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Recognition signal for the C-terminal processing protease of D1 precursor protein in the photosystem II reaction center

An analysis using synthetic oligopeptides

Fumiko Taguchi[a], Yumiko Yamamoto[a], Noritoshi Inagaki[b] and Kimiyuki Satoh[a,b]

[a]*Department of Biology, Okayama University, Okayama 700, Japan* and [b]*National Institute for Basic Biology, Okazaki 444, Japan*

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Synthetic oligopeptides of different chain lengths of 11 to 38 amino acids, corresponding to the carboxyl-terminal sequence of D1 precursor protein of the photosystem II reaction center, were subjected to a proteolytic cleavage by a processing enzyme isolated from spinach, in order to analyze the recognition signal. Practically the same K_m and V_{max} values were obtained for the oligopeptides consisting of more than 19 amino acids; a decrease in affinity, without affecting the V_{max} value, was observed for the peptide consisting of 16 amino acids; no detectable activity was found for the peptide with 11 amino acids. When Asp-342 (12th residue from C-terminus) was replaced with Asn, for the peptide consisting of 16 amino acids, the enzymatic activity was completely abolished. In contrast, replacing Asp-342 with Glu had little effect. The efficiency of these oligopeptides as a substrate mentioned above, together with their effectiveness as an inhibitor, clearly demonstrated that the negative charge on Asp-342 plays a crucial role in the recognition, i.e. binding and cleavage, of the substrate by the processing enzyme, and suggested that the carboxyl-terminal extension consisting of 9 amino acids, by itself is not important in the binding.

C-terminal extension; D1 protein; Photosystem II; Processing protease; Recognition signal; Synthetic oligopeptide

1. INTRODUCTION

The D1 protein is a subunit of the photosystem II (PSII) reaction center [1], and the precursor of this protein is known to furnish a COOH-terminal (C-terminal) extension consisting of 9 to 16 amino acid residues [2–5]. Post-translational removal of this extension is absolutely required for creating the machinery for oxygen evolution in PSII, but not of the primary photochemistry, as demonstrated by the analysis of the LF-1 mutant of *Scenedesmus obliquus* [6,7]. The enzyme involved in the processing has recently been solubilized and partially purified from an alga [8] and higher plants [8–11]. For analyzing the structure and function of this enzyme, D1 precursor proteins synthesized either by the LF-1 mutant of *S. obliquus*, an organism which has no processing enzyme [6], or by an in vitro translation system using wheat germ extracts [9,12], were employed as a substrate. The synthetic oligopeptide, corresponding to the C-terminal 29 amino acids of the D1 precursor protein, was also shown to be a useful substrate for enzymatic analysis [13].

The present study was conducted in order to analyze the recognition signal for the C-terminal processing protease of D1 precursor protein. For this purpose, synthetic oligopeptides of different chain lengths were synthesized and used as substrate. The inhibitory effect of these oligopeptides to the processing activity using D1 precursor proteins translated in vitro was also examined. The results clearly demonstrated that the aspartic side chain (Asp-342) located in close vicinity to the cleavage site, in a highly conserved region of the C-terminal sequence of D1 precursor protein, is essential for recognition, and suggested that the C-terminal extension is not important in the binding of substrate to the enzyme.

Correspondence address: K. Satoh, Department of Biology, Okayama University, Okayama 700, Japan. Fax: (81) (86) 252 6601.

2. MATERIALS AND METHODS

2.1. *Enzyme preparation*

The C-terminal processing enzyme of the D1 precursor protein was partially purified from spinach thylakoids with the method described [11], with some modifications; the enzyme was extracted from thylakoids by sonication, instead of Triton X-100 treatment, and then purified from the extracts by a one-step chromatography using hydroxylapatite, followed by a Sephadex G-75 (SF) gel filtration.

2.2. *Substrates*

Oligopeptides with different chain lengths, corresponding to the C-terminal sequence of the D1 precursor protein, deduced from the nucleotide sequence of spinach *psb*A gene [14], were synthesized by a peptide synthesizer (model, 430A; Applied Biosystems, Foster City). Protecting groups were removed after synthesis, following the manufacturer's protocol, and then impurities in the sample were eliminated by reverse-phase, high-performance liquid chromatography (HPLC). The amino acid sequence of purified oligopeptides was analyzed by a protein sequencer (model, 477A; Applied Biosystems, Foster City).

