., Jordan, D. B. and Diner, B. A. (2000) *Nat. Struct. Biol.* 7, 749–753) has shown that the residues equivalent to Ser-313 and Lys-338, two of the five residues mentioned above, form the catalytic center of this enzyme. Our *in vivo* analysis demonstrates that the three other residues, Asp-253, Arg-255, and Glu-316, are also important determinants of the catalytic activity of CtpA.

(4–6), catalyzes light-induced oxidation of water to molecular oxygen, a critically important process in the biosphere. The life history of PSII is extremely interesting. As a direct consequence of its normal function, D1, one of the catalytic component proteins of PSII, is damaged and subsequently removed. A newly synthesized D1 protein with a carboxyl-terminal extension is then integrated into the PSII complex (7). The processing of the extension peptide on the precursor form of the D1 protein (pD1) is a prerequisite step for the formation of a tetramanganese cluster that is essential for the catalysis of the water oxidation reaction (8, 9).

In the cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803), the carboxyl-terminal extension in pD1 is 16 amino acids long (10). We have recently shown that the presence of this extension is required for optimal photosynthetic performance of *Synechocystis* 6803 cells (11). Mutants that lack catalytically active CtpA are unable to remove the carboxyl-terminal extension of the pD1 protein. As a consequence, they lack the ability to evolve oxygen, presumably because the carboxyl terminus of the mature D1 protein functions as a ligand for the formation of the manganese cluster in PSII (10). The *ctpA* gene was initially identified by genetic complementation analysis of specific photosynthetic mutant strains of *Synechocystis* 6803 (12, 13). Later, the CtpA protease from spinach chloroplasts was identified through biochemical purification techniques (14) followed by cloning and characterization of the plant nuclear gene encoding this enzyme (15). Our initial studies showed that the CtpA proteins from cyanobacteria and green plants share significant sequence similarities (15, 16). However, none of them exhibits sequence homology with other proteases with well defined reaction mechanisms. In addition, *in vitro* inhibitor studies have demonstrated that the CtpA enzyme cannot be classified as an aspartic, cysteine, serine, or metalloprotease (17).

Based on sequence homologies, the CtpA protease was assigned to a newly emerging class of carboxyl-terminal processing proteases that also included the tail-specific protease Tsp from *Escherichia coli* (18, 19). The members of this family of proteases exhibit significant degrees of sequence homology in certain discrete regions of the proteins. These regions of conserved sequences can be classified broadly as two domains, namely 1 and 2 (domains A and B in Ref. 15), respectively.

To understand the functional roles of various conserved amino acid residues and domains of the CtpA protein in its biological activity, we have, in this study, generated 23 tar-

During recent years, a new class of endoproteases with carboxyl-terminal processing activities has been described in various bacteria and organellar systems (1–3). The physiological functions of most of the members of this family of proteases are poorly understood. One notable exception is CtpA, a carboxyl-terminal processing protease found in cyanobacteria and chloroplasts (2, 3). In these photosynthetic organisms, photosystem II (PSII),¹ a large membrane-bound pigment-protein complex

* This research was supported by grants from the National Institutes of Health (GM 45797) and the United States Department of Energy (to H. B. P.), the Ministry of Education, Science and Culture of Japan (Grant-in-aid for Scientific Research B 09440268 and Grants-in-aid for Scientific Research in Priority Areas 09267222 and 09274222 to K. S.), and the Japan-United States Cooperative Photoconversion and Photosynthesis Research Program (to N. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Present address: Laboratory of Photosynthesis, National Institute of Agrobiological Resources, Tsukuba 305-8602, Japan.

** To whom correspondence should be addressed: Dept. of Biology, Box 1137, Washington University, One Brookings Dr., St. Louis, MO 63130-4899. Tel.: 314-935-6853; Fax: 314-935-6803; E-mail: Pakrasi@biology.wustl.edu.

¹ The abbreviations used are: PSII, photosystem II; pD1, precursor form of the D1 protein of PSII; TES, *N*-tris(hydroxymethyl)methyl-2-

aminoethanesulfonic acid; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kb, kilobase(s).

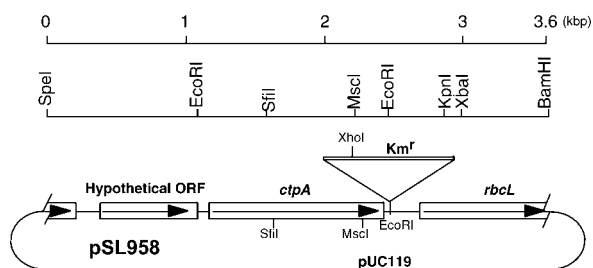


FIG. 1. Construction of pSL958, a recombinant plasmid in which a 1.1-kb kanamycin-resistance (Km^r) cassette was inserted at an *EcoRI* site immediately down-stream of the *ctpA* coding sequence. This plasmid was used for site-directed mutagenesis of the *ctpA* gene in *Synechocystis* 6803.

geted mutant strains by site-directed mutagenesis of *Synechocystis* 6803. Alanine scanning mutations were first introduced at each of 14 residues that are completely conserved among members of this protease family (15). Residues that were thus found to be critical for photoautotrophic growth were subsequently altered by conservative substitutions to gain an in-depth understanding of the catalytic activity of this protease.

