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Partial degradation of the extrinsic 23-kDa protein of the Photosystem II complex of spinach

Mitsue Miyao, Yoko Fujimura and Norio Murata

National Institute for Basic Biology, Myodaiji, Okazaki (Japan)

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Chymotrypsin eliminated nine amino acid residues at the amino-terminal side of the extrinsic 23-kDa protein of the oxygen-evolving Photosystem II complex of spinach. The resultant 22-kDa fragment was able to bind to the Photosystem II complex but with lowered binding affinity. However, once the 22-kDa fragment bound to the complex, it retained most functions of the 23-kDa protein; the fragment provided a binding site for the extrinsic 18-kDa protein, preserved a tight trap for Ca^{2+} in the complex, and shifted the optimum Cl^- concentration for oxygen evolution from 30 to 10 mM, although it was less effective in sustaining oxygen evolution at Cl^- concentrations below 10 mM. These observations suggest that the elimination of nine amino acid residues at the amino-terminal region of the 23-kDa protein does not significantly alter the conformation of the protein, except for partial modification of its binding site and its interaction with Cl^-.

Introduction

The participation of three extrinsic proteins of molecular mass 33, 23 and 18 kDa in photosynthetic oxygen evolution was first demonstrated in the early 1980s [1–3]. Since then, much effort has been made in the study of the oxygen-evolving Photosystem II complex (hereafter, designated as PS II complex) to elucidate the organization of the complex and the functions of extrinsic proteins (for reviews, see Refs. 4, 5). The PS II complex contains one molecule each of the extrinsic proteins per reaction center II [6]. The 33-kDa protein stabilizes two of four Mn atoms which form a catalytic center of oxygen evolution [7–9] and accelerates the S-state transition from S_3 to S_0 in Kok's scheme in Ref. 10. The 23-kDa protein induces a conformational change of the Ca^{2+} trap in the intrinsic part of PS II complex [5,11,12], and reduces the Cl^- requirement of oxygen evolution from 30 to 10 mM [13]. This protein also provides a binding site in the complex for the 18-kDa protein [14], which sustains oxygen evolution at Cl^- concentrations below 3 mM [13,15].

However, information is limited regarding identification of the functional domains and functional amino acids in the three extrinsic proteins. Kuwabara et al. [16], in their study on a degradation product of the 18-kDa protein, have shown that the N-terminal region of the protein forms a domain which binds to the PS II complex. Tanaka and Wada have demonstrated that an intramolecular disulphide bond of the 33-kDa protein is

Abbreviations: Chl, chlorophyll; DNAase, deoxyribonuclease; HPLC, high-performance liquid chromatography; Mes, 4-morpholineethanesulphonic acid; PS II, Photosystem II; SDS, sodium dodecyl sulphate; Z, the secondary electron donor of Photosystem II.

Correspondence: N. Murata, National Institute for Basic Biology, Myodaiji, Okazaki 444, Japan.

necessary for proper folding of the protein [17]. However, no such information has been reported for the 23-kDa protein.

The present study shows that chymotrypsin eliminated nine amino acid residues at the N-terminal side of the 23-kDa protein. The properties of the degraded protein were examined and compared with those of the 23-kDa protein in order to explore the participation of the N-terminal region of the 23-kDa protein.

Materials and Methods

Preparation of PS II membranes

PS II membranes were prepared from spinach thylakoids with Triton X-100 according to the method of Kuwabara and Murata [3] and stored at −196 °C in the presence of 30% (v/v) ethylene glycol [14]. Before use, the membranes were washed three times with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) (designated hereafter as medium A) by centrifugation and resuspension, and finally suspended in the same medium and kept in darkness for 2 h.

PS II membranes depleted of the 23-kDa and 18-kDa proteins were prepared by treating the PS II membranes with 1.0 M NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) for 30 min in darkness, as described previously [14]. The membranes were collected by centrifugation at $35\,000 \times g$ for 20 min and washed once with 200 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) by resuspension and recentrifugation. The resultant pellet of the NaCl-treated membranes was finally suspended in medium A.

Purification of proteins

The 23-kDa and 18-kDa proteins were extracted from untreated PS II membranes by incubation with 1.0 M NaCl/25 mM Mes-NaOH (pH 6.5) for 30 min. The extracted proteins were separated from the membranes by centrifugation at $35\,000 \times g$ for 20 min. The resultant supernatant was passed through a Millipore filter (5 μm), desalted with a gel filtration column (Sephadex G-25, Pharmacia) which had been equilibrated with 2 mM $(NH_4)_2SO_4$/10 mM Mes-NaOH (pH 5.5), and subjected to HPLC in a cation-exchange mode with a column (SP-650S, 14 mm i.d. × 180 mm, Tosoh) equilibrated with 2 mM $(NH_4)_2SO_4$/10 mM Mes-NaOH (pH 5.5). The proteins were eluted with a linear gradient of $(NH_4)_2SO_4$ from 2 to 100 mM in 10 mM Mes-NaOH (pH 5.5) at a flow rate of 3.0 ml · min^{-1}.