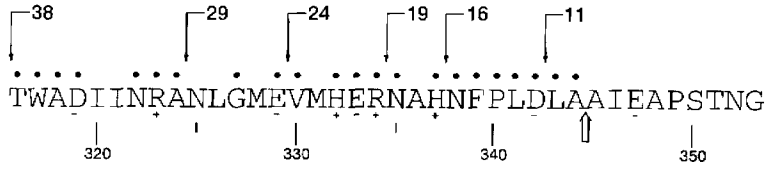

Fig. 1. Carboxyl-terminal sequence of the D1 precursor protein deduced from the nucleotide sequence of spinach *psb*A gene. The chain lengths of six carboxyl-terminal oligopeptides used in this study are indicated on the upper part. An arrow (upward) indicates cleavage site by the processing protease. The conserved residues are indicated for mature protein by dots, and charged residues are indicated by symbols of 'plus' or 'minus'. Numbers on the lower part represent the number of amino acid side chains counted from the N-terminus.

The ^{35}S-labeled D1 precursor protein was obtained by an in vitro transcription/translation system as described [9,12].

2.3. *Enzyme assay*

The standard mixture for enzymatic analysis (20 μl) contained indicated amounts of a synthetic oligopeptide, partially purified enzyme (1.96 μg protein) and 20 mM HEPES-NaOH (pH 7.7) buffer. In a typical experiment, the enzymatic reaction was carried out at 25°C for 4 h. The reaction was terminated by the addition of 18% (w/v) trichloroacetic acid (TCA) to a final concentration of 3.6% and then the mixture was centrifuged at 20,000 × *g* for 20 min at room temperature. To the resultant supernatant (20 μl), 160 μl of 0.1% (v/v) trifluoroacetic acid (TFA) was added, followed by the addition of 2 μl salicylic acid as an internal standard. An aliquot (170 μl) of the resultant solution was subjected to HPLC analysis using a system consisting of an intelligent pump (type, L-6210; Hitachi, Tokyo), an optical detector (type, L-4000UV; Hitachi, Tokyo) and an integrator (type D-2500; Hitachi, Tokyo). A reverse-phase, pre-packed stainless-steel column (4.6 × 250 mm Shin-Pack CLC-C8M, Shimadzu, Kyoto) was used at a flow rate of 1.0 ml · min^{-1} at 25°C. Two solutions were used for elution: Solution A, 0.1% (v/v) TFA; Solution B, 0.1% (v/v) TFA in 70% (v/v) acetonitrile. Elution was conducted according to the following regime: (1) 0–0.5 min, Solution A; (2) 0.5–20 min, increasing linear gradient from Solution A to 20% (v/v) Solution B in Solution A; (3) 20–40 min, increasing linear gradient from the second step to 80% (v/v) Solution B in Solution A; (4) 40–46 min, decreasing linear gradient from the third step to Solution A (recovery process). Elution was monitored at 220 nm.

An enzymatic assay, using in vitro translated D1 precursor protein as the substrate, was conducted as described [9].

3. RESULTS

Fig. 1 shows the sequence of the C-terminal 38 amino acids of the D1 precursor protein deduced from the nucleotide sequence of the spinach *psb*A gene [14]. Six carboxyl-terminal oligopeptides of different chain lengths shown in the figure, all of which start from the predicted C-terminus and contain the cleavage site for the processing enzyme, i.e. the carboxyl-side of Ala-344 (10th position from the C-terminus), were synthesized as described in section 2. They are named as S-11, S-16, S-19, S-24, S-29 and S-38, respectively, depending on the number of amino acids in the sequence, in the following description.

Fig. 2 shows a typical elution profile for the HPLC monitored at 220 nm, after 7 h incubation of S-24 (500 μM), with a partially purified enzyme. The amino acid sequence analysis of individual components observed on the chromatogram demonstrated that a peak at about 27 min (S-24) corresponds to the substrate added to the reaction mixture, and those at about 22 and 10 min (P-15 and P-9) to the cleavage products, i.e. N- terminal and C-terminal fragments, respectively, formed by the enzymatic cleavage. No other polypeptide could be observed in any detectable amount on the chromatogram, even after prolonged incubation for 24 h under the experimental conditions used in this study. The enzymatic reaction proceeded almost linearly during the 24 h under these conditions, and the ratio between the decrement of substrate (S-24) and the increment of the product (P-9) was almost constant over this time range, indicating that the proteolytic cleavage occurs only at a single site. Thus the rate of production of P-9 was used for kinetic analysis.

