A sequential two-step proteolytic process in the carboxyl-terminal truncation of precursor D1 protein in *Synechocystis* sp. PCC6803[1]

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Abstract The D1 protein of photosystem II is synthesized in precursor with a carboxyl-terminal extension. Interestingly, there is quite a range in chain length of the extension, which roughly depends upon the class of organisms. In cyanobacteria, e.g. in *Synechocystis* sp. PCC6803, the extension consists of 16 amino acid residues, seven residues longer than its counterpart in higher plants. In this study, we examined the D1 processing in *Synechocystis* sp. PCC6803 by pulse-chase experiments and detected a proteolytic intermediate of this process. This finding suggests that the elongated extension in this organism is excised with a sequential two-step proteolysis, which differs markedly from the manner observed in higher plants.

Key words: Carboxyl-terminal processing protease; D1 protein; Photosystem II; *Synechocystis* sp. PCC6803

1. Introduction

The D1 protein is known to be an integral subunit of photosystem II (PSII), a pigment–protein complex in the thylakoid membrane in oxygen-evolving photosynthetic organisms. The D1 protein, together with the D2 protein, a homologous counterpart, provides binding sites with an appropriate microenvironment to prosthetic groups involved in the primary reaction of PSII [1,2]. Even in its functional importance, the D1 protein is also known to be an unstable component under conditions of high light intensity. The protein is damaged by reactive species produced as an inevitable consequence of the normal functioning of PSII and is rapidly replaced by a de novo-synthesized copy [3]. The D1 protein is synthesized in precursor form (pD1) with an extended sequence on its carboxyl terminus [4]. The C-terminal extension is removed by a specific endoprotease, carboxyl-terminal processing protease (CtpA) [5,6], as an essential step prior to the assembly of the manganese cluster of the oxygen-evolving complex of PSII [7,8]. A probable explanation for the removal is that the C-terminus of the mature form of the D1 protein (mD1) functions as a ligand for the manganese cluster [9].

The amino acid sequences of D1 protein deposited in the databases are highly conservative [10]. One notable exception is that the chain length of the C-terminal extension of pD1 as well as its sequence shows variability roughly depending upon the class of organisms [11]. In the cyanobacterium, *Synechocystis* sp. PCC6803 (hereafter *Synechocystis* 6803), the extension consists of 16 residues [9], while that of higher plants examined to date consists of nine residues [12]. The physiological significance of this variability at the molecular level is not yet clear; however, it is noteworthy that not only the presence of C-terminal extension but also its sequence might be required for optimal photosynthetic performance in *Synechocystis* 6803 cells [11].

CtpA proteins have been identified from cyanobacteria [5,13], green alga [14], and higher plants [6,15] as a monomeric-active protease of 42–45 kDa. The catalytic center of this protease was shown to be a Ser–Lys dyad, based on a newly emerging catalytic chemistry for hydrolase [16], by site-specific mutagenesis of cyanobacterial CtpA [17] and crystal-structure analysis of algal CtpA [18].

In this article, we report a novel manner for the processing of D1 protein by CtpA in *Synechocystis* 6803. We unexpectedly detected an intermediate stage in the processing reaction in pulse-chase experiments. Further analyses of this intermediate demonstrated that *Synechocystis* CtpA excises the C-terminal extension by a sequential two-step proteolytic process. This is in contrast to higher plants in which the C-terminal extension is removed by a single proteolytic step.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A glucose-sensitive wild-type strain of *Synechocystis* 6803 and its sibling CtpA mutants, S313C and E316D, were used in this study. Details of these strains and basic procedures for the culture are described elsewhere [17]. Cells were grown photoautotrophically in liquid BG-11 medium at 30°C under continuous illumination from fluorescent lamps at 50 μmol photons m^{-2} s^{-1}.