For purification of the 22-kDa fragment of the 23-kDa protein, untreated PS II membranes were incubated with 0.0003% DNAase I (Type IV from bovine pancreas, Sigma), which contained chymotrypsin (see Results), for 5 min in medium A supplemented with 0.3 mM $MgCl_2$. Next, the membranes were collected by centrifugation at $35\,000 \times g$ for 20 min, washed once with medium A by resuspension and recentrifugation, and subjected to the NaCl extraction, gel filtration and HPLC as described above. A fraction of the 22-kDa fragment obtained by HPLC was desalted by gel filtration and again subjected to HPLC.

The purified protein preparations were concentrated, if necessary, by ultrafiltration with an Amicon YM 10 Diaflo membrane and dialyzed against 10 mM Mes-NaOH (pH 6.5) and kept frozen at −80 °C until use. The concentrations of the 23-kDa and 18-kDa proteins were determined using molar absorption coefficients of 22 and 12 mM^{-1} · cm^{-1} at 277 nm for the 23-kDa and 18-kDa proteins, respectively, which were obtained from those previously reported [18] with correction for their molecular masses; molecular masses of 20.2 kDa for the 23-kDa protein and 16.5 kDa for the 18-kDa protein, which were derived from nucleotide sequences of their cDNAs [19], were used instead of those of 24 kDa and 18 kDa estimated by SDS gel electrophoresis [18]. The absorption coefficient of the 22-kDa fragment was assumed to be the same as that of the 23-kDa protein; the difference between the molar absorption coefficients of the 22-kDa fragment and the 23-kDa protein was estimated to be less than 6%, since the 22-kDa fragment lacked only one of the eight tyrosine residues but retained the two tryptophan residues of the 23-kDa protein [19].

Chymotrypsin treatment of the 23-kDa and 18-kDa proteins

Digestion of the purified 23-kDa and 18-kDa proteins by chymotrypsin was investigated by incubating the proteins with α-chymotrypsin (Type VII from bovine pancreas, Sigma), which had

been treated with 1-chloro-3-tosylamide-7-amino-heptanone to inactivate trypsin, as follows. The protein dissolved in 0.1 mM $CaCl_2$/10 mM Mes-NaOH (pH 6.5) at a protein concentration of 20 μM was mixed with one-fifth volume of the chymotrypsin dissolved in 1.0 mM HCl at 1.0 or 0.1 μM to give an enzyme-to-protein ratio of 10^{-2} or 10^{-3} (mol/mol). After incubation at 20°C for 15 min, the reaction was stopped on addition of one-thirtieth volume of 100 mM phenylmethylsulphonyl fluoride in methanol.

Rebinding of the proteins to PS II membranes

To study the binding ability of the 23-kDa protein and 22-kDa fragment to the PS II complex, designated amounts of the purified protein or fragment were added to the NaCl-treated PS II membranes depleted of the 23-kDa and 18-kDa proteins at a Chl concentration of 0.3 $mg \cdot ml^{-1}$. To investigate the ability of the 23-kDa protein and the 22-kDa fragment in providing the binding site for the 18-kDa protein, the NaCl-treated membranes were first incubated with the 23-kDa protein or 22-kDa fragment at a protein-to-Chl ratio of 2:220 (mol/mol) for 30 min, and then designated amounts of the 18-kDa protein were added to the membrane suspension. After being kept for 30 min, the suspension was mixed with 6 vol. of medium A. The reconstituted membranes were collected by centrifugation at $35000 \times g$ for 20 min, washed three times with medium A by resuspension and recentrifugation. All the above procedures were performed at 0–4°C.

Analytical methods

The polypeptides of PS II membranes and digested proteins were analyzed by SDS-urea polyacrylamide gel electrophoresis with a gel containing 6.0 M urea, using the buffer system of Laemmli [20]. The polyacrylamide concentrations of the stacking and separation gels were 5 and 15%, respectively. Polypeptides of the reconstituted membranes were analyzed by SDS-urea polyacrylamide gel electrophoresis, using the buffer system of Chua and Bennoun [21], except that the electrode buffer was 0.1% SDS/25 mM Tris-glycine (pH 8.3). The gel contained 5.0 M urea, and the polyacrylamide concentrations of the stacking and separation gels were 5 and 12%, respectively. Under these conditions, the 23-kDa protein and the 22-kDa fragment were well separated from others on the gel. The gel was stained with Coomassie brilliant blue R-250 and the electrophoretic pattern was photographed or recorded at 560 nm with a TLC scanner (CS930, Shimadzu). The amount of each protein was determined according to the peak heights of the stained bands in the densitogram, with the purified proteins as a standard.

The isoelectric point of the proteins was determined by isoelectric focusing using an automatic gel electrophoresis apparatus (PhastSystem, Pharmacia). The pH gradient used ranged from pH 3 to 10, and the isoelectric point was determined from the migration distance of the protein on the gel using a calibration curve made with an isoelectric focusing calibration kit (pH range 3–10, Pharmacia).