In order to quantitatively estimate the efficiency of the synthetic oligopeptides as a substrate, the reaction

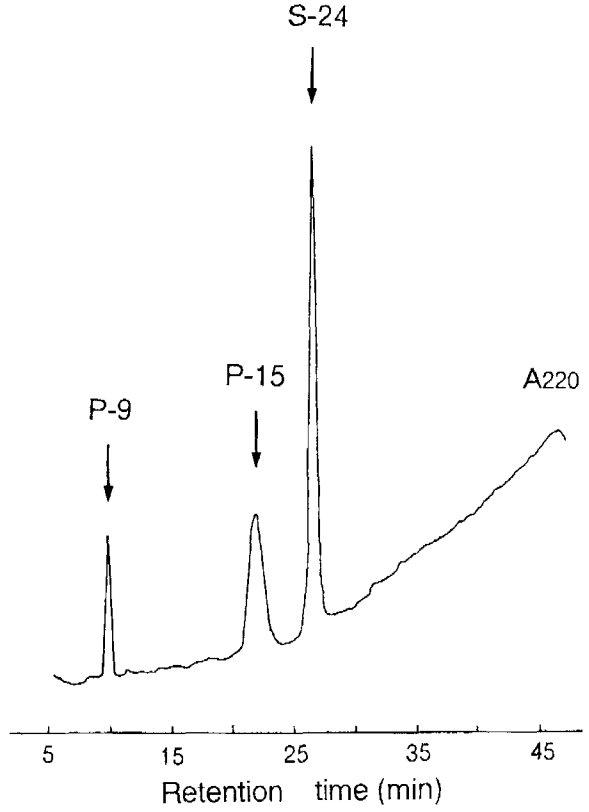

Fig. 2. A typical elution profile of HPLC. See text for further explanations.

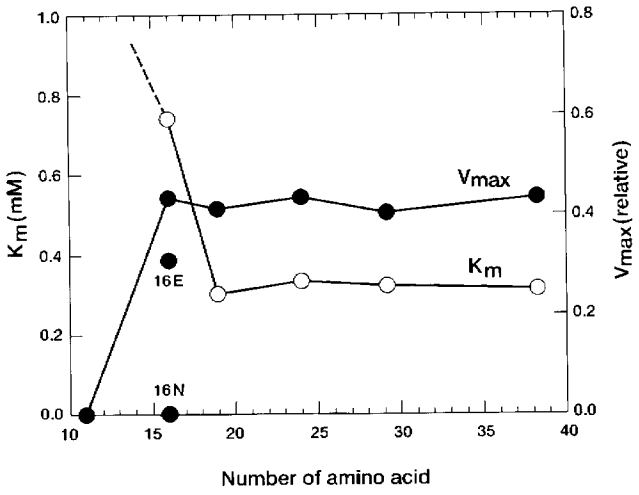

Fig. 3. The K_m (open circles) and V_{max} (closed circles) values for each oligopeptide. At the 342 position, Asp was replaced either with Glu (16E) or with Asn (16N). See text for further explanations.

rate versus concentration relationship was analyzed upto 640 μM for each oligopeptide. By using conventional Lineweaver–Burk's plots of the data obtained, both the affinity (K_m value) and the maximum rate at saturating substrate concentration (V_{max} value) were estimated. As shown in Fig. 3, practically the same K_m value of about 300 μM and the same V_{max} value were obtained for oligopeptides consisting of more than 19 amino acids. However, deletion of 3 amino acids from the N-terminus of the S-19 appreciably decreased the affinity to the enzyme without affecting the V_{max} value. Thus, it may be possible to expect that a decrease in the affinity is the result of the elimination of a positive charge on the His-337 (17th amino acid from the C-terminus), since this is the only charged residue among 3 amino acids deleted. However, the dinitrophenyl-derivative at histidine sites for the S-29, both at the His-332 and the His-337, exhibited identical K_m-values with that of the control (data not shown), suggesting that a decrease in chain length, rather than the loss of a positive charge on histidine, is responsible for the affinity change.