The mutants thus generated were analyzed for their ability of photoautotrophic growth. We also measured PSII-mediated oxygen evolution activity and the cellular CtpA protease content in these mutant strains. Finally, the half-lives of the pD1 protein in these mutants were determined by *in vivo* pulse-chase experiments. Our data indicated that replacements of 9 of the 14 selected residues did not affect the catalytic activity of CtpA but significantly decreased the cellular content of this protein. On the other hand, five residues were critically important for the *in vivo* processing of the pD1 protein in *Synechocystis* 6803 cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals used were of reagent grade. Enzymes for recombinant DNA work were from New England Biolabs (Beverly, MA).

Bacterial Strains and Culture Conditions—*Synechocystis* 6803 wild type and mutant cells were grown at 30 °C under 50 μmol of photons $\text{m}^{-2} \text{s}^{-1}$ of white light in BG11 medium (20). Liquid cultures were grown with vigorous bubbling with air. The medium used for the heterotrophic mutants was supplemented with 5 mM glucose. Solid medium was supplemented with 1.5% (w/v) agar, 0.3% (w/v) sodium thiosulfate, and 10 mM TES-KOH, pH 8.2. A ΔctpA mutant was used as the background strain to generate various site-specific mutations in the *ctpA* gene. The ΔctpA mutant strain was generated by transforming wild type *Synechocystis* 6803 cells with the recombinant plasmid pSL795. To construct the pSL795 plasmid, we used the pSL794 plasmid that has the *ctpA* gene along with its flanking regions as a 6-kb *HindIII*-*BamHI* fragment (12) cloned in the pUC119 vector. A 1.5-kb *EcoRI* fragment containing an erythromycin-resistance cassette was inserted into pSL794 after the plasmid was digested with *EcoRI*, which replaced a 1.4-kb fragment containing the complete coding sequence of the *ctpA* gene by the cassette. The ΔctpA mutant was grown in the BG11 medium supplemented with 5 mM glucose and 3 $\mu\text{g ml}^{-1}$ erythromycin. The CtpA^k strain was used as a positive control in the current study. It was generated by transforming the ΔctpA mutant with the pSL958 plasmid (Fig. 1). The pSL958 plasmid has the *ctpA* gene along with an upstream hypothetical open reading frame and downstream *rbcL* sequences cloned in pUC119. Furthermore, a 1.1-kb kanamycin-resistance cassette was inserted at an *EcoRI* site immediately downstream of the *ctpA* coding sequence. Complete segregation was followed after transformation and confirmed by PCR. The growth of various cyanobacterial strains was monitored by measurements of light scattering at 730 nm on a DW 2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL). *E. coli* strain TG1 (*supE hsd Δ 5 thi Δ (lac-proAB) F' (traD36 proAB⁺ lacI⁹ lacZ Δ M15)*) used for the preparation of various plasmids was grown at 37 °C in Luria-Bertani medium (21).

Site-directed Mutagenesis—Site-specific mutations were generated using a PCR-based method described elsewhere (22). Sequences of upstream and downstream primers are 5'-ATCCCTCGGTACGTT-GAACC-3' and 5'-TCACGAGGCAGACCTCAGCG-3', respectively, and were used to amplify the entire coding sequence of the *ctpA* gene.

Mutagenic primers are listed in Table I. After PCR mutagenesis, the products were digested with *SfiI* and *MscI* (Fig. 2). The resultant fragments were purified and ligated into pSL958 digested with the same pair of restriction enzymes. The presence of the desired mutations in the *ctpA* gene was confirmed by sequencing of the resultant plasmids on an automated sequencer, model 373S (Applied Biosystems, Foster City, CA) using an ABI PRISM dye-primer cycle sequencing kit. The generation of the desired mutants was achieved by transforming ΔctpA cells with individual plasmids using a previously described procedure (23, 24).

Measurement of Oxygen Evolution—The rates of PSII-mediated oxygen evolution from intact *Synechocystis* 6803 cells were measured on a Clark-type oxygen electrode in the presence of 0.5 mM 2,6-dichloro-*p*-benzoquinone and 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ as described previously (25). Samples in BG11 medium were adjusted to a final chlorophyll concentration of 5 $\mu\text{g ml}^{-1}$ (26).

Western Analysis—For the expression of the mature form of the CtpA protein in *E. coli*, a translation initiation codon incorporated into an *NdeI* recognition site was introduced into the *ctpA* gene by PCR. The PCR products were digested with *NdeI* and *EcoRI* and cloned into the pET21a vector (Novagen, Madison, WI) digested with the same pair of restriction enzymes. The resultant plasmid (pSL962) was transformed into the *E. coli* strain BL21(DE3). After induction, the recombinant overexpressed protein was purified by SDS-PAGE (27) and used to raise anti-CtpA antisera in a rabbit. Anti-D1 antisera used during this study were kind gift from Prof. M. Ikeuchi (University of Tokyo). Proteins obtained from sonicated *Synechocystis* 6803 cells were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes. Detection of the CtpA and D1 proteins by Western blotting analysis was carried out using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech Ltd., Little Chalfont, Buckinghamshire, UK). The relative amounts of CtpA was estimated using the Intelligent Quantifier software (BioImage, Ann Arbor, MI).