The partial amino acid sequence at the N-terminal side was determined with a gas-phase protein sequence analyzer (470A, Applied Biosystems). The sequence at the C-terminus was examined by analyzing amino acids released from the protein upon carboxypeptidase digestion as follows. The purified protein was dissolved in 0.1 M pyridine-acetic acid buffer (pH 5.5) containing 0.5% SDS at a protein concentration of 5 μM. Carboxypeptidase Y (from baker's yeast, Sigma) was added to the solution to give an enzyme-to-protein ratio of 0.005 (mol/mol). After various incubation times at 25°C, a portion of the reaction mixture was withdrawn and the reaction was stopped by adding 50% (w/v) trichloroacetic acid to give a final concentration of 3%. Precipitated protein and enzyme were removed by centrifugation at $15000 \times g$ for 10 min and the resultant supernatant was applied to an amino acid analyzer equipped with a fluorometric detection system (835, Hitachi).

Other methods

Oxygen-evolution activity of PS II membranes was measured using a Clark-type oxygen electrode with phenyl-*p*-benzoquinone as an artificial electron acceptor [3]. Chl concentration was determined according to Arnon [22].

Results

Purification and characterization of the 22-kDa fragment of the 23-kDa protein

The 23-kDa and 18-kDa proteins were specifically and almost completely removed from the PS II complex upon treatment of the PS II membranes with 1.0 M NaCl [14]. The resultant NaCl extract from PS II membranes was a good starting material for purification of these proteins, since it was free of other protein components and single-step column chromatography was sufficient for the purification [18]. However, it contained a considerable amount of nucleic acids (see below), which had been adsorbed on the surface of PS II membranes and were released upon NaCl extraction. The nucleic acids formed sticky aggregates in the NaCl extract, made the extract viscous and hampered handling. Although a part of the nucleic acids was removed from the NaCl extract by passing through a Millipore filter, the use of DNAase seemed to facilitate the protein purification procedures.

We treated PS II membranes with DNAase I prior to extraction with 1.0 M NaCl (see Materials and Methods). This made handling of the NaCl extract easy and increased the yield of protein after passage through a Millipore filter and gel filtration. However, the NaCl extract from the DNAase-treated membranes contained three polypeptides of 22, 17 and 10 kDa, in addition to the 23-kDa and 18-kDa proteins (Fig. 1A, lane c). A similar polypeptide pattern was observed when the NaCl extract was incubated with the DNAase (data not shown). This suggests that the polypeptides which emerged upon the DNAase treatment are degradation products of the 23-kDa and/or 18-kDa protein, and that the DNAase used in this study was contaminated with proteinase(s). Hereafter, this DNAase containing the

