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COOH-terminal residues of D1 and the 44 kDa CPa-2 at spinach photosystem II core complex

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The COOH-termini of the 32 kDa D1 and 44 kDa CPa-2 were determined by protein sequencing of peptides from trypsinized photosystem II core complexes. COOH-terminal fragments were isolated by affinity chromatography using anhydrotrypsin-agarose. One peptide had a sequence corresponding to the segment from Asn at position 335 to Ala at position 344 of the sequence deduced from the *psb*A gene coding for D1. Nine amino acids may be cleaved from the COOH-terminus of pre-D1 during maturation. In contrast, CPa-2 was not modified at its COOH-terminus.

Covalent modification; D1 peptide; Photosystem II: (Spinach)

1. INTRODUCTION

In the photosystem II (PS II) core complex containing at least six subunit peptides, the photochemical reaction center may consist of a dimer of two homologous proteins: the 32 kDa D1 (herbicide-binding protein) and 34 kDa D2 [1] proteins. D1 was characterized from the amino acid sequence determined from the *psb*A gene coding for the 32 kDa thylakoid protein [2]. Using the predicted protein structure, the topography of D1 in thylakoid membranes [3] and localization of the herbicide-binding site [4] and primary donor Z [5] have been analyzed. The role of D1 in the PS II reaction center has also been suggested from the sequence homology with a reaction center subunit of a purple photosynthetic bacterium [6].

Newly synthesized 33.5 kDa D1 in illuminated chloroplasts is processed prior to being assembled into the PS II reaction center in the 32 kDa form [7–9]. Mardar et al. [10] proposed the removal of about 12–16 amino acids from the COOH-terminus of pre-D1. Since a mutant alga which fails to process pre-D1 has a reduced content of manganese and show no oxygen-evolving activity [11], the peptide segment near the COOH-terminus is believed to be folded into a form that is essential in oxygen evolution. To determine the COOH-terminus (cleavage site) of D1, we analyzed sequences of COOH-terminal fragments of trypsinized PS II core complexes.

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Abbreviations: Chl, chlorophyll; HPLC, high-pressure liquid chromatography; PS II, photosystem II

2. MATERIALS AND METHODS

PS II membranes were prepared from spinach thylakoids according to Kuwabara and Murata [12]. Membranes (10 mg Chl) were incubated in 10 ml of 60 mM octyl-D-glucoside containing 40 mM 2-(*N*-morpholino)ethanesulfonate-NaOH, pH 6.0, and 10 mM NaCl at 5°C for 10 min and centrifuged at 220000 × *g* at 5°C for 5 h in discontinuous layers comprising 3 ml of 10% sucrose and 2.5 ml of 30% sucrose, both of which contained 60 mM octyl-D-glucoside, 40 mM 2-(*N*-morpholino)-ethanesulfonate-NaOH, pH 6.0, and 10 mM NaCl. PS II core complexes at the bottom and in the 30% sucrose layer [13] were suspended in 50 ml of 0.1 M Tris-Cl^-, pH 8.0, and collected by centrifugation at 220000 × *g* at 5°C for 1 h. The pellets were suspended in 4 ml of 0.1 M Tris-Cl^-, pH 8.0, containing 2 M urea and digested using *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Worthington, NJ) at a substrate/enzyme ratio of about 150 at 37°C for 6 h in the dark with gentle

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stirring. After centrifugation at 220000 × *g* for 30 min, peptides in the supernatant were separated by reverse-phase HPLC with a Cosmosil ODS column (4.6 × 150 mm, 300 Å pore size; Nacalai Tesque, Kyoto) using a 0–60% acetonitrile concentration gradient containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. After lyophilization, tryptic peptides from HPLC were dissolved in 0.8 ml of 50 mM acetate, pH 5.0, containing 20 mM $CaCl_2$, slowly applied to an affinity column (0.8 ml) of anhydrotrypsin-conjugated agarose (Takara Biomedicals, Kyoto) and washed with one column volume of the same buffer. The unadsorbed fraction was injected into the reverse-phase HPLC and each peak was rechromatographed with a 0–60% acetonitrile gradient containing 10 mM triethanolamine-acetate, pH 5.1. Automated Edman degradation of the isolated peptides was carried out on a model 477A protein sequencer with a model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, CA). Peptide fragments were identified using the program IDEAS [14] for searching homologous sequences in the National Biomedical Research Foundation data base.

3. RESULTS AND DISCUSSION

D1 is mostly hydrophobic and assumed to have five transmembrane helices [3]. Other subunits of the PS II core complex would have a similar transmembrane structure to D1. Spinach D1 contains 15 arginine residues in the sequence of 353 amino acids [2]. We anticipated that the peptide segments near the NH_2- and COOH-termini of the intrinsic membrane proteins would be cleaved off by trypsin even if the entire molecule were insoluble in an aqueous medium. Fig.1A shows an analysis of the tryptic digest by reverse-phase HPLC. More than 80 peaks were observed, 7 peptides being abundant. Among the tryptic peptides, those with arginine and lysine residues at the COOH-terminus were adsorbed onto the anhydrotrypsin-agarose [15]: the peptide fragment at the COOH-terminus might pass through the affinity chromatography if it ends on amino acids other than lysine or arginine. About 10 peaks remained in the unadsorbed fraction from the anhydrotrypsin-affinity chromatography (fig.1B). Peak 3 was further separated into two peptides, 3.1 and 3.2, by reverse-phase HPLC at pH 5.1 (fig.2). Peptide 3.2 was 10 amino acids in length and corresponded to the segment from Asn at position 335 to Ala at position 344 of the sequence of D1 deduced from *psb*A (table 1). Marder et al. [10] suggested that 12–16 amino acids have been removed from the COOH-terminus of pre-D1 in *Spirodela* by best fitting the stoichiometry of radiolabelled