2.2. Pulse-chase experiment

Cells in late-log phase were collected, washed twice, and then suspended with BG-11 medium at a final concentration of 1×10^8 cells ml^{-1}. Two hundred microliters of the cell suspension was incubated in the presence of 5 μl of the Redivue™ Pro-mix™ L-[^{35}S] in vitro cell labeling mix (a mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine for cell labeling experiments, in which the ratio of methionine:cysteine is approximately 7:3; >37 TBq $mmol^{-1}$, 530 MBq ml^{-1}; Amersham Pharmacia Biotech) at 15°C for 10 min with gentle shaking under

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Abbreviations: CtpA, carboxyl-terminal processing protease; IB, intermediate band; LEP, Lysyl Endopeptidase; mD1, mature form of D1 protein; pD1, precursor form of D1 protein; PSII, photosystem II

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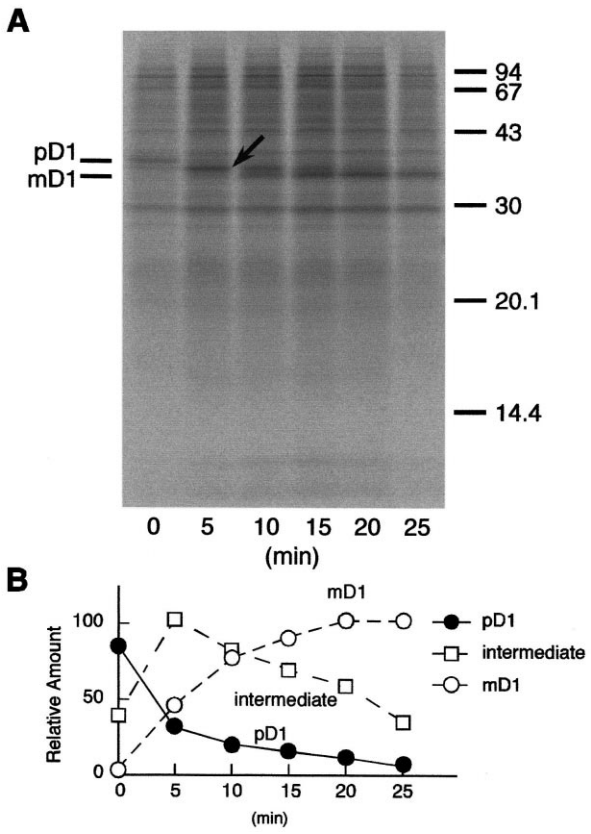

Fig. 1. The appearance of a novel band during a pulse-chase experiment at low temperature. A: *Synechocystis* 6803 cells were labeled at 15°C for 10 min (0 min) and further incubated at 25°C for 5–25 min in the presence of chloramphenicol. Positions of pD1 and mD1 proteins are indicated by bars at the left side of the autoradiogram. An arrow indicates the position of the IB. Bars with numbers at the right side of the column represent molecular mass marker proteins. B: A graph shows a transition of the relative amounts of pD1 (closed circle), the IB (square) and mD1 (open circle), respectively during the pulse-chase experiment shown as A. All data were adjusted by the amount of mD1 at 20 min in the chasing period as 100%.

illumination from heat-absorbed incandescent lamps at 200 μmol photons m^{-1} s^{-1}. After the labeling, the cell suspension was further incubated at 25°C in the presence of 200 μg ml^{-1} chloramphenicol. Cells withdrawn at appropriate intervals were collected by centrifugation and then washed with an ice-cooled 20 mM HEPES/KOH buffer (pH 7.5) containing 10 mM NaCl, 6 mM $MgCl_2$, 1 mM EDTA, and 200 μg ml^{-1} chloramphenicol. The cells were ground in a small glass homogenizer with glass beads (106 μm and finer; Sigma) in 200 μl of SDS–PAGE sample loading buffer.