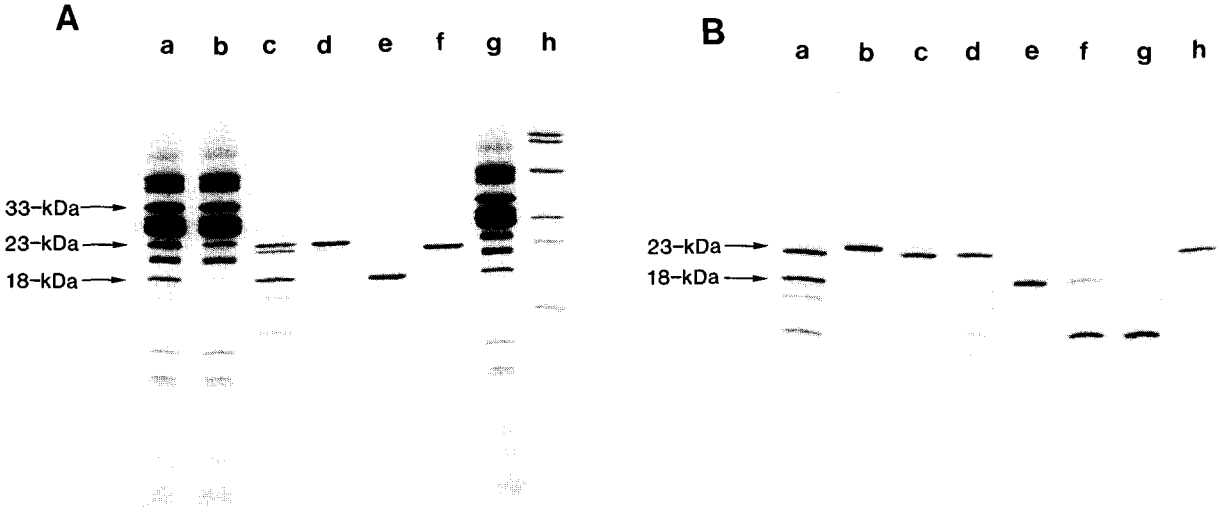

Fig. 1. Degradation of the 23-kDa and 18-kDa proteins with 'DNAase' and by α-chymotrypsin digestion. The polypeptide composition was analyzed by SDS-urea polyacrylamide gel electrophoresis with the buffer system of Laemmli [20]. (A) Degradation of the 23-kDa and 18-kDa proteins in PS II membranes with the 'DNAase' which was contaminated with proteinase activity. Lanes (a) and (g), untreated PS II membranes; (b) NaCl-treated PS II membranes depleted of the 23-kDa and 18-kDa proteins; (c) NaCl extract of PS II membranes which had been treated with the 'DNAase'; (d), (e), and (f), HPLC fractions II, III and IV, respectively, from the NaCl extract of the PS II membranes which had been treated with the 'DNAase' (see Fig. 2); (h) molecular-weight marker proteins: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). (B) Degradation of the purified proteins with α-chymotrypsin. Lanes (a), NaCl extract of PS II membranes which had been treated with the 'DNAase'; (b), purified 23-kDa protein; (c) and (d), the 23-kDa protein incubated with the chymotrypsin at enzyme-to-protein ratios of 10^{-3} and 10^{-2} (mol/mol), respectively; (e), purified 18-kDa protein; (f) and (g), the 18-kDa protein incubated with the chymotrypsin at enzyme-to-protein ratios of 10^{-3} and 10^{-2} (mol/mol), respectively; (h), purified 22-kDa fragment (HPLC fraction IV from the NaCl extract of PS II membranes which had been treated with the 'DNAase').

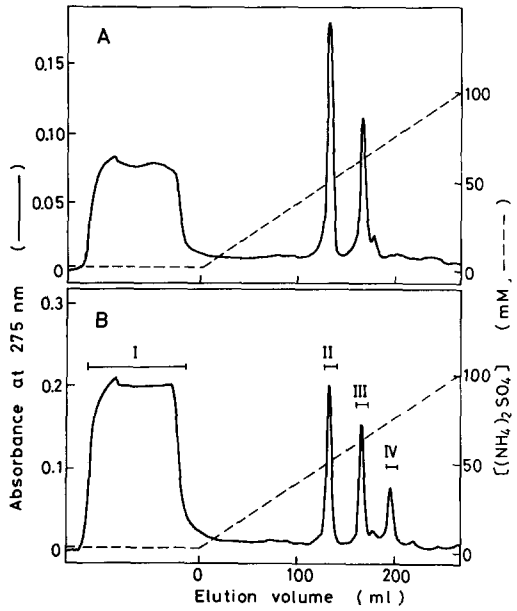

Fig. 2. HPLC of proteins and nucleic acids in the NaCl extract of PS II membranes. PS II membranes untreated or treated with the 'DNAase' corresponding to 40 mg Chl were incubated with 1.0 M NaCl/25 mM Mes-NaOH (pH 6.5) for 30 min. The NaCl extract obtained as a supernatant of centrifugation was desalted by gel filtration and subjected to HPLC in a cation-exchange mode with an SP-650S column (Tosoh). Eluates indicated by bars were collected and designated as fraction I to IV. (A) The NaCl extract of untreated PS II membranes; (B) The NaCl extract of PS II membranes treated with 'DNAase'.

proteinase activity is denoted as 'DNAase' to discriminate the effect of the contaminating proteinase from that of DNAase itself.

Since the 'DNAase' was prepared from bovine pancreas, it is likely that chymotrypsin and/or trypsin was contained as a contaminant. Fig. 1B shows the pattern of digestion of the purified proteins by α-chymotrypsin from bovine pancreas. The 23-kDa protein degraded to polypeptides of 22, 10 and 8 kDa, and the 18-kDa protein to a polypeptide of 10 kDa. Digestion products corresponded in migration distance in the gel with the polypeptides in the NaCl extract from the 'DNAase'-treated PS II membranes (Fig. 1B). This suggests that the degradation of the 23-kDa and 18-kDa proteins upon the 'DNAase' treatment was caused by chymotrypsin which had been contained in the 'DNAase'.

Fig. 2 shows the separation of proteins in the NaCl extract of PS II membranes by HPLC. When the "DNAase" was not used, three elution bands appeared (Fig. 2A). A fraction which was not adsorbed and eluted with 2 mM $(NH_4)_2SO_4$ contained nucleic acids but no protein, since its absorption maximum was around 260 nm and no polypeptide band was detected by SDS gel electrophoresis (data not shown). The 23-kDa and 18-kDa proteins were eluted with 45–50 and 60–65 mM $(NH_4)_2SO_4$, respectively. When PS II membranes were treated with the 'DNAase' prior to NaCl extraction, four elution bands appeared and were collected as fractions I to IV (Fig. 2B). The peak positions of fractions I, II and III in the chromatogram were the same as those in Fig. 2A. Fraction I contained nucleic acids only. Fraction II mainly consisted of the 23-kDa protein and fraction III contained the 18-kDa protein and a polypeptide of 10 kDa (Fig. 1A). Fraction IV which was eluted with about 75 mM $(NH_4)_2SO_4$ was composed of a single polypeptide of 22 kDa (Fig. 1A). A polypeptide of 17 kDa was eluted with 68 mM $(NH_4)_2SO_4$ giving a small elution band after the 18-kDa protein (Fig. 2B). When PS II membranes were incubated with the 'DNAase' for a longer period, the relative amounts of the polypeptides of 22 kDa and 10 kDa in the NaCl extract increased and another elution band consisting of a polypeptide of 10 kDa appeared at 130 mM $(NH_4)_2SO_4$ (data not shown).