Pulse-Chase Analysis of the Half-life of the Precursor Form of the D1 Protein—Cyanobacterial cells (5×10^8) were collected by centrifugation at $3,000 \times g$ for 5 min. The resultant pellet was washed twice with BG11 and resuspended in 100 μl of BG11. Five μl of L-[³⁵S]methionine (37 TBq/mmol, 370 MBq/ml, Amersham Pharmacia Biotech) were added into the suspension, and the cells were incubated for 5 min at 30 °C under 90 $\mu\text{E m}^{-2} \text{s}^{-1}$ of heat-absorbed incandescent light. After this pulse period, chloramphenicol (200 $\mu\text{g ml}^{-1}$) was added to the cells, which were then incubated for various chase periods. Twenty μl of sample were collected after each chase period, and cells were washed once with a lysis buffer containing 20 mM HEPES-KOH pH 7.5, 10 mM NaCl, 5 mM MgCl_2 , 1 mM EDTA, and 1 mM *p*-4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (*p*-ABSF) and resuspended into 20 μl of the same buffer. The cells were then disrupted in a small glass homogenizer with glass beads ($\leq 106 \mu\text{m}$, Sigma). Two hundred μl of sample buffer for SDS-PAGE were added to the homogenate. The resultant mixture was centrifuged at $20,000 \times g$ for 10 min at 25 °C, the supernatant was fractionated by SDS-PAGE, and the labeled D1 protein was visualized by fluorography. The half-life of the precursor form of the D1 protein in each mutant was estimated by analyzing the resultant fluorographs using the Intelligent Quantifier software (BioImage).

RESULTS

Directed Replacements of Conserved Residues in the CtpA Protease—The protein encoded by the *ctpA* gene in *Synechocystis* 6803 has 427 amino acids. To evaluate the contributions of different residues to the *in vivo* activity of CtpA, we chose 14 amino acid residues that are absolutely conserved among different members of this protease family and are, hence, expected to be important for their catalytic activities (Fig. 3b) (15). Among these residues, 10 are localized in two highly conserved domains, named 1 and 2 (domains A and B in Ref. 15), respectively. The extent of conservation of these domains among different members of the family are shown in Fig. 3b. Site-directed mutagenesis was employed to introduce specific substitutions at each of these 14 residues in the CtpA protein of *Synechocystis* 6803. Initially, we introduced alanine at each of these positions. Sites thus determined to be important for the *in vivo* enzymatic activity of CtpA were further subjected to semiconservative substitutions.

Photoautotrophic Competence and CtpA Content of the Alanine Substitution Mutants—We have previously determined

TABLE I
Sequences of mutagenic primers

Italicized and underlined letters indicate substituted bases and target codons, respectively. Shown in parentheses are the sites for restriction enzymes that were introduced in the primers for easy screening of the respective mutants.

GGCTGCAACCCCATGCGCAAATTTTGGCGATCGACGG	D149A	(<i>FspI</i>)
GAATTTACCCCTGACTGCGCAGTTAATTTCCCTCAGTC	R198A	(<i>FspI</i>)
GACGGTTATATCTTGGCGCTGCGTAACAACCCGGTGCG	D253A	(<i>HaeII</i>)
GGTTATATCTTGGATTTGGCCAAACAACCCCGGTGGCTTACTCC	R255A	(<i>MscI</i>)
CTTGGATTTGCGTAAACGCGCCCGGTGGCTTACTCCAGGC	N257A	(<i>HhaI</i>)
CAACCAGGGTACTGCTGCGAGCCAGCGAAATTTTAGCCGGAGC	S313A	(<i>PstI</i>)
GGTACTGCCAGTGCTGCGAGAATTTTAGCCGGAGCTTTGC	S315A	(<i>PstI</i>)
CTGCCAGTGCCAGCGCTATTTTAGCCGGAGCTTTGCAGG	E316A	(<i>Eco47III</i>)
CCGGAGCTTTGCAGGCCAATCAGCGGGCCACTC	D324A	
GAGCTTTGCAGGATAATCAGCGCCCACTCTAGTGGG	R327A	
GGAAAAACCTTTGGAGCGGGTTTGATTCAATCCTTG	K338A	(<i>BsrBI</i>)
GGTAAGGGTTTGATTGCTAGCTTGTGTAACATCCGATGG	Q342A	(<i>NheI</i>)
GCCGGCATTGCCGTCGCGGTGGCCAAATACGAAACC	T356A	(<i>Bsh1236I</i>)
ACTGGGCATTATGCCCTGCGAGAAGTGGTGAGCAACCCC	D376A	(<i>PstI</i>)
GACGGTTATATCTTGGAAATTTGCGTAACAACCCCGGTGGC	D253E	
GACGGTTATATCTTGGAAATTTGCGTAACAACCCCGGTGGC	D253N	
GGTTATATCTTGGATTTGAGAACAACCCCGGTGGCTTACTCC	R255K	
GGTTATATCTTGGATTTGACAACAACCCCGGTGGCTTACTCC	R255H	
CAACCAGGGTACTGCTGTGCCAGCGAAATTTTAGCCGGAGC	S313C	
CTGCCAGTGCCAGCGACATTTTAGCCGGAGCTTTGCAGG	E316D	
CTGCCAGTGCCAGCGCAGATTTTAGCCGGAGCTTTGCAGG	E316Q	
GGAAAAACCTTTGGTACCGGTTTGATTCAATCCTTG	K338H	
GGAAAAACCTTTGGTAGAGGTTTGATTCAATCCTTG	K338R	

