

Competitive inhibition analysis of the enzyme-substrate interaction in the carboxy-terminal processing of the precursor D1 protein of photosystem II reaction center using substituted oligopeptides

Yumiko Yamamoto, Kimiyuki Satoh*

Department of Biology, Okayama University, Okayama 700-8530, Japan

Received 24 March 1998

Abstract A clear parallelism was demonstrated between the efficiency as substrate of the substituted oligopeptides corresponding to the carboxy-terminal (C-terminal) sequence of the precursor D1 protein (pD1) in the in vitro enzymatic assay and their competitive inhibitory capacity toward the proteolytic C-terminal processing of the full-length pD1 integrated in the intact photosystem II complex embedded in the thylakoid membrane of *Scenedesmus obliquus* LF-1 mutant, as shown e.g. by the influence of L343A, A345G and A345V substitutions and the effect of C-terminal fragments. This suggests that the basic mechanism for substrate recognition by the processing protease elucidated in the enzymatic analysis using synthetic oligopeptides is also effective in vivo, although it can sometimes be difficult to detect the consequence of amino acid substitution in the integrated systems.

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Key words: C-terminal processing; Competitive inhibition; D1 protein; Photosystem II; Processing protease; Synthetic oligopeptide

1. Introduction

The D1 subunit of the photosystem II (PSII) reaction center [1] is synthesized as a precursor which is immediately converted into the mature form by excising its carboxy-terminal (C-terminal) extension consisting of 8–16 amino acids. The C-terminal cleavage of the precursor D1 protein (pD1) is catalyzed by a nuclear-encoded endopeptidase, which functions in the lumenal space of thylakoids, in eukaryotic organisms [2,3]. This proteolytic processing of pD1 is indispensable to the integration of the Mn cluster of the water-splitting machinery in the PSII, as demonstrated by studies of an X-ray induced-mutant of *Scenedesmus obliquus* (LF-1) [4] and site-directed mutants of *Synechocystis* sp. PCC 6803 [5]. A deletion analysis of the D1 protein in *Synechocystis* has suggested that the C-terminus of mature D1 protein directly ligates to the Mn cluster, as a possible mechanism [5]. The physiological role of the C-terminal extension in the precursor protein, therefore, is speculated to be the protection of the C-terminus of D1 protein against unfavorable interactions with its surroundings under certain circumstances during the integration of newly synthesized proteins into the active oxygen-evolving complex.

The protease involved in this process has been extracted and purified to near homogeneity from spinach [6] and more recently in its pure state from *Scenedesmus obliquus* [7]. The gene for the protease (*ctpA*) has been identified based

on information regarding the partial amino acid sequence for spinach and *Scenedesmus* proteins, as well as on the genetic complementation analysis for the PSII-deficient mutant in *Synechocystis* sp. PCC 6803 [2,6–8]. A low but significant homology of the deduced amino acid sequence of CtpA with that of a tail-specific protease (Tsp) in *Escherichia coli* has suggested that the enzyme can be classified as a unique, new type of serine protease, which uses the catalytic hydroxyl/amine dyads as its active center [9,10].

In order to analyze the mechanism of this unique enzymatic process and to understand the role of the proteolytic processing in the integration of oxygen-evolving machinery in the PSII, synthetic oligopeptides corresponding to the C-terminal sequence of spinach pD1 have been utilized in in vitro analyses [11,12]. In these studies, substituted C-terminal oligopeptides were synthesized in order to analyze the recognition signal for the protease on pD1 [12]. These in vitro studies demonstrated that the amino acid residue at the cleavage site on pD1 (+1 position, Ala-345 in spinach) largely influences to the rate of proteolysis in the order of Ala, Ser, Cys, Phe > Gly > Val ≫ Pro = 0. On the other hand, we have constructed site-directed mutants of *Chlamydomonas reinhardtii* in which the +1 position of pD1 (Ser in this organism) is replaced by Gly, Val, Cys or Phe, in order to confirm the predicted influence of the reduced rate of C-terminal processing due to the amino acid substitution at the cleavage site in the photosynthetic performances of living cells [13]. However, the result of analysis indicated that the apparent rate of C-terminal processing of pD1 in vivo estimated by a pulse-chase experiment, as well as the steady-state level of D1 protein, appeared to be independent of the +1 substitution. This raises the suspicion that the mechanism of substrate recognition elucidated by the in vitro analysis using synthetic oligopeptides of short chain length may not be effective in the in vivo system with full-length pD1 of about 34 kDa in the multi-subunit PSII complex embedded in the thylakoid membrane.