Fig. 3 shows the partial amino acid sequence of a polypeptide of 22 kDa in fraction IV. The sequence at the N-terminal side from the 1st to the 27th amino acid residues of the 22-kDa polypeptide was the same as that from the 10th to the 36th residues of the 23-kDa protein, and the sequences at the C-terminus of the 22-kDa polypeptide and the 23-kDa protein were identical. This indicates that the 22-kDa polypeptide is a degradation product of the 23-kDa protein which lacks nine amino acid residues at the N-terminus. Hereafter, the 22-kDa polypeptide is designated as a 22-kDa fragment of the 23-kDa protein.

The amino acid sequence at the N-terminal side of a polypeptide of 10 kDa which was eluted with 130 mM $(NH_4)_2SO_4$ was determined up to the 20th amino acid residue (data not shown). This sequence was the same as that from the 34th to the 53rd amino acid residue of the 18-kDa protein reported by Jansen et al. [19], indicating that the

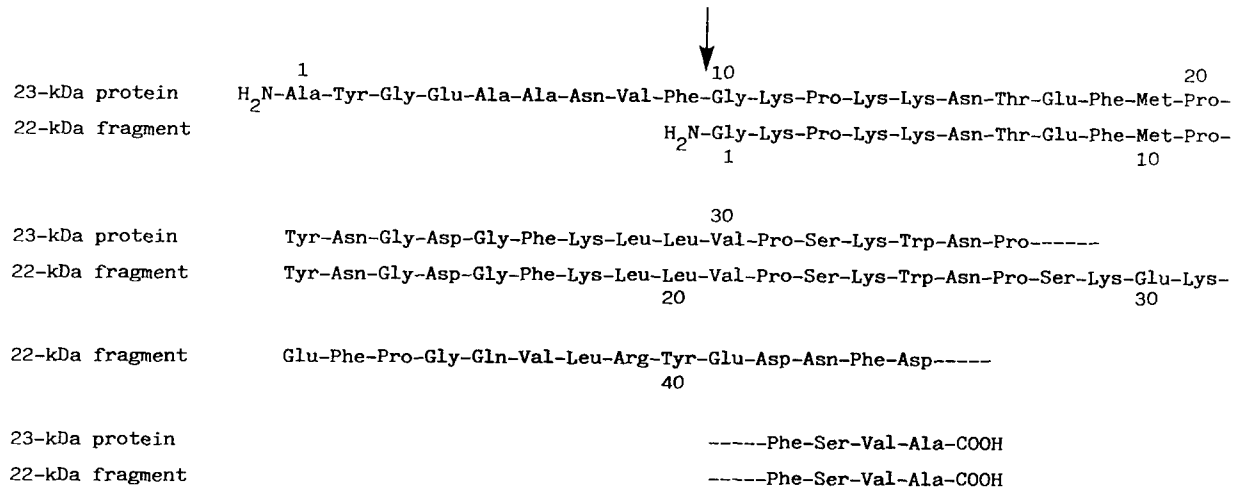

Fig. 3. Partial amino-acid sequences of the 23-kDa protein and the 22-kDa fragment. The arrow indicates the proteolysis site upon the 'DNAase' treatment of PS II membranes.

10-kDa polypeptide was a fragment of the 18-kDa protein. A polypeptide of 17 kDa was also likely to be a degradation product of the 18-kDa protein, which is produced by a proteinase latently bound to the PS II membranes [16], since it appeared even when the 'DNAase' was not used.

A polypeptide of 22 kDa obtained from the purified 23-kDa protein by α-chymotrypsin digestion and that of 10 kDa from the purified 18-kDa protein were also purified by HPLC and their amino acid sequences at the N-terminal side were determined. The sequences of these digestion products were the same as that of the 22-kDa fragment and of the 10-kDa polypeptide, respectively, which emerged upon the 'DNAase' treatment of PS II membranes. Therefore, we conclude that the degradation of the proteins upon 'DNAase' treatment was caused by chymotrypsin contained in the 'DNAase'.

The isoelectric point of the 22-kDa fragment was 6.8, while that of the 23-kDa protein was 6.5. This is consistent with the fact that an acidic amino acid, glutamic acid, was lost in the 22-kDa fragment (Fig. 3). In spite of a slight change in the isoelectric point, the elution band in the chromatogram shifted from 50 to 75 mM $(NH_4)_2SO_4$ upon the conversion into the 22-kDa fragment (Fig. 2). This suggests that the surface of the 22-kDa fragment is more positively charged than that of the 23-kDa protein.