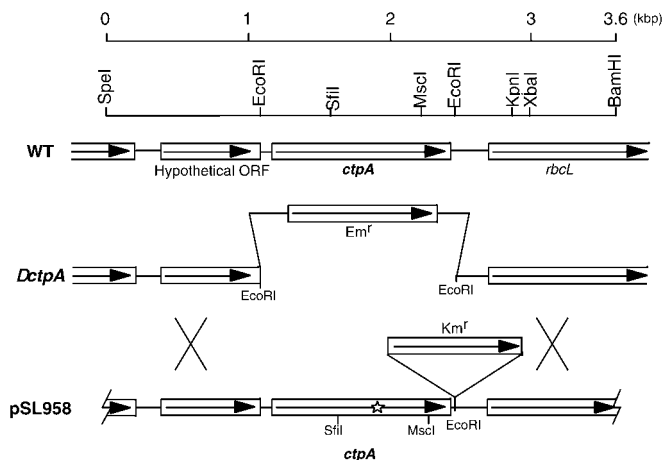


FIG. 2. A schematic diagram illustrating the strategy employed for site-specific mutagenesis of the *ctpA* gene in *Synechocystis* 6803. The top panel depicts a 3.6-kb *SpeI*-*BamHI* fragment of *Synechocystis* 6803 genomic DNA that contains the *ctpA* gene and its surrounding sequences. The middle panel represents the Δ *ctpA* deletion mutant strain in which the *ctpA* gene has been replaced by an erythromycin-resistance (*Em^r*) cassette. *In vitro* mutagenized plasmids (bottom panel; see Fig. 1) were engineered with a *Km^r* gene inserted downstream of the *ctpA* gene for the introduction of site-specific mutations. The asterisk represents a site-specific mutation in the *ctpA* gene (see "Experimental Procedures" for details).

that in the absence of CtpA activity, *Synechocystis* 6803 cells can not grow photoautotrophically (13). The photoautotrophic capabilities of the 14 different alanine substitution mutants are shown in Table II. Using anti-CtpA antisera in Western blot analysis, we determined that the amount of CtpA in wild type *Synechocystis* 6803 cells is 0.0026% of its total cellular protein content (data not shown). Thus, CtpA is a relatively minor protein in these cells, consistent with its regulatory role in the biogenesis of PSII. We determined the cellular CtpA content (Fig. 4 and Table II) and the status of the D1 protein (Table II) in the alanine substitution mutants. First, the CtpA content of the CtpA^k control strain was 53% of that of the wild type strain. In the CtpA^k strain, a kanamycin-resistance cassette was introduced at an *EcoRI* site immediately downstream of the *ctpA* coding sequence, which might have decreased the stability of the corresponding transcript and/or efficiency of its

translation. However, this control strain had normal PSII-mediated oxygen evolution activity as well as a pD1 half-life that was comparable with that in the wild type cells (Table III).

As shown in Fig. 4 and Table II, alanine substitution of each of nine residues, namely Asp-149, Arg-198, Asn-257, Ser-315, Asp-324, Arg-327, Gln-342, Thr-356, and Asp-376 reduced the cellular content of CtpA significantly. However, all of these mutant strains could grow photoautotrophically, and they accumulated the D1 protein in its mature form. It is noteworthy that the CtpA contents of both the A149A and the R198A mutants were less than 5% of the normal amount. Evidently, the presence of very small amounts of functional CtpA enzyme was sufficient to process the pD1 protein in *Synechocystis* 6803 cells. Clearly, none of these nine residues is essential for the catalytic activity of the CtpA protease. In contrast, alanine substitution of each of the remaining five residues resulted in the loss of photoautotrophic growth of the corresponding mutant strains. All of these five mutants accumulated the D1 protein in its precursor form, although a significant amount of the CtpA protease was present in each of them. These five residues are localized in the highly conserved domains 1 and 2 of the CtpA protein (Fig. 3a). Among these residues, Ser-313 and Lys-338 correspond to two active site residues (Ser-430 and Lys-455, respectively) of the Tsp protease from *E. coli*, previously identified by Keiler and Sauer (28). Our data indicated that the amino acid residues Asp-253, Arg-255, Ser-313, Glu-316, and Lys-338 are critically important for the *in vivo* pD1 processing function of the CtpA protease.

Conservative Replacements of Five Critical Residues—The above five critical residues were further subjected to conservative replacement mutagenesis. The acidic residue Asp-253 was changed to Glu, another acidic residue, or to Asn, the corresponding amido group-bearing residue. Similarly, Glu-316 was replaced by either Asp or Gln. The two basic residues Arg-255 and Lys-338 were each replaced by two other (potentially) basic residues. Finally, Ser-313 was substituted by cysteine, because in some proteases cysteine can replace the active site serine with retention of proteolytic activity (28). It is noteworthy that wild type *Synechocystis* 6803 CtpA does not have any cysteine residue. We determined the photoautotrophic growth competence, doubling time, CtpA content, status of the D1 protein (mature or the precursor form), and the rates of PSII-mediated oxygen evolution from the resultant 9 mutant