In this paper, the recognition mechanism for pD1 by the isolated spinach protease was studied by analyzing the competitive inhibitory effect of the C-terminal oligopeptides on the proteolytic C-terminal cleavage, using the following two classes of substrate of full-length size: (1) in vitro transcribed/translated pD1, and (2) pD1 in the PSII complex embedded in the PSII membrane from LF-1 mutant of *Scenedesmus* which lacks the proteolytic activity of C-terminal processing for pD1 [4,7]. The results indicated that the basic conclusion for the enzyme-substrate interaction derived from the previous in vitro analysis using synthetic oligopeptides as substrate is absolutely valid in the proteolytic processing of pD1 integrated into the functional PSII complex.

*Corresponding author. Fax: (81) (86) 251-7862.

E-mail: kimiyuki@cc.okayama-u.ac.jp

2. Materials and methods

2.1. Preparation of enzyme

The processing protease for pD1 of the PSII reaction center was partially purified from sonication extracts of spinach thylakoids by the method which includes hydroxylapatite and Sephadex G-75 gel-filtration chromatographies as described previously [12]. Fractions containing the enzymatic activity were dialyzed against a 25 mM HEPES/KOH (pH 7.7) buffer to remove NaCl, which inhibits the proteolytic cleavage at higher concentrations.

2.2. Preparation of synthetic oligopeptides

The substituted oligopeptides corresponding to the C-terminal sequence of pD1 deduced from the nucleotide sequence of spinach *psbA* gene were synthesized by a peptide synthesizer and purified as described previously [12].

2.3. Preparation of substrate

2.3.1. PSII-enriched membrane with precursor D1 protein. The PSII-enriched membrane containing pD1 was prepared from the LF-1 mutant of *Scenedesmus obliquus*, which was kindly provided by Dr. N.I. Bishop (Oregon State University, Eugene, OR, USA) [4]. The algal cells were grown in the dark at 25°C on NGY medium. The PSII-enriched membrane was prepared from cells in the logarithmic growth phase according to Kuwabara and Murata's method as modified by Metz and Seibert [14].

2.3.2. In vitro transcribed/translated precursor D1 protein. The pD1 was synthesized by in vitro SP6 RNA polymerase transcription of linearized pSPTB28, a clone containing the tobacco *psbA* gene, followed by the translation of capped transcripts in a wheat germ translation system in the presence of ³⁵S-Met [6]. The reaction mixture for the translation which contained radiolabeled pD1 was diluted twice with distilled water and then stored at −80°C until use.

2.4. Proteolytic processing assay

2.4.1. PSII-enriched membrane as substrate. The standard mixture (100 µl) contained 10 µg Chl of PSII-enriched membrane, partially purified spinach enzyme (10 µg protein) and 40 mM Na/K phosphate buffer (pH 6.9). The proteolytic reaction was carried out at 25°C for 20 min in the presence or absence of oligopeptide. After collecting the membrane by centrifugation at 15 000 × g for 3 min at 0°C, the membrane was solubilized by the sample buffer for electrophoresis (125 mM Tris-HCl pH 6.8 at 25°C, 5% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) 2-mercaptoethanol), and then an aliquot of the extracts (2.5 µg Chl of PSII-enriched membrane) was subjected to SDS-PAGE. Following Western blotting on the nitrocellulose membrane, the processing activity was visualized by using an antiserum raised against spinach D1 protein (kindly provided by Dr. M. Ikeuchi, Tokyo University).