Binding of the 23-kDa protein and the 22-kDa fragment to the PS II complex

The 23-kDa protein can stoichiometrically and functionally rebind to the PS II complex which

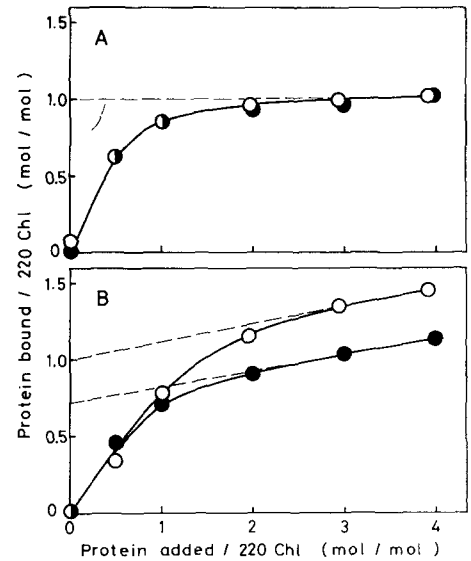

Fig. 4. Rebinding profile of the 23-kDa protein and the 22-kDa fragment to the PS II complex depleted of the 23-kDa and 18-kDa proteins. Designated amounts of the 23-kDa protein or 22-kDa fragment were added to the NaCl-treated PS II membranes in 300 mM sucrose/25 mM Mes-NaOH (pH 6.5) containing 10 mM (○) or 50 mM NaCl (●). After 30 min, the membranes were washed three times with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). (A) Rebinding of the 23-kDa protein; (B) rebinding of the 22-kDa fragment.

has been depleted of the 23- and 18-kDa proteins [14]. As shown in Fig. 4A, rebinding of the 23-kDa protein was saturated at a protein-to-Chl ratio of 1 : 220 (mol/mol), the original level in intact PS II membranes [6], in the presence of either 10 or 50 mM NaCl, indicating a specific binding between the 23-kDa protein and the PS II complex. The 22-kDa fragment also became rebound to the NaCl-treated membranes, but without saturation at the original level with an increase in the added protein; in 10 mM NaCl, the amount of rebound protein increased steeply up to about one molecule per 220 Chl molecules and then continued to increase gradually without reaching saturation (Fig. 4B). The excess binding of the fragment over the original level of the 23-kDa protein seemed to be loose, since this fraction of the bound fragment was released by repetitive washing of the PS II membranes with medium A (data not shown). The binding profile shown in Fig. 4B was observed after the third washing. It is likely that the excess binding was caused by electrostatic interaction between the positively charged fragment and negatively charged surface of PS II membranes. Extrapolation of the straight line of the excess binding to the ordinate (Fig. 4B) reveals that the tight binding of the 22-kDa fragment in 10 mM NaCl was saturated at a protein-to-Chl ratio of 1 : 220 (mol/mol). This suggests that the 22-kDa fragment can stoichiometrically bind to the PS II complex. However, in 50 mM NaCl, not only the non-specific but also the stoichiometric binding of the 22-kDa fragment was reduced, whereas the stoichiometric binding of the 23-kDa protein was unaffected (Fig. 4). These observations suggest that the binding affinity of the 22-kDa fragment to the PS II complex is lower than that of the 23-kDa protein.

Function of the 23-kDa protein and the 22-kDa fragment

The 23-kDa protein acts to modulate the binding of Cl^- and Ca^{2+}, inorganic components essential for oxygen evolution [5,11–13]. Fig. 5 shows the effect of the 23-kDa protein and its 22-kDa fragment on the Cl^- dependence of oxygen-evolution activity in the NaCl-treated PS II membranes. In order to distinguish the effect of the 23-kDa protein, or its 22-kDa fragment, on the Cl^- dependence from that of Ca^{2+}, we added Ca^{2+} exogenously to the assay mixtures. Ca^{2+} at 1 mM sustained the activity at all Cl^- concentrations from 2 to 300 mM but did not affect the optimum Cl^- concentration (Fig. 5). Further addition of the 23-kDa protein shifted the optimum Cl^- concentration from 30 to 10 mM and also supported the oxygen evolution at Cl^- concentrations below 10 mM. The 22-kDa fragment also shifted the optimum Cl^- concentration from 30 to 10 mM but was less effective than the 23-kDa protein in supporting oxygen evolution at Cl^- concentrations below 10 mM.