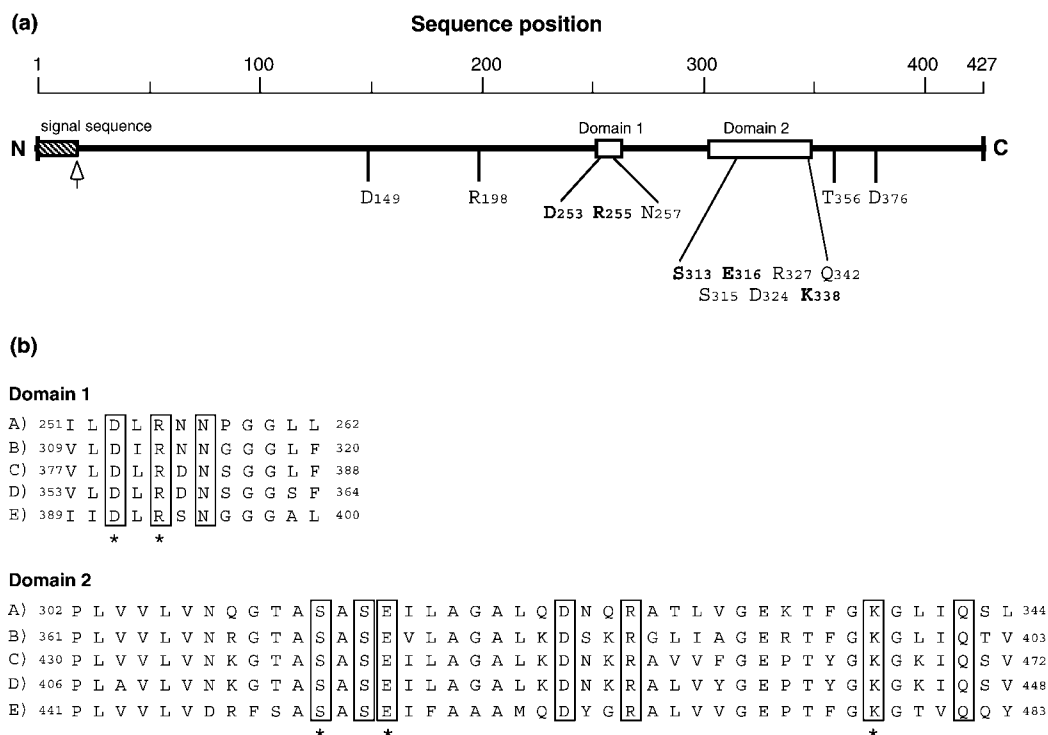


FIG. 3. *a*, a schematic depiction of sequence and domain locations of 14 residues of the CtpA protein that were selected for site-directed mutagenesis. The residues critical for photoautotrophic activity are shown in **bold**. *b*, sequence similarities between two conserved domains of different members of the CtpA family of carboxyl-terminal processing proteases: A, *Synechocystis* 6803 CtpA; B, *Scenedesmus obliquus* CtpA; C, *Spinacia oleracea* CtpA; D, *Arabidopsis thaliana* CtpA; and E, *Escherichia coli* Tsp protease. The boxes with an asterisk below indicate the residues that are critically important for *in vivo* catalytic activity of CtpA, as determined by alanine substitution mutagenesis, and were subjected to further semiconservative replacements. The rest of the boxes indicate the residues that were subjected to alanine substitution mutagenesis only.

TABLE II

Growth property, CtpA protease content, and the status of the D1 protein in mutant strains with alanine substitutions at fourteen selected residues of the CtpA protein

The CtpA^k positive control strain was generated by transforming the Δ ctpA strain with pSL958 (Fig. 1). The Δ ctpA strain served as a negative control in these studies.

Strain	Photoautotrophic growth	CtpA Content	Status of D1 protein ^a
Wild-type	+	187	M
CtpA ^k	+	100	M
Δ ctpA	-	0	P
D149A	+	5	M
R198A	+	2	M
D253A	-	36	P
R255A	-	66	P
N257A	+	33	M
S313A	-	118	P
S315A	+	72	M
E316A	-	96	P
D324A	+	35	M
R327A	+	33	M
K338A	-	88	P
Q342A	+	33	M
T356A	+	36	M
D376A	+	14	M

^a M, mature form of D1; P, precursor form of D1. Results shown are those from a representative experiment.

strains (Table III and Fig. 5). Both D253E and D253N mutations allowed photoautotrophic growth, although at reduced rates. In contrast, the E316D mutant could grow photoautotrophically, whereas the E316Q mutant could not. In addition, the latter mutant strain did not exhibit any PSII activity and accumulated only the precursor form of D1 (Fig. 5). These results indicated that the presence of a negatively charged residue at position 316 is essential for the catalytic activity of

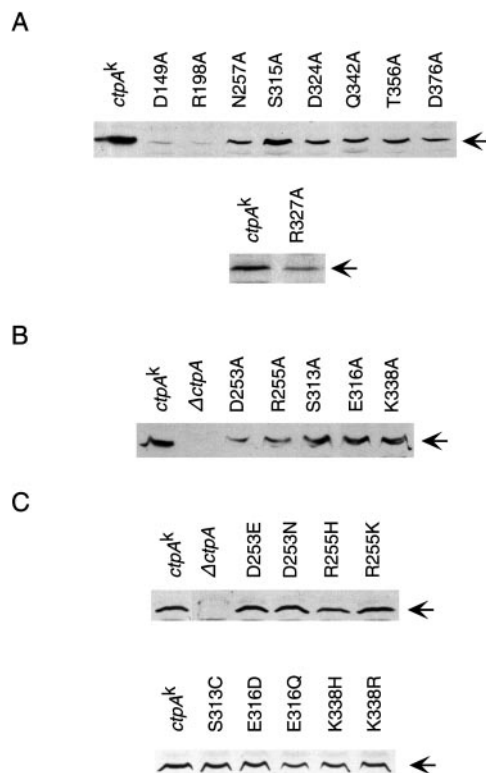


FIG. 4. **Immunological detection of mutant CtpA proteins.** Lysates of mutant cells were prepared as described under "Experimental Procedures." The presence of the CtpA protein in autotrophic (A), heterotrophic (B), and conservatively substituted (C) CtpA mutants was examined by Western blotting analyses using antisera raised against recombinant CtpA protein. The arrows indicate the positions of the CtpA proteins.