2.3. SDS–PAGE and peptide-mapping analysis

Labeled proteins were separated by SDS–PAGE [19] with minor modifications. In this study, separation gels contained 15% (w/v) polyacrylamide and 7 M urea. Peptide-mapping was performed with a method described elsewhere [15] using Lysyl Endopeptidase (LEP; Wako Pure Chemical Industries, Osaka, Japan). After electrophoresis, gels were soaked in a solution containing 20% (v/v) methanol and 5% (v/v) acetic acid and then dried onto filter paper. Visualization and quantitative analysis of labeled proteins were accomplished with an imaging analyzer, model BAS-2000 (Fuji Film, Tokyo, Japan).

3. Results

3.1. Pulse-chase experiment at low temperature

Our previous study showed that the processing of pD1 in *Synechocystis* 6803 proceeded rapidly at 30°C, a normal growth temperature for this organism [17]. To design the pulse-chase experiment, we considered the result reported by Kanervo et al. that the processing in this organism was severely inhibited when cells were cultured at 15°C [20]. Under these conditions, the pD1 in thylakoid, possibly because CtpA activity and/or its accessibility to the cleavage site on substrate, were significantly restricted. Accordingly, we applied 15°C for the labeling of *Synechocystis* 6803 cells in this study. Fig. 1A clearly shows that the D1 protein labeled for 10 min at this temperature was still its precursor form (0 min). This was confirmed by co-electrophoresis with pD1 from a CtpA null mutant (data not shown) [5]. We added chloramphenicol, a translation inhibitor, to the cell suspension and further incubated the suspension at 25°C. During the incubation, the pD1 band rapidly disappeared, and the mD1 band gradually emerged as expected (Fig. 1A). During the chasing period, a sharp band appeared transiently and unexpectedly, migrating as a speed which was intermediate between that of the pD1 and mD1 bands (arrow in Fig. 1A). Fig. 1B shows the transition between relative amounts of these three bands during the chasing period.

To examine whether the intermediate band (IB) was the D1 protein or not, we conducted immunoprecipitation analysis using antiserum raised against the spinach D1 protein (a generous gift from Dr. M. Ikeuchi, University of Tokyo). This antiserum specifically detected the *Synechocystis* D1 protein (data not shown). In this immunoprecipitation analysis, the IB could be precipitated as efficiently as pD1 or mD1 (data not shown), suggesting that the band corresponds to a variant of the D1 protein.

3.2. Peptide-mapping analysis of the IB

The D1 protein of *Synechocystis* 6803 is known to have only one lysine residue; at the 238th position. A lysine-specific protease, LEP, consequently separates the D1 protein into two fragments (Fig. 2A). The resulting N-terminal part is estimated to consist of 238 residues and the remaining C-terminal part is variable depending on the state of processing; the C-terminal fragment from the pD1 is estimated to consist of 122 residues, while that from mD1 is estimated to be 106 residues.

The pD1, intermediate, and mD1 bands isolated from a preparative polyacrylamide gel were subjected to peptide-mapping analysis. Fig. 2B shows that the larger fragments corresponding to the N-terminal part had apparently identical mobilities in the resultant gel. On the other hand, the C-terminal fragment from the IB showed a different mobility from both corresponding parts of pD1 and mD1, respectively. This suggests that the IB was caused by an alteration in its C-terminal fragment.

3.3. Pulse-chase experiment using CtpA mutants

It is well known that cells exposed to low temperature show several aberrant responses. To remove the possibility that the appearance of the IB was induced by incubation at lower temperature, we conducted a pulse-chase experiment at 30°C, a normal growth temperature for this organism. In this experiment, we effectively used mutants in which in vivo activity of the CtpA was significantly reduced by substitution of a critical residue for the catalysis of this protease. Our previous study demonstrated that the catalytic serine (S313