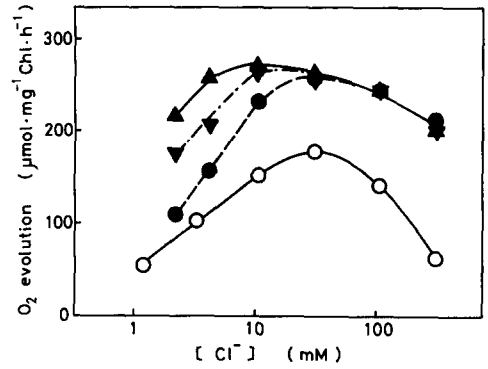

Fig. 5. Effect of Ca^{2+}, the 23-kDa protein and the 22-kDa fragment on the Cl^- dependence of the oxygen-evolution activity of the NaCl-treated PS II membranes. The NaCl-treated PS II membranes were incubated with the 23-kDa protein or 22-kDa fragment at a protein-to-Chl ratio of 3 : 220 (mol/mol) for 30 min, and then the reconstituted membranes were collected by centrifugation and suspended in 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5). The oxygen-evolution activity was measured in 300 mM sucrose/25 mM Mes-NaOH (pH 6.5) containing designated concentrations of Cl^-, in the presence or absence of 1 mM Ca^{2+} after a 5-min incubation at 25 °C. Chloride ions were added as $CaCl_2$ and/or NaCl. (○——○), NaCl-treated membranes, no Ca^{2+} added; (●— — —●), NaCl-treated membranes, 1.0 mM Ca^{2+} added; (▼·–·–·▼), NaCl-treated membranes supplemented with the 22-kDa fragment, 1.0 mM Ca^{2+} added; (▲——▲), NaCl-treated membranes supplemented with the 23-kDa protein, 1.0 mM Ca^{2+} added.

The effect of the 22-kDa fragment on the Ca^{2+} trap was examined by investigating the light-dependent inactivation of oxygen evolution. Previous reports [5,12] have suggested that illumination releases Ca^{2+} from its functional site in the PS II complex lacking the 23-kDa protein, resulting in inactivation of oxygen evolution, and that the 23-kDa protein protects the complex from the

light-dependent Ca^{2+} release. Since Ca^{2+} is necessary for electron transport from the Mn cluster to Z^+ [23,24], the oxygen-evolution activity can be an indirect measurement of the functional Ca^{2+} bound to the complex. Table I shows the effect of the 23-kDa protein and its 22-kDa fragment on the light-induced inactivation of oxygen evolution. When NaCl-treated PS II membranes depleted of the 23-kDa and 18-kDa proteins were incubated under illumination, the oxygen-evolution activity measured with supplementation of the 23-kDa protein was reduced by 40% of that before the incubation, whereas incubation in darkness did not substantially inactivate oxgyen evolution. This light-induced inactivation was not observed when 10 mM Ca^{2+} or the 23-kDa protein was present, indicating the protective effect of the 23-kDa protein against Ca^{2+} release as previously shown [5,12]. Addition of the 22-kDa fragment also suppressed the light-dependent inactivation, that is, the light-dependent Ca^{2+} release, as in the case of the 23-kDa protein. This suggests that the 22-kDa fragment functions to retain Ca^{2+} at the functional site in the PS II complex under illumination.

TABLE I

EFFECT OF THE 23-kDa PROTEIN AND ITS 22-kDa FRAGMENT ON THE LIGHT-DEPENDENT INACTIVATION OF OXYGEN EVOLUTION OF PS II MEMBRANES

NaCl-treated PS II membranes lacking the 23-kDa and 18-kDa proteins were suspended in 4.0 mM EGTA/10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5) with the designated supplement at a final Chl concentration of 0.35 $mg \cdot ml^{-1}$, and incubated for 30 min in darkness or under illumination from an incandescent lamp at 80 $W \cdot m^{-2}$ at 0 °C. After the membrane suspension was diluted 45-fold with 10 mM NaCl/300 mM sucrose/25 mM Mes-NaOH (pH 6.5), the oxygen-evolution activity was assayed. When indicated, the 23-kDa protein was added just before assay of oxygen evolution.

Conditions for incubation		O_2 evolution ($\mu mol \cdot (mg\ Chl)^{-1} \cdot h^{-1}$)
light or dark	supplement	
Before incubation		400 [a] (100%)
Dark	None	380 [a] (95%)
Light	None	240 [a] (60%)
Light	10 mM $CaCl_2$	410 [a] (103%)
Light	23-kDa protein [b]	400 (100%)
Light	22-kDa fragment [b]	380 (95%)

[a] Oxygen-evolution activity was assayed in the presence of the 23-kDa protein at a protein-to-Chl ratio of 3:220 (mol/mol).
[b] Protein-to-Chl ratio, 3:220 (mol/mol).

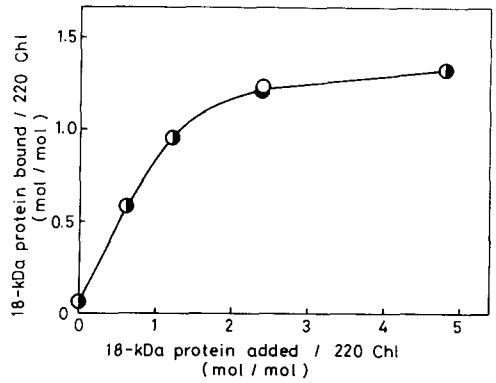

Fig. 6. Effect of the 23-kDa protein and the 22-kDa fragment on rebinding of the 18-kDa protein to the PS II complex. The designated amounts of the 18-kDa protein were added to the NaCl-treated PS II membranes supplemented with the 23-kDa protein (○) or 22-kDa fragment (●) at a protein-to-Chl ratio of 2:220 (mol/mol).