When the S-11 was incubated with the processing enzyme, no detectable amount of cleavage product was observed even at the highest concentration of substrate used in this study (640 μM). The amino acid sequence corresponds to the difference between the S-16 and the S-11, i.e. Asn-Phe-Pro-Leu-Asp, is completely conserved among the 43 *psb*A genes sequenced so far for a wide variety of organisms [15], and thus it is possible to expect that this part plays an essential role in the recognition of the substrate by the processing protease. Within this region, Asp-342 is the only charged residue, and thus this side chain might be responsible for recognition. To investigate the requirement of this residue in recognition, two C-terminal oligopeptides of the D1 precursor proteins of 16 amino acids, with replaced side chains at the position of Asp-342, i.e. Asp into Asn (S-16N) and into Glu (S-16E), were synthesized and subjected to enzymatic cleavage. When S-16E was incubated with the enzyme, the cleavage product (P-9) was formed with an almost comparable rate to that for S-16 (Fig. 3). On the other hand, practically no proteolytic activity was detected when S-16N was used as a substrate. This result suggests that the negative charge on the Asp-342 is crucial for recognition and/or cleavage of substrate by the processing protease. In order to examine whether the negative charge on the Asp is essential for binding, inhibitory effects of S-16N were examined in the study shown in Fig. 4, using S-24 as a substrate. The presence of S-16N in the reaction mixture competitively inhibited the cleavage of S-24 (data not shown), and in the presence of a 10 times higher concentration, compared to that of the substrate, completely prevented cleavage of S-24 by the enzyme (Fig. 4), suggesting that S-16N has a strong affinity for the enzyme, although it cannot be cleaved under the experimental condition used.

There are considerable variations in different species, in the amino acid sequence, as well as in the chain length, in the C-terminal extension of the D1 precursor protein [15]. This may suggest that the extension is not important in the binding of the substrate to the processing enzyme. The fact that the *psb*A gene product of a higher plant is processed by the enzyme in cyano-

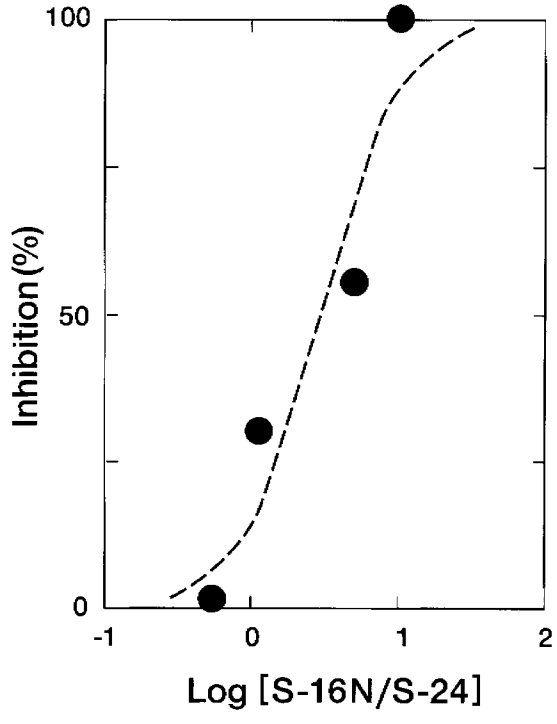

Fig. 4. Percent inhibition by S-16N (Asp-342 is replaced with Asn) of the proteolytic activity using S-24 as substrate. The final concentration of S-24 in the reaction mixture was 320 μM.