2.4.2. In vitro transcribed/translated precursor D1 protein as substrate. The enzymatic activity was assayed by measuring the molecular mass shift of the radiolabeled pD1 into a smaller-sized product, i.e. mature D1 protein. The incubation mixture (27.5 µl) contained 2.5 µl of radiolabeled pD1, partially purified spinach enzyme (3 µg protein) and 25 mM HEPES/KOH (pH 7.7) buffer. After incubation for 3 h at 25°C in the presence or absence of oligopeptide, the proteolytic reaction was terminated by the addition of an equal volume of the sample buffer for electrophoresis, and then radiolabeled proteins were separated by SDS-PAGE and analyzed by autoradiography, as described previously [6].

3. Results and discussion

The upper lane of Fig. 1 shows the C-terminal sequence of 24 amino acids for spinach pD1 [15]. In the in vitro experiment using isolated C-terminal processing protease from spinach, it has been shown that the synthetic oligopeptide corresponding to the C-terminal sequence of pD1 of the size of this chain length can specifically be cleaved at exactly the same position as in vivo, i.e. the carboxy side of Ala-344 [12]. On the other hand, the synthetic C-terminal oligopeptide has also been shown to inhibit the proteolytic processing of

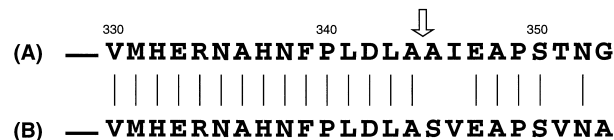


Fig. 1. Deduced C-terminal sequences of pD1 for spinach (A) and *Scenedesmus obliquus* (B) [19]. An arrow indicates the cleavage site for processing protease. Numbers are counted from the N-terminus (Met-1).

the full-length pD1 translated in vitro using wheat germ extracts [16]. This inhibition is competitive in nature, namely, the synthetic oligopeptide functions as a competitive substrate to inhibit the proteolytic C-terminal cleavage of the full-length pD1. Therefore, the degree of inhibition represents the affinity of the synthetic oligopeptide toward the processing protease, relative to the full-length pD1.

The competitive inhibitory effect of the C-terminal fragments of pD1 was analyzed for the processing of full-length pD1 translated in vitro, using the following three kinds of synthetic oligopeptides, i.e. the C-terminal extension consisted of 9 amino acids (C-9), the C-terminal 15 amino acid sequence of the mature D1 protein (N-15) and the C-terminal 24 amino acid sequence of pD1 (S-24) which corresponds to the peptide-linked product between N-15 and C-9. With the addition of S-24 to the reaction mixture for proteolysis, the enzymatic activity of C-terminal cleavage for the full-length pD1 gradually decreased with increasing concentrations of the oligopeptide. In contrast, the addition of N-15 or C-9 to the reaction mixture exhibited no inhibitory effect in the concentration range tested. This result indicates that the C-terminal oligopeptide substrate (S-24) loses its affinity toward the enzyme as a result of cleavage into two molecular moieties, i.e. to N-15 and C-9 fragments, as generally anticipated. Thus, it is concluded that the structure of substrate, formed by a linkage between the C-terminal terminal part of the mature D1 protein and the C-terminal extension of pD1, which is cleaved off by the action of protease, is important for the enzyme in recognizing the substrate.

The D1 protein is synthesized by thylakoid-bound polyosomes on the stromal surface of the membrane and then integrated into the PSII complex consisting of multi-subunits. The proteolytic processing by the enzyme at the C-terminus seems to occur immediately after the insertion of the precursor protein into the multi-subunit PSII complex at the luminal space of thylakoid of acidic pH [17]. It is therefore suspected that the mode of enzyme-substrate interaction in the proteolytic processing of full-length pD1 in vivo which takes place in the acidic luminal space of thylakoid under functional conditions could be significantly different from that in vitro described above and elsewhere [11,12], and this could be the reason why there was no detectable effect of the amino acid substitution at the cleavage site (+1 position) in the in vivo experiment using *Chlamydomonas* [13]. Thus, we repeated the same kind of analysis described above using the pD1 integrated into the PSII complex in the thylakoid membrane as a substrate by utilizing the PSII-enriched membrane from the LF-1 mutant of *Scenedesmus obliquus* (see Fig. 1), in which the processing protease is inactive but the core part of PSII is properly integrated into the thylakoid membrane and functional, as demonstrated by the biophysical analysis [18] and the photo-reactivation study [3]. By incubating with spinach