TABLE III

Photoautotrophic growth, doubling time, CtpA content, PSII-mediated oxygen evolution rate, and half-life of pD1 of mutant strains with semiconservative allelic replacements at five critical residues of the CtpA protein

Results shown are those from a representative experiment.

Strain	Photoautotrophic growth	Doubling time ^a	CtpA content		Rate of O ₂ evolution		pD1 half-life ^b
			h	%	%	min	
Wild type	+	10.5	187	100	100	<5	
<i>ctpA^k</i>	+	12.2	100	100	100	<5	
Δ <i>ctpA</i>	-	NG	0	0	0	>90	
D253E	+	20.5	105	68	68	15	
D253N	+	22.8	102	41	41	30	
R255K	+	34.5	133	27	27	~90	
R255H	-	NG	78	0	0	nd	
S313C	+	18.2	109	82	82	10	
E316D	+	24.8	99	32	32	60	
E316Q	-	NG	53	0	0	nd	
K338H	-	NG	66	0	0	nd	
K338R	-	NG	93	0	0	nd	

^a NG, no growth.

^b nd, not determined because pD1 remains unprocessed (Fig. 5).

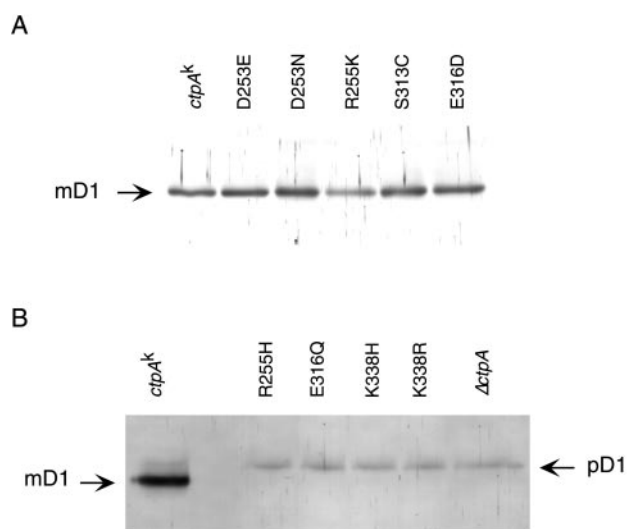


FIG. 5. Immunological detection of the mature (*mD1*) and precursor (*pD1*) forms of the D1 protein in conservatively substituted CtpA mutants. Lysates of mutant cells were prepared as described under "Experimental Procedures." Mutant strains with functional CtpA (Table III) are shown in *panel A*, and those with inactive CtpA are shown in *panel B*.

CtpA. Similarly, the R255K mutant, but not the R255H mutant, could grow photoautotrophically. It is possible that the His residue at this position may remain in its uncharged form, suggesting that the presence of a positively charged residue at position 255 is necessary for CtpA activity. In contrast, both K338R and K338H mutants had no PSII activity and could not grow photoautotrophically. Thus, Lys-338 is an essential residue for CtpA activity. Finally, the S313C mutant exhibited photoautotrophic growth properties and significant PSII-mediated O₂ evolution activity, indicating that a Cys can functionally replace the Ser at the 313 position.

Estimation of the *in Vivo* Half-life of pD1—Radioactive pulse-chase analysis was performed to determine the half-life of the pD1 protein as an estimate of the *in vivo* activity of CtpA in various mutants (Fig. 6 and Table III). All of the non-photoautotrophic alanine substitution mutants had stable pD1 protein. We could not detect any CtpA-dependent processing activity in any of these mutants (data not shown). In the wild type and the *ctpA^k* control strains, the pD1 protein was rapidly converted to its mature form. As described above, five of the nine mutants with conservative replacements could grow autotrophically (Table III). The *in vivo* half-life of pD1 ranged