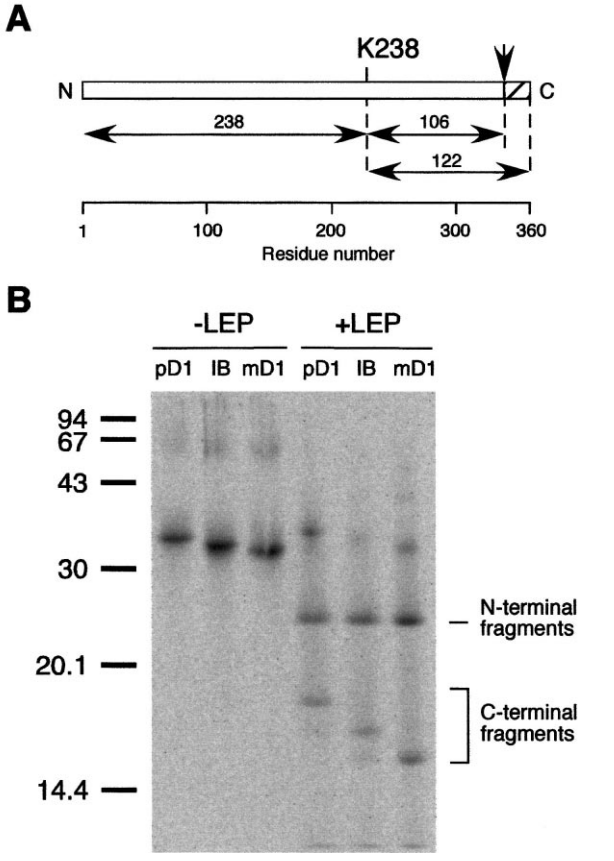

Fig. 2. Peptide-mapping analysis of the IB. A: A schematic drawing of the D1 protein with a speculative fragment pattern after LEP treatment. The downward arrow indicates a C-terminus of mD1, and the shaded box corresponds to the C-terminal extension. B: Three bands, pD1, the IB, and mD1, isolated from a preparative polyacrylamide gel, were re-electrophoresed in the absence (−LEP) and presence (+LEP) of 1.8 μg of LEP.

in *Synechocystis* 6803) is substitutable for cysteine with a moderate reduction of the in vivo activity of CtpA [17]. This mutant still maintains photoautotrophic growth competence, indicating that the substitution did not affect the specificity of the CtpA with respect to substrate recognition and cleavage. The specificity is known to be rigidly connected with formation of the photosynthetic oxygen-evolving machinery of PSII [7–9,17], which is absolutely required for photoautotrophic growth competence. Fig. 3B shows that the IB was observed even at 30°C using the CtpA mutant, S313C. We also detected the IB in another CtpA mutant, E316D (Fig. 3C). The mutant still maintains photoautotrophic growth competence; however, the CtpA activity was more repressed than that in the mutant S313C [17]. These results clearly show that the appearance of the IB is not an artifact caused by incubation at lower temperature. In addition, the results also suggested that both the appearance and disappearance of the IB seem to depend on the in vivo activity of the CtpA in each strain (Fig. 3D). In the wild-type cells, the processing reaction proceeded rapidly at 30°C; therefore, the IB was weakly detected at 0 min and disappeared quickly within 5 min of the chasing period (Fig. 3A). In the mutant S313C, the IB gradually emerged and was more stable than that in wild-type during the chasing period (Fig. 3B). In the mutant E316D, an incubation period of 60 min was necessary to detect the IB, and the mD1 band could not be detected during this period (Fig. 3C). These observations suggested that not only the appearance but also disappearance of the IB was mediated by the action of CtpA.