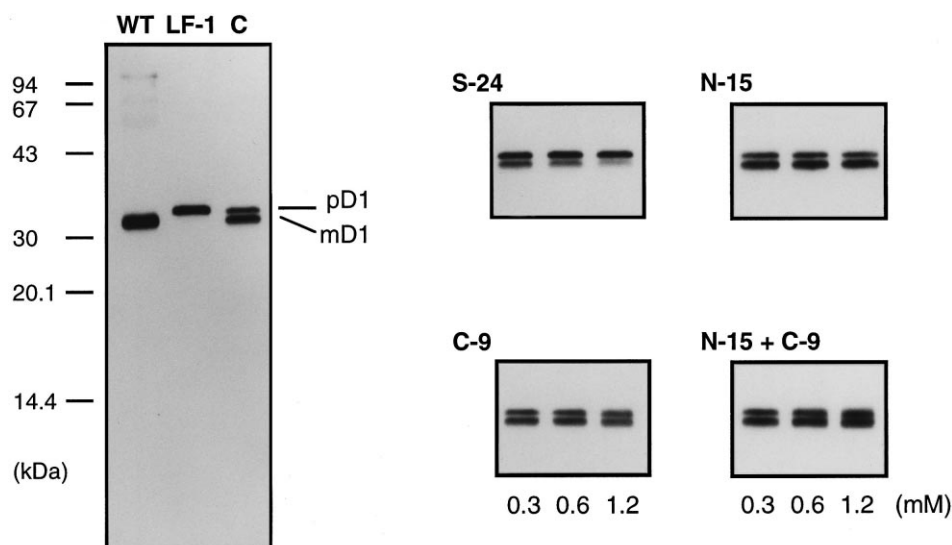


Fig. 2. Inhibitory effect of oligopeptides on the C-terminal processing of pD1 embedded in the thylakoid membrane of *Scenedesmus*. The enzymatic reaction was carried out as described in Section 2, in the presence or absence of oligopeptides. Lanes WT and LF-1 correspond to the non-treated PSII-enriched membrane from wild-type and LF-1 mutant of *Scenedesmus*, respectively. Concentrations of added oligopeptide are indicated for the four panels on the right side, at the bottom. S-24, C-terminal 24 amino acids of the pD1; N-15, C-terminal 15 amino acids of the mature D1 protein; C-9, C-terminal extension consisting of 9 amino acids; C, control (LF-1, treated with protease). pD1, precursor D1 protein; mD1, mature D1 protein.

processing protease for 20 min at 25°C, pD1 of the LF-1 mutant of *Scenedesmus* with a molecular mass of about 34 kDa shifted down to an approximately 2 kDa smaller size, but no further degradation could be observed on the chromatogram of SDS-PAGE (left part of Fig. 2). Thus, the processing of pD1 in the PSII complex of *Scenedesmus* by spinach protease presumably occurs correctly at the C-side of Ala-344, judging from the size of the cleavage product as analyzed by lysyl-endopeptidase treatment (data not shown). The enzymatic processing of the intact, full-length pD1 integrated into the PSII complex was markedly inhibited by the addition of S-24 oligopeptide in a concentration-dependent fashion, but not by N-15, C-9, or N-15 plus C-9, as in the case of in vitro translated pD1 in solution (Fig. 2). Therefore, it can be

concluded that the basic mechanism for substrate recognition in the C-terminal processing of pD1 is identical between systems with the following three classes of substrate of different integrity, i.e. the full-length pD1 in the PSII complex embedded in the thylakoid membrane, the in vitro translated full-length pD1 in buffer solution and the synthetic C-terminal oligopeptide of short chain length in an in vitro system [11,12].