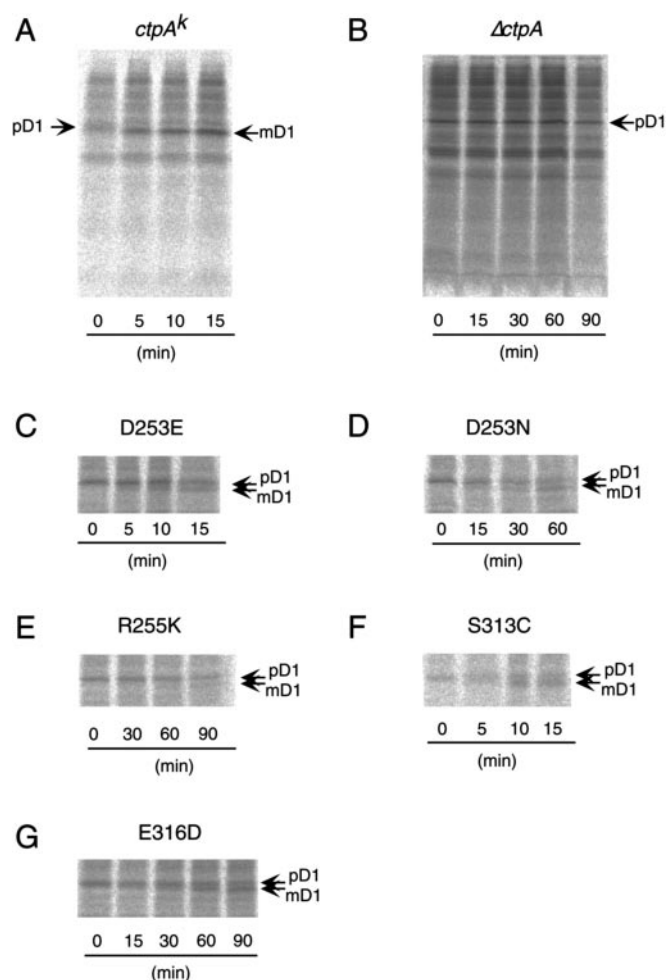


FIG. 6. Pulse-chase analyses to examine D1 processing activity in conservatively substituted CtpA mutants. Cyanobacterial cells were labeled with [³⁵S]methionine for 5 min at 30 °C under illumination and then incubated (chase) in the presence of 200 μg ml⁻¹ chloramphenicol. Cells were collected after the indicated chase periods. Proteins in extracts from such cells were separated by SDS-PAGE. The gels used in this analysis contained 6 M urea for improvement of separation. Labeled proteins were detected by fluorography. The arrows indicate the positions of the precursor form (*pD1*) and the mature form (*mD1*) of the D1 protein, respectively.

between 10 and nearly 90 min in these mutant strains. In particular, the E316D and the R255K mutants had unusually slow D1 processing activities. It is noteworthy that the oxygen

evolution activities and the rates of photoautotrophic growth of these five mutants correlated well with the activities of their CtpA protease, indicating that the processing of D1 is a rate-limiting step during photosynthetic growth of these mutants. In contrast, the other four mutants in this group did not have any CtpA activity, and in these cells, the pD1 protein was stably accumulated (Fig. 5).

DISCUSSION

In the family of carboxyl-terminal processing proteases, CtpA is the only member with a defined and essential physiological activity. The high specific activity of this endoprotease is a critical determinant during the assembly of functionally competent PSII complexes. In this study, we employed site-directed mutagenesis to generate a series of targeted replacement mutants to identify critical residues for *in vivo* catalysis by the CtpA enzyme. Alanine-scanning mutagenesis of 14 selected conserved residues revealed that nine mutants, D149A, R198A, N257A, S315A, D324A, R327A, Q342A, T356A, and D376A, could grow photoautotrophically (Table II). In addition, the D1 protein in these mutants was present in its processed form, indicating that these residues do not significantly contribute to the catalytic activity of CtpA. It is noteworthy that with a single exception (S315A), these mutants had significantly reduced cellular content of CtpA (varying between 2 and 36%). We speculate that a plausible role of these eight conserved residues is maintaining the stable structure of the protease *in vivo*. In contrast, the remaining five residues, namely Asp-253, Arg-255, Ser-313, Glu-316, and Lys-338, are critical determinants for the catalytic activity of CtpA. Alanine substitution of these residues severely disrupted the activity of the CtpA protease. As a consequence, these mutants had unprocessed pD1 protein, lost their oxygen evolution activity, and could not grow photoautotrophically (Table II). All of these five residues are localized in either of the two conserved domains, 1 and 2, of the CtpA protein (Fig. 3a).

Another well studied carboxyl-terminal processing protease is the Tsp enzyme from *E. coli*. In particular, Keiler and Sauer (28) have conducted a detailed *in vitro* study on a series of Tsp allelic replacement mutants to identify the amino acid residues in the active site of this enzyme. They have concluded that catalysis of the Tsp protease is based on a Ser-Lys dyad mechanism, first described for bacterial signal peptidase, an amino-terminal processing enzyme (29, 30). Clearly, Ser-313 and Lys-338, two critical residues in CtpA in *Synechocystis* 6803 (Fig. 3a), are functionally homologous with the two active site residues, Ser-430 and Lys-455, of the Tsp protease (28). In addition, both Tsp (28) and CtpA (Table III) proteases can function when their respective active site Ser residues are replaced by Cys. Our studies also indicated that Lys-338 is an essential residue in CtpA, because all three of the K338A, K338R, and K338H mutants had no CtpA activity. Based only on these data, one may conclude that the catalytic activity of the CtpA protease is based on the Ser-Lys dyad mechanism and hence is similar to that of the Tsp protease as well as the signal peptidase. In this scenario, Ser-313 and Lys-338 in CtpA might act as a nucleophile and a general base, respectively.

Our data also demonstrate that the Asp-253 and Arg-255 residues in the conserved domain 1 were important for the *in vivo* activity of CtpA. A possible explanation for the absence of D1 processing activity in the D253A mutant is its reduced CtpA content (36% of control, Table II). However, mutants such as D149A and R198A, with 5% or less of control CtpA content (Table II), have processed D1 protein. A more reasonable conclusion is that the Asp-253 residue contributes directly to the catalytic activity of CtpA. A similar reasoning is also applicable to the Arg-255 residue. Recent x-ray structural data of the

CtpA protein from the eukaryotic alga *Scenedesmus obliquus* (31) indicate that both the Asp-253 and Arg-255 residues are distant from the catalytic center of CtpA. Interestingly, Liao *et al.* (31) have suggested that the main chain amide of Gly-318 in *Scenedesmus* (the equivalent of Gly-260 of *Synechocystis* 6803 CtpA, Fig. 3b) contributes to the stabilization of a tetrahedral intermediate. We suggest that Asp-253 and Arg-255, two nearby charged residues, indirectly influence such a stabilization process, presumably an important step during the processing reaction catalyzed by CtpA.