4. Discussion

The C-terminal processing of D1 protein is known to be a prerequisite step in the integration of photosynthetic oxygen-evolving machinery in PSII [7–9]. In spite of its functional importance, many aspects of this proteolytic process remain unclear. For instance, a variability found in the chain length of C-terminal extensions among different classes of oxygenic-photosynthetic organisms has yet to be explained. In most cyanobacteria including *Synechocystis* 6803, the C-terminal extension consists of 16 residues [9], seven more than found in its counterpart in higher plants (Fig. 4). However, we could

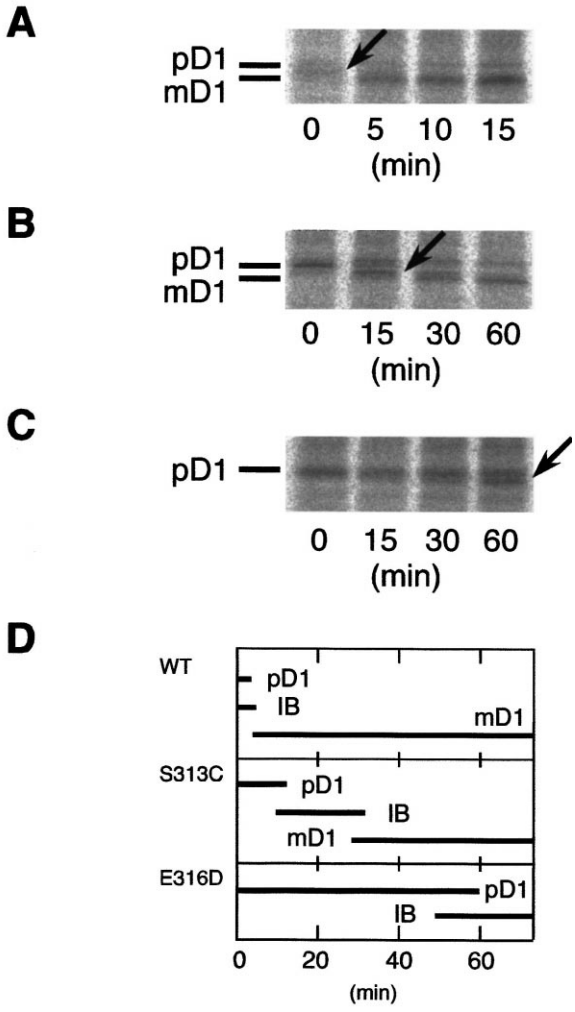

Fig. 3. The appearance of the IB during the pulse-chase experiment using the wild-type (A) and CtpA mutants S313C (B) and E316D (C), at a normal growth temperature. The cells were labeled at 30°C for 10 min (0 min) and further incubated at the same temperature in the presence of chloramphenicol. The bars on the left side indicate the positions of pD1 and mD1. Arrows indicate the position of the IB. D: A schematic drawing of the band compositions during the pulse-chase experiment at 30°C. The bold lines represent the most abundant component at each period in the resultant gels shown in A–C.

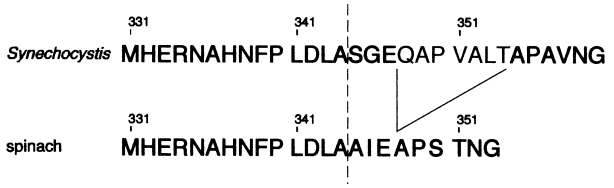

Fig. 4. A sequence comparison of the C-terminal part of the D1 protein. An amino acid sequence alignment of the D1 protein from *Synechocystis* sp. 6803 and *Spinacia oleracea* (spinach). The vertical broken line indicates the C-terminus of mD1. The *Synechocystis* C-terminal extension consists of 16 residues and has the seven extra residues (shown gray) at its middle part.

not clarify the evolutional relationship, i.e. whether the variety was caused by a deletion from the cyanobacterial extension or an insertion into the prototype of higher plant extension. The fact that the extra sequence has been conserved in current cyanobacteria [11] suggests its physiological importance, even though there is as yet no reasonable explanation at molecular level.