Another function of the 23-kDa protein is to provide the binding site for the 18-kDa protein on the PS II complex [14]. Fig. 6 shows the effect of the 23-kDa protein and its 22-kDa fragment on the rebinding of the 18-kDa protein. When the 23-kDa protein was absent, the 18-kDa protein rebound only non-specifically to PS II membranes [14]. This non-specific binding can be afforded by electrostatic interaction between the positively charged protein and negatively charged PS II membranes. When the 23-kDa protein was present, on the other hand, additional rebinding, which was saturated at the original level, occurred giving a biphasic increase in the binding curve (Fig. 6) [14]. The 22-kDa fragment showed the same effect on the rebinding of the 18-kDa protein as the intact protein, suggesting that the 22-kDa fragment acts to provide the binding site for the 18-kDa protein on the PS II complex in the same fashion as that of the 23-kDa protein.

Discussion

The three extrinsic proteins participating in oxygen evolution in the PS II complex have been well characterized and their functions are becoming clear. However, the molecular mechanisms of the functions are still in question. Limited proteo-

lysis represents a powerful technique for the study of functional domains within proteins. In this study, we examined a degradation product of the 23-kDa protein, which was produced in the course of our protein purification procedure.

The NaCl extract from PS II membranes, the starting material for purification of the 23- and 18-kDa proteins, also contained nucleic acids. Although the nucleic acids can be separated from the proteins by cation-exchange HPLC (Fig. 2A), their presence hampered the protein purification and reduced the yield of the protein. Treating PS II membranes with DNAase I prior to NaCl extraction resolved this problem, but, on the other hand, degraded the proteins by proteinase activity present in the 'DNAase' used in this study. The 23-kDa protein degraded mainly to a polypeptide of 22 kDa and the 18-kDa protein to a polypeptide of 10 kDa.

The partial amino acid sequences of the degradation products indicate that the proteolysis sites were the carboxy side of phenylalanine-9 in the 23-kDa protein and that of tyrosine-33 in the 18-kDa protein. This specificity of hydrolysis suggests that the proteinase contained in the 'DNAase' is chymotrypsin. This was confirmed by comparing the partial amino acid sequences of digestion products by the 'DNAase' and by α-chymotrypsin.

The chymotrypsin hydrolyzed the peptide bond at the carboxy side of phenylalanine-9 of the 23-kDa protein, whether it was bound to the complex or dissolved in aqueous solution. This suggests that this part of the protein is exposed to the outer aqueous phase in both bound and soluble forms.

The removal of nine amino acid residues at the N-terminus of the 23-kDa protein increased positive charge on the protein surface, judging from the shift of band position in the chromatogram (Fig. 2B). It probably resulted from the exposure of a region rich in lysine residues adjacent to the proteolysis site (Fig. 3) to the outer phase upon proteolysis.

The elimination of the N-terminal region lowered the binding affinity of the 23-kDa protein to the PS II complex. Stoichiometric rebinding of the 23-kDa protein was achieved at the protein-to-Chl ratio of 1:220 (mol/mol) (Fig. 4A). This indicates that all the added protein could bind to the complex as long as there were vacant binding sites. On the other hand, more than double the amount of protein was necessary for the stoichiometric binding of the 22-kDa fragment (Fig. 4B). In addition, in 50 mM NaCl the binding of the 22-kDa fragment was much more depressed as compared with that of the 23-kDa protein.

Two explanations are possible for the lower binding affinity of the 22-kDa fragment. In one explanation, the N-terminal region of the 23-kDa protein is assumed to be the binding site for the complex. Since the 23-kDa protein binds to the complex by electrostatic interaction [25], it is likely that glutamic acid at the fourth amino acid residue participates in the binding. Removal of the negative charge of glutamic acid-4 and the resulting exposure of the positive charge of lysine-11, -13 and -14 might reduce the binding affinity to the complex. In another explanation, the binding site to the complex is assumed to be different from the N-terminal region. The positive charge of the 22-kDa fragment which is exposed after degradation can produce a repulsive force between the fragment and the complex, resulting in an apparently lower binding affinity. The latter explanation, however, does not agree with the observation in Fig. 4B that the binding of the 22-kDa fragment was much depressed in 50 mM NaCl, for which the electrostatic repulsion should be weaker than in 10 mM NaCl. Therefore, it is likely that the binding domain is near the N-terminal region, and therefore, is affected by the removal of the N-terminal nine amino acid residues.

Once the 22-kDa fragment was stoichiometrically bound to the PS II complex, it functioned as the 23-kDa protein. The fragment shifted the optimum Cl^- concentration of oxygen evolution from 30 to 10 mM, provided a binding site for the 18-kDa protein on the PS II complex, and acted to trap Ca^{2+} at its functional site in the same manner as the 23-kDa protein (Figs. 5 and 6, Table I). These findings suggest that the overall conformation of the 23-kDa protein was not considerably altered by the loss of the N-terminal region.

The conversion of the 23-kDa protein into the 22-kDa fragment reduced the capability to support oxygen evolution at Cl^- concentrations lower than 10 mM (Fig. 5). To fully understand the

molecular mechanisms involved, together with the mechanism of the Cl^- effect, further studies are required.

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