An unexpected and exciting finding in the current study is that Glu-316 is also critically important for CtpA activity. In particular, the replacement of Glu-316 with Gln, an uncharged residue with a similar size, abolishes the enzyme activity, whereas replacement of the same residue with Asp still maintains activity although at a reduced level (Fig. 5 and Table III). These data strongly suggest that the negative charge on Glu-316 is directly involved in the catalytic function of CtpA and stand in sharp contrast with the accepted Ser-Lys dyad mechanism of the related enzyme Tsp (28). In this context, it is noteworthy that in the reported structure of *Scenedesmus* CtpA, the carboxyl group of Glu-375 (the equivalent of Glu-316 in *Synechocystis* CtpA) is in close proximity to both of the catalytic residues, Ser and Lys. As pointed out by Liao *et al.* (31), in the resting state of the *Scenedesmus* enzyme, the carboxylate side chain of the Glu-375 residue forms hydrogen bonds with the main chain nitrogens of Gly-396 and Lys-397 and cannot directly interact with the ϵ -amino group of Lys-397. Although it is possible that the negative charge on this glutamate residue indirectly influences the proposed Ser-Lys dyad activity of the CtpA enzyme, our data also raise the possibility of a Ser-Lys-Glu triad mechanism that may be revealed when the structure of the CtpA enzyme with bound substrate (or substrate analog) is examined at a future time. In any case, further studies are needed to clarify the role of this glutamate residue in CtpA and its close relatives.

In summary, we have developed a simple method to identify amino acid residues that are important for the *in vivo* activity of the CtpA protease in the cyanobacterium *Synechocystis* 6803. Current ongoing studies in our laboratories are focused on exploiting this system for a more detailed understanding of the catalytic mechanism of the CtpA enzyme.

Acknowledgments—We thank Dr. V. V. Bartsevich for generating the Δ ctpA *Synechocystis* 6803 mutant strain T795 and Prof. M. Ikeuchi for the antisera against the D1 protein.

REFERENCES

- Keiler, K. C., and Sauer, R. T. (1998) in *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) pp. 460–461, Academic Press, London
- Pakrasi, H. B. (1998) in *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) pp. 462–463, Academic Press, London
- Satoh, K. (1998) in *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., eds) pp. 463–464, Academic Press, London
- Namba, O., and Satoh, K. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 109–112
- Pakrasi, H. B. (1995) *Annu. Rev. Genet.* **29**, 755–776
- Zouni, A., Witt, H. T., Kern, J., Fromme, P., Krauss, N., Saenger, W., and Orth, P. (2001) *Nature* **409**, 739–743
- Zhang, L., Paakkari, V., van Wijk, K. J., and Aro, E. M. (1999) *J. Biol. Chem.* **274**, 16062–16067
- Diner, B. A., Ries, D. F., Cohen, B. N., and Metz, J. G. (1988) *J. Biol. Chem.* **263**, 8972–8980
- Bowyer, J. R., Packer, J. C. L., McCormack, B. A., Whitelegge, J. P., Robinson, C., and Taylor, M. A. (1992) *J. Biol. Chem.* **267**, 5424–5433
- Nixon, P. J., Trost, J. T., and Diner, B. A. (1992) *Biochemistry* **31**, 10859–10871
- Ivleva, N. B., Shestakov, S. V., and Pakrasi, H. B. (2000) *Plant Physiol.* **124**, 1403–1411
- Shestakov, S. V., Anbudurai, P. R., Stanbekova, G. E., Gadzhiev, A., Lind, L. K., and Pakrasi, H. B. (1994) *J. Biol. Chem.* **269**, 19354–19359
- Anbudurai, P. R., Mor, T. S., Ohad, I., Shestakov, S. V., and Pakrasi, H. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8082–8086
- Fujita, S., Inagaki, N., Yamamoto, Y., Taguchi, F., Matsumoto, A., and Satoh, K. (1995) *Plant Cell Physiol.* **36**, 1169–1177

15. Inagaki, N., Yamamoto, Y., Mori, H., and Satoh, K. (1996) *Plant Mol. Biol.* **30**, 39–50
16. Oelmüller, R., Herrmann, R. G., and Pakrasi, H. B. (1996) *J. Biol. Chem.* **271**, 21848–21856
17. Yamamoto, Y., Inagaki, N., and Satoh, K. (2001) *J. Biol. Chem.* **276**, 7518–7525
18. Silber, K. R., Keiler, K. C., and Sauer, R. T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 295–299
19. Keiler, K. C., Waller, P. R. H., and Sauer, R. T. (1996) *Science* **271**, 990–993
20. Allen, M. M. (1968) *J. Phycol.* **4**, 1–4
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, New York
22. Picard, V., Erdsdal-Badju, E., Lu, A., and Bock, S. C. (1994) *Nucleic Acids Res.* **22**, 2587–2591
23. Dzelzkalns, V. A., and Bogorad, L. (1988) *EMBO J.* **7**, 333–338
24. Bartsevich, V. V., and Pakrasi, H. B. (1995) *EMBO J.* **14**, 1845–1853
25. Mannan, R. M., and Pakrasi, H. B. (1993) *Plant Physiol.* **103**, 971–977
26. Lichtenthaler, H. K. (1987) *Methods Enzymol.* **148**, 350–382
27. Laemmli, U. K. (1970) *Nature* **227**, 680–685
28. Keiler, K. C., and Sauer, R. T. (1995) *J. Biol. Chem.* **270**, 28864–28869
29. Paetzel, M., and Dalbey, R. E. (1997) *Trends Biochem. Sci.* **22**, 28–31
30. Paetzel, M., Dalbey, R. E., and Strynadka, N. C. (1998) *Nature* **396**, 186–190
31. Liao, D.-I., Qian, J., Chisholm, D. A., Jordan, D. B., and Diner, B. A. (2000) *Nat. Struct. Biol.* **7**, 749–753