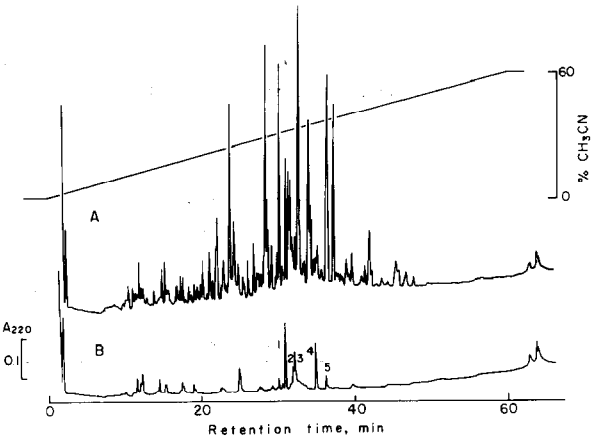

Fig.1. Separation of tryptic peptides by reverse-phase HPLC. The concentration gradient of acetonitrile containing 0.1% trifluoroacetic acid increased linearly from 0 to 60% at a rate of 1%/min. Absorbance was monitored at 220 nm. (A) Tryptic peptides of spinach PS II core complexes; (B) unadsorbed peptides from the anhydrotrypsin-agarose affinity chromatography of (A).

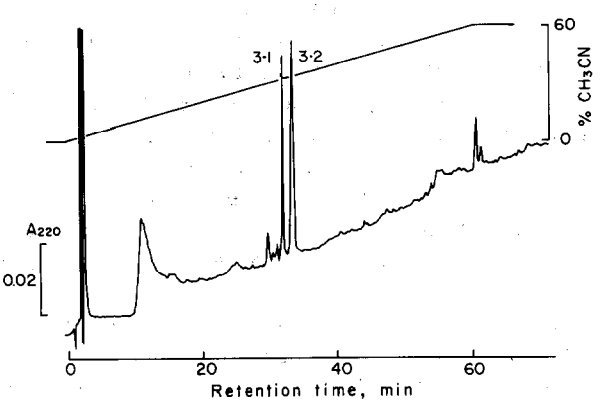

Fig.2. Rechromatography of the COOH-terminal fragment of D1. Peak 3 in fig.1B was separated as in fig.1 except that the solvent system was a 0–60% acetonitrile gradient containing 10 mM triethanolamine-acetic acid, pH 5.1.

amino acids incorporated into the papain digests of D1 to the distribution of amino acids in the sequence of D1. Diner et al. [11] observed no immunobinding of antibodies raised against a synthetic peptide of the last 14 amino acids of pre-D1 with the mature D1. In support of their suggestion, our findings show that the last 9 amino acids from Ala at position 345 to Gly at position 353 were missing in D1 at the PS II reaction center complex. It should be noted that this COOH-

Table 1

Comparison of amino acid sequences of peptides 3.2, 4 and 5 from the anhydrotrypsin-agarose affinity chromatography with those in the COOH-terminal region of D1 and CPa-2 deduced from the respective nucleotide sequences of *psb*A [2] and *psb*C [18]

	335	340	345	350
D1	Arg-Asn-Ala-His-Asn-Phe-Pro-Leu-Asp-Leu-Ala-Ala-Ile-Glu-Ala-Pro-Ser-Thr-Asn-Gly			
Peptide 3.2	Asn-Ala-His-Asn-Phe-Pro-Leu-Asp-Leu-Ala			
	460	465	470	
CPa-2	Lys-Gly-Ile-Asp-Arg-Asp-Phe-Glu-Pro-Val-Leu-Ser-Met-Thr-Pro-Leu-Asn			
Peptide 4	Asp-Phe-Glu-Pro-Val-Leu-Ser-Met-Thr-Pro-Leu-Asn			
Peptide 5	Gly-Ile-Asp-Arg-Asp-Phe-Glu-Pro-Val-Leu-Ser-Met-Thr-Pro-Leu-Asn			

terminal extension is already omitted from *psb*A in *Euglena* [16].

Considering that the NH_2-terminal of the mature D1 is Thr at position 2 [17], the molecular mass of D1 is 37980 Da if contributions due to phosphorylation and acetylation [17] are not counted.

Peptides 4 and 5 corresponded to the COOH-terminal sequence of CPa-2 deduced from *psb*C of spinach chloroplasts (table 1). Comparing the sequences of peptides 4 and 5 to that of CPa-2 derived from *psb*C [18] one observes that CPa-2 is not modified at the COOH-terminus. In contrast, Michel et al. [16] reported that the first 14 amino acids were processed at the NH_2-terminus of CPa-2. Isolation of peptides 4 and 5, which had not been cleaved at sites other than lysine and arginine, also proved that pseudotrypsin or endogenous proteases were not activated during trypsin treatment of PS II core complexes.

In conclusion, D1 is processed on the COOH side of Ala at position 344, releasing the last 9 amino acids of pre-D1 in spinach chloroplasts. The sequence of CPa-2 ends as deduced from the *psb*C genome.

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