In order to further analyze the recognition signal on substrate for the C-terminal processing protease of pD1 in its native state, we designed a series of synthetic C-terminal oligopeptides of 19 amino acids with substitution(s) around the processing site; (i) Leu-343 at the -2 position, which is completely conserved among the *psbA* genes so far sequenced for

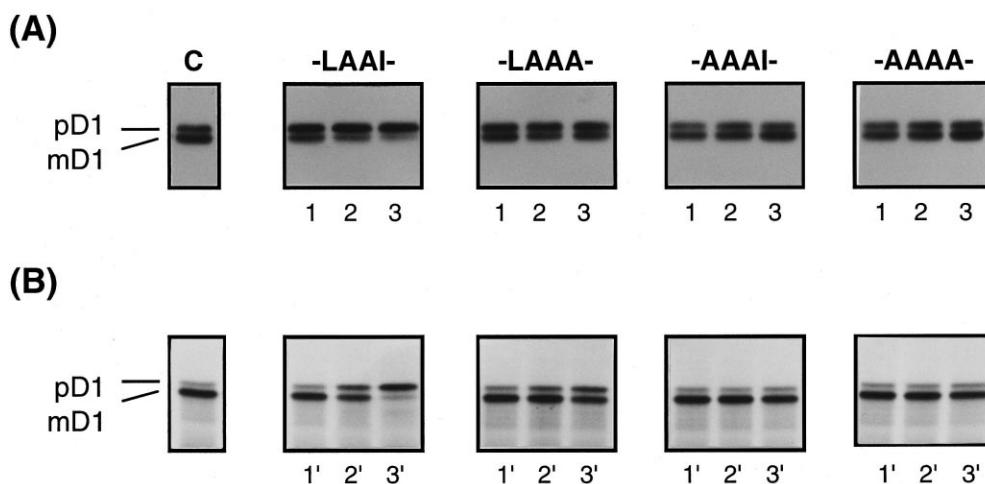


Fig. 3. Inhibitory effect of substituted oligopeptides on the C-terminal processing of pD1. pD1 integrated into the thylakoid membrane of *Scenedesmus* (A) and in vitro translated pD1 (B) were treated with protease in the presence of the following oligopeptides: LAAI, C-terminal 19-mer of pD1 without substitution; LAAA, with I346A substitution; AAAI, with L343A substitution; AAAA, with both L343A and I346A substitutions. Concentrations of oligopeptide; 0.3 mM (lane 1), 0.6 mM (lane 2), 1.2 mM (lane 3), 0.181 mM (lane 1'), 0.545 mM (lane 2'), 1.09 mM (lane 3'). C, no addition.

a wide variety of organisms, ranging from cyanobacteria to higher plants, was replaced by Ala (L343A); (ii) Ile-346 at the +2 position (in spinach), which is conservatively substituted to Val or Gly in some organisms, was altered to Ala (I346A); (iii) both Leu-343 and Ile-346 were altered to Ala (L343A and I346A) [15]. In our previous study using these substituted C-terminal oligopeptides as a substrate for the partially purified protease from spinach, L343A, L343A and I346A could not be cleaved, whereas I346A could be cleaved although the efficiency was appreciably lower than that for the control [12]. Fig. 3 shows the competitive inhibitory effect of these substituted oligopeptides on the enzymatic C-terminal cleavage of pD1 under two different conditions described above, i.e. pD1 integrated into the PSII complex in the thylakoid membrane (A) and that in buffer solution, in vitro translated pD1 (B). The addition of oligopeptide without substitution (LAAl) to the reaction mixture exhibited a pronounced inhibitory effect to the proteolytic cleavage of pD1, irrespective of the state of substrates. The addition of I346A, which can be used as a substrate for the processing protease in vitro, appreciably reduced the cleavage rate in proportion to its effectiveness as substrate, whereas L343A or L343A and I346A exhibited no inhibitory effect on the proteolysis for the full-length pD1 either integrated into the intact thylakoid membrane of *Scenedesmus* (A) or in buffer solution (B). This result confirms the conclusion, derived from the previous analysis using these oligopeptides as a substrate [12], that Leu at position 343 is crucial in the enzyme-substrate interaction and further convinces us that the principle for substrate recognition by the processing protease is basically identical in entirely different systems, i.e. with the small-sized C-terminal synthetic oligopeptide in solution and the full-length pD1 integrated into the PSII complex in the thylakoid membrane. Thus, the in vitro enzymatic system using synthetic oligopeptides can be taken to mimic the in vivo state in the recognition of substrate by the processing protease.