In this study, we followed the details of the processing reaction in a cyanobacterium, *Synechocystis* 6803 using pulse-chase experiments. Unexpectedly, we detected a novel transient band in an SDS–polyacrylamide gel, migrating at a speed intermediate between that of pD1 and mD1 (Fig. 1). Fig. 1B showed that the increment of the IB for the first 5 min of the chasing period was likely to correlate with the decrement of pD1 band reciprocally. The data could be interpreted that the newly detected IB appeared as a consequence of partial truncation of pD1. Similar reciprocity was observed between intermediate and mD1 during the further incubation, suggesting that the intermediate finally converted into mD1 in a sequential process. Peptide-mapping analysis (Fig. 2B) supported the idea that D1 protein is subjected to stepwise truncation at its C-terminus. Furthermore, pulse-chase experiments at a normal growth temperature using CtpA mutants (Fig. 3) revealed that both the appearance and disappearance of the IB were tightly associated with in vivo activity of the CtpA. Therefore, we interpret that the newly detected band is a processing intermediate of the D1 protein. It also suggests that a single protease, i.e. *Synechocystis* CtpA, excises the C-terminal extension in a sequential two-step proteolysis.

A series of analyses on spinach CtpA, a well-defined orthologous protease in a higher plant, using synthetic oligopeptides as substrates demonstrated that the CtpA in this organism specifically cleaves the peptide bond between Ala-344 and Ala-345 of D1 protein [21,22]. No intermediate could be detected in previously conducted pulse-chase experiments in higher plants [23,24], suggesting that the C-terminal extension in higher plants is removed by the CtpA in a single proteolytic step. Thus, our finding that the C-terminal extension in *Synechocystis* 6803 is excised with a sequential two-step manner was unexpected, and very interesting from the view point that the manner might be based on the unique feature of the CtpA and/or the C-terminal extension in this organism.

Unfortunately, the first cleavage site in this sequential two-step processing has not been determined in this study. However, the band separation in peptide-mapping analysis (Fig. 2B) suggested that the site is near the center of the C-terminal extension, possibly located in the middle of the seven extra residues (Fig. 4). The spinach CtpA is known to be a highly specific protease, which recognizes the successive sequence around the cleavage site of pD1 (PLDLA:A) for specific cleavage at the scissile peptide bond [21,22]. The sequence of *Synechocystis* D1 protein around the final cleavage site, the peptide bond between Ala-344 and Ser-345, shows significant homology with the spinach sequence (Fig. 4). In the middle part of the *Synechocystis* extension, a sequence from Pro-350 and Ala-355 (PVALT A) is markedly homologous with the spinach sequence around the cleavage site. The sequence consists of a proline, hydrophobic residues (V and L), and relatively smaller residues (T and A), which are ranged with similar manner of the spinach sequence (PLDLA:A); therefore, the the peptide bond between Thr-354 and Ala-355 is the most probable candidate of the first cleavage site in the sequential two-step processing. However, it is noteworthy that negative charge at P3 position (Asp-342 in the spinach sequence), which was elucidated to be especially critical for the cleavage by the spinach CtpA [21,22], is not conserved in this sequence. This suggests that the *Synechocystis* CtpA may have a different property about recognition of the P3 residue for the first cleavage.

The fundamental question remaining to be answered is why the endoprotease CtpA cleaves the C-terminal extension in a two-step manner. We speculated a simple possibility that the seven extra residues in the extension restrict accessibility of CtpA to the final cleavage site possibly by steric hindrance. The alternative possibility is that the manner might be caused by the specific motif in CtpA, i.e. PDZ-domain, which provides an ability for binding to the C-terminus of the substrate [18,25]. The C-terminal extension fixed by the PDZ-domain might be truncated stepwise. Further analysis is evidently needed to clarify the molecular mechanism and significance of the sequential two-step processing in cyanobacteria.

In conclusion, we reported a novel and unexpected manner of processing the D1 protein in *Synechocystis* 6803. Further analyses of this phenomenon will provide clues to elucidate not only the molecular mechanism of the process but also the other remaining questions, e.g. the evolution of the C-terminal extension and its processing.

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