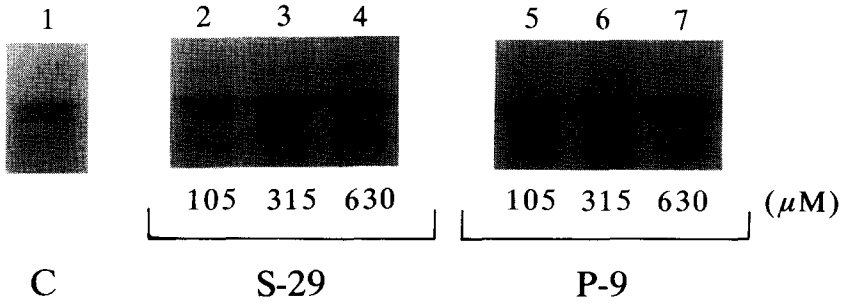

Fig. 5. Inhibitory effect of a synthetic oligopeptide to the processing activity using an in vitro translated D1 precursor protein as substrate. The ^{35}S-labelled precursor protein (pD1) was incubated with the enzyme for 2 h at 25°C under different conditions and then the enzymatic activity was visualized by autoradiography. C, no addition; S-29, addition of S-29; P-9, addition of a synthetic oligopeptide corresponding to the C-terminal extension consisting of 9 amino acids of the D1 precursor protein.

bacterial cells [16], the D1 precursor protein of which contains largely different C-terminal extension compared to that of higher plants, further supports this interpretation. In order to test this possibility, the inhibitory effect of a polypeptide, corresponding to the C-terminal 9 amino acids (P-9) to the processing activity, using an in vitro translated D1 precursor protein, was examined in the experiment shown in Fig. 5. As a control, the effect of the S-29 on the enzymatic activity with an in vitro translated D1 precursor protein as substrate was examined (S-29). As shown in the figure, the S-29 inhibited the enzymatic activity in a concentration-dependent fashion as expected. On the other hand, the addition of P-9 to the reaction mixture exhibited no effect, even at the highest concentration used (630 μM). This result, together with the fact that S-11 is inactive as a substrate, confirms that recognition of the substrate by the processing enzyme is based mainly on the highly conserved sequence to the N-terminal side of the cleavage site, and that Asp-342 plays an essential role for that event, although we have not yet examined the other possibility that the processing enzyme may recognize some other regions, in addition to Asp-342, in the lumenal extension of the D1 protein, such as the sequence between transmembrane helix I and II, or transmembrane helix III and IV, which consist of highly conserved residues [17]. This possibility can easily be examined by comparing the rate of processing in vivo and that in vitro using synthetic oligopeptides as a substrate.

4. DISCUSSION

Debus et al. [18,19] and Nixon and Diner [5,20] conducted systematic analyses on the function of C-terminal extension of the D1 precursor protein with a technique of site-directed mutagenesis using a transformable cyanobacterium, *Synechocystis* 6803. They showed that when Asp-342 was replaced with Gln, Ala or Val, photoautotrophic growth and photosynthetic oxygen evolution of the organisms were completely abolished. In contrast, replacing Asp-342 with Glu had little effect. Based on these observations they suggested that Asp-342 could potentially coordinate Mn or Ca^{2+} ions. However, other effects caused by these mutations, such as a destabilized binding of the peripheral proteins, such as the 33 kDa protein, or conformational changes in the PSII core, might also account for the impairment or abolition of photoautotrophic growth and oxygen evolution [18–20]. The dependence on the nature of amino acid side chain at position Asp-342 of the processing activity observed in this study, also supports this other possibility, namely photoautotrophic growth was abolished in their study, because of the inhibition of C-terminal processing, which is necessary for creating the machinery for oxygen evolution, due to the elimination of a negative charge at the 342 position on the substrate. Nixon and Diner also investigated the Mn-depleted PSII complex isolated from a mutant of *Synechocystis* 6803, having Asp-342 replaced with Val. The results indicated that, in this mutant, the high affinity Mn^{2+} binding site is intact, but that no functional Mn complexes can be assembled [5]. Thus, the properties of this mutant resemble the *S. obliques* LF-1 mutant, which has no processing enzyme [6]. This fact also supports our interpretation presented in this study that the presence of Asp at the 342 position is essential for recognizing the substrate by the processing enzyme, rather than Mn-binding.

Recently, Nixon and Diner have shown that oxygen evolution is blocked in a cyanobacterial mutant, *Synechocystis* 6803, in which the C-terminus of D1 protein is Leu-343 [5]. They emphasized the importance of the C-terminus of the D1 protein in assembling the functional Mn cluster. If the free carboxyl group at the C-terminus of the mature D1 protein by itself is a ligand to the Mn cluster, the presence of the C-terminal extension may have an important role in the protection of this crucial site before and during the processes of its assemblage into a functional complex. Further studies evidently are needed in order to understand the functional meanings of this process.

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