Among organisms, there are considerable differences both in the size and in the sequence of the C-terminal extension of pD1, which is cleaved off by the processing protease, in contrast to the sequence upstream the cleavage site, i.e. the C-terminal sequence of the mature protein (see, for example, Fig. 1) [15]. However, Ala at the +1 position, i.e. the N-terminus of the C-terminal extension, is highly conserved in different species, with exceptions where Ala is replaced by Ser in some cyanobacteria, *Chlamydomonas reinhardtii*, *Scenedesmus obliquus* etc. [19]. As mentioned in Section 1, our previous in vitro study using synthetic oligopeptides as a substrate for the isolated processing protease from spinach indicated that the amino acid residue at the +1 position significantly influences the rate of C-terminal cleavage, in the order of Ala, Ser, Cys, Phe > Gly > Val >> Pro = 0 [12]. In contrast, in the previous in vivo analysis using *Chlamydomonas* cells, we could not detect any significant influence of these substitutions to the apparent rate of C-terminal processing in vivo estimated by the pulse-chase experiment, as well as the steady-state level of the mature D1 protein, at least under the experimental conditions used [13]. Therefore, in the present analysis, we examined the inhibitory effect of a series of oligopeptides consisting of the C-terminal 19 amino acids of pD1, in which Ala at position 345 was altered to the corresponding amino acids, i.e. to Gly, Ser, Pro, Val and Cys, on the proteolytic C-terminal processing of pD1 of full-length size. For this purpose, we

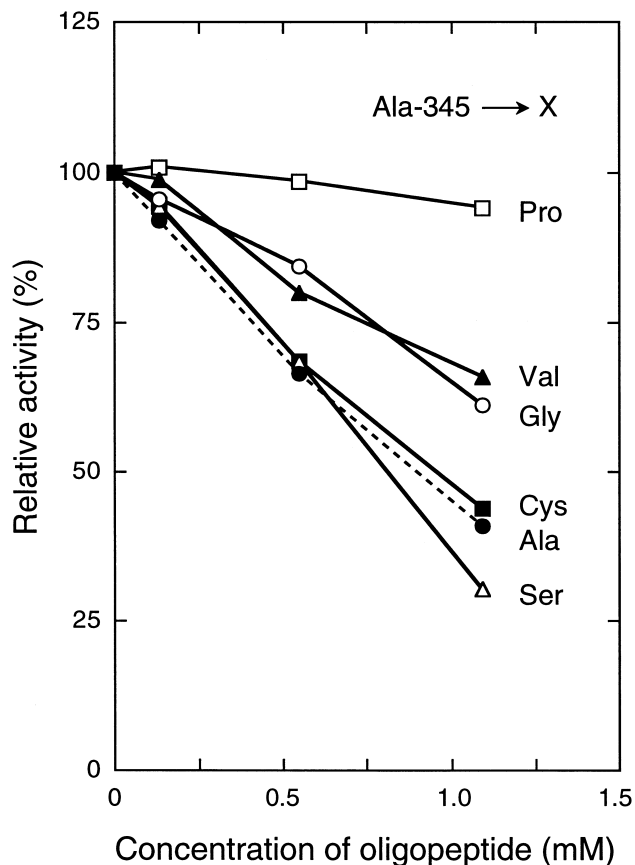


Fig. 4. Inhibitory effect of oligopeptides with substitution at the cleavage site (+1 position). The processing activities are indicated by the relative ratio of the amounts, normalized at zero concentration of oligopeptide, between mature D1 protein and total D1 protein estimated by the reactivity to specific antibodies.

again utilized the PSII membrane of *Scenedesmus* where pD1 is integrated into the PSII complex in the intact thylakoid membrane which can be photo-activated to establish the oxygen-evolving PSII machinery [3]. By the addition of an oligopeptide which can be used as an efficient substrate in the in vitro analysis, i.e. A345S or A345C, an inhibitory effect comparable to that of the control (A345A) could be observed for the proteolytic cleavage of pD1 integrated into the PSII complex in the thylakoid membrane (Fig. 4). On the other hand, by the addition of A345G or A345V oligopeptide, the proteolytic cleavage of full-length pD1 in the PSII complex was only partly reduced, consistent with the fact that these two polypeptides are less efficient substrates as shown by the in vitro analysis [12]. Furthermore, A345P had practically no inhibitory effect on the proteolytic C-terminal processing of full-length pD1 in the PSII complex, suggesting that the blockage of the proteolytic cleavage in the A345P substitution demonstrated in the previous study [5,12] was due to the reduced affinity of the substrate to the enzyme, rather than to the tolerance of the Ala³⁴⁴-Pro³⁴⁵ bond to the proteolytic cleavage.

The results of three series of experiments presented in this study demonstrate that there is a clear parallel between the efficiency of proteolytic cleavage of C-terminal oligopeptides by the processing protease in the in vitro enzymatic assay and the degree of their competitive inhibitory effect against the C-

terminal processing of full-length pD1, either in buffer solution or integrated into the intact PSII complex embedded in the thylakoid membrane. This fact convinces us that the basic mode of recognition by the enzyme in the C-terminal cleavage is identical for substrates of different integrity, i.e. the synthetic C-terminal oligopeptide of short chain length, the in vitro translated full-length pD1 in buffer solution and the pD1 integrated into the intact PSII complex in the functional thylakoid membrane. Consequently, we attribute the discrepancy between the results obtained by the in vitro enzymatic analysis using synthetic oligopeptides [12] and the in vivo study using *Chlamydomonas* [13], for the +1 substitutions at the cleavage site, to the rate-limiting step in the in vivo pulse-chase analysis, rather than to the change in the basic mode of enzyme-substrate interaction(s).

The D1 subunit of the PSII reaction center is synthesized as a precursor form on ribosomes attached to the stromal surface of the thylakoid membrane in chloroplasts, and the C-terminus is co-translationally translocated through the membrane into the luminal space. These processes are highly coordinated in vivo with a series of successive processes of integration of the PSII complex, which include the insertion of precursor proteins into the multi-subunit complex accompanied by the binding of various co-factors, in addition to the C-terminal processing and the ligation of Mn cluster to establish the functional oxygen-evolving complex of PSII [17]. It is therefore possible to speculate that the apparent rate of C-terminal processing of pD1 in vivo estimated by a pulse-chase analysis under certain experimental conditions using intact cells may not represent the actual rate of the enzymatic process of C-terminal cleavage in vivo. This might be the reason why we could not detect any significant effect of the amino acid substitution at the cleavage site in the in vivo analysis using *Chlamydomonas* [13]. However, since it is evident that the amino acid at the cleavage site is evolutionally conserved among a wide variety of photosynthetic organisms, it can be reasonably expected that we could detect the difference due to the reduced rate of C-terminal processing of pD1 resulting from the amino acid substitution in the viability of organisms under certain conditions. Long-term mixed culture growth analysis would be a convenient method for detecting small differences in the viability of different mutants (paper in preparation).

Although this paper has emphasized that the basic mechanism for the enzyme-substrate interaction in the C-terminal processing of pD1 is identical between in vivo and in vitro systems, it is also important to note that there are marked differences in the enzymatic properties between these two conditions, such as the pH dependence and the apparent

K_m value for the proteolytic process (data to be published). Thus, further analysis both in vivo and in vitro is evidently needed to understand the molecular mechanism of the proteolytic C-terminal processing in the integration of water splitting machinery in the PSII complex of oxygenic photosynthetic organisms.

Acknowledgements: This work was supported by Grants-in-Aid for Scientific Research (B) (09440268) and Scientific Research on Priority Areas (09267222 and 09274222) from the Ministry of Education, Science, Sports and Culture. The authors want to express their thanks to Dr. N. Inagaki for his kind help and discussion.

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